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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

by Bradford's method [32] using bovine serum albumin (BSA) (Sigma Chem. Co., USA) as the standard protein. The protein content was expressed as mg per gram of leaf fresh weight (mg g^{-1} FW).

CAT activity was analyzed according to the method of Hadwan and Abed [33], with slight modifications. The reaction mixture consisted of 20 μL of crude extract, 200 μL of 100 mM H_2O_2 in 50 mM Na_2KPO_4 buffer (pH 7.4). The reaction was incubated at 37 °C for 3 min and then stopped by adding 800 μL of 64.8 mM ammonium molybdate. CAT activity was detected by measuring the decrease in absorbance at 415 nm. One unit of CAT activity was defined as 1 μmole of H_2O_2 was used in 1 min. The activity was expressed as units per gram of leaf fresh weight (U g^{-1} FW).

POD activity was measured based on the method of Liu and coworkers [34], with slight modifications. The reaction mixture contained 100 μL of crude extract, 100 μL of 0.25% (v/v) guaiacol, 100 μL of 0.1 M H_2O_2 and 2.7 mL of 10 mM phosphate buffer (pH 7.0). The reaction was allowed to proceed for 1 min by measuring the increase in absorbance at 470 nm. One unit of POD activity was defined as the amount of enzyme that caused an increase in absorbance for 0.01 in 1 min. POD activity was presented as units per gram of leaf fresh weight (U g^{-1} FW).

PPO activity was assayed following the method of Murr and Morris using catechol as substrate [35], with minor modifications. The activity of PPO was measured by adding 40 μL of crude extract to 2.96 mL of a solution containing 0.1 M phosphate buffer (pH 6.5) and 0.5 M cetechol. The absorbance was measured at 420 nm for 10 s and one unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance for 0.01 in 1 min. The PPO activity was exhibited as units per gram of leaf fresh weight (U g^{-1} FW).

PAL activity was assayed as described by the method of D′ Cunha and coworkers [36], with slight modifications. An enzyme extract (70 μL) was incubated with 930 μL of 50 mM Tris-HCl (pH 8.9) containing 0.1 M L-phenylalanine for 1 h at 37 °C. The reaction was stopped with 160 μL of 6 N HCl then the absorbance was determined at 290 nm with a spectrophotometer. One unit of PAL activity was defined as the change in absorbance for 0.01 in 1 min at 290 nm. PAL activity was shown as units per gram of leaf fresh weight (U g^{-1} FW).

Each sample of the extract was measured in duplicate for each replicate. All experiments involved four replicates per treatment.

2.5. Callose and lignin detection

Callose deposition was detected using the method of Ton and Mauch-Mani [37], with slight modifications. Rubber tree seedlings were sprayed with CHT or ABA and kept for 48 h. Leaf fragments (1.5 × 1.5 cm) were cut out from primary fully expanded leaves and immersed in 95% ethanol until the green color of the leaf fragments was disappear. Then, leaf fragments were washed for two times with 70% ethanol and stained with 0.01% aniline blue in 0.15 M phosphate buffer solution (pH 9.0). The leaf fragments were observed immediately under a fluorescence microscope with UV filter (Olympus CKX53, Japan). Callose deposition was visualized as yellowish green fluorescent.

Lignin deposition was detected according to the method of Jensen's [38]. Leaf fragments were immersed in 2% (w/v) phloroglucinol containing 20% HCl for 10 min and observed under a microscope (Olympus E330 camera, Japan). Lignin deposition was visualized as the red color.

2.6. Measurement of endogenous ABA and SA levels

Determination of ABA and SA contents in leaf tissues was carried out on plants treated with CHT, AA or DW after their foliar application. Leaf samples were used for extraction according to the method of Ederli and coworkers [39], with minor modifications. The samples were homogenized in 90% methanol and centrifuged at 12,000 rpm for 10 min at 4 °C. After that, the supernatant was mixed with 50% trichloroacetic acid, and then filtered through a 0.2 μm cellulose acetate fiber membrane. The extracts were analyzed by high performance liquid chromatography (HPLC; Agillant1100, Waldbronn, Germany) using a column ZORBAX Eclipse XDB-C18 (250 × 4.6 mm), 5 μm particle size, a diode array detector for ABA (254 nm), fluorescence detector for SA (Ex 294 nm, Em 426 nm). The mobile phase consisted of acetronitrile with 0.1% (v/v) formic acid (15:85) was run at a flow rate of 1.0 mL min^{-1}. Each sample was performed in HPLC with three replicates.

2.7. Expression analyses of defense-related genes

2.7.1. RNA extraction and cDNA synthesis

The rubber tree tissues (young leaves) were immediately frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Total RNA was extracted from ground samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The quality and concentration of the extracted total RNA were analyzed by agarose gel electrophoresis and measured by a spectrophotometer (MaestroGen, Hsinchu, Taiwan), respectively. The first-strand cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The remaining RNA was removed from the cDNA products with RNase H (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was kept at −20 °C until use.

2.7.2. Cloning of partial sequence of HbNCED gene

Degenerate primers for *HbNCED* gene were designed based on the conserved sequences of the available published sequences of *NCED* genes from ten plants, *Vitis vinifera* (GenBank accession no. NM_001281270.1), *Brachypodium distachyon* (GenBank accession no. XM_003579073), *Setaria italica* (GenBank accession no. XM_004955423.1), *Ricinus communis* (GenBank accession no. XM_002517778.1), *Cucumis sativus* (GenBank accession no. XM_004134863.2), *Erythranthe guttatus* (GenBank accession no. XM_012981017.1), *Populus euphratica* (GenBank accession no. XM_011031241.1), *Eucalyptus grandis* (GenBank accession no. XM_010064424.1), *Nicotiana sylvestris* (GenBank accession no. XM_009799644.1) and *Solanum tuberosum* (GenBank accession no. NM_001288174.1), in GenBank. The PCR reaction was carried out using EmeraldAmp® PCR Master Mix (Takara, Otsu, Shiga, Japan), 0.25 μM of each degenerate primer (forward: 5′-GGBCAC-CAYYTCTTCGACGGCGACGGYATG-3′ and reverse: 5′-YTCC-CAMGCRTTCCAGAGRTGGAARCAGAA-3′), and 0.5 μL of the first-strand cDNA. PCR reaction was performed under the following conditions: initial denaturation step at 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min, and final elongation step was incubated at 72 °C for 10 min before holding at 4 °C. The expected DNA band was cut, gel-purified by the Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan), and then ligated into pGEM®T-EasyVector (Promega, Madison, WI, USA). The recombinant plasmid was transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). The positive colonies were selected on MacConkey plates consisting 50 μg mL^{-1} ampicillin. The recombinant plasmid was purified from 3 mL of bacterial culture using the E.Z.N.A.®Plasmid Mini Kit I (OMEGA, bio-tek, Norcross, GA) and subjected to sequencing by the Macrogen DNA sequencing service (Seoul, South Korea). The obtained sequence was compared with other database in the GenBank via the basic alignment search tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein translation was performed using the

Translate tool (ExPAsy; http://web.expasy.org/translate/).

