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Induction of peroxidase, scopoletin, phenolic compounds and resistance in *Hevea brasiliensis* by elicitin and a novel protein elicitor purified from *Phytophthora palmivora*

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ABSTRACT

Elicitin and a new protein 75 kDa elicitor were purified from the culture filtrate of *Phytophthora palmivora*, a pathogen of *Hevea brasiliensis* (rubber plant). Elicitin was obtained by using a one step of DEAE cellulose chromatography and the new elicitor was obtained by two steps of chromatography: a DEAE cellulose column followed by a hydrophobic column. Both elicitors were stable to heat and a wide range of pH values, but were sensitive to ProteaseK. Both elicitors induced scopoletin, peroxidase isozymes (with substrate *o*-dianisidine and scopoletin) and total phenolic compounds in cell suspension of *H. brasiliensis* with similar kinetics. In addition, both elicitors induced peroxidase enzyme (*o*-dianisidine), total phenolic compounds and enhanced local resistance against *P. palmivora* on young rubber tree seedlings. However, the increase of peroxidase enzyme and total phenolic compounds in rubber tree seedlings was different from those in cell suspension. Furthermore, during the expression of local resistance the zoospore of *P. palmivora* induced the peroxidase enzyme (*o*-dianisidine) more rapidly and with higher level than the control plants. *H. brasiliensis* is more responsive to the new elicitor than elicitin in triggering defense responses. That is the new elicitor was active at a concentration lower than those required for elicitin, about a 30-fold decrease for activation defense responses in cell suspension. For induction of peroxidase enzyme (*o*-dianisidine), phenolic compounds and local resistance of rubber plants against *P. palmivora*, the 75 kDa protein was active at about a 2-fold lower concentration when compared to elicitin.

1. Introduction

The rubber tree (*Hevea brasiliensis*) is an economically important crop in Thailand, the products of which are exported worldwide and produce significant revenue for the country. *Phytophthora palmivora* is the causative agent of "leaf fall" and "black stripe" in the rubber plant. It attacks the petioles, causing mature leaves to fall prematurely and attacks the tapping surface resulting in poor latex production.

Plant defense mechanism consists of passive (constitutive defense mechanism) and active responses (induced disease resistance). The passive defense mechanisms are already present prior to contacting with the pathogen, while the active defense mechanisms are activated only after recognition of the pathogen [14]. The passive responses include the natural physical and chemical barriers such as cuticle, stomatal aperture and phytoanticipins. The active defenses consist of two classes of responses that include the local resistance such as cell wall reinforcement, hypersensitive response (HR) and phytoalexin accumulation and the systemic resistance such as production of pathogenesis-related proteins (PR-proteins) and systemic acquired resistance (SAR).

The hypersensitive response is one of the defense mechanisms in plants. In a resistant plant the infected cells and those surrounding it turn brown, with the characteristics of a burn-like lesion after cells die. This reduces the amount of nutrient available to the pathogen inside the dead tissue and limits the growth of the pathogen and its further spread. Hypersensitive cell death is a multifaceted defense mechanism, active against viruses, fungi and bacteria [23]. *P. palmivora* and other *Phytophthora* spp. produce a family of structurally related extracellular proteins, known as elicitins, that can induce hypersensitive cell death and other biochemical changes associated with defense responses in *Nicotiana* spp. [21,22,27,28,31]. A 32-kDa glycoprotein isolated from *Phytophthora megasperma* also displays a similar necrotic action on tobacco leaves [2]. Harpins isolated from *Pseudomonas* species also induce necrotic zones when infiltrated into tobacco leaves in a similar way to those produced by the proteins isolated from *Phytophthora* spp. [1].

Phytoalexins are low molecular weight antibiotics synthesized by plants following infection or stimulation by elicitors. The

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response is localized to the sites of infection and surrounding areas [8]. The first evidence of an active defense response in *H. brasiliensis* was reported by Tan and Low [38]. They detected a blue fluorescent compound in leaf tissue after infection with *Colletotrichum gloeosporioides*. Later, this compound was induced in *H. brasiliensis* through infection by *Microcyclus ulei* and was identified as scopoletin (Scp), a hydroxycoumarin [13]. Scp is considered to be a phytoalexin which is toxic to leaf fungal pathogens of the rubber tree [6,10,11].

Some pathogenesis-related proteins such as β-1,3 glucanase, chitinase and peroxidase (POD), are related to plant defenses while others are proteinase inhibitors that disrupt pathogen nutrition. PR-proteins are sometimes present in low levels before infection and are also induced by stress. Peroxidase (POD) (E.C. 1.11.1.7) is an enzyme associated with the plant defense pathway and is elicited when challenged with elicitors [26,29]. In sunflower, the role of a peroxidase enzyme in the metabolism of the phytoalexin has been investigated in leaf discs by using scopoletin as substrate. The disappearance of scopoletin was associated with the increased activity of the peroxidase that converted the coumarin to a colored insoluble metabolite [9].

Phenolic compounds are plant metabolites widely spread throughout the plant kingdom. They are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Phenolic compounds have been found to be strong antioxidants against free radicals and other reactive oxygen species [24].

