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Association of the *Tobacco mosaic virus* 126 kDa replication protein with a GDI protein affects host susceptibility

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ABSTRACT

An interaction between the *Tobacco mosaic virus* (TMV) 126 kDa replication protein and a host-encoded Rab GDP dissociation inhibitor (GDI2) was identified and investigated for its role in infection. GDI proteins are essential components of vesicle trafficking pathways. TMV infection alters the localization of GDI2 from the cytoplasm to ER-associated complexes. Partial silencing of GDI2 results in significant increases in the number of TMV infection foci observed in inoculated tissues. However, GDI2 silencing does not affect TMV accumulation at the infection site, cell-to-cell movement, or susceptibility of the host to mechanical inoculation. Furthermore, increases in the number of successful infection foci were specific to TMV and correlated with the appearance of vesicle-like rearrangements in the vacuolar membrane. Tissue infiltrations with brefeldin A, an inhibitor of vesicle trafficking, also enhanced host susceptibility to TMV. Combined these findings suggest that the 126 kDa–GDI2 interaction alters vesicle trafficking to enhance the establishment of an infection.

Introduction

For simple positive-strand RNA viruses one essential step in the establishment of an infection involves the association of replication-associated components with elements of the host's endomembrane system (Netherton et al., 2007; Miller and Krijnse-Locker, 2008). The successful completion of this step is generally recognizable by the formation of membrane associated virus replication complexes (VRCs). While the form and cellular location of these membrane complexes can vary dramatically between different viruses, they all function to concentrate replication factors and mask nascent viral RNA synthesis from cellular defenses. To date, a number of virus and host components have been linked to the membrane rearrangements associated with VRC assembly (Salonen et al., 2005; Netherton et al., 2007; Miller and Krijnse-Locker, 2008). For example, the 3A protein of Poliovirus, a member of the *Picornaviridae*, uses the GTPase cellular secretory element Arf1 and its guanine exchange factor GBF1 to selectively recruit phosphatidylinositol-4-kinase IIIβ, which catalyzes the production of phosphatidylinositol-4-phosphate (PI4P) enriched membrane lipids (Hsu et al., 2010). PI4P enriched membranes subsequently attract the poliovirus 3D polymerase protein, initiating VRC assembly and virus replication. In contrast, the 1a protein of *Brome mosaic virus* (BMV), type member of the *Bromoviridae*, contains domains that include an amphipathic α-helix that mediate both membrane and self interactions (Liu et al., 2009). The self-assembly of 1a is thought to produce a shell-like structure that combined with its ability to associate with membranes produces the spherule-shaped invaginations that are characteristic of the BMV VRC. From these studies it is evident that the structural make-up and physiology of VRC associated endomembrane systems are reprogrammed during infection. Knowledge as to how these endomembrane systems are altered by the virus is thus essential to understanding the infection process.

In this study, we utilized *Tobacco mosaic virus* (TMV) as a model positive-strand RNA virus to identify and investigate the role of a Rab GDP dissociation inhibitor (GDI) in the establishment of infection. TMV is the type member of the genus *Tobamovirus* and encodes two replication proteins (126 and 183 kDa) as well as a coat protein and movement protein (Goelet et al., 1982). Both replication proteins contain methyltransferase and helicase domains. The 183 kDa replication protein contains an additional RNA-dependent RNA-polymerase domain that is produced via the read-through of an amber stop codon at the end of the 126 kDa protein (Pelham, 1978). Early in infection both 126 and 183 kDa proteins associate with the endoplasmic reticulum (ER), forming small membrane associated cytoplasmic VRCs (Heinlein et al., 1998; Mas and Beachy, 1999; dos Reis Figueira et al., 2002). As the infection progresses these smaller VRCs condense to form larger ER-derived membrane structures known as viroplasms or X-bodies (Esau and Cronshaw, 1967; Hills et al., 1987; Saito et al., 1987; Mas and Beachy, 1999). Evidence

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suggests that the 126 kDa replication protein is primarily responsible for the formation of these bodies as its expression alone results in the formation of both early and late stage VRC-like cytoplasmic bodies (dos Reis Figueira et al., 2002; Wang et al., 2010). Association of the 126 kDa replication protein with the hosts endomembrane system is thought to occur via an interaction with the transmembrane protein, TOM-1 (tobamovirus multiplication 1) (Yamanaka et al., 2000). TOM-1 and the closely related TOM-3 protein are required for virus replication and have been identified as possible membrane tethers for replication complexes (Yamanaka et al., 2002). Interestingly, TOM proteins have been shown to predominantly localize to the plant cell vacuolar membrane, suggesting that either these proteins localize differently during infection or that the vacuolar membrane plays a role in infection (Hagiwara et al., 2003).

Using a yeast two-hybrid approach we identified an interaction between the helicase domain of the TMV 126 kDa protein and the Arabidopsis encoded Rab GDP dissociation inhibitor protein 2 (GDI2). GDI proteins regulate the activity of Rab proteins and are essential components of vesicle trafficking within the cell. Rab proteins are members of the Ras superfamily of small GTPases that function to regulate intracellular vesicle trafficking pathways (Pfeffer, 2003; Barr, 2009). GDI proteins bind to Rab proteins in their inactive, GDP-bound state and recycle them to their donor membrane for use in further vesicle trafficking (Seabra and Wasmeier, 2004; Ali and Seabra, 2005). There are 57 identified Rab proteins in *Arabidopsis thaliana*, but only two identified GDI proteins (Bock et al., 2001; Pereira-Leal and Seabra, 2001). Both Arabidopsis GDI proteins share 95% identity and show similarly high levels of identity with GDI proteins in tomato, 82%, and tobacco, 84%. This level of homology suggests that these proteins are functionally conserved within a range of TMV hosts.

Since GDI proteins play an essential role in vesicle trafficking and because TMV replication occurs on vesicle-like membrane bodies, the interaction between GDI2 and the TMV 126 kDa replication protein was further investigated. Studies revealed that TMV infection alters the localization of GDI2 from the cytoplasm to ER-associated membrane bodies of similar size and shape to those produced during infection. Interestingly, silencing GDI2 prior to infection significantly increased the number of TMV infection foci produced upon inoculation, indicating that GDI2 regulates factors involved in host susceptibility. This enhanced susceptibility was specific to TMV and correlated with the appearance of vesicle-like rearrangements in the vacuolar membrane. Combined, these findings suggest that the TMV–GDI2 interaction modulates factors within the cell's vesicle transport system that contribute to the infection process.

