

Characterization of mobile mRNA from host plants into the parasitic weed *Cuscuta pentagona*

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Characterization of mobile mRNA from host plants into the parasitic weed *Cuscuta pentagona*

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Abstract

The obligate stem parasite *Cuscuta pentagona* is able to take up host plant mRNA through a specialized organ known as the haustorium. Direct cell-to-cell symplastic connections between two different organisms are rare, and the translocation mechanisms and fate of these RNAs in the parasite is not understood. To characterize this phenomenon, mobile *Arabidopsis* and tomato mRNAs were identified from microarray and transcriptome sequencing projects and quantified in the host-parasite system. Mobile RNAs were quantified using real time (qRT)-PCR and were found to vary substantially in their rate of uptake and distribution in the parasite. Transcripts of tomato Gibberellic Acid Insensitive (*SIGAI*) and Cathepsin D Protease Inhibitor (*SIPI*) can be traced over 30-cm of parasite stem. *SIPI* was abundant in the *C. pentagona* stem, but the number of copies decreased substantially within the first eight hours post detachment. Additional studies of mobile RNAs from *Arabidopsis*, Translationally Controlled Tumor Protein (*AtTCTP*), Auxin Response Factor (*AtARF*) and a Salt-inducible Zinc Finger Protein (*AtSZFP*) supported the idea that mRNA molecules differ in their mechanisms of uptake and mobility between host and parasite. Known phloem-mobile RNAs (*SIGAI* and *AtTCTP*) have uptake patterns that differ from each other as well as from other RNAs that are not reported to be phloem mobile (*SIPI* and *AtSZF1*). The function of RNAs in plants extend beyond protein translation to include post transcriptional gene silencing or long distance signaling, and mobile RNA in *C. pentagona* systems offers novel insights into this aspect of plant biology. Studies of cell-to-cell trafficking

of RNAs and other macromolecules would be facilitated by the ability to manipulate individual cells. To this end, work was initiated to explore alternative approaches to understanding single cell biology using laser-mediated approaches. Optoperforation, or the use of multiphoton processes to form quasi-free electron plasmas to initiate transient pore formation in plasma membranes, has been demonstrated, but not in cells of an intact plant. This work details a protocol for optoperforation of *Arabidopsis* epidermal cells to allow for uptake of external dye-labeled dextrans and retention for up to 72 hours, and has the potential for transformation and molecular tagging applications.

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Attributions

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Timecourse video confirmation of symplastic uptake of cascade blue dye over 5 minutes.

. LeBlanc_ML_f4

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Chapter 1: RNA trafficking in parasitic plant systems

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Abstract

As sessile organisms, the local and long-distance coordination of plant development and response to the environment is facilitated, in part, by mobile RNA signaling. However, since it is difficult to trace RNA from its cell of origin to its destination, investigations of mobile RNA identity and function are challenging as they involve sampling phloem sap or detecting trafficked transcripts among a very large pool of non-mobile RNAs. Some parasitic plants form symplastic connections to host plants and can be used as an alternate system for studying RNA trafficking. The haustorial connections of *Cuscuta* and *Phelipanche* species are similar to graft junctions in that they are able to transmit mRNAs, viral RNAs, siRNAs and proteins from the host plants to the parasite. In contrast to other graft systems, these parasites form connections with host species that span a wide phylogenetic range, such that a high degree of nucleotide sequence divergence may exist between host and parasites and allow confident identification of most host RNAs in

the parasite system. This allows for the use of genomics approaches to understanding RNA trafficking. Further research is needed to better understand the host-parasite interaction but parasitic plants may provide an intriguing perspective on RNA trafficking.

Keywords: *Cuscuta*, haustorium, host-parasite interactions, Orobanchaceae, *Orobanche*, *Phelipanche*, RNA trafficking

Introduction

RNA trafficking in plants has received increasing attention in recent years. It is an important phenomenon in that it suggests that plants operate at the level of supracellular organisms, meaning that mRNA is not restricted to the cell in which it was synthesized, but rather moves from cell to cell and even over long distances through the phloem (Lucas & Lee, 2004a). Under this model mRNAs function in cells distant from their point of origin by carrying information throughout the plant and act as part of a system for coordinating plant development.

However, the study of RNA movement between cells of a single organism presents tremendous challenges. It is difficult to confidently determine the cell of origin of an RNA molecule when the genotypes of source and destination cells are identical. This inability to distinguish mRNA origins hampers research, and while it is currently thought that there are about 1,100 proteins and hundreds of mRNAs that undergo long distance movement (Atkins *et al.*, 2011) the actual scope of RNA trafficking over short and long distances is unknown.

Parasitic plants are relevant to the consideration of RNA trafficking because certain species have the ability to form symplastic unions with host species wherein the connections allow the transfer of RNA. In the case of lespedeza dodder (*Cuscuta pentagona*), the connections permit transfer of mRNA from host to parasite (Roney, J *et al.*, 2007; David-Schwartz R, 2008), and this raises exciting prospects for understanding RNA trafficking in plants. Because *Cuscuta* has a relatively wide host range and can effectively parasitize a number of species from a diverse range of plant families, this parasite can act as a sink for host mobile RNA from many different species.

Furthermore, the evolutionary distance between *Cuscuta* and most of its hosts means that the majority of mRNAs synthesized in a host have sequences that are divergent from those of *Cuscuta*, thus simplifying the process of bioinformatically recognizing host mRNA that has

trafficked into the parasite. To the extent that *Cuscuta* connections to hosts approximate normal cell-to-cell connections within plants, *Cuscuta* can serve as an exceptionally wide heterograft to facilitate studies of mobile RNA. This review will examine the nature of host-parasite connections and consider the advantages and disadvantages of using parasites for studies of RNA trafficking in plants.

Parasitic Plant Connections: The Perfect Graft?

The connection between parasitic plants and their hosts has been compared to “the perfect graft” (Kuijt, 1983). The analogy of parasitic plant connections to graft unions is appropriate in that both involve fusing together separate plants to forge new cellular connections and vascular continuity. Both grafts and parasite connections establish symplastic connections (Although this is not true of all parasite species, it is accepted for *Cuscuta* and *Phelipanche* spp.), and have the ability to transmit RNA (Westwood, James H *et al.*, 2009; Harada, 2010). However, whereas man-made grafts are the result of joining cut tissues, the parasitic connection involves a highly coordinated biological invasion (Joel, 1994; Lee, 2007). Although parasitism may elicit defense responses from the host (Borsics & Lados, 2002; Griffitts *et al.*, 2004; Swarbrick *et al.*, 2008), compatible reactions display little tissue necrosis and haustorial connections are characterized by close association of live cells from both species.

Another difference between graft unions and parasite connections is the greater breadth of compatibility between parasites and hosts compared to graft compatibilities. Parasites are able to form connections with plant species that are phylogenetically distant from themselves, which stands in contrast to grafting where success is greatest when stock and scion are from the same or closely related species (Mudge *et al.*, 2009). For example, a hetero-graft may consist of a pepper

scion on a tomato stock, but both species are members of the Solanaceae family. Parasites in contrast, commonly connect to host plants that are phylogenetically distant from themselves, with an excellent example being *Striga hermonthica*, a dicotyledonous member of the Orobanchaceae family that attacks grass (Poaceae) hosts. The host range of parasitic plants may also vary substantially, and some species are able to parasitize a wide range of host species while others are limited to just a single genera. Host-specificity is variable among parasite species and certain parasites have adapted to attack relatively broad ranges of crop plants and are economically important agricultural weeds (Parker & Riches, 1993b; Westwood *et al.*, 2010).

Parasitic plants live by tapping into the vascular system of a host plant and withdrawing the necessary water and nutrients needed to provide part or all of the parasite nutritional needs. Parasites connect to their hosts using a specialized structure, the haustorium, which penetrates host tissue and forms a bridge to the vascular system of the host. Haustoria vary substantially in their anatomy and function among different parasitic plant species, and are generally characterized by whether they form connections to the xylem only or to both xylem and phloem (Irving & Cameron, 2009). Xylem-feeding parasites primarily withdraw water and dissolved solutes from the host and are generally hemiparasitic in that they are able to photosynthesize to produce at least part of their carbon needs. Parasites that form both xylem and phloem connections are often holoparasitic, relying on their hosts for all nutritional needs. Among this latter group are the dodders (*Cuscuta* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.), two genera with relatively well-characterized haustoria. RNA trafficking to parasitic plants has been best characterized in these species, particularly *Cuscuta*, but even here the level of understanding of RNA movement is far from complete.

With respect to *Cuscuta*, the overwhelming physiological data indicates that the parasite absorbs phloem contents from the host. However, the exact mechanism is not clear as no direct phloem connections have been demonstrated (Vaughn, 2006). Rather, cells of the parasite searching hyphae that encounter host sieve elements appear to grow around the phloem cells of the host (Felsenstein, 1978). These parasite cells differentiate in a manner consistent with development of sieve elements, although they also contain an elaborate network of smooth endoplasmic reticulum (ER) proximal the host cell, a feature of transfer cells (Christensen *et al.*, 2003). For this reason it remains a formal possibility that *Cuscuta* may acquire host resources by apoplastic transfer, although this seems to fall short of explaining the ability of *Cuscuta* to readily absorb macromolecules such as mRNA, proteins and viruses from their hosts. Physiological continuity of host and parasite phloem is sufficient to transfer the symplastic marker carboxyfluorescein within two hours of dye being applied to the host (Birschwilks, M. *et al.*, 2006). This dye, as well as GFP-tagged viral movement protein, moved readily through the phloem of established haustoria, yet was not observed extensively in host parenchyma cells outside the vascular bundle, suggesting that phloem comprises the major connection. The cell wall structure of *Cuscuta* phloic hyphae is extremely loose such that it could permit the passage of larger molecules via an apoplastic mechanism (Vaughn, 2006), but more research will be needed to definitively settle the question of phloem transfer.

In contrast to the scant anatomical evidence for direct phloem connections, *Cuscuta* has well documented plasmodesmata (PD) connections with host cells (Vaughn, 2003; Birschwilks, M. *et al.*, 2006). These occur along the cell walls of searching hyphae of the *Cuscuta* haustorium that traverse the host cortex to reach the host vascular tissue. The searching hyphae grow through host cells in a manner that does not puncture them, but rather forms what amounts to a tunnel

through a host cell and results in the formation of new cell wall on either side, creating a chimeric structure composed of host and parasite cell walls (Vaughn, 2003). PD in these walls may take simple or branched form and are spanned by desmotubules typical of PD (Vaughn, 2003; Birschwilks, M. *et al.*, 2006). It is possible that these PD contribute to the transfer of materials between host and parasite, but the question has never been addressed experimentally.

The symplastic connections between broomrapes and their hosts differ from those of *Cuscuta* in terms of anatomy, but appear to share many of the same physiological functions. In contrast to *Cuscuta*, direct connections between sieve elements of *Orobanche crenata* and those of its host *Vicia narbonensis* have been imaged using electron microscopy (Dorr & Kollmann, 1995). PD between these species have also been documented and are proposed to lead to formation of sieve pores between adjacent sieve elements (Dorr & Kollmann, 1995). Anatomical differences between Convolvulaceae and Orobanchaceae parasites are not surprising given their different evolutionary origins of parasitism (Barkman *et al.*, 2007). Broomrapes also contrast with *Cuscutas* in the host organ targeted, as broomrapes attack roots while *Cuscutas* parasitize stems and leaves.

Host-parasite movement of macromolecules

Parasitic plants acquire a wide range of macromolecules from their hosts that are relevant to the current discussion (Table 1). The capacity of *Cuscuta* to acquire mRNAs from hosts was first demonstrated for mRNAs of specific genes known to be mobile in pumpkin phloem (Roney, J *et al.*, 2007). Total RNA was isolated from *Cuscuta* stems near the point of host attachment and used in reverse transcriptase (RT)-PCR and the resulting amplified products were then sequenced to confirm that the mRNA detected in the parasite was identical to that of the host gene.

Additional experiments used tomato hosts and hybridized RNA extracted from *Cuscuta* onto a tomato microarray, leading to the identification of 474 putatively mobile mRNAs. Confirmation tests on a subset of these using RT-PCR, along with an additional study (David-Schwartz R, 2008) bring the current total of confirmed trafficked mRNAs into *Cuscuta* to 27 (Westwood, James H *et al.*, 2009). Mobility of mRNA into *Cuscuta* from three different hosts (alfalfa in addition to tomato and pumpkin) provides evidence that the phenomenon is not specific to just one host-parasite interaction.

Host mRNAs trafficked into the parasite cover a range of biological functions that are typical of those reported in other mobile transcriptomes. For example, the list of host mobile mRNAs in *Cuscuta* include transcription factors such as the *CmNACP* and *CmWRKYP* (Ruiz-Medrano, R *et al.*, 1999) and *GIBBERELLIC ACID INSENSITIVE* (GAI) (Haywood, V. *et al.*, 2005; Huang & Yu, 2009a). In addition, *Cuscuta* contains host RNAs associated with protein synthesis such as translation initiation factors and ribosomal proteins have been found, the presence of which in phloem of other species has fueled speculation over the possibility of protein translation in sieve elements (Kragler, 2010a). Finding mRNAs for genes associated with defense responses such as a cathepsin D proteinase inhibitor (Ryan, 1990) are not surprising given that the system is based on a pathogenic attack of one plant on another. One potential paradox is the finding of both large and small subunits of host RuBisCO mRNA in *Cuscuta* (David-Schwartz R, 2008), which is surprising because the small subunit is generally thought to be immobile and has been used as an indicator of non-phloem contamination in studies of phloem-mobile RNAs (Ruiz-Medrano, R. *et al.*, 1999).

The finding of host *rbcS* mRNA in *Cuscuta* supports the hypothesis that *Cuscuta* is accessing contents of parenchyma cells as well as phloem. In an *in situ* RT-PCR experiment, mRNA of the

tomato phosphofructokinase gene was shown to be concentrated in host parenchyma as well as adjacent *C. pentagona* parenchyma cells closest to the host (David-Schwartz R, 2008). This, along with detection of transcripts localized to sieve element and companion cell regions in the parasite, suggests cell-to-cell translocation of the transcript mediated by parenchyma connections. It is possible that transcripts cross multiple layers of parenchyma cells to ultimately reach the parasite phloem, but it is also possible that the *Cuscuta* searching hyphae cells themselves differentiate into phloem or take on the role of assisting phloem cells, which would simplify the pathway to long-distance movement. Host mRNAs do move long distances as they have been found distant from the point of haustorial attachment. RT-PCR with specificity to the host transcript showed that tomato phosphofructokinase mRNA was detected in *Cuscuta* shoots up to 30 cm away from the point of contact with the host, but no target mRNA was detected beyond that point (David-Schwartz R, 2008). This would seem to indicate a limit to mRNA translocation, although it is premature to draw any conclusions based on findings from a single gene. Additional studies are needed that characterize mobility of multiple transcripts, ideally using quantitative techniques that can reveal the dynamics of host to parasite transfer as well as long distance movement and fate of host mRNA in the parasite.

The mobility of host mRNAs into *Cuscuta* suggests that a similar transfer would occur into broomrapes from their hosts. However this has not been demonstrated, and studies with *P. aegyptiaca* growing on pumpkin did not detect mRNA transfer even though conditions were identical to those used to demonstrate mRNA transfer into *Cuscuta* (Roney, J *et al.*, 2007). More recently, data from an EST sequencing project (The Parasitic Plant Genome Project, <http://ppgp.huck.psu.edu/>) revealed no host mRNAs in the shoots of attached *P. aegyptiaca*, suggesting that host mRNA mobility into this parasite may be limited at the point of haustorial

transfer into the parasite or between the tubercle and shoot of the parasite. Further experiments are needed to specifically answer this question.

Other examples of host-parasite exchange of RNA include viruses, viroids and siRNA signals (Table 1). *Cuscuta* has long been recognized for the ability to accept and transmit viruses with its hosts and in fact has often been used as a vector for transmitting plant viruses between different plants (Bennet, 1944). Fifty-six viruses have been reported to thus move through *Cuscuta* (Hosford, 1967). More recent work quantified potato virus Y isolate N (PVYN) transmitting through a *Cuscuta* bridge between two *Nicotiana tabacum* plants, yet showed little virus accumulation in the *Cuscuta* bridge itself compared to recipient host plant and suggesting that virus movement occurs in *Cuscuta* without multiplication in the parasite (Birschwilks, M. *et al.*, 2006). In *P. aegyptiaca* the uptake of three positive ssRNA viruses Cucumber Mosaic Virus (CMV), Tomato Mosaic Virus (ToMV), Potato Virus Y (PVY) and the ssDNA virus Tomato Yellow Leaf Curl Virus (TYLCV) were shown to move into the parasite from infected hosts and, in the case of CMV, is able to replicate in the parasite (Gal-On *et al.*, 2009).

Viroid transport is useful to understanding long distance RNA trafficking in general (Wang & Ding, 2010), and is interesting to consider with respect to movement into parasitic plants. Potato Spindle Tuber Viroid (PSTVd) was shown to be taken up from a tomato host by *Phelipanche ramosa* and translocated through the tubercle and into stems of floral shoots (Vachev *et al.*, 2010). The viroid in this study appeared to replicate in the parasite but was not mobile in the reverse direction back into the host, suggesting a dominant sinkward flow.

RNA-based silencing signals also move from host into parasite where they are able to influence gene expression. The facultative parasite *Tryphysaria versicolor* was transformed to express the

β -glucuronidase (GUS) reporter gene and then allowed to parasitize lettuce plants that expressed a fragment of the GUS gene in a hairpin orientation (Tomilov *et al.*, 2008). Parasites attached to siRNA-expressing host plants showed a decrease in GUS staining in the root tissue near the point of host attachment, indicating transmission of the silencing signal. However, the silencing signal did not move into tissues distant from the site of attachment, suggesting that movement was somehow restricted. The signal was able to move through a *T. versicolor* bridge and into a second host plant expressing GUS. As with *Cuscuta* bridges that transmit viruses from one plant to another, it seems that parasites are able to transfer silencing signals. A similar approach was taken to silence mannose 6-phosphate reductase (M6PR) in *P. aegyptiaca* using constructs expressed in a tomato host (Huang & Yu, 2009a). This caused a decrease in the expression of the parasite gene and raises the prospect of trans-specific gene silencing as a potential strategy for controlling parasitic weeds through engineered hosts.

In addition to RNAs, proteins have also been shown to traffic from hosts to parasitic plants. The characterization of protein mobility employed transgenic host plants expressing green fluorescent protein (GFP) controlled by the phloem targeting *Arabidopsis* SUC2 promoter (AtSUC2-GFP) (Imlau *et al.*, 1999). This construct was expressed in tobacco and the GFP signal translocated into *Cuscuta reflexa*, confirming the symplastic connection between host and this parasite as well with evidence of unloading of the fluorescent protein in sink tissues (Haupt *et al.*, 2001a). The same construct was used to study host protein uptake by *P. aegyptiaca* parasitizing transgenic tomato. Although an endoplasmic reticulum-targeted version of the protein did not move, a soluble version accumulated in tubercles of the parasite (Aly *et al.*, 2011).

Parasitic tissue is not the final destination of every host molecule taken up. The leafhopper-borne yellows disease, originally believed to originate from a virus, is caused by a mycoplasma-like

bacteria that flows through plant phloem (Doi *et al.*, 1967). In two studies these bacteria, called phytoplasmas, have been shown to traverse *Cuscuta odorata* bridges, and infect healthy secondary *Catharanthus roseus* (periwinkle) hosts. *Cuscuta* bridges connecting Alder Yellows (ALY) infected alder to non-infected periwinkle were able to transfer the phytoplasma, and symptoms were slowly manifested four months after the *Cuscuta* bridge was established (Marcone *et al.*, 1997). In another study phytoplasmas were transmitted in 50% of the cases from *Lilium* (hybrid Casablanca) to periwinkle through the *Cuscuta* bridge, with the recipient plant developing symptoms of stunting and flower bud deficiency 2-3 weeks following connection by *Cuscuta* (Kamińska & Korbin, 1999)

The final example of host-parasite macromolecule exchange is Horizontal Gene Transfer (HGT). Although HGT and RNA trafficking both involve movement of nucleic acids, it is not certain whether they use similar mechanisms. HGT has been reported between several non-parasitic plants, but seems to occur at a higher frequency in parasitic associations (Richardson & Palmer, 2007). The high frequency of transfer events to or from parasites is probably attributable to the greater opportunity for nucleic acid exchange provided by the close physical association of the parasitic interaction. HGT events are generally discovered during phylogenetic studies in which genes that are otherwise reliable indicators of species phylogenies instead show high homology to versions in distant families. All HGT events described to date are the result of transfers that occurred thousands of years ago, and little is known about the frequency with which HGT events occur, or the frequency at which the transferred genes are introgressed into the recipient genome.

The precise mechanism of HGT remains unresolved, but two possibilities exist. One mechanism that relates directly to the topic of mRNA trafficking is suggested by an HGT event in which a gene of unknown function moved from a grass host into *Striga* spp. (Yoshida *et al.*, 2010). The

introduced gene in *Striga* has high homology to the version in grass, but lacks introns and seems to have the remnant of a poly-A sequence, suggesting that the transfer occurred via an mRNA intermediate. In contrast, most other reported HGT events involve mitochondrial genes (e.g., *atp1*, *atp6* and *matK*), and these have been proposed to transfer between plants as large sections of mitochondrial DNA (Mower *et al.*, 2010). The transfer of DNA across graft junctions has been shown to occur in both directions, although the movement of DNA is limited to the region of the graft so is likely a cell-to-cell movement that does not involve phloem (Stegemann & Bock, 2009). This may be similar to what happens in parasitic plant and other natural grafts, but limited mobility of the transgenes would require development of a shoot from the graft junction to incorporate the foreign gene into the germline of the recipient plant. The study of HGT will benefit from increased sequencing of all plants, including parasitic species.

RNA trafficking

A model for cell-to-cell RNA transport to parasites

As a starting point, we base our understanding of host-parasite symplastic connections on what is known from cell-to-cell connections in autotrophic plants. In land plants intracellular connections need to be small enough to allow structural integrity of cell walls, while allowing for macromolecular movement throughout the plant. Cell-to-cell movement of RNA occurs through PD which are complex structures embedded in cell walls between cells and are composed of plasma membrane lined with microtubules and a continuous span of ER (Wolfe & dePamphilis, 1998; Hyun *et al.*, 2011). Water and solutes are able to passively travel via concentration gradients but movement is limited to molecules below 1 kDa. Facilitated movement of larger

molecules is selective and usually requires localization to, and interaction with, PD (Wolfe & dePamphilis, 1998; Hyun *et al.*, 2011).

In order for mRNA to move in a non-cell-autonomous manner, recognition of signal sequences, or motifs, such as 3'untranslated regions (UTRs), interact with specific RNA binding proteins much like the movement proteins (MP) of RNA viruses (discussed below). The MPs bind RNAs, repress translation, and enable movement of the ribonuclearprotein (RNP) complex to a PD (Baillaud, 1953; Hyun *et al.*, 2011). Mutational studies have determined that cell-to-cell movement of RNA is also motif-dependent in order for RNPs to associate with PD trafficking proteins. NON-CELL-AUTONOMOUS PATHWAY PROTEIN1 (NCAPP1) acts as a receptor for pumpkin (*Cucurbita maxima*) Phloem Protein 16 (CmPP16) in order to enter the phloem stream (Xoconostle-Cazares *et al.*, 1999), and requires phosphorylation and glycosylation (of Tyr63 and Ser66, respectively) for entrance to PD (Lee *et al.*, 2003). SHORTROOT (SHR) requires multiple motifs for mobility, but a singular specific sequence that confers mobility has not been identified (Gallagher & Benfey, 2009).

One of the best characterized mobile mRNAs is KNOTTED1 (KN1). The KNOTTED1-like family of transcription factors are ubiquitous plant cell constituents responsible for regulating pattern formation of the apical meristem. The KN1 homeodomain protein complexed to its own mRNA was detected in phloem and was associated with determining cell fate (Lucas *et al.*, 1995a). Fluorescently-labeled *E. coli* KNOTTED1 mRNA transcript was microinjected into maize mesophyll adjacent to vascular tissue, and was seen to migrate from cell to cell, presumably via PD connections. Mutated KN1 proteins were microinjected to show that the homeodomain protein was necessary for translocation.

Given this understanding of cellular RNA transport, we propose a model for physical interaction and RNA trafficking between host and parasite cells (Figure 1). We focus on host-parasite connections via a shared PD because in the case of *Cuscuta* there is both physical and experimental evidence for trafficking through PD (discussed above) and for broomrapes the PD may initiate the formation of sieve pores between species (Dorr & Kollmann, 1995). The model is based on the assumption that chimeric PD are congruent to self PD in terms of structure and function. Therefore, host RNP complexes destined for transport to another cell would associate with appropriate chaperone and PD-associated proteins and be directed through the PD. Once in the parasite cell, the fate of the RNP complex is unknown, but presumably could be processed in a manner similar to other translocated RNAs. Of course one of the interesting aspects of host parasite interactions is that the interaction is not necessarily equal and while the parasite requires an open PD for nutrient acquisition, the host would benefit by shutting down the connection. One or both plants must maintain the shared PD, with its associated plasma membrane, ER and embedded proteins, but nothing is known about this aspect of the interaction.

Virus movement as a model for mRNA trafficking

The mechanism of intercellular virus delivery, facilitated by MPs in cell-to-cell movement, informs our understanding of RNA transmission into and through parasitic plant attachments. Most viruses make MPs and coat proteins which assemble onto viral RNA and form viral nucleoprotein complexes (vNPCs) to facilitate passage through PD (Lough & Lucas, 2006; Lucas *et al.*, 2009; Hyun *et al.*, 2011). Two models demonstrate the mechanism of MP-mediated intercellular transmission of viral RNA through PD. First, the MP of a well-characterized phloem mobile pathogen, Tobacco Mosaic Virus (TMV), may associate with ER, F-actin and

microtubules (MTs) for delivery of vNPCs to PD. The MP may send a PD dilation signal to adjacent cells to allow vNPCs transport (Waigmann *et al.*, 1994b; Waigmann & Zambryski, 1995; Kawakami *et al.*, 2004), and has been shown to bind to calreticulin in the ER to aid in cytoskeletal trafficking (Chen *et al.*, 2005). A second mechanism for virus transmission based on cell-to-cell trafficking of potato virus X (PVX) presents some of the complexities of movement of vNPCs to PD. PVX encodes three viral proteins, called a triple-gene block (TGB) that is associated with cell-to-cell movement of vNPCS to PD. TGBp2 and TGBp3 are integral endomembrane ER proteins and TGBp1 forms a TGBp1-viral RNA complex to increase SEL of PD. Anchoring TGBp1-viral RNA complex to TGBp2 and TGBp3 mediates the delivery of the complex to the PD. In both TMV and PVX, movement proteins encounter SEL-binding motifs on PD proteins, which results in dilation of the channel and movement of the virus-protein complex through the PD (Haupt *et al.*, 2005).

Long distance RNA transport

Generally, long distance transport of RNA occurs when mRNAs from companion cells are loaded into sieve elements and are thus able to move systemically in the phloem stream. The RNP complex is stabilized as the sieve elements do not contain ribosomes for translation and lack RNase activity that would lead to degradation, making an ideal conduit for transport of mRNAs (Kragler, 2010a). mRNAs may be specifically unloaded at their destination (Haywood, V. *et al.*, 2005) based on a hypothesized targeting signal (or “zip code”) incorporated into the RNA or accompanying protein. Mounting evidence indicates that RNA motifs enhance long-distance trafficking, specifically the UTRs of mRNA and chaperone proteins that direct movement through the symplast. UTRs from *StBEL5* fused to the coding sequence of a less

mobile BEL-homolog increased mobility (Hannapel, 2010a) and the 3'UTR of *GAI* in *Arabidopsis* has been shown to be required for transcript movement (Huang & Yu, 2009a)

Another important example is *FLOWERING LOCUS T (FT)* RNA, which travels by means of a *cis*-acting element on the transcript, independent of the essential FT protein, from leaf to shoot apical meristem to induce flowering and suggests that mRNA has a role in systemic signaling of major developmental transitions (Li *et al.*, 2009; Li *et al.*, 2011). If parasites form compatible cellular connections with their hosts it is possible to infer these mechanisms of RNA mobility function in much the same way in a heterologous systems, and raising questions about the potential for direct information exchange across species.

Parasitic plants as tools for studying RNA trafficking

Studying RNA mobility over short and long distances in plants is technically challenging because of the need to pinpoint source and destination cells of the mRNA. Techniques range from the elegant application of transgenics, phloem-sap collection (either directly or using aphid stylets), grafting, or combinations of these (Atkins *et al.*, 2011). Each approach has advantages, but they are also subject to limitations in scope and potential for artifacts. We propose that parasitic plants can be added to this suite of techniques.

Studies of short-distance movement of mRNA have relied on transgenics and the use of genes with high specificity of expression (Kim *et al.*, 2005), microinjection (Xoconostle-Cazares *et al.*, 1999) or particle bombardment (Bennet, 1944). These approaches are excellent for demonstrating cell-to-cell movement and characterizing the features controlling trafficking of specific genes. However, these experiments are laborious and target individual genes, so are less useful for genomic-scale studies.

Investigations of long distance trafficking are somewhat simpler because RNAs identified from phloem sap are assumed to be in the process of moving from source to destination. Direct collection of phloem sap exuded from stem incisions is the ideal, but relatively few herbaceous species are copious exuders like cucurbits (Liere & Maliga, 2001) and legumes (Sharkey & Pate, 1976). The phloem stream of most plant species is rapidly shut down by callose plugs resulting in insufficient quantities of sap. To circumvent this limitation, phloem sap can be gathered by placing the cut stem in a solution of EDTA, a chelating agent that delays formation of the calcium cation callose plug that clogs the sieve plates (Turgeon & Wolf, 2009). However, in all cases of phloem sap collection, care must be taken to avoid contamination with cellular contents from the cut cells at the incision site. This is generally done by blotting the wound surface immediately after the incision and discarding the first several μl of sap exudate, but this minimizes contamination rather than completely eliminating it.

Another ingenious approach for sampling phloem is aphid stylectomy, which uses the ability of aphids to precisely insert their stylets into plant sieve elements. Subsequent severing of the aphid from its stylet leaves a tube that exudes small droplets of nearly pure phloem sap (Fisher & Frame, 1984; Doering-Saad *et al.*, 2002). Disadvantages of this method are that it is technically challenging and yields low volumes of sap, generally in the nano- or microliter range (Atkins *et al.*, 2011). A relatively high throughput method for collecting barley phloem sap has been used that attached microcapillary tubes to the embedded stylets of 600 aphids on 30 plants, and over the course of 6 hours captured 10 μl of phloem sap for protein and mRNA analysis (Gaupels *et al.*, 2008).