2.7.3. Semi-quantitative reverse transcription polymerase chain reaction (semi-qRT-PCR)

The expression of defense-related genes was analyzed by semi-qRT-PCR. The *H. brasiliensis's mitosis protein YLS8* gene (*HbMito*), a housekeeping gene (GenBank accession no. HQ323250) was used as the reference gene [40]. The specific primers for *HbNCED*, *HbPR1*, *HbGLU*, *HbASI* and *HbCAT* genes were designed based on the *HbNCED* gene, the *HbPR1* gene (GenBank accession no. KM514666) [41], the *HbGLU* (GenBank accession no. AY325498) [42], the *HbASI* (GenBank accession no. KM979450) [43] and the *HbCAT* gene (GenBank accession no. MF383167), respectively. The PCR reaction was carried out using EmeraldAmp® PCR Master Mix (Takara,Otsu, Shiga, Japan), 0.25 μM of each gene-specific primer (supplemental Table 1) and 0.5 μL of cDNA template. The reaction was performed with an initial denaturation step at 94 °C for 4 min; followed by 35 cycles (for *HbNCED*), 25 cycles (for *HbPR1*, *HbGLU* and *HbCAT*), and 40 cycles (for *HbASI*) of denaturing at 94 °C for 1 min; annealing at 55 °C for 30 s (for *HbGLU*) and 60 °C for 30 s (for *HbNCED*, *HbPR1*, *HbCAT* and *HbASI*) and extension at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. PCR products were separated by electrophoresis in 2.0% (w/v) agarose gel, then visualized under the UV transilluminator and photographed by a Gel Document and densitometry values of the DNA bands were analyzed with the Image-Using VisionWorksLS (UVP BioSpectrum® MultiSpectral Imaging System ™, Cambridge, UK).

2.8. Statistical analysis

The data were performed by one-way analysis of variance (ANOVA) at $p < 0.05$. Significant means were compared by Duncan's multiple range test using SPSS Statistics 17.0 software.

3. Results

3.1. Effect of exogenous chitosan and ABA applications on rubber tree resistance against P. palmivora

Exogenous chitosan and ABA applications to rubber trees significantly decreased disease symptom and disease severity in response to *P. palmivora* inoculation (Fig. 1A and B). Rubber tree seedlings pretreated with chitosan or ABA revealed a significant reduction in infection development, showing a disease severity of 48.89% and 51.11% at 5 days after inoculation, respectively. On the other hand, the control plants (AA or DW) revealed a gradually increased disease symptom, showing a disease severity of nearly 90% after inoculation. The result suggested that exogenous chitosan and ABA treatments could effectively enhance resistance in rubber tree against *P. palmivora* invasion.

3.2. Induction of defense-related enzymes by exogenous chitosan and ABA

To evaluate the effect of exogenous chitosan and ABA treatments on rubber tree, we sprayed leaves of rubber tree seedlings with either chitosan or ABA and subsequently determined the activity of defense-related enzymes.

No significant difference was found in the appearance of leaves after treatment with either elicitor when compared to the control (AA or DW) for 48 h (Fig. 2A). The protein contents and enzyme activities of CAT, POD, PPO and PAL were measured in rubber tree leaves at 0, 3, 6, 12, 24 and 48 h after treatments (Fig. 2B–F). After spraying with exogenous chitosan or ABA, the protein content started to increase at 3 h to at 6 h and reached the highest level at 12 h when compared to the control (AA or DW) (Fig. 2B).

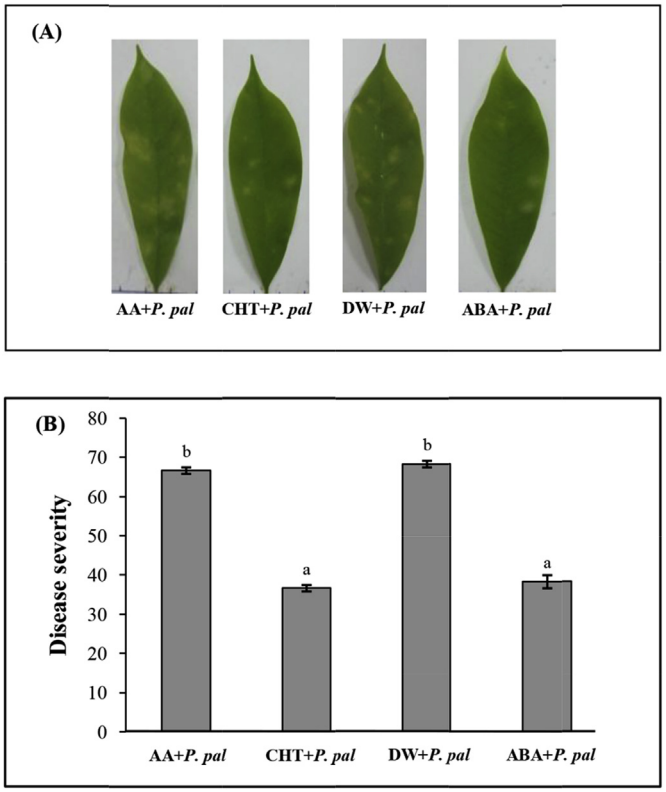

Fig. 1. Protection of rubber tree against *P. palmivora* infection by exogenous chitosan and ABA: disease symptom (A) and disease severity (%) (B) of rubber tree leaf seedlings pre-treated for 1 day with 10 mM acetic acid pH 5.6 (AA; control in CHT treatment), 0.2% (w/v) chitosan (CHT), distilled water (DW; control in ABA treatment) or 50 μM abscisic acid (ABA) before inoculated with *P. palmivora*. Disease symptom and disease severity were evaluated at 5 days after inoculation. Disease severity index (DSI) was calculated from each treatment. Data bars were the means (±SE.) of three replicates, each replicate contained 10 seedlings. Significant differences, according to Duncan's multiple range test ($p \leq 0.05$), were presented by differences among treatments. All the leaves were detached from the plants at 5 days after inoculation for better pictorial representation.