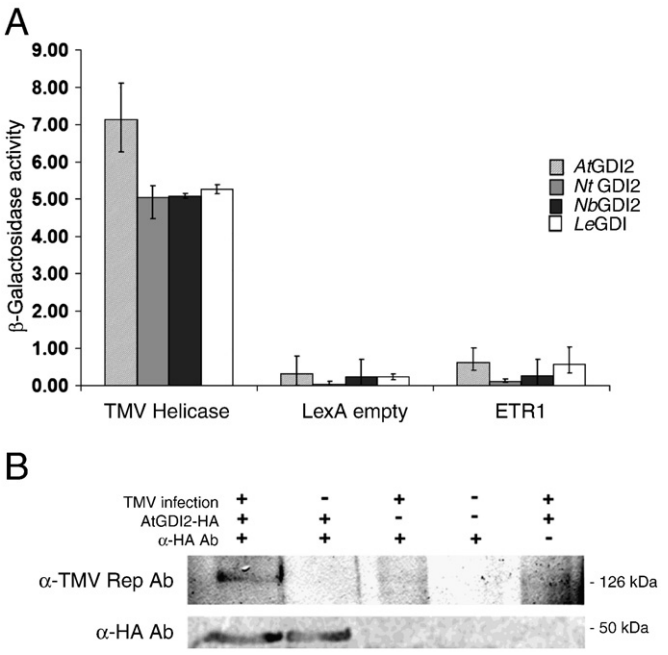

Fig. 1. Interaction between the TMV 126 kDa replication protein and GDI2. (A) Quantitative β-galactosidase assays displaying an interaction between the 126 kDa helicase domain (a.a. 814–1116) and the GDI proteins from Arabidopsis (*At*GDI2), *N. tabacum* (*Nt*GDI2), *N. benthamiana* (*Nb*GDI2) and tomato (*Le*GDI2). The ethylene receptor component ETR1 is used as a non-interacting control. (B) Co-immunoprecipitation of *At*GDI2-HA and the full-length TMV 126 kDa replication protein within TMV-infected tissues.

Results

Rab GDP dissociation inhibitor 2 (GDI2) interacts with the TMV 126 kDa protein

The helicase domain of the TMV 126/183 kDa replication proteins, amino acids 814–1116, was used in a two-hybrid assay to probe an *Arabidopsis thaliana* ecotype Nossen cDNA library for replicase interacting proteins. Of several interacting *Arabidopsis* clones, three were found to encode the C-terminal 110 amino acids of *At*GDI2 (At3g59920). To further confirm this interaction the full-length 445 amino acid (49.5 kDa) *At*GDI2 open reading frame (ORF) was cloned from the highly TMV susceptible Arabidopsis ecotype Shahdara (Dardick et al., 2000) and demonstrated via two-hybrid assay to interact with the viral helicase domain (Fig. 1A). Subsequent sequence comparisons revealed *At*GDI2 shares significant sequence identity with GDIs found in other important TMV hosts including *Solanum lycopersicum* (82%; TIGR gene index TC162880), *Nicotiana tabacum* (84%), and *Nicotiana benthamiana* (84%). Full-length GDIs cloned from these hosts were also found to interact with the TMV helicase domain via the two-hybrid assay (Fig. 1A), suggesting that this interaction is conserved among diverse TMV hosts.

To confirm the GDI2–TMV helicase interaction occurs within the context of an infection the *At*GDI2 ORF was fused to the hemagglutinin (HA) epitope and cloned into an *Agrobacterium* expression vector to create p35S::HA*At*GDI2. Agroinfiltration of p35S::HA*At*GDI2 into TMV or mock infected leaf tissue was subjected to pull-down assays using anti-HA antibodies. Results indicated that complexes containing HA*At*GDI2 and the full-length TMV 126 kDa replication protein were recovered only from tissues that were both p35S::HA*At*GDI2 infiltrated and TMV infected (Fig. 1B). Thus, HA*At*GDI2 is capable of interacting with the full-length 126 kDa replicase protein within the context of an active virus infection.

TMV infection alters AtGDI2-GFP localization

The effects of a TMV infection on the localization and accumulation of GDI2 were investigated using 35S::*At*GDI2-GFP transgenic *N. benthamiana* plants expressing the *At*GDI2 ORF fused to GFP. Low magnification fluorescent microscopy indicated that within uninfected tissues *At*GDI2-GFP fluorescence is localized to the cytoplasm of epidermal leaf cells (Fig. 2A). This is consistent with the localization of related GDI proteins and their role in vesicle transport (Ullrich et al., 1993; Seabra and Wasmeier, 2004). In contrast, we noted that within TMV-infected tissues *At*GDI2-GFP derived fluorescence was substantially altered, suggesting that infection changes either the localization or accumulation of the *At*GDI2-GFP protein (Fig. 2A). Western immunoblots for the detection of GFP were used to monitor the accumulation of *At*GDI2-GFP in both mock and TMV-infected tissues. Analysis of two independent transgenic 35S::*At*GDI2-GFP lines indicated no noticeable difference in the accumulation of *At*GDI2-GFP in either mock or infected tissues (Fig. 2B). Higher magnification confocal microscopy studies with the addition of Rhodamine B hexyl ester, an ER/mitochondria stain (Rashid and Horobin, 1990; Terasaki

