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Chitosan enhances resistance in rubber tree (*Hevea brasiliensis*), through the induction of abscisic acid (ABA)

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ABSTRACT

Chitosan is considered as a natural biodegradable compound that has potential to control plant diseases. In this work, we investigated the effect of chitosan in stimulating defense responses in rubber tree against *Phytophthora palmivora*. Foliar spraying of either 0.2% (w/v) chitosan or 50 μM abscisic acid (ABA) were efficient in the reduction of disease severity in *P. palmivora* infected rubber tree. In addition, the increases of enzyme activities, i.e. catalase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, the expression of *HbPR1*, *HbGLU*, *HbASI* and *HbCAT* genes and the deposition of callose and lignin were observed in chitosan- or ABA-treated rubber tree leaves. Besides, a partial cDNA sequence for 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme involved in ABA biosynthesis was firstly isolated from rubber tree using RT-PCR (designated *HbNCED*). The *HbNCED* gene fragment comprised of 753 bp and the deduced *Hb*NCED protein showed 94% similarity to the *NCED5* from *Ricinus communis*. Moreover, the up-regulation of *HbNCED* gene by exogenous chitosan was correlated with the induction of endogenous ABA measured by HPLC. Our results suggested that the exogenous chitosan could effectively reduce disease severity through the up-regulation of defense-related genes and ABA-biosynthesis gene, which led to the activation of defense-related proteins and ABA level in rubber tree.

1. Introduction

Para rubber tree (*Hevea brasiliensis* Muell. Arg) is one of many economically crops in Thailand. During the nursery phase of cultivation, rubber tree seedling can be easily attacked by various pathogens. *Phytophthora palmivora* is an oomycete disastrous pathogen causing leaf fall and black stripe diseases on rubber tree [1].

Plants have an innate immune system to defend themselves against pathogen attacks by activating defense mechanisms, including chemical, physical and enzymatic responses. The plant innate immune system can be induced in plants when cell membrane recognizes pathogen associated molecular patterns (PAMPs) during the pathogenesis process through the specific pattern recognition receptors (PRRs), resulting in the onset of PAMP-triggered immunity (PTI). Nevertheless, pathogens utilize various effectors that inhibit PTI, leading to disease occurrence, and this process is referred to as effector-triggered susceptibility (ETS). On the other hand, plants have evolved a multitude of strategies to produce resistance (*R*) proteins that recognize and inhibit pathogen effectors, restoring immunity and this process is referred to as effector-triggered immunity (ETI). Hence, the activation of PTI and/or ETI enhances plant disease resistance and suppresses pathogen invasion [2–4]. In addition, plants possess a systemic resistance, which consists of two major forms: (i) systemic acquired resistance (SAR) and (ii) induced systemic resistance (ISR). SAR is salicylic acid (SA)-dependent and involves the accumulation of pathogenesis-related (PR) proteins. On the other side, ISR is dependent on the biosynthesis of abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), and the activation of protease inhibitors (PIs) [5–7]. SA and JA influence each other through a complex network of synergistic and/or antagonistic interplay [8].

Nowadays, many biological products are used to enhance plant immunity for controlling plant diseases. Chitosan has become a promising non-chemical alternative agent for potential application in biocontrol of plant pathogens. Besides exerting an antimicrobial activity, it elicits plant defense responses and enhances plant

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capacity to tolerate abiotic stresses [9–11]. Chitosan is a deacetylated derivative of chitin (deacetylation degree, DD, usually ranging from 40 to 98%), a co-polymer that contains a group of heteropolysaccharides composing of β-1,4 linked D-glucosamine and N-acetyl D-glucosamine residues. It is a nontoxic, a natural polysaccharide found widely in the outer shell of crustaceans (crabs, shrimps and crayfishes) including in fungal cell walls.

Chitosan has been identified to act as PAMPs or a general elicitor, stimulating non-host resistance and priming plant systemic immunity [12]. The enhanced defense responses by chitosan application include the enlargement of hydrogen peroxide (H_2O_2) via the octadecanoid pathway and nitric oxide (NO) in the chloroplast, the activation of MAP-kinases, oxidative burst, and hypersensitive responses (HR) [13,14]. In addition, chitosan has been proven to induce enzyme activities of reactive oxygen species (ROS) detoxification enzymes, such as catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO); defense-related enzymes, such as phenylalanine ammonia lyase (PAL), chitinase (CHI) and β-1,3 glucanase (GLU); the accumulation of PR-proteins, such as PR1 protein and PIs and defense-related secondary metabolites, such as callose and lignin [15,16]. Moreover, it has been documented that chitosan can trigger the synthesis of JA and ABA [17].

It is well known that the ABA plays a key role in plant growth and development, for instance germination, seed dormancy, embryo maturation and stomatal aperture. Besides, ABA can stimulate plant adaptive response to abiotic stress, including drought, low temperature and salinity leading to stomatal closure and compatible osmolyte accumulation [18]. In addition, increasing evidences have indicated that ABA is involved in plant responses to pathogen attacks [19,20]. For examples, ABA induced the expression of defense-related genes, such as *PR1*, *POD*, *PPO* and *GLU* in tomato seedlings [21]. Moreover, the exogenous ABA application enhanced the activities of CAT, POD, PPO and PAL in leaves of maize seedlings [22]. Typically, SA and JA signalings often behave antagonistically in plant defense against biotrophs and necrotrophs, respectively. The accumulation of ABA can promote JA biosynthesis, whereas being an antagonist of SA biosynthesis [23–25].