Grafting is an excellent method for detecting phloem-mobile molecules where the combination of stock and scion enable differentiation of the mobile signal. For example, wide grafts (or heterografts) between related species such as pumpkin and cucumber have been used in several key studies of RNA trafficking (Lucas *et al.*, 1995a; Lee *et al.*, 2003; Ham *et al.*, 2009).

Solaceous species are conducive to grafting and have been used in experiments that showed that tuber formation in potato is regulated by a mobile transcription factor, *StBEL5* (Banerjee *et al.*, 2006), and that tuber formation is increased in grafts with transgenic overexpressing scions.

Where one member of the graft carries a mutation or transgene, it is possible to demonstrate trafficking of a specific mRNA and coincident transmission of a phenotype as was done for *StBEL5* in potato (Rosin *et al.*, 2003) and the leaf shape phenotype Mouse ears (ME) in tomato (Kim *et al.*, 2001). A procedure for grafting together different *Arabidopsis* plants has been used for detecting flowering signals, but the success rate is relatively low compared to other graft systems at approximately 11% (Yunker, 1932). A disadvantage of grafting is the relatively narrow range of plants that can be joined together. Even wide grafts are restricted to members of the same plant family (Mudge *et al.*, 2009), which leaves ambiguity in distinguishing mobile RNAs between stock and scion.

Parasites form connections to their hosts that have many of the features of grafts as well as specific advantages. Chief among these is the ability of parasites to form interspecific grafts with a wide range of species. Given the ability of parasites to form symplastic connections with many of the species commonly used for RNA trafficking studies such as *Arabidopsis* (Westwood & Foy, 1998; Birschwilks *et al.*, 2007), tomato, and cucurbits (Roney, J *et al.*, 2007), it is possible to conduct comparative studies on different plants using the parasite as a common “scion”. More importantly, the phylogenetic differences between parasites and these hosts facilitates the use of

genomics approaches to understand RNA trafficking. *Cuscuta* that was grown on host species with sequenced genomes yields a mixture of host and self RNA that can be distinguished using microarrays (Roney, J *et al.*, 2007) and it presents an excellent application for next-generation sequencing. Even without an extensive *Cuscuta* genome, the host sequences can be determined for most genes based on exact matches to known host sequences. An extensive database for expressed genes of Orobanchaceae parasites exists (Westwood *et al.*, 2012), which will improve confidence in distinguishing host and parasite RNAs. The broad host range of *Cuscuta* will also facilitate studies on species that do not graft well or from which phloem exudates are difficult to obtain.

A concern over analyses of phloem sap obtained from incisions is that the sudden release in pressure would create artifacts such as dislodged macromolecules that would otherwise not be mobile in sieve elements (Atkins *et al.*, 2011). Parasitic plants would avoid this concern because the process of parasitism unfolds gradually, so presumably is accomplished without the artifacts arising from sudden pressure changes. Of course the parasite creates a strong sink that draws material from the host, but with negligible leaf surface area (for *Cuscuta* and *Phelipanche*), the process of withdrawing host vascular contents is likely within the range of normal plant translocation.

As with other methods of studying RNA trafficking, host-*Cuscuta* connections have certain disadvantages. Among these are the technical issues of establishing connections on specific host locations and at specific times. Parasites are unpredictable, and generating uniform tissues from synchronized attachment points can be challenging. In *Cuscuta*, the haustoria also follow a developmental progression through stages of host penetration, vascular connection, and eventually occlusion (Vaughn, 2003), and although the period of open transfer of

macromolecules likely spans many days or weeks, there is little information on how to distinguish actively translocating haustoria from those that are too young or old to function well. Thus, the most important limitation may well be the dearth of information on the precise functioning of haustorial connections. Finally, the interaction of host and parasite is ultimately one of pathogen and host, and it is reasonable to expect that control of the PD and macromolecule exchange is a point of contention between the two species. One consequence of this is that the mRNAs trafficked from host to parasite will be enriched in pathogen/defense response functions, but this is also true to some degree for all other methods of sampling phloem contents.

Potential significance of trans-species RNA trafficking

The most intriguing questions regarding cross species movement of RNA are whether and how RNA from one species functions in another species. Answering this question is difficult because mechanisms of action of native trafficked RNAs are not fully understood. If mRNAs function through translation into protein as indicated for *KN1* (Kim *et al.*, 2005), then parasites should be able to process host transcripts as well as they process their own mobile mRNA (Figure 1). On the other hand, if trafficked RNA acts in a sequence specific manner, then function in a parasite would depend on the presence of sufficiently homologous genes in the parasite. Many trafficked mRNA may not have a metabolic or regulatory function and may simply serve as a nutrient source for the parasite. Of course all three of these possibilities may occur, depending on the transcript in question.

Mobile RNAs and proteins have been shown to influence leaf shape (Kim *et al.*, 2001; Haywood, V. *et al.*, 2005), tuberization (Banerjee *et al.*, 2006), and flowering (Li *et al.*, 2011). There is

little evidence for transmission of a host phenotype in parasites, as the general morphology of the parasite does not depend on which host is used. Of course, parasites may be deficient in key regulatory pathways, for example those that lack expanded leaves would not be expected to perceive and respond to altered signals for leaf shape. A particularly intriguing area is flowering, and some authors have suggested that *Cuscuta* flowering time depends on timing of host flowering, thus invoking the possibility that *FT* RNA and protein from the host are capable of inducing flowering in the parasite (Fratianne, 1965; Mortazavi *et al.*, 2008). If the trafficked mRNA is a transcription factor, it is possible that the delivery of just a few molecules to the right cells could effectively throw the switch to turn on a new developmental program (Kragler, 2010a).

From an evolutionary standpoint, it is reasonable to think that parasites would be under selective pressure to decode and use information from host mRNAs and proteins that reveal the host physiological status, thereby enabling the parasite to respond to any changes in the host system. For example, by recognizing when the host is preparing to flower or senesce, the parasite can complete its own reproductive cycle in time. Considering that the host forms a dominant feature of the parasite's environment, it would not be surprising if the parasites were found to have mechanisms to monitor the health and developmental status of their hosts.

One mechanism for communication that has empirical support is post transcriptional gene silencing. The ability to silence GUS in transgenic *Triphysaria* and M6PR in *Phelipanche* indicates that the process works across species as long as the RNAi construct matches the target gene in the parasite. We have no information to date on whether parasites contain homologs of silencing targets known from other plant species, but we hypothesize that parasites may encounter miRNA signaling molecules that are generated in stressed plants (Stefanovic *et al.*,

2002; Lu & Huang, 2008). Of the many identified miRNAs, miRNA399, a phosphate starvation response signal, has been shown to move into grafted tissues (Stefanovic *et al.*, 2002), so likely passes into parasites. It will be interesting to learn whether parasites have sufficiently conserved homologs to be affected by this type of regulation. For perspective we asked this question of four *C. pentagona* genes with sequences available in the 1KP Project (<http://www.onekp.com>). Genes for a pentatricopeptide repeat-containing protein (Soly01g081290.2.1), DNA mismatch repair protein (Soly02g082660.2.1), clathrin heavy chain (Soly06g051310.2.1) and ATPase subunit 1 (Soly11g039980.1.1) are 80% (464/583), 83% (166/199), 85% (720/852) and 96% (251/261) identical, respectively, at the nucleotide level between tomato and *C. pentagona* (Bombarely *et al.*, 2011). This suggests that for certain genes it is likely that a highly homologous 21 nt silencing signal, such as miRNA, could act between host and parasite.

Because parasites are foremost feeding on their hosts, it is possible that the RNA is taken up as a nutrient and carried with the bulk movement of solutes from the host plant with no informational significance for the parasite. The host trafficked mRNA in parasites may undergo catabolism to provide inorganic phosphate and sugars for conservation of cellular and organismal homeostasis of the parasite or to maintain haustorial function. However, for this to be true, the parasite must have a mechanism to distinguish host trafficked RNA from its own. This might be possible given sufficient differences between host and parasite RNA and protein sequences if the limitation on function was at the level of uptake into parasite cells. By this hypothesis, parasite PD would discriminate and only allow uptake of self RNPs. However, if such precise recognition is possible, it would suggest that the parasite ability to differentiate RNPs would decrease when parasitizing closely related species, or for certain highly conserved genes. No evidence exists that parasite host ranges are specifically aimed at targeting the most distantly related species.

There may be an evolutionary benefit to trafficking of nucleic acids into the parasite, such as for horizontal gene transfer (Yoshida *et al.*, 2010). Many parasites lineage including Rafflesiaceae, Orobanchaceae, *Cuscuta*, Mitrastemonaceae, Santalales, and *Pilosyles* (Apodanthaceae) show evidence of HGT events in phylogenetic studies (Table 1). However, the functional significance of most HGT events involving parasites is unclear and a benefit to the parasite has not yet been demonstrated for any gene acquired in this way.

Finally, it is interesting to speculate whether RNAs from the parasite could be used as pathogenic factors in establishing and maintaining host connections. Although an assay for parasite-to-host movement of a *C. pentagona* PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE β subunit mRNA was negative (David-Schwartz R, 2008), the movement of viruses and RNAi signals through parasite bridges from one host into another suggests that bidirectional movement of mRNA is also possible (Birschwilks, M. *et al.*, 2006; Tomilov *et al.*, 2008). If RNA trafficking is important in cell-to-cell communication, it would be reasonable to expect that parasites have evolved a way to use this system to their advantage.

Conclusions and future directions

Parasitic plants that form symplastic connections with their hosts provide a new perspective on cell-to-cell and long-distance trafficking of RNAs. The haustorium creates a union with the host that resembles a graft in many ways, but is established through invasive growth of the parasite and is coordinated with host cells and therefore presents a unique type of junction.

Macromolecules including RNAs, proteins and DNAs, are able to traffic between the plants and the system presents new opportunities for studying RNA movement. Parasitic plants are ultimately pathogens, and the interactions may not be precisely equivalent to the connections

between two cells of the same plant, and it is almost certain that parasitic plants have adapted the normal structure and function of PD and RNA trafficking machinery to meet their needs.

Unraveling the intricacies of the parasite-host interface is likely to contribute to understanding these phenomena in the same way that studies of viruses high-jacking the RNA trafficking system played a major role in elucidating components of PD function (Carrington *et al.*, 1996).

The use of parasitic plants for understanding RNA trafficking will benefit from deeper understanding of haustorial function. The interface between two plants involves coordination at many levels, from structural (shared PD) to signaling. Further research on this interaction will provide new insights into cell-cell interactions

An important feature of the haustorial connection is the ability to connect to diverse host species and this leads to a unique system in which the mobile transcriptome of one species is mixed into that of another. The situation raises intriguing questions about the functions of RNA trafficking in plants. Which RNAs are trafficked, are they targeted specifically, and how do they function at their destination? Are they translated into protein, do they modify gene expression, or are they recycled into raw material for nutrition of the recipient cells? Many of these questions can be addressed using parasitic plants. The ability to readily distinguish host and parasite sequences facilitates identification of mobile transcripts and their movement and fate in the parasite can be tracked using quantitative methods. Next generation sequencing provides the power to reconstruct mobile transcriptomes of hosts from parasite-derived RNA populations.

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References

- Aly, R., Hamamouch, N., Abu-Nassar, J., Wolf, S., Joel, D., Eizenberg, H., Kaisler, E., Cramer, C., Gal-On, A., and Westwood, J. (2011). Movement of protein and macromolecules between host plants and the parasitic weed *Phelipanche aegyptiaca* Pers. *Plant Cell Rep* 30, 2233-2241.
- Atkins, C., Smith, P., and Rodriguez-Medina, C. (2011). Macromolecules in phloem exudates—a review. *Protoplasma* 248, 165-172.
- Ayre, B.G., and Turgeon, R. (2004). Graft transmission of a floral stimulant derived from CONSTANS. *Plant Physiol* 135, 2271-2278.
- Baillaud, L. (1953). Effect of temperature on the period of nutation of the voluble stems of *Cuscuta*. *CR Hebd Acad Sci* 236, 1986-1988.
- Banerjee, A., Chatterjee, M., Yu, Y., Suh, S.-G., Miller, W., and Hannapel, D. (2006). Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. *Plant Cell* 18, 3443-3457.
- Barkman, T.J., Mcneal, J.R., Lim, S.H., Coat, G., Croom, H.B., Young, N.D., and Depamphilis, C.W. (2007). Mitochondrial DNA suggests at least 11 origins of parasitism in angiosperms and reveals genomic chimerism in parasitic plants. *BMC Evol Biol* 7.
- Bennet, C.W. (1944). Studies of dodder transmission of plant viruses. *Phytopathology* 34, 905-932.
- Birschwilks, M., Haupt, S., Hofius, D., and Neumann, S. (2006). Transfer of phloem-mobile substances from the host plants to the holoparasite *Cuscuta* sp. *J Exp Bot* 57, 911-921.
- Birschwilks, M., Sauer, N., Scheel, D., and Neumann, S. (2007). *Arabidopsis thaliana* is a susceptible host plant for the holoparasite *Cuscuta spec.* *Planta* 226, 1231-1241.

- Bombarely, A., Menda, N., Tecle, I.Y., Buels, R.M., Strickler, S., Fischer-York, T., Pujar, A., Leto, J., Gosselin, J., and Mueller, L.A. (2011). The sol genomics network (solgenomics.net): growing tomatoes using Perl. *Nucleic Acids Res* 39, D1149-D1155.
- Borsics, T., and Lados, M. (2002). Dodder infection induces the expression of a pathogenesis-related gene of the family PR-10 in alfalfa. *J Exp Bot* 53, 1831-1832.
- Buhtz, A., Pieritz, J., Springer, F., and Kehr, J. (2010). Phloem small RNAs, nutrient stress responses, and systemic mobility. *BMC Plant Biol* 10, 64.
- Burch-Smith, T.M., and Zambryski, P.C. (2012). Plasmodesmata paradigm shift: regulation from without versus within. *Annu Rev Plant Biol* 63, 239-260.
- Carrington, J.C., Kasschau, K.D., Mahajan, S.K., and Schaad, M.C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8, 1669-1681.
- Chen, M.-H., Tian, G.-W., Gafni, Y., and Citovsky, V. (2005). Effects of calreticulin on viral cell-to-cell movement. *Plant Physiol* 138, 1866-1876.
- Christensen, N.M., Dörr, I., Hansen, M., Van Der Kooij, T.a.W., and Schulz, A. (2003). Development of *Cuscuta* species on a partially incompatible host: induction of xylem transfer cells. *Protoplasma* 220, 131-142.
- Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of arabidopsis. *Science* 316, 1030-1033.
- Crafts, A.S. (1932). Phloem anatomy, exudation, and transport of organic nutrients in cucurbits. *Plant Physiol* 7, 183-225.

- David-Schwartz R, R.S., Townsley B, Machuka J, Sinha N (2008). Long-distance transport of mRNA via parenchyma cells and phloem across the host-parasite junction in *Cuscuta*. *New Phytol* 179, 1133-1141.
- Davis, C., Anderson, W., and Wurdack, K. (2005). Gene transfer from a parasitic flowering plant to a fern. *Proc R Soc Lond B Biol Sci* 272, 2237 - 2242.
- Davis, C.C., and Wurdack, K.J. (2004). Host-to-parasite gene transfer in flowering plants: phylogenetic evidence from Malpighiales. *Science* 305, 676-678.
- Doering-Saad, C., Newbury, H.J., Bale, J.S., and Pritchard, J. (2002). Use of aphid stylectomy and RT-PCR for the detection of transporter mRNAs in sieve elements. *J Exp Bot* 53, 631-637.
- Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. (1967). Mycoplasma- or PLT Group-like Microorganisms Found in the Phloem Elements of Plants Infected with Mulberry Dwarf, Potato Witches' Broom, Aster Yellows, or Paulownia Witches' Broom. *Ann Phytopath Soc Japan* 33, 259-266.
- Dörr, I. (1972). Contact of *Cuscuta* hyphae with sieve tubes of its host plants. *Protoplasma* 75, 167-184.
- Dorr, I., and Kollmann, R. (1995). Symplasmic sieve element continuity between *Orobanche* and its host. *Bot Acta* 108, 47-55.
- Fisher, D.B., and Frame, J.M. (1984). A guide to the use of the exuding-stylet technique in phloem physiology. *Planta* 161, 385-393.
- Fratianne, D.G. (1965). The interrelationship between the flowering of dodder and the flowering of some long and short day plants. *Am J Bot* 52, 556-562.

- Gal-On, A., Naglis, A., Leibman, D., Ziadna, H., Kathiravan, K., Papayiannis, L., Holdengreber, V., Guenoune-Gelbert, D., Lapidot, M., and Aly, R. (2009). Broomrape can acquire viruses from its hosts. *Phytopathology* 99, 1321-1329.
- Gallagher, K.L., and Benfey, P.N. (2009). Both the conserved GRAS domain and nuclear localization are required for SHORT-ROOT movement. *Plant J* 57, 785-797.
- Gaupels, F., Buhtz, A., Knauer, T., Deshmukh, S., Waller, F., Van Bel, A., Kogel, K.-H., and Kehr, J. (2008). Adaptation of aphid stylectomy for analyses of proteins and mRNAs in barley phloem sap. *J Exp Bot* 59, 3297 - 3306.
- Griffitts, A.A., Cramer, C.L., and Westwood, J.H. (2004). Host gene expression in response to Egyptian broomrape (*Orobanche aegyptiaca*). *Weed Sci* 52, 697-703.
- Ham, B.-K., Brandom, J.L., Xoconostle-Cazares, B., Ringgold, V., Lough, T.J., and Lucas, W.J. (2009). A Polypyrimidine Tract Binding Protein, Pumpkin RBP50, Forms the Basis of a Phloem-Mobile Ribonucleoprotein Complex. *Plant Cell* 21, 197-215.
- Hannapel, D.J. (2010). A Model System of Development Regulated by the Long-distance Transport of mRNA. *J Integr Plant Biol* 52, 40-52.
- Harada, T. (2010). Grafting and RNA transport via phloem tissue in horticultural plants. *Sci Hortic-Amsterdam* 125, 545-550.
- Haupt, S., Cowan, G.H., Ziegler, A., Roberts, A.G., Oparka, K.J., and Torrance, L. (2005). Two plant viral movement proteins traffic in the endocytic recycling pathway. *Plant Cell* 17, 164-181.
- Haupt, S., Oparka, K.J., Sauer, N., and Neumann, S. (2001). Macromolecular trafficking between *Nicotiana tabacum* and the holoparasite *Cuscuta reflexa*. *J Exp Bot* 52, 173-177.

- Haywood, V., Yu, T.S., Huang, N.C., and Lucas, W.J. (2005). Phloem long-distance trafficking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development. *Plant J* 42, 49-68.
- Hosford, R.M.J. (1967). Transmission of plant viruses by dodder. *Bot Rev* 42, 387-406.
- Huang, N.-C., and Yu, T.-S. (2009). The sequences of Arabidopsis GA-INSENSITIVE RNA constitute the motifs that are necessary and sufficient for RNA long-distance trafficking. *Plant J* 59, 921-929.
- Hyun, T.K., Uddin, M.N., Rim, Y., and Kim, J.Y. (2011). Cell-to-cell trafficking of RNA and RNA silencing through plasmodesmata. *Protoplasma* 248, 101-116.
- Imlau, A., Truernit, E., and Sauer, N. (1999). Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell* 11, 309-322.
- Irving, L.J., and Cameron, D.D. (2009). You are what you eat: Interactions between root parasitic plants and their hosts. *Adv Bot Res* 50, 87-138.
- Joel, D.M., Losner-Goshen D (1994). "Early host-parasite interaction: models and observations of host root penetration by the haustorium of Orobanche". (Proceedings of the 3rd International Workshop on Orobanche and Related Striga Research: Amsterdam: Royal Tropical Institute).
- Kamińska, M., and Korbin, M. (1999). Graft and dodder transmission of phytoplasma affecting lily to experimental hosts. *Acta Physiol Plant* 21, 21-26.
- Kawakami, S., Watanabe, Y., and Beachy, R.N. (2004). Tobacco mosaic virus infection spreads cell to cell as intact replication complexes. *P Natl Acad Sci USA* 101, 6291-6296.

- Kim, J.Y., Rim, Y., Wang, L., and Jackson, D. (2005). A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. *Gene Dev* 19, 788-793.
- Kim, M., Canio, W., Kessler, S., and Sinha, N. (2001). Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. *Science* 293, 287-289.
- Kragler, F. (2010). RNA in the phloem: A crisis or a return on investment? *Plant Sci* 178, 99-104.
- Kuijt, J. (1983). "Tissue compatibility and the haustoria of parasitic angiosperms," in *Vegetative Compatibility Responses in Plants*, ed. R. Moore. (Waco, TX: Baylor University), 1-12.
- Lee, J.-Y., Yoo, B.-C., Rojas, M.R., Gomez-Ospina, N., Staehelin, L.A., and Lucas, W.J. (2003). Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. *Science* 299, 392-396.
- Lee, K.B. (2007). Structure and development of the upper haustorium in the parasitic flowering plant *Cuscuta japonica* (Convolvulaceae). *Am J Bot* 94, 737-745.
- Li, C., Gu, M., Shi, N., Zhang, H., Yang, X., Osman, T., Liu, Y., Wang, H., Vatish, M., Jackson, S., and Hong, Y. (2011). Mobile FT mRNA contributes to the systemic florigen signalling in floral induction. *Sci Rep* 1.
- Li, C., Zhang, K., Zeng, X., Jackson, S., Zhou, Y., and Hong, Y. (2009). A cis Element within Flowering Locus T mRNA Determines Its Mobility and Facilitates Trafficking of Heterologous Viral RNA. *J Virol* 83, 3540-3548.
- Lough, T., and Lucas, W. (2006). Integrative plant biology: role of phloem long-distance macromolecular trafficking. *Annu Rev Plant Biol* 57, 203 - 232.

- Lu, X.-Y., and Huang, X.-L. (2008). Plant miRNAs and abiotic stress responses. *Biochem Biophys Res Commun* 368, 458-462.
- Lucas, W.J., Bouche-Pillon, S., Jackson, D.P., Nguyen, L., Baker, L., Biao, D., and Hake, S. (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270, 1980-1983.
- Lucas, W.J., Ham, B.-K., and Kim, J.-Y. (2009). Plasmodesmata - bridging the gap between neighboring plant cells. *Trends Cell Biol* 19, 495-503.
- Lucas, W.J., and Lee, J.-Y. (2004). Plasmodesmata as a supracellular control network in plants. *Nat Rev Mol Cell Biol* 5, 712-726.
- Lucas, W.J., Yoo, B.-C., and Kragler, F. (2001). RNA as a long-distance information macromolecule in plants. *Nat Rev Mol Cell Biol* 2, 849-857.
- Marcone, C., Ragozzino, A., and Seemuller, E. (1997). Dodder transmission of alder yellows phytoplasma to the experimental host *Catharanthus roseus* (periwinkle). *Eur J Forest Pathol* 27, 347-350.
- Moore, M.J. (2005). From birth to death: The complex lives of eukaryotic mRNAs. *Science* 309, 1514-1518.
- Mower, J., Stefanovic, S., Hao, W., Gummow, J., Jain, K., Ahmed, D., and Palmer, J. (2010). Horizontal acquisition of multiple mitochondrial genes from a parasitic plant followed by gene conversion with host mitochondrial genes. *BMC Biol* 8, 150.
- Mower, J.P., Stefanovic, S., Young, G.J., and Palmer, J.D. (2004). Plant genetics: gene transfer from parasitic to host plants. *Nature* 432, 165-166.
- Mudge, K., Janick, J., Scofield, S., and Goldschmidt, E.E. (2009). "A History of grafting," in *Horticultural Reviews*. John Wiley & Sons, Inc.), 437-493.

- Park, J.M., Manen, J.F., and Schneeweiss, G.M. (2007). Horizontal gene transfer of a plastid gene in the non-photosynthetic flowering plants *Orobanche* and *Phelipanche* (Orobanchaceae). *Mol Phylogenet Evol* 43, 974-985.
- Parker, C., and Riches, C.R. (1993). *Parasitic weeds of the world: biology and control*. Wallingford, Oxon: CAB International.
- Richardson, A.O., and Palmer, J.D. (2007). Horizontal gene transfer in plants. *J Exp Bot* 58, 1-9.
- Roney, J., Khatibi, P., and Westwood, J. (2007). Cross-species translocation of mRNA from host plants into the parasitic plant dodder. *Plant Physiol* 143, 1037 - 1043.
- Rosin, F.M., Hart, J.K., Horner, H.T., Davies, P.J., and Hannapel, D.J. (2003). Overexpression of a Knotted-like homeobox gene of potato alters vegetative development by decreasing gibberellin accumulation. *Plant Physiol* 132, 106-117.
- Ruiz-Medrano, R., Xoconostle-Cazares, B., and Lucas, W. (1999a). Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* 126, 4405 - 4419.
- Ruiz-Medrano, R., Xoconostle-Cazares, B., and Lucas, W.J. (1999b). Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* 126, 4405-4419.
- Ryan, C.A. (1990). Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annual Review of Phytopathology* 28, 425-449.
- Sharkey, P.J., and Pate, J.S. (1976). Translocation from leaves to fruits of a legume, studied by a phloem bleeding technique: Diurnal changes and effects of continuous darkness. *Planta* 128, 63-72.

- Stegemann, S., and Bock, R. (2009). Exchange of genetic material between cells in plant tissue grafts. *Science* 324, 649-651.
- Swarbrick, P.J., Huang, K., Liu, G., Slate, J., Press, M.C., and Scholes, J.D. (2008). Global patterns of gene expression in rice cultivars undergoing a susceptible or resistant interaction with the parasitic plant *Striga hermonthica*. *New Phytol* 179, 515-529.
- Tomilov, A.A., Tomilova, N.B., Wroblewski, T., Michelmore, R., and Yoder, J.I. (2008). Trans-specific gene silencing between host and parasitic plants. *Plant J* 56, 389-397.
- Turgeon, R., and Wolf, S. (2009). Phloem Transport: Cellular Pathways and Molecular Trafficking. *Annu Rev Plant Biol* 60, 207-221.
- Vachev, T., Ivanova, D., Minkov, I., Tsagris, M., and Gozmanova, M. (2010). Trafficking of the potato spindle tuber viroid between tomato and *Orobanche ramosa*. *Virology* 399, 187-193.
- Van Dorst, H.J.M., and Peters, D. (1974). Some biological observations on pale fruit, a viroid-incited disease of cucumber. *Eur J Plant Pathol* 80, 85-96.
- Vaughn, K.C. (2003). Dodder hyphae invade the host: a structural and immunocytochemical characterization. *Protoplasma* 220, 189-200.
- Vaughn, K.C. (2006). Conversion of the searching hyphae of dodder into xylem and phloem hyphae: A cytochemical and immunocytochemical investigation. *Intl J Plant Sci* 167, 1099-1114.
- Waigmann, E., Lucas, W.J., Citovsky, V., and Zambryski, P. (1994). Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proc Natl Acad Sci U S A* 91, 1433-1437.

- Waigmann, E., and Zambryski, P. (1995). Tobacco mosaic virus movement protein-mediated protein transport between trichome cells. *Plant Cell* 7, 2069-2079.
- Wang, Y., and Ding, B. (2010). Viroids: Small probes for exploring the vast universe of RNA trafficking in plants. *J Integr Plant Biol* 52, 28-39.
- Westwood, J.H., Depamphilis, C.W., Das, M., Fernandez-Aparicio, M., Honaas, L., Timko, M.P., Wafula, E.K., Wickett, N.J., and Yoder, J.I. (2012). The Parasitic Plant Genome Project: new tools for understanding the biology of *Orobanche* and *Striga*. *Weed Sci* 60, 295-306.
- Westwood, J.H., and Foy, C.L. (1998). "Arabidopsis thaliana can be a model host for *Orobanche* research", in: *Current Problems of Orobanche Research, Proceedings of the Fourth International Workshop on Orobanche*. (eds.) K. Wegmann, L.J. Musselman & D.M. Joel. (Albena, Bulgaria: Institute for Wheat and Sunflower "Dobroudja").
- Westwood, J.H., Roney, J.K., Khatibi, P.A., and Stromberg, V.K. (2009). RNA translocation between parasitic plants and their hosts. *Pest Man Sci* 65, 533-539.
- Westwood, J.H., Yoder, J.I., Timko, M.P., and Depamphilis, C.W. (2010). The evolution of parasitism in plants. *Trends Plant Sci* 15, 227-235.
- Xoconostle-Cazares, B., Xiang, Y., Ruiz-Medrano, R., Wang, H.-L., Monzer, J., Yoo, B.-C., Mcfarland, K.C., Franceschi, V.R., and Lucas, W.J. (1999). Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. *Science* 283, 94-98.
- Yoshida, S., Maruyama, S., Nozaki, H., and Shirasu, K. (2010). Horizontal gene transfer by the parasitic plant *Striga hermonthica*. *Science* 328, 1128.

Table 1. Macromolecules demonstrated to transfer between hosts and parasitic plants.

