Plant Biotechnology 33, 111–115 (2016)
DOI: 10.5511/plantbiotechnology.16.0503a

Short Communication

Silencing of *Nicotiana benthamiana* SEC14 phospholipid transfer protein reduced jasmonic acid dependent defense against *Pseudomonas syringae*

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Received April 18, 2016; accepted May 3, 2016 (Edited by Y. Nagano)

Abstract We previously identified *SEC14*, phospholipid transfer protein superfamily gene, in *Nicotiana benthamiana* (*NbSEC14*) that was closely related to phospholipid signaling as well as jasmonic acid-dependent defense responses during plant immune responses against *Ralstonia solanacearum*. To examine effect of *NbSEC14*-silencing on basal plant defenses, we used two other bacterial pathogens with different virulent strategies, *Pseudomonas syringae* pv. *tabaci* and pv. *mellea*. *NbSEC14*-silenced plants showed accelerated growth of *P. syringae* pv. *tabaci* and pv. *mellea*, and formation of necrotic lesions. Induction of JA-related *PR-4* gene was compromised in *NbSEC14*-silenced plants, which was supported by reduced jasmonic acid levels in *NbSEC14*-silenced plants. These results suggested that NbSEC14 might be regulating plant basal resistance against plant pathogenic Pseudomonads via jasmonic acid-dependent signaling pathway.

Key words: Basal resistance, jasmonic acid, *Pseudomonas syringae*, SEC14 phospholipid transfer protein, virus-induced gene silencing.

Phospholipid-based signaling cascades are common signal transduction mechanisms in plant immune responses. For example, induction of phospholipase D genes occurred after elicitor treatment of tomato and rice cells (Laxalt et al. 2001; Yamaguchi et al. 2005). An avirulent strain of *Xanthomonas oryzae* induced the expression of phospholipase C and phospholipase D genes in rice (Young et al. 1996), and in tomato, phospholipase C genes (*SlPLC4* and *SlPLC6*) were required for general immune responses (Vossen et al. 2010). Phospholipid metabolism and signaling are therefore important for plant immune responses, although the molecular mechanisms involved in regulation of phospholipid synthesizing enzymes remained largely elusive. Previously, we identified a gene from *Nicotiana benthamiana* related to the *SEC14* superfamily (*NbSEC14*) that encoded a phosphatidylinositol transfer protein (PIPTs). Sec14-like proteins, one of the major PIPTs, possess in vitro phosphatidylinositol and phosphatidylcholine transfer activities were found in plants, yeast, invertebrates, and mammals (Aravind et al. 1999; Cleves et al. 1991; Wirtz 1991). Their function is involved in essential biological processes, such as phospholipid metabolism, membrane trafficking, polarized membrane growth, signal transduction, and stress responses (Dove et al. 1997; Phillips et al. 2006; Saito et al. 2007). Although PIPTs are abundant throughout all eukaryotic organisms, there is remarkably little information regarding their precise roles in plant defenses. We previously showed that phospholipid turnover was significantly reduced in *NbSEC14*-silenced plants in response to *Ralstonia solanacearum*. In addition, reduction of jasmonic acid (JA)-dependent *PR-4* expression and acceleration of disease development of bacterial wilt, and growth of *R. solanacearum* pathogen were observed in the *NbSEC14*-silenced plants. Therefore, we hypothesized that NbSEC14 may act as an important regulator of phospholipid turnover, which contributes to induction of JA-dependent plant defense responses against *R. solanacearum* (Kiba et al. 2012, 2014). We here addressed role of NbSEC14 in basal plant resistance in

Abbreviations: CFU, colony forming unit; JA, jasmonic acid; PIPT, phosphatidylinositol transfer protein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; VIGS, virus-induced gene silencing.

This article can be found at http://www.jspcmb.jp/
Published online June 11, 2016

N. benthamiana, employing two compatible bacterial pathogens with different virulent strategies, *Pseudomonas syringae* pv. *tabaci*, a causal agent of wildfire disease, and *P. syringae* pv. *mellea*, a causal agent of Wisconsin leaf blight disease.

