SEC14 Phospholipid Transfer Protein Is Involved in Lipid Signaling-Mediated Plant Immune Responses in *Nicotiana benthamiana*

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Abstract

We previously identified a gene related to the SEC14-gene phospholipid transfer protein superfamily that is induced in *Nicotiana benthamiana* (NbSEC14) in response to infection with *Ralstonia solanacearum*. We here report that NbSEC14 plays a role in plant immune responses via phospholipid-turnover. NbSEC14-silencing compromised expression of defense-related PR-4 and accumulation of jasmonic acid (JA) and its derivative JA-Ile. Transient expression of NbSEC14 induced PR-4 gene expression. Activities of diacylglycerol kinase, phospholipase C and D, and the synthesis of diacylglycerol and phosphatidic acid elicited by avirulent *R. solanacearum* were reduced in NbSEC14-silenced plants. Accumulation of signaling lipids and activation of diacylglycerol kinase and phospholipases were enhanced by transient expression of NbSEC14. These results suggest that the NbSEC14 protein plays a role at the interface between lipid signaling-metabolism and plant innate immune responses.

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Introduction

Plants have evolved innate immune responses to detect and respond quickly to foreign infections [1]. Plants use transmembrane pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) at the cell surface. Plants perceive bacterial flagellin, EF-Tu, and fungal chitin oligomers through their cognate receptors FLS2, EFR, and CERK1, respectively [2,3]. Plants also recognize avirulent gene products as pathogen infections by polymorphic receptors typically containing nucleotide-binding leucine-rich repeat resistance (R) proteins [4].

After recognition events, intracellular signaling cascades, such as changes in ion fluxes, cytoplasmic Ca2⁺ levels, oxidative burst, protein phosphorylation, and the production of stress-related hormonal substances, are required for the establishment of plant immune responses [5,6]. The increase in Ca2⁺ concentration and activation of Ca2⁺-dependent protein kinases induces an oxidative burst in the potato after PAMPs recognition [7]. The generation of reactive oxygen species (ROS) and nitric oxide (NO) is also implicated in defense-related gene expression mediated by both PRRs and R proteins [8,9,10]. In *Nicotiana* plants, members of the mitogen activated protein kinase (MAPK) family, SIPK, WIPK, and NTf6, are involved in defense induction in response to PAMPs, INF1 and HWC [11,12]. Both WIPK and SIPK are also sufficient to induce N-gene mediated resistance to the tobacco mosaic virus [13]. In tomato, LeMKK2, LeMKK3, and LeMPK3 are required for Pto-mediated resistance against *Pseudomonas syringae pv. tomato* carrying AvrPto [14]. In *Arabidopsis* plants, members of the MAPK family, MPK3 and MPK6, are implicated in PRRs and R protein-mediated defense responses [15,16]. Plant defense responses are also controlled by a complex, interconnected signaling network that includes the hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) with antagonistic interaction of the JA and SA signaling pathways [17]. In *Arabidopsis thaliana*, an SA-dependent cascade is critical for biotrophic immune responses against *Pseudomonas syringae pv. tomato* DC3000. In contrast, ET/IA pathways are required for necrotrophic resistance against *Alternaria brassicicola* [18].

Phospholipids-based signaling cascades are common signal transduction mechanisms during plant immune responses. Phospholipases are activated during defense signal transduction. For example, induction of phospholipase D genes (PLD) occurs after elicitor treatment of tomato cells [19]. Similarly, treatment of *N*-acetyl chitooligosaccharide elicitor could induce rapid activation of PLD, resulting in the accumulation of phosphatidic acid (PA) in rice cells [20]. An avirulent strain of *Xanthomonas oryzae* induced the expression of both phospholipase C (PLC) and PLD genes in rice [21]. Isoforms of tomato *PLC* are required for Cf-4-dependent immune responses, whereas *SPLC4* and *SPLC6* are required for general immune responses [22]. Among the phospholipids, PA has been shown as intracellular signaling molecule leading to plant immune responses. In tomato suspension-cultured cells, PA and...
diglycerol pyrophosphate accumulate in response to a xylanase elicitor [23]. PA also accumulates in tomato cells in response to a race-specific Avr4 elicitor in a Cf-4 dependent manner [24].

Phospholipid metabolism and signaling are important in plant immune responses, although the molecular regulatory mechanisms of phospholipid-synthesizing enzymes have remained elusive. Previously, we identified a gene related to the SEC14-gene superfamily from N. benthamiana (NbSEC14). NbSEC14 rescued temperature-sensitive growth mutant of sec14 in yeast, and NbSEC14 protein showed phospholipid transfer activity. Moreover, acceleration of disease development of bacterial wilt and growth of R. solanacearum were observed in the NbSEC14-silenced plants [25]. SEC14 protein belongs to the large yet under-characterized Sec14-protein superfamily (1550 proteins) originally isolated from Saccharomyces cerevisiae [26,27,28]. SEC14 protein functions as phospholipid transfer protein and acts in the phospholipid metabolism and phosphoinositide signaling pathways involved in diverse cell functions [29,30,31]. However, there is no information about role of SEC14 phospholipid transfer protein in plant immune responses. In this study, we analyzed the role of NbSEC14 phospholipid transfer protein in plant immune responses. In addition, we also discuss a possible relationship between phospholipid turnovers and NbSEC14 protein that leads to plant immune responses.