Material	Examples	Parasite	Host(s)	Reference
mRNA	Many	<i>Cuscuta</i>	Tomato Pumpkin Alfalfa	(Roney, J <i>et al.</i> , 2007; David-Schwartz R, 2008)
siRNA	GUS silencing M6PR silencing	<i>Triphysaria</i> <i>Phelipanche</i>	Lettuce Tomato	(Tomilov <i>et al.</i> , 2008) (Huang & Yu, 2009a)
Viruses	Many	<i>Cuscuta</i>		(Hosford, 1967; Birschwilks, M. <i>et al.</i> , 2006)
	ToMV, PVY, TYLCV, CMV HSVd	<i>Phelipanche</i> <i>Cuscuta</i>	Tomato Cucumber	(Gal-On <i>et al.</i> , 2009) (van Dorst & Peters, 1974)
Viroids				
Phytoplasmas	PSTVd Yellows disease	<i>Phelipanche</i> <i>Cuscuta</i>	Tomato Alder to Periwinkle Lily to Periwinkle	(Vachev <i>et al.</i> , 2010) (Marcone <i>et al.</i> , 1997; Kamińska & Korbin, 1999)
Protein	GFP	<i>Cuscuta</i>	Tobacco, <i>Arabidopsis</i>	(Haupt <i>et al.</i> , 2001a; Birschwilks, M. <i>et al.</i> , 2006)
DNA	GFP <i>nad1B-C</i>	<i>Phelipanche</i> Rafflesiaceae	Tomato Vitaceae	(Aly <i>et al.</i> , 2011) (Davis & Wurdack, 2004)
	<i>atp1</i> , <i>atp6</i> , <i>matR</i> <i>atp1</i>	<i>Cuscuta</i> Orobanchaceae, Convolvulaceae	<i>Plantago</i> <i>Plantago</i>	(Mower <i>et al.</i> , 2004; Mower <i>et al.</i> , 2010) (Mower <i>et al.</i> , 2004)
	<i>atp1</i>	<i>Pilostyles</i> Rafflesiaceae <i>Cytinus</i> <i>Mitrastema</i>	Legumes Vitaceae <i>Helianthemum</i> <i>Fagus</i>	(Barkman <i>et al.</i> , 2007)
	<i>rps2</i>	<i>Orobanche</i> and <i>Phelipanche</i>	unknown	(Park <i>et al.</i> , 2007)
	<i>ShContig9483</i> <i>nad1B-C</i> , <i>matR</i>	<i>Striga</i> Santalales	grass <i>Botrychium</i> <i>virginianum</i>	(Yoshida <i>et al.</i> , 2010) (Davis <i>et al.</i> , 2005)

Figure 1

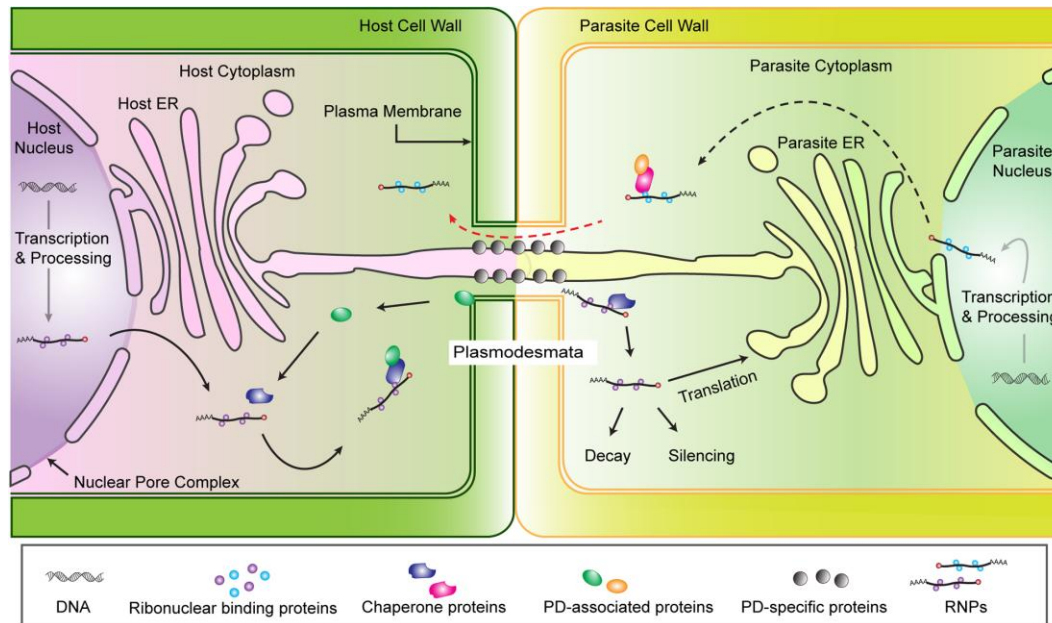


Figure1. Hypothesized model of RNA trafficking from host to *Cuscuta* via plasmodesmata.

Mature RNAs (mRNA, miRNA or siRNA) associate with RNA binding proteins and are targeted for export from the nucleus and post-transcriptional regulation. The ribonuclearprotein complex (RNP) includes the RNA molecule plus proteins that help facilitate RNP export through the nuclear pore complex (NPC) into the cytoplasm (Lucas *et al.*, 2001; Moore, 2005) . The RNPs in the cytoplasm may be translated into protein, degraded or translocated into adjacent cells.

Selective mechanisms for PD transport of non-cell-autonomous proteins (NCAPs) or RNPs proposed by Lucas *et al.* (2001, 2009) suggest chaperone proteins and/or PD-associated proteins carry NCAPs or RNPs to a PD docking protein which dilates the PD channel to transport the RNP to the cytoplasm of neighboring cells (Kim *et al.*, 2005). Nothing is known about the fate of a host RNP after it reaches the parasite cell, but formal possibilities include translation, degradation, or modulation of parasite gene expression (e.g., silencing). The existence of similar

mechanisms that would allow RNP trafficking from parasite to host are expected in parasite cells (Red dashed arrow).

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Chapter 2: Characterization of tomato and *Arabidopsis* mobile RNAs trafficking into the parasitic plant *Cuscuta pentagona*

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SUMMARY

- The cross-species movement of mRNA from hosts to the parasitic plant *Cuscuta pentagona* has been previously reported, but not characterized quantitatively or with attention to uptake patterns and fate of specific mRNAs.
- Real-Time PCR and RNA-Seq approaches were used to identify and characterize mobile transcripts from tomato and *Arabidopsis* hosts into *C. pentagona*.
- Tomato transcripts of Gibberellic Acid Insensitive (*SIGAI*) and Cathepsin D Proteinase Inhibitor (*SIP1*) differed significantly in rate of uptake into the parasite, but were then distributed over the length of the parasite shoot. When parasite shoots were detached from hosts, the *SIP1* transcript concentrations in the parasite showed greatest decrease within the first eight hours. *Arabidopsis* transcripts also varied in mobility into the parasite and assay of

specific regions of a Salt-inducible Zinc Finger Protein (*AtSZFP*) transcript revealed distinct patterns of abundance in the parasite.

- The uptake and distribution of host mRNAs into *C. pentagona* appears to vary among mRNAs, and perhaps even the region of the mRNA under investigation. We propose that mRNAs traffic into the parasite via multiple routes, or that other mechanisms for selective uptake and mobility exist between host and parasite.

Keywords: mRNA trafficking, parasitic plant, *Cuscuta pentagona*, lespedeza dodder, haustorium, Real-time PCR

INTRODUCTION

Cuscuta pentagona (Engelm) is a vining holoparasite of the family Convolvulaceae. The vegetative body of the plant lacks roots or expanded leaves, so consists primarily of a yellow-orange stem that winds around the shoots of host plants (Parker & Riches, 1993a; Press & Graves, 1995). The parasite initiates haustoria at the points of contact with hosts and these specialized organs link the two plants in a physiological continuum that allows translocation of water, nutrients and metabolites into the parasite. In addition to small molecules, a range of macromolecules including RNAs and proteins are mobile into *C. pentagona* (LeBlanc *et al.*, 2012). However, aside from initial reports of RNA mobility, nothing is known about the specifics of trafficking, function, and fate of host RNAs in *C. pentagona*.

The key organ in host-parasite interactions is the haustorium, which penetrates host tissues and forms vascular connections to the host. The *Cuscuta* haustorium develops from specialized cells, termed searching hyphae, which grow between and through host cells, and form parasite-to-host cytoplasmic connections via plasmodesmata (PD) in the chimeric host-parasite cell walls that form during searching hyphae growth (Vaughn, 2003). These PD can be simple or branched and contain desmotubules that traverse the channel (Birschwilks, M. *et al.*, 2006). The searching hyphae that contact host vascular tissue differentiate into xylem or phloem elements, depending on which conductive tissue they contact (Dorr, 1990). Water and dissolved nutrients move freely via xylem connections between the host and parasite, but the nature of the phloem-phloem connections is less well understood. There is strong physiological evidence of a symplastic transfer mechanism (Haupt *et al.*, 2001b; Birschwilks, M. *et al.*, 2006), but no anatomical evidence for direct sieve element connections.

Cell-to-cell and long distance trafficking of RNAs (including mRNA, miRNA and siRNA) appears to form a system for communication in plants that functions in coordinating plant development (Jorgensen, 2002; Lucas & Lee, 2004b; Kruszka *et al.*, 2012). RNA transcripts have been shown to be present in the phloem of *Arabidopsis* (Deeken *et al.*, 2008), *Ricinus* (Doering-Saad *et al.*, 2006), *Cucurbita* (Xoconostle-Cazares *et al.*, 1999) and *Oryza* (Sasaki *et al.*, 1998), and more than 1,000 phloem mobile RNAs were found in cucurbits (Ruiz-Medrano, R. *et al.*, 1999). Long-distance mobile RNAs have been implicated in the regulation of plant development. For example, grafting experiments have shown that tuber formation in potato is regulated by a mobile transcription factor, *StBEL5* (Banerjee *et al.*, 2006; Hannapel, 2010b) and leaf shape is influenced by the phloem mobile mRNA of the *Gibberellic Acid Insensitive* (*GAI*) gene (Haywood *et al.*, 2002).

Movement of RNAs into the phloem stream for long-distance transport is regulated by PD and pore plasmodesmal units between companion cells and sieve elements (Kehr & Buhtz, 2008), and the selective nature of specific long distance mRNA delivery suggests a mechanism other than bulk flow at work (Kragler, 2010b). In order for mRNAs to traffic in the phloem, they must associate in ribonucleoprotein complexes with non-cell autonomous proteins such as the movement proteins (Ham *et al.*, 2009) associated with virus infection that interact with PD to alter size exclusion limits (Wolf *et al.*, 1991; Waigmann *et al.*, 1994a). Mobile RNA-protein complexes have been identified for *StBEL5* (Banerjee *et al.*, 2006) and *KNOTTED-1* (Lucas *et al.*, 1995b).

Previous research has identified several RNAs with host-parasite mobility, including *GAI* and a Cathepsin D Proteinase Inhibitor (PI) (Westwood, James H. *et al.*, 2009), but no quantitative measures of mobile RNA have been made. A non-quantitative assay of pyrophosphate-dependent

phosphofructokinase transcripts suggests that levels are highest near the point of host attachment and decrease until the signal is lost beyond 30 cm away from the point of attachment (David-Schwartz *et al.*, 2008) but nothing is known about the dynamics of RNA movement and degradation. Here we use quantitative Reverse Transcriptase PCR (qRT-PCR) and RNA-seq to study the transfer, distribution, and disappearance of host mRNA in *C. pentagona*. Our data show that host transcript abundance varies among sections of *C. pentagona* over distance and time, and that specific transcripts have different patterns of uptake into the parasite. Additionally, host transcripts disappear over the course of several hours following parasite detachment from hosts. These data suggest that RNA trafficking into parasitic plants is a regulated process that differentiates between specific host mRNAs.

MATERIALS AND METHODS

Plant Material and Tissue Sampling

Tomato (*Solanum lycopersicum*, cv Rutgers) and *Arabidopsis thaliana* (Columbia) were grown in MetroMix 360 potting medium in a 20°C growth room under 12 h or 8 h light cycles, respectively. *Cuscuta pentagona* seeds were scarified in concentrated sulfuric acid for 60 min followed by three rinses with distilled water, and planted in the medium at the base of two-week old tomato seedlings or four-week old *Arabidopsis* seedlings. When necessary, the parasite stems were encouraged to attach to the host by placing growing tips near main shoots of the host. Established *C. pentagona* with at least two coils around healthy host stems were used in these studies.

For experiments on long distance translocation of host mRNA, stems of *C. pentagona* growing on tomato, each approximately 30 cm long, were harvested by cutting at the site of attachment and wiped with 100% ethanol to remove any surface host DNA. Three 30 cm stems were cut into 10 cm sections representing host and parasite stem at the attachment site, first 10 cm of parasite stem, the next 10 cm, and the final 10 cm including the tip of the parasite (Fig. 1a). Similar regions were pooled and RNA was extracted. All samples were replicated and the entire experiment was repeated multiple times with consistent results.

For the experiment on shorter distance distribution of host RNAs in the parasite, young, unbranched stems grown on tomato were cut into 2.5 cm sections representing the host-parasite connection region, the basal portion of *C. pentagona* stem toward the main body of the parasite, and four sequential sections of the parasite leading to its shoot tip (Fig. 2a). Each tissue was pooled in groups of three for RNA extraction and each tissue extraction and assay was replicated three times.

Disappearance of host RNA in the parasite was analyzed by removing shoots of *C. pentagona* from 8-wk old tomato hosts, then further subdivided as necessary to create uniform sections averaging 5 cm in length. Sections were randomly assigned to treatment groups consisting of about 0.15 g tissue for each replicate of each time point, covered with foil and placed in the dark for 0, 4, 8 and 24 h prior to RNA extraction. All treatments were replicated three times and the entire experiment was repeated.

For characterization of *Arabidopsis* transcripts, triplicate samples were harvested from the parasite alone near the point of haustorial attachment (10-15 cm long sections of *C. pentagona*

stem), the region of parasite-host haustorial attachment (up to 3 cm long), and the host bolt just above the attachment site (3 cm) (Fig. 4a).

DNA and RNA Extraction

DNA was extracted using the CTAB method (Doyle & Doyle, 1987) with approximately 0.2 g of tomato, *Arabidopsis* or *C. pentagona* tissue disrupted under liquid nitrogen. DNA samples were stored at -20°C until use. RNA was extracted using RNeasy Plant Kit (Qiagen) modified to start from material ground in sterile micro glass tissue grinders in a minimum of 450 µl of Buffer RLT for 0.05 g of tissue. All RNA was treated with RNase-free DNase (Qiagen) to remove DNA and stored at -80°C until assayed.

PCR and qRT-PCR

Quantification of tomato transcripts (*SIPI* and *SIGAI*) as well as the *C. pentagona* putative Heat Shock Protein 70 (*CpHSP*) was done using custom TaqMan® Gene Expression Assays (Applied Biosystems) probes and the TaqMan® One-Step RT-PCR kit (Applied Biosystems). Primers and probes for each gene are given in Table 1.

For use as an endogenous parasite reference gene, a homolog to *Ipomoea nil* (Japanese morning glory) chloroplast heat shock protein 70 (HSP70, EU409605.1) was identified in *C. pentagona* sequencing data. The region targeted by the TaqMan primer/probe set showed 56% identity in amino acid sequence to this protein (ABZ04080.1). Tests of the primer/probe set showed stable expression across seedling, mature attachment, 24 hr-post detachment stem, and flower tissues of *C. pentagona*.

Transcripts mobile from *Arabidopsis* into *C. pentagona* were identified using next-generation sequencing of RNA from *Arabidopsis*, host-parasite interface, and *C. pentagona* tissues as

described above (Fig. 4a). Two independent biological replicates of each set of tissues were sequenced using the Illumina Genome Analyzer IIx for one replicate and HiSeq 2000 for the second. In all, 1.3 billion 75- and 100-bp reads were generated and host reads in the parasite were identified by mapping them to the *Arabidopsis* cDNA reference (TAIR10) allowing 2 mismatches. Expression of the mobile transcripts were normalized using Reads/Kilobase of exon model/Million mapped reads (RPKM) (Mortazavi *et al.*, 2008)

Three genes with mobile transcripts from *Arabidopsis* into *C. pentagona* were selected for further characterization: *Auxin Response Family (AtARF)*, *Translationally Controlled Tumor Protein (AtTCTP)*, and *Salt inducible Zinc Finger Protein (AtSZFP)*. The primer sequences and annealing temperatures used for qRT-PCR are summarized in Table 1. Specificity of primers for *Arabidopsis* mobile transcripts was conducted on *Arabidopsis* genomic DNA using GoTaq® Green PCR Mastermix (Promega, Inc.). Quantification of transcripts was conducted on cDNA made from RNA using the High Capacity cDNA RT-PCR kit (Applied Biosystems, Foster, CA, USA). Quantitative PCR of three biological replicates from each was carried out in an ABI 7300 System (Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems). Standard curves with dilutions of 10^4 to 10^8 copies were generated with $R^2 > 0.97$ for each assay. Absolute quantities of transcripts were calculated per 100 ng of starting cDNA from the average of two technical replicates.

Statistical Analysis

All extractions were performed in triplicate. Mean separation for short distance mobility and decay qRT-PCR results was carried out using Tukey-Kramer HSD test performed in JMP (SAS

Institute, Inc. Cary, NC, USA). Data for characterization of *Arabidopsis* transcripts were log transformed to normalize variance prior to statistical analysis.

RESULTS

Long distance movement of host RNA in the parasite

Previous work described mobility of 21 RNAs from tomato into *C. pentagona* (Roney, JK *et al.*, 2007; Westwood, James H. *et al.*, 2009). We selected two candidates for further characterization based on either known phloem mobility of homologs in other systems (*SIGAI*), or high expression in the host-parasite system (*SIPI*). Transcript copy numbers for these were determined in consecutive 10 cm sections of *C. pentagona* growing on tomato and in the tomato stem at the point of haustorial attachment (Fig. 1a).

Transcript levels of *SIPI* and *SIGAI* determined through qRT-PCR were highest in the regions of host-parasite connection and lower in the parasite shoot (Fig. 1b). Levels of *SIPI* and *SIGAI* differed from each other, both in overall magnitude and in the number of transcripts found in *C. pentagona* shoots compared to the host tissue. An average of nearly 520,000 transcripts/ μ g RNA of *SIPI* were found in the host and 168,000 transcripts/ μ g RNA in the first 10 cm of parasite stem, an approximately three-fold decrease. In contrast, 7,700 transcripts/ μ g RNA of *SIGAI* were found in the host and less than 15 transcripts/ μ g RNA in the first 10 cm of parasite, a negative fold change of more than 500. Host RNA transcript numbers appear to be highest in the parasite near the point of host attachment and lowest in the middle of the parasite stem (undetected for *SIGAI* transcripts), although no statistical differences separate the 20 cm from 30 cm sections of *SIPI*. An endogenous *C. pentagona* gene, *CpHSP*, shows constant expression

levels over the length of the parasite stem and indicates uniform extraction of RNA from the tissues.

Specific distribution of SIPI in the C. pentagona stem

The *SIPI* transcript was selected for more detailed characterization based on its high level of mobility into the parasite. RNA was extracted from *C. pentagona* shoot branches of uniform development consisting of a relatively new attachment point (minimum of two coils around host stem) and a growing shoot of about 10 cm (Fig. 2a). *SIPI* transcript levels were again highest in the host-parasite connection tissue, with neighboring sections of parasite in both basal and apical directions having decreased transcript levels approximately five-fold less than that in the host stem (Fig. 2b). Transcript levels in the *C. pentagona* shoot from the attachment point to the apical tip had a declining trend with the furthest section statistically different from the rest of the stem.

Host RNA Stability in the parasite

In order to measure the stability of host RNA in the parasite, *C. pentagona* stems were disconnected from tomato hosts and held in the dark for 0, 4, 8 and 24 h time periods, followed by RNA extraction and quantification of *SIPI* and *CpHSP*. After severing from the host there is a significant decrease in the number of *SIPI* transcripts, with the quantity halved in the first 4 h, and further reduced by 8 h (Fig. 3). A low level of *SIPI* transcripts were still detected at 24 h post detachment. The *C. pentagona* stem has a thick cuticle and may survive for several days after being severed from a host. Nevertheless, to account for potential parasite senescence, *CpHSP* expression was used as an indicator of parasite viability and a measure of RNA integrity, and the

SlPI levels were normalized to their 0 h levels. *CpHSP* transcript levels were unchanged or slightly elevated over the 24 h course of the experiment.

Transcriptome analysis identifies mobile RNAs from Arabidopsis

Previous work has used microarrays to identify host transcripts in the parasite (Roney, JK *et al.*, 2007). With next generation sequencing technologies it is now possible to rapidly screen for mobile RNAs in a host-parasite system. RNA from *Arabidopsis*, *C. pentagona*, and the host-parasite interface tissues (Fig. 4a) were sequenced using Genome Analyzer IIX and HiSeq 2000 platforms. Host RNAs that had moved into parasite tissues were identified by mapping back to *Arabidopsis* cDNA. Based on this analysis, three *Arabidopsis* transcripts that were abundant in parasite tissue, *AtSZF1*, *AtARF* and *AtTCTP*, were selected for further characterization and *Arabidopsis* gene-specific primers were developed and validated (Data not shown). The movement of mRNA from these genes into the parasite was then confirmed by RT-PCR showing that product amplification occurs from RNAs derived from *Arabidopsis* and *C. pentagona* grown on *Arabidopsis*, but not from tomato or *C. pentagona* grown on tomato (Fig. 4b).

Transcript levels were quantified by qRT-PCR on RNA extracted from host, interface or parasite tissues, with highest levels always occurring in the pure host tissue (Fig. 4c-e). Fewer transcripts were found in interface regions, and fewer still in the parasite tissue, with the exception of *AtSZF1*. The decreasing transcript levels across these tissues are consistent with decreasing proportions of host RNA in the tissues. Another measure of transcript abundance is the number of reads generated by the Illumina sequencing. Read counts mapped to *AtSZF1*, *AtARF* and *AtTCTP* from GAIIX and HiSeq 2000 generally agree with each other and the qRT-PCR data (Fig. 4c-e).

The Illumina data provides additional insight because the expression of transcripts can be normalized to the total number of host transcripts using data expressed as RPKM (Reads Per Kilobase per Million mapped reads) (Mortazavi *et al.*, 2008). This avoids the issue of host RNA dilution by parasite RNA and reflects the relative levels of specific host transcripts as a proportion of the total host transcriptome (in the host tissue) or the mobile host transcriptome (in the parasite tissue). Analyzed in this way, the proportion of *AtTCTP* appears to remain constant across the three different tissues (from 3,000 to 6,000 RPKM), while *AtARF* and *AtSZF1* occur at higher proportions in the interface and parasite tissues than in the host tissue (Fig. 5). Additional biological replications would be needed to provide strong statistical support for these conclusions, but K-means clustering for all transcripts for each tissue results in a number of patterns for genes with similar expression profiles, two of which are exemplified by these transcripts (data not shown).

Discrete regions of single host transcript detected differentially in parasite

To determine whether different sections of a single transcript behaved in the same manner, three separate primer sets were developed that correspond to distinct sections of the *AtSZF1* transcript spanning varying regions of paired-end read coverage (light blue bars, Fig. 6a). Absolute quantification of these transcript sections (Fig. 4a) showed consistent levels of all three sections in the host and host-parasite interface tissues. However, the levels of the three sections varied in the parasite tissue, with significantly higher transcript abundance of *AtSZF1-a* compared to *AtSZF1-b* or *AtSZF1-c*, with differences spanning two orders of magnitude between highest and lowest levels (Fig. 6b). Sequencing data can show irregularities in read coverage over the length of a transcript, however, these results show that there may be regions of a given transcript that

are more or less detectable, but that sequencing data irregularities are more to do with library construction and not indicative of biological variation.

DISCUSSION

Host transcripts show quantitative differences in mobility into C. pentagona

This study quantified movement of five host transcripts into *C. pentagona*. The tomato transcripts *SIPI* and *SIGAI* differed substantially in their overall levels of expression and in the proportion of total mapped reads that entered into the parasite (Fig. 1). The *SIPI* transcripts move in greater abundance from tomato into the parasite, in contrast to *SIGAI* transcripts that were detectable at levels over two orders of magnitude lower in the parasite. These differences in mobility suggest different routes of movement or other regulation of trafficking and are interesting considering that *SIGAI* has been documented to be phloem mobile (Ruiz-Medrano, R. *et al.*, 1999; Haywood, Valerie *et al.*, 2005). Once inside the parasite, copies of the *SIPI* transcript were detected along the length of a 30-cm stem and did not differ significantly in concentration. The *SIGAI* transcript was not detected in the middle section of the dodder stem, but was detected in the apical section, supporting the idea that accumulation of mobile transcript in the meristem region is possible (Haywood, Valerie *et al.*, 2005). However, *SIGAI* was the lowest expressed transcript studied and more detailed analysis of *SIPI* suggested steady or decreasing transcript numbers towards the tips of young *C. pentagona* shoots (Fig. 2). Further evidence that transcripts differ in trafficking dynamics was provided by the transcripts from *Arabidopsis*. *AtTCTP*, *AtARF*, and *AtSZF1* displayed differing rates of uptake as measured by qRT-PCR and by RPKM data from RNA-seq analysis (Figs. 4 and 5).

The biological basis for differential transcript movement from host to parasite is unknown. It could be a product of the nature of the haustorial connections, with mRNAs that would normally only move intercellularly short distances being taken up into the parasite phloem and trafficked long distances. Finding host *TCTP* and *GAI* transcripts in the attached *C. pentagona* supports phloem transfer as both have been shown to associate with phloem proteins and be transported in the phloem (Ruiz-Medrano, R. *et al.*, 1999; Aoki *et al.*, 2005; Haywood, Valerie *et al.*, 2005; Berkowitz *et al.*, 2008a; Huang & Yu, 2009b). Similar transfer of host macromolecules into the phloem stream of *Cuscuta* has been demonstrated (Birschwilks, M. *et al.*, 2006). However, phloem transfer may not be the only route – or the most efficient route – of movement into the parasite.

It is not known whether transcripts of *ARF*, *SZF1* and *PI* are loaded into phloem, but it is also possible that they may enter the parasite due to sheer abundance of their production in host parenchyma cells connected to the parasite haustoria through PD. In terms of function, *ARF* is a growth-responsive gene (Neuteboom *et al.*, 1999; Cai & Lashbrook, 2008), *SZF1* a transcription factor (Laity *et al.*, 2001), and *PI* a defense gene (Orr *et al.*, 1994; Leplé *et al.*, 1995; Lisón *et al.*, 2006). *PI* is induced in host cells in the region of haustoria ingress (data not shown), and may “spill over” into haustorial cells through joint PD. This phenomenon has been suggested with the small subunit of RuBisCO, which has been considered to be non-phloem mobile but was detected by RT-PCR in *C. pentagona* haustoria cells (David-Schwartz *et al.*, 2008). When *AtARF* and *AtSZF1* numbers are expressed on an RPKM basis, they indicate induced expression in the host-parasite interface tissue and proportionate flow into the parasite (Fig. 5).

Host transcript fate in the parasite

A key to understanding the functional significance of host mRNAs in the host-parasite interaction is determining the fate of host transcripts in the parasite. Possible fates include translation into protein, targeted degradation, or some other process of RNA turnover. Studies of *SLPI* transcript persistence in detached parasite stems showed that host transcript numbers declined over the first 8 hours, but were still detectable 24 h after detachment (Fig. 3). This was not due to senescence-associated RNA decay because the native *CpHSP70* transcripts remained constant or even increased during the span of the experiment. The lifetime of mRNA in eukaryotic cells can be as short as 20 minutes or as long as 24 hours (Aloni, 1987), so host transcripts fit within this broad range. The disappearance of *SLPI* transcripts appears to be biphasic, with an initial period of decay showing a half-life of about 4 hours, but some transcript persists. It is possible that translocation and unloading of phloem contents in *C. pentagona* is greatly reduced several hours after being severed from the host and the remaining RNA in the phloem is protected from decay.

Additional insight into transcript fate comes from the analysis of *AtSZF1* using three primer sets specific to different regions of the mRNA. The *AtSZF1-a* transcript, located near the 3' end, was detected most in the parasite, so that may be the most stable part of the molecule or it may reflect a region of mRNA protection by an RNA binding protein (Fig. 6). Primer sets designed for other parts of the transcript were found at lower abundance and suggests that specific regions of the mRNA are selectively degraded in the parasite. The three segments were present at similar levels in the host and host-parasite interface tissues, suggesting that the degradation process is specific to the transcripts in the parasite. mRNA decay in plants is not as well understood as it is in fungal and mammalian organisms, but dynamic RNA turnover events are necessary for maintaining rapid responses to developmental cues (Belostotsky, 2008). Most non-defective mRNA

degradation pathways are deadenylation dependent and proceed in the 5'-to-3' direction; however, endosomal degradation is common for small RNA decay by RISC proteins. The quantification patterns for *AtSZF1-a* indicate preservation of the 3'-end, with some type of decay occurring at the 5'-end (*AtSZF1-b*) and toward the mid portion (*AtSZF1-c*). The cause(s) of transcript disappearance is the subject of ongoing and future experiments.

These data indicate that primer location is important in assaying transcript abundance and that caution is needed in interpreting results from only one primer set. The RNA-seq data, in principle, should give an indication of transcript degradation in the parasite based on counts of reads over the length of the transcript (Fig. 6a). However, Illumina sequencing results in differential efficiency of sequencing across mRNAs that result in uneven sequencing depths (Oyola *et al.*, 2012).

Next generation sequencing can identify mobile RNA in chimeric system

Our work shows the efficacy of next-generation sequencing to identify host transcripts moving into the parasite, as host sequences are generally easy to identify due to their distinct genetic differences from the parasite. The agreement between Illumina sequencing results and qPCR results were generally good, with the exception of the interface samples of *AtSZFP1* and *AtTCTP* (Fig. 4). This is especially remarkable given the high degree of biological variation in host-parasite connections and the methodological differences between the techniques.

One significant advantage of Illumina sequencing is the ability to accurately divide mixed host-parasite transcriptomes into distinct host and parasite groups. qRT-PCR, in contrast, is expressed in terms of total RNA concentrations that cannot distinguish host from parasite. The Illumina

method enables expression of data as RPKM and is useful for understanding the level of a host transcript in the interface or parasite as a proportion of the host transcriptome from that tissue. For example, Figure 5 shows that *AtTCTP* transcripts were nearly equally abundant as a proportion of the host RNA populations in each tissue, while *AtSZF1* and *AtARF* are less abundant in the pure host than in the interface and parasite tissues. This provides insight that is not easily obtained using qRT-PCR.

Future Work

The data presented here suggest that trafficking and fate of host RNA into parasites is complex, with specific transcripts moving by different routes or different mechanisms of regulation. More work is needed to understand precise mechanisms of transcript exchange and functional significance of these transcripts in the parasite. Two potentially important tools are based on host-parasite mobility of RNAi signals. One is a control strategy for parasitic weeds using RNA silencing signals transmitted from hosts into parasites (Yoder *et al.*, 2009). Another uses the same technique as a research tool to study *Cuscuta* genes by specific silencing via the host (Alakonya *et al.*, 2012). Both techniques will benefit from greater understanding of the mechanisms regulating host-parasite RNA movement.