N. benthamiana was grown in the growth room as described previously (Maimbo et al. 2007). *P. syringae* pv. *tabaci* 6605, and *P. syringae* pv. *mellea* MAFF302303 were cultured in PY medium containing 20 μg/ml rifampicin. The density of bacterial suspension was adjusted to 1.0×10^8 CFU/ml and inoculated by leaf infiltration method as described previously (Maimbo et al. 2010). Virus-induced gene silencing (VIGS) of *NbSEC14* was carried out with pPVX201 vector, and a 389-bp cDNA fragment of the 3′-terminal region of *NbSEC14*. VIGS experiments were performed with method as described previously (Kiba et al. 2014). Quantitative real time PCR (qRT-PCR) was done with SYBR Premix EX Taq II (Takara; Shiga Japan) and an Applied Biosystems 7300 real time PCR instrument (Kiba et al. 2014). Phytohormone contents were determined by previously described method (Kiba et al. 2014). Briefly, purified extracts (10 μl) were subjected to measurement on a triple quadrupole LC-MS/MS 6410 (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax SB-C18 column [2.1 mm i.d.×50 mm, (1.8 μm), Agilent Technologies]. Multiple reaction-monitoring (MRM) mode was used to measure JA content relative to a known amount of added deuterated JA standard (25 ng) prior to sample extraction.

To analyze the role of NbSEC14 in defense responses against *P. syringae* pv. *tabaci* and *P. syringae* pv. *mellea*, we first observed the expression pattern of *NbSEC14* following the inoculation with both bacteria. Expression of *NbSEC14* was induced 1 to 3 days after inoculation with *P. syringae* pv. *tabaci*. A significant induction of *NbSEC14* was also observed in *P. syringae* pv. *mellea* inoculated *N. benthamiana*, with a maximum of expression observed at 3 days after inoculation (Figure 1A). We have previously shown that NbSEC14 is closely related to JA-mediated defenses against *R. solanacearum* (Kiba et al. 2014). Then, we analyzed the *PR-4* expression, marker gene for JA signaling, in *N. benthamiana* infected with both compatible bacterial pathogens. In *P. syringae* pv. *tabaci* inoculated plants, expression of *PR-4* was significantly induced 1 and 3 days after inoculation. A similar expression pattern of *PR-4* was observed in *N. benthamiana* inoculated with *P. syringae* pv. *mellea* (Figure 1B). These results suggested that NbSEC14 and JA signaling were both induced by infection with *P. syringae* pv. *tabaci* and *P. syringae* pv. *mellea.*

To clarify the relationship between NbSEC14 and JA signaling, we analyzed JA-dependent *PR-4* expression and JA levels in *NbSEC14*-VIGS plants. In control plants inoculated with both *P. syringae* pv. *tabaci* and pv. *mellea*,

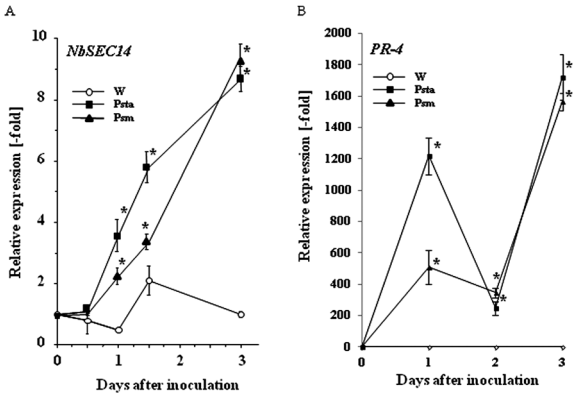

Figure 1. Expression pattern of *NbSEC14* and *PR-4*. Total RNA was isolated from *N. benthamiana* leaves inoculated with water (W), *P. syringae* pv. *tabaci* (Psta; 10^4 CFU ml^{-1}) or *P. syringae* pv. *mellea* (Psm; 10^4 CFU ml^{-1}). Expression values of *NbSEC14* (A) and *PR-4* (B) are shown as relative to the absolute non-treated control, and normalized with actin in control plants. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from water-infiltrated control plants (* $p<0.05$, Student's *t*-test).

expression of *PR-4* showed peaks of expression at 2 and 3 days after inoculation, respectively (Figure 2A). Expression level of *PR-4* transcript was greatly reduced in *NbSEC14*-VIGS plants. The accumulation of JA was observed in control plants 2 days after inoculation with both *P. syringae* pv. *tabaci* and pv. *mellea*, whereas significant reduction of JA levels was observed in *NbSEC14*-silenced plants (Figure 2B).

