Trigalactosyldiacylglycerol 3 protein orthologs are required for basal disease resistance in Nicotiana benthamiana

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Abstract  Phosphatidic acid plays an important role in Nicotiana benthamiana immune responses against phytopathogenic bacteria. We analyzed the contributions of endoplasmic reticulum-derived chloroplast phospholipids, including phosphatidic acid, to the resistance of N. benthamiana against Ralstonia solanacearum. Here, we focused on trigalactosyldiacylglycerol 3 (TGD3) protein as a candidate required for phosphatidic acid signaling. On the basis of Arabidopsis thaliana TGD3 sequences, we identified two putative TGD3 orthologs in the N. benthamiana genome, NbTGD3-1 and NbTGD3-2. To address the role of TGD3s in plant defense responses, we created double NbTGD3-silenced plants using virus-induced gene silencing. The NbTGD3-silenced plants showed a moderately reduced growth phenotype. Bacterial growth and the appearance of bacterial wilt disease were accelerated in NbTGD3-silenced plants, compared with control plants, challenged with R. solanacearum. The NbTGD3-silenced plants showed reduced both expression of allene oxide synthase that encoded jasmonic acid biosynthetic enzyme and NbPR-4, a marker gene for jasmonic acid signaling, after inoculation with R. solanacearum. Thus, NbTGD3-mediated endoplasmic reticulum—chloroplast lipid transport might be required for jasmonic acid signaling-mediated basal disease resistance in N. benthamiana.

Key words: basal disease resistance, Nicotiana benthamiana, phosphatidic acid, trigalactosyldiacylglycerol, virus-induced gene silencing.

Phospholipid-mediated signaling cascades are important for the establishment of plant defense responses (Canonne et al. 2011; Munnik 2001; Testerink and Munnik 2005). Among the phospholipids, phosphatidic acid (PA) functions as a signal molecule in responses to both biotic and abiotic stresses (Hong et al. 2016; Liu et al. 2013; Zhao 2015). The production of PA is activated by osmotic stresses, such as salinity, drought, and hyperosmosis, and by treatments with the drought-related stress hormone abscisic acid (Munnik 2001). PA is also formed after leaf wounding in various plants, including soybean, sunflower, pepper, broad bean, and castor bean (Munnik 2001; Testerink and Munnik 2005). The biotic chitin elicitor induces PA biosynthesis, leading to phytoalexin accumulation in rice cells (Yamaguchi et al. 2005). Additionally, PA is produced during the Cf-4 mediated defense responses in tomato (de Jong et al. 2004). Therefore, PA plays important roles in protecting plants from abiotic and biotic stresses.

Recently, we identified the SEC14 gene, encoding a phospholipid transfer protein, in Nicotiana benthamiana (NbSEC14). The suppression of phospholipase C and D activities and a reduction in the PA content have been observed in NbSEC14-silenced plants, resulting in compromised resistance against phytopathogenic bacteria (Kiba et al. 2012, 2014, 2016). We also reported that phoshoinositide-specific phospholipase C2 in N. benthamiana participates in basal disease resistance (Kiba et al. 2020). Conversely, the silencing of PA phosphatase causes an over accumulation of PA, resulting in enhanced resistance against Ralstonia solanacearum (Nakano et al. 2013, 2015). A subcellular localization analysis showed that the PA phosphatase localizes to chloroplasts in N. benthamiana (Nakano et al. 2013). These results suggest an important role for PA, namely PA accumulation in chloroplasts, in N. benthamiana resistance against phytopathogens. In 18:3 plants, a major source of PA in chloroplast is reportedly synthesized in endoplasmic reticulum (ER) through the eukaryotic lipid metabolic pathway, and transport to chloroplast (LaBrant et al. 2018; Michaud and Jouhet 2019; Negia et al. 2018; Zhang et al. 2019). However, role of PA transport from ER to chloroplast in plant immune responses is largely obscure. Here, we report

Abbreviations: CFU, colony forming units; ER, endoplasmic reticulum; JA, jasmonic acid; PA, phosphatidic acid; qRT-PCR, quantitative real time-polymerase chain reaction; Rs, Ralstonia solanacearum; TGD, trigalactosyldiacylglycerol; VIGS, virus-induced gene silencing.
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the contributions of trigalactosyldiacylglycerol (TGD) protein complex as a candidate required for PA signaling to induce the resistance against *R. solanacearum* in *N. benthamiana*.