Treatment with chitosan and ABA showed a continuous increase in CAT activity within 12 h and the trend was similar for both elicitors. At 12 h, leaves treated with chitosan and ABA enhanced the activity of CAT by 2.13-fold and 1.79-fold, respectively, when compared to the control (AA and DW). After that, the activity remained high throughout the experimental period of 48 h (Fig. 2C).

For POD, chitosan induced the POD activity and reached a maximum level of 2.24-fold at 12 h, and then decreased slightly when compared to the control (AA) (Fig. 2D). Whereas the treatment with ABA, the POD activity raised more rapidly, becoming 2.54-fold higher than that of the control plants (DW) at 6 h, then the activity decreased, however remaining higher than that of the control plants up to 48 h (Fig. 2D).

In plants treated with chitosan, PPO activity was enhanced significantly within 6 h and raised again at 48 h comparing to the control (AA), while plants treated with ABA, PPO activity gradually increased from 3 h to 12 h and enhanced again at 48 h comparing to the control (DW) (Fig. 2E).

The application of exogenous chitosan and ABA resulted in a significant increase of PAL activity in rubber tree leaves. PAL activity registered a 9.24-fold and 2.46-fold, respectively, after treatment at 48 h when compared to the control (AA or DW) (Fig. 2F).

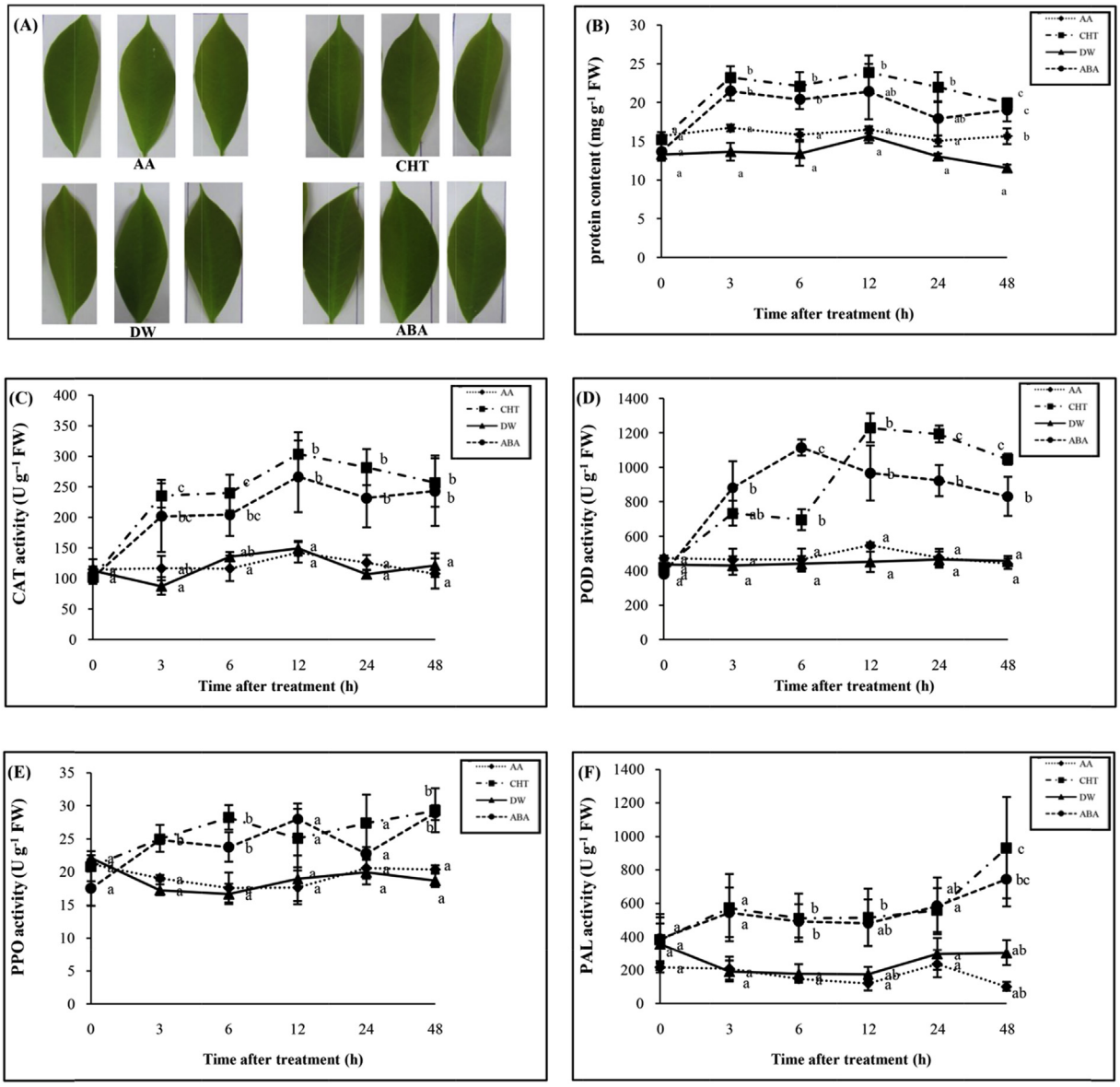

Fig. 2. Physio-morphological traits of rubber tree leaves at 48 h after treatment with chitosan or ABA (A). Chitosan and ABA elicited changes in protein content (B) and activities of CAT (C), POD (D), PPO (E) and PAL (F) at various times. Each experiment of rubber trees was treated with 10 mM acetic acid pH 5.6 (AA; control in CHT treatment), 0.2% (w/v) chitosan (CHT), distilled water (DW; control in ABA treatment) or 50 μM abscisic acid (ABA). Data were means ± SE. Different letters among treatments within the same time point indicated significant differences in Duncan's test ($p = 0.05$). All the leaves were detached from the plants at 48 h after each treatment for better pictorial representation.

3.3. Callose and lignin deposition

After rubber tree seedlings were treated with exogenous chitosan or ABA, the leaf fragments showed callose deposition as yellowish green fluorescent spots, while the control plant (AA or DW) showed a slight callose deposition (Fig. 3). Chitosan and ABA treated fragments showed lignin deposition as appearing of the red color spot under bright field microscope, while the control plants (AA or DW) were almost unstained (Fig. 4). These results indicated that exogenous chitosan and ABA treatments could be effective callose and lignin elicitors in rubber tree.

