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Tobacco mosaic virus infection disproportionately impacts phloem associated translatomes in Arabidopsis thaliana and Nicotiana benthamiana

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ABSTRACT

In this study we use vascular specific promoters and a translating ribosome affinity purification strategy to identify phloem associated translatome responses to infection by tobacco mosaic virus (TMV) in systemic hosts *Arabidopsis thaliana* ecotype Shahdara and *Nicotiana benthamiana*. Results demonstrate that in both hosts the number of translatome gene alterations that occurred in response to infection is at least four fold higher in phloem specific translatomes than in non-phloem translatomes. This finding indicates that phloem functions as a key responsive tissue to TMV infection. In addition, host comparisons of translatome alterations reveal both similarities and differences in phloem responses to infection, representing both conserved virus induced phloem alterations involved in promoting infection and virus spread as well as host specific alterations that reflect differences in symptom responses. Combined these results suggest phloem tissues play a disproportion role in the mediation and control of host responses to virus infection.

1. Introduction

The ability of a plant virus to move systemically throughout its host is often essential to its biological success as well as a key factor in its ability to cause disease. Systemic movement generally requires the virus to gain access to the plant's vascular phloem. As the plant's main transport tissue, phloem functions in the systemic movement of a diverse set of molecules that include sugars, lipids, amino acids, nucleic acids, proteins, and phytohormones (Carella et al., 2016; Lucas et al., 2013; Turgeon and Wolf, 2009; Turnbull and Lopez-Cobollo, 2013). Many of these phloem mobile molecules are essential to the maintenance of plant physiology, development and the sensing and activation of stress and defense responses (Carella et al., 2016; Fu and Dong, 2013). Thus, the movement of molecules into and out of the phloem is highly regulated and represents a significant barrier to virus infection. Clearly, plant viruses have evolved to use phloem transport systems to establish infection in tissues distal to the original site of infection. Yet despite the importance of this transport tissue, relatively little is known about the molecular mechanisms of virus phloem loading or how the host phloem is altered during infection. Here we report on the translational gene alterations that occur within host vascular tissues in response to tobacco mosaic virus (TMV) infection.

The vascular phloem consists of sieve elements (SEs) and companion cells (CCs) surrounded by an array of support cells that include bundle sheath (BS) and phloem parenchyma (PP) (Knoblauch and Oparka, 2012; Turnbull and Lopez-Cobollo, 2013). At maturity CCs provide the genetic and metabolic capabilities to the conductive anucleate SEs via specialized branched plasmodesmata (PD) known as plasmodesmata pore units (PPUs) (van Bel, 1996). Virus access into the phloem requires cell-to-cell movement through PD connecting the surrounding support cells and CCs followed by movement into the SEs through the PPUs (Hipper et al., 2013). Passage through each of these cell types and their connecting PD thus represent a potential barrier to systemic virus movement. As an example, resistance in soybean against cowpea chlorotic mottle virus has been linked to the blockage of virus movement from BS cells to phloem cells, suggesting the connection between these unique cell types represents a barrier to the systemic movement of this virus (Goodrick et al., 1991). Once in the SEs virus systemic movement occurs predominately via translocation, following the source to sink route of photosynthate (Gibbs, 1976; Hipper et al., 2013; Santa Cruz, 1999). Thus, modulating access to the vascular phloem appears to be a key determinant influencing systemic virus movement.

For viruses such as TMV, the type member of the genus *Tobamovirus*, cell-to-cell movement through PD requires both virus movement (MP) and replication proteins with PD transit occurring as a

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ribonucleoprotein (vRNP) complex, for review see (Carrington et al., 1996; Heinlein, 2015; Liu and Nelson, 2013). For systemic movement TMV also requires a functional capsid protein with virion assembly likely occurring within the SE (Callaway et al., 2001; Nelson and van Bel, 1998; Scholthof, 2005). TMV systemic movement is also impacted by host factors, including several components that bind the virus MP (Heinlein, 2015; Liu and Nelson, 2013; Ueki et al., 2010). For example, increased callose deposition within the vascular phloem has been associated with the overexpression of a cadmium-induced glycine-rich protein (cdiGRP), leading to the inhibition of *Tobamovirus* systemic movement (Ueki and Citovsky, 2002). Furthermore, reductions in PD associated callose deposition have been associated with a TMV MP - ankyrin repeat host protein interaction and can enhance virus movement (Ueki et al., 2010). Several lines of evidence also indicate that transcriptional reprogramming is essential for effective *Tobamovirus* systemic movement (Chen et al., 2013; Collum et al., 2016; Levy et al., 2013; Sasaki et al., 2009). Most recently, the targeted disruption of the CC expressed IAA26, an auxin indole, acidic acid (Aux/IAA) transcriptional regulator was found to enhance TMV phloem loading and systemic movement (Collum et al., 2016). IAA26 disruption was associated with reductions in defense-associated responses including genes involved in the regulation of callose deposition. Combined these studies indicate that viruses such as TMV use an array of approaches to modulate PD and phloem characteristics in order to gain access to the plant's vasculature.

To date the specific impact of virus infections on the vascular phloem has been difficult to determine in part due to the technical difficulty of isolating such complex tissues. CCs and SEs form a pressurized system, and disruption of this system, as is done in many phloem sampling techniques can lead to the introduction of contaminates (Turgeon and Wolf, 2009). Other methods such as laser dissection require specialized equipment and can be costly to establish. In this study, we used translating ribosome affinity purification (TRAP) which utilizes His-FLAG-tagged ribosomal proteins expressed from tissue specific promoters (Mustroph et al., 2009; Reynoso et al., 2015). These tagged ribosomes are used to immuno-purify mRNA-ribosome complexes, referred to as the translatome. An advantage of this method is that it does not require invasive techniques or expensive equipment prior to mRNA harvesting. In addition, mRNAs associated with ribosomes are more likely to be in the process of translation and thus better represent the cellular condition than total cellular mRNA.