*Nicotiana benthamiana* was grown in a cultivation room as reported previously (Maimbo et al. 2007). *Ralstonia solanacearum* strain OE1-1 (RsOE1-1), and *Agrobacterium tumefaciens* were cultured as described previously (Maimbo et al. 2010). The bacterial suspensions were infiltrated using syringes without needles (Maimbo et al. 2010).

Total RNA was isolated using a NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). A 1-µg sample of total RNA was used as the template for reverse transcription with a ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd. Tokyo, Japan). The sequence analysis was performed using reagents from the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3100 Avant Automated Sequencer (Applied Biosystems, Warrington, UK). The sequence analysis was carried out using DNASIS (version 3.6; Hitachi, Yokohama, Japan) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al. 1990). In silico analysis of protein localization was analyzed with PSORT WWW Server (https://psort.hgc.jp/).

cDNA fragments from the conserved region of the *NbTGD3-1* and *NbTGD3-2* sequences were amplified with specific primer pairs (Supplementary Figure S1; Supplementary Table S1) and then cloned independently into pPVX201 (Kiba et al. 2012). The pPVX201 plasmid lacking any insert was used as a control. The binary plasmids were transformed individually into *A. tumefaciens* strain GV3101, and the transformants were inoculated independently into *N. benthamiana* leaves as described previously (Maimbo et al. 2007).

The qRT-PCR was performed using the method described in Maimbo et al. (2007). Briefly, the qRT-PCR was carried out in 20-µl reaction mixtures, containing 1 µl of cDNA template, 10 pM of the respective primers (Supplementary Figure S1; Supplementary Table S1), and THUNDERBIRD qPCR MIX (Toyobo Co.), on an Applied Biosystems 7300 real-time PCR instrument. All the values were normalized to those of the actin gene (Maimbo et al. 2007), which was used as an internal standard in each cDNA stock. Expression analyses were performed with at least three biological replications to ensure that expression patterns were reproducible, and representative data are presented. Standard deviations and differences between expression ratios of controls and other samples were tested for statistical significance using the Student t-test.

Bacterial suspensions (10⁸ colony forming units (CFU)/ml) of RsOE1-1 were inoculated into *N. benthamiana* leaves. The bacterial populations were determined by plating on Hara-Ono medium-containing plates (Hara and Ono 1984). Plants inoculated with RsOE1-1 were labeled and inspected daily for 17 d to assess wilting symptoms. For each plant, the disease index, on a scale of 0–4, was calculated as described previously (Maimbo et al. 2010). The statistical analysis was carried out using two-sided t-tests.

The TGD protein complex is composed with five TGD proteins. Among the TGD proteins, TGD3 encodes an ATP-binding protein required as energy source of transporter complex (Lu et al. 2007). Then, we focused on TGD3. On the basis of the TGD3 sequences from *Arabidopsis thaliana*, we searched for TGD3 orthologs in *N. benthamiana* through the Sol genomics network (https://solgenomics.net/). We identified two TGD3 orthologs in the *N. benthamiana* genome. Deduced amino acid sequences of these TGD3 orthologs showed high similarities to chloroplast-localized TGD3s from *Nicotiana* and other plant species (Figure 1A). Therefore, TGD3 proteins are conserved among the wide variety of plant species. Consequently, we designated them *NbTGD3-1* (Niben101Scf00072) and *NbTGD3-2* (Niben101Scf00094). The deduced amino acid sequence of the full-length cDNA of *NbTGD3-1* contained sulfate ABC transporter, ATP-binding protein conserved domain, including A-loop, Walker A, Q-loop, C-motif and Walker B domains. However, that of *NbTGD3-2* lacked the conserved A-loop and Walker A domain (Figure 1B).

To determine the expression profiles of *NbTGD3-1* and *NbTGD3-2* in response to *R. solanacearum*, the total RNAs were isolated from *N. benthamiana* leaves at 0, 6, 9, 12, 18, 24, and 48 h after inoculation with RsOE1-1. The expression of *NbTGD3-1* was transiently induced 9 h after inoculation with RsOE1-1. In contrast, the expression of *NbTGD3-2* was reduced from 9 to 48 h after inoculation (Figure 1C).

