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A critical analysis of phosphatidic acid mediated resistance response in *Sinapis alba* against *Alternaria brassicicola*

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ABSTRACT

The enhanced expression of *PLD* genes and the quantitative increase in the level of phosphatidic acid (PA) in resistant *Sinapis alba* compared to the downregulated or unchanged expression of the *PLD* genes and decreased PA level in susceptible *Brassica juncea* when challenged with *Alternaria brassicicola*, indicated a positive relationship between PA-mediated signalling and resistance against this necrotrophic pathogen. Furthermore, spraying PA onto the susceptible species *B. juncea* increased resistance and enhanced expression of ABA-responsive genes, which was comparable to the expression of these genes in *S. alba* when challenged with *A. brassicicola*.

- The enhanced expression of several *PLD* genes in resistant *S. alba* was observed compared to *B. juncea* upon challenge with *A. brassicicola*.
- An increase in the PA level in *S. alba* compared to *B. juncea* following interaction with the pathogen.
- Leaves of *B. juncea* incubated on PA-soaked filter paper did not show resistance.
- Spraying *B. juncea* leaves with PA led to resistance as well as to the enhanced expression of ABA-responsive genes.
- A temporal increase in the PA level is associated with increased resistance against *A. brassicicola*.

1. Introduction

Oil seed mustard is a very important source of edible oil and of great economic importance. Therefore, increasing the production of this crop by minimizing the yield loss is one of the priority areas of crop improvement in the world. Black spot disease caused by the necrotrophic fungal pathogen *Alternaria brassicicola* is one of the most damaging and widespread fungal diseases of Indian mustard (*Brassica juncea*) and other oilseed mustard species. The non-availability of a source of resistance against this pathogen in the oilseed mustard germplasm makes oilseed mustard a very good target for *Alternaria* black spot disease. However, the two species *Sinapis alba* and *Arabidopsis thaliana* (Col-0), belonging to the *Brassicaceae* family, show non-host resistance against this pathogen, but the exact mechanism of resistance is unknown [9,20]. In this context, lipids are very promising signalling compounds that are involved in various stress (abiotic and biotic) responses and development in plants [25]. Different environmental factors stimulate the hydrolysis of membrane phospholipids, which give rise to various classes of lipid and signal messengers, such as free fatty acids (FFAs), phosphoinositides, inositol polyphosphates, phosphatidic acid (PA), diacylglycerol (DAG), DAG-pyrophosphate (DGPP) and lysophospholipids [3,5,29]. The transiently increased production of such lowly abundant signalling lipids and their rapid

Abbreviations: S. alba, *Sinapis alba*; *B. juncea*, *Brassica juncea*; *A. brassicicola*, *Alternaria brassicicola*; PLD, phospholipase D; PA, phosphatidic acid; hai, hours after inoculation; ABA, abscisic acid; ROS, reactive oxygen species; PCR, polymerase chain reaction; IAA, indole acetic acid; SA, salicylic acid; JA, jasmonic acid; GA, gibberellic acid; CK, cytokinin; BR, brassinosteroids.

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turnover activates several downstream signalling pathways that are specific to cellular events and physiological responses [28]. Among these signalling lipids, PA is considered to be one of the most potent signal-transduction activator/regulator in almost all eukaryotes, including mammals, plants, yeast and insects [8,18,24,27]. Intracellular PA levels increase following pathogen elicitation, wounding, freezing, hyperosmotic stress and water-deficit conditions [22]. Phosphatidic acid is a lipid that has been implicated in the regulation of numerous physiological and cellular effects, many of which involve ABA responses [11]. Phosphatidic acid is produced from structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine by phospholipase D (*PLD*) enzyme or via the phosphorylation of diacylglycerol (DAG) by DAG kinases (*DAGK*s) [7]. Plants possess six classes of *PLD* homologues (α, β, γ, δ, ε and ζ), according to their sequence homology, gene structure, domain structure, biochemical properties and in vitro enzymatic activity [30], but humans possess only two *PLD* enzymes and yeast has only one. The *Arabidopsis* genome encodes 12 *PLD* members and rice, 17 members, which indicates a specialised function of individual *PLD*s in the plant kingdom. Different members of the *PLD* gene family have been reported to be induced by various abiotic stresses and during developmental processes in different plant species [25]. In *Arabidopsis*, *PLDε* (also known as *PLDα4*) mediates hyperosmotic stress responses in saline and dehydrated conditions [11]. The external application of PA not only upregulates the expression of pathogenesis-related genes, but also promotes salicylic acid- (SA)-mediated cell death in plants [22,2]. The effect of PA was extremely specific and was not found with other types of phospholipids [22]. Kalachova et al. [13]; showed that the activation of *PLD* in a definite pattern is specific for different kinds of stress. In this respect Zhao et al. [33], observed that *PLDβ1*-deficient plants are more susceptible to the necrotrophic fungal pathogen *Botrytis cinerea* than wild-type plants. The direct and indirect links between the expressions of various isoforms of *PLD* and plant growth hormones such as IAA (auxin), ABA (abscisic acid), SA and JA (jasmonic acid) have been well documented. Phosphatidic acid is a positive regulator of the ABA response and ABA stimulates specific *PLD* activity [10]. In vivo functions, such as the transient stimulation of *PLD* in guard cells of *Vicia faba* [12] and during *Arabidopsis* seed germination [15], indicate the positive role of PA in ABA responses. In addition Krinke et al. [17], reported that SA could activate different *PLD* isoforms. The negative regulator of ABA responses, *ABI1* (PP2C), is selectively inhibited once PA binds to it [32]. Phytohormones play a pivotal role in plant–pathogen interactions, depending on the pathogen's lifestyle. In addition to the well-known defence-related hormones, such as SA, JA and ethylene (ET), growth-related hormones such as IAA, ABA, gibberellic acid (GA), cytokinin (CK) and brassinosteroids (BR) have also been implicated in plant defence [4]. Jasmonate is the key player in resistance against necrotrophic pathogens and SA acts against biotrophs [13]. The increased ABA level in *S. alba* as part of the resistance mechanism [20], as well as the susceptibility of *Arabidopsis* ABA mutants [1] to *A. brassicicola* suggests the role of ABA in resistance. Furthermore, mutations in auxin-responsive genes in *Arabidopsis* were found to be detrimental to resistance against necrotrophs such as *Botrytis cinerea* and *Plectosphaerella cucumerina* [19]. Mutations in genes involved in the ABA response [1] and the auxin response [16] led to reduction in the JA level, which is the most important factor for resistance against necrotrophs. The role of PA in resistance against *A. brassicicola* has been investigated here, since ABA responses are controlled by PLD-derived PA during stress. The temporal enhancement of the PA level by spraying the leaves of *B. juncea* with PA suspension upon challenge with *A. brassicicola* causes resistance due to increased ABA responses. However, prolonged exposure to PA by maintaining the leaves on PA-soaked filter paper caused more cell death and did not show resistance compared to untreated *B. juncea* plants upon challenge with the necrotrophic pathogen *A. brassicicola*.