3.4. Effect of chitosan on abscisic acid and salicylic acid contents

After chitosan treatment, the ABA level was increased at 6 h, with an ABA content of 5.47 ng g^{-1} FW; thereafter, ABA content declined until 24 h and peaked again at 48 h (Fig. 5A). On the other hand, SA level increased remarkably at 24 h, with an SA content of 4.09 ng g^{-1} FW, after that SA level decreased rapidly (Fig. 5B). The control plants treated with acetic acid (AA) or distilled water (DW) did not significantly change the endogenous ABA and SA levels in plant tissues (Fig. 5). The result indicated that the exogenous chitosan could induce the biosynthesis of endogenous ABA, which seemed to antagonize the synthesis of endogenous SA in rubber tree.

3.5. Cloning of NCED gene from H. brasiliensis

A 753 bp fragment was obtained from RT-PCR using the degenerate primers. The nucleotide sequence showed similarities to the *NCED* genes of other plants, designated as *HbNCED*. This partial cDNA of *HbNCED* has been deposited in the National Center for Biotechnology Information under the accession no. MF375917. The deduced HbNCED amino acid sequence (251 amino acid residues) revealed a high similarity with *R. communis* (NCED5) at 94%

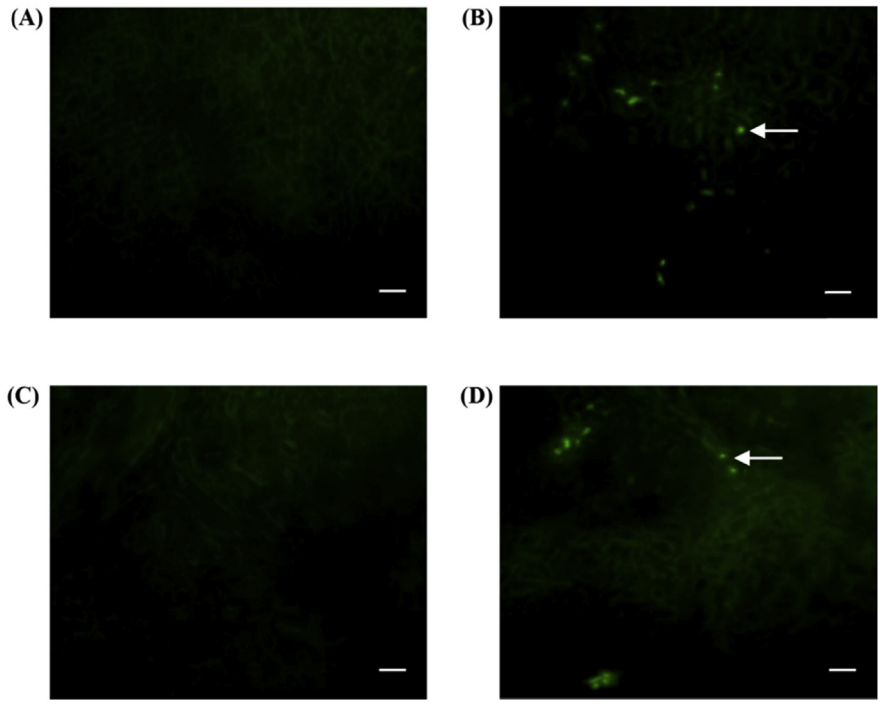

Fig. 3. Callose staining with aniline blue in rubber tree leaves sprayed with 10 mM acetic acid pH 5.6 (AA) (A), 0.2% (w/v) chitosan (CHT) (B), distilled water (DW) (C) or 50 μM abscisic acid (ABA) (D). Chitosan and ABA triggered yellowish green fluorescent spots (arrow) due to callose deposition in rubber tree leaf tissues at 48 h (scale bars = 20 μm).

(GenBank accession no. XP_002519665.1), *Jatropha curcas* (NCED5) at 90% (GenBank accession no. XP_012072117.1), and *Gossypium raimondii* (NCED5) at 89% (GenBank accession no. XP_012452721.1), respectively (Fig. 6).

3.6. Expression analysis of 9-cis-epoxycarotenoid dioxygenase (NCED) gene in H. brasiliensis

The expression of the *HbNCED* gene was induced remarkably in rubber leaves at 6, 12 and 48 h after treatment with chitosan, displaying 9.95-fold, 6.04-fold and 6.73-fold, respectively, when compared to the control (AA) (Fig. 7A). At the same time point (6, 12 and 48 h), ABA accumulation was also higher after chitosan application (Fig. 5A). For ABA-treated plants, the *HbNCED* gene expression at 3 and at 6 h was significantly increased earlier than that in chitosan treatment, displaying 3.11-fold and 3.91-fold, respectively, when compared to the control plants (DW), thereafter the expression declined and was suppressed at 24 h and at 48 h

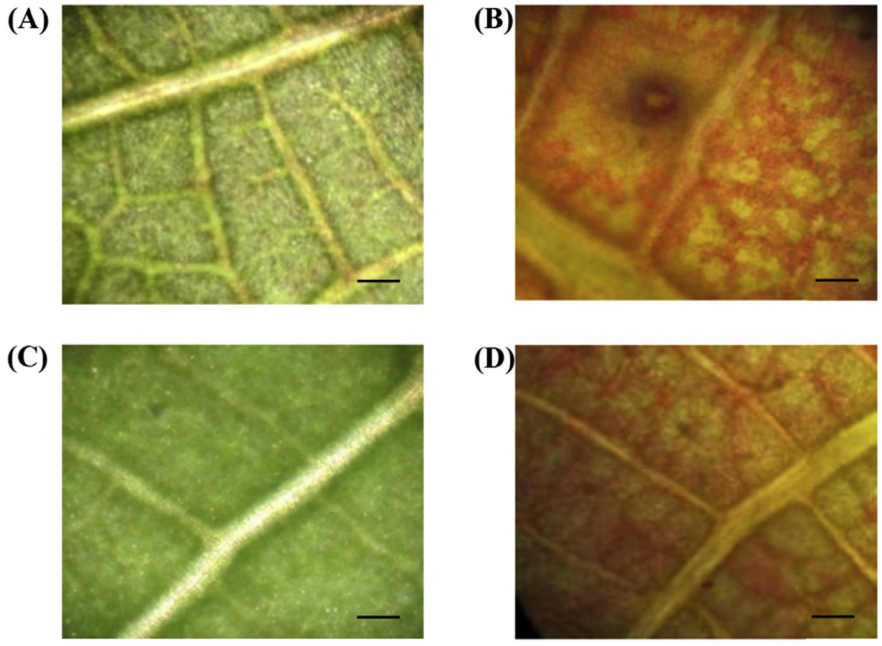

Fig. 4. Lignin staining with phloroglucinol in rubber tree leaves sprayed with 10 mM acetic acid pH 5.6 (AA) (A), 0.2% (w/v) chitosan (CHT) (B), distilled water (DW) (C) or 50 μM abscisic acid (ABA) (D). Chitosan and ABA triggered red color spots due to lignin deposition in rubber tree leaf tissues at 48 h (scale bars = 100 μm).