As mentioned above, VIGS of *NbSEC14* resulted in reduction of JA responses, suggesting the involvement of JA in regulation of defense responses against *P. syringae* pv. *tabaci* and pv. *mellea* by NbSEC14. Next, we examined the effect of *NbSEC14*-VIGS on bacterial growth and disease development. In *NbSEC14*-silenced plants, bacterial growth was elevated 5 to 10-fold (log CFU cm^{-2}) at 12 to 48 h after inoculation with both bacteria compared to controls (Figure 3A). Inoculation with *P. syringae* pv. *tabaci* induced necrotic lesions within 2 days of inoculation in the silenced plants, and the leaves completely died within 6 days. For *P. syringae* pv. *mellea* inoculation, lesion extensions were observed at 3 days, and inoculated leaves completely died after 7 days in the silenced plants. In contrast, the control leaves were still alive even at 7 days after inoculation with both *P. syringae* pv. *tabaci* and pv. *mellea* (Figure 3B, C). These results suggested that NbSEC14 is essential for defense responses against phytopathogenic bacteria with different virulent strategies. Thus, we proposed that NbSEC14 is a universal plant factor required for basal resistance against wide variety of bacterial pathogens.

In our previous reports, *NbSEC14*-silencing impaired the accumulation of JA and JA-dependent *PR-4* expression, as well as it promoted faster growth of both avirulent and virulent *R. solanacearum*. Acceleration of disease development was also observed in case of virulent

