Note

Silencing of phosphoinositide dependent protein kinase orthologs reduces hypersensitive cell death in *Nicotiana benthamiana*

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Abstract Phosphatidic acid plays an important role in plant immune responses against phytopathogenic bacteria in *Nicotiana benthamiana*. Here we focused on phosphoinositide dependent protein kinases (PDKs) as a candidate required for phosphatidic acid signaling. Based on *Arabidopsis* PDK sequences, we identified four putative PDK orthologs in *N. benthamiana* genome. To address the role of PDKs in plant defense responses, we created all four *NbPDKs*-silenced plants by virus-induced gene silencing. the *NbPDKs*-silenced plants showed a moderately reduced growth phenotype. Induction of hypersensitive cell death was compromised in the *NbPDKs*-silenced plants challenged with *Ralstonia solanacearum*. The hypersensitive cell death induced by bacterial effectors was also reduced in the *NbPDKs*-silenced plants. the *NbPDKs*-silenced plants showed decreased production of salicylic acid, jasmonic acid and jasmonoyl-L-isoleucine, as well as hydrogen peroxide after inoculation with *R. solanacearum*. These results suggest that *NbPDKs* might have an important role in the regulation of the hypersensitive cell death via plant hormone signaling and oxidative burst.

Key words: effector-triggered immunity, hypersensitive cell death, *Nicotiana benthamiana*, phosphoinositide dependent protein kinase, *Ralstonia solanacearum*, virus-induced gene silencing.

It has been shown that phospholipid-based signaling cascades are important for plant immune responses (Canonne et al. 2011; Munnik 2001; Testerink and Munnik 2005). Recently, we identified the *SEC14* gene encoding a phospholipid transfer protein from *N. benthamiana* (*NbSEC14*). Suppression of phospholipase C and phospholipase D activity, and production of diacylglycerol and phosphatidic acid (PA) were observed in *NbSEC14*-silenced plants, resulting in compromised disease resistance against phytopathogenic bacteria (Kiba et al. 2012, 2014, 2016). Silencing of PA phosphatase caused over accumulation of PA, resulting in enhanced disease resistance against *Ralstonia solanacearum* (Nakano et al. 2013). Induction of hypersensitive cell death (HCD) was also accelerated in PA phosphatase-silenced plants (Nakano et al. 2015). These results suggest an important role of PA in the induction of immune responses and HCD in *N. benthamiana*. To this end, PA functions as a signal molecule in responses to both biotic and abiotic stresses (Liu et al. 2013).

Previously, PA production was activated by osmotic stresses, such as salinity, drought, hyperosmotic stress, and treatment with the drought-related stress hormone abscisic acid (Munnik 2001). PA is also formed after wounding of leaves in various plants, including soybean, sunflower, pepper, broad bean, and castor bean (Munnik 2001; Testerink and Munnik 2005). The chitin elicitor induces the PA biosynthesis, leading to phytoalexin accumulation in rice cells (Yamaguchi et al. 2005), and PA is also produced after Cf-4 mediated pathogen recognition (de Jong et al. 2004). Taken together, PA has an important role in protection of plants against abiotic and biotic stresses. Recently, PA-binding proteins have been characterized as the targets of PA, being involved in the regulation of plant growth, development, and stress responses (Liu et al. 2013; McLoughlin and Testerink 2013). While many proteins with PA-binding properties have been identified mainly in *Arabidopsis thaliana*, it is still poorly understood how PA-binding proteins function in *N. benthamiana* and *R.*

Abbreviations: DAB, 3,3'-diaminobenzidine; ETI, Effector-triggered immunity; HCD, hypersensitive cell death; PA, phosphatidic acid; PDK, phosphoinositide dependent protein kinase; Rs, *Ralstonia solanacearum*; VIGS, virus-induced gene silencing.

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solanacearum interaction. In this study, we focused on phosphoinositide dependent kinases (PDKs) from *N. benthamiana* as candidates for PA-mediated signaling components required for immune responses against *R. solanacearum*, namely HCD induction.