Materials and Methods

Plant Materials

N. benthamiana was grown in a plant growth room as described before [32].

Bacterial Isolates, Culture Conditions, and Inoculation

Bacterial strains used in this study are listed in Table S1. Ralstonia solanacearum strains 8107 (R8107), Pseudomonas cichorii SPC9018 were cultured in PY medium containing 20 µg/mL rifampicin. The density of bacterial suspension was adjusted to 1.0x10⁸ CFU/mL and inoculated by leaf infiltration as described in Maimbo et al., [32].

Primers and Plasmids

Primers and plasmids used in this study are listed in Tables S2 and S3, respectively.

RNA Isolation

Total RNA was prepared from N. benthamiana leaves with RNAiso (Takara Bio, Shiga, Japan) according to the manufacturer’s procedure. RNA samples were then treated with DNase I (RNase-free; Takara) to degrade contaminating genomic DNA as described previously [32].

Quantitative Real Time PCR

Quantitative real time PCR was performed by the method described in Maimbo et al., [32]. Reverse transcription was performed with 1 µg total RNA using PrimeScript RT reagent Kit (Takara), and qRT-PCR with 20 µL of a reaction mixture containing 1 µL of cDNA template, and 10 pM of the respective primers using the SYBR GreenER qPCR Reagent System (Invitrogen, Tokyo, Japan) and an Applied Biosystems 7300 real time PCR instrument. Cycling parameters were the same for all primers: an initial 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve runs were performed at the end of each PCR reaction to verify the specificity of primers by the presence of a single product. The expected single DNA product and its molecular weight were verified by agarose gel electrophoresis. We also checked the sequence of amplified DNA products by direct sequencing with an upper primer. Relative quantification of gene expression was performed according to the instructions for the Applied Biosystems 7300 real-time PCR system using the comparative cycle threshold [Ct] method for the calculation of Qty value. All values were normalized to the expression values of the actin gene used as an internal standard in each cDNA stock. Expression analyses were performed with at least two biological replications to ensure that expression patterns were reproducible, and representative data are presented. Standard deviations and differences between expression ratios of non-treated controls and other samples were tested for statistical significance using the Student t-test.

Vector Constructs and Seedling Infection for Virus-induced Gene Silencing

A 389-bp cDNA fragment of the 3'-terminal region of NbSEC14 was used for Virus-Induced Gene Silencing (VIGS) experiments as described previously [25]. Construct for NbGol1-silencing was prepared as described previously [33]. Plasmid pPVX201 with no insert was used as a control. All binary plasmids were transformed into A. tumefaciens strain GV3101 [34] and inoculated into N. benthamiana leaves as described previously [32]. Specificity of NbSEC14-silencing was tried to confirm by NbSEC14-silencing with two different parts of NbSEC14 cDNAs (Figure S1) and Southern blot analysis with cDNA fragment (sec14P1) as probe. According to results of Southern blot, we could observed single band, suggesting specific silencing of NbSEC14 [25]. However, we found out two different contigs (Nbs00015170g0012.1 and Nbs00058777g0001.1) in Sol Genomics database (http://solgenomics.net/). Because they have more than 92% nucleotide identities with NbSEC14, we judged that silencing might affect all members of NbSEC14 family, and we therefore observed an overall effect of gene silencing of NbSEC14 family on plant immune responses.

Plasmid Construction for Agrobacterium-mediated Transient Expression

A full-length open reading frame (ORF) of NbSEC14 with a FLAG tag was amplified with secORF-S and secFlag-A primers using pGEMNbSEC14 [25] as a template, and the PCR product cloned into pGEM-Easy vector (pGEMNbSEC14Flag). pGEMNbSEC14Flag was then digested with BamHI and SacI (Takara), and insert was cloned into the pBI121 vector (CLONTHEC, Tokyo, Japan) digested with the same enzymes. The final construct was designated pBI-NbSEC14. For agroinfiltration experiments, we also used the binary vector p35S-INF1 [35]. The binary vector p35S-GUS containing the GUS gene [36] was used as a control. These binary plasmids were transformed into A. tumefaciens strain GV3101, and inoculated into N. benthamiana leaves as described previously.

