Journal of Plant Physiology 171 (2014) 1757–1762

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

Linoleic acid-induced expression of defense genes and enzymes in tobacco

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ARTICLE INFO

Article history:
Received 28 May 2014
Received in revised form 27 August 2014
Accepted 27 August 2014
Available online 6 September 2014

Keywords:
Defense enzymes
Defense genes
Induced systemic resistance (ISR)
Linoleic acid (LA)
Pectobacterium carotovorum subsp. *carotovorum* (PCC)

ABSTRACT

Linoleic acid (LA) is a naturally occurring fatty acid (FA) found to elicit induced systemic resistance (ISR) of tobacco against the bacterial soft rot pathogen, *Pectobacterium carotovorum* subsp. *carotovorum* (PCC). In this study, we examined effects of six doses of exogenous LA on the induction of defense genes and enzymes. The optimum ISR activity was observed in plants treated with 0.1 mM LA where the effect of LA on membrane permeability was minimal. The application of LA as a root drench enhanced the activity of defense enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO) and induced the expression of β-glucuronidase (GUS). PAL and POD activities were increased in a concentration dependent manner while the maximum PPO activity was observed after treatment with 0.01 mM LA. An RT-PCR analysis of the defense-related genes, *Coi1*, *NPR1*, *PR-1a* and *PR-1b*, of tobacco plants treated with 0.1 mM LA revealed an association of LA with elicitation of ISR in tobacco.

Introduction

Plants interact with a large array of different compounds and regulatory molecules in order to defend themselves against pathogens and stresses. Fatty acids (FAs) are essential macromolecules present in all living organisms, and FAs modulate a variety of plant responses to biotic and abiotic stresses. Evidences show that FAs play important roles in plant defense, including cross talk with various phytohormones that are major mediators of plant defense signaling pathways (Hammerschmidt, 1999; Spoel and Dong, 2008; Rafaele et al., 2009). FAs and their derivatives can significantly alter plant gene expression and metabolism, thus influencing the outcome of plant–microbe interactions (Upchurch, 2008; Kachroo and Kachroo, 2009). FAs and many other organic compounds released as root exudates provide nutrients for plant growth-promoting rhizobacteria (PGPR) and other beneficial microbes in the rhizosphere (Upchurch, 2008). PGPR augment plant growth and immunity through a wide range of mechanisms and production of secondary metabolites (Kloepper et al., 2004). Linoleic acid (LA) (18:2) is a naturally occurring major FA utilized by plants for the production of jasmonic acid (JA), a major signaling compound involved in plant defense and development. Activation of the JA signaling pathways is involved in the induction of below ground plant defenses because it alters the composition of organic compounds released by the roots, which in turn influences the structure of the rhizosphere's microbial community (Carvalhais et al., 2013).

LA is produced and metabolized by various microorganisms (Gentile et al., 2003; McKellar and Nelson, 2003). LA and its derivates are also known to possess a direct biocidal effect against bacteria and phytopathogenic fungi (Walters et al., 2004; Zheng et al., 2005; Liu et al., 2008). LA was found to be one of the ISR determinants of *Ochrobactrum lupini* KUDC1013, a PGPR extensively used in the authors' laboratory (Sumayo et al., 2013). Thus, the objective of this study is to determine the effect of exogenous LA application on tobacco plants and the possible molecular mechanisms involved in LA-elicited induced systemic resistance (ISR). Studies on plant–FA–pathogen interactions are limited and information on the direct effect of exogenous LA on induction of plant defenses is scant especially in regards to its ability to elicit ISR against the bacterial soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum*. We examined the effect of exogenous LA on the activity of some defense enzymes; phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenoloxidase (PPO) and β-glucuronidase

Abbreviations: FA, fatty acid; GUS, β-glucuronidase; ISR, induced systemic resistance; LA, Linoleic acid; PAL, phenylalanine ammonia-lyase; PCC, *Pectobacterium carotovorum* subsp. *carotovorum*; PGPR, plant growth-promoting rhizobacteria; POD, peroxidase; PPO, polyphenol oxidase.

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http://dx.doi.org/10.1016/j.jplph.2014.08.015
0176-1617/

(GUS). To better understand the defense response of tobacco, we also analyzed the expression of signaling regulatory genes, *Coil* and *NPR1* along with the defense genes, *PR1-a* and *PR1-b*. The results obtained are important to get a better understanding of how plants react to or perceived exogenous FAs and their direct utilization for defense.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) seeds were sterilized by soaking in 1.2% sodium hypochlorite (NaOCl) for 30 min followed by extensive washing with sterile distilled water (SDW). Seeds were transferred in 90 mm × 15 mm petri dishes containing half-strength Murashige and Skoog salt (MS) medium containing 0.8% agar and supplemented with 1.5% sucrose and allowed to germinate for 2–3 days at 25 °C in the absence of light (Murashige and Skoog, 1962). Equally germinated seeds were transferred to MS agar in the following culture containers for each experimental uses: 24-well microtiter plates (SPL, Korea) (1.5 mL MS media/well) for induction of systemic resistance and histochemical assay; Incu Tissue 72 mm × 72 mm × 100 mm culture boxes (SPL, Korea) (150 mL/box) to obtain leaf samples for membrane permeability, enzyme activity assays and RT-PCR. Plants were allowed to grow in growth chambers at 25 °C with a 14 h/10 h light/dark conditions.

