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Lipidomic analysis reveals differential defense responses of *Taxus cuspidata* cells to two elicitors, methyl jasmonate and cerium (Ce^{4+})

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Abstract

Methyl jasmonate (MeJA) and cerium (Ce^{4+}) elicitation share common features of increasing taxol accumulation of *Taxus cuspidata* cells. Interestingly, Ce^{4+} induces programmed cell death (PCD), but this phenomenon is not observed with MeJA elicitation. Here, using a lipidomic approach to measure more than 100 membrane glycerophospholipids of *T. cuspidata* cells quantitatively, we discovered that lysophosphatidylcholine (LysoPC), phosphatidic acid (PA) and phosphatidylcholine were three potential lipid markers that were responsible for the differences between Ce^{4+}-induced cells and MeJA-induced cells. Compared with MeJA elicitation, marked increase of phospholipase D (PLD) activity was observed following Ce^{4+} elicitation, suggesting that the PLD activation and high concentrations of PA production might mediate the PCD. Rapid increase of phospholipase A_2 (PLA_2) activity caused the release of fatty acids and LysoPC following Ce^{4+} elicitation, which enhanced endogenous jasmonic acid (JA) accumulation. In contrast, PLA_2 activity was poorly induced following MeJA elicitation. PLA_2 inhibitor suppressed not only JA accumulation but also taxol production, suggesting that the PLA_2 activation mediated Ce^{4+}-induced taxol production partially through a JA-dependent signaling pathway. These results demonstrate that differential alternation of glycerolphospholipids caused by phospholipases constitutes an important step in cell death response to Ce^{4+} and increasing taxol production.

Keywords: Lipidomics; Programmed cell death; Phospholipase A_2 and D; Jasmonic acid; Elicitation; *Taxus cuspidata* cells

1. Introduction

Plant cell culture has been developed as a promising alternative for the production of secondary metabolites, such as taxol, an excellent anticancer drug [1]. Recently, low production yield of taxol from plant cell cultures has prompted intense efforts to develop strategies for the drug production [1–4]. Methyl jasmonate (MeJA), a substance used in plant defense, has been used as one of the most effective elicitors in the production of taxol [1,5]. *Taxus* metabolomic analysis and molecular cloning analysis revealed that the metabolic profile of taxoids and many key enzymatic steps involved in taxol biosynthesis are affected following MeJA elicitation [6,7]. It has been reported that rare earth elements (cerium or lanthanum) have widespread biological effects on development, differentiation and lesion in plants and animals [8–10]. In our previous studies, the addition of Ce^{4+} ($Ce(NH_4)_2(NO_3)_6$) to *Taxus cuspidata* cells has been found to be very effective for strengthening taxol biosynthesis and increasing its release. And Ce^{4+} was more effective than other rare earth ions (La^{3+} and Ce^{3+}) [2]. Moreover, this metallic elicitor induced not only the production and release of taxol, but also programmed cell death (PCD) [11–14]. Interestingly, with MeJA elicitation, the same high-level of taxol biosynthesis was obtained, but *T. cuspidata* cell viability reduction did not appear to be caused by PCD [15]. MeJA and Ce^{4+} elicitation share common features of increasing taxol accumulation, but cause different physiological changes in *T. cuspidata* cells. It becomes important to understand the differences of their overall effects on the cellular components.

Abbreviations: AA, Aristolochic acid; EYTA, 5, 8, 11, 14-eicosatetraynoic acid; GPCho, Glycerophosphocholine; GPEtn, Glycerophosphoethanolamine; GPGro, Glycerophosphoglycerol; GPIns, Glycerophosphoinositol; GPSer, Glycerophosphoserine; JA, Jasmonic acid; LysoPC, Lysophosphatidylcholine; MeJA, Methyl jasmonate; NBD-PtdEt, NBD-phosphatidylethanol; OPLS, Orthogonal partial least squares; PLA_2, Phospholipase A2; PA, Phosphatidic acid; PCA, Principal component analysis; PCD, Programmed cell death; PLD, Phospholipase D

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Secondary metabolites are almost universally derived from primary metabolic pathways. In plants, the lipids and its downstream products are especially required for the developmental and cell death processes and proper responses to biotic and abiotic stresses [16]. PCD leads to blebbing and eventual disruption of plasma membranes and other organelle membranes [17]. It is well known that the perturbation of glycerophospholipid homeostasis, including hydrolysis of structural glycerophospholipids and increases of phosphatidic acid (PA) and lysophosphsphatidylcholine (LysoPC), plays pivotal roles in regulating cell death response [18]. Despite the important roles of membrane glycerophospholipids in defense response, to our knowledge, the roles of glycerophospholipids and related phospholipases in mediating elicitor-induced valuable secondary metabolite biosynthesis is still not well discerned. Therefore, it is necessary and important to identify the lipid metabolic pathways and specific metabolizing enzymes that are involved, which may help improve the processes of taxol production in induced cultures.

In plants, an important defense response is the generation of oxylipins, which include a variety of oxygenated fatty acid-derived compounds [19]. The best known oxylipins are jasmonate family of molecules, such as jasmonic acid and its methyl ester, MeJA. A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite production has been well characterized in cells of *Catharanthus roseus* and *Hypericum perforatum* [20,21]. Increasing evidences demonstrate that the release of linolenic and linoleic acid, which are needed for the synthesis of JA, requires phospholipase A_2 (PLA_2) and phospholipase D (PLD) [22,23]. In the hypersensitive response (HR) of tobacco leaves, an obvious increase in PLA_2 activity was found to precede the accumulation of JA and cell death [24]. In *Arabidopsis,* the linolenic acid that is used for wound-induced JA synthesis was found to be derived directly from PA released by activated PLD [25]. Recently, a comparative lipidomic analysis has suggested that the activation of PLD and the production of PA acted as a positive step in regulating spontaneous PCD and taxol biosynthesis in *Taxus chinensis* var. *mairei* cells [26]. Whether the changes of PLA_2 and PLD activity could also act as key signals mediating elicitor-induced taxol production remains to be investigated.