2. Materials and methods

2.1. The growth conditions of plants and fungal strain and the harvesting of inoculated tissue

S. alba and *B. juncea* were grown under optimal environmental conditions in a green house at 24 °C and 60% relative humidity under long-day conditions of 16 h light:8 h darkness [20]. *Alternaria brassicicola* strain MUCL 20297 (obtained from Willem Broekaert, Katholieke Universiteit Leuven, Belgium) was grown on half-strength potato dextrose agar as mentioned in Mazumder et al. [20]. Spores were harvested in sterile water and the concentration of the conidia was adjusted to 5×10^5 spores/mL after determining the conidial concentration with a haemocytometer. Leaves of three-week-old plants were spotted either with 5 μL fungal spores for inoculation or only with 5 μL water for the controls, according to Mazumder et al. [20]. Leaves were collected at 24, 48 and 72 h after inoculation (hai), from three different plants and were snap-cooled in liquid nitrogen and stored at −80 °C for future use.

2.2. Phosphatidic acid estimation by gas chromatographic (GC) analysis

Lipid extraction was carried out using 2 g of each leaf sample in chloroform, according to the protocol of Welti et al. [31]. The lipid samples were dried under a flow of nitrogen gas and were dissolved in 500 μL of chloroform: methanol (2:1 v/v). The extracted lipid samples were separated by preparative TLC on a Merck-60 silica gel plate using the solvent system chloroform:methanol:acetic acid (70:25:3). The bands from the lipid samples that co-migrated with the PA standard (1, 2-Dipalmitoyl-*sn*-glycero-3-phosphate monosodium salt from Sigma- Aldrich), were scraped from the TLC plates (at R_f = 0.6, approximately), dissolved in chloroform:methanol:ammonium hydroxide (70:35:4) and incubated for 1 h at 37 °C. The samples were filtered using filter papers presoaked in acetone that were dried under nitrogen gas. Lipid samples were converted to methyl esters by incubation in 0.5 N methanolic HCl (1 mL each) overnight at 50 °C. After neutralisation with NaOH, 1 mL water and 2 mL DCM (di-chloro methane) were added to each sample and the DCM phase was separated, followed by washing twice with water. Samples were dried and dissolved in 100 μL DCM for GC analysis. In addition to the inoculated leaf samples, the PA standard (1, 2-Dipalmitoyl-*sn*-glycero-3-phosphate monosodium salt from Sigma- Aldrich) was also processed similarly. To standardise the estimation of the PA level by GC, the methyl ester derivative of the pure standard [1 mg of 1, 2-Dipalmitoyl-*sn*-glycero-3-phosphate monosodium salt (Sigma-Aldrich)] was synthesised using methanolic HCl and was dissolved in chloroform:methanol (2:1 v/v). After purification of the methyl derivative of PA, 2 μL, 4 μL or 6 μL of the methyl ester of PA dissolved in chloroform:methanol (2:1 v/v) was injected into a Thermo Scientific GC machine with a TIR-WAX capillary column (30 m × 0.25 mm i.d. × 0.25 μm) at 260 °C. The oven temperature was initially 50 °C for 1 min, then increased by 10 °C/min to 260 °C, and was then maintained for 30 min. The major peaks at about 15.57 min showed a linear increase in peak area with an increase in the amount of PA methyl ester. Therefore, the peak areas at a retention time of 15.57 min of all the standard samples were calculated to create the standard curve (Supplementary Fig. 1).