Disruption of Chloroplast Function Through Downregulation of *Phytoene Desaturase* Enhances the Systemic Accumulation of an Aphid-Borne, Phloem-Restricted Virus

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Chloroplasts play a central role in pathogen defense in plants. However, most studies explaining the relationship between pathogens and chloroplasts have focused on pathogens that infect mesophyll cells. In contrast, the family *Luteoviridae* includes RNA viruses that replicate and traffic exclusively in the phloem. Recently, our lab has shown that *Potato leafroll virus* (PLRV), the type species in the genus *Polerovirus*, forms an extensive interaction network with chloroplast-localized proteins that is partially dependent on the PLRV capsid readthrough domain (RTD). In this study, we used virus-induced gene silencing to disrupt chloroplast function and assess the effects on PLRV accumulation in two host species. Silencing of phytoene desaturase (*PDS*), a key enzyme in carotenoid, chlorophyll, and gibberellic acid (GA) biosynthesis, resulted in a substantial increase in the systemic accumulation of PLRV. This increased accumulation was attenuated when plants were infected with a viral mutant that does not express the RTD. Application of GA partially suppressed the increase in virus accumulation in *PDS*-silenced plants, suggesting that GA signaling also plays a role in limiting PLRV infection. In addition, the fecundity of the aphid vector of PLRV was increased when fed on *PDS*-silenced plants relative to PLRV-infected plants.

Alteration of chloroplast function is a common strategy used by most plant pathogens during host infection (Bhattacharyya and Chakraborty 2017; Bilgin et al. 2010; Zhao et al. 2016), as these organelles play a central role in regulating many different pathways including cellular metabolism, hormone biosynthesis, cell-wall dynamics, and production of defense molecules, such as reactive oxygen species (DeBlasio et al. 2015a; de Torres Zabala et al. 2015; Di Carli et al. 2012; Matsumura et al. 2003; Rodrigo et al. 2012; Whitham et al. 2006). Studies regarding

chloroplast function during viral infection have centered on viruses that replicate in mesophyll cells, in which chloroplasts are abundant. Accumulation of the *Tobacco mosaic virus* (TMV) coat protein (CP) on chloroplast inner membranes inhibits photosynthetic electron transport, presumably by binding to photosystem II (PSII) (Banerjee et al. 1995; Hodgson et al. 1989; Reinero and Beachy 1989). Conversely, some plant viruses that replicate in the cytoplasm inhibit photosynthesis by binding to and interfering with the transport of nuclear encoded polypeptides of the oxygen-evolving complex (OEC), which associates with PSII in the chloroplast (Balasubramaniam et al. 2014; Kong et al. 2014; Takahashi et al. 1991, 1992). In most cases, inhibition of chloroplast function by viral proteins leads to the development of disease symptoms, such as chlorosis (Balasubramaniam et al. 2014; Banerjee et al. 1995; Jang et al. 2013; Kong et al. 2014; Takahashi and Ehara 1992; Takahashi et al. 1991). Thus, it has been proposed that disruption of chloroplast function is an indirect effect of viral replication and overtaking host resources (Pallas and García 2011). However, the effects observed on virus accumulation of *Alfalfa mosaic virus* (AMV) and *Rice stripe virus* (RSV) when the levels of PsbP, (a component of the OEC) are experimentally manipulated suggests that photosynthesis or chloroplast-localized proteins act as an active inhibitor of virus accumulation (Balasubramaniam et al. 2014; Kong et al. 2014). Exactly how photosynthesis acts as a barrier to virus infection remains unclear, although biosynthesis of plastid-derived molecules as a plant defense strategy has been implicated in plant-bacterial interactions (Zurbriggen et al. 2009). In accordance with the host-manipulation hypothesis (Holmes and Bethel 1972), plant viruses may actively target chloroplasts as a means to ubiquitously inhibit plant defense pathways at their source (Maule et al. 2002).

The role of chloroplasts during infection with phloem-restricted viruses is not known, which is surprising given the diversity of host phenotypes induced by these viruses that suggests the involvement of chloroplast signaling. Viruses in the family *Luteoviridae*, commonly known as luteovirids, are among the best-studied phloem-restricted pathogens (Gray et al. 2014). Luteovirids are positive-sense, single-stranded RNA viruses that replicate in phloem companion and parenchyma cells (Shepardson et al. 1980). They are transmitted exclusively by aphids, insects in the order Hemiptera, in a persistent circulative manner (Gray et al. 2014). These viruses move systemically in plants as icosahedral shaped virions comprised of two structural proteins, the CP, which makes up the majority of the capsid subunits, and a minor structural component known

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as the readthrough protein (RTP), which is translated via readthrough of the CP stop codon to form an extension that protrudes from the surface of the virion, referred to as the readthrough domain (RTD) (Brault et al. 2000, 2003; Filichkin et al. 1994; Lee et al. 2005; Kaplan et al. 2007; Peter et al. 2008, 2009). The major visual symptoms of a luteovirid infection are interveinal chlorosis, in which the mesophyll tissue surrounding the phloem becomes chlorotic while phloem tissue appears healthy, and inhibition of plant growth (Peter et al. 2009; Taliany et al. 2003). Symptoms increase with increased light intensity (Jones 1981) and luteovirid protein expression leads to a rise in foliar carbohydrate levels (Gonçalves et al. 2005; Herbers et al. 1997) with starch deposition in chloroplasts (Shepardson et al. 1980). Virions can also be observed localized to the outer membrane of chloroplasts in infected phloem cells and, occasionally, detected within these organelles along with the viral movement protein P17 (Golinowski et al. 1987; Link et al. 2011; Schmitz et al. 1997; Shepardson et al. 1980). The biological importance of these associations in the luteovirid infection cycle remains unclear.

The RTD provides specificity for some interactions with the chloroplast and chloroplast-localized proteins (Boissinot et al. 2014; Brault et al. 2000; DeBlasio et al. 2015b and a; Peter et al. 2009). Mutational studies have shown that truncation of the PLRV RTD C-terminus in the nonincorporated form of the RTP enhances localization of virions along the chloroplast outer membrane in mesophyll cells when mutant virus unloads from the phloem in young, developing sink leaves after pruning (Peter et al. 2009). For virus mutants in which the RTD is not expressed, photosynthesis-associated proteins, including photosystem I and PSII, phosphoribulokinase (PRK), PsbP—a protein subunit of the OEC, and proteins involved in chloroplast import have reduced affinity for virus protein complexes (DeBlasio et al. 2015a). In addition, cross-linking mass spectrometry experiments identified six host proteins with functions in photosynthesis that form physical interactions with the PLRV structural proteins or with the viral P1 polypeptide (DeBlasio et al. 2015c). Structural modeling guided by cross-linker distance constraints placed residues for one of these proteins, PsbQ (another component of the OEC), under diversifying selection at an interfacial region with the surface-exposed orientation of the arginine-rich domain (Torrance 1992) of a PLRV CP dimer (DeBlasio et al. 2015c). Downregulation of *PSBQ* expression resulted in a decrease in PLRV accumulation early in the infection cycle, suggesting that, contrary to what has been observed for other plant pathogens, this specific chloroplast-associated protein acts as a positive regulator of PLRV.

In the current study, we used *Tobacco rattle virus* (TRV)-induced gene silencing (VIGS) of phytoene desaturase (*PDS*), a chloroplast-associated enzyme, to investigate the effect of chloroplast disruption on PLRV infection in two host species. Conventionally used as a visual marker for the onset of VIGS (Baulcombe 1999; Brigneti et al. 2004; Lu et al. 2003), downregulation of *PDS* results in severely reduced levels of chlorophyll and carotenoids, turning leaves from green to white, arrestment of chloroplast development at a proplastid-like stage, and a decrease in gibberellic acid (GA) biosynthesis resulting in plant growth inhibition (Liu et al. 2014; Qin et al. 2007). Collectively, our data reveal a functional role for the chloroplast and GA in suppressing the accumulation of PLRV in systemically infected tissues. The role of chloroplasts in luteovirid biology may extend beyond systemic host infection to include interactions with its aphid vector. Downregulation of *PDS* also led to an increase in the fecundity of the PLRV vector *Myzus persicae*. Our results support the hypothesis that disease symptoms associated with luteovirid infection, i.e., interveinal

chlorosis and plant dwarfism, are a consequence of deliberate deactivation of these pathways by the virus at very precise levels during infection. Consequences of this manipulation lead to an increase in its own replication and its vector's propagation within susceptible host plants. To our knowledge, this is the first report to show that normal chloroplast function can negatively regulate the systemic accumulation of a phloem-limited virus as well as the fecundity of its insect vector.