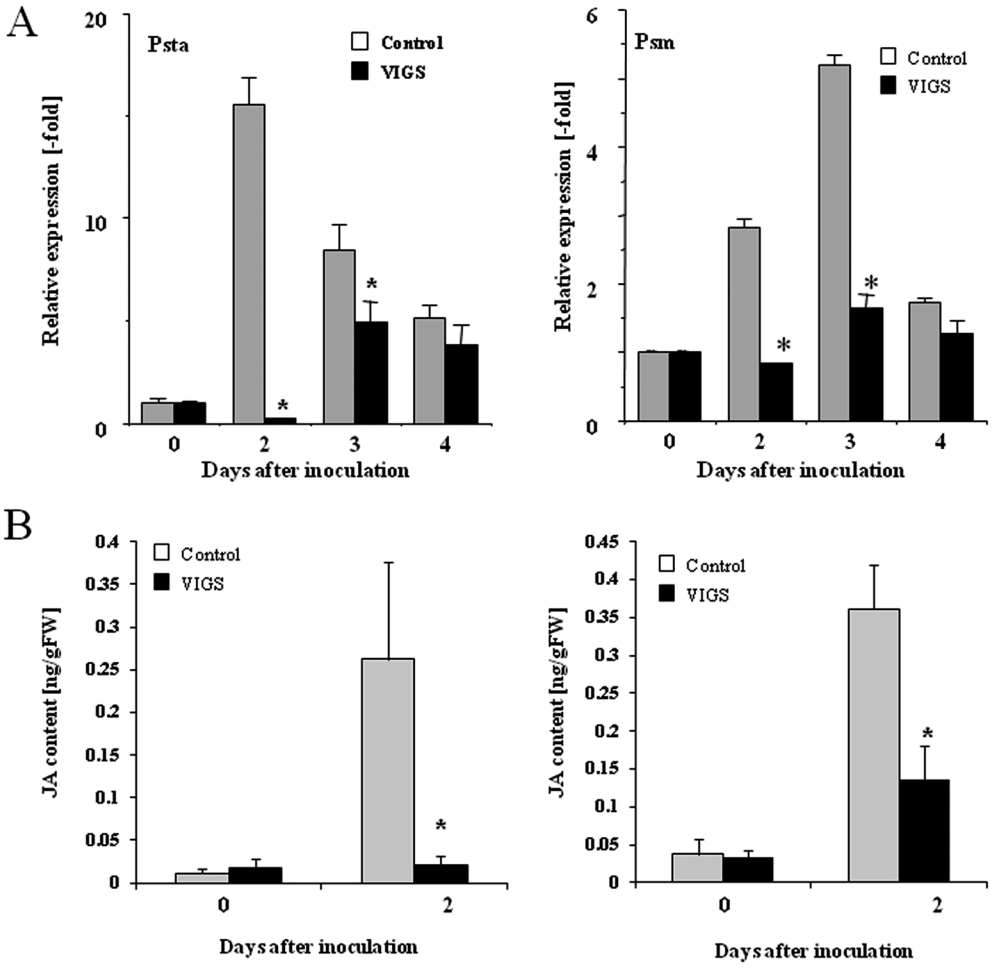

Figure 2. Virus-induced gene silencing of *NbSEC14* and its effect on jasmonic acid biosynthesis and jasmonic acid-dependent *PR-4* gene expression. Control (Control; gray box) and *NbSEC14*-silenced leaves (VIGS; black box) were infiltrated with *P. syringae* pv. *tabaci* (Psta; left panel 10^4 CFU ml^{-1}) and *P. syringae* pv. *mellea* (Psm; right panel 10^4 CFU ml^{-1}). (A) Relative expression of *PR-4* was shown as relative to the absolute non-treated control, and normalized with actin in control or *NbSEC14*-silenced plants. Values represent the means and SD from triplicate experiments. (B) Jasmonic acid was determined at designated time points by LC-MS/MS. Values represent the means and SD from replicated experiments ($n=5$). Asterisks denote values significantly different from empty PVX controls (* $p<0.05$, Student's *t*-test).

R. solanacearum (Kiba et al. 2012, 2014). In present study, we observed strong induction of *NbSEC14* and *PR-4* expression in *N. benthamiana* inoculated with both *P. syringae* pv. *tabaci* and pv. *mellea* (Figure 2). We assume that NbSEC14 should be involved in the regulation of defense responses upstream of JA biosynthesis, which then promotes disease development and accelerates growth of both *P. syringae* pv. *tabaci* and pv. *mellea* (Figures 1, 3) in *NbSEC14*-silenced plants.

The activation of the JA-signaling pathway is essential for resistance against necrotrophic pathogens (Antico et al. 2012), such as those used in this study. *P. syringae* pv. *tabaci* is the causal agent of wildfire disease in tobacco plants, whereas disease symptom is promoted by tabtoxinine-β-lactam, a host non-specific bacterial toxin produced by *P. syringae* pv. *tabaci*. Tabtoxinine-β-lactam causes death of plant cells through the inhibition of glutamine synthetase, which leads to an abnormal accumulation of ammonium ions and the characteristic necrotic wildfire lesions (Turner and Debbage 1982). *P. syringae* pv. *mellea*, a causal agent of Wisconsin leaf blight disease, also causes necrotic lesion in tobacco by yet unknown mechanism. Thus, our results show that NbSEC14 may be regulating JA-dependent immune responses against necrosis-inducing *P. syringae*.

Sec14 is reportedly involved not only in essential biological processes including phospholipid metabolism, membrane biogenesis and stress responses (Dove et al. 1997; Phillips et al. 2006; Saito et al. 2007). Sec14 was amongst proteins that have higher expression in cold-resistant cabbage under light chilling treatment, suggesting a function of Sec14 in cold resistance (Lee et al. 2013). We previously reported that NbSEC14 likely regulates phospholipid turnover, resulting in induction of JA-dependent immune response against *R. solanacearum* (Kiba et al. 2014). Taken together with our current results, we can now propose even more versatile role of NbSEC14 in stress responses, not only against abiotic but