Phytohormone Analysis

Phytohormone contents were measured by the method described previously [37]. Extracted samples were subjected to measurement on a triple quadrupole LC-MS/MS 6410 (Agilent Technologies, USA) equipped with a Zorbax SB-C18 column [2.1 mm id×50 mm, (1.8 µm), Agilent Technologies]. Hormone amounts were calculated from the ratio of endogenous hormone peak and known amount of internal standards spike, and related to actual fresh mass of the samples used for extraction.
Protein Analysis

Preparation of crude protein fractions and protein analysis were performed as described [38]. Crude protein fractions isolated from *N. benthamiana* leaves were separated by 12.5% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Labs., Hercules, CA). The blots were subjected to western blot analyses with a monoclonal antibody raised against the Flag-tag sequence (Sigma-Aldrich, Tokyo, Japan). Cross-reacting proteins were visualized with a goat alkaline phosphatase secondary antibody (BioRad) conjugated with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Nacalai Tesque, Kyoto, Japan). Equal loading of protein fractions was estimated by Coomassie brilliant blue staining of Rubisco large subunit.

**In vivo 

*N. benthamiana* leaves were detached and leaf petioles were dipped in water containing 0.59 Mbq carrier-free [³²P]orthophosphate (Muromachi Chemical, Tokyo, Japan) and incubated at 25°C for 12 h.

Phospholipid Extraction, Separation and Analysis

Total lipids were extracted in CHCl₃:MeOH:HCl (50:100:1, v/v/v) according to the method described in Munnik *et al.* [38]. Total lipid extracts were dried by vacuum centrifugation, dissolved in CHCl₃, and separated by thin layer chromatography (TLC) with Silica 60 TLC plate (Merck, Darmstadt, Germany) using three different solvents. An alkaline TLC [CHCl₃:MeOH:25% NH₄OH:H₂O (45:35:2:8, v/v/v/v)] was used to isolate phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylglycerol (PG) and PI, and an acidic TLC [CHCl₃:MeOH:CH₃COOH:H₂O (40:15:14:13.7:5, v/v/v/v/v/v)] for PC, as described by Munnik *et al.* [39,40]. An ethyl acetate solvent system [the organic upper phase of ethyl acetate/sooctane/formic acid/H₂O (13:2:3:10, v/v/v/v/v)] was used to separate PA from the other phospholipids [40]. Radiolabelled lipids were visualized and quantified by autoradiography, with densitometry scans performed on a GE Storm 860 with ImageQuant TL (GE Healthcare, Tokyo, Japan). Relative amount of phospholipid was calculated as relative to the nontreated sample.

**DAG Quantification by DAG Kinase Reaction**

Quantification of DAG levels was performed by measuring DAG [³²P]-phosphorylation using *Escherichia coli* DAG kinase as described by Zien *et al.*, with slight modifications. First, leaf-extracted lipids were dried for a short time period under nitrogen. Second, micelles (20 µl) containing 7.5% octyl-β-D-glucopyranoside (Nacalai) and 20 mg mL⁻¹ of 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (Funakochi, Tokyo, Japan) were added to the plant lipid mixture. Third, a reaction mixture (50 µl) containing 10 mM Tris-HCl (pH 6.0) containing 10 µM CaCl₂ and 200 µM PiP₂ spiked with 0.86 Kbq [³H]-PiP₂. Reactions were stopped by addition of chloroform:methanol (2:1, v/v), 0.2 M HCl. Samples were centrifuged at 10,000 x g, and radioactivity in the water-soluble upper phase counted with a liquid scintillation system.

Statistical Analysis

Statistical analysis was carried out using t-test.

**Results**

NbSEC14 Protein Regulates the Expression of Defense-related Genes

NbSEC14-silencing was carried out independently by NbSEC14-silencing with two different parts of the NbSEC14 cDNA (P1, P2), showing very similar effect on immune responses in both cases (Figure 1, Figure S1). Therefore, we only used SEC14P1 fragment for further analysis. To determine the role of NbSEC14 in plant innate immunity, we focused on characteristic immune responses, including hypersensitive response (HR), salicylic acid (SA)-dependent and jasmonic acid (JA)-dependent signaling pathways. We could not observe any visible differences in HR induction in both control and NbSEC14-silenced plants (Figure S2). In control leaves (empty vector VIGS) inoculated with Rs8107, expression of NbSEC14 and PR-4, a marker gene for JA signaling, showed peaks of expression at 12 and 24 hours after inoculation (HAI), respectively, but PR-4 transcript levels were greatly reduced in NbSEC14-silenced leaves. In contrast, expression of PR-1a, a marker gene for SA signaling, was rather enhanced in the silenced plants 48 HAI with Rs8107. In the case of *hin1*, the HR-related gene, the expression was much less affected by NbSEC14-silencing (Figure 1). These results suggested a possible involvement of JA in NbSEC14-related immune responses. Then, we confirm JA and JA-Ile contents as well as SA content. As shown Figure 2, accumulation of JA and JA-Ile was observed in control plants challenged with Rs8107, whereas significant reduction of both hormone contents was observed in NbSEC14-silenced plants. In contrast, we could detect SA accumulation and hyper-accumulation of SA was observed in the silenced plants compared to control plants.