Plant defense reactions can be triggered by elicitors present in the cell walls of microorganisms or in their extracellular media. All *Phytophthora* species except *Phytophthora parasitica* var. *Phytophthora nicotianae* produce and secrete proteins called elicitins when cultured in appropriate culture media [18]. Elicitin is a non-glycosylated protein with a molecular weight of 10 kDa. Elicitins can stimulate plant responsive activities such as phytoalexin accumulation, synthesis of pathogenesis-related proteins and the occurrence of hypersensitive cell death or cell necrosis [19]. Palmivorein, a member of the elicitin family, has been purified in our laboratory from the culture filtrate of *P. palmivora* isolated from the *H. brasiliensis*. Elicitin was obtained by ammonium sulfate precipitation and further purified using ion-exchange and gel filtration. It is classified as an elicitin according to its acidic p*I* and a valine residue at position 13 [5]. Like other elicitins, the *P. palmivora* elicitin causes tissue necrosis on tobacco leaves as shown in this study. It also causes hypersensitive necrosis and Scp accumulation in leaves of *H. brasiliensis* (submitted manuscript).

Phytophthora species produce not only elicitins but also other proteins that have elicitor activity such as a polypeptide of 34 kDa found in the culture media of *Phytophthora nicotianae* [3] and a 90 kDa protein (PB90) in the culture medium of *Phytophthora boehmeriae* [40]. The PB90 induced the hypersensitive reaction in tobacco leaves and triggered systemic acquired resistance (SAR) to TMV, *Alternaria alternata*, *P. parasitica* and *Ralstonia solanacearum*. In addition, it caused hypersensitive necrosis in leaves of the Chinese cabbage and induced SAR to *Collectorium higginsianum* [40].

In this study, we purify and characterize elicitin (palmivorein) and a new elicitor from the culture filtrate of *P. palmivora* then compare the elicitor activities between elicitin and the new elicitor by observing the synthesis of Scp, peroxidase enzymes, phenolic compounds and test for the induction of local resistance.

2. Materials and methods

2.1. Pathogen and plant host

P. palmivora, isolated from *H. brasiliensis*, was maintained on Potato Dextrose Agar (PDA) medium at 25 °C in the dark. For elicitor production, *P. palmivora* was grown for 15 days in Henninger medium [16] on a rotary shaker at 100 rpm at 25 °C.

Tobacco plants (*Nicotiana tabacum*) and rubber plants (RRIM600 cultivar) were grown in a growth chamber with a photoperiod of 12 h of light and 12 h of dark at 25 °C. Cell suspension generated from an integument derived callus of *H. brasiliensis* (BPM-24 cultivar) was grown in Murashige and Skoog's (MS) medium supplemented with 2 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.1 mg/l of thidiazuron (TDZ), at pH 5.7 on a rotary shaker at 100 rpm and 25 °C for 14 days. Fourteen-day-old cell suspension was grown in 5% MS, 10 mM 4-morpholineethanesulfonic acid (MES) and 3% sucrose for 3 days before treatment. It was grown in the same medium that was used for equilibrating cell suspension during treatment.

2.2. Purification of the elicitors from P. palmivora

Proteins with elicitor activity were purified from the culture filtrate of *P. palmivora*. A 15-day-old, fungal culture, was filtered through two layers of Whatman filters (no. 1). Proteins were precipitated by the addition of 61.1 g of $(NH_4)_2SO_4$ per 100 ml of culture filtrate at 4 °C overnight. The pellet was collected by centrifugation at 12,000 g for 20 min at 4 °C and then resuspended with distilled water. The solution was desalted by loading onto PD-10 column and eluted with distilled water. The fractions from the PD-10 column were monitored for protein content at an absorbance of 280 nm. The eluted fractions with a high protein content were pooled and further purified by DEAE cellulose and hydrophobic columns. The amount of protein was measured at 280 nm.

2.3. Column chromatography

The fractions with a high protein content from the PD-10 column were pooled and purified using a 30 ml DEAE cellulose column, previously equilibrated with 20 mM Tris–HCl buffer pH 7.5 at 4 °C. The DEAE cellulose column was washed with the equilibration buffer until the washed fractions had no protein. The fractions were monitored for protein at an absorbance of 280 nm. Elution was achieved with the same buffer containing 0.1 M NaCl at a flow rate of 30 ml/h, 1 ml/fractions were collected and measured for their protein contents. The fractions with high protein content were checked for the purity of elicitin by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).

Further fractions eluted with the same buffer containing 0.3 M NaCl were pooled and adjusted to 1 M $(NH_4)_2SO_4$ then loaded onto a HiTrap Phenyl FF (high sub) column that had been equilibrated with 20 mM Tris–HCl pH 7.5 containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer then followed with 20 mM Tris–HCl pH 7.5 containing 0.5 M $(NH_4)_2SO_4$ to wash off impurities. The purified protein with high elicitor activity was eluted with 20 mM Tris–HCl pH 7.5 containing 0.3 M $(NH_4)_2SO_4$ and checked for purity by SDS-PAGE.

The purified elicitin and the purified novel elicitor were desalted by loading onto a PD-10 column before use.

2.4. Polyacrylamide gel electrophoresis

Tricine–SDS-PAGE was performed on 16.5% (w/v) polyacrylamide gels according to Schagger and Von Jagow [32]. After electrophoresis, gels were stained with silver nitrate (GE Healthcare, Bio-Sciences). A value of the relative molecular mass (M_r) of the protein band was estimated by comparison with a broad molecular weight marker (6.5–200 kDa).