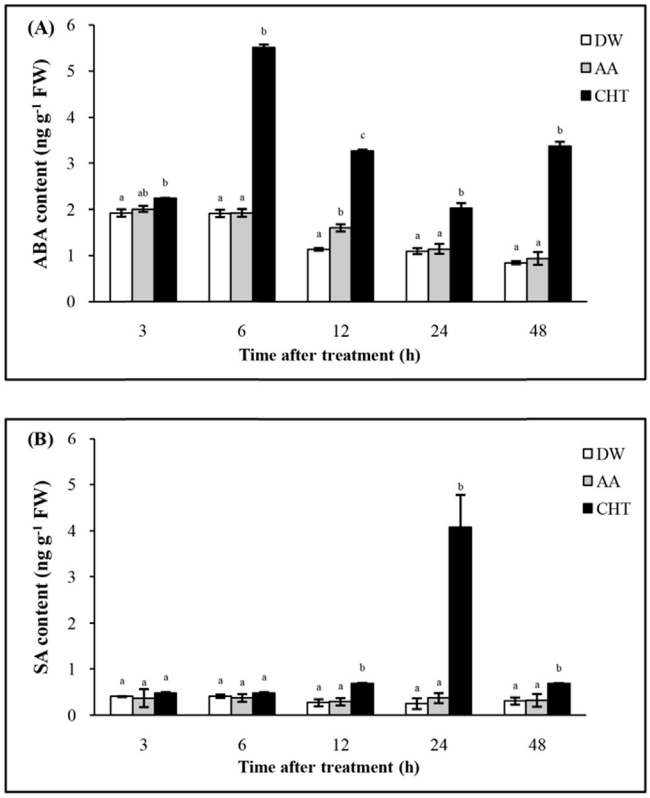

Fig. 5. Determination of abscisic acid (ABA) (A) and salicylic acid (SA) (B) contents (ng g^{-1} FW) by HPLC after spraying rubber tree leaves with 0.2% (w/v) chitosan (CHT) comparing to distilled water (DW) and 10 mM acetic acid pH 5.6 (AA). Data were means ± SE. Different letters among treatments within the same time point indicated significant differences in Duncan's test ($p = 0.05$).

(Fig. 7A). The result suggests that the exogenous chitosan could induce the biosynthesis of endogenous ABA through the transcription of *HbNCED* gene. Even though the endogenous ABA was not measured by HPLC after spraying with exogenous ABA, the synthesis of endogenous ABA may be induced as well since the transcription of *HbNCED* gene was obviously up-regulated after ABA application (Fig. 7A).

3.7. Effect of exogenous chitosan and ABA on defense gene expression in H. brasiliensis

In order to investigate the effect of exogenous chitosan and ABA on rubber tree defense gene expression, the expression pattern of *HbPR1*, *HbGLU*, *HbASI* and *HbCAT* genes was determined in chitosan and ABA-treated plants, comparing to the control plants (AA for CHT treatment and DW for ABA treatment).

After treatment with exogenous chitosan, the expression of *HbPR1* gene was gradually increased from 3 h and reached the highest level at 24 h, with a significant increase 3.46-fold when compared to the control plants (AA) and then slightly decreased at 48 h (Fig. 7B). Whereas, ABA-treated plants stimulated the expression of *HbPR1* gene at 3 h, with a significant increase 4.82-fold when compared to the control plants (DW) and then was suppressed at 24 h to at 48 h (Fig. 7B). The results indicated that chitosan and ABA-treated plants up-regulates the expression of *HbPR1* genes at different time points and the chitosan could stimulate the expression of *HbPR1* gene stronger than the ABA treatment.

The expression of *HbGLU* gene in chitosan-treated rubber tree leaves was rapidly induced at 3 h and slightly increased at 6 h to at 24 h reached the maximum level at 48 h, with a significant increase 3.70-fold when compared to the control plants (AA) (Fig. 7C). While, the expression of *HbGLU* gene in ABA-treated rubber tree leaves was slightly induced at 3 h and thereafter there was no substantial difference between ABA treatment and the control plants (DW) over the time course (Fig. 7C).

After treatment with exogenous chitosan, the expression of *HbASI* gene was up-regulated at 3 h and reached the peak at 48 h, with a significant increase 9.43-fold when compared to the control plants (AA) (Fig. 7D). For treatment with exogenous ABA, the expression of *HbASI* gene was up-regulated at 6 h and continuously increased at 12 h to at 24 h and remarkably increased at 48 h, with a significant increase 34.80-fold when compared to the control plants (DW) (Fig. 7D).

For *HbCAT* gene, the exogenous chitosan caused an increase of *HbCAT* transcript at all time points except at 24 h and peaked at 48 h after treatment, with a significant increase 4.70-fold when compared to the control plants (AA) (Fig. 7E). For ABA-treated plants, the expression of *HbCAT* gene reached the highest level at 6 h, with a significant increase 3.52-fold when compared to the control plants (DW) and then gradually declined over the time course (Fig. 7E). The result demonstrates that the exogenous ABA stimulated the expression of *HbCAT* gene earlier than the chitosan treatment.

These results suggest that both exogenous chitosan and ABA treatments could induce some defense-related genes in rubber tree, at least the tested ones (*HbPR1*, *HbGLU*, *HbASI* and *HbCAT*).

4. Discussion

Chitosan as a polycationic polymer shares inductive properties with polycationic protein, such as histones, protamine and synthetic polymers of ornithine, arginine and lysine [42]. The molecular and biochemical changes in plants treated with chitosan include the stimulation of MAP-kinases, oxidative burst [13,14], lignification, callose deposition [44], the enhancement in PR proteins [45], the occurrence of hypersensitive response (HR), the accumulation of phytoalexins [46], and in some systems cause the production of ABA, JA, H_2O_2 [18], and also a priming of SAR [47]. The chitosan mechanism of action starts from binding to the cell membrane of plant after that chitosan initiates a secondary messenger signal in the plant cell and generates H_2O_2 via the octadecanoid pathway in the chloroplast.