To address the role of *NbTGD3* in plant resistance, we created constructs to obtain double *TGD3*-silenced *N. benthamiana* plants. The suppression of both *NbTGD3*s after the initiation of VIGS was assessed by qRT-PCR (Supplementary Figure S2A). The *NbTGD3*-silenced plants showed a moderately reduced growth phenotype (Supplementary Figure S2B, C) as reported for TGD3 proteins in Arabidopsis (Fan et al. 2015; Xu et al. 2005). The expression of *NbTGD3-1* was transiently induced 9 h after inoculation with RsOE1-1. In contrast, the expression of *NbTGD3-2* was reduced from 9 to 48 h after inoculation (Figure 1C).

To address the role of NbTGD3 in plant resistance, we created constructs to obtain double *TGD3*-silenced *N. benthamiana* plants. The suppression of both *NbTGD3*s after the initiation of VIGS was assessed by qRT-PCR (Supplementary Figure S2A). The *NbTGD3*-silenced plants showed a moderately reduced growth phenotype (Supplementary Figure S2B, C) as reported for TGD3 proteins in Arabidopsis (Fan et al. 2015; Xu et al. 2005).

Next, the RsOE1-1 suspension was inoculated into *NbTGD3*-silenced and control plants, and development of disease symptom was observed. An increase in wilting symptoms was observed in *NbTGD3*-silenced plants compared with control plants. *NbTGD3*-silenced plants were completely wilted by 17 d after inoculation with RsOE1-1. However, even though the wilting symptoms were visible from 6 d after inoculation, the control plants were still alive at 17 d (Figure 2A, B). Consistent with disease symptom, RsOE1-1 was more abundant
in \( NbTGD3 \)-silenced plants 18–24 h after inoculation, showing an approximate 10-fold increase, in comparison with control plants (Figure 2C). Thus, \( NbTGD3 \) might play important roles in basal defenses against \( R. solanacearum \).

We previously reported that phospholipid turnover is closely associated with jasmonic acid (JA) signaling and plays an important role in defense responses against \( RsOE1-1 \) (Kiba et al. 2014; Nakano et al. 2013). Thus, we investigated the influence of the \( NbTGD3 \) silencing on JA signaling. Total RNA was extracted from \( NbTGD3 \)-silenced and control leaves 0, 18, 24 and 48 h after inoculation with \( RsOE1-1 \), and analyzed the expression patterns of allene oxide synthase (\( NbAOS \)) that encoded JA biosynthetic enzyme. As shown in Figure 2D, expression of \( NbAOS \) was induced in control plants 24 h after the inoculation with \( RsOE1-1 \). In contrast, reduction of \( NbAOS \) expression was observed in \( NbTGD3 \)-silenced plants challenged with \( RsOE1-1 \) compared with control plants (Figure 2D). In addition to \( NbAOS \), the expression level of \( PR-4 \), a marker gene for the JA-signaling pathway, was also increased by 48 h after the inoculation of control plants with \( RsOE1-1 \). Conversely, the expression of \( PR-4 \) was significantly reduced in \( NbTGD3 \)-silenced plants challenged with \( RsOE1-1 \) compared with control plants (Figure 2E). Thus, \( NbTGD3 \) might be involved in JA-mediated defense responses against \( R. solanacearum \).

The biogenesis of chloroplast membranes in higher plants requires an extensive supply of lipids from the ER. Five TGD proteins (TGD1, -2, -3, -4, and -5) have been implicated in this lipid transfer process. Together, TGD1, -2, and -3 constitute an ATP-binding cassette transporter complex. Two proteins, TGD1 and TGD2, encode the permease and substrate-binding component, respectively (Fan et al. 2015; Michaud and Jouhet 2019; Roston et al. 2012). A third protein, TGD3, is a small ATPase proposed to be part of the translocator (Lu et al. 2007). TGD4 encodes a PA-binding protein required for lipid import into chloroplasts (Wang et al. 2013). TGD5 is a small glycine-rich protein that physically interacts with TGD1, -2, -3, and -4, facilitating lipid transfer from the outer to the inner plastid envelope (Fan et al. 2015). In this experiment, we successfully isolated two cDNAs having high similarities with chloroplast-localized TGD3s from several plant species (Figure 1A). The deduced amino acid sequence of \( NbTGD3-1 \) contained complete conserved sequence required for TGD3s.
function. In contrast, NbTGD3-2 lacked part of the conserved domain (Figure 1B). Thus, NbTGD3-1 may have a central role in transporting the lipid supply from the ER to chloroplasts in N. benthamiana. Furthermore, the expression of NbTGD3-1 was up-regulated in response to infection with R. solanacearum (Figure 1C). Therefore, NbTGD3-1 might also participate in plant defense responses.