RESULTS

Silencing of *PDS* in *Nicotiana benthamiana* differentially affects the accumulation of wild-type PLRV and a mutant that lacks the RTD.

To investigate the effect chloroplast function has on the systemic accumulation of PLRV, *N. benthamiana* plants were simultaneously infected with a TRV-based VIGS construct targeting *PDS* (TRV:*PDS*) and infectious clones of either wild-type (WT) PLRV or a mutant that does not express the RTD (Δ RTD) (Liang et al. 2004). To avoid transgene silencing of the *Cauliflower mosaic virus* 35S promoter, infections of PLRV and TRV were initiated, using *Agrobacterium tumefaciens*-mediated transformation (DeBlasio et al. 2015b) into separate leaves (Supplementary Fig. S1). Coinfection of plants with PLRV and TRV:*GFP* was used as a negative control. At 1.5 weeks postinfiltration (wpi), *N. benthamiana* plants expressing TRV:*PDS* exhibited photobleaching and dwarfism typically associated with downregulation of *PDS* (Qin et al. 2007; Senthil-Kumar et al. 2007). These symptoms were independent of PLRV infection and were not observed in plants infected with TRV:*GFP* plus PLRV or PLRV alone (Supplementary Fig. S2). At 1.5 wpi, the average titer of WT PLRV, measured by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), was 5.3-fold higher in systemic-infected leaves silenced for *PDS* relative to levels in TRV:*GFP* control plants (Fig. 1). Newly emerged leaves on *PDS*-silenced plants continued to exhibit increased levels of WT PLRV at 2.5, 3.5, and 5 wpi, with virus antigen levels reaching a peak fold change of 7.5 at 3.5 wpi, as compared with TRV:*GFP* control plants (Fig. 1). Although levels of the Δ RTD mutant in *PDS*-silenced leaves were significantly increased approximately fourfold in *PDS*-silenced leaves at all timepoints tested (Fig. 1), the Δ RTD virus titers remained lower than WT levels, despite Δ RTD virus levels being similar to WT in TRV:*GFP* control plants (Fig. 1). The observed increases in PLRV titer in *PDS*-silenced plants had no additional, detrimental effect on the morphology of plants, compared with *PDS*-silenced plants that were not infected with PLRV. There was no observable effect on the rate of systemic movement of PLRV (WT or Δ RTD) in *PDS*-silenced plants. All plants tested positive for PLRV starting at 1.5 wpi (data not shown), suggesting that severe disruption of chloroplast function affects PLRV infection at the level of viral replication, assembly, or stability, singly or in combination, and not long-distance movement. In addition, occupation of the silencing machinery by endogenous *PDS* transcripts, which would not occur in TRV:*GFP* control plants, did not have an indirect effect on PLRV titer by making these proteins less available for silencing of PLRV viral RNA (vRNA), as silencing of *ANXD1* starting at 1.5 wpi had no effect on PLRV titer (Supplementary Fig. S3). Thus we continued to use TRV:*GFP* as a control for subsequent experiments.

Droplet digital polymerase chain reaction (ddPCR) (Hayden et al. 2013; Hindson et al. 2013) was used to quantify the absolute levels of WT PLRV subgenomic RNA1 (sgRNA1) and TRV RNA 2 at 3.5 wpi in a subset of plants that were tested by DAS-ELISA (Fig. 2). Using primers to specifically amplify a region located in the PLRV CP open-reading frame of sgRNA1

(Supplementary Fig. S4), levels of WT PLRV vRNA were measured as approximately 3.3-fold higher in leaves when *PDS* was silenced compared with TRV:*GFP* control leaves (Fig. 2A). Conversely, there were no significant effects on TRV2 vRNA in *PDS*-silenced leaves, regardless of PLRV coinfection (Fig. 2B, $P = 0.17$). These data show that severe disruption of chloroplast function regulates PLRV infection at the level of transcription since PLRV vRNA levels are increased in *PDS*-silenced leaves compared with negative controls. Down-regulation of chloroplast function had no significant effect on the levels of TRV, a plant virus whose replication and movement is not limited to phloem.

Positive correlation between degree of photobleaching in *PDS*-silenced leaf sectors and PLRV accumulation in two host species.

Since host-specific effects on systemic movement, tissue tropism, and aphid transmission have previously been observed for PLRV (Kaplan et al. 2007; Lee et al. 2005; Peter et al. 2008), we tested whether silencing of *PDS* would have the same effect on PLRV accumulation in *Solanum sarrachoides*, a natural weed host of PLRV (Alvarez and Srinivasan 2005). Coinfection of *S. sarrachoides* with WT PLRV and TRV:*PDS*,

following the same workflow used for *N. benthamiana*, resulted in a wide variation in the extent of photobleaching observed in individual *S. sarrachoides* leaves. Whereas infiltration of *N. benthamiana* with TRV:*PDS* resulted in near-complete photobleaching of all newly emerging leaves starting at 1.5 wpi, photobleaching in *S. sarrachoides* was first observed in newly emerging leaves approximately 5.5 wpi and was often non-continuous, resulting in delineated green and white sectors within the same leaf (Fig. 3A). This “mosaic-like” silencing phenotype was independent of coinfection with PLRV (data not shown) and is typical of symptoms observed in other solanaceous plants when heterologous gene sequences are used for silencing (Senthil-Kumar et al. 2007), such as the TRV:*PDS* tomato construct used in this study (Liu et al. 2002). Simultaneous infiltration of *S. sarrachoides* plants with WT PLRV and TRV constructs at the four- to five-leaf stage resulted in PLRV accumulating in systemically infected leaves ahead of the onset of *PDS* silencing. Visible symptoms of PLRV infection (i.e., interveinal chlorosis) were first observed at 3 wpi, a full 2.5 weeks ahead of the first signs of photobleaching from the silencing of *PDS* (data not shown). By 6 wpi, most leaves exhibited severe chlorosis and necrosis due to PLRV infection (data not shown). Therefore, to synchronize the onset

Fig. 1. Potato leafroll virus (PLRV) accumulation in systemically infected leaves is significantly higher in *Nicotiana benthamiana* silenced for phytoene desaturase (*PDS*) compared with plants infected with a *Tobacco rattle virus* green fluorescent protein silencing construct (TRV:*GFP*). Bar graphs show the normalized average change (Δ) in absorbance (405 minus 490 nm) values for double antibody sandwich enzyme-linked immunosorbent assay measuring the accumulation of wild type (WT) or a mutant form of the virus that does not express the readthrough domain (Δ RTD) in newly emerging *N. benthamiana* leaves silenced for *PDS* at A, 1.5 weeks postinfiltration (wpi), B, 2.5 wpi, C, 3.5 wpi, and D, 5 wpi. Mean values were normalized to the mean value measured for plants coinfecting with WT PLRV and TRV:*GFP*. Error bars represent $\pm$ standard error for 20 (1.5 wpi), 15 (2.5 wpi), 10 (3.5 wpi), or five (5 wpi) plants. Lowercase letters represent significant differences ($P < 0.05$) measured by two-way analysis of variance and Tukey's highly significant difference post hoc test, with the interaction effect between TRV and PLRV conditions having a $P < 0.05$ for all timepoints. Data represents two to three independent experiments.

of *PDS* silencing with the early asymptomatic stages of WT PLRV systemic infection, *S. sarrachoides* plants were first infected with TRV constructs at the four- to five-leaf stage. Six weeks later, when *PDS* silencing was visible, plants were infiltrated with the WT PLRV infectious clone in *Agrobacterium tumefaciens*. Since the foliar disease symptoms induced by the Δ RTD mutant are negligible in *S. sarrachoides* (Peter et al. 2009), the effect of *PDS* silencing on Δ RTD accumulation was assessed in plants that were simultaneously infiltrated with the TRV constructs at the four- to five-leaf stage, as done in the experiments with *N. benthamiana*.

Taking advantage of the discontinuous silencing phenotype, we compared the titer of PLRV (WT and Δ RTD) in photobleached (white) sectors to the level of virus in the non-photobleached (green) sectors from the same *S. sarrachoides* leaf (Fig. 3A) by DAS-ELISA (Fig. 3B and C). Comparison of PLRV titer in different sectors from plants coinfecting with PLRV and TRV:*GFP* was used as an additional control. At 3 wpi, WT virus

levels were similar in the white sectors of *PDS*-silenced leaves as compared with the green sectors from the same leaf and from leaves coinfecting with TRV:*GFP* (Fig. 3B, $P = 0.57$ and 0.37 , respectively). In contrast, the average level of the Δ RTD mutant was 3.3-fold higher in white sectors compared with green sectors within *PDS*-silenced leaves and approximately 14-fold higher than Δ RTD virus levels in TRV:*GFP* leaves (Fig. 3C), although overall levels still remained lower than those measured for WT PLRV (Fig. 3B versus C). Green sectors from *PDS*-silenced leaves also had higher levels of Δ RTD PLRV (approximately 4.2-fold, $P = 0.051$) than samples from TRV:*GFP* control plants (Fig. 3C), suggesting that even though these sectors were visibly green the *PDS*-mediated signaling that regulates virus levels (directly or indirectly) is occurring at some low level within the green sectors in TRV:*PDS* leaves.