Nicotiana benthamiana was grown in a cultivation room as before (Maimbo et al. 2007). *Ralstonia solanacearum* strain 8107 (Rs8107), which is an incompatible pathogen inducing HCD on *N. benthamiana* (Kiba et al. 2003) and *Agrobacterium tumefaciens* were cultured as described previously (Maimbo et al. 2010). The bacterial suspensions were infiltrated by syringes using standard procedures (Maimbo et al. 2010). Total RNA was isolated using a NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). A 1- μ g 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 with the reagents for the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) and an Applied Biosystems 3100 Avant Automated Sequencer (Applied Biosystems, Warrington, UK). cDNA fragments for the conserved region of *NbPDK2-1* to *NbPDK2-4* sequences (*NbPDKs*) were amplified with specific pairs of primers (Supplementary Figure S1, Table S1), and then cloned into the pPVX201 (Kiba et al. 2012). The binary plasmids were transformed into *A. tumefaciens* strain GV3101, and the transformant was inoculated into *N. benthamiana* leaves as described previously (Maimbo et al. 2007). qRT-PCR was performed using the method described in Maimbo et al. (2007). Briefly, the qRT-PCR was carried out in 20 μ l of reaction mixture, containing 1 μ l of cDNA template, 10 pM of the respective primers (Supplementary Table S1) and THUNDERBIRD qPCR MIX (Toyobo Co.), on an Applied Biosystems 7300 real-time PCR instrument. All values were normalized to the expression values of the actin gene, which was used as an internal standard in each cDNA stock. Cell death was measured by ion conductivity method (Ito et al. 2014a, b) using a Twin Cord B-173 conductivity meter (HORIBA, Kyoto, Japan). Phytohormone contents were determined by a triple quadrupole Liquid Chromatography/Mass Spectrometer LC-MS/MS 6410 (Agilent Technologies, USA) equipped with a Zorbax SB-C18 column [2.1 mm i.d. $\times$ 50 mm, (1.8 μ m), Agilent Technologies]. Hormone amounts were calculated from the ratio of the endogenous hormone peak and the known amounts of stable isotope labeled internal standards, and related to the actual fresh mass of the samples used for extraction (Kiba et al. 2014). Visualization of H₂O₂ in situ was carried out by 3,3'-diaminobenzidine (DAB) staining as described by Nakano et al. (2013).

Based on PDK1 sequences from *Arabidopsis thaliana*, we searched for PDK orthologs in *N. benthamiana* using

the sol genomics network (<https://solgenomics.net/>). We identified 4 PDK orthologs in the *N. benthamiana* genome. Database analysis of PDK orthologs showed high similarity to PDK2 from *Nicotiana* and other plants, and we therefore designated them as *NbPDK2-1*, *NbPDK2-2*, *NbPDK2-3* and *NbPDK2-4* (Supplementary Figure S1A, B). Deduced amino acid sequences of the full-length cDNAs of the *NbPDK2-1*, *NbPDK2-2* and *NbPDK2-3* contained STKc-PDK1 and PH domains. In contrast, *NbPDK2-4* lacked half of STKc-PDK1 and PH domains (Supplementary Figure S1A, C). In *Arabidopsis*, PDK1 was activated by binding of PA to PH domain, and STKc-PDK1 was required for the enzymatic activity (Anthony et al. 2004). In the present study, *NbPDK2-1*, 2 and 3 contained conserved both STKc-PDK1 and PH domain, suggesting that *NbPDK2-1*, 2 and/or 3 act as signaling component downstream of PA (Supplementary Figure S1).

Then, we created constructs for obtaining all four PDKs (*NbPDKs*)-silenced plants (Supplementary Figure S2). Suppression of all *NbPDKs* after initiation of VIGS was estimated by qRT-PCR (Supplementary Figure S3A). The *NbPDKs*-silenced plants showed moderately dwarf phenotype (Supplementary Figure S3B, C), consistent with previous reports on phospholipid signaling having a role in plant growth (Xue et al. 2007). Therefore, our *NbPDKs* might also act as downstream phospholipid signaling components during plant growth. To address the role of *NbPDKs* in plant defense responses, we inoculated Rs8107 to clarify the silencing effect of *NbPDKs* on HCD. The induced cell death appeared in the control plants 18–24 h after inoculation, whereas suppression of cell death was observed in *NbPDKs*-silenced plants (Figure 1A). The induced level of HCD marker *hin1* gene was also suppressed in *NbPDKs*-silenced plants (Figure 1B). These results suggested that *NbPDK2s* act as positive regulator for HCD in *N. benthamiana* (Figure 1). In tomato, PDK1 phosphorylates cell death inhibitor *Adi3*, and gene silencing of *Adi3* caused MAPK dependent cell death. Chemical inhibitor of PDK1, OSU-03012, also reduced viability of tomato protoplasts (Devarenne et al. 2006), indicating a negative regulation of HCD by PDK1 in tomato cells. Therefore, the role of PDKs in HCD regulation may be different between *N. benthamiana* plants and tomato plants. In addition, silencing the *NbPDKs* also had inhibitory effect on the induction of HCD by the *Agrobacterium*-mediated transient expression of *R. solanacearum* effectors, *AvrA* and *PopP1* (Poueymiro et al. 2009, Figure 1C). Plants have evolved the innate immune system to recognize pathogens effector proteins by their cognate resistance proteins, resulting in the initiation of effector-triggered immunity (ETI) (Gassmann and Bhattacharjee 2012). ETI is characterized as specific, sustainable and robust immune