Because silencing of *NbSEC14* reduced PR-4 expression, but not PR-1a and *hin1*, we tested the effect of the transient expression of *NbSEC14* in *N. benthamiana* leaves on the expression of these genes (Figure 3a). qRT-PCR and western blot analysis confirmed a higher level of overexpressed *NbSEC14*, up to 8 times more relative to β-glucuronidase (*GUS*) gene-expressing control leaves at 48 hours (Figure 3b, c). Expression of *PR-1a* was significantly reduced, but *hin1* expression was not affected. In contrast, *PR-4* expression was significantly elevated in *NbSEC14*-overexpressing plants compared to control plants (Figure 3d). To strengthen our

Assay for Phospholipase Enzymatic Activities

PLD activity was measured as the production of phosphatidylbutanol (PBut), as described previously [43,44]. Briefly, *N. benthamiana* leaves were prelabelled with [³²P] for 12 h, and infiltrated with Rs8107 suspension with 0.25% n-butanol. Incubations were stopped and lipids extracted as described above. [³²P]-labelled PBut was separated by an ethyl acetate TLC system, and its radioactivity visualized and quantified as described above. Relative phospholipase activity was calculated relative to the absolute PVX at time 0.

In vitro phosphoinositide-specific phospholipase C activity was measured by the hydrolysis of [³H]PiP₂ as described previously [45,46]. Briefly, total protein fractions were incubated at 25°C for 30 min in Tris-Malate (pH 6.0) containing 10 µM CaCl₂ and 200 µM PiP₂ spiked with 0.86 Kbq [³H]-PiP₂. Reactions were stopped by addition of chloroform:methanol (2:1, v/v), 0.2 M HCl. Samples were centrifuged at 10,000 x g, and radioactivity in the water-soluble upper phase counted with a liquid scintillation system.
hypothesis of NbSEC14 interacting with JA signaling, we focused on NbCoi1 gene, which encodes F-box protein and have been well known as positive regulator of JA signaling [47]. Then, we examined the effect of NbCoi1-silencing on PR-4 induction by transient expression of NbSEC14. A strong reduction of PR-4 expression controlled by NbSEC14 in NbCoi1-silenced plants was observed (Figure S3). Taken together, NbSEC14 protein may associate with the expression of defense-related genes related to JA-dependent pathway.

NbSEC14 Protein Regulates Phospholipid Signaling during Plant Immune Responses

NbSEC14 protein exhibits phospholipid transfer activities [25], and may be involved in plant immune response. Given these results, we evaluated the role of NbSEC14 protein in plant phospholipid metabolism in relation to its role in immunity. Among the phospholipids, we first focused on signaling phospholipids, diacylglycerol (DAG) and PA, and determined changes of DAG and PA in control and NbSEC14-silenced plants challenged with Rs8107. The formation of DAG and PA increased in Rs8107-inoculated control leaves, with peak DAG levels 12 HAI and peak PA levels at 24 HAI, whereas both phospholipid content was significantly reduced in NbSEC14-silenced plants (Figure 4a). In addition, NbSEC14-expressing plants showed increased DAG (24 HAI) and PA (48 HAI) compared to GUS-expressing controls (Figure 4b).

Regulation of Phospholipase Activities by NbSEC14 Protein

Diacylglycerol kinase (DGK)-PLC and PLD pathways are the two major metabolic pathways that produce DAG and PA [46]. A supporting pharmacological experiment showed that immune responses might be indeed related to PLC and PLD, since population of Rsf8107 was stimulated in the concomitant presence of PLC and PLD inhibitors (Figure S3). We therefore analyzed the relationship between NbSEC14 protein and these phospholipid

![Figure 1. Virus-induced gene silencing of NbSEC14 and its effect on the expression of defense-related genes.](image-url)
metabolic enzymes during immune responses. DGK activity was enhanced 12–24 HAI, whereas activation of DGK activity was compromised in NbSEC14-silenced plants. NbSEC14-silencing blocked the increase in PLC activity at 6–24 HAI induced by Rs8107. NbSEC14 silencing also blocked the increase in PLD activity, as measured by 32P-phosphatidylbutanol production, that occurred from 6–12 HAI (Figure 5a). In contrast, overexpression of NbSEC14 in N. benthamiana increased all DGK, PLC and PLD activities (Figure 5b).

Figure 2. Effect of silencing of NbSEC14 on plant hormone contents. Control (PVX) and NbSEC14-silenced (PVX:sec14P1) N. benthamiana plants were infiltrated with Rs8107. Plant hormone contents of jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile) and salicylic acid (SA) were determined at designated time points by LC-MS/MS. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*; P<0.05).