To determine if this localized effect on PLRV accumulation could be observed in *N. benthamiana*, mosaic-like silencing of *PDS* was induced by simultaneously infiltrating plants with TRV constructs and PLRV at 6 weeks postgermination, when plants were showing signs of inflorescence shoot emergence. At 1.5 wpi, white-green sectoring was visible on cauline leaves of TRV:*PDS*-infected plants (Fig. 3D). Levels of WT PLRV were significantly increased approximately twofold in white, photobleached sectors compared with green sectors on the same *N. benthamiana* leaf and 7.8-fold higher than levels measured in TRV:*GFP* plants at 3.5 wpi (Fig. 3E). Similar to what was observed for the Δ RTD mutant in *S. sarrachoides* (Fig. 3C), the levels of WT PLRV were also significantly higher (approximately fourfold) in the green sectors of *PDS*-silenced leaves compared with levels in *N. benthamiana* infected with TRV:*GFP* (Fig. 3E). For the Δ RTD mutant, virus levels in white sectors were increased 2.3-fold at 3.5 wpi compared with green sectors from the same *PDS*-silenced *N. benthamiana* leaf (Fig. 3F). However, levels of the Δ RTD mutant in leaf samples from TRV:*GFP* coinfecting plants were statistically similar to virus levels measured in both the white and green sectors of TRV:*PDS* leaves (Fig. 3F). Overall, these results show the same trend for both host species. PLRV titer is highest in sections of leaf that are completely photobleached compared with areas of the same leaf that still appear green, regardless of host or the presence of the RTD. In addition, the accumulation defects of the Δ RTD mutant could only be partially rescued by silencing of *PDS* in both the model and natural host.

An increased number of PLRV-infected phloem foci are observed in photobleached leaf sectors of *PDS*-silenced plants.

It has previously been reported that phloem unloading of RTD C-terminal truncation mutants in *S. sarrachoides* results in higher virus titers as virus invades and presumably replicates in mesophyll cells (Peter et al. 2009). Although host proteins that participate in restricting PLRV to phloem cells have yet to be identified, it is known that chloroplast-derived signals can affect plasmodesmata gating and trafficking (Benitez-Alfonso et al. 2009; Burch-Smith et al. 2011). Therefore, to determine if the increased levels of PLRV observed in *PDS*-silenced tissue could be the result of phloem unloading, tissue immunoblot analysis (Lee et al. 2002) was used to visualize the localization of PLRV (WT and Δ RTD) within white and green sectors of *N. benthamiana* and *S. sarrachoides* source leaves exhibiting discontinuous silencing of *PDS* (Fig. 4). Plants were not pruned to avoid phloem unloading due to the Δ RTD mutation itself (Peter et al. 2009). At the infection timepoints tested, both WT (Fig. 4A and B, E and F) and Δ RTD mutant (Fig. 4C and D, G and H) virus localized to punctate foci in leaf sections from

Fig. 2. Levels of viral RNA are increased in phytoene desaturase (*PDS*)-silenced *Nicotiana benthamiana* leaves systemically infected with *Potato leafroll virus* (PLRV). Bar graphs show the average absolute copies per microliter of **A**, wild-type (WT) PLRV and **B**, *Tobacco rattle virus 2* (TRV2) viral RNA in total RNA extracted from PLRV-infected *N. benthamiana* leaves silenced for *PDS* compared with plants coinfecting with TRV:*GFP* monoinfected with **A**, WT PLRV or **B**, with TRV constructs, at 3.5 weeks postinfiltration. Primers used for droplet digital polymerase chain reaction amplified unique regions in the coat protein of PLRV and TRV2. Error bars represent $\pm$ standard error for eight plants. Lowercase letters represent significant differences ($P < 0.01$) measured by one-way analysis of variance and Tukey's highly significant difference post hoc test.

plants coinfecting with TRV:*GFP* as well as green and white sectors of *N. benthamiana* and *S. sarrachoides* leaves expressing TRV:*PDS*, indicating that PLRV was still phloem-limited, since we did not observe the perfuse staining of leaves associated with phloem unloading (Peter et al. 2009). Qualitatively, in TRV:*PDS* expressing plants, the intensity of staining and the number of PLRV infected foci per area of leaf appeared greater in sections of leaf that were either devoid of all pigmentation or located near the border of a green and white sector (Fig. 4A, C, E, and G). For *N. benthamiana*, this was most evident in plants coinfecting with WT PLRV (Fig. 4A), which exhibited the largest increase in PLRV titer due to silencing of *PDS* when measured by DAS-ELISA compared with the Δ RTD mutant (Figs. 1 and 3E). A similar trend was also observed in *S. sarrachoides* between TRV:*PDS* green and white sectors coinfecting with either WT (Fig. 4E, 3 wpi) or Δ RTD (Fig. 4G, 8 wpi) PLRV. The Δ RTD-infected foci in TRV:*GFP* expressing *S. sarrachoides* leaves were barely detectable compared with TRV:*PDS* sectors that were completely photobleached (Fig. 4G

to H). Again, it was the Δ RTD mutant, which exhibited the largest change in titer due to silencing of *PDS* in *S. sarrachoides* (Fig. 3C).

Quantification of PLRV infection foci per area of leaf showed that on average, foci number was significantly increased two and 29-fold in photobleached (completely white) sectors of TRV:*PDS*-silenced *S. sarrachoides* plants coinfecting with WT PLRV and the Δ RTD mutant, respectively, compared with green leaves from TRV:*GFP* coinfecting plants (Fig. 5A). In white sectors of TRV:*PDS*-silenced leaves of *N. benthamiana* coinfecting with WT PLRV, we observed a 3.7-fold increase in the average number of infection foci compared with TRV:*GFP* negative control plants (Fig. 5B). We did not observe a difference in the number of infection foci between TRV:*GFP* leaves and photobleached areas of TRV:*PDS*-silenced *N. benthamiana* plants coinfecting with the Δ RTD mutant at the timepoint tested (Fig. 5B). These data are consistent with the trends we observed in virus titer in white sectors of these same TRV:*PDS*-silenced plants compared

Fig. 3. Levels of wild type (WT) and the readthrough domain mutant (Δ RTD) of Potato leafroll virus (PLRV) are increased within photobleached (white) sectors compared with green sectors on **A** to **C**, *Solanum sarrachoides* and **D** to **F**, *Nicotiana benthamiana* leaves exhibiting mosaic silencing of phytoene desaturase (*PDS*). Representative images show green and white sector areas (black dotted circles) that were sampled on a single *S. sarrachoides* (**A**) and *N. benthamiana* (**D**) leaf exhibiting discontinuous silencing of *PDS*. Bar graphs show the average change (Δ) in absorbance (405 minus 490 nm) values for double antibody sandwich enzyme-linked immunosorbent assay analysis of WT (**B** and **E**) and Δ RTD PLRV (**C** and **F**) accumulation in green and white sectors on *S. sarrachoides* (**B** and **C**) and *N. benthamiana* (**E** and **F**) leaves expressing TRV:*PDS* at 3.5 to 6 weeks postinfiltration (wpi) and 3.5 wpi, respectively. Levels of PLRV in plants coinfecting with TRV:*GFP* are also shown. Error bars represent $\pm$ standard error for two to eight leaf discs sampled from eight to 10 *S. sarrachoides* plants and five to nine *N. benthamiana* plants. Lines above bars represent significant differences (one asterisk [*] for $P < 0.05$ and two [**] for $P < 0.01$) measured by one-way analysis of variance and Tukey's highly significant difference post hoc test. Data represents two independent experiments.

with TRV:*GFP* plants, as measured by DAS-ELISA (Fig. 3B and C, E and F). Although we did not observe the wide-spread phloem unloading associated with the PLRV SYG C-terminal PLRV mutant described by Peter et al. (2009), we mechanically inoculated healthy *N. benthamiana* plants using sap

(Peter et al. 2009) from leaf tissue coinfecting with WT PLRV and TRV:*PDS* or TRV:*GFP*, to assess whether spread into a small number of vascular-associated cells could be occurring in TRV:*PDS*-infected plants, which may not be discernable at the resolution level of a tissue print. Studies have shown that