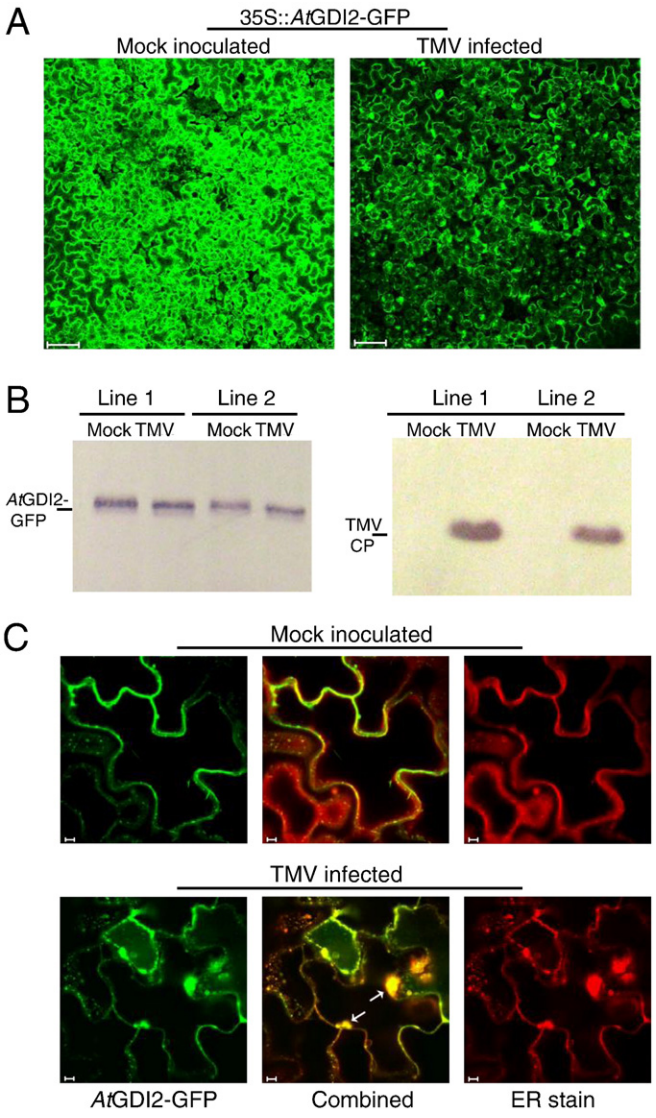

Fig. 2. TMV directed relocalization of *At*GDI2-GFP. (A) Mock and TMV-infected transgenic *N. benthamiana* expressing *At*GDI2-GFP fusion protein. Both images were taken with the same image settings. (B) Western immunoblots for the detection of *At*GDI2-GFP and the TMV coat protein (CP) from two independent 35S::*At*GDI2-GFP transgenic lines either mock or TMV infected. (C) Confocal microscopy image of mock or TMV-infected 35S::*At*GDI2-GFP transgenic leaf tissue with GFP or ER Rhodamine B derived fluorescence. Bars = 10 μm.

and Reese, 1992), demonstrated that in TMV-infected tissues *At*GDI2-GFP is predominantly relocalized to ER-associated inclusions (Fig. 2C) similar to those that occur in response to a TMV infection or the transient expression of the TMV 126 kDa replicase protein (Mas and Beachy, 1999; dos Reis Figueira et al., 2002). Combined these data indicate that TMV infection does not significantly alter the accumulation of *At*GDI2-GFP protein but instead changes its cellular localization.

Over-expression of AtGDI-GFP does not affect TMV infection

TMV induced disruptions in the localization of GDI2 suggest that functions of this host protein play a role during virus infection. To address this possibility transgenic 35S::*At*GDI2-GFP plants were used to investigate the effect of *At*GDI2-GFP overexpression on TMV infection. A recombinant TMV construct, TMV30BdsRed, expressing the fluorescent dsRed protein from a heterologous coat protein subgenomic promoter was used to identify virus-infected tissues in these plants (Shivprasad et al., 1999). TMV30BdsRed infection foci were monitored for spread and TMV coat protein accumulation. In comparison to non-transgenic control plants the appearance of TMV30BdsRed infection foci were not significantly altered in terms of numbers or the accumulation of TMV coat protein (Fig. 3). This suggests that over-accumulation of GDI prior to infection does not significantly alter the infection process.

Silencing of NbGDI2 enhances host susceptibility to TMV infection

A *Tobacco rattle virus* (TRV) gene silencing system was used as a means to investigate GDI2 function during infection. TRV produces mild symptoms in tobacco and tomato and has been developed as a viral induced gene silencing (VIGS) vector to knockdown the expression of targeted endogenous genes (Dinesh-Kumar et al., 2003). TRV-VIGS knockdown of the *Nicotiana benthamiana Nb*GDI2 gene resulted in a significant reduction (>77%) in the level of *Nb*GDI2 mRNA in comparison to a TRV vector only control construct (Fig. 4A). *Nb*GDI2 VIGS silenced plants produced characteristic mild TRV symptoms indicating that the partial disruption of this gene was not significantly detrimental to cell survival. The number of expressed GDI genes in *N. benthamiana* is unknown; however, based on the strong sequence similarity between *At*GDI1 and *At*GDI2 (95%) it seems likely that VIGS induced GDI silencing could disrupt the accumulation of all related GDI sequences within *N. benthamiana.*

In initial studies, purified TMV30BGFP was mechanically inoculated onto 29 fully expanded leaves of 15 *N. benthamiana* plants infected with the TRV *Nb*GDI2 silencing construct and 29 leaves of 15 plants infected with the unmodified TRV vector construct. The average number of fluorescent infection foci observed in the VIGS *Nb*GDI-silenced leaves (mean = 81, SE = 10) was significantly more than that of the infection foci observed in the vector only leaves (mean = 29,

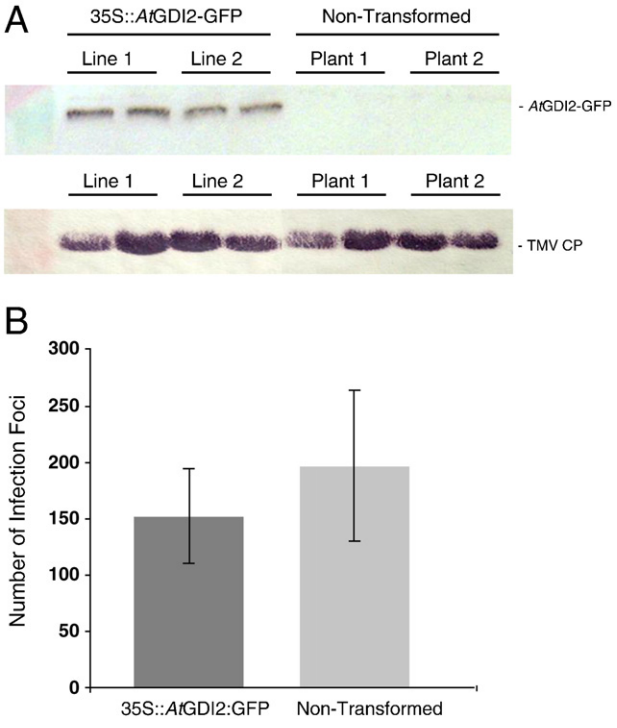

Fig. 3. Effects of *At*GDI2 over-accumulation on TMV infection. (A) Western immunoblots for the detection of *At*GDI2-GFP and TMV coat protein (CP) in 35S::AtGDI2-GFP and non-transformed *N. benthamiana* plants. Lanes represent protein levels in individual TMV30BdsRed fluorescent infection foci at 8 dpi. (B) Number of TMV30BdsRed infection foci in 35S::*At*GDI-GFP transgenic and non-transgenic *N. benthamiana* plants at 8 dpi. Data was taken from 10 different leaves on five different plants. The *t* test value for the number of TMV30BdsRed infection foci is $P(T \leq t) = 0.15$.