Figure 3. Transient expression of NbSEC14 and induction of defense-related genes expression. (a) Diagram illustrating constructs used for transient expression of NbSEC14. (b) Total protein extracts were prepared from N. benthamiana leaves inoculated with Agrobacterium carrying NbSEC14 (NbSEC14) and GUS control (GUS), and separated by SDS-PAGE. Equal amounts of loaded protein fractions were estimated by Coomassie Brilliant Blue staining of the Rubisco large subunit (CBB), and NbSEC14 protein: FLAG detected by western blot using monoclonal antibodies raised against the Flag-tag (Anti-Flag). (c) Total RNA was isolated from N. benthamiana leaves inoculated with GUS and NbSEC14 expressing Agrobacterium. Relative expression of PR-4, PR-1a, and hin1 transcripts were normalized with actin and calculated as relative to the non-treated control. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from GUS-expressing controls (*; P<0.05).
Discussion

In this experiment, we could observe that NbSEC14-silencing caused dramatic changes of signaling phospholipids after plants were challenged with Rs8107 (Figure 4). Among the phospholipids, DAG is well known as a signaling phospholipid and is reportedly shown to play a crucial role in the response of tobacco cells to aluminum ions [48]. DAG is likely to act as a signaling molecule in tobacco pollen tubes [49]. PA is also recognized as a signaling phospholipid [45]. PA is produced in response to xylanase treatments, and the accumulation of PA induces ROS production and cell death in tomato cells and Arabidopsis [50,51,52,53]. Wound-induced PA accumulation causes JA accumulation in Arabidopsis plants [54]. Here, we showed that NbSEC14-silencing blocked increases in JA contents and JA-dependent PR-4 genes, but did not affect hin1 gene and subsequent HR (Figure 1, 2 and Figure S2). Expression of SA-dependent PR-1a was rather enhanced in the silenced plants. Transiently increasing NbSEC14 protein enhanced the expression of defense-related PR-4, whereas PR-1a expression was significantly reduced (Figure 5). In addition, up-regulation of PR-4 gene expression was reduced in NbCoil-
Figure 5. Regulation of phospholipase activities by NbSEC14. (a) Effect of NbSEC14-silencing on activities of DGK, PI-PLC and PC-PLD. Control (PVX; gray box) and NbSEC14-silenced (PVX:Sec14P1; black box) N. benthamiana leaves were inoculated with Rs8107 and incubated at 25°C for indicated times. Phospholipid quantity was calculated relative to the absolute PVX at time 0. (b) DGK and phospholipase activity in NbSEC14-expressed plants. Total protein extracts were prepared from N. benthamiana leaves inoculated with NbSEC14 (NbSEC14) and GUS control (GUS) expressing Agrobacterium. Phospholipid levels are calculated relative to the non-treated control samples. Values represent the means and SD of the results from triplicate experiments. Asterisks denote values significantly different from controls (0) (*; P<0.05). doi:10.1371/journal.pone.0098150.g005
silenced plants (Figure S3). Therefore, these results suggested the direct or indirect relation of NbSEC14 on JA-dependent signaling pathway. NbSEC14 silencing caused faster growth of avirulent and virulent bacteria, and acceleration of disease development by virulent bacteria, and reduction PR-4 expression was also observed in the silenced plants in response to virulent bacteria [25]. The competitive interactions between SA and JA, and negative effects of JA on SA have been already described by Pieterse et al. [53] and others. Therefore, NbSEC14 protein may be closely associated with plant immunity related to the JA pathway, with interference of SA signaling by competitive interaction of the JA and SA signaling pathways.

Several plant SEC14-like proteins were reportedly shown to play a key role in the lipid-mediated signaling, and PA, DAG, and phosphoinositides (PIPs) regulate important cellular functions. AtSEC14p transfers phosphatidylinositol (PI) and phosphatidylycholine (PC) in vitro, in addition to stimulating intracellular and plasma membrane migration in a polarized pattern that focuses membrane trafficking, Ca²⁺ signaling, and cytoskeleton functions at the growing root hair apex [56]. AtPATL1, a novel cell-plate-associated protein, regulates membrane lipid composition (PI and PC) to activate PLD [57]. Ssh1p directly activates PI-3-kinase and PI-4-kinase in response to hyperosmotic stress [58]. Schaaf et al., [59] have shown that Sec14 protein is capable of stimulating the production of PIPs by presenting PI to PtdIns 4-kinase. PIp can then serve as a substrate for a PIP kinase to make another class of lipid signaling molecules, PtdIns-4,5-P₂ (PIP₂), and PIP₂ can then be a substrate for PLC and generate DAG and PA. PC can be hydrolyzed by either PLD to generate PA [60] or by PLC-PC to generate phosphocholine and DAG in plants [61]. In animals, PLC produces DAG as a second messenger [60]. NbSEC14-silencing reduced PLC and PLD activity in response to Rs8107 inoculation, whereas transient expression of NbSEC14 activated both enzyme activities (Figure 5). We could observe drastic changes of signaling phospholipids in NbSEC14-silenced plant as well as NbSEC14-expressing plants (Figure 4). NbSEC14 protein transfers PC and PI in vitro [25]. Unfortunately, although we could not determine actual substrate(s) for NbSEC14 protein in plants, we speculated that NbSEC14 protein affected lipid signaling-mediated plant immune systems in Nicotiana through PLC and PLD activities.