Figure 1. Effect of *NbPDKs*-silencing on HCD induction. Control and *NbPDKs*-silenced *N. benthamiana* plants were infiltrated with *R. solanacearum* 8107 (Rs8107), *Agrobacterium* harboring 35S-AvrA (AvrA) and 35S-PopP1 (PopP1). (A) Cell death induced by *R. solanacearum* was determined by measurement of ion conductivity. Values represent mean with SD ($n=5$). (B) Total RNA was isolated from *R. solanacearum* inoculated plants, and relative expression of *Nbhin1* transcripts relative to the absolute non-treated control, and normalized with actin (*hin1*). Values represent the means and SD from triplicate experiments. (C) Cell death induction by AvrA and PopP1 were determined by measurement of ion conductivity. Values represent mean with SD ($n=5$).

Figure 2. Effect of *NbPDKs*-silencing on oxidative burst and plant hormone synthesis. Control and *NbPDKs*-silenced *N. benthamiana* plants were infiltrated with *R. solanacearum* 8107. (A) Hydrogen peroxide was visualized 18h after inoculation with *R. solanacearum* by DAB staining as described in Materials and Methods. (B) Total jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile), and salicylic acid (SA) content were determined at designated time points. Values represent the means and SE from replicated experiments ($n=5$). Asterisks denote values significantly different from those of control plants (*; $p<0.05$, t -test).

response with HCD (Jones and Dangl 2006). Therefore, our results demonstrate that *NbPDKs* contributes to induction of HCD in responses to Rs8107, as well as effectors-induced HCD, suggesting the involvement of *NbPDKs* in ETI induction.

During the HCD induction by Rs8107 inoculation, hydrogen peroxide production was detected in control plants. In contrast, reduced hydrogen peroxide levels were observed in *NbPDKs*-silenced plants (Figure 2A). Reactive oxygen species reportedly play a role in HCD induction (Torres 2010; Yoshioka et al. 2003; Zhang et al. 2009). It has been reported that activated PDK1

stimulates OXI1/AGC2-1 required for oxidative signaling in *A. thaliana* (Anthony et al. 2006). The PA-PDK1 signaling cascade also induces a respiratory burst in plants (Rentel et al. 2004; Yu et al. 2010). Our present data confirm the involvement of *NbPDKs* in triggering reactive oxygen production (Figure 2A), and therefore, *NbPDKs* might be playing an important role in HCD regulation via oxidative signals.

As shown in Figure 2B, jasmonic acid (JA), JA-L-isoleucine, as well as salicylic acid (SA) accumulated in control plants by 18 and 24h after inoculation with Rs8107, whereas reduced levels of all plant hormones

were observed in *NbPDKs*-silenced plants. Plant hormonal signals are critical for HCD induction. In tomato plants, JA signaling pathway is reportedly required for HCD-inducible β -cryptogein signaling (Starý et al. 2019). The increased resistance against powdery mildew was correlated with cell death, H_2O_2 accumulation, and upregulated expression of SA-dependent defense genes in *N. benthamiana* (Guo et al. 2019). Now, our present data also show involvement of *NbPDKs* in JA and SA signaling at the level of biosynthesis of these hormone (Figure 2B). Therefore, *NbPDKs* might be having an important role in HCD induction, via regulation of JA and SA biosynthetic pathways.

In summary, we demonstrate that *NbPDKs* contribute to induction of HCD in plants. From our findings, while undergoing ETI induction, *NbPDKs* may be activating oxidative burst, JA and SA-mediated signaling in plants, during HCD induction. Further studies will be required to clarify the role of individual PDKs (*NbPDK2-1* to *2-4*) and complex mechanisms by which the *NbPDK* proteins are engaged in HCD induction.

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Author contributions

A.K., K.O. and Y.H. designed the research; A.K., K.F., M.M., I.G. and Y.H. performed the research; A.K., I.G. and T.S. analyzed the data and wrote the article.

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