Journal of Plant Physiology 184 (2015) 15–19

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Short communication

Cell death-inducing stresses are required for defense activation in DS1-phosphatidic acid phosphatase-silenced *Nicotiana benthamiana*

Masahito Nakano[a], Hirofumi Yoshioka[b], Kouhei Ohnishi[c], Yasufumi Hikichi[a], Akinori Kiba[a,*]

[a] *Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kochi University, Nankoku, Kochi, Japan*
[b] *Laboratory of Defense in Plant-Pathogen Interactions, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya, Japan*
[c] *Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi, Japan*

ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 23 June 2015
Accepted 23 June 2015
Available online 2 July 2015

Keywords:
Hypersensitive response
DS1-phosphatidic acid phosphatase
Nicotiana benthamiana
Phosphatidic acid
Reactive oxygen species

ABSTRACT

We previously identified DS1 plants that showed resistance to compatible *Ralstonia solanacearum* with accelerated defense responses. Here, we describe activation mechanisms of defense responses in DS1 plants. After inoculation with incompatible *R. solanacearum* 8107, DS1 plants showed hyperinduction of hypersensitive response (HR) and reactive oxygen species (ROS) generation. Transient expression of PopP1 and AvrA induced hyperinduction of HR and ROS generation. Furthermore, *Pseudomonas cichorii* (Pc) and a type III secretion system (TTSS)-deficient mutant of *P. cichorii* showed accelerated induction of HR and ROS generation. Chitin and flg22 did not induce either HR or ROS hyperaccumulation; however, INF1 accelerated HR and ROS in DS1 plants. Activation of these defense responses was closely associated with increased phosphatidic acid (PA) content. Our results show that DS1 plants exhibit PA-mediated sensitization of plant defenses and that cell death-inducing stress is required to achieve full activation of defense responses.

1. Introduction

Plants recognize pathogen-associated molecular patterns (PAMPs), such as flagellin, flg22, elf18, and chitin, and induce PAMP-triggered immunity (PTI) (Bittel and Robatzek, 2007; Boller and He, 2009). In addition, plants recognize effector molecules secreted via the type III secretion system (TTSS), invoking effector-triggered immunity (ETI). ETI is a rapid and strong defense response accompanied by programmed cell death (hypersensitive response, HR) (Rathjen and Moffett, 2003; Jones and Dangl, 2006).

Phospholipid-based signaling cascades are common signaling components during plant immune responses. Expression of phospholipase D (PLD) genes has been reported to be induced after elicitor treatment in tomato cells (Laxalt et al., 2001). Treatment of chitin has been reported to induce rapid activation of PLD (Yamaguchi et al., 2005). Isoforms of phospholipase C (PLC) are required not only for ETI but also for general immune responses in tomatoes (Vossen et al., 2010). We previously identified SEC14 phospholipid transfer protein (NbSEC14) that regulates PLC and PLD activity, resulting in regulation of phospholipid signaling-mediated plant innate immune responses (Kiba et al., 2014).

Among the phospholipids, phosphatidic acid (PA) has been shown to be an important intracellular signaling molecule during plant immune responses. In tomato suspension-cultured cells, accumulation of PA was observed in response to xylanase elicitor treatment (van der Luit et al., 2000). PA also accumulates in tomato cells in response to a race-specific Avr4 elicitor in a Cf-4-dependent manner (de Jong et al., 2004). Recently, we have identified a gene-silenced *Nicotiana benthamiana* plant showing resistance to *Ralstonia solanacearum*, designated as a DS1 (disease suppression 1) plant. The gene associated with the DS1 phenotype encodes phosphatidic acid phosphatase (DS1-PAP). DS1 plants have been shown to cause increased amounts of PA, resulting in accelerated immune responses including accelerated cell death,

Abbreviations: ETI, effector-triggered immunity; GUS, β-glucuronidase; HA, hemagglutinin; HR, hypersensitive response; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PAMPs, pathogen-associated molecular patterns; PCR, polymerase chain reaction; PLC, phospholipase C; PLD, phospholipase D; PTI, PAMPs-triggered immunity; Pc, *Pseudomonas cichorii* SPC9018; PchrcC, type III secretion system mutant of *P. cichorii* SPC9018; ROS, reactive oxygen species; Rs8107, *Ralstonia solanacearum* 8107; Rs8107Y, type III secretion system mutant of *R. solanacearum* 8107; RT-PCR, reverse transcription-polymerase chain reaction; TTSS, type III secretion system; VIGS, virus-induced gene silencing.

* Corresponding author at: Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kochi University, Nankoku 783-8502, Japan.
E-mail address: akiba@kochi-u.ac.jp (A. Kiba).

http://dx.doi.org/10.1016/j.jplph.2015.06.007
0176-1617/

hyperaccumulation of reactive oxygen species (ROS), and hyperinduction of *PR-4* expression (Nakano et al., 2013). In this study, we focused on activation mechanisms of PA-mediated plant immune responses accelerated in DS1 plants.

2. Materials and methods

2.1. Plant growth conditions and pathogen inoculation

Nicotiana benthamiana was grown as previously described. *Ralstonia solanacearum* 8107 (Rs8107), *Pseudomonas cichorii* SPC9018 (Pc), and their TTSS mutants (Rs8107Y, PchrcC) were cultured in the previously described peptone-yeast medium containing antibiotics (Hojo et al., 2008; Maimbo et al., 2010). Bacterial inoculation was performed by leaf infiltration (Maimbo et al., 2010).