In this work, we have studied the effect of 0.2% (w/v) chitosan and 50 μM ABA on the activity of defense-related enzymes and the expression of defense-related genes in rubber tree leaves.

4.1. Stimulation activity of defense-related enzymes

It has been shown that chitosan and ABA induce defense responses and resistance against pathogens in different plant species [14,21]. In this study, the pretreatment of chitosan or ABA significantly reduced the disease severity in rubber tree after inoculated with *P. palmivora* (Fig. 1). Similarly, the studies in tea plants showed that chitosan reduced the blister blight disease incidence and also induced the activity of defense-related enzymes [48]. ABA reduced the disease severity in tomato plant when challenged with *Alternaria solani* [21] and also suppressed the development of necrotic lesion in *Brassica juncea* leaves when inoculated with *A. brassicicola* [49].

The enhancement of antioxidant enzyme is one of the most effective defense mechanisms in plant through the conversion of O_2 free radicals into H_2O_2. H_2O_2 is a reactive oxygen species (ROS) which contributes to stimulation of plant defense. However, high

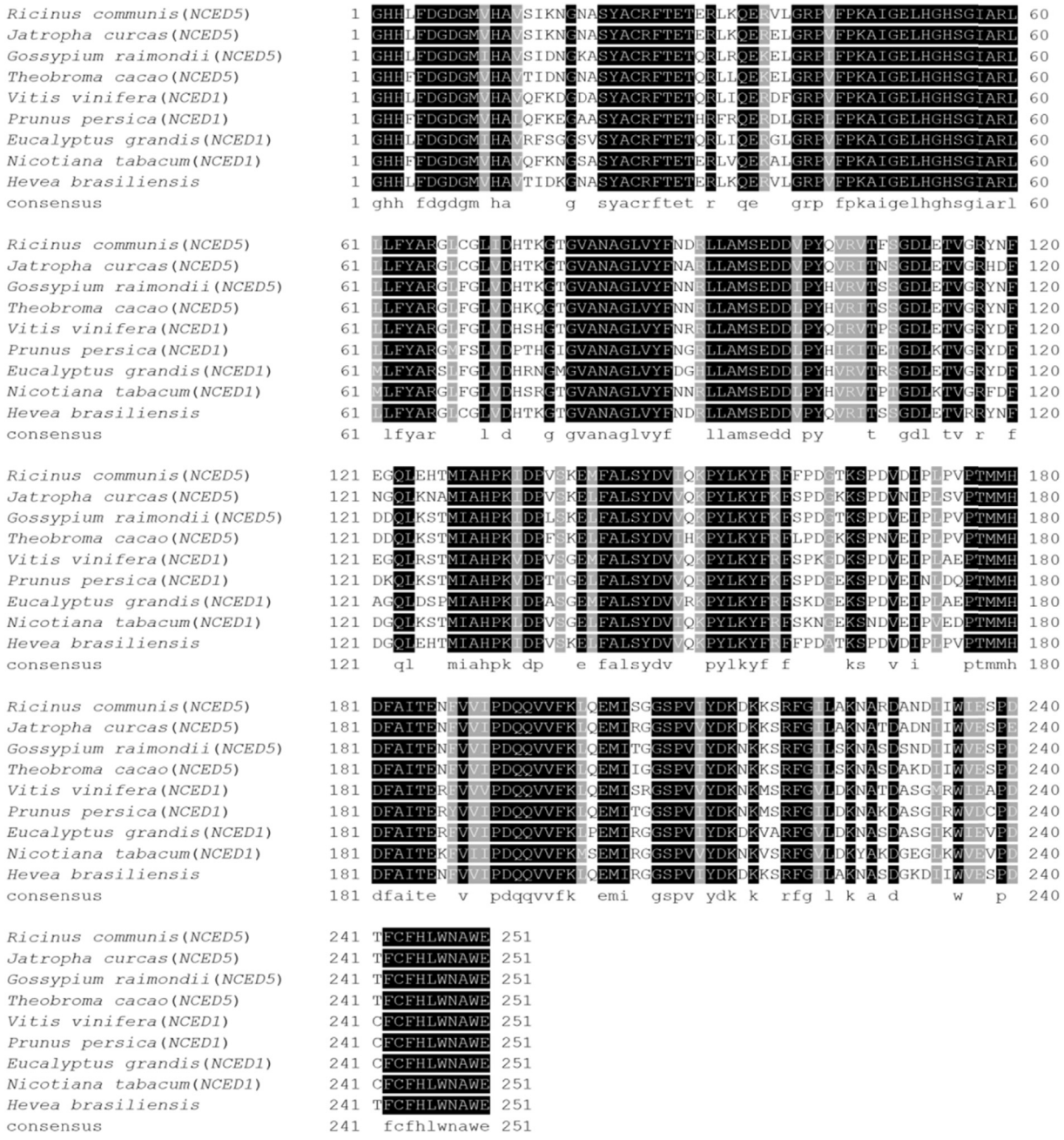

Fig. 6. Alignment of the deduced amino acid sequences of HbNCED (GenBank accession no. MF375917), NCED5 from *R. communis* (GenBank accession no. XP_002519665.1), NCED5 from *J. curcas* (GenBank accession no. XP_012072117.1), NCED5 from *G. raimondii* (GenBank accession no. XP_012452721.1), NCED5 from *Theobroma cacao* (GenBank accession no. XP_007025058.2), NCED1 from *V. vinifera* (GenBank accession no. NP_001268199.1), NCED1 from *Prunus persica* (GenBank accession no. XP_007214657.1), NCED1 from *E. grandis* (GenBank accession no. XP_010062726.1), and NCED1 from *N. tabacum* (GenBank accession no. XP_001312598.1) using ExPAsy program. The conserved amino residues were shaded dark, while highly conserved amino residues were shaded grey.