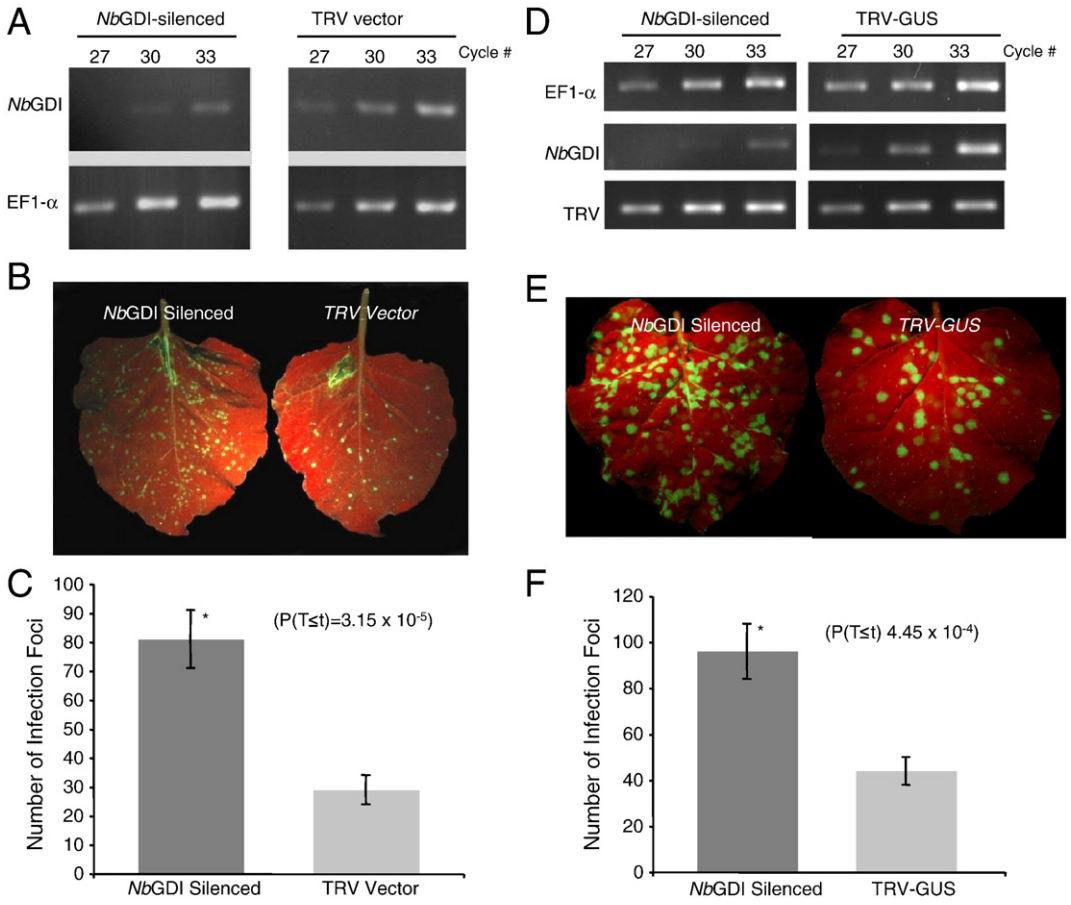

Fig. 4. Enhanced susceptibility to TMV30BGFP in *Nb*GDI2-silenced leaf tissue. (A and D) Representative semiquantitative RT-PCR analysis of *Nb*GDI2-silenced and control *N. benthamiana* tissues. (B and E) Results of infectivity assays with TMV30BGFP in *Nb*GDI2-silenced and control leaves. Photos taken at 8 dpi (B) and 9 dpi (C). (C and F) Number of TMV30BGFP infection foci averaged from 29 (C) and 21 (F) leaves per treatment.

SE = 5) ($P(T \leq t) = 3.15 \times 10^{-5}$; Fig. 4B and C). Analysis of virus spread, measured as the average diameter of TMV30BGFP fluorescent infection foci, and virus accumulation, measured as the level of detectable coat protein in individual TMV30BGFP foci, were found to be similar for *Nb*GDI-silenced and TRV vector control plants (data not shown). Thus, the knockdown of *Nb*GDI2 mRNA levels did not appear to affect the biology of the virus once the initial infection was established.

To further confirm this observation a second VIGS control construct that expresses a segment of the β-glucuronidase gene (GUS) was created as a means to mimic any effect conferred by the addition of a sequence to the TRV-VIGS vector. RT-PCR analysis of *Nb*GDI2 mRNA again showed a significant reduction in the *Nb*GDI2 VIGS silenced leaf tissue in comparison to tissue infected with the TRV-GUS vector construct (Fig. 4D). We also monitored the level of TRV genomic RNA present in both *Nb*GDI2 and GUS TRV infected tissues and found that neither the *Nb*GDI2 sequence nor its silencing adversely affected the accumulation of the TRV silencing vector (Fig. 4D). To monitor TMV infections, two sets of 21 leaves from 10 *N. benthamiana* plants infected with either the *Nb*GDI2 or GUS TRV-VIGS constructs were inoculated with TMV30BGFP. Results again demonstrated that silencing of *Nb*GDI2 produces a greater than 2-fold increase in TMV30BGFP infection foci (mean = 96, SE = 12) over the control TRV-GUS (mean = 44, SE = 6) ($P(T \leq t) = 4.45 \times 10^{-4}$; Fig. 4E and F).

GDI silencing does not alter the efficiency of mechanical inoculations

One possible explanation for enhanced TMV susceptibility in GDI2 silenced tissues is that these tissues are simply easier to infect by mechanical wounding. In this hypothesis, GDI2 silencing affects cellular membranes such that they are more susceptible to mechanical inoculations or recover more slowly from wounding and thus remain accessible to virus infection over a longer period of time. To test these possibilities we first determined if the enhanced susceptibility observed in *Nb*GDI2 silenced tissues was specific to TMV. For these experiments *Nb*GDI2 silenced leaf tissues were challenged with *Tobacco necrosis virus* (TNV). TNV is a member of the *Tombusviridae* family of plant viruses and shares no specific sequence homologies with TMV. TNV also produces a distinctive necrotic lesion at the site of infection in leaves of *N. benthamiana* making it possible to assess numbers of infection foci. Results from TNV inoculations showed no significant difference in the number of TNV lesions observed in either *Nb*GDI2 (mean = 96, SE = 4) or GUS-silenced (mean = 72, SE = 10) plants ($P(T \leq t) = 0.37$; Fig. 5A), suggesting that the silencing of GDI2 is specific to TMV infection.