Fig. 4. Representative immunoblots of **A to D**, *Nicotiana benthamiana* and **E to H**, *Solanum sarrachoides* leaves coinfecting with *Potato leafroll virus* (PLRV) (WT and Δ RTD) and TRV:*PDS* (A, C, E, and G) at 3.5 weeks postinfiltration (wpi) and 3.5 to 6 wpi, respectively. Areas positive for PLRV are indicated by purplish-blue staining, and green sectors in TRV:*PDS* leaves are outlined from white sectors with a black dotted line. PLRV-infected foci in green sectors are highlighted with black arrowheads for easy visualization. Virus localization and levels in leaves coinfecting with TRV:*GFP* (B, D, F, and H) are also shown. Labeling with the PLRV antibody was not observed in healthy controls or leaves infected with TRV constructs only (data not shown). Bar size is equal to 1 mm.

phloem unloading of PLRV, either through mutation or coinfection with umbraviruses, facilitates the mechanical transmissibility of PLRV, which is never observed when WT PLRV is phloem-restricted (Peter et al. 2009; Ryabov et al. 2001). Although all mechanically inoculated plants became infected with TRV, showing signs of *PDS*-silencing starting at 1.5 to 2 wpi, none of the plants became infected with PLRV (data not shown). Collectively, these data show that the increased PLRV titer in photobleached sectors of *PDS*-silenced host plants is not due to replication of virus in the mesophyll but a result of an increase in the number of infected phloem bundles.

Application of exogenous GA partially suppresses PLRV accumulation in *PDS*-silenced *N. benthamiana* leaves.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) expression analyses of the *pds3*-null mutant in *Arabidopsis thaliana* showed that, in addition to downregulation of genes associated with chlorophyll and carotenoid biosynthesis, loss of *PDS* expression leads to a decrease in the expression of genes involved in GA biosynthesis, a phytohormone that acts as a positive regulator of plant growth (Qin et al. 2007). These included *geranylgeranyl pyrophosphate synthase* (*GGPS*), which codes for the enzyme that produces an important precursor molecule, and *copalyl diphosphate synthase* (*CPS*), the first gene in the committed GA pathway. Addition of GA to growth media partially rescues the dwarf phenotype observed in *pds* null mutants, even though plants still remained albino, indicating that chloroplast development or function was still perturbed. Similarly, silencing of *phytoene synthase* (*PSY*), the enzyme directly upstream of *PDS3* in the carotenoid pathway, leads to a four-fold decrease in endogenous levels of GA in orchids (Liu et al. 2014). To assess the possibility that the increased accumulation of PLRV in *PDS*-silenced plants may be a result of GA deficiency and not chloroplast dysfunction, *N. benthamiana* plants coinfecting with WT PLRV and TRV constructs were sprayed with 0.1 mM GA₃ in 0.02% Tween 20 for 1 week, starting at 2 wpi when newly emerging leaves on TRV:*PDS*-infected plants started to exhibit near-complete photobleaching. In line with what has previously been reported (Xu et al. 2013), application of GA₃ resulted in an increase in stem elongation and leaf area in both *PDS*-silenced and TRV:*GFP* control plants coinfecting with WT PLRV, as compared with plants sprayed with 0.02% Tween 20 (Fig. 6). As observed for the *pds* null mutant in *A. thaliana* (Qin et al. 2007), *PDS*-silenced *N. benthamiana* plants exposed to GA₃ remained photobleached and stunted compared with TRV:*GFP* plants sprayed with GA₃. Growth of TRV:*PDS* plants exposed to GA₃ was comparable to the overall growth of TRV:*GFP* control plants that were sprayed with 0.02% Tween 20 (Fig. 6; Supplementary Fig. S5). Interestingly, the titer of WT PLRV was significantly decreased 1.8-fold in *PDS*-silenced leaves exposed to GA₃ compared with *PDS*-silenced leaves sprayed with Tween alone (Fig. 7). Yet, levels of PLRV were still 7.5- and 4.7-fold higher in TRV:*PDS* plants sprayed with GA₃ than TRV:*GFP* plants also sprayed with or without the hormone (respectively) suggesting that application of GA₃ can only partially suppress PLRV accumulation in the absence of *PDS* activity. TRV:*GFP* plants exposed to GA₃ showed no significant change in PLRV titer compared with TRV:*GFP* plants sprayed with 0.02% Tween 20 (Fig. 7, Tukey highly significant difference, $P = 0.9$).

Silencing of *PDS* increases the fecundity of aphids that transmit PLRV.

Aphid growth, longevity, and fecundity are all increased when insects are reared on PLRV-infected potato plants compared with those that are infected with *Potato virus Y*, a stylet-

borne potyvirus that only requires a brief association with the insect in order to be transmitted (Castle and Berger 1993), supporting the hypothesis that pathogens that are highly dependent on their vector for transmission have evolved ways to modify host physiology that benefit their vectors to increase dissemination (Holmes and Bethel 1972). To determine the effect silencing of *PDS* and increased PLRV titer would have on aphid fecundity, *M. persicae* nymphs were allowed to develop on *PDS*-silenced *N. benthamiana* leaves with or without WT PLRV coinfection. The cumulative number of progeny produced by a single aphid was measured after 8 and 11 days (Fig. 8). Control conditions included nymphs reared on healthy plants and those that were monoinfected with WT PLRV or coinfecting with TRV:*GFP*. In contrast to what has previously been reported for aphids reared on PLRV-infected potato (Castle and Berger 1993), we observed a 2.6- and ninefold decrease in the survival and fecundity, respectively, of *M. persicae* at 8 days of development on *N. benthamiana* plants monoinfected with WT PLRV compared with healthy plants and those expressing TRV:*PDS* or TRV:*GFP* (Fig. 8A and B). In the

Fig. 5. Silencing of phytoene desaturase (*PDS*) leads to an increase in the number of *Potato leafroll virus* (PLRV) infection foci in photobleached leaf sectors compared with green leaves coinfecting with a *Tobacco rattle virus* green fluorescent protein silencing construct (TRV:*GFP*). **A**, The average number of PLRV (WT and ΔRTD) infection foci per square millimeter of leaf area in white sectors of TRV:*PDS*-silenced (hatched bars) *Solanum sarrachoides* and **B**, *Nicotiana benthamiana* leaves compared with plants coinfecting with TRV:*GFP* (green bars). Leaf area and PLRV infection foci were manually quantified from immunoblot images, using Image J with five to 13 plants (one leaf per plant) for each infection condition. Error bars represent $\pm$ standard error with lines above bars indicating significant differences (one asterisk (*) for $P < 0.05$ and two (**) for $P < 0.01$) measured by Student *t* test within a PLRV infection condition. Data represents three independent experiments.

absence of PLRV infection, downregulation of *PDS* significantly enhanced aphid fecundity at 8 and 11 days compared with all other plant conditions (Fig. 8B and C) while aphid survival was unaffected (Fig. 8A). After 8 days of development, aphids reared on plants monoinfected with TRV:*PDS* had approximately twofold more progeny than aphids reared on healthy, TRV:*GFP*-monoinfected plants and TRV:*PDS* plants coinfecting with WT PLRV. Aphid fecundity on TRV:*PDS* plants was enhanced approximately 15- and fivefold compared with plants monoinfected with PLRV or plants coinfecting with PLRV and TRV:*GFP*, respectively (Fig. 8A). This trend continued even after 11 days of development, with aphids still producing more progeny on TRV:*PDS* plants compared with all other infection conditions (Fig. 8B). We also observed an approximately three- to sixfold increase in aphid fecundity on TRV:*GFP*- and *PDS*-silenced plants coinfecting with PLRV compared with PLRV single infections at both 8 and 11 days, although the change was not statistically significant (Fig. 8A and B).

DISCUSSION

This study presents the first evidence that chloroplast function negatively regulates the transcription and accumulation of PLRV, a biotrophic virus with a tropism for phloem. Like mesophyll cells, phloem cells also contain functional chloroplasts that are smaller in size. These organelles react differently to phytohormones and are more stable reservoirs for starch storage compared with their mesophyll counterparts (Esau 1973; Paramonova et al. 2002). Within infected phloem cells, WT PLRV virions associate with the chloroplast membrane and, to a small extent, within the chloroplast themselves (Golinsowski et al. 1987; Schmitz et al. 1997; Shepardson et al. 1980). We observed that severe disruption of chloroplast function via downregulation of *PDS* significantly enhanced the systemic accumulation of WT and Δ RTD PLRV virions in *N. benthamiana* leaves that exhibited near-complete loss of pigmentation (photobleaching) compared with control plants and leaf sectors on the same plant that still remained green. This enhancement was sustained over several weeks of infection. WT PLRV vRNA was also elevated in *PDS*-silenced *N. benthamiana* leaves at this same timepoint during the infection cycle, indicating that regulation of PLRV by chloroplast function is occurring during replication as well as virion production. However, it is unclear from our data if virion levels are increased merely

due to increased levels of sgRNA1 or if downregulation of chloroplast function affects different stages of the PLRV infection cycle. To our knowledge, no evidence in the literature exists that explains the stoichiometric relationship between levels of PLRV sgRNA1 and capsid proteins translated within an infected cell.