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References

- Alakonya A, Kumar R, Koenig D, Kimura S, Townsley B, Runo S, Garces HM, Kang J, Yanez A, David-Schwartz R, Machuka J, Sinha N. 2012.** Interspecific RNA Interference of SHOOT MERISTEMLESS-Like Disrupts *Cuscuta pentagona* Plant Parasitism. *Plant Cell*.
- Aloni R 1987.** The Induction of Vascular Tissues by Auxin. In: Davies P ed. *Plant Hormones and their Role in Plant Growth and Development*: Springer Netherlands, 363-374.
- Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H. 2005.** Destination-Selective Long-Distance Movement of Phloem Proteins. *Plant Cell* **17**(6): 1801-1814.
- Banerjee AK, Chatterjee M, Yu Y, Suh S-G, Miller WA, Hannapel DJ. 2006.** Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. *Plant Cell* **18**(12): 3443-3457.
- Belostotsky DA. 2008.** State of Decay: An Update on Plant mRNA Turnover. *Nuclear Pre-Mrna Processing in Plants* **326**: 179-199.
- Berkowitz O, Jost R, Pollmann S, Masle J. 2008.** Characterization of TCTP, the Translationally Controlled Tumor Protein, from *Arabidopsis thaliana*. *Plant Cell* **20**(12): 3430-3447.
- Birschwilks M, Haupt S, Hofius D, Neumann S. 2006.** Transfer of phloem-mobile substances from the host plants to the holoparasite *Cuscuta* sp. *Journal of Experimental Botany* **57**(4): 911-921.
- Cai S, Lashbrook CC. 2008.** Stamen Abscission Zone Transcriptome Profiling Reveals New Candidates for Abscission Control: Enhanced Retention of Floral Organs in Transgenic

- Plants Overexpressing Arabidopsis ZINC FINGER PROTEIN2. *PLANT PHYSIOLOGY* **146**(3): 1305-1321.
- David-Schwartz R, Runo S, Townsley B, Machuka J, Sinha N. 2008.** Long-distance transport of mRNA via parenchyma cells and phloem across the host-parasite junction in *Cuscuta*. *New Phytologist* **179**(4): 1133-1141.
- Deeken R, Ache P, Kajahn I, Klinkenberg J, Bringmann G, Hedrich R. 2008.** Identification of Arabidopsis thaliana phloem RNAs provides a search criterion for phloem-based transcripts hidden in complex datasets of microarray experiments. *The Plant Journal* **55**(5): 746-759.
- Doering-Saad C, Newbury H, Couldridge C, Bale J, Pritchard J. 2006.** A phloem-enriched cDNA library from Ricinus: insights into phloem function. *Journal of Experimental Botany* **57**(12): 3183-3193.
- Dorr I. 1990.** Sieve Elements in haustoria of parasitic angiosperms. In H-D Behnke, R-D Sjölund, eds, *Sieve Elements: Comparative Structure, Induction and Development*. Berlin: Springer.
- Doyle J, Doyle J. 1987.** A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**(1): 11-12.
- Ham B-K, Brandom JL, Xoconostle-Cazares B, Ringgold V, Lough TJ, Lucas WJ. 2009.** A Polypyrimidine Tract Binding Protein, Pumpkin RBP50, Forms the Basis of a Phloem-Mobile Ribonucleoprotein Complex. *Plant Cell* **21**(1): 197-215.
- Hannapel DJ. 2010.** A Model System of Development Regulated by the Long-distance Transport of mRNA. *Journal of Integrative Plant Biology* **52**(1): 40-52.

- Haupt S, Oparka KJ, Sauer N, Neumann S. 2001.** Macromolecular trafficking between *Nicotiana tabacum* and the holoparasite *Cuscuta reflexa*. *Journal of Experimental Botany* **52**(354): 173-177.
- Haywood V, Kragler F, Lucas WJ. 2002.** Plasmodesmata: Pathways for Protein and Ribonucleoprotein Signaling. *Plant Cell* **14**(90001): S303-325.
- Haywood V, Yu T-S, Huang N-C, Lucas WJ. 2005.** Phloem long-distance trafficking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development. *The Plant Journal* **42**(1): 49-68.
- Huang N-C, Yu T-S. 2009.** The sequences of Arabidopsis GA-INSENSITIVE RNA constitute the motifs that are necessary and sufficient for RNA long-distance trafficking. *The Plant Journal* **59**(6): 921-929.
- Jorgensen RA. 2002.** RNA traffics information systemically in plants. *Proceedings of the National Academy of Sciences* **99**(18): 11561-11563.
- Kehr J, Buhtz A. 2008.** Long distance transport and movement of RNA through the phloem. *Journal of Experimental Botany* **59**(1): 85-92.
- Kragler F. 2010.** RNA in the phloem: A crisis or a return on investment? *Plant Science* **178**(2): 99-104.
- Kruszka K, Pieczynski M, Windels D, Bielewicz D, Jarmolowski A, Szweykowska-Kulinska Z, Vazquez F. 2012.** Role of microRNAs and other sRNAs of plants in their changing environments. *Journal of Plant Physiology* **169**(16): 1664-1672.
- Laity JH, Lee BM, Wright PE. 2001.** Zinc finger proteins: new insights into structural and functional diversity. *Current Opinion in Structural Biology* **11**(1): 39-46.

- LeBlanc ML, Kim G, Westwood JH. 2012.** RNA trafficking in parasitic plant systems. *Frontiers in Plant Science* 3.
- Lep   JC, Bonad  -Bottino M, Augustin S, Pilate G, L   T  n VD, Delplanque A, Cornu D, Jouanin L. 1995.** Toxicity to *Chrysomela tremulae* (Coleoptera: Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. *Molecular Breeding* 1(4): 319-328.
- Lis  n P, Rodrigo I, Conejero V. 2006.** A Novel Function for the Cathepsin D Inhibitor in Tomato. *PLANT PHYSIOLOGY* 142(3): 1329-1339.
- Lucas WJ, Bouch  -Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S. 1995.** Selective Trafficking of KNOTTED1 Homeodomain Protein and Its mRNA Through Plasmodesmata. *Science* 270(5244): 1980-1983.
- Lucas WJ, Lee J-Y. 2004.** Plasmodesmata as a supracellular control network in plants. *Nature Reviews Molecular Cell Biology* 5(9): 712-726.
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. 2008.** Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods* 5(7): 621-628.
- Neuteboom LW, Ng JMY, Kuyper M, Clijdesdale OR, Hooykaas PJJ, van der Zaal BJ. 1999.** Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation. *Plant Molecular Biology* 39(2): 273-287.
- Orr GL, Strickland JA, Walsh TA. 1994.** Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. *Journal of Insect Physiology* 40(10): 893-900.
- Oyola SO, Otto TD, Gu Y, Maslen G, Manske M, Campino S, Turner DJ, Macinnis B, Kwiatkowski DP, Swerdlow HP, Quail MA. 2012.** Optimizing Illumina next-

- generation sequencing library preparation for extremely AT-biased genomes. *BMC Genomics* **13**: 1.
- Parker C, Riches CR. 1993.** *Parasitic weeds of the world : biology and control*. Wallingford, Oxon: CAB International.
- Press MC, Graves J. 1995.** *Parasitic plants*. London: Chapman & Hall.
- Roney JK, Khatibi PA, Westwood JH. 2007.** Cross-Species Translocation of mRNA from Host Plants into the Parasitic Plant Dodder. *PLANT PHYSIOLOGY* **143**(2): 1037-1043.
- Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ. 1999.** Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* **126**(20): 4405-4419.
- Sasaki T, Chino M, Hayashi H, Fujiwara T. 1998.** Detection of Several mRNA Species in Rice Phloem Sap. *Plant and Cell Physiology* **39**(8): 895-897.
- Vaughn KC. 2003.** Dodder hyphae invade the host: a structural and immunocytochemical characterization. *Protoplasma* **220**(3-4): 189-200.
- Waigmann E, Lucas WJ, Citovsky V, Zambryski P. 1994.** Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proceedings of the National Academy of the Sciences* **91**(4): 1433-1437.
- Westwood JH, Roney JK, Khatibi PA, Stromberg VK. 2009.** RNA translocation between parasitic plants and their hosts. *Pest Management Science* **65**(5): 533-539.
- Wolf S, Deom CM, Beachy R, Lucas WJ. 1991.** Plasmodesmatal Function Is Probed Using Transgenic Tobacco Plants That Express a Virus Movement Protein. *Plant Cell* **3**(6): 593-604.

Xoconostle-Cazares B, Xiang Y, Ruiz-Medrano R, Wang H-L, Monzer J, Yoo B-C, McFarland KC, Franceschi VR, Lucas WJ. 1999. Plant Paralog to Viral Movement Protein That Potentiates Transport of mRNA into the Phloem. *Science* **283**(5398): 94-98.

Yoder JI, Gunathilake P, Wu B, Tomilova N, Tomilov AA. 2009. Engineering host resistance against parasitic weeds with RNA interference. *Pest Management Science* **65**(5): 460-466.

Table 2. Genes of interest.

Name	Organism	Transcript name	Accession #	Sequence	T _m	Product (bp)
SIPI	<i>Solanum lycopersicum</i>	Cathepsin D Proteinase Inhibitor	AJ295638	F: 5'-CACGATCCGAATTGTAACGAAACA-3'	*	*
				R: 5'-GGTGAGTCCCCGAATTCAGATG-3'		
				Probe: 5'-CCCTTGTCCAAATGGTG-3'		
SIGAI	<i>Solanum lycopersicum</i>	Gibberellic Acid Insensitive	NM_001247436	F: 5'-TCCGCCTGGAAGCTATTGA-3'		
				R: 5'-CCACCCACCCGAGTTTACA-3'		
				Probe: 5'-ACTCGACTTCTCCGGCGCC-3'		
CpHSP	<i>Cuscuta pentagona</i>	Heat Shock Protein	KC854407	F: 5'-CCGAGACCGGAGCAATACTTG-3'		
				R: 5'-GAGCAATCGGACGTTTTTCGTT-3'		
				Probe: 5'-CCAAGAGCAATTTC-3'		
AtARF	<i>Arabidopsis thaliana</i>	Auxin Response Family	AT5G35735	F: 5'-AAATGCGGTCAGCTGGGGAGT-3'	64.5	140
				R: 5'-ACCATGTTGGATCGGCGAAGACT-3'		
AtTCTP	<i>Arabidopsis thaliana</i>	Translationally Controlled Tumor Protein	AT3G16640.1	F: 5'-GCGGAGGCAACGTCTCTTGGG-3'	60	163
				R: 5'-TCGCTGCGACCGACCACAGT-3'		
AtSFP1-a	<i>Arabidopsis thaliana</i>	Salt inducible Zinc Finger Protein 1	AT3G55980	F: 5'-ACTCCACCGGCTTTCAGCTC-3'	66	110
				R: 5'-TGCCAAAACCGCGGAGTCTCA-3'		
AtSFP1-b	<i>Arabidopsis thaliana</i>	Salt inducible Zinc Finger Protein 1	AT3G55980	F: 5'-TCCTCGAATTCGCTGCTTG-3'	62	84
				R: 5'-ACCCTGACTCATCCAAATCAAGCCC-3'		
AtSFP1-c	<i>Arabidopsis thaliana</i>	Salt inducible Zinc Finger Protein 1	AT3G55980	F: 5'-TGTGAACAGAGCTTGTGGTGA-3'	62	61
				R: 5'-ACAGCCAGCAACAGCACAGTGA-3'		

* T_m and product length determined by TaqMan® probe design (Applied Biosystems).

Figures

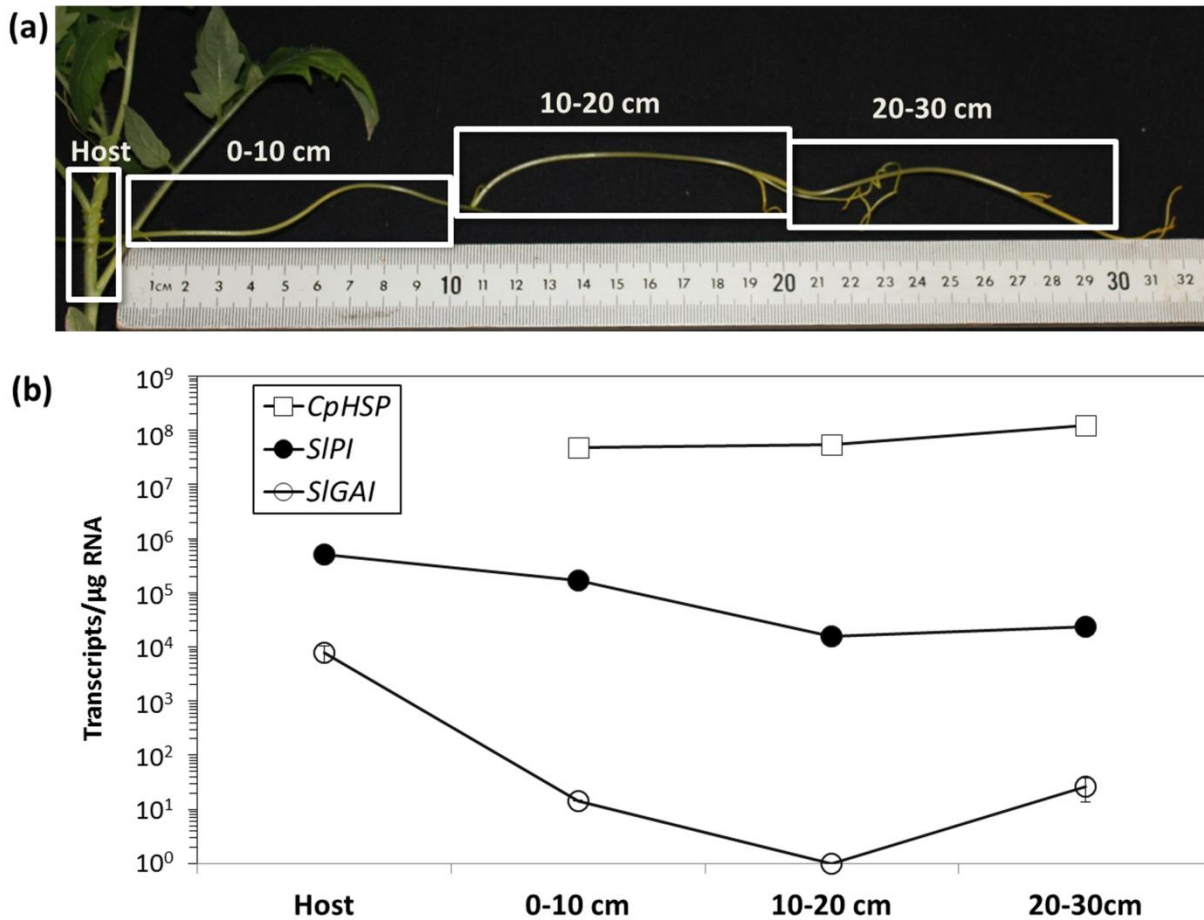


Fig. 1 Quantification of RNA transcripts in tomato host and along a 30 cm length of *C. pentagona* attached to host. (a) Illustration of harvesting scheme consisting of host and parasite tissues at the point of connection (host) and consecutive 10-cm sections of *C. pentagona*. (b) Copies of tomato RNAs Cathepsin D Proteinase Inhibitor (*SIPI*) and Gibberellic acid insensitive (*SIGAI*) expression. A *C. pentagona* Heat Shock Protein, *CpHSP*, is included for comparison. Data are means $\pm$ SE from three biological replicates.

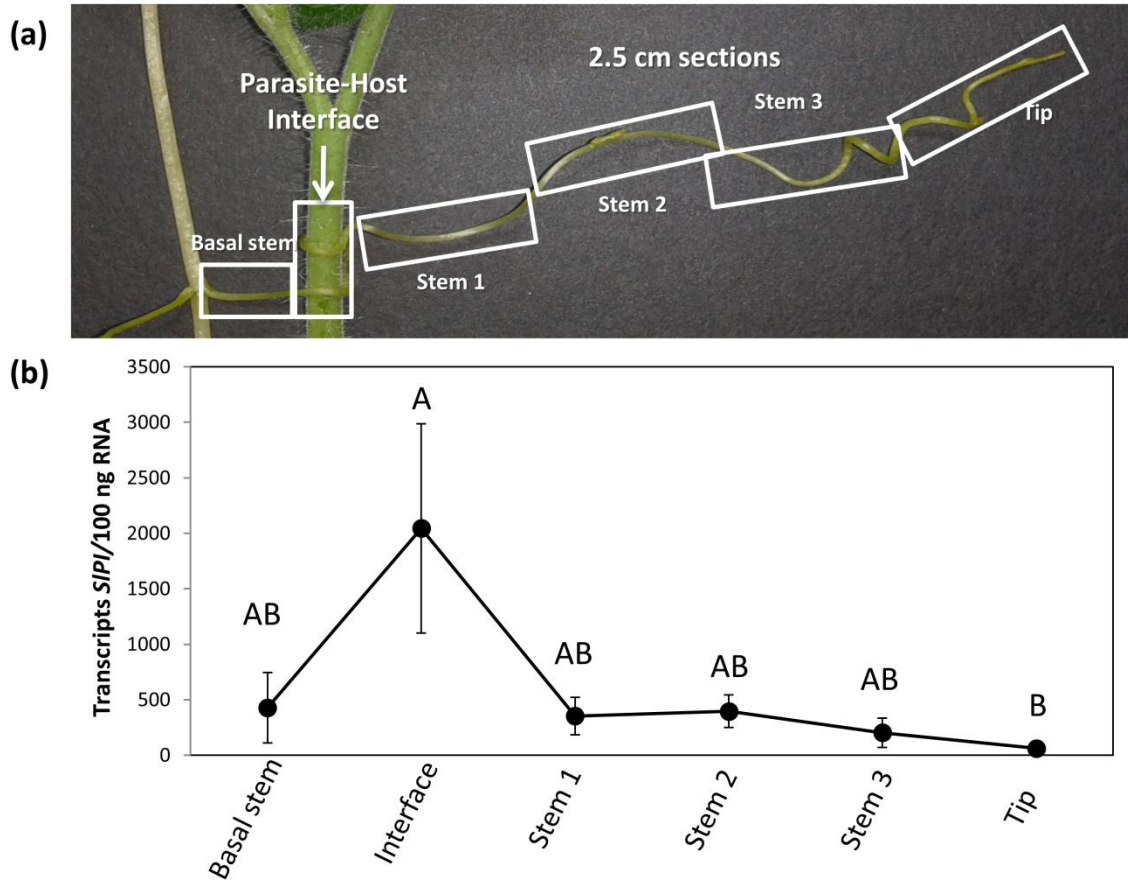


Fig. 2 Tomato transcript detection in basal and apical *C. pentagona* stems. (a) Extraction sampling from branched *C. pentagona* parasitizing tomato for detecting RNA flow in juvenile and mature tissue. (b) Copies of tomato Cathepsin D Proteinase Inhibitor (*SIPI*) were highly abundant in the interface and decreased in the apical growth of the parasite as the distance from the haustorium increased. Data points are copies detected per ng RNA $\pm$ SE of three biological replicates with significance calculated by Tukey-Kramer HSD test.

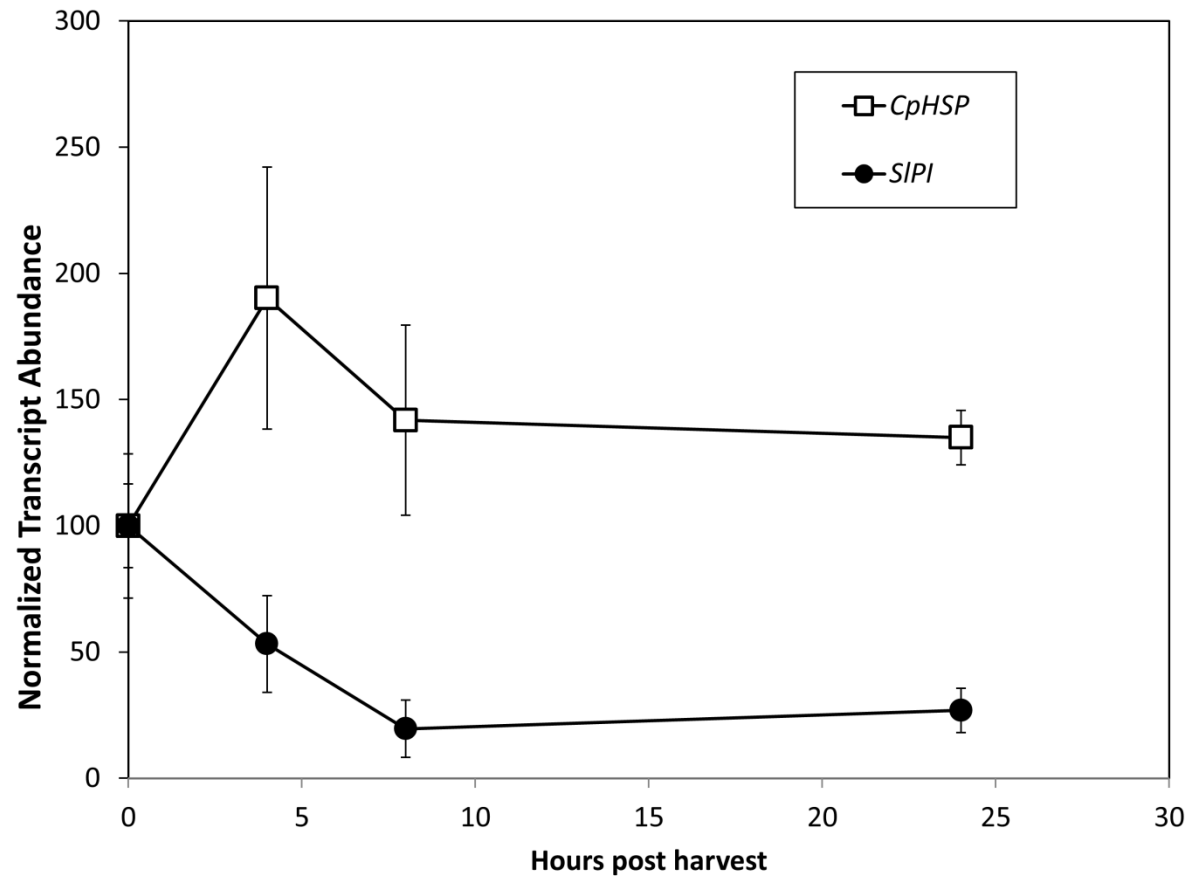


Fig. 3 RNA transcripts of tomato Cathepsin D Proteinase Inhibitor (*SIPI*) decrease significantly in the parasite severed from the host connection over a 24 hour time period relative to *C. pentagona* Heat Shock Protein (*CpHSP*) in absolute quantification real time PCR. ANOVA showed significant difference between hourly *SIPI* transcripts levels at $0 < 0.05$.

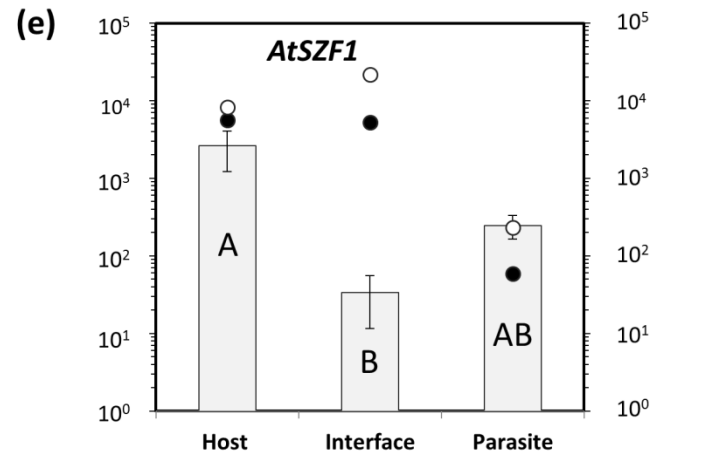
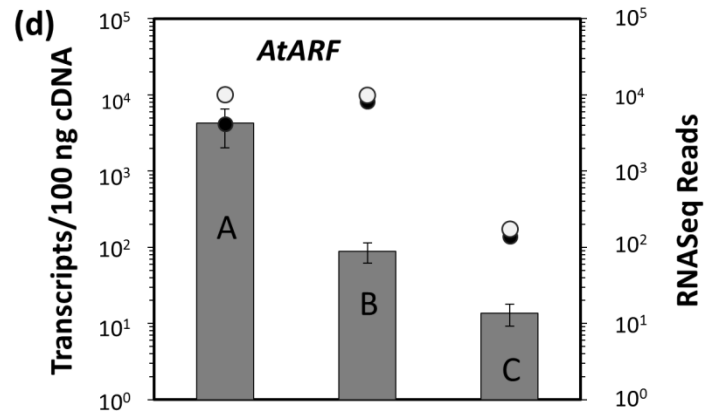
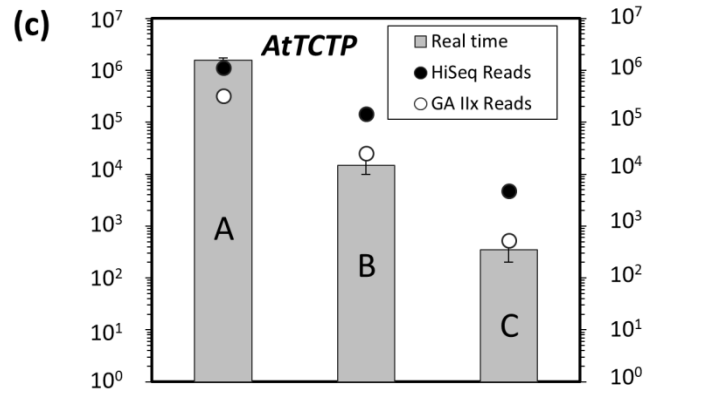
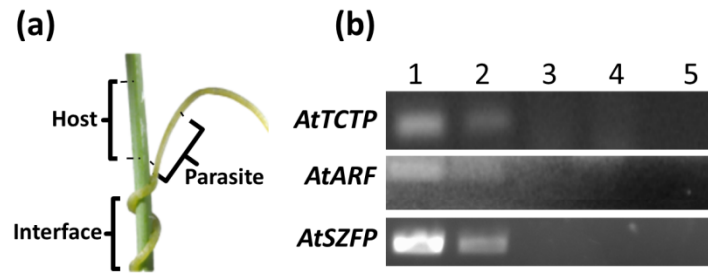


Fig. 4. Transcripts identified from sequence analysis were found to have comparable abundance profiles when assayed by absolute quantitation real time PCR (qRT-PCR). Sequence analysis identified transcripts of varying abundance in (a) RNA extracted from Arabidopsis (Host) stem above the site of attachment, the interface and the apical stem of *C. pentagona* growing away from the host (Parasite). (b) Gel of RT-PCR confirmation of host-specificity of three of the most abundant transcripts: Arabidopsis Auxin Response Family (*AtARF*), Translationally Controlled Tumor Protein (*AtTCTP*), and Salt inducible Zinc Finger Protein (*AtSZF1*). Lane 1 is *Arabidopsis* RNA; 2 is RNA from *C. pentagona* attached to Arabidopsis; 3, Tomato RNA; 4, RNA from *C. pentagona* attached to tomato; 5, non-template control. (c) Transcript reads identified in sequencing data from HiSeq 2000 (closed circles) and Genome Analyzer IIX (GAIIx, open circles) expressed mapped reads in Host, Interface and Parasite tissues follow trends similar to copies detected in qRT-PCR per 100 ng RNA (bars).

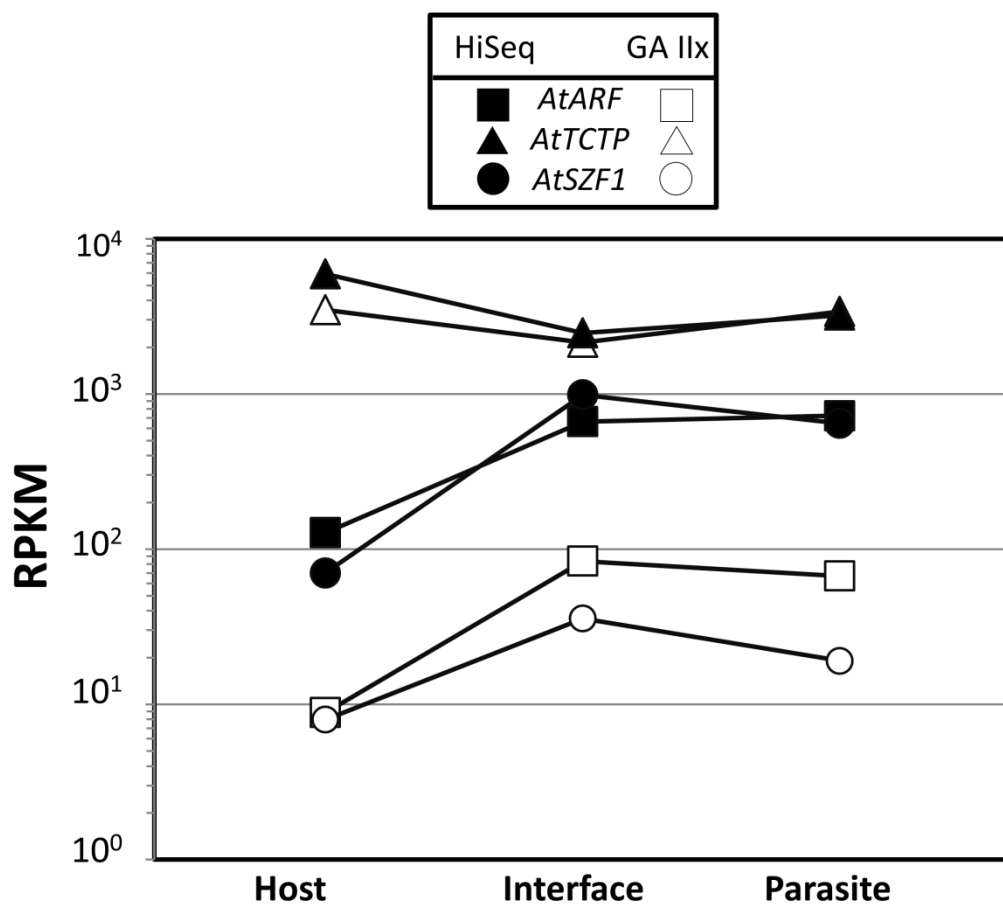


Fig. 5. Reads per Kilobases per Million mapped reads (RPKM) values of Arabidopsis Auxin Response Family (*AtARF*), Translationally Controlled Tumor Protein (*AtTCTP*), and Salt inducible Zinc Finger Protein (*AtSZF1*) from Genome Analyzer IIX and HiSeq 2000 assay data from mapped Host, Interface and Parasite RNA populations.