Chemical treatment preparation

LA was purchased from Sigma–Aldrich (Korea). The working solution was prepared by dilution of LA with SDW to achieve concentrations of 0.0001, 0.001, 0.01, 0.1, 0.25 and 0.5 mM LA. As reported in a previous study (Sumayo et al., 2013), 1.0 mM of LA is phytotoxic to tobacco seedlings thus, experiments were performed applying the LA concentrations lower than 1.0 mM. Control plants were treated with only SDW.

Pathogen growth condition

The soft rot pathogen, PCC was cultured in Luria–Bertani (LB) agar plates at 30 °C. For experimental use, colonies from a 24 h grown culture were suspended in 0.85% saline solution and adjusted to a final concentration of 10^8 CFU/mL. Bacterial concentration was determined by dilution plating on LB agar plates. The strain was stored in 15% (v/v) glycerol at −70 °C for long term storage.

Assessment of the phytotoxicity of LA by membrane permeability assay

Spectrophotometric monitoring of membrane permeability was performed using Evans blue dye (Coventry and Dubery, 2001). Second and third expanded leaves were collected from tobacco plants (four to six expanded leaves stage) and sterilized in 70% ethanol (30 s), 1.2% NaOCl (3 min) followed by rinsing several times with SDW. One cm leaf strips were obtained, placed in 0.25× MS liquid medium supplemented with different concentrations of LA and incubated at 30 °C with shaking at 160 rpm for 24 h. The strips were submerged in 0.25% Evans blue dye for 20 min with shaking at 80 rpm. Strips were washed with SDW until no further dye was eluted and were homogenized with 0.5% aqueous sodium dodecylsulphate (SDS). After centrifugation at 8000 × *g* for 3 min, the homogenate was collected and the optical density was determined at 600 nm (UV-1650PC Spectrophotometer, Shimadzu, Japan). Each treatment has two replicates with 4 leaf strips per replicate.

Elicitation of ISR on tobacco plants pre-inoculated at the root level with LA

Seven days after transplanting in 24-well plates, LA treatment was carried out by drenching 10 μL of LA solutions on the root part of each tobacco seedling (four expanded leaves stage). After 7 days, the plants were challenged with PCC. Five μL of pathogen suspension was drop inoculated onto each four leaves per tobacco seedling. The soft rot symptom was determined by visual inspection of wilting in the spot infected 24 h after infection. The number of symptomatic leaves per seedling was counted as a measure of disease incidence (Ryu et al., 2004). The experiment was repeated three times with three replicates per treatment using 24 plants per replicate.

Assay of defense enzyme activities

Three weeks after transplanting in culture boxes, tobacco plants (four to six expanded leaves stage) were drenched on the root part with 1 mL of different LA solutions. One week after treatment, leaf tissues were ground with liquid nitrogen and the resulting powder was transferred to a micro tube and added with ice cold 50 mM sodium phosphate extraction buffer (pH 7.5) containing 1 mM polyethyleneglycol, 1 mM phenylmethylsulfonyl fluoride, 8% (w/v) polyvinylpyrrolidone and 0.01% Triton X-100. The homogenates were centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatants (crude enzyme extracts) were immediately assayed for enzyme activities. Protein content in the enzyme extracts was determined based on bicinchoninic acid assay using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Three independent experiments were performed with three replicates per treatment.

PAL activity was determined by measurement of the conversion of L-phenylalanine to trans-cinnamic acid (CA) using the method of Kováčik and Klejdus (2012). PAL specific activity was expressed as μmol of CA synthesized min^{-1} mg^{-1} protein.

POD activity was determined following the oxidation of guaiacol in the presence of hydrogen peroxide using the method of Altunkaya and Gökmen (2011). POD activity was monitored at 420 nm. One unit of POD was expressed as an increase in absorbance of 0.01 min^{-1} mg^{-1} protein.

PPO activity was determined spectrophotometrically following the oxidation of catechol using the method by Aydemir and Akkanli (2006). PPO activity was expressed as changes in absorbance at 420 nm min^{-1} mg^{-1} protein. One unit of PPO was defined as the amount of enzyme that caused an increase in the absorbance of 0.01 min^{-1}.

Histochemical assay for GUS enzyme activity

The expression of plant defensin promoter to the GUS reporter gene in transgenic tobacco (PDF1.2::GUS *Nicotiana tabacum*) plants was observed with histochemical staining method performed following the method described by Xu et al. (1994). Seven days after treatment with LA, whole transgenic tobacco plants (four expanded leaves) were carefully uprooted, immersed in staining solution [100 mM Na_2HPO_4, 10 mM EDTA, 2.0 mM X-Gluc (5-bromo-4-choloro-3-indolyl-β-D-glucuronic acid) in dimethyl formamide, 1.0 mM $K_3Fe(CN)_6$ and 0.1% Triton X-100, pH 7.0] and incubated 37 °C in the dark for 48 h. During this process, extra care was taken to avoid injury to the plants. After incubation, the staining solution was replaced with 50% ethanol to remove chlorophyll. The ethanol solution was replaced with fresh 50% ethanol at every 12 h until chlorophyll was completely removed. Leaves from all treatments