The PLC and PLD pathways are crucial in plant defense. Indeed, treatment of an N-acetyl chitooligosaccharide elicitor could induce rapid activation of PLD and the accumulation of PA, increasing elicitor-responsive genes as well as phytoalexin biosynthesis in rice cells [20]. Phytoalexin production induced by treatment with the glycoprotein elicitor from Mycosphaerella pinodes is mediated by a PIP2-PLC pathway [61,62]. Pharmacological experiments suggested that PLC and PLD might have an important role in the plant immune response against R. solanacearum (Figure S3). Inversely, NbSEC14-silencing did not affect HR induction, but did affect resistance to both virulent and avirulent bacteria (Figure S2) [25]. These results further imply that NbSEC14 protein may influence HR-independent defense via phospholipase-mediated phospholipid metabolism.

In conclusion, we have speculated that NbSEC14 protein may influence on PLC and PLD activities, as well as downstream PA and DAG production, associated with innate immune responses during bacterial infections (Figure S5). With the capacity of NbSEC14 protein to change the expression of defense-related genes via JA signaling, further studies will be required to clarify the complex mechanism by which NbSEC14 protein is engaged in plant immunity, and to characterize the phospholipases and/or kinases involved in the signaling cascades.

Supporting Information

Figure S1 Alignment of NbSEC14 with its homologues in N. benthamiana genome, and virus-induced gene silencing of NbSEC14. (a) Alignment of NbSEC14 with its homologues in N. benthamiana genome. cDNA fragments used for VIGS experiments are shown in gray boxes (Sec14P1) and black box (Sec14P2). Bold characters with underlines show primer sequences used for qRT-PCR (secrpF and secrpR). Dashed lines show nucleotide sequences that are not presented. (b) VIGS of NbSEC14 with Sec14P2 cDNA, and the effect of NbSEC14 on NbSEC14 and PR-4 expression by inoculation with avirulent R. solanacearum. Asterisks denote values significantly different from PVX controls (*; P<0.05, t-test). (TIF)

Figure S2 Effect of NbSEC14-silencing on HR induction. Control and NbSEC14-silenced N. benthamiana plants were infiltrated with HR-inducible Rs8107, P. cichorii (Pc) or Agrobacterium harboring 35S-GUS (control GUS) or 35S-IN1 (IN1). (a) Control samples (gray box) and NbSEC14-silenced plants (VIGS; back box) N. benthamiana plants were infiltrated with Rs8107, P. cichorii (Pc) or Agrobacterium harboring 35S-IN1 (IN1). Cell death was determined by Evans blue staining (OD600 nm disk⁻¹). (TIF)

Figure S3 Role of jasmonic acid pathway in NbSEC14-induced PR-4 gene expression. Total RNA was isolated from control (PVX), NbCoi1-silenced (Coi) N. benthamiana leaves inoculated with GUS and NbSEC14 expressing Agrobacterium. Relative expression of NbSEC14 and PR-4 transcripts were normalized with actin and calculated as relative to the GUS-expressing control. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty vector (PVX)-expressing controls (*; P<0.05). (TIF)

Figure S4 PLC and PLD activity is required for defense responses in N. benthamiana. (a) Schematic of phospholipase-dependent reactions in plant cells. (b) N. benthamiana leaves were infiltrated with Rs8107 (10⁶ CFU ml⁻¹) in the absence (Mock) or concomitant presence of 50 µM 1-[6-((17b-3-Methoxyestra-1,3,5(10)-tri-en-17-yl)amino)hexyl]-1-H-pyrrrole-2,5-dione (U73122; PLC inhibitor) and 0.1% normal-butanol (n-ButOH; PLD inhibitor) or 50 µM 1-[6-((17b-3-Methoxyestra-1,3,5(10)-tri-en-17-yl)amino)hexyl]-2,5-pyrrolinedione (U73343) + 0.1% 2-butanol (2-ButOH) (inactive analogue) (Kirik and Mudgett 2010), and bacterial population was determined by plating at specified time points. Values are means of four replicate experiments with SD. Asterisks denote values significantly different from control leaves (*; P<0.05). (TIF)
Figure S5  Function of NbSEC14 protein in the induction of plant immunity. NbSEC14 is expressed in response to bacterial infections. NbSEC14 protein initiates the binding/transfer of phospholipids, leading to changes in membrane lipid composition, synthesis of phospholipids and/or phospholipases. The subsequent generation of phospholipid-derived secondary messengers regulates other defense-related genes and the induction of plant immune responses to pathogen infection.

(TIF)

Table S1  List of bacteria used in this study.

(TIF)

Table S2  List of primers used in this study.

(TIF)

References

1. Jones DA, Takemoto D (2004) Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules.Curr Opin Immunol 16: 48–62.

2. Gohe V, Robatzek S (2008) Breaking the barriers: Microbial effector molecules subvert plant immunity. Ann Rev Phytopathol 46: 189–215.

3. Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol 20: 10–16.

4. Jones JD, Dans J (2006) The plant immune system. Nature 444: 325–329.

5. Boller T, Felix G (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Ann Rev Plant Biol 60: 379–406. 

6. Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immun Rev 203: 249–266.

7. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujisawa M, et al. (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19: 1065–1080.

8. Gecz T, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Sci 118: 17–20.

9. Asai S, Yoshioka H (2009) Nitric oxide as a partner of reactive oxygen species derived second messengers regulates other defense-related genes and the induction of membrane lipid composition and substrate supply for lipid kinases and/or phospholipases. The subsequent generation of phospholipid-derived secondary messengers regulates other defense-related genes and the induction of plant immune responses to pathogen infection.

10. Asai S, Yoshioka H, Kobayashi M (2009) Molecular mechanisms of generation for nitric oxide and reactive oxygen species, and role of the radical burst in plant immunity. Mol Cells 28: 321–329.

11. Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, et al. (2003) Nicotiana benthamiana homologs of Hsp90 homologue, Hsp90Δ and Hsp90Δ participate in Hsp90 accumulation and resistance to Phytophthora infestans. Plant Cell 15: 706–718.

12. Asai S, Ohita K, Yoshioka H (2008) MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in Nicotiana benthamiana. Plant Cell 20: 1789–1806.

13. Jin H, Liu Y, Yang KY, Kim CY, Baker B, et al. (2003) Function of mitogen-activated protein kinase pathway in V gene-mediated resistance in tobacco. Plant J 35: 719–731.

14. Engren SK, Liu Y, Schiff M, Dinesh-Kumar SP, Martin GB (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pseudomonas-mediated disease resistance in tomato. Plant J 36: 905–917.

15. Pedley KF, Martin GB (2005) Role of mitogen activated protein kinase proteins in plant immunity. Curr Opin Plant Biol 8: 541–547.

16. Pitzschke A, Schikora A, Hirt H (2009) MAPK cascade signaling networks in plants. Plant Cell 21: 489–508.

17. Bankaitis VA, Bargmann BOR, de Wit PJGM, Joosten MHA, et al. (2006) The Sec14 superfamily and phospholipid transfer proteins and functional specification of lipid signaling pools. Adv Enzyme Reg 47: 27–40.

18. Ohashi M, de Vries JK, Frank R, Snook G, Bankaitis VA, et al. (1995) A role for phospholipid transfer protein in secretory vesicle formation. Nature 377: 544–547.

19. Simon JP, Morimoto T, Bankaitis VA, Gottlieb TA, Ivanov IE, et al. (1998) An essential role for phospholipidtransferase protein in the scission of COP1-coated vesicles from trans-Golgi network. Proc Natl Acad Sci USA 95: 11181–11186.

20. Munnik T, Irvine RF, Musgrave A (1994) Rapid turnover of phosphatidylinositol transfer protein in yeast. Biochim Biophys Acta 1220: 236–248.

21. Atsumi H, Gomi S, Watanabe Y, Kawahara M, et al. (2003) Diverse biological functions of phosphatidylinositol transfer proteins in eukaryotes. Biochem Mol Biol 41: 21–49.

22. Maimbo M, Ohnishi K, Hikichi Y, Yoshioka H, Kiba A (2007) Induction of a small heat shock protein and its functional roles in Nicotiana plants in the defense response against Botrytis cinerea. Plant Physiol 145: 1580–1599.

23. Nakano M, Nishihara M, Yoshioka H, Takahashi H, Sawasaki T, et al. (2013) Suppression of D3i phosphatidylphosphate phosphatase confers resistance to Botrytis cinerea in Nicotiana tabacum. PLoS One 8, e75124.

24. Huitema E, Vleeshouwers VGAA, Cakir C, Kamoun S, Govers F (2005) Differences in intensity and specificity of hypersensitive response induction in Nicotiana spp. by EN1, EN1A2, and EN2B of Phytophthora infestans. Mol Plant-Microbe Interact 18: 183–193.

25. Maimbo M, Ohnishi K, Hikichi Y, Yoshioka H, Kiba A (2007) Soyacylprotein-like protein regulates defense responses in Nicotiana plants against Botrytis cinerea. Plant Physiol. 152, 2023–2035.

26. Katou S, Yamamoto A, Yoshioka H, Kawakita K, Doke N (2003) Functional analysis of potato mitogen-activated protein kinase gene, SMMEK1. J Gen Plant Pathol 69: 161–168.

27. Fukushima K, Alamgir KMd, Yamashita Y, Morita I, Inui K, et al. (2013) Response of rice to insect elicitors and the role of OsJAR1 in wound and herbivory-induced JA-defense accumulation. J Integr Plant Biol 55: 775–784.

28. Munnik T, Murgave A, de Vrije T (1994) Rapid turnover of polyphosphoinositides in carnation flower petals. Planta 193: 89–98.