Silencing of *PDS* in *N. benthamiana* but not *S. sarrachoides* resulted in a significant increase in WT PLRV titer, TRV:*PDS* indicating that chloroplast regulation of WT PLRV accumulation is host-dependent. Host-specific effects have been previously reported for PLRV. For example, the P17 movement protein is required for the initiation of a systemic infection in *Physalis floridana* and *Solanum tuberosum* but not in *Nicotiana* species (Lee et al. 2002). Similarly, luteovirids with mutations in their CP domain also exhibit varying phenotypes among host plants (Kaplan et al. 2007; Lee et al. 2005). Our data are consistent with previous observations showing that PLRV infects different solanaceous host plants using partially distinct mechanisms. Yet, we did observe an increase in the titer of the Δ RTD mutant in both *N. benthamiana* and *S. sarrachoides* plants silenced for *PDS*, suggesting that regulation of PLRV by the chloroplast occurs to some degree in *S. sarrachoides*. Interestingly, the Δ RTD mutant in *PDS*-silenced plants always accumulated to lower levels compared with WT PLRV in both host species. Studies have shown that in the absence of the RTD, poleroviruses show reduced accumulation in some host species compared with others (Brault et al. 1995; Bruyère et al. 1997; Mutterer et al. 1999; Peter et al. 2008, 2009). Our data show that accumulation of the Δ RTD is differentially affected by the loss of chloroplast function independent of host environment. This is compelling, given the fact that coimmunoprecipitation (co-IP) experiments with the Δ RTD mutant revealed an enrichment of chloroplast-related proteins whose interaction was lost or weakened in the absence of this domain (DeBlasio et al. 2015a). Twelve of these host proteins (Table 1) were orthologous in sequence to proteins whose gene expression was found to be downregulated in a null mutant of *PDS*

Fig. 6. Application of exogenous gibberellic acid (GA) to *Nicotiana benthamiana* plants coinfecting wild-type *Potato leafroll virus* (WT PLRV) results and with *Tobacco rattle virus* (TRV)-based virus-induced gene silencing constructs in shoot and leaf elongation. Plants were sprayed every other day for 1 week with 0.1 mM GA₃ in 0.02% Tween 20 (GA+) or 0.02% Tween 20 (GA-) starting at 2 weeks postinfiltration with virus clones. Representative images of plant growth after application with GA₃ compared with plants not exposed to GA₃ are shown.

Fig. 7. Application of exogenous gibberellic acid (GA) partially suppresses increased levels of *Potato leafroll virus* (PLRV) induced by silencing phytoene desaturase (*PDS*) in *Nicotiana benthamiana*. The bar graph shows the average change (Δ) in absorbance (405 minus 490 nm) values for double antibody sandwich enzyme-linked immunosorbent assay analysis of wild-type (WT) PLRV accumulation in newly emerging *N. benthamiana* leaves silenced for *PDS* at 3 weeks postinfiltration with virus and 1 week of application with 0.1 mM GA₃ in 0.02% Tween 20 (GA+) or 0.02% Tween 20 (GA-). Error bars represent $\pm$ standard error for two leaf discs sampled from five plants. Lowercase letters represent significant differences ($P < 0.01$) measured by two-way analysis of variance and Tukey's highly significant difference post hoc test, with the interaction effect between *Tobacco rattle virus* (TRV) and hormone conditions having a $P < 0.01$.

(Qin et al. 2007). The inability of the Δ RTD mutant to reach WT levels in *PDS*-silenced leaves suggests that virus replication or assembly or both may, in part, be regulated by interactions between the RTD and these chloroplast-related proteins. Alternatively, strong association of the Δ RTD mutant virus with the plastid outer envelope (Peter et al. 2009), which is still intact even in the undifferentiated plastids of *PDS* and *PSY*-silenced leaves (Liu et al. 2014; Qin et al. 2007), could be inhibiting short-distance movement of the virus. This would result in a lower number of infected phloem cells and, thus, lower levels of measured titer. Indeed, a reduced number of infection foci, especially in the internal phloem of petioles and stems, has been reported for the Δ RTD mutant compared with WT-infected *S. sarrachoides* (Peter et al. 2009). That same trend was also observed between WT and Δ RTD PLRV-infected leaves in this study (Figs. 4 and 5).

Collectively, our data show that normal chloroplast function can act as a negative regulator of PLRV infection in two host species, although the magnitude of the effect is host and viral dependent. This contrasts with nonphloem limited viruses that use the chloroplast as a site for replication and protection against the host RNA silencing machinery (Zhao et al. 2016). A dramatic increase in vRNA and virion titer in *PDS*-silenced plants for PLRV supports the general observation that *Luteoviridae* replication occurs in the cytoplasm (Gill and Chong 1979; Nass et al. 1998) and is not dependent on a fully functioning, developed chloroplast. Our results for PLRV are more in line with cytosolic-replicating viruses like AMV and RSV, whose replication is inhibited by an interaction with nuclear-encoded, chloroplast proteins in the cytoplasm (Balasubramaniam et al. 2014; Kong et al. 2014). However, not all cytosolic replicating viruses are affected by chloroplast function. In this study, we observed that accumulation of TRV, a tobamovirus that replicates and moves in mesophyll cells (Harrison et al. 1976), was largely unaffected in *PDS*-silenced plants coinfecting with PLRV. This result shows that regulation of PLRV by chloroplast function is not just a ubiquitous response against all plant viruses.

In the context of previous work, regulation of PLRV titer by the chloroplast is nuanced and dynamic. Downregulation of *PSBQ*, a functional component of the OEC, resulted in a decrease in PLRV titer in locally infected leaf tissue (DeBlasio et al. 2015c), demonstrating that, prior to systemic movement, at least one chloroplast-associated protein can have a positive effect on PLRV accumulation. Evidence in the literature demonstrates instances in which the same host protein has opposite functions at different timepoints during pathogen infection (Shen et al. 2010), which could also be extended to organelles like the chloroplast. This includes luminal binding protein, a molecular chaperone of the endoplasmic reticulum, which we have shown can act as an activator early on and later as a

repressor of PLRV (DeBlasio et al. 2015c). It should also be noted that *PSBQ*-silenced plants had no visual effects on pigment levels or plant growth (DeBlasio et al. 2015c) like those observed in *PDS*-silenced plants, suggesting that chloroplast pathways one or both associated with and regulating these two processes may be having a dampening effect on systemic levels of PLRV that is independent of *PSBQ*.

Tissue immunoblots revealed that the effect of *PDS*-silencing on PLRV accumulation was not an indirect result of virus

Fig. 8. Silencing of phytoene desaturase (*PDS*) enhances *Myzus persicae* fecundity on *Nicotiana benthamiana* while infection with *Potato leafroll virus* (PLRV) decreases nymph survival and number of progeny produced. **A**, The average number of nymphs that survived to adulthood after 8 days and **B**, the average, cumulative number of progeny produced at 8 and **C**, 11 days on *N. benthamiana* plants mono- or co-infected with wild-type PLRV and *Tobacco rattle virus* (TRV) virus-induced gene silencing constructs (TRV:GFP and TRV:PDS). Aphids reared on healthy plants are included as an additional control. Error bars represent $\pm$ standard error of 37 to 39 aphids on day 8 of development and four to 18 aphids on day 11. Aphids that did not survive to day 8 were counted as having zero progeny. Only progeny produced by surviving aphids were counted on day 11. Lowercase letters represent significant differences ($P < 0.05$) measured by two-way analysis of variance and Tukey's highly significant difference post hoc test. The interaction between TRV and PLRV conditions was not significant ($P > 0.1$) for all panels. Data represent three independent experiments.

phloem unloading, since the distribution pattern of PLRV infection in albino sectors was still detected as punctate foci (Peter et al. 2009; Ryabov et al. 2001). Thus, PLRV phloem restriction is not dependent on normal functioning chloroplasts and suggests that regulation of PLRV by *PDS* occurs either directly in phloem or via signaling between the *PDS*-silenced mesophyll tissue and the virus within phloem. We did, however, observe an increase in the number of PLRV-positive foci in photobleached sectors of *PDS*-silenced leaves compared with the green tissue of TRV:*GFP* control plants, suggesting that downregulation of chloroplast function leads to an increase in the number of phloem cells that become infected. It is known that downregulation of the chloroplast localized protein