We also utilized a detached leaf assay to determine if *Nb*GDI2 silenced tissues were slower to recover from mechanical wounding (Furumoto and Wildman, 1963). Specifically, detached *N. benthamiana* leaves were mechanically wounded by rubbing with carborundum and at various times post-wounding inoculated by dipping into a solution of 0.005 or 0.001 mg/ml of TMV30BGFP. Results showed increased TMV30BGFP infection sites in *Nb*GDI2 silenced leaves vs. GUS-silenced control leaves (Fig. 5B). However, when the number of infection sites at each time point is calculated as a percent of the total infection sites produced at time zero no specific differences in the overall rate of infectivity is observed between treatments (Fig. 5C). Thus, recovery from mechanical wounding appears to occur equally well in *Nb*GDI2 silenced and non-silenced tissues. Combined these findings indicate that alterations in host susceptibility to mechanical

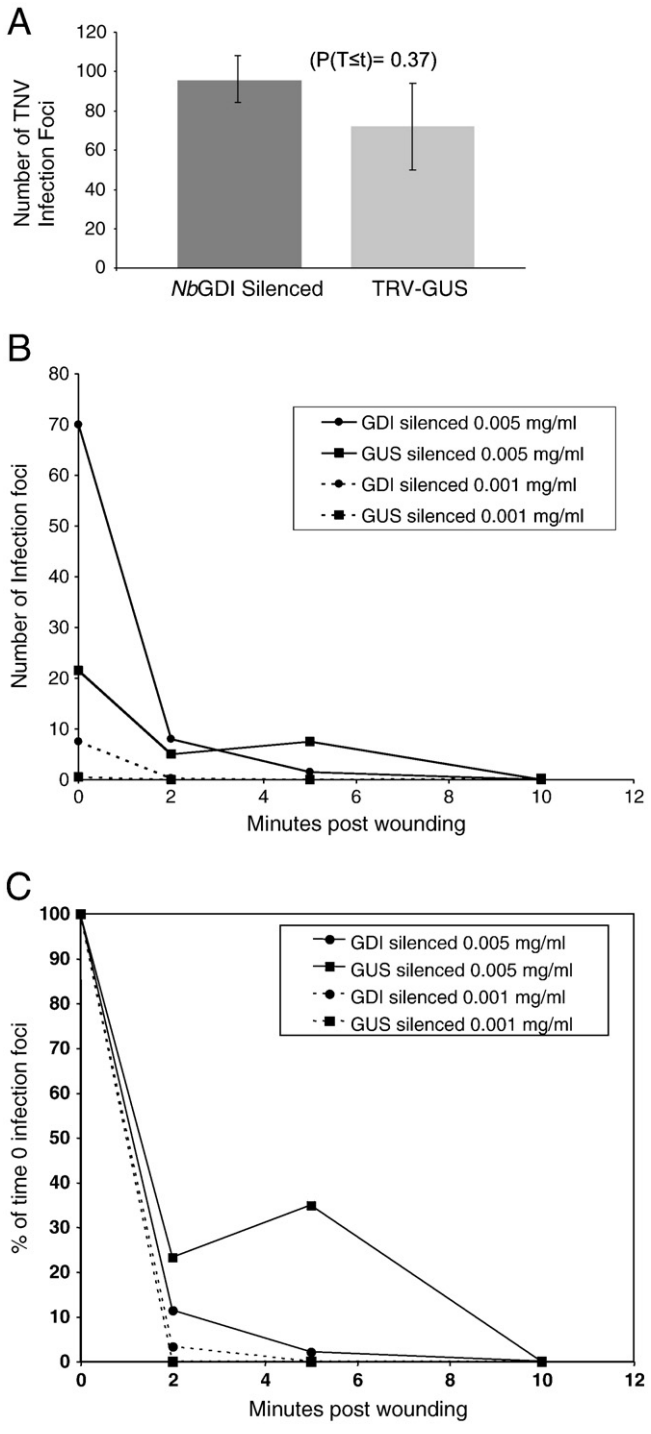

Fig. 5. GDI silencing does not affect the efficiency of mechanical inoculations. (A) Number of TNV infection foci observed in *Nb*GDI2 silenced and control leaf tissues. (B) Number of TMV30BGFP infection foci observed in silenced and control *N. benthamiana* leaf tissues at times post-wounding. Leaves were wounded and then immersed in a solution of 0.005 mg/ml or 0.001 mg/ml purified TMV30BGFP at the specified time points. (C) Data in B presented as the percent of the total number of infection foci observed at time zero for each treatment.

inoculations are not a significant factor in the enhanced TMV susceptibility observed in GDI2 silenced tissues.

Brefeldin A enhances susceptibility to TMV

Increased susceptibility to TMV in GDI2 silenced tissues could have been linked to membrane perturbations that enhanced the availability of host components needed to initiate VRC formation. Since GDI proteins function in the recycling of proteins involved in vesicle trafficking, we reasoned that reductions of available GDI2 proteins disrupt the transport of vesicles and their cargo within the cell. To test this hypothesis we used the drug, brefeldin A (BFA) as an alternative means to disrupt vesicle trafficking. BFA targets the Sec7 domain of guanine nucleotide exchange factors that activate the Arf family of small GTPases involved in the regulation of vesicle transport. Disruption of Arf function inhibits vesicle retrograde trafficking from the ER to the Golgi, affecting the distribution of components within the endomembrane network (Klausner et al., 1992; Shin and Nakayama, 2004). Half-leaf assays in which half of the leaf was infiltrated with 1% DMSO and the other half of the leaf was infiltrated with 1% DMSO containing 5 μg/ml of BFA were compared for susceptibility to infection by TMV30BGFP. Within the leaf halves treated with BFA we noted a 40% ($P(T<=t)=0.0089$) increase in the number of fluorescent infection foci as compared to the DMSO only infiltrated leaf half (Fig. 6). This finding suggests that interference in the trafficking of proteins within the endomembrane system, either by disrupting GDI2 function or BFA treatment has a positive impact on the establishment of TMV infection sites.

Silencing NbGDI2 produces endomembrane changes in the vacuolar membrane similar to that observed during TMV infection

Since GDI proteins function in vesicle trafficking and TMV is known to remodel ER membranes during replication we reasoned that TMV interference in GDI2 function may have a role in this remodeling. To monitor changes to the endomembrane system,

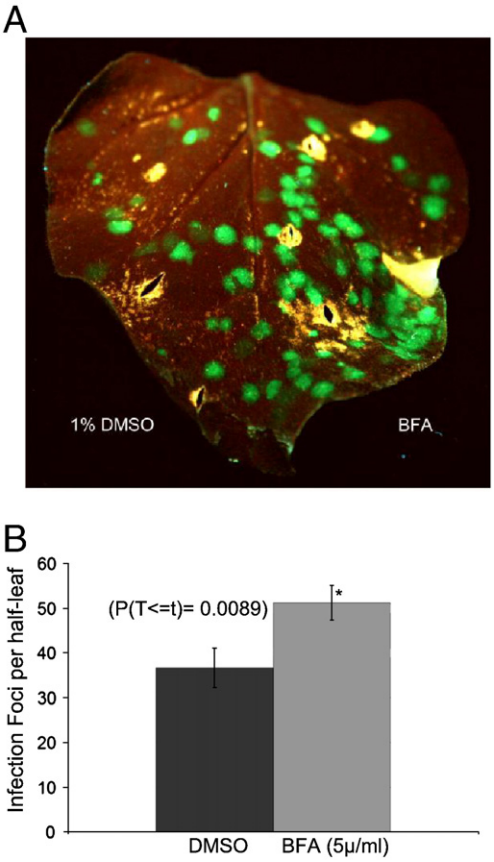

Fig. 6. Brefeldin A treatment enhances susceptibility to TMV30BGFP in *N. benthamiana*. (A) DMSO only and DMSO plus 5 μg/ml brefeldin A (BFA) treated half leaf assay inoculated with TMV30BGFP. (B) TMV30BGFP infection foci per half leaf averaged from 12 leaves.