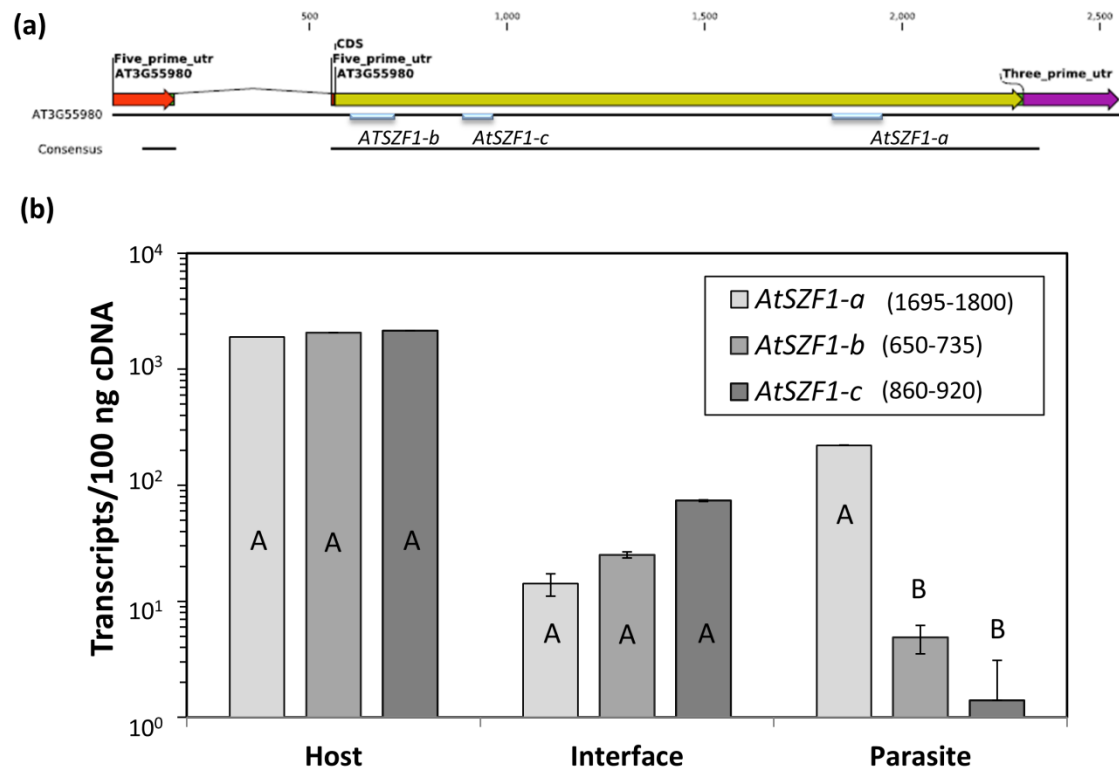


Fig. 6. Differential uptake of discrete sections of a single transcript showed variability of detection in host, interface, and parasite RNA populations. (a) *AtSZF1a-c* primer pairs cover separate sections (light blue bars) that fall on the *AtSZF1* transcript that are covered differentially by RNA-seq paired end reads (blue lines) from Genome Analyzer IIx data. (b) Real time PCR quantification of these transcripts on cDNA generated from host, attached parasite and joint parasite-host tissue (interface) RNA shows a distribution of transcripts most abundantly in host, less detectable in the interface and lowest in the parasite except in the *AtSZF1a* section of the *AtSZF1* transcript, which accumulates in the parasite more so than in the interface. Mean copies/100ng cDNA +/- SE.

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Chapter 3: Optoperforation of single, intact *Arabidopsis* cells for uptake of extracellular dye-conjugated dextran.

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Abstract: A plant science research goal is to manipulate single cells in an intact organism in order to study their interactions with neighboring cells. Based on a technique previously demonstrated in isolated plant cells, mammalian cells and cyanobacteria, *Arabidopsis* epidermal cells were optoperforated to allow for uptake of external cascade blue-labeled dextrans. Adverse organelle responses were determined to be minimal and dye retention was demonstrated for at least 72 hours. This technique overcomes the physical challenges presented by the plant cell wall and demonstrates the feasibility of *in situ* optoperforation.

Keywords: Laser mediated optoperforation, cascade blue, fluorescein, dextran, *Arabidopsis*, multiphoton events, confocal microscopy

Introduction

Optoperforation (OP) can be found in scientific literature under many names: nanoinjection, optical injection, phototransfection, photoporation, or micropuncture; all of which refer to the technique of using lasers to form a quasi-free electron plasma at a cellular boundary to increase its permeability, ultimately allowing uptake of materials from a solution surrounding the cell (Stevenson *et al.*, 2010). Optical cellular manipulation has been applied to mammalian cells for ophthalmic surgery as long ago as the 1960s (Senz & Müller, 1989). Unfortunately, plant science has been unable to utilize this technique in intact plant systems due the unyielding nature of the plant cell wall. Instead, cultured and protoplast cells have been the subjects for a number of optoperforation studies (Weber, et al., 1990; Badr et al. 2005; Fernando et al., 2006). , many of which used this optical approach as a replacement for the otherwise cumbersome techniques available for the introduction of transgenes, such as biolistics or *Agrobacterium*-mediated transformation.

A principle that many optoperforation experiments exploit is a difference in tonicity between the intra- and extra-cellular domains that allows for a concentration gradient, which facilitates movement from one region to the other. For example, UV-laser irradiated *Brassica napus* cultures in hypertonic solution with a β -glucuronidase (*gusA*) expressing construct had a 50% survivability rate of perforated cells two days post laser exposure and *gusA* expression was detected in 71% of survivors after 4 days (Weber *et al.*, 1990). Cultured cells of *Brassica napus* have been shown to remain viable following UV (343 nm, 15 ns) laser irradiation followed by uptake of bizbenzimidazole-labelled pBR322 plasmid, with cytoplasmic streaming visible one hour post treatment and similar survival rates (Weber *et al.*, 1990). Cultured wheat cells were also

transformed with a *gusA* expression vector by a UV laser (6 ns, 308 nm with pulse energies up to 13 mJ) with confirmation of transformation seen in 3 out of 600 samples (Badr *et al.*, 2005).

In addition, pollen has been used for laser mediated transformation due to its ease of culture, but transfection efficiency was low (Eapen, 2011). Specifically, *Lilum hybrid* pollen walls were perforated with irradiances of 1.42 J/cm^2 at 0.6 ms from a near infrared (NIR) laser (Ti:Sapphire, 1kHz) and infiltrated with a mitochondrion-targeted red fluorescent protein construct [6]. Atomic force microscopy showed formation of a hole $1.1 \text{ }\mu\text{m}$ wide and $0.9 \text{ }\mu\text{m}$ deep, and overall transformation efficiency was only 4.4%, slightly higher than the 4.1% efficiency typical for biolistics methods (Fernando *et al.*, 2006).

Protoplasts of *Nicotiana tabacum*, Bright Yellow-2 (BY-2), were subjected to 1064 nm (Nd:YAG) picosecond pulses with energies $<5 \text{ mJ}$ and were found to take up peroxisome-localized Yellow Fluorescent Protein (YFP) (Schinkel *et al.*, 2008). Fluorescence was detected 22 to 48 hours post irradiation in five out of 200 cells (2.5% efficiency).

A recent experiment using *Anabaena* cyanobacteria was the first to demonstrate bacterial cell wall penetration using femtosecond laser pulses in the presence of gold nanoparticles, which increase the localized electromagnetic field of the laser and allow perforations to occur at lower pulse energies and shorter pulse trains than previous attempts (Barbashov *et al.*, 2011).

Laser mediated incorporation of a post transcriptional gene silencing signal, such as siRNA, is another technique with value to plant science. Callus cultures of three species, slash pine (*Pinus elliottii*), cotton (*Gossipyum hirsutum*) and rice (*Oryza sativa*), which had each been transformed to express Green Fluorescent Protein (GFP), were used to test uptake of a GFP siRNA construct (Tang *et al.*, 2006). The calli were subjected to a laser induced stress wave (LISW) originating

from a rubber sheet adjacent to the tissue, which caused the affected calli to become permeable to the siRNA construct. This resulted in stable silencing of GFP expression starting as early as five days post LISW and lasting for greater than two months, with a total efficiency of 80-95% silencing (Tang *et al.*, 2006).

Although optoperforation may substitute for other methods of single cell manipulation, the true value of the technique is in manipulating specific cells of intact plant organs, particularly meristematic tissues, instead of callus and cultured cells lacking a rigid cell wall (protoplasts). We could find no reports of the transformation of cells in living, intact plants using optoperforation. Here we show NIR femtosecond laser-mediated infiltration of a membrane impermeable dextran-conjugated fluorophore into cells of live *Arabidopsis thaliana* seedling stems, along with data on the laser effects to cellular structures. This method is ideal for studies of cell-to-cell interactions and potentially paves the way for introducing transgenes to specific cells within a plant.

Materials and Methods

Plant preparation

Arabidopsis thaliana (cv. Colombia) seeds were plated on Murashige and Skoog (MS) + sucrose media (Murashige & Skoog, 1962) and grown in 12 hr light/dark cycles for 1 week. Seedlings were prepared for laser irradiation and dye uptake as described in Weber, et al. (Weber *et al.*, 1988) by submersion in 1X phosphate buffered saline (PBS) with either a control solution containing 50% v/v 70 kDa dextran-fluorescein (Life Technologies Co.) or a treatment solution of 50% 10 kDa dextran-cascade blue (Life Technologies Co.) in a glass bottomed 35 mm dish (Mattek Co., P35G-1.5-20C) and covered with a plastic coverslip. Transgenic *Arabidopsis* plants

expressing vacuole-targeted Cyan Fluorescent Protein (CFP) (Nelson *et al.*, 2007) were used for laser refractory tests.

Following the optoperforation procedure, plants were rinsed with sterile distilled water with minimal movement of the overlying coverslip. Water supplemented with 50% 1/4X Hoagland's Solution (Hoagland & Arnon, 1950) was introduced under the coverslip, and the housing dish was capped with a lid and sealed with parafilm to prevent desiccation.

Optoperforation Apparatus

The laser source used for both the fluorescence imaging and optoperforation portions of this work was a Kerr lens mode locked Ti:Sapphire laser (Chameleon, Coherent Inc. Santa Clara, CA) operating in a wavelength range of 690-1064 nm with a pulse duration of approximately 140 fs, a repetition rate of 80 MHz, and a TEM00 spatial mode. Before being coupled to the laser port of a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss Inc., Thornwood, NY), the laser pulses pass through an acousto-optical modulator, providing both laser beam attenuation and beam-blanking. After arriving at the laser port, the pulses continued onward to a galvano scanning mirror unit, then to a 660 nm edge short-pass dichroic beamsplitter, and were finally focused on a sample by a telecentric water-immersed objective lens (NA 1.2 /40x, Carl Zeiss MicroImaging, Inc. Thornwood, NY). Laser power was measured at the sample plane.

Fluorescence microscopy and image processing

In order to measure the fluorescence of either nonreactive dye or fluorescent proteins, a multiphoton (MP) excitation configuration was used. To monitor cascade blue (CB) uptake, the sample was excited by 750 nm at 20 mW (2.6×10^{12} W/cm²), the ensuing fluorescence was

collected by the objective, and passed through the dichroic mirror and a 427/37 nm (CWL/BW) bandpass filter. Similarly, the fluorescence of CFP was observed by exciting the sample with 840 nm at 25 mW (2.6×10^{12} W/cm²) and passing said fluorescence through the dichroic mirror and a 487/44 nm bandpass filter. Images were captured by a PMT and processed by the LSM software (©Carl Zeiss Microimaging, GmbH. 1997-2006) on a PC. Efficacy of dye retention was determined by measuring the differential fluorescent intensity between targeted and untargeted cells. For time-lapse analysis of CB uptake, the fluorescence intensity was integrated over a region enclosed by the monitored cell.

Optoperforation Protocol

A glass bottom dish containing the *Arabidopsis* seedling was mounted on the cross-slide stage of the microscope, and the horizontal movement of the stage was actuated manually by a coaxial drive. A built-in piezoelectric step motor in the microscope controlled the vertical position of the objective and, by extension, the focal point. Through this motion control, the positions of the dish and objective were adjusted until the cells closest to the glass interface were visualized in brightfield images.

At this point, cells of the stem epidermal layer that were in contact with the surrounding medium and had a uniform structure that were amenable to visualization were targeted. The Z position of the objective was adjusted until a cell wall surface was in the focal plane (only cell walls salient to the surrounding solution were targeted in this work). Then the injection site was selected and its XY position was recorded using the spot scan feature of the Zeiss software, which directs the laser at a desired point in the focal plane during the scan process. This is in contrast to a planar scan, where the entire focal plane is scanned in a raster pattern. Once the injection site was

acquired, the objective was moved 1.5 μm below the cell wall plane. The laser was then directed at the specified XY location, exposing that location to a given perforation power for a pulse train duration of 0.64 μs . Following the perforation, a planar image was immediately taken. The objective was moved upwards in 0.5 μm increments, repeating the inject-scan procedure for each increment until either a notable response from the cell to the perforating laser was observed or the objective moved a total distance of 3 μm . This procedure was applied to additional cells of the same *Arabidopsis* stem such that the total number of cells targeted on the same plant ranged from 5 to 10.

For time lapse analysis of the optoperforation process, the protocol was the same except instead of capturing a solitary planar image immediately after injection, planar images were taken continuously for five minutes. From these images, the temporal development of CB uptake was determined by measuring the average fluorescence intensity in a region of interest confined to the perforated cell.

Post Optoperforation Measurements

For ascertaining cell viability and long term dye retention, cells were imaged from 24 to 72 hours following the optoperforation event. Planar image stacks in increments of 1 μm were taken of regions encompassing targeted cells. Cells were visually inspected for general vitality and morphological changes induced by the optoperforation process. In addition, for cells containing CB within the confines of their cell wall, the distribution of the fluorophore was quantified.

SEM Imaging

Scanning Electron Microscopy (SEM) for determining cell wall structural changes due to optoperforation was carried out on a Quanta 600 FEG system (FEI, Hillsboro, OR, USA).

Results and Discussion

Optoperforation mechanism for intact plant cells

To explain the mechanisms responsible for femtosecond laser optoperforation, we rely on the theoretical framework proposed by Vogel *et. al* (Vogel *et al.*, 2005). In this framework, when femtosecond laser pulses are tightly focused on a cell in an aqueous environment, sufficiently high intensities can be produced in the focal volume such that quasi-free electrons are generated through multiphoton ionization and subsequent avalanche ionization (Fig. 1a and b). Since such requisite intensities are only found in the focal volume, the generated quasi-free electron plasma is relegated within this volume. At sufficiently low densities, this plasma reacts photochemically with the cell wall and plasma membrane, dissociating biomolecules (such as lignin, cellulose, or phospholipids) to create a pore (Fig. 1c). As the free electron density increases, cumulative heating and thermoelastic stress effects emerge, resulting in cavitation bubble formation and tensile shockwaves. As such, to achieve spatial accuracy of pore formation and avoid collateral cell damage, laser intensities must operate below the point in which cavitation bubbles emerge, referred to as the optical breakdown threshold, while still achieving sufficient plasma formation for pore creation (see discussion below).

In order to compare this work to the aforementioned theoretical findings, our optoperforation results need to be described in the context of laser irradiance at the focal plane. We approximate the spatial irradiance distribution in this plane as Gaussian, such that

$$I(r) = I(0) \text{Exp} \left[-2 \frac{r^2}{\alpha^2} \right] \quad (1)$$

where r is the coordinate in the radial direction, $I(0)$ is the on-axis irradiance, and α is the radius at the $1/e^2$ irradiance level. The measured average power, P_{avg} , can be related to the peak on-axis irradiance, $I_{\text{peak}}(0)$, by

$$I_{\text{peak}}(0) = \frac{2P_{\text{peak}}}{\pi\alpha^2} = \frac{2P_{\text{avg}}}{v\tau\pi\alpha^2} \quad (2)$$

where v is the repetition rate of the laser, τ is the laser pulse duration, and α is the $1/e^2$ radius.

Due to temporal broadening of the confocal system, the pulse duration will be larger than the 140 fs initially produced. According to the manufacturer's specifications, the confocal system incurs a group-delay dispersion at 750 nm of approximately 7500 fs², providing a broadened pulse duration of $\tau \approx 200$ fs (Konig, 2000).

The $1/e^2$ diameter of the image spot from a truncated Gaussian input beam passing through a diffraction-limited lens is given by (Tsampoula *et al.*, 2007),

$$d = K_{1/e^2} \cdot \lambda \cdot f/\# \quad (3)$$

where K_{1/e^2} is a constant that depends on the truncation ratio, λ is the wavelength of light, and $f/\#$ is the f-number of the objective. The truncation ratio is defined as $T = D_B/D_L$, where D_B is the $1/e^2$ diameter of the input beam and D_L is the diameter of the limiting aperture of the

objective. In our system, the truncation ratio was measured to be 0.77 (which corroborates our assumption regarding the Gaussian focal-plane irradiance distribution), ultimately giving a $K_{1/e}^2$ of 1.64 (Gerald F. Marshall, 2005). For NA=1.2, the corresponding f-number is 0.24. Since $\alpha=d/2$, $\alpha=180$ nm.

Hence, the average powers used in this study, ranging between 5-100 mW, correspond to on-axis peak intensities in the range of 6.4×10^{11} to 1.3×10^{13} W/cm².

Due to the fact that the OP mechanics are confined to the focal volume, precisely co-aligning the focal plane with the cell wall is paramount. In fact, a misfocus of a Gaussian beam by as little as 3 μ m was shown to reduce the efficacy of OP-mediated transfection by more than 50% (Gerald F. Marshall, 2005). To address such concerns, we adopted a gradational OP protocol whereby a 3 μ m long axial section centered on the cell-wall plane was exposed to successive perforation intensities, as described above. While this approach incontrovertibly exposes targeted cells to more optical doses, we did not observe a reduction in cell viability as a result of using this method.

In addition to this gradational approach, the imaging scheme also had a role to play in the overall OP efficacy. Since the seedlings were submerged in a fluorescent medium, the imaging technique needed to reject fluorescence outside the region of interest, namely the interface of the cell wall and fluorescent dye, to ensure sufficient contrast. This is a problematic task for conventional fluorescence microscopy where upstream fluorescence can obscure in-plane emissions. Hence, an imaging technique that veritably complements the OP procedure is one that also relies on nonlinear optical effects: two photon excitation (TPE) microscopy.

TPE relies on the simultaneous absorption of two photons to induce a fluorophore into an excited state. In the case of OP, TPE produces nonlinear effects that result in the plasma being constrained to the focal volume where the photon density is sufficiently high. Thus, optical sectioning can be achieved since fluorescence is permitted only in the focal plane. When considering a fluorophore with a visible emission, the TPE source generally resides in the infrared region. As a result, beyond its optical sectioning capabilities, TPE possesses a large Stokes shift to allow proper optical filtering of the excitation source from the ensuing emissions, suffers less attenuation due to a reduced scattering cross section compared to shorter wavelengths, and enjoys improved depth discrimination compared to its single photon counterpart (So *et al.*, 2000). This latter attribute is particularly apt for our specimens because symplastic uptake of dye needs to be monitored throughout a $\sim 50\ \mu\text{m}$ thick cell. Although the use of longer wavelengths results in a two-fold reduction in resolution for TPE compared to single photon excitation, this loss of resolution is inconsequential for the application at hand, since features to be resolved are on the order of $1\ \mu\text{m}$ or more.

While the infrared tunability of the Ti:sapphire laser offers the ability to use a swathe of visible-emitting fluorophores, CB in particular was included in the hypertonic solution to monitor the diffusion of exogenous material into the cell upon the optical creation of a pore. Since the two-photon absorption cross section of CB indicates that this dye is readily excitable by $750\ \text{nm}$ (Li *et al.*, 2010), the same wavelength was useful for both optoperforation and imaging of fluorescent dye infiltration. Hence, the laser cavity required no adjustment when these two functions were performed in succession, allowing immediate imaging of the cell after exposure to a perforating laser pulse train. In addition, the high energy emission of CB avoids the issue of emission cross-talk with autofluorescence sources such as lignin, cellulose and plastids.

To ensure that the infiltration of this nonreactive dye into a targeted cell was solely due to the OP process, the dye was linked to membrane-impermeable dextrans and a series of control experiments were performed. Unbound fluorescent dyes, such as CB and fluorescein, are small enough to be taken up through the root vasculature and spread throughout the water-carrying tissues of the plant. Conjugation to a dextran of sufficient size prevents this passive diffusion. For instance, intact stem and root cells take up fluorescein-tagged dextran molecules up to 10 kDa (not shown); in contrast, 10- and 70 kDa dextrans proved to be non-diffusible and, as such, were used for these studies.

Due to dextran conjugation, dye uptake was not observed in the absence of a perforating laser. Only cells exposed to the OP protocol showed an increase in measurable CB fluorescence within the confines of the cell wall. Furthermore, this intracellular increase in CB fluorescence was due to the presence of dye, as no CB fluorescence was observed from cells subjected to the OP protocol that were in a medium devoid of CB (data not shown).

In order to measure destructive laser ablation effects on the cell wall, we applied the OP technique to a TEMPO-oxidized nanocellulose material with properties similar to the natural cell wall [21]. Treated material was imaged by Scanning Electron Microscopy (SEM) to determine the size of the hole generated, indicating that an incident irradiance of $4.6 \times 10^{13} \text{ W/cm}^2$ lasting 5.1 ms produced an ablation area 37.8 μm in diameter with a clearance hole of 6.3 μm (Fig. 2). Following this laser exposure, long-lived vapor bubbles with diameters $>10 \mu\text{m}$ were observed and lingered for several seconds before their dissipation. The presence of these bubbles indicates that thermal accumulation effects are largely responsible for the scope of the observed damage, particularly when considering the 180 nm spot size of the laser. Based on OP use on mammalian cells (Vogel *et al.*, 2005), the OP laser parameters used here correspond to the onset of

immediate cell death in those studies. However, since the typical dimensions of a plant cell can be as little as 10 μm wide and up to 100 μm long, we would expect this cell death onset for *Arabidopsis* cells at much lower irradiances and reduced exposure times. The latter parameter is more important in this regard due to its integral role in accumulated thermal effects. Hence, these findings warranted reducing the exposure time by more than three orders of magnitude to 0.64 μs .

To develop an OP procedure that preserves cell vitality, we must determine the laser irradiance threshold that demarcates destructive and non-destructive perforations. Indicators of the former kind are the byproducts of localized high plasma densities, such as transient cavitation bubbles and observable damage beyond the focal volume due thermoelastic tensile stress waves. However, as the perforation laser irradiance is reduced toward the threshold level, the presence of these indicators is diminished to the point where the vitality of the cell is compromised without the aforementioned visual cues in brightfield images. In order to avoid these surreptitious effects and ascertain the threshold in which they occur, we observed in real time the impact of laser irradiances on the integrity of the cell vacuole by monitoring the fluorescence from *Arabidopsis* plants expressing CFP targeted to the vacuole (Fig. 3). At $1.2 \times 10^{13} \text{ W/cm}^2$, the vacuole either shifted and retracted away from the perforation site, indicating a rapid incursion of exogenous material (Fig. 3a, LeBlanc_ML_f2), or split and reformed into discrete compartments (Fig. 3b, LeBlanc_ML_f3). The observed damage to the vacuole corresponded to leakage of the infiltrated dye and cell death, indicating that the long-term integrity of the plasma membrane was compromised. For irradiances at or below $9.0 \times 10^{12} \text{ W/cm}^2$, no response by the vacuole was observed.

Exchange of contents between the cytoplasm and the surrounding solution was observed for irradiances above $5.3 \times 10^{12} \text{ W/cm}^2$ (data not shown), implying that pores of sufficient size are produced above this lower bound. Hence, we conclude that optimum perforation irradiances lie in the range of 5.3×10^{12} - $9.0 \times 10^{12} \text{ W/cm}^2$. However, not all of these irradiances exhibited similar OP efficiencies. Successful OP, in which targeted cells displayed CB retention 72 hours post OP event along with vital morphological criteria (such as plasma streaming), was observed in 50 out of 92 optoperforated cells (54% efficiency) for $9.0 \times 10^{12} \text{ W/cm}^2$ and in 90 out of 133 optoperforated cells (68% efficiency) for $7.7 \times 10^{12} \text{ W/cm}^2$. As such, $7.7 \times 10^{12} \text{ W/cm}^2$ was used for the remaining OP procedures of this work.

At this irradiance, bright field images indicate that the pore formed in the cell wall is between 2 and $2.5 \mu\text{m}$ in diameter (Fig 4). In comparison to the laser spot size, the observed pore dimension indicates that the laser-generated tensile stress is not confined to the focal volume at this irradiance for cell walls. The rigid nature of the cell wall constituents (cellulose, lignin), in contrast to those of the plasma membrane, may be more susceptible to these stress waves, facilitating larger pore formation.

By monitoring the temporal evolution of the fluorescent emission within a targeted cell after an optoperforation event, the observed rate of diffusive flow can indicate the time scale of the repair mechanisms for the plasma membrane. To this purpose, time-lapse images were taken over a five minute period in which the OP protocol was applied to single cells of the *Arabidopsis* stem surround by a 10 kDa-dextran cascade blue conjugate (Fig. 5, LeBlanc_ML_f4). Dye is incorporated through the cell wall and what appears to be the cytoplasm as defined by the outline of the intact vacuolar membrane and organelles that are distinct from the background.

In this region (Fig. 6a), the fluorescence emission increases monotonically for the first 90 seconds until reaching an upper limit and then, at the 300 second mark, gradually recedes from that limit (Fig. 6b). The initial increase in fluorescence is indicative of a rapid influx of dye through the laser-formed pore in the cell wall and plasma membrane. Since the increase stalls at an intensity below the saturated intensity in the exogenous medium, this behavior suggests that the pore in the plasma membrane has been sealed at the onset of the upper limit. The subsequent decrease in irradiance at 300 s is a consequence of the dye diffusing within the cytoplasm and/or diffusion of unincorporated dye from the apoplast to the extracellular space by means of turgor pressure-associated forces.

In order to confirm that extracellular dye was incorporated into the cytoplasm and not just in the apoplastic space between cell wall and plasma membrane, cells were treated with 5 M NaCl solution to plasmolyse the cells of a seedling 24 hrs post optoperforation. Prior to addition of salt solution, the dye-dextran conjugate is seen in discrete yet dispersed areas, possibly due to compartmentalization for degradation (Fig. 7a). The effect of the salt solution was rapid, so imaging was done immediately following addition of the solution. After treatment the dye aggregates as a result of plasmolysis, the shrinking of the plasma membrane in response to osmotic stress (Fig. 7b).

To test the ability of cells to recover from OP, cells that had taken up CB dye were imaged immediately after the procedure and then again 72 h later. Over this 72 hour period, CB dye remained within the cellular boundary of perforated cells (Fig. 8). Cytoplasmic streaming was visible in treated cells as well as incorporation of dye into discrete structures, presumed to be peroxisomes or other organelles, which indicate normal cellular functions post OP. Eight cells, denoted by asterisks, were targeted (Fig. 8a) and it is possible to detect fluorescence emission

after 72 h in six of those cells (Fig. 8b, c), indicating an efficiency of approximately 85% for this set of cells.

Conclusions

The formation of a transient pore within the plasma membrane of an intact plant cell is a unique accomplishment, particularly when utilizing common microscopy equipment. This work has demonstrated the efficacy of laser mediated optoperforation for pinpoint accurate targeting of cells within a plant stem for long-term symplastic retention of an extracellular dye conjugate as confirmed by repeated imaging (Figs. 4 & 8) and saline treatment (Fig. 7). From our observations, we conclude that the focused fs NIR laser beam creates a hole in the cell wall and allows for a temporary breach of the plasma membrane sufficient for extracellular material to be incorporated into the cell with 68% efficiency.

It is notable that commonplace confocal microscope setups with the addition of a fs laser source can be used for this relatively simple “point-and-shoot” technique. Only minimal technical knowledge is required for a researcher to generate such remarkable results.

The technique has multiple applications related to determining cell function and fate. Specific cells can be loaded with dyes or proteins that traffic into neighboring cells or otherwise influence function of surrounding cells. Long-lived, immobile dyes could be used as a kind of “molecular tattoo” to mark cell fate in developing tissues over time. Ideally, the approach can be used to specifically inject plasmids containing transgenes to transform single cells with new genes or silencing constructs to investigate the role of specific cellular context in plants. OP can therefore be an important tool in the emerging field of single-cell biology.

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References

- Badr YA, Kereim MA, A.Yehia M, Fouad OO, Bahieldin A. 2005.** Production of fertile transgenic wheat plants by laser micropuncture. *Photoch. Photobio. Sci.* **4**(10): 803-807.
- Barbashov Y, Zalesskii A, Aibushev A, Sarkisov O, Radtsig M, Khmel' I, Koksharova O, Nadtochenko V. 2011.** Femtosecond optoperforation of the cell wall of cyanobacterium *Anabaena* sp. PCC 7120 in the presence of gold nanoparticles. *Nanotech. Russ.* **6**(9): 668-675.
- Eapen S. 2011.** Pollen grains as a target for introduction of foreign genes into plants: an assessment. *Physiol. Mol. Biol. Plant* **17**(1): 1-8.
- Fernando D, Richards J, Kikkert J. 2006.** *In vitro* germination and transient GFP expression of American chestnut (*Castanea dentata*) pollen. *Plant Cell Rep.* **25**(5): 450-456.
- Gerald F. Marshall GES. 2005.** "Handbook of Optical and Laser Scanning." New York: Marcel Dekker.
- Hoagland DR, Arnon DI. 1950.** The water-culture method for growing plants without soil. *Circular. California Agricultural Experiment Station* **347**(2nd edit): 32 pp.
- Konig K. 2000.** Multiphoton microscopy in life sciences. *J Microsc-Oxford* **200**: 83-104.
- Li Z, Renneckar S, Barone J. 2010.** Nanocomposites prepared by in situ enzymatic polymerization of phenol with TEMPO-oxidized nanocellulose. *Cellulose* **17**(1): 57-68.
- Murashige T, Skoog F. 1962.** A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant* **15**(3): 473-497.
- Nelson BK, Cai X, Nebenführ A. 2007.** A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. *Plant J.* **51**(6): 1126-1136.

- Schinkel H, Jacobs P, Schillberg S, Wehner M. 2008.** Infrared picosecond laser for perforation of single plant cells. *Biotechnol. Bioeng.* **99**(1): 244-248.
- Senz R, Müller G. 1989.** Laser in medicine. *Berich. Bunsen Gesell.* **93**(3): 269-277.
- So PT, Dong CY, Masters BR, Berland KM. 2000.** Two-photon excitation fluorescence microscopy. *Annu Rev Biomed Eng* **2**: 399-429.
- Stevenson DJ, Gunn-Moore FJ, Campbell P, Dholakia K. 2010.** Single cell optical transfection. *J. R. Soc. Interface* **7**(47): 863-871.
- Tang W, Weidner DA, Hu BY, Newton RJ, Hu X-H. 2006.** Efficient delivery of small interfering RNA to plant cells by a nanosecond pulsed laser-induced stress wave for posttranscriptional gene silencing. *Plant Sci.* **171**(3): 375-381.
- Tsampoula X, Garces-Chavez V, Comrie M, Stevenson DJ, Agate B, Brown CTA, Gunn-Moore F, Dholakia K. 2007.** Femtosecond cellular transfection using a nondiffracting light beam. *Appl. Phys. Lett.* **91**(5).
- Vogel A, Noack J, Hüttman G, Paltauf G. 2005.** Mechanisms of femtosecond laser nanosurgery of cells and tissues. *Appl. Phys. B-Lasers O.* **81**(8): 1015-1047.
- Weber G, Monajembashi S, Greulich KO, Wolfrum J. 1988.** Microperforation of plant tissue with a UV laser microbeam and injection of DNA into cells. *Naturwissenschaften* **75**(1): 35-36.
- Weber G, Monajembashi S, Wolfrum J, Greulich K-O. 1990.** Genetic changes induced in higher plant cells by a laser microbeam. *Physiol. Plantarum* **79**(1): 190-193.