INCREASED SIZE EXCLUSION LIMIT (ISE)2, which also leads to impaired plastid development and severe chlorosis similar to but not as extreme as the photobleaching observed in *PDS*-silenced plants, increases the frequency of secondary plasmodesmata formation between epidermal and mesophyll cells, with *ISE2*-silenced tissue exhibiting increased cell-to-cell movement of fluorescent probes (Burch-Smith and Zambryski 2010; Burch-Smith et al. 2011). Both silencing and overexpression of *ISE2* leads to an increase in the intercellular spread of TMV to noninoculated leaves with silencing slightly increasing the size of infection foci and overexpression reducing their size (Ganusova et al. 2017). It is possible that, in *PDS*-silenced plants, in which chloroplast function is also severely

Table 1. Orthologous genes of *Nicotiana benthamiana* proteins found in complex with *Potato leafroll virus* (PLRV) are differentially regulated in *Arabidopsis thaliana pds3*

Protein ^a	Accession no. ^b	PLRV co-IP fold enrichment ^c		Ortholog ^d	<i>pds3</i> Gene expression ^e
		WT/control	WT/ARTD		
Interactions dependent on the readthrough domain (RTD)					
GSTU19	NbS00049661g0007.1	+/-	2.1	AT1G78380	Decreased
RGP1	NbS00002592g0008.1	+/-	2.8	AT3G02230	Decreased
MTO3	NbS00016613g0001.1	+/-	2.8	AT3G17390	Decreased
CK1I	NbS00018784g0006.1	+/-	4.0	AT4G14340	Decreased
LOS2	NbS00000250g0010.1	141	11.8	AT2G36530	Decreased
CHLP	NbS00008675g0003.1	63.1	2.0	AT1G74470	Decreased
LHCA4	NbS00052312g0004.1	46.4	+/-	AT3G47470	Decreased
PRK	NbS00007805g0017.1	27.2	5.2	AT1G32060	Decreased
SHD	NbS00013845g0026.1	14.9	2.8	AT4G24190	Decreased
GLDP2	NbS00007101g0012.1	13.7	2.5	AT2G26080	Decreased
GLDP1	NbS00022684g0008.1	12.3	2.3	AT4G33010	Decreased
CAB1	NbS00014580g0002.1	10.7	2.7	AT1G29930	Decreased
Interactions dependent on coat protein					
HTH	NbS00039424g0014.1	+/-	0.9	AT1G72970	Decreased
NPHP3	NbS00000891g0006.1	+/-	1.2	AT4G10840	Decreased
EIF3A	NbS00047179g0008.1	+/-	1.5	AT4G11420	Decreased
RPT2a	NbS00039466g0005.1	+/-	1.8	AT4G29040	Decreased
P5CS1	NbS00022823g0015.1	+/-	1.7	AT2G39800	Decreased
ATS9	NbS00048446g0001.1	+/-	1.5	AT1G29150	Decreased
CSY2	NbS00001219g0008.1	+/-	1.0	AT3G58750	Decreased
EIF4A-III	NbS00047703g0010.1	+/-	ns	AT3G19760	Decreased
AHA1	NbS00001908g0012.1	202.4	1.1	AT2G18960	Decreased
RS3A	NbS00004044g0002.1	42.2	1.0	AT4G34670	Decreased
CAS	NbS00026008g0003.1	25.7	1.7	AT5G23060	Decreased
EIF4A1.1	NbS00025364g0011.1	20.3	1.5	AT3G13920	Decreased
CA2	NbS00029322g0004.1	13.6	1.4	AT5G14740	Decreased
MAB1	NbS00060745g0006.1	11.4	0.8	AT5G50850	Decreased
RPL24	NbS00016248g0010.1	9.3	0.9	AT3G53020	Decreased
RK24	NbS00006992g0007.1	7.7	1.3	AT5G54600	Decreased
RPS18C	NbS00011076g0013.1	7.3	1.0	AT4G09800	Decreased
GAPA-2	NbS00019521g0023.1	6.4	1.6	AT1G12900	Decreased
CA1	NbS00016159g0006.1	6.3	1.3	AT3G01500	Decreased
FBA2	NbS00028064g0012.1	6.0	1.5	AT4G38970	Decreased
RBS8	NbS00041372g0009.1	3.4	1.3	AT5G38420	Decreased
RBS8	NbS00022486g0001.1	2.9	1.2	AT5G38410	Decreased
SUS2	NbS00027551g0008.1	+/-	1.3	AT1G80070	Increased
SUVH3	NbS00002523g0003.1	+/-	0.7	AT1G71310	Increased
PLD	NbS00035704g0012.1	195.0	1.2	AT3G15730	Increased
RPL21A	NbS00001165g0003.1	102.1	1.0	AT1G09690	Increased
GRP2	NbS00023063g0001.1	68.7	1.0	AT4G38680	Increased
EXL2	NbS00004697g0005.1	39.0	0.6	AT5G64260	Increased
RPS5A	NbS00009842g0001.1	17.8	0.9	AT3G11940	Increased
RPL6	NbS00002188g0022.1	9.1	0.9	AT1G74060	Increased
TSJT1	NbS00027299g0010.1	8.4	ns	AT5G43830	Increased

^a Gene symbols for *N. benthamiana* proteins previously identified as in complex (high confidence) with PLRV (DeBlasio et al. 2015b and c).

^b Gene accession numbers manually curated from an in-house database of protein sequences from the *N. benthamiana* draft genome (DeBlasio et al. 2015b and c).

^c Average fold enrichment of *N. benthamiana* proteins in α -PLRV coimmunoprecipitations (co-IP) from tissue infected with WT PLRV compared with negative control co-IP as reported by DeBlasio et al. (2015b) (WT/control) and with the Δ RTD mutant as reported by DeBlasio et al. (2015c) (WT/ Δ RTD). ns = PLRV-plant interaction that was not detected as a high confident interaction in the analysis.

^d *A. thaliana* ortholog sharing the most significant sequence identity with the *N. benthamiana* protein identified in the PLRV-plant interactome.

^e Direction of differential gene expression in the *pds3* *A. thaliana* mutant as reported by Qin et al. (2007).

downregulated, there could be an increase in the number of secondary plasmodesmata between phloem companion and parenchyma cells that increases short-distance movement of PLRV into more phloem cells.

Alternatively, chloroplasts are also a major site for the synthesis and regulation of most of the phytohormones and defense compounds found in plants (Serrano et al. 2016). Some symptoms of virus infection can be attributed to changes in the biosynthesis or activity of phytohormones (Jameson and Clarke 2002); however, the role of phytohormones during luteovirid infection is poorly understood. Russell and Kimmins (1971) concluded that stunting of plants infected with *Barley yellow dwarf virus*, another luteovirid, is caused by a decrease in the mitotic activity of the meristem. A decrease in gibberellic-like activity was consistently found in infected plants compared with healthy plants while levels of auxin remained unchanged, suggesting involvement of endogenous GA in these virus-induced changes. However, application of exogenous GA₃, although able to partially reverse the stunting phenotype, did so equally in infected and healthy plants by increasing cell elongation and not cell division, which was the pathway affected by the presence of virus (Russell and Kimmins 1971). The impact of these hormonal changes on luteovirid titer and systemic movement was not addressed in this previous study. Our data show that GA₃ is a negative regulator of PLRV when chloroplast function is disrupted but is not sufficient to completely suppress the enhancement of PLRV accumulation induced by the downregulation of *PDS*. This other pathway affected by silencing of *PDS* must also regulate PLRV levels in phloem cells. Early work with potexviruses shows that systemic levels of (+) single-stranded RNA virus replication can be inhibited by application of GA₃ (Clarke et al. 1998). In addition, the TMV CP negatively regulates antiviral defense responses by stabilizing the accumulation of DELLA proteins, which act as negative regulators of GA signal transduction (Rodriguez et al. 2014). PLRV and related viruses may work to circumvent plant defense by altering levels of GA to inhibit growth, which favors their replication. Besides GA₃, it is possible other phytohormones and defense molecules, such as salicylic acid (SA), jasmonic acid (JA), and levels of H₂O₂, may be functioning in PLRV regulation. Numerous studies have shown that the function of signaling molecules in plant defense is dependent on the type of pathogen (Collum and Culver 2016) with hormones like SA and JA often acting antagonistically to one another (Alazem and Lin 2015; Takahashi et al. 2004). Yet, even though chloroplast development is inhibited in the *A. thaliana pds3*-null mutant, genes involved in JA and SA signaling were not reported to be affected by this mutation (Qin et al. 2007). In addition, H₂O₂, a key trigger of programmed cell death during pathogen defense (Gechev and Hille 2005), can still rapidly accumulate in *PDS*-silenced wheat cells compared with nonsilenced control plants and response to infection with the fungal pathogen *Zymoseptoria tritici* (Lee et al. 2015). However, it is possible that specific changes in the levels of these defense molecules in response to PLRV infection in the *PDS*-silenced background could also contribute to the increases in virus titer that we observed. Further experimentation into the exact mechanism of regulation of PLRV by chloroplast function, either through defense-related pathways, structural changes to plasmodesmata in phloem cells, or both, is needed to discern between these different hypotheses.