H_2O_2 level is harmful for plant tissues and must be scavenged by antioxidant enzymes, including CAT and POD [50,51]. Our data showed that chitosan and ABA treatments induced the increase in protein accumulation (Fig. 2B) and also the activities of CAT and POD (Fig. 2C and D) which suggested that both elicitors might cause an increase of endogenous ROS levels, especially H_2O_2. In addition, the exogenous chitosan and ABA elicited the activities of PPO and PAL in rubber tree leaves (Fig. 2E and F). PPO in plant can catalyze the polymerization of phenolic compounds which leads to lignin synthesis [52]. PAL is an important enzyme in phenylpronanoid pathway, which is directly involved in the synthesis of phenols, phytoalexins, and lignin that is associated with the localized resistance processes [53]. Other researchers reported that 0.1% chitosan stimulated protein contents also activities of POD and PPO by foliar spraying of the turmeric plant (*Curcuma Longa* L.) [54], 0.2% chitosan increased the activity of POD by coating on orange fruit [55] and also 1 kDa of chitosan triggered the activities of CAT and guaiacol peroxidase under cadmium stress in *B. rapa* L [56]. As the combination of chitosan and methyl jasmonate (MeJA) pre-treatment, the activities of POD, PPO and PAL were raised in cherry tomato fruit after *A. alternata* infection [57]. Lin and coworkers [13] demonstrated that low molecular weight chitosan could trigger an

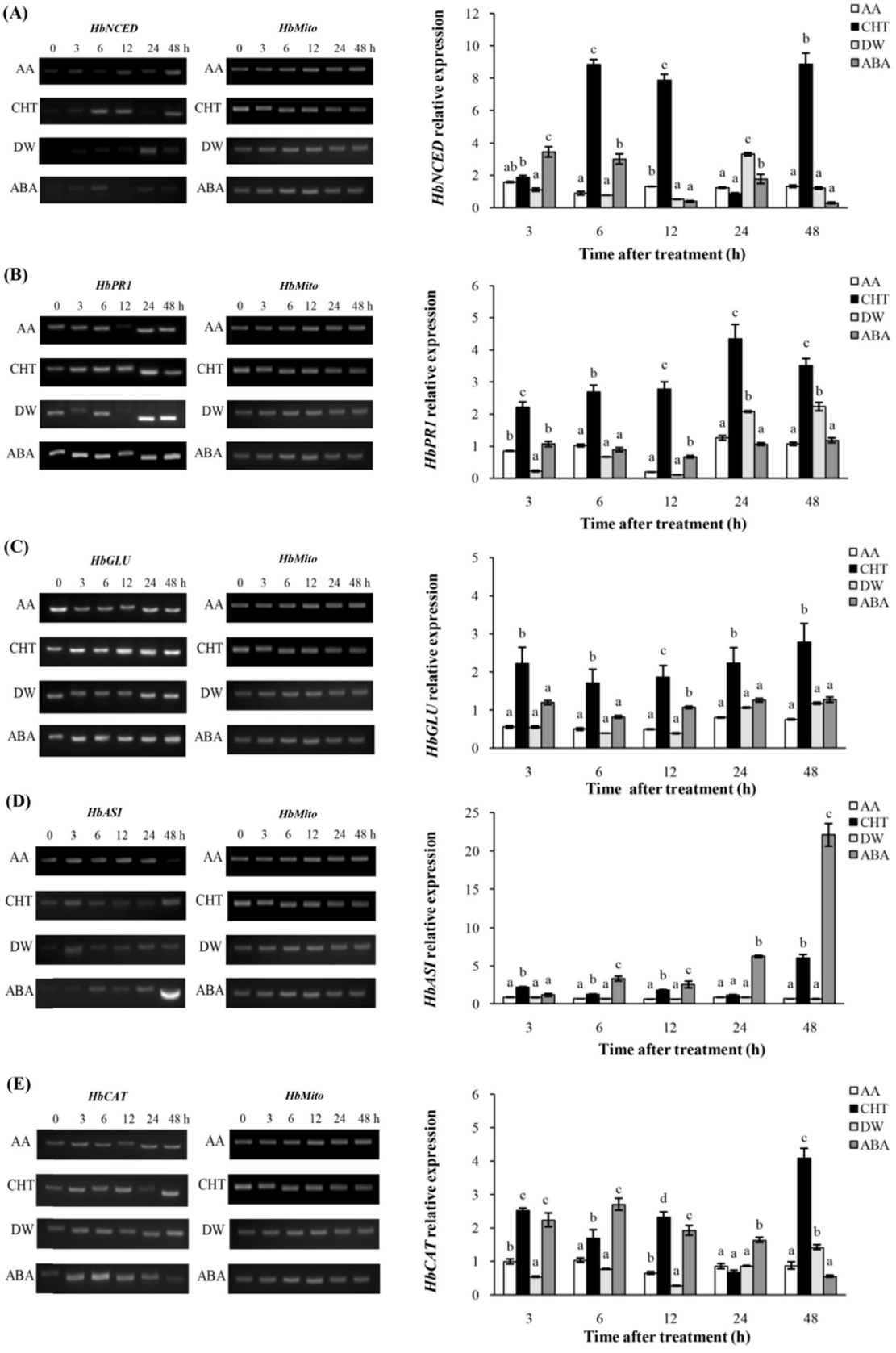

Fig. 7. Time course inductions of *HbNCED* (A), *HbPR1* (B), *HbGLU* (C), *HbASI* (D) and *HbCAT* (E) expressions by semi-qRT-PCR. Expression was measured in leaves of rubber plants after the following treatment: 10 mM acetic acid pH 5.6 (AA; control in CHT treatment), 0.2% (w/v) chitosan (CHT), distilled water (DW; control in ABA treatment) or 50 μM abscisic acid (ABA). Data were calculated as gene expression relative to *HbMito* gene expression. Bars indicated means ± SE. Different letters among treatments indicated significant differences in Duncan's test $p \leq 0.05$ as compared with control AA or DW.

increase of PAL activity. Meanwhile, a higher degree of acetylation (DA) of chitosan induced PAL activity in tobacco plant during protection against *P. nicotianae* [58]. For ABA, the exogenous 10 and 100 μM ABA induced the activity of CAT in leaves of maize seedlings [22]. By treating leaves of tomato seedlings with 7.58 μM of ABA, it could enhance the activities of POD, PPO and PAL [21].

Moreover, our results showed that chitosan and ABA are involved in the accumulation of defense-related metabolites as indicated by callose induction (Fig. 3) and lignin (Fig. 4) deposition. Accordingly, it has been reported in some papers that chitosan with molecular weight of 76 and 120 kDa were the most effective in inducing callose synthesis, particularly 76 kDa chitosan, with a 95% reduction of disease lesions [59], 0.01% chitosan caused callose deposition in *Arabidopsis* [60], and also 0.15% (w/v) chitosan or 0.1 mM ABA triggered callose deposition in tobacco plant when challenged with tobacco necrosis virus (TNV) [17]. It has also been shown that chitosan treatment could synthesize the precursors of lignin and phenolic acids with antimicrobial activity in wheat seeds [61]. A partially acetylated chitosan hydrolysate could trigger the deposition of lignin at the wound margins of wheat leaves and caused a significant increase in the activities of PAL, POD and CAT [62].