Figures

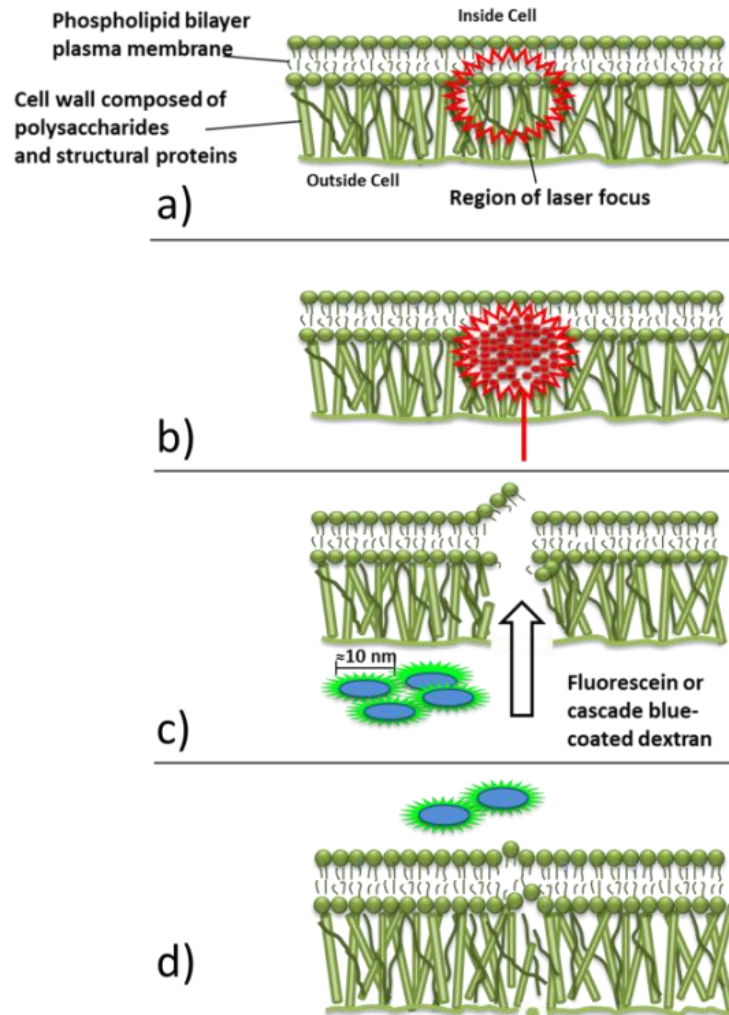


Fig. 1. Illustration of laser-induced plasma formation at cell wall allowing for uptake of fluorophores through a transient plasma membrane pore. (a) Representation of cell structure within intact plant tissue at the site of laser focus. (b) Approximation of ideal laser ablation. (c) Broken cell wall and perforated plasma membrane allow external macromolecules to flow into the cellular space along a concentration gradient. (d) The transient nature of the plasma membrane pore allows it to re-seal, leaving slight damage to the cell wall but internalization of extracellular material.

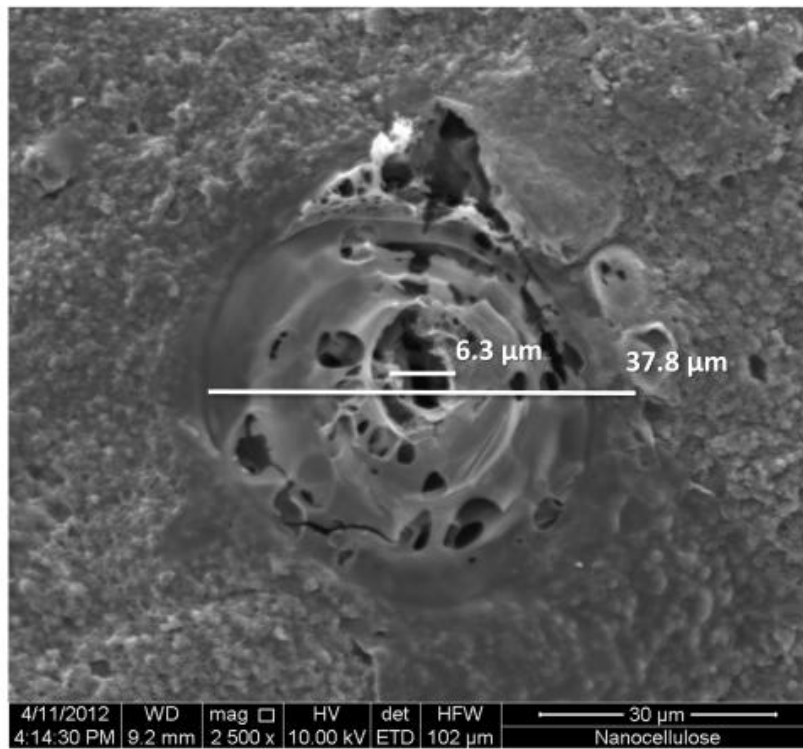


Fig. 2. Scanning electron microscope image of the hole and peripheral effects generated at $4.6 \times 10^{13} \text{ W/cm}^2$ irradiance for 5.1 ms exposure time in PCA-TEMPO nanocellulose (2500X magnification).

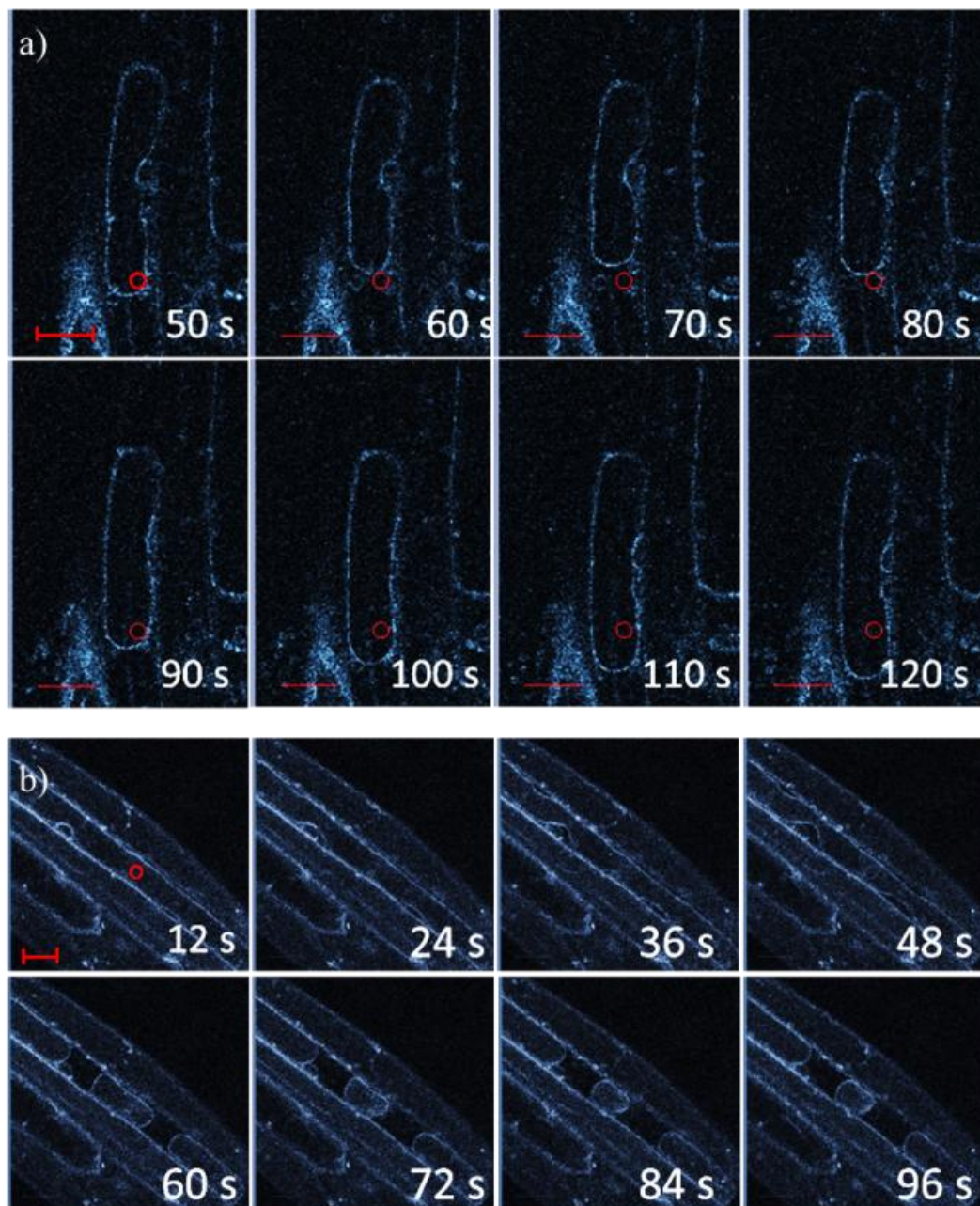


Fig. 3. Timecourse images of *Arabidopsis* expressing CFP targeted to the vacuole membrane. (a) Over a two minute time period following $1.2 \times 10^{13} \text{ W/cm}^2$ laser scan the vacuole shrinks and then rebounds. (b) A scan with the same power laser causes the vacuole to contract and break into three discrete units. Red circle = site of laser scan. Bar = 20 μm .

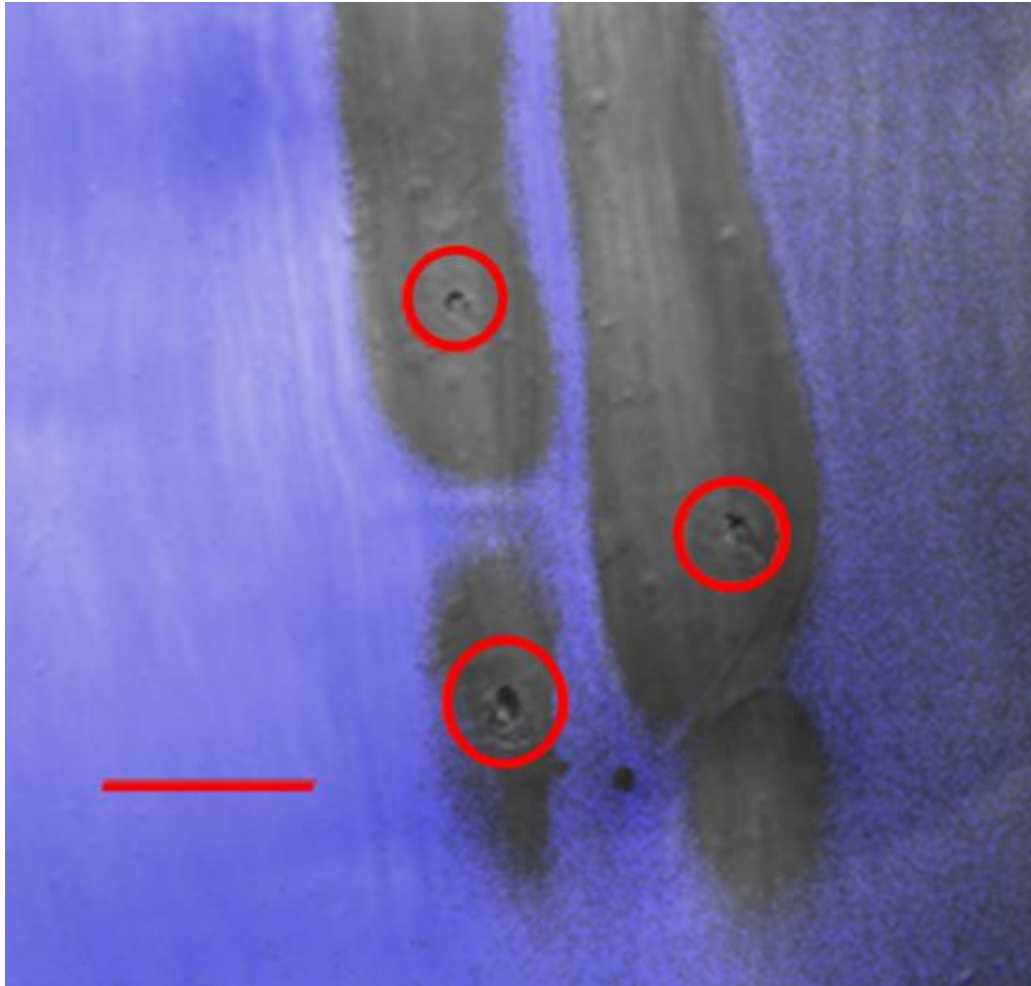


Fig. 4 *Arabidopsis* stem epidermal cells subjected to laser optoperforation at $7.7 \times 10^{12} \text{ W/cm}^2$ for $0.64 \mu\text{s}$ showed hole formation, where indicated by red circles, less than $2.5 \mu\text{m}$ in diameter. Bar = $20 \mu\text{m}$.

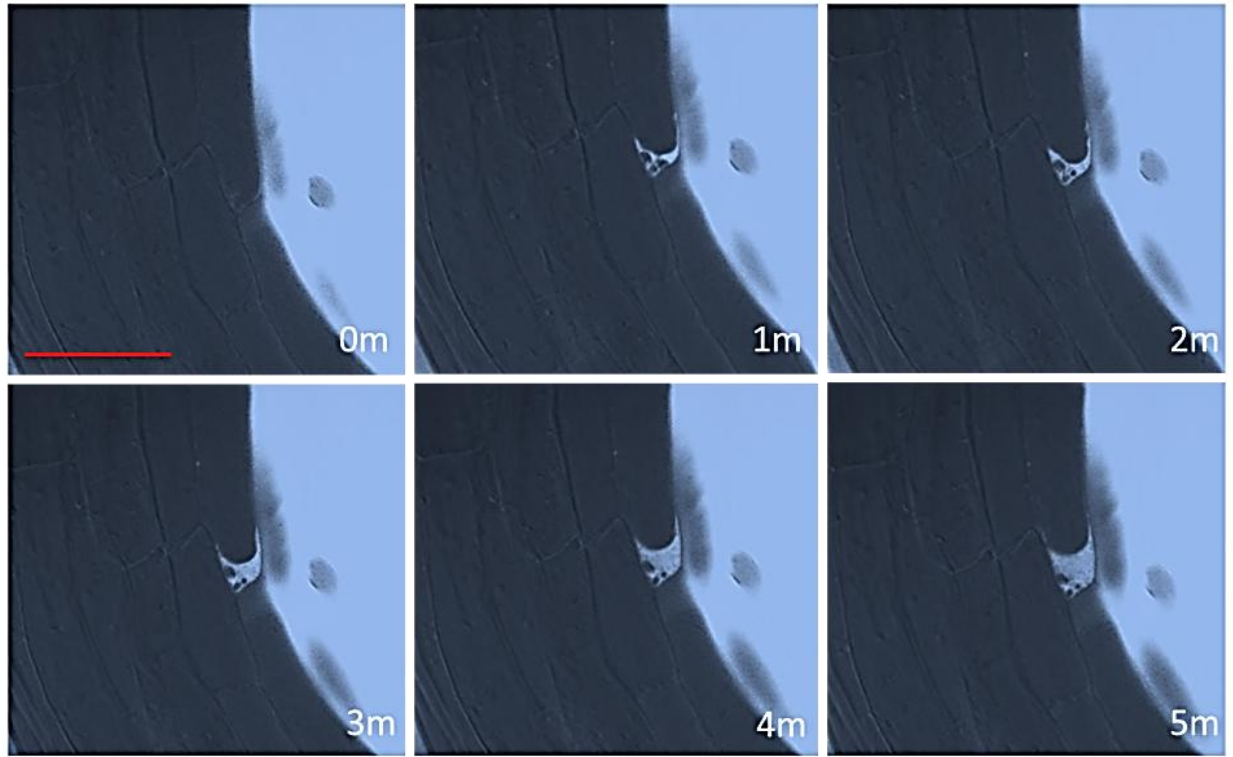


Fig. 5. Confirmation of symplastic uptake of cascade blue dye over 5 minutes. Vacuolar membrane is visible as well as the outline of other organelles, suggesting dye is dispersed inside the plasma membrane. Bar = 50 μm .

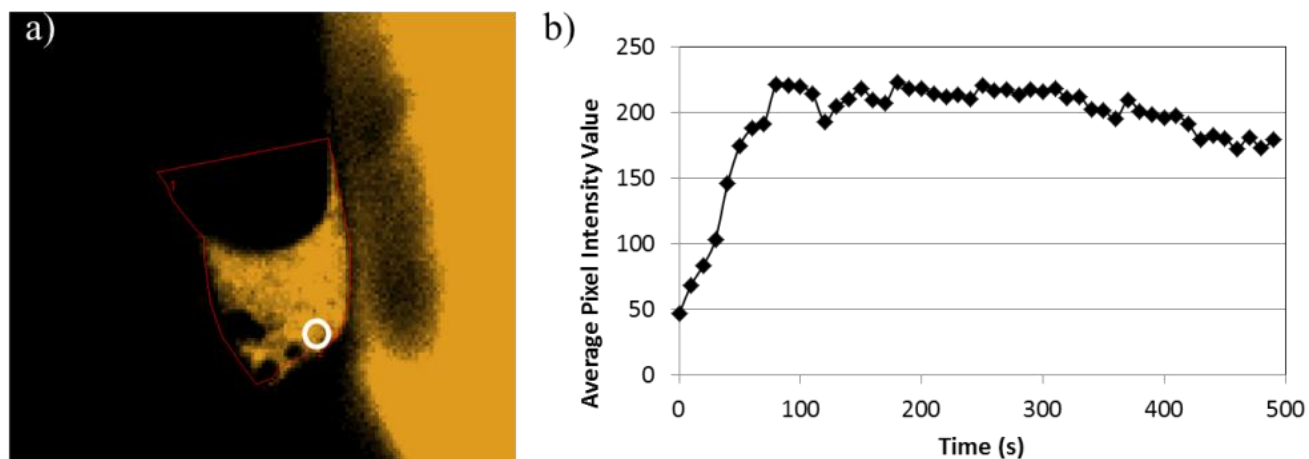


Fig.6. Time-resolved cascade blue conjugated dextran fluorescent intensity within optoperforated cell (a), indicating rapid influx of dye through artificial pore, and subsequent pore sealing and dye redistribution throughout cellular confines (b).

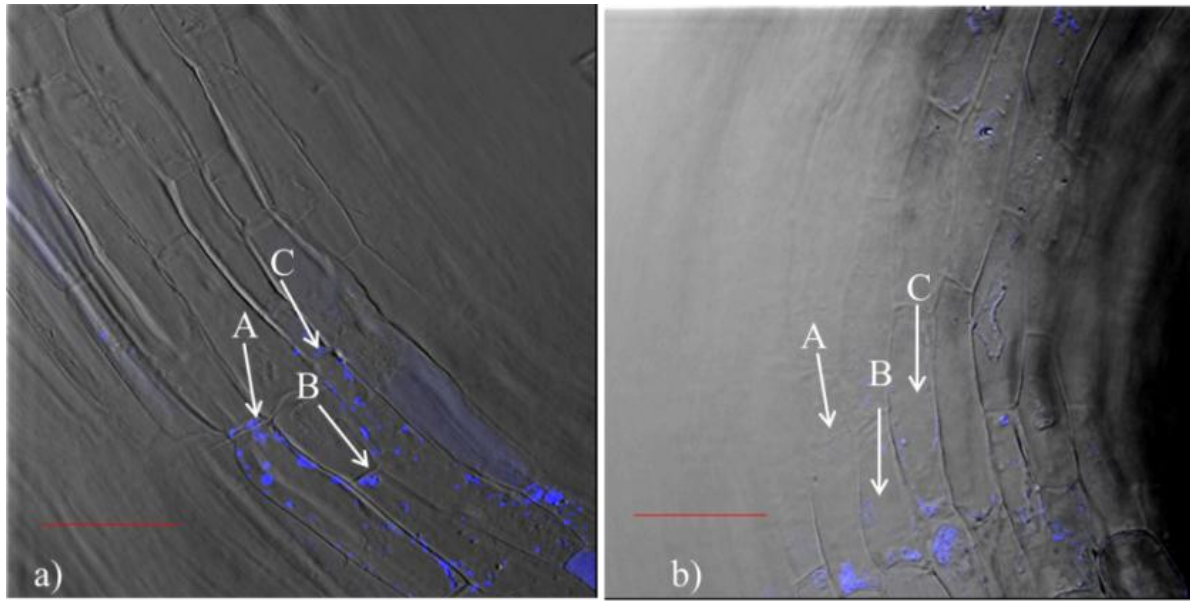


Fig. 7. Cascade blue-10 kDa dextran appearance within cells before (a) and after (b) treatment with 5 M NaCl. The addition of saline solution changes the focal plane and orientation of the root so cells of interest are denoted by letters A, B, and C. Bar = 50 μ m.

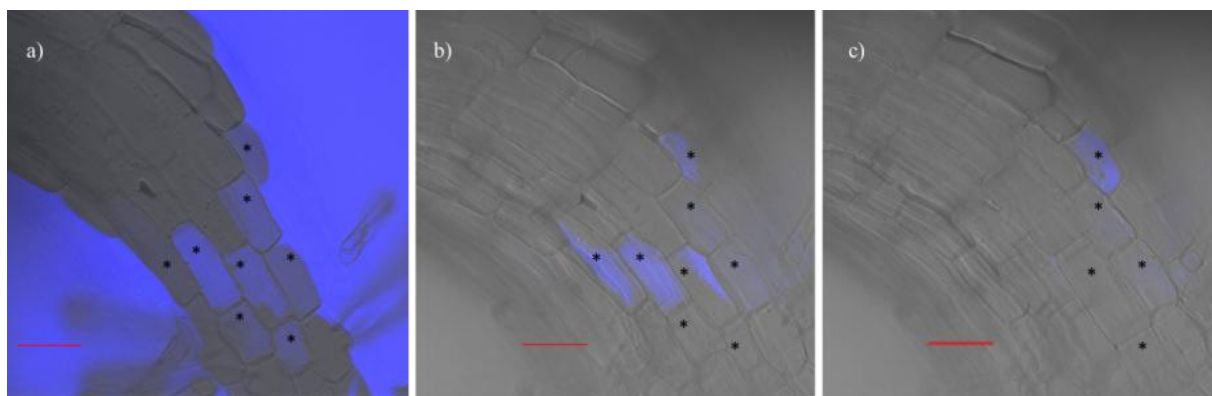


Fig. 8. Optoperforated cells exhibiting 10 kDa dextran-cascade blue dye conjugate uptake 0.5 h post laser exposure (a), and 72 h post exposure (b, c). Panel (b) is at the zero Z-plane of focus and (c) is at -14. Asterisks = OP cells visible in plane. Bar = 50 μm .

Chapter 4: Alternative approaches to visualizing host-parasite interactions

Abstract: The goal of this research is to better understand the phenomena of cell to cell movement of RNA, particularly in host-parasite systems. Useful techniques for *in situ* hybridization with RNA probe conjugated-Quantum dots, tracing transgenic proteins, and selecting microscopic tissue sections for metabolomics will be presented in this chapter. These approaches utilize exciting technical applications that may have impacts in future research.

Keywords: Quantum dots, RNA trafficking, Real Time PCR, Fluorescent protein conjugates, Laser microdissection, cryosection, *in situ* hybridization

Introduction

In order to study RNA and protein signals mobile within a plant, researchers have been limited to either phloem-sap collection or grafting experiments. Both present certain disadvantages: the process of sap collection can damage adjacent cells and invite contamination of the sample, whereas grafting can be a difficult process with strict limitations on stock-scion combinations (Kragler, 2010). The parasite-host system for studying trafficking provides a distinctive advantage in that the interspecies vascular connections are symplastic in nature (Dorr, 1990) and given the distinct genetic differences between parasite and host, allow for a high throughput sequencing approach to mobile transcriptome analysis. The dynamic interface between host and parasite in particular lends itself to creative techniques of study, particularly in regards to imaging RNA and proteins within the matrix of the cell.

One method to visualize specific mRNAs is by using probes labeled with Quantum Dot (QD) fluorophores. QDs have come to the fore as having distinct advantages over traditional fluorophores for single molecule tracing in biological applications (Pinaud *et al.*, 2010). Sustained light transmission, discrete emission spectra and the availability of biological tags have increased the biological applications of QDs (Medintz *et al.*, 2005). *In situ* hybridizations to fixed tissues have pinpointed RNA transcripts in animal (Tholouli *et al.*, 2006) and plant tissues (Riegler *et al.*, 2008), providing the possibility for imaging phloem mobile mRNAs at the dodder-host connection site. Plant-based uses for QDs have been less common than animal-based assays, with a majority of literature available on the topic related to biological applications in animal cells. Adhesion protein localization using QDs has been successfully carried out in

cultured plant pollen tube cells *in vitro* with results similar, if not better, to those using fluorescein isothiocyanate (FITC) (Ravindran & et al., 2005).

Another method for visualization of RNA originating in the host in parasite tissues is the utilization of fluorescent proteins expressed in a transgenic plant. Protein translation under the control of a phloem specific promoter, such as SUT1 (Kühn *et al.*, 1997), allows for the characterization of proteins within the vasculature (Imlau *et al.*, 1999) such as when the expression of a Green Fluorescent Protein (GFP) was used to identify vascular continuity between host and *Cuscuta* (Birschwilks *et al.*, 2006). The tagging of single RNAs with fluorophores is now possible with the discovery of a small molecule GFP-mimic called Spinach, which has been used to characterize intracellular RNA dynamics in a living human embryonic kidney (HEK) cells (Paige *et al.*, 2011). This could have potential as a RNA tracing mechanism for plant cells in the future.

Approaches using fluorescent probes, either nanoparticles or proteins, require microscopy on specially fixed and sectioned tissues. Some sections can be made free hand and imaged immediately, while more invasive *in situ* hybridizations require paraffin embedding and sectioning. Traditional fixation followed by paraffin embedding has given way to cryosectioning, a faster technique for sectioning that avoids harsh chemical fixation or long embedding processes. Tissues can be embedded in a matrix and frozen, and then sectioned in a frozen state. Subsequent dissection of tissues using automated laser assistance is a way for accurate sampling of microscopic regions, such as the cells of the haustorial connection. There are advantages and disadvantages to these approaches, but the desired outcome is visual confirmation of cell-to-cell

transmission of RNA or protein. This chapter will present data on efforts to visualize RNA and mobile proteins from a host into *C. pentagona*.

Results and Discussion

A. In planta QD applications

QD are a relatively new tool for probing biological tissues for *in situ* localization. The nanoparticles can be 20-40 nm across with a MW of ~150 kDa and are made of a cadmium selenide core surrounded by a zinc sulfide shell (Tholouli, 2008). The particles were initially made for the semiconductor industry but were shown to have excellent fluorescent properties. Because of their inorganic nature, they are not prone to photobleaching and can be subjected to stronger excitation intensity which aids in detecting very low levels of signal (Medintz *et al.*, 2005)

Their optical properties differ from those of organic fluorophores because of their unique crystalline shape, in that they can absorb light over a longer range of wavelengths while emitting light within a fairly narrow range. This large distance between excitation and emission spectra (for example, peak excitation at 535 nm and emission peak at 600 nm for QD 605) makes these ideal for visualization using spectral unmixing techniques, which rely on digital imaging microscopy to separate signals pixel-by-pixel for any given wavelength (Farkas *et al.*, 1998; Tholouli *et al.*, 2006). Because of the small size and bright signal given off, single QD tracking

has been of recent interest as a highly sensitive tool for imaging specific cellular events (Pinaud *et al.*, 2010).

Successful *in situ* hybridization studies have been carried out for probing mRNA transcripts in mouse brain stem (Chan, Yuen *et al.* 2005) and bone marrow (Tholouli *et al.*, 2006) by exploiting the biomolecular binding ability of the QD. In order to hybridize the fluorophore to mRNA targets of interest, thiol-labeled ssDNA have been linked to QDs via a metal-thiol bond (Sheng-Mei Wu, 2006; Lu Ma & Li, 2008) and other work has used streptavidin tags attached to the exterior of the nanoparticle to allow for conjugation to biotin-tagged oligonucleotides (Chan *et al.*, 2005; Muller *et al.*, 2006; Tholouli *et al.*, 2006). Experiments using QD-probe hybridization in plant cells are rare, with only brief mention in the literature (Riegler *et al.*, 2008).

Previous *in situ* hybridization experiments using QDs for mRNA detection utilized commercially available QDs which were conjugated to biotin-linked oligonucleotides (Tholouli *et al.*, 2006). The strong affinity between streptavidin and biotin allowed for the formation of an efficient, if somewhat ungainly, probe (Fig. 1). QDs tagged with approximately twenty streptavidin molecules were chosen for this experiment based on an emission spectrum of 605nm. This emission spectrum is ideal in plant tissues as autofluorescence overlap is minimal at this wavelength. The addition of biocytin as a blocking agent to unbound streptavidin sites ensures a 1:1 probe:QD ratio (Chan *et al.*, 2005).

Streptavidin-coated QDs bound to biotinylated single stranded cDNA probes complementary to mRNA sequences of known phloem-mobile elements, specifically *GAI* and *PI* were designed (See Chapter 2 for information on these genes). A scrambled, nonsense probe with a 50% GC

content that was determined through BLAST search to not have complementarity to our targets was assembled as a control. For proof of concept, a 50-base string of thymidines with the same 3'biotin tag was assembled. This should provide a strong signal as it should hybridize to a long adenine-rich RNA sequences of the poly-A tail of mRNA transcripts.

First, experimentation focused on testing the QD-oligonucleotide probe conjugates (QD-Probe) to single stranded (ss) target DNA strands using a gel shift assay. Single-stranded DNA targets were generated through an asymmetrical PCR procedure (Gyllenstein & Erlich, 1988). Using primers specific to the GAI target sequence, ssDNA products were generated and equal concentrations of QD-probe to target sequences are incubated overnight in Southern hybridization solution. The Southern hybridization solution has constituents appropriate for the binding of ssDNA molecules to one another (Southern, 1975). These conjugates were then subjected to low voltage electrophoresis in a 0.7% agarose gel. Migration patterns varied between unconjugated QDs (little or no migration due to their large size and lack of charge) and QD-probe to target conjugates (rapid migration due to pull by the positively charged probe and DNA targets) (Fig. 2).

In order to locate host mRNAs in parasite tissue, this work aimed to use *in situ* hybridization of an mRNA probe in paraffin embedded fixed tissue (Hejatko *et al.*, 2006). This work used RNase-free cross and longitudinal sections of host, dodder and host-dodder haustorial connections fixed to slides for hybridization per Tholouli et al (2006). Unconjugated QDs were included as a control. Imaging was carried out on a Zeiss Laser Confocal Scanning Microscope (LCSM) using Zen software. Spectral unmixing is a unique property offered by the LCSM that

allows for a scan of emission spectra and allows for pinpointing exact localizations of the signal of interest.