In addition to the downregulation of genes involved in carotenoid, GA, and chlorophyll biosynthesis, disruption of *PDS3* in *A. thaliana* leads to the differential expression of hundreds of plant genes that could be classified into 20 different metabolic pathways (Qin et al. 2007). We found a total of 43 *N. benthamiana* proteins that were identified as interacting with

WT PLRV, using co-IP coupled to mass spectrometry (DeBlasio et al. 2015b and a) that were orthologs of *A. thaliana* proteins whose genes were found differentially expressed in the *pds3 A. thaliana* null mutant compared with WT (Table 1). Twelve of these proteins were found to have a weaker affinity for PLRV in the absence of the RTD, showing that their interaction with PLRV-host protein complexes is dependent on this domain (DeBlasio et al. 2015a). Corresponding orthologous genes for all 12 of these proteins were found to be downregulated twofold or more in *pds3* (Table 1). These proteins include, reversibly glycosylated polypeptide-1 (RGP1), a plasmodesmata or Golgi localized protein involved in synthesis of cell-wall polysaccharides known to regulate the cell-to-cell transport of virus and viral movement proteins (De Storme and Geelen 2014; Delgado et al. 1998; Zavaliev et al. 2010), enolase 2 (LOS2), a glycolytic enzyme required for normal plant growth and development (Eremina et al. 2015), and PRK, an enzyme in the photosynthetic carbon reduction (Calvin) cycle, which we previously identified as forming a direct interaction with the PLRV CP, using cross-linker technology (DeBlasio et al. 2015c). The remaining 31 *N. benthamiana* proteins sharing functional similarity to proteins whose genes were differentially expressed in *pds3* were enriched equally in α -PLRV co-IPs from *N. benthamiana* leaf tissue infected with WT or Δ RTD PLRV compared with controls. This suggests that the interaction with PLRV is dependent on domains in the CP (DeBlasio et al. 2015a and b). Twenty-two orthologs within this group were downregulated due to disruption of *PDS3*, while nine were found upregulated (Table 1). These include the abiotic stress-responsive protein GRP2 (glycine-rich protein 2) (Mangeon et al. 2010), SUVH3 [Su(var)3-9 homolog 3], a key regulator in heterochromatin repression (Casas-Mollano et al. 2006), and EXL2 (EXORDIUM-LIKE 2), a protein putatively involved in the control of plant growth and carbon allocation (Schröder et al. 2012). Interaction with PLRV and their differential regulation when *PDS* expression is disrupted make these 43 proteins potential targets that may be involved in regulating accumulation of PLRV in host plants. It should be noted that *PDS3* was not one of the host proteins identified as coimmunoprecipitating with PLRV.

The vector manipulation hypothesis explains the evolution of plant viruses to alter the behavior of its insect vector to promote spread to new hosts (Ingwell et al. 2012). In the case of luteovirids, which are transmitted in a circulative-persistent manner, aphids must feed for prolonged periods of time (6+ h) for efficient transmission (Kotzampigikis et al. 2010). Virus-induced changes to host physiology, such as yellowing of leaves and generation of volatile chemoattractants, lure healthy aphids to infected plants and induce arrestment of the insect (Eigenbrode et al. 2002; Macias and Mink 1969). These behaviors are not observed when plants are infected with a non-persistent virus that can be acquired within seconds of probing (Castle et al. 1998). Aphids that transmit luteovirids exhibit increased growth rates and produce more offspring on infected plants compared with plants that are healthy (Bosque-Pérez and Eigenbrode 2011; Castle and Berger 1993; Ingwell et al. 2012; Jiménez-Martínez et al. 2004). These positive impacts on vector performance have been shown to be host dependent (Pinheiro et al. 2014, 2017). Eventually, host preference switches and viruliferous aphids become more attracted to healthy plants (Medina-Ortega et al. 2009). Our observation that aphid survival and fecundity significantly decreased on PLRV-infected *N. benthamiana* when insects were caged and prevented from dispersing, support the notion that, over time, virus-infected plants become poor-quality hosts. However, our understanding of the underlying molecular pathways that

regulate the phenotypic effects luteovirids have on their aphid vectors is still limited. Yet, our study shows that deregulation of chloroplast function through *PDS* silencing mimics the initial benefit luteovirid infection has on aphids. Analysis of genes differentially expressed in *pds3* *A. thaliana* mutants shows that loss of chloroplast function leads to the upregulation of ethylene biosynthesis and ethylene-responsive genes (Qin et al. 2007), which has been reported by Casteel et al. (2015) to be the mechanism involved in enhancing aphid fecundity when aphids are reared on *A. thaliana* plants infected with *Turnip mosaic virus* (TuMV), a single-stranded positive-sense RNA virus (Potyviridae) that is also transmitted by *M. persicae* but in a nonpersistent manner. Interestingly, the CP of TuMV also accumulates in chloroplasts in which it affects photosynthesis by inhibiting PSII (Fu et al. 2004).

Conclusions.

Pathogens use a myriad of tricks to bypass the host natural defenses to create a cellular environment that promotes their replication and transmission (Abbink et al. 2002; Bhat et al. 2013; de Torres Zabala et al. 2015; Lee et al. 2015). Given that downregulation of chloroplast function leads to such a dramatic increase in the levels of PLRV that is only partially suppressed by exogenous application of GA₃, it is our hypothesis that chloroplast function actively limits PLRV accumulation through another mechanism. It is less likely that virus accumulation in *PDS*-silenced leaves is an indirect effect of lower energy levels being available to initiate plant defense strategies due to loss of photoassimilate production, as this would result in decreased levels of virus not increased as we observed in the *PDS*-silenced leaves. Furthermore, the *pds3* mutant does result in upregulation of genes involved in stress tolerance, indicating that not all pathways related to defense are compromised when chloroplast function is so severely perturbed (Qin et al. 2007). It is an intriguing possibility that manifestation of disease symptoms like stunting and interveinal chlorosis are not merely secondary effects of competition for host resources but a targeted means for PLRV to bypass chloroplast-regulated defense mechanisms to increase their propagation to a level that favors transmission and attracts insects and yet maintains an adequate level of host health that promotes insect feeding and reproduction.

MATERIALS AND METHODS

Plant infection and analysis of virion titer.

N. benthamiana and *S. sarrachoides* plants were coinoculated with TRV:*PDS* (Liu et al. 2002) or TRV:*GFP* (Kandath et al. 2007) pTRV2 constructs (mixed 1:1 with pTRV1) and PLRV infectious clones (Liang et al. 2004) at the four- to five-leaf-stage, using *Agrobacterium tumefaciens*-mediated transformation as previously described by Lee et al. (2005) and DeBlasio et al. (2015b), with TRV (in GV2260) and PLRV (in LB4404) being infiltrated into separate leaves. For analysis of the effect of *PDS* silencing on WT PLRV in *S. sarrachoides*, plants were first inoculated with TRV constructs at the four- to five-leaf-stage, followed by vein-inoculation of three or four green leaves with WT PLRV at 5.5 wpi with TRV, the timepoint when some leaves exhibited visible signs of *PDS* silencing. To produce mosaic silencing of *PDS* in *N. benthamiana* cauline leaves, plants were coinoculated with TRV and PLRV 6 weeks postgermination, as described above. All *N. benthamiana* plants were grown in 16-h days at 23°C and *S. sarrachoides* plants under fall greenhouse conditions (approximately 21°C and 12-h days). Images of silencing phenotypes and morphological changes in plants were taken with an EOS Rebel T6s camera (Canon).

PLRV titer was measured in newly expanding leaves at various timepoints postinfiltration by DAS-ELISA, using commercially available α -PLRV antibodies (Agdia), as described by Lee et al. (2002), with the following exceptions: 3- (green versus white sectors) or 5-mm discs from one or two leaves per plant were homogenized in 150 or 200 μ l, respectively, of 1× phosphate-buffered saline (PBS), pH 7.4, using a Mixer Mill 400 (Retsch) at a frequency of 30 Hz for 2.5 min, absorbance at 405 and 490 nm was measured 1 or 2 h after addition of substrate. Each biological replicate (100 μ l) from all infection conditions being compared per week were analyzed on the same 96-well microtiter plate with sample positions randomized. Values were normalized to the background absorbance values for 1× PBS and healthy tissue, which were equal (data not shown).