In the present study, we used a lipidomic strategy combined with multivariate analysis and biochemical methods to investigate the differential responses of *T. cuspidata* cells to two elicitors, MeJA and Ce^{4+}.

2. Materials and methods

2.1. Materials

Ammonium cerium nitrate ($Ce(NH_4)_2(NO_3)_6$) and methyl jasmonate (95%) were purchased from Aldrich (Milwaukee, MI, USA). Cerium nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$), 1,2-Dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) (14:0/14:0 PA), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0/14:0 GPEtn), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0 GPCho), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine sodium salt (16:0/16:0, GPSer), 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (sodium salt) (14:0/14:0 GPGro), L-α-phosphatidylinositol (PI) ammonium salt solution from bovine liver, 1-heptadecanoyl-*sn*-glycero-3-phosphocholine (17:0), jasmonic acid standards, 5,8,11,14-eicosatetraynoic acid (EYTA) and aristolochic acid (AA) were purchased from Sigma (St. Louis, MO, USA). 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (18:1-12:0 NBD-GPCho) were from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine (α,β-bis-BODIPY-GPCho) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-mono-BODIPY-GPCho) were from Invitrogen (Molecule Probes, Eugene, OR, USA). Chloroform and methanol were HPLC grade from Merck (Darmstadt, Germany). Ammonium hydroxide (28%–30%) was from J & K Chemical. All other chemicals, with noted exceptions, were obtained from Sigma.

2.2. Cell culture and elicitation

The cell lines were derived from young stems of *T. cuspidata* and sub-cultured onto solid B5 medium at 25 °C in the dark [12]. Cell suspensions were cultured every 10 days for a total of five generations in freshly modified B5 medium containing sucrose (25 $g \cdot L^{-1}$), naphthylacetic acid (2 $mg \cdot L^{-1}$) and 6-benzyl aminopurine (0.15 $mg \cdot L^{-1}$). The suspensions were maintained in 250 mL shake flasks at 25 °C with continuous shaking (110 rpm) in the dark. The pH of the medium was adjusted to 5.8.

Ce^{4+} and Ce^{3+} were dissolved in distilled water to obtain a 0.5 M stock solution, respectively. 100 μL of above stock solutions were added to 50 mL cultures in the exponential growing stage (at the 10th day) to make a final concentration of 1 mM Ce^{4+} and 1 mM Ce^{3+}, respectively. A stock solution of methyl jasmonate was prepared as a mixture of MeJA: ethanol: distilled water (40:460:500, v/v/v). At the 10th day, 28.7 μL of this mixture was added to 50 mL cultures to make a final concentration of 100 μM MeJA. The PLA_2 inhibitors, EYTA and AA, were dissolved in dimethyl sulfoxide (DMSO). 20 μM EYTA and 20 μM AA were applied to the culture system 1 h prior to the addition of various elicitors. In all experiments, the final concentration of DMSO was approximately 0.1% (v/v).

2.3. Lipid extraction

Total lipid extraction of *T. cuspidata* cells was performed following the method of Welti et al. with minor modifications [27]. Briefly, fresh cells (approximate 0.6 g) were immediately placed in 3 mL isopropanol with 0.01% butylated hydroxytoluene (BHT) at 75 °C. Appropriate amounts of mixed internal standards were added during lipid extraction. The tubes were incubated at 75 °C for 15 min, and 1.5 mL chloroform and 0.6 mL ultrapure water were added. The tubes were shaken for 1 h followed by removal of the extract. The cells were re-extracted with chloroform/methanol (2:1) containing 0.01% BHT five times with 30 min of agitation each time. The combined extracts were washed once with 1 mL 1 M KCl and once with 2 mL ultrapure water. The solvent was then evaporated under nitrogen and the resulting lipid samples were stored at −20 °C. The remaining cells were heated overnight at 105 °C and weighed. Prior to analysis, the extracted lipid samples were redissolved in 2 mL chloroform/methanol (1:1, v/v).

2.4. Analysis of glycerophospholipids by LC-MS

Analysis of glycerophospholipids was performed on either a Finnigan LC/ESI/MS^n system (LCQ Advantage, Thermo Electron, San Jose, CA, USA) as previously described by Yang et al. [26] or on a Waters LC-MS system consisting of a 600E HPLC with an autosampler coupled with Quattro Micro API triple-quadruple mass spectrometer (Micromass, Manchester, UK) as described below.

The lipids were separated on a Lichrosphere Si60 column (125 mm × 4 mm, 5 μm, Merck) with a guard column made of the same packing material. The column temperature was kept at 25 °C. Mobile phase A was chloroform/methanol/ ammonium hydroxide (30%) (89.5:10:0.5, v/v/v) and mobile phase B was chloroform/methanol/ ammonium hydroxide (30%) /water (55:39:0.5:5.5, v/v/v/v). The following linear gradient was used: 5%–20% B for 7 min, 20% B for 3 min, 20%–30% B for 5 min, 30%–50% B for 30 min, 50%–5% B for 5 min, 5% B for 5 min. The total elution time was 55 min at 1 $mL \cdot min^{-1}$. Injection volume was 10 μL.