After following exacting *in situ* hybridization protocols, starting from paraffin embedded sections of tomato stems, no QD signals were detected under microscopic analysis specific to cells of interest. Rather, QDs would bind to the slide or peripheral cells (not shown). We considered the possibility that the sheer size of the QD was too large for the small 50-mer RNA conjugate to effectively bind. However, size exclusion chromatography through a 200 Superdex column as explained by Chan et. al (Chan *et al.*, 2005) confirmed that conjugated and non-conjugated QDs eluted at the same time (results not shown). Upon contacting authors of previous work (Sealfon, 2010) it was learned that the QD manufacture process had changed and previous work was not able to be replicated. Without the capacity to manufacture or obtain suitable QDs for this work, further efforts were focused in other research areas.

B. Tracing protein from mobile mRNA

The large amount of research conducted on *Arabidopsis* has created a wealth of research materials for understanding gene expression and function. For example, many *Arabidopsis* lines are available that have various fluorescently labeled cell structures which are useful for localizing cell constituents for imaging (Nelson *et al.*, 2007). Other research groups have produced *Arabidopsis* lines with reporter gene fusions to genes of potential value in studying host-parasite trafficking of proteins. One such gene is Translationally Controlled Tumor Protein (TCTP), which we characterized as having relatively high quantities of mobile transcripts in *C. pentagona* stems grown on *Arabidopsis* (see Chapter 2). Previous work identified the TCTP protein as having various roles in signaling, including regulation of the duration of the mitotic

cell cycle, but not cellular expansion (Turnbull & Lopez-Cobollo, 2013). Interestingly, TCTP has been shown to co-immunoprecipitate with the pumpkin phloem protein CmPP16 (Aoki *et al.*, 2005) and is proposed to have a role in the long distant movement of phloem proteins. Since finding that the protein is phloem mobile and the *TCTP* transcripts move into attached *Cuscuta*, this gene may be useful in understanding host-parasite connections. Transgenic *Arabidopsis* expressing GFP-tagged TCTP under control of the native promoter have been reported (Berkowitz *et al.*, 2008), potentially allowing for *C. pentagona* to be established on this transgenic host. Detection of the TCTP-GFP protein in the parasite could be useful in understanding uptake of the translated form of a known mobile RNA or translation of a host-originated mRNA.

Seeds of transgenic *Arabidopsis* expressing TCTP-GFP under control of the TCTP promoter transformed with a construct including a ~1kb region upstream of the TCTP open reading frame and two introns of the neighboring phosphatase pleiotropic regulator gene (PRL2, At5g16650) plus a 300 bp intergenic region were obtained courtesy of Dr. Josette Masle (Berkowitz *et al.*, 2008). After crossing, homozygosity of a single insert of the TCTP-GFP construct was confirmed with immunoblotting and real time PCR (Berkowitz *et al.*, 2008). Plants were grown from these seeds to the point of bolting and then seeds of *C. pentagona* were introduced. Once *C. pentagona* seedlings had germinated and coiled around the host stem at least twice (to ensure enough time for a well-developed haustorium) RNA was extracted from TCTP-GFP *Arabidopsis* plants and the parasite. To detect the RNA transcript of *AtTCTP*, reverse transcription (RT-)PCR was performed with primers specific to *AtTCTP* (Chapter 2, Table 1; results not shown). Once the presence of *AtTCTP* confirmed in host and parasite, RT-PCR was done to detect the GFP transcript in *Arabidopsis* host, *C. pentagona* parasitizing the host, as well as tomato and *C.*

pentagona on tomato for controls (Fig. 3). The very faint smear in the lane corresponding to *C. pentagona* on TCTP-GFP host suggested the possibility that untranslated *TCTP-GFP* was mobile into the parasite.

To confirm the possibility of TCTP-GFP RNA mobility suggested by RT-PCR, we felt GFP could be detected in the parasite, either from translocation of TCTP-GFP protein movement or from parasite-synthesized GFP if the parasitic translational machinery was active on this host transcript. Unfortunately, repeated imaging attempts were inconclusive for finding GFP expression in the host as well as the parasite. It was determined that the expression of GFP was most detectable in root cells and in very early developmental events, not at the bolting stage at which we were conducting our experiments.

C. Laser microdissection for small scale tissue extraction

Laser capture microdissection (LCM) in conjunction with pressure catapulting (LMPC) utilizes advanced microscopy and laser technologies for specific cell extraction for downstream analysis, such as genomics, transcriptomics, metabolomics and proteomics. The advantage lies in the specific nature of single-cell or tissue sampling that is possible, as opposed to whole tissue extractions, which give an average snapshot of many cell types sampled.

Traditional methods for obtaining the RNA, DNA, proteins or other cell constituents for ‘omics generally require the harvesting of a great deal of tissue without specificity. *In situ* hybridizations were the standard for determining transcription activity or spatial localization of molecules of interest. However, cellular integrity can be compromised in the fixation process and non-specific binding of probes can give false results. LMPC is an alternative process that doesn’t rely on time consuming hybridization techniques.

For plant research the advantages of LMPC are numerous. A recent paper reviewed the use of LMPC in looking at changes in gene expression in response to plant-microbe and plant-nematode invasions (Ramsay *et al.*, 2006). Such attacks induce the expression of defense compounds and invasion-prevention cascades, and by monitoring gene expression changes in specific cells/tissues researchers can better understand these interactions and possibly find novel mechanisms of control. In maize, RNA from epidermal and vascular bundles were extracted via LMPC for downstream microarray for global gene expression (Nakazono *et al.*, 2003).

Plants rely on long-distance signaling for developmental regulation, and increasing evidence exists for phloem-mobile mRNA to be necessary in that system (Kehr & Buhtz, 2008). The ability to specifically extract single sections of phloem tissue helps to overcome the problems associated with studying these molecules; specifically difficulty in sap collection, generating successful interspecies grafts, or RNA extracted from whole plant tissues interfering with signals specifically in the vascular system. It has been demonstrated that LCM is indeed a reliable tool for sampling phloem cells directly and assaying for RNA and detecting known phloem mobile signals such as BEL5 and GAI in potato (Yu *et al.*, 2007).

The technology for LMPC has come a long way since the first archival kidney tissue slides were subjected to laser ablation and RNA extraction (Emmert-Buck *et al.*, 1996). Two major organizations dominate the laser capture market, Zeiss and Leica, and although methods for cell sampling vary, the basic procedure remains the same; namely cells of interest are observed on a slide mounted with a fixed tissue, a laser is used to “cut out” certain cells and these cells can then be used for downstream analysis. Some companies have developed specialized kits for the small-scale extractions that this procedure dictates.

Tissue preparation for plants has to take into account the large vacuoles that tend to take up much of the cellular space. Pressure from the tonoplast on the plasma membrane during microdissection may cause contamination from adjacent cells (Kerk *et al.*, 2003). It has been demonstrated that incubation in a plasmolysing media will shrink vacuoles and concentrate the cytoplasmic contents for improved RNA yields in maize.

The balance between maintaining morphology while preserving the native molecular characteristics of the cell is key to successful downstream applications. Coagulating or precipitative fixatives, such as ethanol and acetone, will denature proteins while chemical crosslinkers like paraformaldehyde fuse cellular components *in situ* (Nakazono *et al.*, 2003). Each has their benefits and drawbacks, with the downstream method of analysis determining which fixative to use. Protein extractions would discourage the use of denaturing fixatives while tissue and RNA quality for plant cell LMPC studies has been shown to be best when fixed in ethanol-acetate (Kerk *et al.*, 2003; Yu *et al.*, 2007).

The embedding medium is also of importance, and usually dictated by the method of analysis. Many hospitals keep archived histology samples for later disease research and these well-preserved slides have yielded quality DNA and RNA (Bonner *et al.*, 1997). Some argue the value for paraffin embedding lies in its long term stability, particularly for plants, and advise that de-paraffinization immediately prior to LMPC is best for collecting RNA from plant cells (Kerk *et al.*, 2003).

Cryosectioning can be used for LMPC sample prep as it is much faster and maintains cellular integrity better than in paraffin embedding and sectioning. A technique for embedding in a sugar media followed by freezing in liquid nitrogen and sectioning on a cryojane system has been

demonstrated for vascular tissue sampling by LMPC in maize (Nakazono *et al.*, 2003). One caveat to cryopreservation is the chance of air spaces being present which, when frozen, allows for ice crystal formation that can degrade cell morphology. This is a problem specific to plant tissue cryosectioning because the intercellular volume is largely air.

Either UV-range or infrared-range light is used for cutting out the cells from sectioned tissue. Ultraviolet photons have the ability to cut through many media provided the power is strong enough, however damage to adjacent tissue and potential harm to the user is possible. Infrared light is non-damaging to adjacent tissues and, via nonlinear effects of pulsed lasers, two or more low energy photons can combine to create a high energy pulse at the focal plane to slice through the sample (Vogel *et al.*, 2007). Driving this is a robotic stage in conjunction with software for selecting regions of interest and directing the path of the laser beam.

Extracting the cut cells from the slide can be done in a number of ways. The first laser microdissection researchers had to hand-pour their own acetate transfer films and use them to press onto the slide surface to remove the section (Emmert-Buck *et al.*, 1996). This method is still used today (using mass produced sticky films), but the incidence of including adjacent cells and contaminants is higher than with other methods. Another film-based extraction technique uses membrane coated slides to which the samples are adhered and cut sections can be removed from the slide by removing the whole membrane. Both of these techniques then use melting and centrifugation steps to remove the membrane from the sample.

Extraction without contact is another method and it is what puts the PC in LMPC. Following ablation on Leica's upright microscope the membrane and adhered sample drops by gravity down into a collection tube. Pressure catapulting utilizes the same laser-based plasma formation

principle described above except that instead of focusing on the sample, the beam is directed below it, on the glass slide, and a burst of electron dense matter launches the sample upwards. The PALM system is equipped with a stage-mounted arm to hold a microfuge tube which will collect the cells as they leap from the slide a few centimeters away. Another catapulting technique uses a film that can be launched directly onto a sticky membrane mounted above the slide.

Once cells are isolated the next step is extracting molecules of interest. As sample volumes are incredibly small, some usual techniques for RNA, DNA and protein extraction have to be modified and some downstream applications that require large volumes of cellular material (i.e. microarray, sequencing) are not possible to use without pooling. However, the usual procedure of breaking the cell down, precipitating the molecule of interest followed by washing and collection are all the same. Plant cells in particular have the added challenge of a cell wall to break down which can alter the extraction protocol and diminish the quality of results. As of now no prepackaged one-step kit exists specifically for plant cell analysis whereas direct PCR and RT-PCR can be done in microdissected mammalian cells.

Despite the small volumes of material extracted it is still possible to obtain very high quality RNA, DNA, protein and other metabolites from LMPC tissues. Depending on tissue type, approximately 50 ng RNA 100 cells⁻¹ have been collected from both plant and mammalian sections, (Kerk *et al.*, 2003).

One way to overcome the obstacle of small sample size is to pool single catapulted samples. Caps from multiple catapulting events can be maintained on ice and then interchangeably snapped onto one main tube that goes through multiple lysing steps.

In potato stems, the laser mediated dissection of phloem, xylem and epidermal cells yielded RNA of very high quality was able to be used in a reverse transcription PCR to monitor expression of phloem mobile RNAs (Yu *et al.*, 2007). The LMPC procedure in this tissue is the same as for animal cells.

In a collaborative effort with Dr. Eva Collakova's lab, LMPC was used in order to analyze metabolomic profiles in Egyptian broomrape (*Phelipanche* = *Orobanchae aegyptiaca*) tubercles. LMPC was used to isolate a $1.5 \times 10^6 \mu\text{m}^2$ of designated cells from preserved haustoria that was sliced and affixed to slides using the Cryojane system. Following progressive desiccation washes in ethanol, samples were cut as shown in Fig. 4. After collection these tissues were held in HPLC Grade methanol and centrifuged at 14K rpm to pellet. The supernatant was removed and the pellet was dried for storage at -80°C . Analysis on Ultra GC/MS/MS in an associated lab is on hold for future assay.

As a side project, the techniques described here (cryosectioning and laser capture microdissection) were demonstrated on video for archival purposes to allow for future students to understand the process. Links are available to this content on the Molecular Plant Sciences YouTube page here: <http://www.youtube.com/user/VTechMPS/> .

References

- Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H. 2005.** Destination-Selective Long-Distance Movement of Phloem Proteins. *Plant Cell* **17**(6): 1801-1814.
- Berkowitz O, Jost R, Pollmann S, Masle J. 2008.** Characterization of TCTP, the Translationally Controlled Tumor Protein, from *Arabidopsis thaliana*. *The Plant Cell Online* **20**(12): 3430-3447.
- Birschwilks M, Haupt S, Hofius D, Neumann S. 2006.** Transfer of phloem-mobile substances from the host plants to the holoparasite *Cuscuta* sp. *Journal of Experimental Botany* **57**(4): 911-921.
- Bonner RF, Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA. 1997.** Laser Capture Microdissection: Molecular Analysis of Tissue. *Science* **278**(5342): 1481-1483.
- Chan P, Yuen T, Ruf F, Gonzalez-Maeso J, Sealfon SC. 2005.** Method for multiplex cellular detection of mRNAs using quantum dot fluorescent in situ hybridization. *Nucleic Acids Research* **33**(18): e161.
- Dorr I. 1990.** Sieve Elements in haustoria of parasitic angiosperms. In H-D Behnke, R-D Sjölund, eds, *Sieve Elements: Comparative Structure, Induction and Development*. Springer, Berlin.
- Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. 1996.** Laser Capture Microdissection. *Science* **274**(5289): 998-1001.

- Farkas DL, Du C, Fisher GW, Lau C, Niu W, Wachman ES, Levenson RM. 1998.** Non-invasive image acquisition and advanced processing in optical bioimaging. *Computerized Medical Imaging and Graphics* **22**(2): 89-102.
- Gyllenstein UB, Erlich HA. 1988.** Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA Locus. *Proceedings of the National Academy of Sciences of the United States of America* **85**(20): 7652-7656.
- Hejatko J, Blilou I, Brewer PB, Friml J, Scheres B, Benkova E. 2006.** In situ hybridization technique for mRNA detection in whole mount Arabidopsis samples. *Nat. Protocols* **1**(4): 1939-1946.
- Imlau A, Truernit E, Sauer N. 1999.** Cell-to-Cell and Long-Distance Trafficking of the Green Fluorescent Protein in the Phloem and Symplastic Unloading of the Protein into Sink Tissues. *Plant Cell* **11**(3): 309-322.
- Kehr J, Buhtz A. 2008.** Long distance transport and movement of RNA through the phloem. *Journal of Experimental Botany* **59**(1): 85-92.
- Kerk NM, Ceserani T, Tausta SL, Sussex IM, Nelson TM. 2003.** Laser Capture Microdissection of Cells from Plant Tissues. *PLANT PHYSIOLOGY* **132**(1): 27-35.
- Kragler F. 2010.** RNA in the phloem: A crisis or a return on investment? *Plant Science* **178**(2): 99-104.
- Kühn C, Franceschi VR, Schulz A, Lemoine R, Frommer WB. 1997.** Macromolecular Trafficking Indicated by Localization and Turnover of Sucrose Transporters in Enucleate Sieve Elements. *Science* **275**(5304): 1298-1300.

- Lu Ma S-MW, Jing Huang, Ya Ding,, Li D-WPL. 2008.** Fluorescence in situ hybridization (FISH) on maize metaphase chromosomes with quantum dot-labeled DNA conjugates. *Chromosoma* **117**: 181-187.
- Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. 2005.** Quantum dot bioconjugates for imaging, labelling and sensing. *Nature Materials* **4**(6): 435-446.
- Muller F, Houben A, Barker PE, Xiao Y, Kas JA, Melzer M. 2006.** Quantum dots--a versatile tool in plant science? *Journal of Nanobiotechnology* **4**: 5.
- Nakazono M, Qiu F, Borsuk LA, Schnable PS. 2003.** Laser-Capture Microdissection, a Tool for the Global Analysis of Gene Expression in Specific Plant Cell Types: Identification of Genes Expressed Differentially in Epidermal Cells or Vascular Tissues of Maize. *The Plant Cell Online* **15**(3): 583-596.
- Nelson BK, Cai X, Nebenführ A. 2007.** A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. *Plant J.* **51**(6): 1126-1136.
- Paige JS, Wu KY, Jaffrey SR. 2011.** RNA Mimics of Green Fluorescent Protein. *Science* **333**(6042): 642-646.
- Pinaud F, Clarke S, Sittner A, Dahan M. 2010.** Probing cellular events, one quantum dot at a time. *Nature Methods* **7**(4): 275-285.
- Ramsay K, Jones MGK, Wang Z. 2006.** Laser capture microdissection: a novel approach to microanalysis of plant-microbe interactions. *Molecular Plant Pathology* **7**(5): 429-435.
- Ravindran S, et al. 2005.** Quantum dots as bio-labels for the localization of a small plant adhesion protein. *Nanotechnology* **16**(1): 1.
- Riegler J, Ditengou F, Palme K, Nann T. 2008.** Blue shift of CdSe/ZnS nanocrystal-labels upon DNA-hybridization. *Journal of Nanobiotechnology* **6**: Article No.: 7.

Sealfon S 2010. Re: QD Conjugation difficulties. In LeBlanc M. Email.

Sheng-Mei Wu XZ, Zhi-Ling Zhang, Hai-Yan Xie, Zhi-Quan Tian, Jun Peng, Zhe-Xue Lu,

Dai-Wen Pang, Zhi-Xiong Xie,. 2006. Quantum-Dot-Labeled DNA Probes for
Fluorescence In Situ Hybridization (FISH) in the Microorganism *Escherichia coli*.
ChemPhysChem 7(5): 1062-1067.

Southern EM. 1975. Detection of specific sequences among DNA fragments separated by gel
electrophoresis. *J Mol Biol* 98(3): 503-517.

Tholouli E, Hoyland JA, Di Vizio D, O'Connell F, MacDermott SA, Twomey D, Levenson

R, Yin JAL, Golub TR, Loda M, Byers R. 2006. Imaging of multiple mRNA targets
using quantum dot based in situ hybridization and spectral deconvolution in clinical
biopsies. *Biochemical and Biophysical Research Communications* 348(2): 628-636.

Tholouli ES, E Barrow, V Clay, JA Hoyland, RJ Byers,. 2008. Quantum dots light up
pathology. *The Journal of Pathology* 216(3): 275-285.

Turnbull CGN, Lopez-Cobollo RM. 2013. Heavy traffic in the fast lane: long-distance
signalling by macromolecules. *New Phytologist* 198(1): 33-51.

Vogel A, Horneffer V, Lorenz K, Linz N, Hüttmann G, Gebert A 2007. Principles of Laser
Microdissection and Catapulting of Histologic Specimens and Live Cells. In: Michael
WB, Karl Otto G eds. *Methods in Cell Biology*: Academic Press, 153-205.

Yu Y, Lashbrook C, Hannapel D. 2007. Tissue integrity and RNA quality of laser
microdissected phloem of potato. *Planta* 226(3): 797-803.

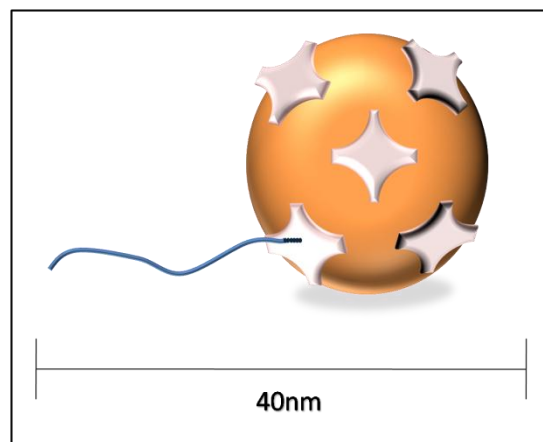


Fig. 1. Illustration of a 50-mer oligonucleotide probe with 3'Biotin tag conjugated to Streptavidin-coated CdSe Quantum Dot.

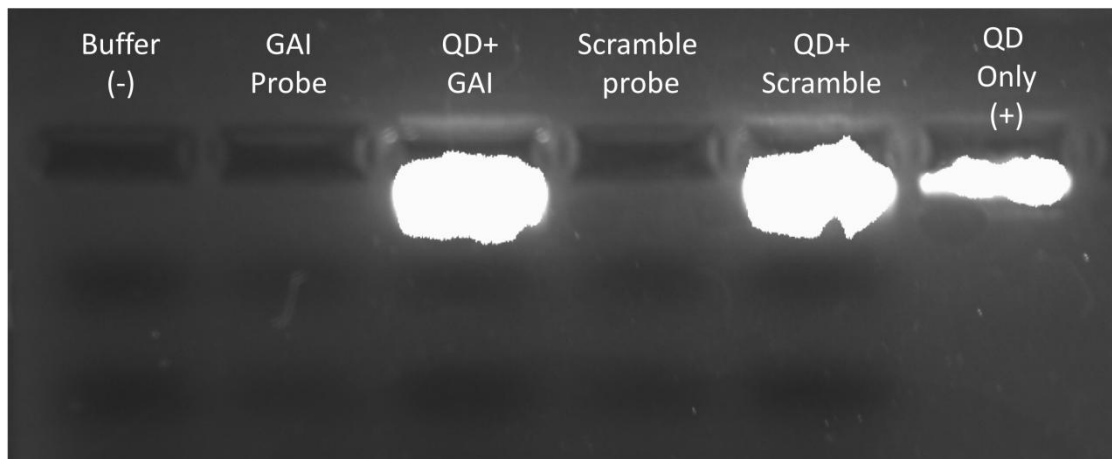


Fig 2. Gel shift assay shows slightly more migration in Quantum Dots conjugated to a *SIGAI* or random (Scramble) probe compared to QD alone. Note the exceptionally bright signal given off by the QD under UV light.

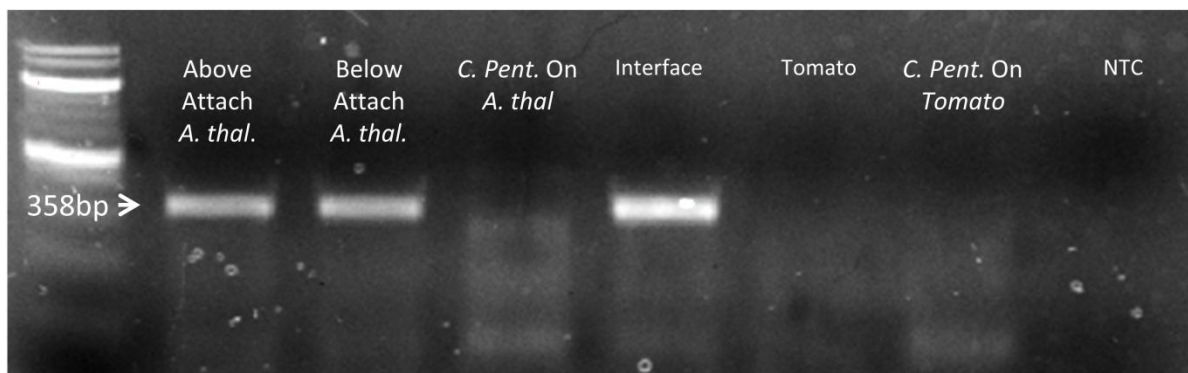


Fig. 3. RT-PCR results for *GFP* RNA in host tissue above and below the haustorium, the parasitic stem and the interface between the two organisms.

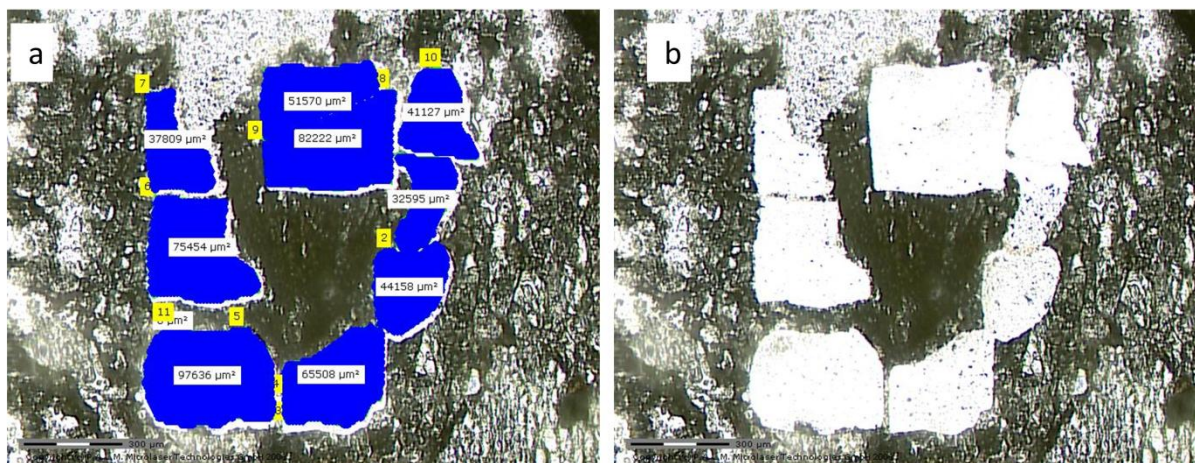


Fig. 4.(a) *Orobanche aegyptiaca* tubercle and the area targeted for ablation surrounding a vascular bundle (blue shapes) and the area post ablation (b). Total area of highlighted area is calculated for each region (a).

Chapter 5. *Cuscuta pentagona* Sequencing and the Search for Controls

Abstract: Next generation sequencing technology is making the characterization of the genome or transcriptome of any organism possible. Our lab has been progressing towards generating the *Cuscuta pentagona* transcriptome and work is underway for de novo assembly of its genome (Kim et al., unpublished). Even before these data sets are completed, there is a need for identifying two types of genes that are useful in post translational gene silencing for parasitic weed control or to identify housekeeping reference genes for Real Time PCR controls. Here, two such data mining attempts yielded a potential *C. pentagona* transcript target for silencing by RNA interference and characterization of a housekeeping gene for use in PCR applications.

Keywords: RNA-Seq, transcriptome, assembly, housekeeping, *Cuscuta pentagona*, RNAi, Heat-shock protein 70, Granule bound starch synthase I

Introduction

Transcriptome profiling of *Cuscuta pentagona* is underway in the Westwood lab (Kim et al., unpublished) which has heretofore not been sequenced. Transcripts from *C. pentagona* growing on tomato and *Arabidopsis* hosts were sequenced using Illumina technology and assembled contigs were BLAST searched against multiple plant genome databases to identify putative gene candidates. This new database opened up many research possibilities, but the most relevant to the needs of the present study were a potential RNAi-target and housekeeping gene candidates. This chapter details the work to identify and characterize these gene candidates.

Housekeeping genes

A good reference gene represents total RNA or cDNA in a sample and must be stably expressed across developmental stages so that it can be used to normalize against variations in quality or quantity of sample and rate of biosynthesis or degradation at the time of sampling (Brunner *et al.*, 2004; Bustin *et al.*, 2005; Guénin *et al.*, 2009). Because most commonly used reference genes were characterized in very early work by northern blotting, they need to be confirmed accurate for quantitative real time PCR applications which are more accurate with even the smallest of samples to avoid data misinterpretations (Brunner *et al.*, 2004; Bustin *et al.*, 2005). In just two plant studies (of many), 11 and 9 common controls candidates were screened against 27 tomato and 23 cotton tissue samples respectively (Exposito-Rodriguez *et al.*, 2008; Artico *et al.*, 2010). Out of each set, only 2 tomato and 4 cotton housekeeping genes were deemed to be exceptionally accurate controls for quantitative PCR applications.

The identification of housekeeping genes is of great interest to parasitic plant science as these are important references needed for the characterization of parasitism using PCR based methods.

Common control genes for plant based qPCR have yet to be identified for parasitic plants, particularly *C. pentagona*. The host-parasite system presents a unique challenge to developing reference genes because hosts can share highly conserved genes and the phenomenon of mobile host RNA moving into the parasite may confound housekeeping that are not species-specific.

RNAi for control of parasitic weeds

Transcriptome sequencing of parasitic plants may yield useful strategies for control by RNA interference (RNAi). It has been demonstrated that small interfering RNA (siRNA) and microRNA (miRNA) are propagated via phloem and play a role in long distance signaling (Yoo *et al.*, 2004). Given our understanding of the host-parasite phloem continuity, RNAi for post transcriptional gene silencing has the potential for being a good control mechanism provided appropriate targets can be identified (Yoder *et al.*, 2009; Bandaranayake & Yoder, 2013).

Post transcriptional gene silencing (PTGS) in plants utilizes a pathway of single-stranded RNA degradation to cause unproductive RNA turnover and, as an added consequence, disrupt the production of certain proteins (Depicker & Van Montagu, 1997). RNA interference (RNAi) utilizes small 12-25 nucleotide strands of micro (mi)RNA or small interfering (si)RNA complementary to mRNA for protein translation (Watanabe, 2011). When the complementary short RNAs bind to a homologous sequence of RNA, the targeted sequence is degraded by a complex of Dicer-like (DCL) proteins which bind dsRNA and Argonaute (AGO) proteins that cut the nucleotides where antisense silencing RNA binds to mRNA.

Short sequences of tandem inverted RNAs separated by a short spacer can be placed into a modified pHellsgate Gateway cloning vector to be transcribed to form a hairpin structure which gets targeted for degradation by DCL and then allows for complementary binding to a natural

RNA for silencing (Helliwell & Waterhouse, 2003; Watson *et al.*, 2005). These hairpin constructs are relatively easy to produce and can be developed to target any number of genes. Further research has shown that small RNAs moving through the phloem can direct epigenetic silencing events in grafted *Arabidopsis* (Molnar *et al.*, 2010).

Systemic mobility and sequence specificity of silencing signals make them intriguing methods of parasitic weed control. Research indicates the possibility of parasitic uptake of a phloem mobile silencing signal by showing that a *GUS* siRNA can translocate into *GUS*-expressing root parasite *Triphysaria* via transgenic lettuce (Tomilov *et al.*, 2008). The *GUS* expression is silenced at the site of attachment and in the direction of symplastic flow for a short time. Recently, Alakonya *et al.* (2012) identified a homolog to a member of the KNOX-1 transcription factor, which regulates leaf development by controlling cytokinin biosynthesis in the shoot apical meristem (SAM) called Shoot Meristemless-like (STM) in *Cuscuta* (Alakonya *et al.*, 2012). RNAi for the *CpSTM* transcript under the control of the SUC2 promoter in tobacco transferred into parasitizing *C. pentagona* and resulted in decreased vigor and early flowering.