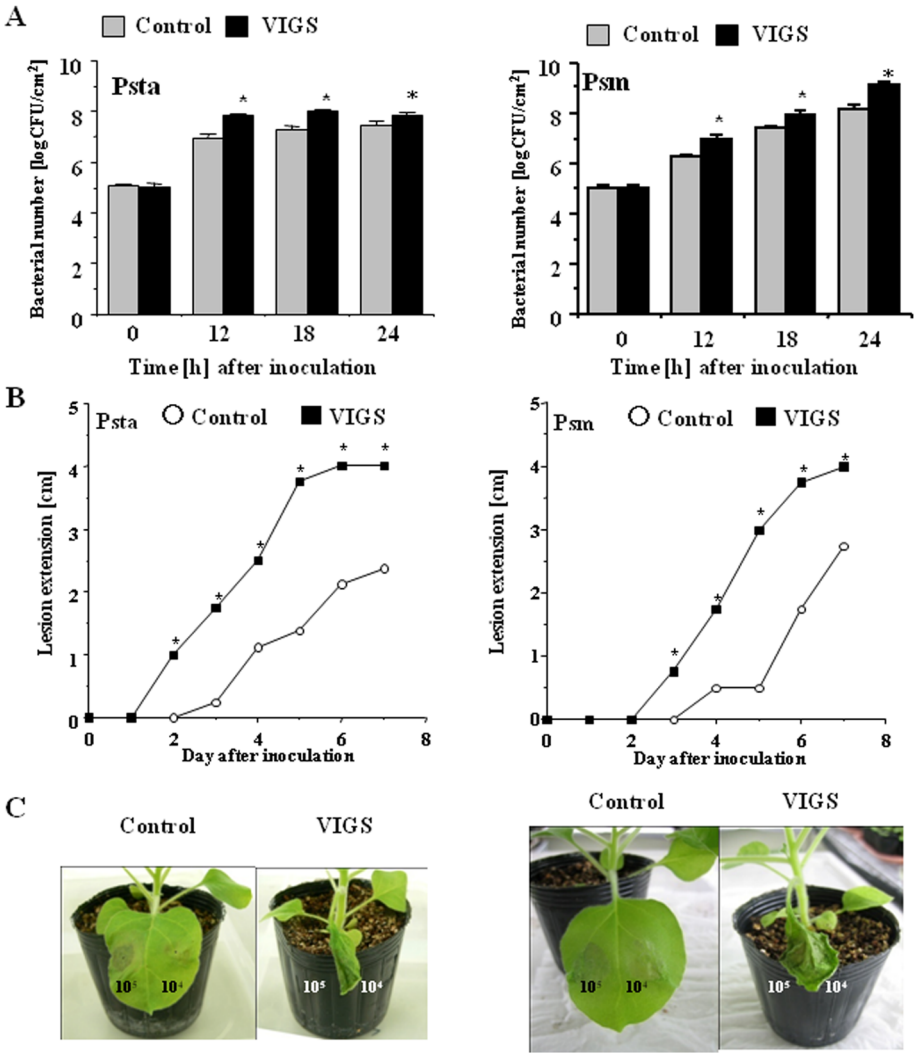

Figure 3. Effect of *NbSEC14*-silencing on growth of phytopathogenic bacteria and disease development. Control (Control; gray box) and *NbSEC14*-silenced (VIGS; black box) leaves were infiltrated with *P. syringae* pv. *tabaci* (Psta; 10^4 CFU ml^{-1}) or *P. syringae* pv. *mellea* (Psm; 10^4 CFU ml^{-1}). (A) The bacterial population was determined by plating at specified time points. Values are the means of four replicate experiments, with their corresponding SD. Asterisks denote values (A, B) significantly different from empty PVX controls (* $p<0.05$, Student's *t*-test). (B) Lesion extension of wildfire (Psta) and Wisconsin blight (Psm) as measured daily in control (Control; open circles) and *NbSEC14*-silenced plants (VIGS; solid circles). Values are the means of four replicate experiments. Asterisks denote values (A, B) significantly different from empty PVX controls (* $p<0.05$, Student's *t*-test). (C) Characteristic symptoms in *N. benthamiana*. Left panel: Pictures taken 5 days after inoculation with *P. syringae* pv. *tabaci* (left side; 10^5 CFU ml^{-1} and right side; 10^4 CFU ml^{-1}). Right Panel: Pictures taken 5 days after inoculation with *P. syringae* pv. *mellea* (left side; 10^5 CFU ml^{-1} and right side; 10^4 CFU ml^{-1}).

also biotic stress. It can be thus concluded that NbSEC14 is required for basal stress responses against wide variety of environmental stresses.

Acknowledgements

The authors thank Dr. David C. Baulcombe of the Sainsbury Laboratory, John Innes Centre, for providing the PVX vector. This work was supported by Japan Society for the Promotion of Science KAKENHI to AK (16780031 and 18780029) and to YH (15028214 and 16380037). AK is also grateful for financial support from The Asahi Glass Foundation, The Agricultural Chemical Research Foundation and Sapporo Bioscience Foundation. This research was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) as part of the Joint Research Program implemented at the Institute of Plant Science and Resources, Okayama University in Japan, and LC-MS/MS instrumentation was supported by Japan Advanced Plant Science Network.

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