To investigate the effects of virus infection on the phloem translatome we expressed tagged ribosomes from two phloem specific promoters (pSUC2 and pSULTR2;2) as well as from the ubiquitously expressed cauliflower mosaic virus 35 S promoter in two TMV systemic hosts, *Arabidopsis thaliana* ecotype Shahdara and *Nicotiana benthamiana*. Results from these studies demonstrate that leaf vascular phloem tissue is disproportionately regulated in response to TMV infection as compared to non-phloem tissues, confirming at the molecular level the importance of modulating the vascular phloem barrier during virus infection. These studies also reveal both similarities and differences in host phloem responses to TMV infection. Genes showing similar phloem translatome alterations in both systemic hosts included those involved in the transport of the systemic acquired resistance (SAR) mobile signal and the production of phloem mobile siRNAs. These similarly altered phloem translatome genes likely represent conserved virus induced responses involved in modulating the phloem environment and/or promoting systemic virus movement. In contrast, host differential translatome responses such as those involved in the generation of reactive oxygen species likely reflect unique host responses that contribute to observed differences in symptoms, including the development of systemic necrosis in *N. benthamiana*. To our knowledge this study is the first profiling of the phloem translatome in response to virus infection.

2. Materials and methods

2.1. Translatome constructs and plant lines

Translatome constructs *p35S::HF-RPL18*, *pSUC2::HF-RPL18* and *pSULTR2;2::HF-RPL18* were kindly provided by Dr. J. Bailey-Serres, University of California, Riverside, CA, USA (Mustroph et al., 2009). For GUS expression constructs, upstream promoter sequences covering 2 kb upstream of the pSUC2 and pSULTR2;2 open reading frame were amplified from the provided translatome constructs using promoter-specific primers (Table S1). Cloned promoter fragments were moved into pBI101.1 (Clontech) directly upstream of the GUS reporter ORF via primer-generated Sal1 and BamH1 restriction sites to create *pSUC2::GUS* and *pSULTR2;2:GUS*. All constructs were introduced into the *Agrobacterium tumerfaciens* strain GV3101 (Holsters et al., 1978). *A. thaliana* ecotype Shahdara plants were transformed by the floral dip method (Clough and Bent, 1998). *N. benthamiana* plants were transformed by leaf disc transformation method (Horsch et al., 1985). Transgene insertions were confirmed by qRT-PCR using primers specific for the His6-FLAG-RPL18 transcript (Table S1**).** Plants were maintained in growth chambers for a 12-h photoperiod, light 100 μmol s-1 m-2, at 24 °C. No abnormalities in seedling growth or plant development were observed in any of the transgenic lines.

2.2. Virus inoculations and tissue immuno-prints

Four week old plants were used for all virus inoculations. Arabidopsis and *N. benthamiana* leaves were dusted with carborundum and mechanically inoculated with TMV (1 mg/mL) or mock infected with sterile water. Inoculated leaf tissue was collected after six days. For Arabidopsis the leaf tissue from 20 plants was combined for each biological replicate. For *N. benthamiana* leaf tissue from 9 plants was combined for each biological replicate. Two biological replicates were generated for each promoter construct. Biological replicates were grown in different months under the same growth chamber conditions (12-h photoperiod, light 100 μmol s-1 m-2 at 24 °C).

To compare virus accumulations and spread inoculated leaves were placed onto nitrocellulose sheets and sandwiched between two sheets of filter paper leaving an imprint of the leaf. Tissue prints were washed in buffer (50 mM Tris pH 7.6, 150 mM NaCl) and blocked in 5% nonfat dry milk for 20 min at room temperature. Prints were then probed with rabbit anti-CP antiserum followed by incubation with alkaline phosphatase conjugated goat anti-rabbit antibodies (Sigma Chemical Company, St. Louis, MO USA). CP accumulation was visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium, as previously described (Knecht and Dimond, 1984).

2.3. Ribosomal associated mRNA purifications

Isolation of polysomes from *promoter::HF-RPL18* lines was done as previously described (Lin et al., 2014; Mustroph et al., 2009; Reynoso et al., 2015) with slight modifications. Frozen tissue was homogenized in Polysome Extraction Buffer (PEB; 200 mM Tris-HCl, pH 9.0, 200 mM KCl, 25 mM ethylene glycol tetraacetic acid (EGTA) pH 8.0, 35 mM $MgCl_2$, 1% (v/v) octylphenyl-polyethylene glycol (Igepal CA-630), 1% (v/v) polyoxyethylene 10 tridecyl ether, 1% (v/v) sodium deoxycholate, 5 mM dithiothereitol (DTT), 1 mM PMSF, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 0.5 mg/mL heparin) using 10 mL PEB per 5 g of tissue. Homogenates were clarified by centrifugation at 16,000*g* for 15 min and filtered with cheesecloth. For *N. benthamiana* samples, the supernatant was loaded onto an 8 mL 1.6 M sucrose cushion. Samples were ultracentrifuged at 170,000 g for 18 h at 4 °C to pellet polysomes. 1 mL of PEB was used to resuspend the pellet.

IP of polysomes from *promoter::HF-RPL18* plants was done as previously described (Mustroph et al., 2009; Reynoso et al., 2015) with