Virus-induced gene silencing of *Annexin D1*.

A sequence fragment (289 bp) targeting *N. benthamiana* *Annexin D1* (Niben101Scf00260g00003.1) mRNA was designed using the Sol Genomics VIGS tool (Fernandez-Pozo et al. 2015) and corresponded to nucleotide positions 161 to 449 in the mRNA sequence. This fragment, amplified from healthy *N. benthamiana* cDNA (discussed below) was cloned into the pTRV2 attR2-attR1 binary vector described by Liu et al. (2002), using Gateway technology. VIGS pTRV2 plasmids were transformed into *Agrobacterium tumefaciens* GV3101 and were coinfiltrated with one or both pTRV1 and WT PLRV, as described above. PLRV titer in systemic leaves was measured at 1.5, 2.5, and 3.5 wpi, as described above.

Semiquantitative and ddPCR.

Total RNA was extracted and cDNA was synthesized, following the protocol described by DeBlasio et al. (2015c), from a pool of two to three leaf discs per systemic leaf (two or eight plants) collected at 1.5 (*ANXD1* analysis) or 3.5 wpi, with the following exceptions: tissue was cryo-milled, using a Mixer Mill 400 (Retsch); an additional DNase treatment was done on-column during RNA extraction using RNase free DNase kit (Qiagen), following manufacturer instructions; cDNA synthesis was performed on 1 μ g of total RNA using the SuperScript III First-Strand synthesis system (Invitrogen), following the random hexamers protocol.

Primers used for semi-qPCR analysis of *ANXD1* were designed to amplify a region in Niben101Scf00260g00003.1 spanning an intron: NbANXD1_forward (5'-ATGGCTAGTCT TACTGTTCCG-3') and NbANXD1_reverse (5'-CTTCCTTAG CCAATAGGCATC-3'). Primers amplifying a region in *L23*: L23_forward (5'-AAGGATGCCGTGAAGAAGATGT-3') and L23_reverse (5'-GCATCGTAGTCAGGAGTCAAC-3') were used as an endogenous control. PCR reactions consisted of 10 μ l of REDTaq Ready Mix (Sigma-Aldrich), 10 μ M of forward and reverse primers and 2 μ l of 1:10 diluted template cDNA in a 20- μ l reaction volume. Thermocycling conditions started at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min, in a Bio-Rad T100 Thermal Cycler. Quantification of PCR products were made using ImageJ software. Primers for ddPCR were designed to amplify unique regions in the CP open-reading frame of PLRV and TRV2 (Ratcliff et al. 2001): PLRV CP_forward (5'-CTAACAGAGTTCAGCCAGT GG-3') and PLRV CP_reverse (5'-TGTCCTTTGTAAACACG AATGTC-3'), TRV2 CP_forward (5'-ACTCACGGGCTAAC AGTGCT-3') and TRV2 CP_reverse (5'-TCGTAACCGTTGT GTTTGGA-3'). Primer annealing temperatures and cDNA dilutions were optimized using REDTaq and were verified to be specific by semi-qPCR, following manufacturer instructions. Absolute quantification of viral cDNA was performed on the

QX100 Droplet Digital PCR system (Bio-Rad), using EvaGreen SuperMix (Bio-Rad), following manufacturer instructions. Total cDNA was diluted 1:800 in Ultrapure diethylpyrocarbonate-treated H₂O (Invitrogen) and 2 µl was used per reaction. Forward and reverse primers within each primer set were mixed 1:1 and 1 µl of the mix was used in separate ddPCR reactions for PLRV and TRV. Thermocycling conditions (Applied Biosystems 2720 Thermo cycler) started at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, 1 cycle at 4°C for 5 min, 1 cycle at 90°C for 5 min, and ending at 12°C. The ddPCR droplet data was analyzed using the QuantaSoft analysis software (Bio-Rad), which presents the target results as copies per microliter of PCR mixture.

Localization and quantification of PLRV infection foci.

Tissue immunoblot analysis was performed based on the protocol described by Lee et al. (2002) on fully expanded, *N. benthamiana* (4 wpi with virus) and *S. sarrachoides* (8 wpi with ΔRTD and 3 wpi with WT PLRV) leaves. The epidermal layer from the abaxial surface of leaves was removed, using tweezers, and the exposed spongy mesophyll layer was evenly pressed onto calcium chloride (0.2 M)-treated nitrocellulose membrane (Nitrobind, 0.45 micron, Maine Manufacturing, LLC). Blots were stored at 4°C in a sealed container until they were processed. Tissue blots were blocked for 2 h in 5% nonfat dried milk (NFDm) (Best Yet) dissolved in 1× PBS supplemented with 0.5% Tween 20 at room temperature. PLRV antigen was detected by first incubating blots with an in-house, polyclonal α-PLRV immunoglobulin (DeBlasio et al. 2015b), diluted 1:2,500 in 1× PBST, 0.4% NFDm, overnight at 4°C with gentle agitation. Blots were washed three times for 10 min in 1× PBST (PBS with Tween 20), followed by a 2-h incubation at room temperature, with a 1:2,500 dilution of goat antirabbit immunoglobulin conjugated to alkaline phosphatase in 1× PBST, 0.1% NFDm, as the secondary antibody (Sigma-Aldrich). Blots were washed as described above with a final 5-min wash in 1× PBS. Alkaline phosphatase was detected using the 1-Step NBT/BCIP system (Pierce).

Images were captured using an Olympus SZX-12 stereo microscope, using a DFPLFL 1.6× PF objective coupled with 10× eyepieces and a zoom range of 20×. From these images, the total area of completely white, photobleached sectors in TRV: *PDS*-silenced plants and green leaf sections of TRV: *GFP*-infected plants were measured using the Freehand Area Selection tool in ImageJ 1.47t (64-bit) to manually trace around white sectors in each immunoblot. Area was then computed using the ImageJ Measurement tool with the scale set for the appropriate number of pixels per millimeter, using the scale bar as a reference for each image. Rounded, PLRV infection foci (indicated by purple-blue alkaline phosphatase staining) in a given area were manually counted regardless of foci size or stain intensity. Staining in longitudinal veins was not counted.

Application of exogenous GA₃.

N. benthamiana plants were coinoculated with TRV constructs (TRV: *PDS* or TRV: *GFP*) or the WT PLRV infectious clone, singly or in combination, at 4 weeks postgermination, as described above. At 2 wpi with virus clones, plants were misted three times with 0.1 mM GA (MP Biomedicals, LLC) in 0.02% Tween 20 every 2 days for 1 week (Xu et al. 2013). Control plants were sprayed with 0.02% Tween 20 only. At 1 week post-application, PLRV titer was measured in 5-mm discs sampled from two newly expanding leaves per plant, by DAS-ELISA (described above). Leaf length and inflorescence height were measured manually at this same timepoint to confirm effects of

GA₃ application on plant growth. Images of plant growth were taken 2 weeks postapplication of GA₃, using a Canon EOS Rebel T6s camera.

Aphid fecundity assays.

All experiments were done using a *N. benthamiana*-adapted red strain of the green peach aphid (*Myzus persicae*) maintained by the C. Heck lab (U.S. Department of Agriculture, Emerging Pests and Pathogens Unit). Aphids were reared on healthy *N. benthamiana* 2 to 3 weeks at 18 to 20°C, under 16-h days. Apterous adult aphids were placed on the underside of healthy, detached *N. benthamiana* leaves for 48 h to allow for the production of nymphs. Five nymphs were then placed in plastic cages on the adaxial side of leaves of *N. benthamiana* plants 2.5 wpi with WT PLRV only, TRV clones only (TRV: *PDS* or TRV: *GFP*), or coinfecting with WT PLRV and TRV clones. Healthy plants were used as a negative control. After 48 h, all nymphs but one were removed. This single nymph was allowed to develop and progeny were counted after 8 and 11 days.

Statistical analysis.

Data were analyzed by two-tailed Student *t* test in Excel and one- or two-way analysis of variance, followed by a Tukey's honestly significant difference post hoc test, using the web-server calculator or Rstudio (version 3.1.3), respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Tukey's honestly significant difference webserver calculator:
http://astatsa.com/OneWay_Anova_with_TukeyHSD

Rstudio (version 3.1.3) open studio software: <http://www.rstudio.com>