several known membrane marker proteins were chosen based on their target membranes and expressed as GFP fusion proteins. The marker proteins selected were (GFP-HDEL [ER] (Haseloff et al., 1997), GFP-Vam3 [vacuole] (Uemura et al., 2002), Sar1-GFP [ER exit site] (daSilva et al., 2004), Pad1-GFP [20S proteosome] (Fu et al., 1998) and ERD2-GFP [Golgi]. Using the TRV-VIGS system, transgenic *N. benthamiana* lines for each of the GFP marker proteins were silenced for *Nb*GDI2 and examined by fluorescence microscopy for changes in the localization of the marker proteins in comparison to TRV vector only treated plants. *Nb*GDI2 silencing in ER (GFP-HDEL and Sar1-GFP), proteosome (Pad1-GFP) or Golgi (ERD2-GFP) backgrounds did not produce a noticeable difference in the localization or intensity of GFP derived fluorescence (Supplemental Fig. 1). In contrast, *Nb*GDI2 silencing in vacuolar GFP-Vam3 plants resulted in the production of small (3–9 μm) vesicle-like bodies (Fig. 7B and E). In Z-stacks these bodies appeared to be distributed throughout the cytoplasm of the cells (Supplemental Fig. 2). In contrast, control TRV vector only plants did not display similar types of GFP-Vam3 vesicle-like bodies (Fig. 7A; Supplemental Fig. 2). The appearance of GFP-Vam3 labeled vesicles suggests that the transport of this vacuolar marker protein is altered in response to *Nb*GDI2 silencing.

Although TMV infection has not previously been shown to alter the vacuolar membrane, the accumulation of Vam3 vesicle-like bodies in GDI2-silenced tissues was of interest since the 126 kDa-interacting TOM host proteins have been shown to predominantly localize to the vacuolar membrane (Hagiwara et al., 2003). When we compared TMV-infected and mock-inoculated GFP-Vam3 plants, we observed discrete Vam3-GFP labeled vesicles similar to those observed in GDI2 silenced Vam3-GFP tissues (Fig. 7C and D). These findings suggest that interference in GDI2 function, either by silencing or virus infection, produces a similar effect on the vacuolar membrane system.

Discussion

For viruses, the cellular environment of the initially infected cell represents an important determinant in the establishment of infection. Within compatible hosts, the initiation of virus infection generally occurs rapidly upon cell entry, suggesting that the cellular components needed to establish an infection must be readily available and in sufficient quantity prior to virus entry. In this study we identified GDI2, a Rab GDP dissociation inhibitor protein, as a host component that modulates cell susceptibility to the establishment of a TMV infection. *At*GDI2 can functionally replace the yeast GDI protein, Sec19, and interact with specific Arabidopsis Rab proteins, demonstrating that *At*GDI2 is a functional Rab GDP dissociation inhibitor protein (Ueda et al., 2006). Rab GDP dissociation inhibitor proteins play essential roles in the regulation of numerous small Rab GTPase proteins that in turn are involved in vesicle formation, vesicle movement and membrane fusion, functions that are essential in the intracellular trafficking of proteins. The ability of TMV replication proteins to interact with and disrupt GDI2 localization indicates that virus infection alters vesicular trafficking within the infected cell. Furthermore, enhanced susceptibility to TMV infection in GDI2 silenced leaf tissues suggests a link between GDI2 directed cellular trafficking and the establishment of cellular conditions that favor infection.

The establishment of a virus infection involves a series of steps. For TMV the infection process first requires cellular entry through healable wounds caused by mechanical damage. Upon cellular entry the higher pH and reduced calcium ion concentrations within the cell result in the disassembly of the rod-shaped virion from the virus 5′ RNA end (Namba et al., 1989; Culver 2002). This exposes the 5′ end CAP and ribosome initiation site within the virus RNA. Subsequent translation results in the production of the 126/183 kDa replication proteins that in turn recognizes the disassembled 3′ RNA end and initiates minus strand RNA synthesis. Christensen et al. (2009) have recently shown that microinjected fluorescently labeled TMV RNA forms distinct packets that rapidly associate with the motile cortical actin/ER network within the cytoplasm. The 5′ RNA end CAP which is required for infectivity was also required for formation of these packets. This information suggests that the initial translation and attachment of TMV to ER sites of replication occurs via a CAP dependent translation process (Christensen et al., 2009). This is consistent with other eukaryotic mRNAs that have been shown to be localized to specific cell structures (Meignin and Davis, 2010). To establish an active virus replication complex TMV replication proteins must associate with the ER. Within Arabidopsis the membrane spanning proteins TOM1 and 3 are required for efficient virus

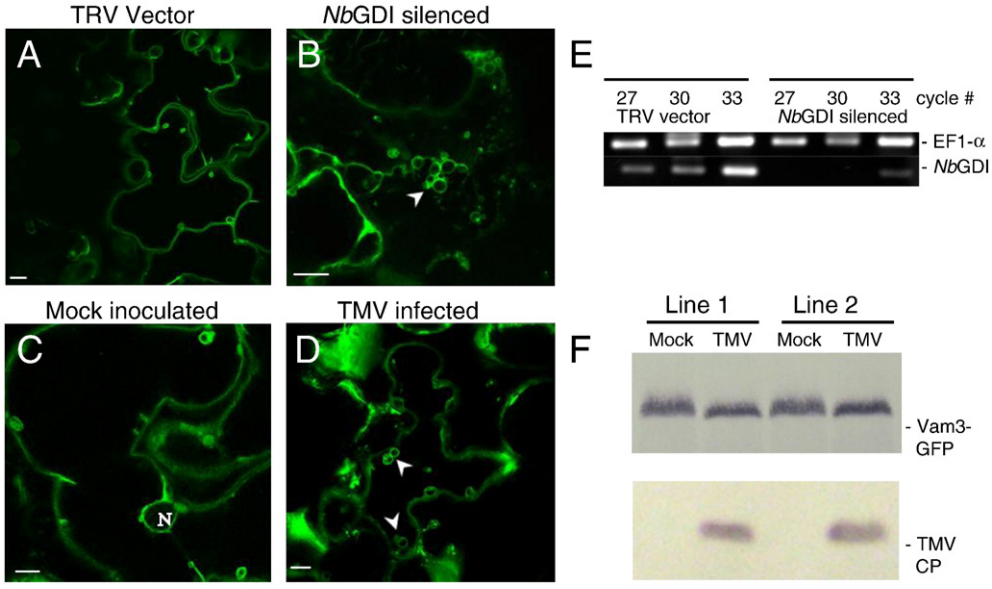

Fig. 7. Effects of GDI silencing and TMV infection on the localization of the vacuolar marker protein Vam3. (A–D), Confocal images of 35S::GFP-Vam3 transgenic *N. benthamiana* plants including the vector control, silenced for *Nb*GDI2, mock-inoculated or infected with TMV. Arrows in B and D indicate vesicle-like structures. (E) Semiquantitative RT-PCR analysis of *Nb*GDI2-silenced and control tissues. (F) Western immunoblot for the detection of the GFP-Vam3 marker protein and the TMV coat protein (CP) in mock and infected leaf tissues from two independent transgenic lines.