Interestingly, our finding also showed that exogenous chitosan induced the ABA biosynthesis (Fig. 5A), except during SA content was significantly enhanced at 24 h (Fig. 5B), inferring the antagonistic action between ABA and SA in rubber tree. It was reported that chitosan induced ABA content for 3-fold, and also H_2O_2 production in bean (*Phaseolus vulgaris* L.) leaves, which might be signals for stomatal closure [63]. Mazumder and coworkers [49] reported that *S. alba* (resistant cultivar) initiated an enhancements of ABA and JA response, as the SA level was suppressed when challenging with necrotrophic fungal pathogen *A. brassicicola*. Instead, SA was stimulated in *B. juncea* (susceptible cultivar) when challenging with the same pathogen.

4.2. Expression of defense-related genes

In plants, ROS can be produced as by-products of metabolism, and also generated through enzymatic pathway, including plasma membrane-localized NAD(P)H oxidase, oxalate oxidase, apoplastic peroxidase and amino oxidase [64]. ROS, especially H_2O_2, can regulate ABA biosynthesis and also ABA signaling which is a direct signal for the expression of gene in plant defense [65]. Meanwhile, ABA causes an increase of H_2O_2 production in guard cells, which is a positive feedback loop [66]. The abundant evidences indicate that both chitosan and ABA actions might relate to oxidative stress which cause an increase of H_2O_2, also induce the expression of defense-related genes [13,67,68].

In this study, the *NCED* gene, encoding enzyme 9-*cis*-epoxycarotenoid dioxygenase which is a rate limiting step in ABA biosynthesis, was firstly isolated from rubber tree leaves. The *HbNCED* gene fragment comprised of 753 bp, encoded 251 amino acid residues. The deduced *Hb*NCED protein showed 94% similar to the *NCED5* from *R. communis* (Fig. 6). Zhang and coworkers [29] reported that the *NCED* gene fragments were cloned from peach (*PpNCED1* and *PpNCED2*, each 740 bp) and grapes (*VVNCED1*, 741 bp). Our finding revealed that the exogenous chitosan could cause an enhancement of endogenous ABA (Fig. 5A) in concordance with the expression of *HbNCED* gene (Fig. 7A). Thus, it suggested that the exogenous chitosan stimulated ABA biosynthesis through the expression of *HbNCED* gene. In *Arabidopsis*, it was found that the expression of *NCED3* gene was induced by exogenous ABA in shoots and roots [69]. Whereas some researchers reported that the expression of *NCED* gene was not induced by exogenous ABA in tomato plant [70] and in cowpea [71]. In addition, increasing ABA by the over-expression of *SgNCED1* gene in tobacco resulted in enhanced transcription and activity of antioxidant enzymes, such as CAT [72]. Similarly, we found that the exogenous ABA could induce the expression of the *HbCAT* gene (Fig. 7E) as well as the activity of CAT (Fig. 2C).

Chitosan-mediated induction resulted in the rapid activation of pathogenesis-related genes, generally considered as the genes that functionally develop disease resistance [73]. Our finding demonstrated that the exogenous chitosan and ABA stimulated the expression of *HbPR1*, *HbGLU*, *HbASI* and *HbCAT* genes (Fig. 7B–E). Some researchers reported that low molecular weight chitosan was more effective than high molecular weight to induce PR1 accumulation in rice seedlings [13], while ABA treatment induced the expression of the *PR1* gene and the *GLU* gene at 24 h and enhanced resistance against *A. solani* in tomato seedlings [21]. In *Arabidopsis*, ABA pretreatment before challenge with *Leptosphaeria maculans* suppressed the expression of β-1,3-glucanase (*PR2*) gene and increased callose deposition. Meanwhile, over-expression of the ABA biosynthesis gene (*NCED3*) resulted in decreased expression of *PR2* gene and induced callose deposition [74]. Accordingly, our results revealed that chitosan treatment on rubber tree seedlings resulted in the increase of *HbNCED* gene expression and the reduction of *HbGLU* gene expression at 6 h to at 12 h (Fig. 7A and C), which led to the enhancement of callose deposition.

The expression of *HbASI* gene was found to be increased during treatment with chitosan and ABA (Fig. 7D). This is in accordance with Koiwa and coworkers [75] which proposed that chitosan and ABA stimulate the induction of *PI* gene through the octadecanoid signal pathway, by catalyzing the breakdown of linolenic acid and the biosynthesis of JA.

Regarding *CAT* gene, it has been demonstrated that chitosan and ABA could induce H_2O_2 generation [13,67,68] and also the expression of antioxidant gene encoding CAT [76]. In our study, it was shown that the exogenous chitosan and ABA activated the expression of *HbCAT* gene (Fig. 7E). Some researchers reported that chitosan treatment induced the highest expression of the *CAT* gene in tea plants [48]. In addition, Xing and coworkers [77] suggested that H_2O_2 might be involved in ABA induced *CAT* expression and the obtained CAT enzyme was possibly implicated in its feedback regulation of the H_2O_2 signaling apart from its ROS scavenging action. Therefore, ABA might regulate defense gene expression and defense responses through the callose deposition, production of reactive oxygen intermediates.

In summary, exogenous chitosan and ABA applications could effectively induce resistance to *P. palmivora*-infected rubber tree through the induction of defense-related enzymes, such as CAT, POD, PPO, PAL and the up-regulation of defense-related genes, such as *HbPR1*, *HbGLU*, *HbASI* and *HbCAT*, including the deposition of callose and lignin. In addition, the up-regulated *HbNCED* gene by chitosan treatment is correlated with the enhancement of ABA content, which results in a primed deposition of callose and lignin. Finally, our results revealed that chitosan- and ABA-treated rubber tree leaf at the developmental B2C stage of seedlings prior to subsequent challenge with *P. palmivora* are effective in controlling the initial onset of infection, though the practical application of these products need further investigation.

4.3. Contributions

NC designed and planned the experiments. AK, ND and KK performed the experiments, measurements and analyzed the data. NC, AK and KE wrote the manuscript and performed editing and corrections. NC, KE and AK revised and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pmpp.2017.12.001.

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