ABA is synthesized both in roots and leaves of plants. It is a terpenoid hormone derived from cleavage of C_{40} carotenoids which is initiated from the plastidal 2-C-methyl-D-erythritol-4-phosphate pathway. The ABA precursor is zeaxanthin, which is converted into xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED), the key enzyme in the ABA biosynthetic pathway. Then, xanthoxin is oxidized to ABA in cytoplasm [26,27]. Although, the *NCED* gene was cloned and characterized in various plant species, such as *Arabdopsis*, grapes, peach and pear [28–30], it has not been studied in rubber tree.

In this work, we investigated the effects of chitosan on induced defense responses in rubber tree against *P. palmivora*. In addition, we isolated a partial cDNA sequence encoding for 9-*cis*-epoxycarotenoid dioxygenase from rubber tree using RT-PCR method (designated as *H. brasiliensis 9-cis-epoxycarotenoid dioxygenase* (*HbNCED*); GenBank accession no. MF375917). Our results suggested that chitosan could stimulate the biosynthesis of ABA through the up-regulated expression of *NCED* gene. Both chitosan and ABA applications could activate enzyme activities of CAT, POD, PPO and PAL, including callose and lignin depositions. Moreover, the expression of *H. brasiliensis* pathogenesis-related protein1 (*HbPR1*), *H. brasiliensis* β-1,3-glucanase (*HbGLU*), *H. brasiliensis* α-amylase/subtilisin inhibitor (*HbASI*) and *H. brasiliensis* catalase (*HbCAT*) genes were significantly induced by chitosan treatment. Our study, therefore, revealed that the induced resistance in rubber tree by chitosan involves the elicitation of defense-related enzymes, the accumulation of callose and lignin, and the stimulation of ABA biosynthesis.

2. Materials and methods

2.1. Phytophthora palmivora zoospore preparation

P. palmivora was isolated from a diseased rubber tree, kindly provided by the Songkhla Rubber Research Center, Thailand, and grown in potato dextrose agar (PDA) plate at 25 °C. To prepare zoospore suspension, the mycelium was transferred onto the surface of V8 juice agar and cultured at 25 °C for 1 week. After that, the culture was added to distilled water, incubated at 4 °C for 15 min, and then shaken at 25 °C for 15 min to release the zoospores. Suspensions of zoospores were measured with a hemocytometer under microscope and adjusted to a concentration of 1×10^5 zoospores mL^{-1}.

2.2. Plant material and treatments

Bud-grafted rubber tree seedlings, cultivar RRIM600, were grown in a field for 3 weeks and then transferred to a controlled room at 12 h/12 h light/dark photoperiod. Leaves at the developmental B2C stage of 22 day-old seedlings were sprayed with: (I) 0.2% (w/v) chitosan (CHT) [(Sigma-Aldrich, Darmstadt, Germany), 60–120 kDa with 60% deacetylation degree (DD)], dissolved in 10 mM acetic acid (pH 5.6 adjusted with 1 N NaOH): (II) 10 mM acetic acid (AA; pH 5.6) as a control for CHT treatment: (III) 50 μM abscisic acid (ABA) (*Phyto*Technology Laboratories®, Lenexa, KS) dissolved in distilled water: (IV) distilled water (DW) as a control for ABA treatment.

2.3. Induced resistance bioassays

One day after the application of exogenous CHT or ABA to the leaves, the seedlings were sprayed with 1×10^5 zoospores mL^{-1} of *P. palmivora*. After that, the inoculated plants were incubated at room temperature. The disease evolution was observed for 5 days after pathogen inoculation. The disease severity index (DSI) was scored by following the method of Parry [31] on a four-point category (where 0 = no disease, 1 = light infection, 2 = moderate infection, 3 = severe infection) and the DSI was calculated using the formula below. Ten rubber tree seedlings were scored for each treatment. The experiments involved three replicates per treatment and repeated two times.

$$\text{Disease severity index (DSI)} = \{[(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d)] \times 100\} / \times (a + b + c + d) \times m$$

(where 0, 1, 2 and 3 are infection categories; a, b, c and d are numbers of plant that fall into the infection categories; m is the highest disease category which is 3).

2.4. Protein extraction and enzyme assays

Rubber tree leaves were sprayed with the previously described solutions (CHT, AA, ABA and DW) and harvested at various time intervals (0, 3, 6, 12, 24 and 48 h). After that, each treatment was frozen immediately in liquid nitrogen and stored at −20 °C until utilized for protein and enzyme extractions. Leaf samples (0.5 g fresh weight) were ground in liquid nitrogen and then homogenized with 1 mL of 0.1 M Tris-HCl buffer (pH 7.0) containing 3% (w/v) polyvinylpolypyrrolidone (PVPP). The extracts were centrifuged at 12,000 rpm for 30 min, and then the supernatant (crude extract) was analyzed for protein concentration and enzyme activities. All steps were carried out at 4 °C. Protein concentration was analyzed