replication (Yamanaka et al., 2000, 2002). Specifically, TOM proteins interact with the TMV 126/183 kDa replication proteins and are thought to function as tethers, linking the viral replicase to host endomembrane system (Yamanaka et al., 2000). The availability of these components is therefore a critical link in establishing active replication complexes.

Disruption of GDI2 function affected only the susceptibility of the host to TMV infection and not TMV movement or virus accumulation at the infection site. This finding suggests that GDI2 silencing alters the availability of cellular components necessary to establish an infection but does not represent a rate-limiting step once an infection is initiated. Thus, the identified interaction between GDI2 and TMV replication proteins most likely represents a mechanism to modulate the composition of the host's endomembrane system during the establishment of an infection. Rab GDP dissociation inhibitor proteins are essential in the recycling and regulation of Rab proteins, affecting an array of vesicle functions, including formation, docking and fusion. Disruption of these functions is likely to affect the processing, movement and localization of numerous host-encoded proteins via the endomembrane system. One possible outcome of this disruption could be alterations in vesicle and protein transport that result in the cell's membranes becoming more susceptible to wounding via mechanical inoculations. However, infectivity studies done at varying time points post-wounding indicate that only the number of infection sites increased in GDI2 silenced tissues but not the specific rate of infectivity. This suggests that observed changes in cell susceptibility are due to alterations in the ability of the virus to establish an infection not in the ability of TMV to gain access to the cell. Additionally, GDI2 silencing did not affect the number of infection sites obtained via mechanical inoculation of the non-related virus, TNV. Enhanced susceptibility to TMV but not TNV further indicates that the effects of silencing GDI2 are specific to TMV and not the result of general changes in membrane properties.

Another possible explanation for enhanced TMV susceptibility observed in GDI2 silenced tissues involves the reallocation of host components needed to establish an infection. By altering the location and/or concentration of needed host components TMV could increase the probability that an infecting virus RNA can initiate an infection. Previous studies have indicated that a minimum of ~72 virus particles are needed to establish a TMV infection by microinjection (Halliwell and Gazaway, 1975). Although these microinjection studies do not rule out the possibility that one virus particle can cause an infection they do suggest that not every particle entering a cell will always establish an infection. Thus, access and availability to host components required for replication likely play an important role in establishing an infection. GDI2's role in vesicle transport would suggest that interference in its function could alter the distribution of host components needed for replication within the endomembrane system. The ability of BFA treatments to similarly enhance susceptibility to TMV provides additional support to indicate that alterations in the composition of the endomembrane system brought on by the disruption of normal vesicle trafficking can enhance the ability of TMV to establish an infection.

It is likely that disruption of vesicle trafficking results in altered distributions of numerous host proteins. Consistent with this possibility we noted that GDI2 silencing enhanced the production of small vesicle-like bodies in vacuolar labeled Vam3-GFP labeled tissues. We also noted the enhanced production of similar Vam3-GFP labeled bodies in TMV-infected tissues. Vam3 functions as a t-SNARE protein on the surface of the vacuolar membrane and is thus involved as a vesicle transport receptor (Sato et al., 1997). The observed Vam3-GFP labeled bodies would suggest that in GDI2 silenced or TMV-infected tissues the targeting of Vam3-GFP to the vacuolar membrane or its maintenance within the vacuolar membrane is altered. To our knowledge this is the first report to identify TMV induced alterations in a vacuolar marker protein. This is of interest since it has previously been demonstrated that the identified replicase interacting membrane protein TOM1 primarily localized to the vacuolar membrane (Hagiwara et al., 2003). The predominant localization of TOM proteins to the vacuolar membrane while TMV replication occurs on the ER suggests that these proteins may be redirected or blocked in their transport to the vacuolar membrane during infection. Disruption of vesicular trafficking through the disruption of GDI2 function could provide a route for this to occur. However, additional studies tracking changes in the localization of TOM proteins and other vacuolar proteins are needed to confirm this possibility.

Materials and methods

Plant material, virus constructs and infectivity analysis

All plants were grown in LCI potting mix (SunGro Inc., Bellevue, WA) at 28 °C with a 12 h light/dark cycle and 80% humidity. TMV30BGFP and TMV30BdsRed cDNA constructs contain the GFP or dsRed ORF inserted downstream of the TMV coat protein subgenomic promoter via PCR generated *Pac*1 and *Xho*1 restriction sites (Shivprasad et al., 1999). TMV30BGFP and TMV30BdsRed virions were purified from transcript infected plants as previously described (Gooding and Hebert, 1967).

Virus infections were accomplished via rub inoculations of carborundum dusted leaves using 20 μL of 0.5 mg/ml purified virions for TMV30BGFP and 20 μL of 0.75 mg/ml of TMV30BdsRed. A *Tobacco necrosis virus* (TNV) strain isolated by Dr. K. Corbett, University of Maryland, was similarly inoculated using 20 μL of 0.025 mg/ml purified virions. The time course infectivity analysis was performed as previously described with the following modifications (Furumoto and Wildman, 1963). Carborundum dusted detached *N. benthamiana* leaves were rub wounded and then dipped at specific times post-wounding in a 1 μg/ml or 5 μg/ml suspension of TMV30BGFP. Leaves were kept moist between wounding and inoculation times. Inoculated leaves were kept in a humidity chamber prior to imaging for fluorescent infection foci at 6–8 days post-inoculation.

Yeast two-hybrid and in vivo pull-down assays

Yeast two-hybrid screens between an Arabidopsis ecotype Nossen cDNA library (ABRC, Columbus, OH) and the TMV helicase domain, amino acids 814–1116, were performed as previously described (Padmanabhan et al., 2005). Full-length GDI2 ORFs were cloned into the yeast vector pGAD10 from cDNA derived from *Lycopersicon esculentum* cv Pilgrim, *Arabidopsis thaliana* ecotype Shahdara, *Nicotiana tabacum* cv Xanthi, and *N. benthamiana* via PCR generated *Xho*I and *EcoR*I restriction sites. Full-length pGAD-GDI2 clones were subsequently co-transformed into yeast strain L40 with either pLexA-Helicase, the empty pLexA vector or one encoding an ethylene receptor component, ETR1 (Clark et al., 1998). Two-hybrid derived β-galactosidase activity was quantified in liquid culture as previously described (Miller, 1972; Goregaoker et al., 2001).