29. Munnik T, Irvine RF, Murgave A (1994) Rapid turnover of phosphatidylinositol 3-phosphate in the green algae Chlamydomonas reinhardtii: signs of phosphatidylinositol 3 kinase activity in lower plants? Biochem J 298: 269–273.

30. Munnik T, Van Hünberslag JAJ. Ter Kiert B, Braun JJ, Irvine RF, et al. (1998) Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipase C and D in Chlamydomonas cells treated with non-permeabilizing concentration of mastoparan. Planta 207: 133–145.

31. Zien CA, Wang C, Wang X, Welth R (2001) In vivo substrates and the contribution of the common mammalian Dcppx to wound-induced metabolism of lipids in Arabidopsis. Biochim Biophys Acta 1530: 236–248.

32. Bligh EG, Dyer WJ (1959) A rapid method for total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.
43. Munnik T, Meijer HJG, Riet B, Hirt H, Frank W, et al. (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant J 22: 147–154.
44. Munnik T (2001) Phosphatidic acid: an emerging plant lipid second messenger. Trend Plant Sci 6: 227–233.
45. Drobak BK, Watkins PAC, Valenta R, Dove SK, Lässjö CW, et al. (1994) Inhibition of plant plasma membrane phosphoinositide phospholipase C by the actin-binding protein, profilin. Plant J 6: 309–400.
46. Testerink CT, Munnik T (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trend Plant Sci 10: 1360–1385.
47. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: A Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility. Science 280: 1091–1094.
48. Pejchar P, Potocky M, Novotna Z, Veselkova S, Kocourkova D, et al. (2010) Aluminium ions inhibit the formation of diacylglycerol generated by phosphatidylcholine-hydrolysing phospholipase C in tobacco cells. New Physiol 100: 150–160.
49. Helling D, Possart A, Cottier S, Klähre U, Kost B (2006) Pollen tube tip growth depends on plasma membrane polarization mediated by tobacco PLC3 activity and endocytic membrane recycling. Plant Cell 18: 3519–3534.
50. Zhang Y, Zhu H, Zhang Q, Li M, Yan M, et al. (2009) Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in Arabidopsis. Plant Cell 21: 2357–2377.
51. Zhang W, Qin C, Zhao J, Wang X (2004) Phospholipase Dα1-derived phosphatic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci U S A 101: 9508–9513.
52. Laxalt AM, Raho N, ten Have A, Lamattina L, (2007) Nitric oxide is critical for inducing phosphatidic acid accumulation in xylanase-elicited tomato cells. J Biol Chem 282: 21160–21168.
53. Testerink C, Larsen BP, van der Does D, van Himenjen J, Munnik T (2007) Phosphatidic acid binds to and inhibits the activity of Arabidopsis CTR1. J Exp Bot 58: 3905–3914.
54. Wang C, Zien CA, Aftihile M, Welti R, Hildebrand DF, et al. (2000) Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in Arabidopsis. Plant Cell 12: 2237–2246.
55. Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nature Chem Biol 5: 308–316.
56. Vincent P, Chua M, Nogue F, Fairbrother A, Mekel H, et al. (2005) A Sec14p-nodulin domain phosphatidylinositol transfer protein polarizes membrane growth of Arabidopsis thaliana root hairs. J Cell Biol 168: 801–812.
57. Peterman TK, Ohod YM, McReynolds LJ, Luna EF (2004) Patellin1, a novel Sec14-like protein, localizes to the cell plate and binds phosphoinositides. Plant Physiol 136: 790–794.
58. Monks DE, Agborum K, Courtney PD, DeWald DB, Dewey RE (2001) Hyperosmotic stress induces the rapid phosphorylation of a soybean phosphatidylcholine-hydrolysing phospholipase C in tobacco cells. New Physiol 100: 150–160.
59. Wimalasekera R, Pejchar P, Polotoky M, Novotna Z, Veselkova S, Kocourkova D, et al. (2010) Aluminium ions inhibit the formation of diacylglycerol generated by phosphatidylcholine-hydrolysing phospholipase C in tobacco cells. New Physiol 100: 150–160.
60. Testerink CT, Munnik T (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trend Plant Sci 10: 1360–1385.
61. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: A Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility. Science 280: 1091–1094.
62. Pejchar P, Potocky M, Novotna Z, Veselkova S, Kocourkova D, et al. (2010) Aluminium ions inhibit the formation of diacylglycerol generated by phosphatidylcholine-hydrolysing phospholipase C in tobacco cells. New Physiol 100: 150–160.
63. Testerink CT, Munnik T (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trend Plant Sci 10: 1360–1385.
64. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: A Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility. Science 280: 1091–1094.
65. Pejchar P, Potocky M, Novotna Z, Veselkova S, Kocourkova D, et al. (2010) Aluminium ions inhibit the formation of diacylglycerol generated by phosphatidylcholine-hydrolysing phospholipase C in tobacco cells. New Physiol 100: 150–160.