2.2. Preparation of PAMPs

Chitin from shrimp shells and synthetic flg22 peptide were purchased from Sigma–Aldrich (Tokyo, Japan) and Funakoshi Chemicals (Tokyo, Japan), respectively. *Phytophthora infestans* INF1 protein was prepared from *Escherichia coli* carrying either pFB52 or pFB53 as described by Yoshioka et al. (2003).

2.3. RNA isolation and cDNA synthesis

RNA isolation and cDNA synthesis were performed as described previously (Nakano et al., 2013). Briefly, total RNA was isolated from *N. benthamiana* leaves using a NucleoSpin RNA plant kit (Macherey-Nagel, Düren, Germany). Further, cDNA was synthesized with 1 μg total RNA using a PrimeScript RT reagent Kit (Takara Shuzo, Shiga, Japan).

2.4. Semiquantitative RT-PCR

DS1 expression was determined by semi-qRT-PCR using Ex-Taq (Takara Shuzo) as described previously (Nakano et al., 2013). *NbEF-1a* was used as endogenous control. The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

2.5. Virus-induced gene silencing (VIGS)

A virus-induced gene silencing vector for *DS1* was created as described previously (Nakano et al., 2013). The plasmid pPVX201 with no insert was used as a control for virus infection. *Agrobacterium tumefaciens* strain GV3101 isolates carrying control and VIGS vectors were inoculated into *N. benthamiana* leaves as described previously (Maimbo et al., 2010). Semi-qRT-PCR analysis showed that the expression of *DS1* was completely abolished in *DS1*-silenced plants (Fig. S1A).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.06.007

2.6. A. tumefaciens-mediated transient expression

A. tumefaciens strain GV3101 carrying β-glucuronidase (GUS) AvrA, PopP1, and INF1 were prepared as described previously (Kamoun et al., 2003; Katou et al., 2003; Poueymiro et al., 2010). These constructs were transiently expressed in *Nicotiana* plants as described previously (Maimbo et al., 2010). The same amount of GUS protein was detected between control and DS1 plants, suggesting that the efficacy of *Agrobacterium*-mediated gene expression was barely affected by *DS1* silencing (Fig. S1B and C).

2.7. ROS measurement

ROS measurements were performed as described previously (Nakano et al., 2013). For pathogens or HR elicitor, *N. benthamiana* leaves were infiltrated with 0.5 mM L-012 solution (10 mM MOPS-KOH, pH 7.4) using a needleless syringe. Chemiluminescence was detected using a photon image processor equipped with ImageQuant LAS-4000 mini (GE Health Care, Tokyo Japan), and quantified with Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan). For PAMPs treatment, *N. benthamiana* leaf discs (6 mm in diameter) were floated on water overnight and then treated with 0.5 mM L-012 solution containing 100 nM flg22 or 100 μg/mL chitin. Chemiluminescence was continuously monitored using a GloMax 20/20 luminometer (Promega, Tokyo, Japan).

2.8. Evans blue staining

To visualize and quantify cell death induction, Evans blue staining was performed as described previously (Nakano et al., 2013). Briefly, leaf discs were submerged in 0.25% Evans blue dye for 30 min. The stained discs were washed with water to remove excess dye and homogenized in 1% SDS. The homogenate was centrifuged at 12,000 rpm for 5 min, and the supernatant was measured spectrophotometrically at OD_{600}.

2.9. Phosphatidic acid (PA) measurement

Leaf petioles were dipped in water containing 0.59 MBq carrier-free [^{32}P] orthophosphate (Muromachi Chemical Co., Tokyo, Japan) and incubated at 25 °C for 12 h. Total lipids were extracted in $CHCl_3$:MeOH:HCl (50:100:1, v/v/v) using the method described previously (Nakano et al., 2013). Lipid extracts were separated on a TLC plate with ethyl acetate solvent. Radiolabeled lipids were visualized by autoradiography, and densitometry scans of autoradiograms were performed using FLA-3000 (GE Healthcare, Tokyo, Japan).

2.10. Preparation of protein fraction and immunoblot analysis

Total protein was extracted from *N. benthamiana* leaves 3 days after inoculation with *A. tumefaciens* carrying HA-tagged GUS. Leaf tissues were homogenized in extraction buffer (0.35 M Tris–HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, and 0.012% bromophenol blue) using a pestle and centrifuged at 20,000 × *g* for 20 min. The resulting supernatant was subjected to estimation of protein concentration with BSA as a standard (Maimbo et al., 2010). The total proteins (20 μg) were separated by 10% SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad). The membrane was probed with Anti-HA antibody (1:4000, Roche) as primary antibody, and then with goat anti-mouse IgG (H + L)-AP conjugate (1:3000, Bio-Rad) as secondary antibody. Immunocomplexes were visualized by chemiluminescence with CDP-Star (Applied Biosystems) according to the manufacturer's manual.

2.11. Statistical analysis

Statistical analysis was performed using *t*-test ($^*P < 0.05$) (Nakano et al., 2013).

3. Results and discussion

We previously showed that DS1 plants showed hypergeneration of ROS, a key mediator for plant defenses (Nakano et al., 2013). Therefore, we analyzed defense activation in DS1 plants using ROS generation as a molecular marker. Among the plant immune responses, because HR is the most characterized immune