In vivo pull-down assays were performed as described (Wang et al., 2009). Briefly, the full-length cDNA of *At*GDI2 was cloned downstream of the double 35S promoter and fused via the C-terminus to the hemagglutinin (HA) tag of the pPily cassette vector using PCR generated *BamH*I and *EcoR*I restriction sites (Ferrando et al., 2000). The pPily expression cassette was subsequently cloned into pBIN19-Plus using a unique *Kpn*I site to create pBIN*At*GDI2 and transformed into *Agrobacterium tumefaciens* strain GV3101 (Holsters et al., 1978; van Engelen et al., 1995; Ferrando et al., 2000). Leaves of *N. benthamiana* were inoculated with 0.5 μg/ml of wild-type TMV and at 2 days post-infection infiltrated with *Agrobacterium* carrying the pBIN*At*GDI2. Leaf tissue was harvested and extracted in buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 0.5% TritonX-100, 0.2% 2-mercaptoethanol, 5% glycerol) with the addition of 1 mM PMSF and

proteinase inhibitor cocktail (Sigma, St. Louis, MO). Tissue extracts were incubated overnight at 40 °C with 5 μL of anti-HA antibody (Santa Cruz Biotechnologies) followed by an additional 3-h incubation with protein A agarose (Invitrogen, Carlsbad, CA). Precipitated immune complexes were resuspended in 2× Laemmli buffer and separated by SDS-PAGE (Laemmli, 1970). Resolved proteins were visualized via Western blot using HA and TMV replicase specific antibodies.

GDI2 silencing assays

The *Tobacco rattle virus* VIGS vectors (pTRV1 and pTRV2) were kindly provided by Dr. S. P. Dinesh-Kumar, University of California, Davis (Liu et al., 2002). Nucleotides 888–1284 of *Nb*GDI2 cDNA and *At*GDI2 cDNA were PCR-modified to contain 5′ *EcoR*1 and 3′ *Kpn*I sites, ligated into similarly digested pTRV2 plasmids and transformed into *A. tumefaciens* strain GV3101. Nucleotides 9–409 of the β-glucoronidase gene were similarly PCR amplified to contain unique *EcoR*I and *Kpn*I sites, ligated into the pTRV2 vector and transformed into the GV3101 strain. For VIGS assays, *A. tumefaciens* containing pTRV2:GDI2 or pTRV2:GUS or the unmodified pTRV2 vector were mixed with an equal volume of cells containing the pTRV1 vector and infiltrated into *N. benthamiana* plants at the four leaf stage.

For mRNA analysis, total RNA was extracted as per the manufacturer's protocol using Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was pretreated with RQ1 DNase (Promega, Madison, WI) and then reverse-transcribed using the SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Four microliters of each reaction was used for PCR analysis. For semiquantitative analysis, a 50 μL PCR reaction was used to amplify gene fragments. *Nb*GDI2 primers were designed to amplify the endogenous gene between nucleotides 300 and 700 using the following primers: NbGDI 300 FWD (CTTTAAAGCAGTTGATGGCAG) and NbGDI 700 REV (GCTCTCCTAATCCATACAAAG). Four microliters of sample was removed at the end of 27, 30, and 34 cycles. These samples were visualized via agarose gel electrophoresis, photographed and analyzed for pixel density using an AlphaImager system (Alpha Innotech, Santa Clara, CA). GDI amplified products were normalized to the EF1-α internal standard and compared for differences in pixel density.

Transgene constructs, production of transgenic plants and analysis

The binary expression vector pBI121 (Clontech, Mountain View, CA) was modified and used to express the following ORFs as GFP fusions: GFP-HDEL (Haseloff et al., 1997), GFP-*At*Vam3 [AT5G46860] (Uemura et al., 2002), *At*Sar1-GFP [AT1G56330] (daSilva et al., 2004), *At*Pad1-GFP [AT3G51260] (Fu et al., 1998), *At*ERD2-GFP [AT1G29330]. A multi-cloning site (MCS) linker oligomer (5′-*Xba*I-*Xho*I-*Spe*I-*Sal*I-*Pac*I-*Kpn*I-*Hpa*I-*Sac*I-3′) was inserted into the *Xba*I and *Sst*I sites of pBI121. GFP-*At*Vam3 was constructed as an N-terminal GFP fusion protein using the *Spe*I and *Pac*I linker sites with the GFP ORF inserted at the *Xho*I and *Spe*I sites. *At*Sar1-GFP, *At*Pad1-GFP, and *At*ERD2-GFP were made as C-terminal GFP fusion proteins in a two-step process. In the first step *At*Sar1, *At*Pad1, and *At*ERD2 ORFs were PCR amplified to contain 5′ *Xho*I and 3′ *BsiW*I restrictions sites. Each ORF was then ligated downstream to the GFP ORF via the *BsiW*I site. In the second step each fusion construct was moved into the modified pBI121 using the *Xho*I and *Kpn*I linker sites. The GFP-HDEL construct was made by PCR amplifying the GFP ORF with C-terminal codons for the amino acids HDEL and an N-terminal signal peptide as described by Horsch et al. (1985). The PCR product was inserted into the modified pBI121 MCS using the *Xho*I and *Kpn*I linker sites.

Surface sterilized leaf disks from *N. benthamiana* were cocultivated with *A. tumefaciens*, strain GV 3101, carrying the modified pBI121 marker protein plasmids grown in LB media containing 25 μg/ml gentamicin, 50 μg/ml rifampicin, and 50 μg/mL kanamycin. Transformed cells were selected for kanamycin resistance and regenerated into plants as previously described (Horsch et al., 1985). Regenerated plants were placed in soil and maintained in planter flats with domes to increase humidity for 2 weeks and gradually introduced to the humidity of the growth chambers. All chambers were maintained at 23 °C with a 12-h photoperiod at 55% humidity.

Whole leaf and cell imaging

Whole leaves were removed from the plant and imaged using a long wavelength UV lamp and a Tiffen™ 58 mm Yellow2 wavelength filter on a Canon EOSD60 digital camera with a tripod. For confocal imaging, leaf epidermal tissue was mounted using water and imaged using a Zeiss LSM510 laser scanning confocal microscope system with 10× NA 0.8 dry and 63× NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thornwood, NY). Excitation sources were 488 nm for GFP and 543 nm for dsRed. Images were modified in Zeiss LSM Imager Examiner and processed for printing in Adobe Photoshop CS (Grand Prairie, TX).

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