The TGD protein complex-mediated lipid transport from the ER to chloroplast membranes is required for chloroplast membrane biogenesis in higher plants (LaBrant et al. 2018). Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) constitute approximately 50% and 30% of chloroplast membrane lipids, respectively (Li and Yu 2018). These galactolipids are synthesized from lipids transported by TGD protein complex (LaBrant et al. 2018). These galactolipids are important for not only photosynthesis, but also abiotic stress tolerance. The gene SENSITIVE TO FREEZING 2 encodes a galactolipid:galactolipid galactosyltransferase that transfers a galactose from MGDG to MGDG, DGDG, or higher oligogalactoglycerolipids, and it is required for freezing tolerance in cold-acclimated Arabidopsis (Moellering et al. 2010). Reducing the level of MGDG may facilitate the maintenance of the lamellar chloroplast outer envelope membrane, resulting in the protection of cellular membranes against freezing stress (Moellering and Benning 2011; Moellering et al. 2010). The stability of the MGDG/DGDG ratio and changes in the unsaturation levels of MGDG and DGDG may help protect the chloroplast membrane's integrity under salt-stress conditions (Bejaoui et al. 2016). Therefore, the translocation of PA from the ER to chloroplasts may aid in protecting against cellular damage caused by abiotic stresses.

In addition to preventing abiotic stress-associated cellular damage, the translocation of PA from the ER to chloroplasts is required for signaling events during stress responses. Mutations in DGD1 (dgd1), the major DGDG-synthesizing enzyme, severely reduce the DGDG content and induce JA overproduction (Yu et al. 2020). Further analyses suggest that JA biosynthesis in dgd1 plants is initially activated through the increased expression of genes encoding 13-lipoxygenases and phospholipase A-Ig3, a plastid lipase with a high substrate specificity for MGDG. JA biosynthesis is sustained by the up-regulation of lipoxygenases and allene oxide cyclase at transcript and protein levels (Lin et al. 2016). Recently, MGDG and DGDG homeostasis was linked to JA production, suggesting that MGDG acts as a major substrate for JA biosynthesis (Li and Yu 2018).

Figure 2. Responses of NbTGD3-silenced plants against Ralstonia solanacearum. Ralstonia solanacearum OE1-1 (RsOE1-1) was inoculated into control and NbTGD3-silenced plants. (A) The development of bacterial wilt disease was rated daily on a 0–4 disease index scale in control (Control; white circles) and NbTGD3-silenced (VIGS; black squares) plants (n=10). (B) Characteristic symptoms on control (Control) and NbTGD3-silenced (VIGS) plants caused by RsOE1-1. Photograph taken 10 d after inoculation. (C) The bacterial populations of RsOE1-1 from control (Control; gray bar) and NbTGD3-silenced (VIGS; black bar) plants were determined by plating at specified time points. Values are means±SDs of five replicate experiments. (D, E) Total RNA was isolated, and the expression levels of NbAOS and NbPR-4 relative to the absolute non-treated control were determined and normalized against actin. Values represent the means±SDs from triplicate experiments. Asterisks indicate values significantly different from those of control plants (*; p<0.05, t-test).
2018). Here, we showed that the silencing of \textit{NbTGD3} affected JA biosynthetic AOS and JA-mediated defensive \textit{PR-4} gene expression levels and disease resistance (Figure 2). Reduction of \textit{NbAOS} expression might result in reduction of JA biosynthesis in \textit{NbTGD3} silenced plants. Based on reduction of \textit{NbPR-4} expression, these results also suggested that suppression of JA signaling in \textit{NbTGD3} silenced plants. Therefore, we hypothesize that reduction of JA substrates or changes in chloroplast membrane composition, led to the reduction in JA synthesis and JA-dependent resistance in \textit{NbTGD3}-silenced plants.

We demonstrated that \textit{NbTGD3} contributes to basal disease resistance in plants. Our findings indicate that \textit{NbTGD3} may be required for the activation of JA-mediated signaling in plants during the establishment of basal resistance. As far as we know, this is the first report regarding a relationship between TGD proteins and disease resistance. Further studies will be required to clarify the complex mechanisms by which the \textit{NbTGD3} proteins are engaged in disease resistance.

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