Results and Discussion

A. Potential RNAi target: granule bound starch synthase homolog

Targets for PTGS can be in any metabolic pathway or developmental control point as long as a detectable change is induced in the parasitic interaction (to evaluate the effect of silencing), and it would be ideal for parasite viability to be affected. For silencing in *Cuscuta*, I had proposed that cuscutain, a cysteine protease associated with cell wall breakdown during *C. reflexa* haustorial development (Bleischwitz *et al.*, 2010), would be an ideal target as it would potentially decrease attachment viability. Using this transcript for an RNAi target for a host-

borne hairpin construct, though, would not be valuable as it wouldn't be transmitted from host to parasite until after haustorial penetration.

From our contig-level data set, a *C. pentagona* starch synthase homolog has been identified based on homology to *Ipomoea batatas* (sweet potato SpSS67, Genbank accession U44126,) and may prove to be a viable candidate for RNAi silencing. One characteristic of *Cuscuta* species is a high concentration of starch granules, easily visible under low power microscopy and comprising up to 10% of tissue dry weight (Singh *et al.*, 1968). A transgenic host expressing an RNAi hairpin of a homolog of SpSS67 may confer some type of phenotypic change or decrease parasitic viability for controlling dodder development.

Based on the RNA-Seq data set for the *C. pentagona* transcriptome, homologs of this granule bound starch synthase were amplified by PCR (data not shown) and RT-PCR. Faint amplification was evident in a tomato RNA sample (Fig. 1a lane 2) suggesting that tomato may be an inappropriate host in this experiment. The absence of product in *Arabidopsis* RNA indicates it would be a viable host, especially since transformation by floral dip is relatively easy. The product was sequenced and shown to have 29.7% amino acid sequence identity to the *I. batatas* homolog (Fig. 1b).

Unfortunately, due to both the publication of a similar experiment carried out by another lab and the delay caused by time commitments to other efforts, this work was put on hold indefinitely. In order to fully explore this project, transgenic host plants would need to be produced and parasitized, and uptake of the silencing RNA would have to be detected via PCR-based assays as well as confirmed phenotypically.

B. *Cuscuta pentagona* housekeeping gene: heat shock protein-70 homolog

To find reference gene candidates in *C. pentagona*, annotated transcripts were researched from Japanese morning glory, *Ipomoea nil*, a close relative to *C. pentagona* (Stefanovic *et al.*, 2003). We queried *C. pentagona* RNA-Seq data sets for *I. nil* homologs to identify potential housekeeping genes and design primers for them based off of commonly used controls in plant research (Guénin *et al.*, 2009). *I. nil* Actin 8 (Act8), Beta-tubulin (β T), Heat shock protein-17 (HSP17) and Heat shock protein-70 (HSP70) genes all had homologs in *C. pentagona* transcript sets. PCR amplification of HSP70 resulted in identification of primer sets specific to *C. pentagona* exclusively and not *Arabidopsis* or tomato (Fig. 2). Reverse transcription of the CpHSP70 primer set yielded high quality amplification (data not shown). Sequenced product was used to develop TaqMan probes (Applied Biosystems) for use as a control in real time PCR applications (Fig. 3a). Translated protein alignments between *I. nil* and *C. pentagona* using Geneious alignment showed 27.6% sequence identity (Fig. 3b).

In order for a housekeeping gene to be used reliably in a RT-PCR experiment, expression should be consistent across all developmental stages with no tissues expressing the gene in a statistically significant way (Brunner *et al.*, 2004). Expression of *CpHSP70* was shown to be stably expressed in RNA from *C. pentagona*-tomato interface RNA; parasite on *Arabidopsis* (At); before, early and late-stage attachments on tomato; flower; and 10 and 14 days post detachment from a tomato host (Fig. 4). However, the statistical separation of means using Tukey's HSD was significantly different in RNA extracted from the apical tip as compared to all other tissues. It can be speculated that the growing tip is a unique developmental region and studies dealing specifically with shoot apices would require different or additional reference genes. *CpHSP70*

primers were used reliably in the work of Chapter 2. An expanded library of primer sets would be a valuable toolbox for further work.

References

- Alakonya A, Kumar R, Koenig D, Kimura S, Townsley B, Runo S, Garces HM, Kang J, Yanez A, David-Schwartz R, Machuka J, Sinha N. 2012.** Interspecific RNA Interference of SHOOT MERISTEMLESS-Like Disrupts *Cuscuta pentagona* Plant Parasitism. *Plant Cell* **24**(7): 3153-3166.
- Artico S, Nardeli SM, Brilhante O, Grossi-de-Sa MF, Alves-Ferreira M. 2010.** Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. *BMC Plant Biology* **10**: 49.
- Bandaranayake PC, Yoder JI. 2013.** Trans-Specific Gene Silencing of Acetyl-CoA Carboxylase in a Root-Parasitic Plant. *Molecular Plant Microbe Interactions* **26**(5): 575-584.
- Bleischwitz M, Albert M, Fuchsbauer HL, Kaldenhoff R. 2010.** Significance of Cuscutain, a cysteine protease from *Cuscuta reflexa*, in host-parasite interactions. *BMC Plant Biology* **10**: 227-227.
- Brunner A, Yakovlev I, Strauss S. 2004.** Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biology* **4**(1): 1-7.
- Bustin SA, Benes V, Nolan T, Pfaffl MW. 2005.** Quantitative real-time RT-PCR – a perspective. *Journal of Molecular Endocrinology* **34**(3): 597-601.
- Dawson JHMLJ, Wolswinkel P., Dorr I. 1994.** Biology and control of *Cuscuta*. *Reviews of Weed Science* **6**: 265-317.
- Depicker A, Van Montagu M. 1997.** Post-transcriptional gene silencing in plants. *Current Opinion in Cell Biology* **9**(3): 373-382.

- Exposito-Rodriguez M, Borges AA, Borges-Perez A, Perez JA. 2008.** Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biology* **8**: 131.
- Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. 2009.** Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of Experimental Botany* **60**(2): 487-493.
- Helliwell C, Waterhouse P. 2003.** Constructs and methods for high-throughput gene silencing in plants. *Nature Methods* **30**(4): 289-295.
- Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC. 2010.** Small Silencing RNAs in Plants Are Mobile and Direct Epigenetic Modification in Recipient Cells. *Science* **328**(5980): 872-875.
- Runyon JB, Mescher MC, Felton GW, De Moraes CM. 2010.** Parasitism by *Cuscuta pentagona* sequentially induces JA and SA defence pathways in tomato. *Plant, Cell & Environment* **33**(2): 290-303.
- Singh M, Singh DV, Misra PC, Tewari KK, Krishnan PS. 1968.** Biochemical Aspects of Parasitism by the Angiosperm Parasites: Starch Accumulation. *Physiologia Plantarum* **21**(3): 525-538.
- Stefanovic S, Austin DF, Olmstead RG. 2003.** Classification of convolvulaceae: A phylogenetic approach. *Systematic Botany* **28**(4): 791-806.
- Tomilov AA, Tomilova NB, Wroblewski T, Michelmore R, Yoder JI. 2008.** Trans-specific gene silencing between host and parasitic plants. *The Plant Journal* **56**(3): 389-397.

- Watanabe Y 2011.** Overview of Plant RNAi. In: Kodama H, Komamine A eds. *RNAi and Plant Gene Function Analysis*: Humana Press, 1-11.
- Watson JM, Fusaro AF, Wang M, Waterhouse PM. 2005.** RNA silencing platforms in plants. *FEBS Letters* **579**(26): 5982-5987.
- Yoder JI, Gunathilake P, Wu B, Tomilova N, Tomilov AA. 2009.** Engineering host resistance against parasitic weeds with RNA interference. *Pest Management Science* **65**(5): 460-466.
- Yoo B-C, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ. 2004.** A Systemic Small RNA Signaling System in Plants. *Plant Cell*: tpc.104.023614.

Figures

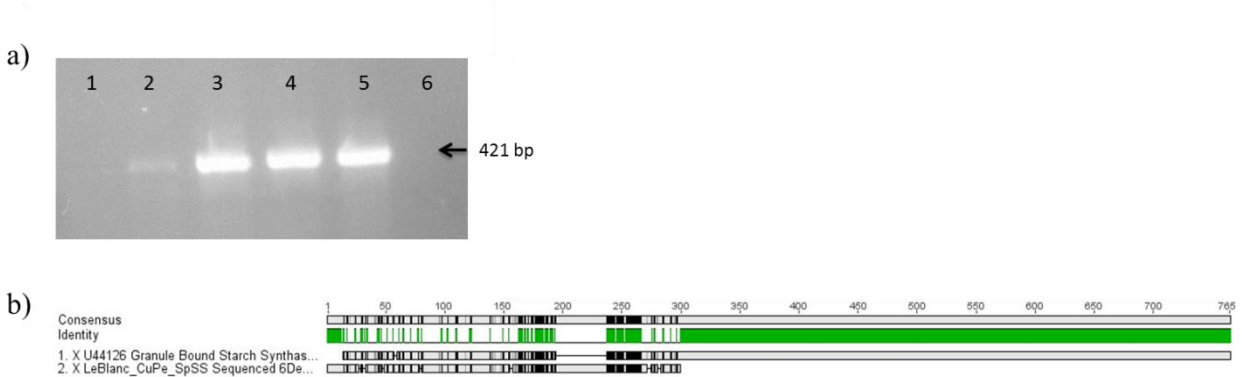


Figure 1. *Ipomoea batatas* granule bound starch synthase (U44126) homolog identified in *Cuscuta pentagona*. (a) RT-PCR shows no product amplified from *Arabidopsis* RNA (lane 1), faint amplification from tomato RNA (2), and amplification from three stages of *C. pentagona*: seedling, early stage attachment, and mature attachment to tomato (lanes 3-5 respectively), and no product in the non-template control (6). (b) Protein sequence alignment using Genious shows 29.7% amino acid sequence identity. Image file from Geneious version 6.1 created by Biomatters, available from <http://www.geneious.com>.

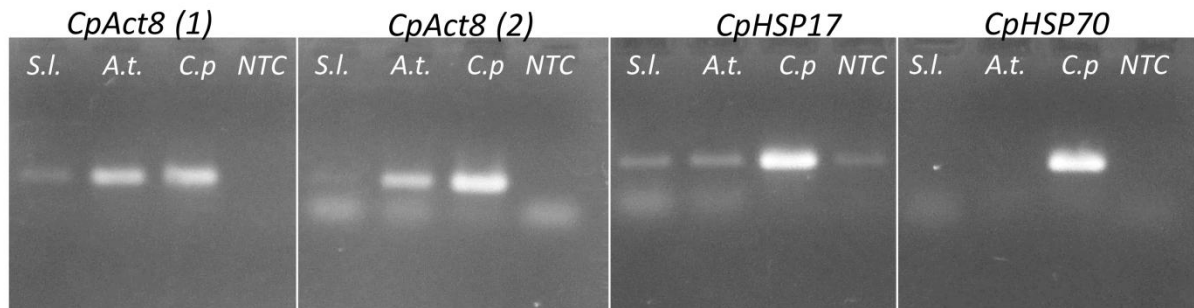


Figure 2. PCR amplification from tomato (*S.l.*), *Arabidopsis* (*A.t.*) and *Cuscuta pentagona* DNA showed cross reactivity to primers for homologs to two sections of *Ipomoea nil* Actin8 (*CpAct8* 1 and 2) and heat shock protein-17 (*CpHSP17*). Primers for the *C. pentagona* homolog to *I. nil* heat shock protein-70 (*CpHSP70*) had specificity to only the parasite.

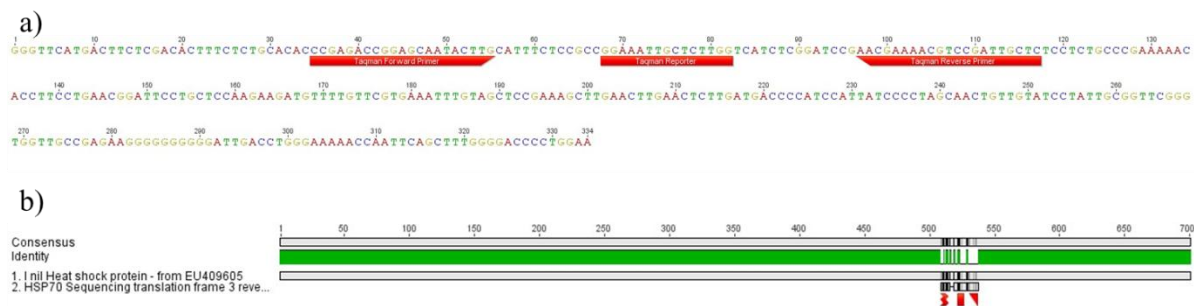


Figure 3. Sequenced PCR product of the *Ipomoea nil* heat shock protein-70 (HSP70) homolog in *Cuscuta pentagona* (a) with TaqMan probes indicated in red. (b) Translated CpHSP70 has 27.3% sequence homology to the translated *I. nil* protein, EU409605, using Geneious alignment (b). Image file from Geneious version 6.1 created by Biomatters, available from <http://www.geneious.com>.

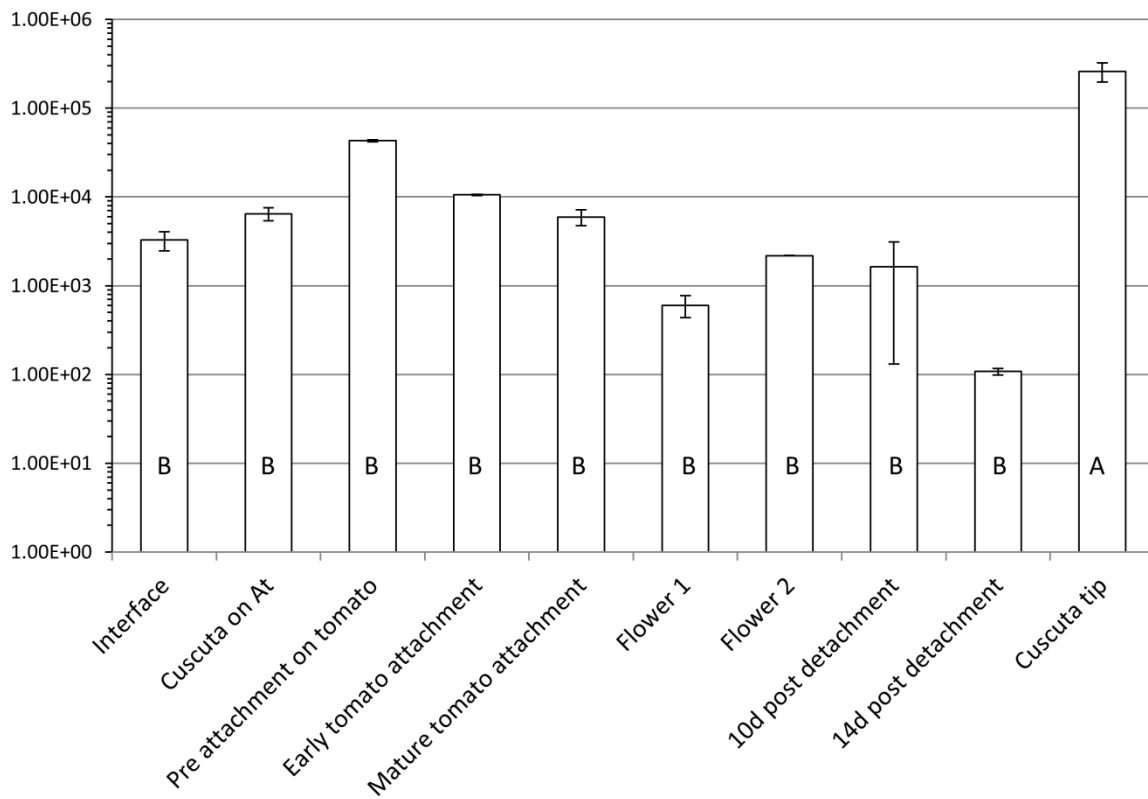


Figure 4. Stability of *C. pentagona* HSP70 across multiple tissue types suggesting suitability as a reference gene for RT-PCR studies. *CpHSP70* amplification levels using a TaqMan probe are statistically the same in real time PCR with the exception of the *C. pentagona* tip. Mean copies per 100 ng RNA $\pm$ SE, means separation by Tukey's HSD.

Chapter 6: Reflection and Future Work

Keywords: RNA trafficking, haustorium, plasmodesmata, miRNA, laser capture microdissection, interdisciplinarity

An overview of host-*Cuscuta* RNA trafficking

The sources of trafficked RNA

From an evolutionary perspective, parasitic plants are fascinating. The haustorium of a parasite such as *Cuscuta* spp. has evolved to allow it to collect all required nutrients from the host plant. Xylem connections have been demonstrated between host and *Cuscuta* and provide for the uptake of water and minerals, and any other material that traffics apoplastically from the roots, such as amino acids, (Dawson, 1994). As the parasite lacks its own root system, the xylem connections are able to provide all water needs. Phloem connectivity is also essential for the uptake of sugars and other materials, however, vestiges of photosynthesis genes remain in their plastid genome (McNeal *et al.*, 2007). This may indicate that phloem-phloem continuity is beneficial to most of these plants but some could be able to survive without this vascular link (Westwood *et al.*, 2010).

Given that *Cuscuta* requires a phloem connection for survival and that RNA is moving between the two plants through the haustorial connection, it is interesting to consider the biological function of RNA trafficking from the host. Are RNAs a byproduct of the process of photosynthate uptake or are they a necessity for other roles in the parasite, such as for signaling? As a signaling molecule, RNA from the host could have an effect on parasite development. For example, the mobile transcript for Flowering locus T (Jackson & Hong, 2012), which signals the plant to move from vegetative to reproductive stages, has been proposed to influence the flowering time of the parasite (Fratianne, 1965). A host-transcribed RNAi hairpin construct has been shown to have post transcriptional gene silencing activity in parasitizing *Cuscuta*, which is

indicative of the parasite utilizing non-native RNAs (Alakonya *et al.*, 2012), but this phenomenon has not been demonstrated for actual host small RNAs.

My work has begun to elucidate some of the means of RNA translocation between species. Since the haustorium is a unique heterotrophic cellular composition that allows for movement of molecules into and out of each organism, the routes of uptake could be through both plasmodesmatal channels as well as direct phloem connections. This symplastic continuity can be described in more detail given some of the information regarding transcript types and region of origin presented in this dissertation.

Plasmodesmata: Plasmodesmata vs. Phloem: Phloem Connectivity

During haustorial development searching hyphae invade the stem of a host plant and come into contact with parenchyma cells and form plasmodesmal (PD) connections. This is followed by further development until connections with vascular tissues are formed (Dawson, *et al.*, 1994). Once the early haustorium is established, non-vascular parenchyma cells on the periphery of the haustoria come in direct contact with the parenchyma cells of the host, such that molecules are transported between the cells of the two organs, including defense (and offense) compounds, nutrients and water. It is possible that a single haustorium, over the potentially hundreds of cells at the interface between host and parasite could include hundreds of thousands of PD channels. The volume of material that could move over that many PD may be quite large, and when considering the multiple haustoria that make up the entire host-parasite interface, the number of PD connections could be in the millions.

At a certain point phloem continuity between host and parasite is established. While it has been experimentally demonstrated that this connection should exist, direct evidence of the phloem

connection between host and parasite has not been shown (Birschwilks *et al.*, 2006; Roney *et al.*, 2007; David-Schwartz R, 2008). Phloem is composed of companion cells (CC) that bridge surrounding cells to the conducting sieve element (SE). Once material (i.e. sugars or RNA) is loaded into the SE, long distance movement is possible practically without impediment. In order to offload the material, it is again transferred to a CC to distribute it to adjoining cells.

Experimental data has not shown whether the parasite makes CC-like connections to host sieve elements or if the parasite has direct SE-SE connectivity (Vaughn, 2003). Given the supposed purpose of this connection (trafficking of host nutrients for growth) the latter is likely the case.

The long-distance trafficking of RNAs may be limited between these two routes as they have unique trafficking dynamics. Because of the nature of PD transport such that in order for RNA to mobilize into a cell it must be conjugated to a protein chaperone and each PD acts as a gate to mobility because a ribonuclear protein (RNP) complex has to conjugate to PD-associated proteins in order to move. While it's not impossible for RNA to move long-distance via this pathway, it may be inefficient for massive RNA translocation. In order for RNA to be taken up in large quantities and moved long distance rapidly, a better conduit is necessary.

Conclusions from my work

In order to make the distinction between phloem or parenchyma sources of trafficked RNA into the haustorium, the rate of flow and the types of RNA moving may provide clues. Future work could query the mobile transcriptome over multiple time points in the development of a strong host-parasite union. These data, with proper annotation and gene ontology categories, could explain the types and provenance of RNAs taken up by a parasite.

Microdissection of this interface for RNA extraction, as described in Chapter 5, would be particularly useful and similar work has already been carried out on vascular bundles of *Arabidopsis* (Deeken *et al.*, 2008). The transcriptome of a microdissected section of the host-parasite interface, such as searching hyphae cells or those on the periphery of the haustorium, could tell us about dynamics of movement based on what proportion of RNAs are from host or parasite.

To determine the percentage of known cell-to-cell localized RNA signals compared to long-distance movers, an organized search of annotated transcripts would have to be carried out. It has been shown there are approximately 1,000 phloem-mobile RNAs (Ruiz-Medrano *et al.*, 1999) and if every one of these are detectable in the mobile transcriptome it would indicate a majority of transcripts move via phloem. If it looks like a majority of transcripts are from localized cell-to-cell movement events, it would signify the host transcripts in the parasite are from those (possibly) millions of PD connections.

My data suggests that the source of host RNA in the parasite is through both PD and SE connections. In the case of tomato proteinase inhibitor (*SIP1*), which is produced locally and not known to be phloem mobile, and Gibberellic acid insensitive (*SIGAI*), a transcript known to move long distance via phloem, our data indicates that *SIP1* moves much more abundantly into the parasite and persists over longer distance compared to *SIGAI* (Chapter 2, Figure 2). This is almost counterintuitive that the phloem-mobile transcript is less detectable over a long distance than the localized one, but the distance travelled into the parasite likely has to do with transcript levels in the host, where more actively expressed genes are more represented in the transcriptome and are detectable over longer distances based of their abundance.

Work in *Arabidopsis* showed that a phloem mobile RNA for transcriptionally controlled tumor protein (*AtTCTP*) was highly detectable as well as RNAs that have not been shown in the literature to move in the vascular system, such as Auxin response family (*AtARF*) and Salt-inducible zinc finger protein1 (*AtSZF1*; Chapter 2, Figure 4c-e). Those experiments indicated that *AtTCTP* and *AtARF* moved into the parasite in equal proportions even though they likely did not enter the haustorium through the same channels.

Despite the disparity in detection levels for each transcript, it is apparent that the phloem conductive tissue in the parasite readily takes up these RNAs, no matter their source, based on data presented here. It appears that the number of connecting PD are as important to contributing to host RNA abundance in the parasite as true phloem associations are.

Also of great importance, is the evidence that parasitic host-RNA trafficking varies with each individual RNA, being that some are more readily mobilized in the parasite compared to others and even certain regions of a given transcript are more likely to travel long distance (Chapter 2, Figure 6b). This may be due to protein chaperone preference by parasitic PD or because there is a mechanism in place for the plant to recognize the value of RNA as a signaling molecule and treating it as such, allowing certain transcripts (or just motifs) to mobilize to more active and developing tissues at the tip.

As far as whether RNA uptake is a byproduct or a direct purpose of haustorial invasion I think that the opinion of most scientists will be molded as we learn more about RNA as a signaling molecule. Early research focused on sugar and nutrient uptake and RNA was just a part of an enriched metabolite flow and not subject to scrutiny. However, future work may prove that RNA

is a key control for many developmental purposes and it could be proven that a parasite would naturally search out an RNA source.

A brief statement on interdisciplinarity

I have been honored to be a part of Virginia Tech's first NSF-IGERT program, Exploring Interfaces through Graduate Education and Research (EIGER). It was the goal of the program to train PhD students to work collaboratively across disciplines to better understand interfaces, both the physical (between two materials, like the surface of atoms in a nanomaterial) and the metaphorical (between schools of thought).

In my time as an EIGER Fellow I benefited greatly from coursework in team psychology and grant writing as well as work with the Physics department that yielded a fine publication. I feel I have a better understanding than most graduates about what working across disciplines requires and the benefits it can give to science in general, such as the joining of computer science and biology to create the field of bioinformatics (the subject of much of this paper).

It is first important to recognize that as we are trained we are indoctrinated into a set of beliefs, due to the core curricula we took for our major as undergrads to the memes common to our field. This has been the standard teaching method and in itself is harmless and has produced countless qualified researchers. This can cause inadvertent biases, though, when people do team research as it can cause unproductive friction. In order to overcome this singular mentality, a researcher has to recognize the value of all inputs and that everyone can bring a unique perspective to a project.

I like to compare this to being a tourist. A bad tourist would expect to go to a foreign country and be frustrated that no one is taking the time to speak to them in their language, and they become upset because things aren't done the way they are back home (sound like anyone you know?). A bad collaborator does the same thing: expects the core set of skills to be universal across disciplines and they become frustrated because their collaborator writes funny or values one type of experiment over another. A good collaborative researcher (and tourist, as well) is benefitted by learning a little of the language of their team – not enough to have a conversation but enough to orient themselves. Just a picking up an Italian guidebook benefits the Roman holiday-goer, perusal of recent literature or a refresher on some basics in the collaborator's field helps to make a good research connection.

While this is an oversimplification of the work that goes into interdisciplinary collaboration it is a philosophy that's benefitted me in my work here and I will no doubt use it after graduation.

Future work

miRNA in the host parasite interaction

Micro-RNA (miRNA) are small (21- to 24-nt) RNAs that are capable of blocking translation by degrading the target transcript through RNAi mechanisms (Chen, 2008). The distinction between miRNA and other RNAi templates is that miRNA comes from a single stranded precursor while small interfering RNAs come from long double-stranded RNAs or single-stranded hairpin RNAs. A large database now exists from deep sequencing and bioinformatics approaches of known miRNA targets. In plants, these silencing signals are now understood to play a role in the rapid turnaround of transcription in response to external cues.

A brief project started in the Westwood lab by a rotation student querying a database of known *Arabidopsis* miRNAs found many of the targets of these transcripts were present in our host-parasite transcriptome data and transcription was up- or down-regulated in the interface compared to in the host stem. This phenomenon could be because the parasite has homologs to these host miRNAs that target the transcripts in the host at the site of attachment.

In order to test this theory, a screen for homologs in the parasite would have to be carried out from a transcriptome data set generated from much more specialized sequencing technology. As it stands, our data was generated from 75-base long reads, which is much longer than the short (under 25-base) lengths of small RNAs.

Is the haustorial structure a stem or root?

This has been a question posed by many a parasitic plant biologist: the haustorium of *Cuscuta* has some physical as well as molecular traits that would make it either a stem or a root. It would be valuable to answer this question once and for all.

The development of a haustorium begins by an expansion of the epidermal cells outward (Dawson *et al.*, 1994) Those epidermal cells are highly metabolically active and phenotypically resemble the expanding cells of an adventitious root. The function of the final structure is also in taking up water through the xylem connection, as a root would.

While anatomical studies indicate the haustorium is root-like, it is also much like a stem as it moves photosynthates into main stem, sinkward, like a shoot. Recent work has shown that the haustorium depends on cytokinins for development and the expression of Shoot Meristemless-like further indicates shoot-like traits (Alakonya *et al.*, 2012).

It is likely, though that the organ is of mixed developmental origins but to answer the question definitively a bioinformatics analysis could prove useful. A number of papers have been published identifying genes specifically up- or down-regulated in shoot or root tissues (Birnbaum *et al.*, 2003; Czechowski *et al.*, 2004; Che *et al.*, 2006). It is possible to search for *C. pentagona* homologs to these in our data sets. Ideally, the expression profiles of the transcripts could be identified in the bioinformatics data but they could also be assayed by real time PCR. A fold change in expression in the haustorium compared to the parasitic stem might further explain the nature of the organ.

References

- Alakonya A, Kumar R, Koenig D, Kimura S, Townsley B, Runo S, Garces HM, Kang J, Yanez A, David-Schwartz R, Machuka J, Sinha N. 2012.** Interspecific RNA Interference of SHOOT MERISTEMLESS-Like Disrupts *Cuscuta pentagona* Plant Parasitism. *Plant Cell* **24**(7): 3153-3166.
- Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN. 2003.** A gene expression map of the Arabidopsis root. *Science* **302**(5652): 1956-1960.
- Birschwilks M, Haupt S, Hofius D, Neumann S. 2006.** Transfer of phloem-mobile substances from the host plants to the holoparasite *Cuscuta* sp. *Journal of Experimental Botany* **57**(4): 911-921.
- Che P, Lall S, Nettleton D, Howell SH. 2006.** Gene expression programs during shoot, root, and callus development in Arabidopsis tissue culture. *Plant Physiology* **141**(2): 620-637.
- Chen X. 2008.** MicroRNA metabolism in plants. *Current Topics in Microbiological Immunology* **320**: 117-136.
- Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. 2007.** FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science* **316**(5827): 1030-1033.
- Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK. 2004.** Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *The Plant Journal* **38**(2): 366-379.
- David-Schwartz R RS, Townsley B, Machuka J, Sinha N. 2008.** Long-distance transport of mRNA via parenchyma cells and phloem across the host-parasite junction in *Cuscuta*. *New Phytologist* **179**(4): 1133-1141.

- Dawson JHMLJ, Wolswinkel P., Dorr I. 1994.** Biology and control of *Cuscuta*. *Reviews of Weed Science* **6**: 265-317.
- Deeken R, Ache P, Kajahn I, Klinkenberg J, Bringmann G, Hedrich R. 2008.** Identification of *Arabidopsis thaliana* phloem RNAs provides a search criterion for phloem-based transcripts hidden in complex datasets of microarray experiments. *The Plant Journal* **55**(5): 746-759.
- Fratianne DG. 1965.** The Interrelationship between the Flowering of Dodder and the Flowering of some Long and Short Day Plants. *American Journal of Botany* **52**(6): 556-562.
- Jackson SD, Hong Y. 2012.** Systemic movement of FT mRNA and a possible role in floral induction. *Front Plant Sci* **3**: 127.
- McNeal J, Kuehl J, Boore J, de Pamphilis C. 2007.** Complete plastid genome sequences suggest strong selection for retention of photosynthetic genes in the parasitic plant genus *Cuscuta*. *BMC Plant Biology* **7**(1): 57.
- Roney JK, Khatibi PA, Westwood JH. 2007.** Cross-Species Translocation of mRNA from Host Plants into the Parasitic Plant Dodder. *PLANT PHYSIOLOGY* **143**(2): 1037-1043.
- Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ. 1999.** Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* **126**(20): 4405-4419.
- Vaughn KC. 2003.** Dodder hyphae invade the host: a structural and immunocytochemical characterization. *Protoplasma* **220**(3-4): 189-200.
- Westwood JH, Yoder JI, Timko MP, dePamphilis CW. 2010.** The evolution of parasitism in plants. *Trends in Plant Science* **15**(4): 227-235.