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Overexpression of β-1,3-Glucanase Gene in Response to *Phytophthora palmivora* Infection in Leaves of *Hevea brasiliensis* Clones

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Abstract

The β-1,3-glucanase (β-glu) gene belongs to pathogenesis-related protein family 2 (PR2), which is induced by pathogens. Leaf fall disease caused by *Phytophthora palmivora* is the most serious disease affecting Para rubber (*Hevea brasiliensis* Müll. Arg.) seedlings. In this study, we examined the development of necrotic lesions and the molecular responses shown by *H. brasiliensis* clones RRIT 251 and RRIM 600 from infection by *P. palmivora*. The expansion of necrotic lesions on Para rubber leaves was observed around points of inoculation. The expression of β-glu was analyzed by RT-PCR, and the accumulation of β-1,3-glucanase protein was determined by denatured SDS-PAGE. The inoculation tests suggested that RRIT 251 clones have a better resistance than RRIM 600 clones, with infection occurring in 33.34 and 100 % of inoculations, respectively. RNA analysis by RT-PCR demonstrated that both RRIT 251 and RRIM 600 clones expressed the β-glu gene during *P. palmivora* infection, and protein analysis by denatured SDS-PAGE displayed no obvious differences between the clones. Although expression of the β-glu gene occurred in both clones, the clones differed in phenotype. The better resistance of RRIT 251 relative to RRIM 600 against infection by inoculation may be associated with other defense mechanisms against *Phytophthora* infection.

Keywords: β-1,3-glucanase, *Hevea brasiliensis*, leaf fall disease, *Phytophthora palmivora*, PR protein

Introduction

Plants have a broad range of defense mechanisms against pathogen infections. Responses to pathogen invasion include an oxidative burst of cells, leading to programmed cell death, and the synthesis of compounds like phytoalexin and pathogenesis-related proteins (PR). PR proteins are the main sources for the conferring of a pathogen-specific resistance to the plant [1,2]. The PR proteins accumulate locally in the infected and adjacent cells, limiting the spreading of a pathogen, and the production of PR proteins in uninfected cells can prevent progress of infection [3,4]. To date, more than 17 different PR proteins have been catalogued, along with their properties and functions [5]. Among these are proteins PR2 and PR3, with functions similar to β-1,3-glucanase (β-glu) and chitinase, respectively, and they play an important role in many plant species against various pathogens.

The protein β-glu in plants belongs to the PR2 family, and is an important component in defense mechanisms against pathogens [6-9]. β-glu proteins have molecular masses in a range from 33 to 44 kDa [10,11], and a molecular mass of 41 kDa in *Hevea brasiliensis* [12]. These proteins are hydrolytic enzymes that can cleave 1,3-β-D linkages in β-1,3-glucans, which are cell wall components in several pathogenic fungi [13,14] and are often the major components of a cell wall [15,16]. For example, *Phytophthora*, in the Class Oomycete, has 80 - 90 % β-1,3-glucan in its cell wall. The β-glu can act in 2

ways; directly, by degrading the cell walls of the pathogen, or indirectly, by releasing cell wall-derived materials that elicit active defense reactions in the plant [17].

Para rubber (*Hevea brasiliensis* Müll. Arg.) is an important economic crop in Thailand. Effective and efficient propagation of *H. brasiliensis* is done by bud grafting. The *Hevea* clones RRIT 251 and RRIM 600 are the most cultivated clones in Thailand, giving high yields in the Thai environment. Many high-yield clones are susceptible to diseases such as Para rubber leaf fall, caused by the *Phytophthora* species. The β-glu gene was first isolated from *Hevea* [18], and the main work on β-glu from rubber trees has focused on its allergenic properties [19,20]. The role of β-glu in plant response to pathogen infection is poorly known in *Hevea*. The objective of this study was to determine the effects of infection on expression of the β-glu gene of the *Hevea* clones RRIT 251 and RRIM 600, in connection with necrotic lesion development during *Phytophthora palmivora* infection. RT-PCR was used to observe the expression of the β-glu gene, while SDS-PAGE was employed to determine the accumulation of β-glu. The relation of β-glu gene expression to necrotic symptoms will be discussed.

Materials and methods

Plant materials, fungal isolates, and inoculations

The bud-grafted *H. brasiliensis* clones RRIT 251 and RRIM 600 were raised in polyethylene bags in greenhouse conditions. The pathogen used was a highly virulent isolate of *P. palmivora*. The fungi were stored in Potato Dextrose Agar (PDA) slants at 4 °C. Plates containing PDA medium were inoculated with the stock culture and incubated at room temperature for 5 days. For sporulation, zoospore suspension was prepared by rinsing V8 culture plates growing *Phytophthora* with 15 ml sterilized DW, then incubating the rinse suspension at 4 °C for 15 min, and keeping it at room temperature for 30 min to release zoospores. For inoculation, Para rubber leaves were wounded with sterile needles, and zoospore suspension was dropped onto the wounds. The necrotic lesions caused by *P. palmivora* were observed at 24, 48 and 72 h post inoculation (hpi).

RNA extraction

Total RNA was extracted from young leaves of both RRIT 251 and RRIM 600 clones using Tri Reagent (Sigma-Aldrich, Steinheim, Germany), according to the manufacturer's instructions. Tissue samples of Para rubber leaves were ground with liquid nitrogen in a small mortar with a pestle. The ground tissues were homogenized in Tri Reagent as 0.1 g sample in 1 ml. The aqueous phase was transferred to a fresh tube and incubated at room temperature. Some $CHCl_3$ (0.2 ml) was added to the sample and mixed thoroughly, then incubated at room temperature (25 °C). After centrifugation at 13,000 rpm, the aqueous phase was collected and added to 100 % isopropanol. The solution was centrifuged at 13,000 rpm to precipitate nucleic acid, and the pellet was collected and dried. The pellet was re-suspended into RNase-free DEPC-treated distilled water. The concentration of RNA was measured, and the sample stored at –20 °C.

Analysis of β-glu mRNA expression by RT-PCR

Total RNA (500 ng) extracts from Para rubber leaves were reverse-transcribed to single stranded cDNA using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, life technologies) and specific primers. About 200 base pair (bp) β-glu genes were amplified using specific primers for ß-1,3-glucanase: HbPR2-F16 and HbPR2-R210 (**Table 1**). The sequences of primer pairs were acquired from the GenBank (http://www.ncbi.nlm.nih.gov) nucleotide sequence database, based on the *H. brasiliensis* clone PRII105 accession number DQ989337, and designed using Primer3 software (University of Massachusetts Medical School, USA). The housekeeping gene β-actin, included in upstream and downstream primers [21], was used as an internal control (**Table 1**). The cDNA was synthesized with one cycle at 55 °C for 30 min, and pre-denaturation at 94 °C for 2 min, followed by the polymerase chain reaction (PCR). In PCR, 40 cycles of amplification were performed under the following conditions: denaturation at 94 °C for 0.15 min, annealing at 55 °C for 0.30 min, extension at 68

°C for 1 min, and a final extension at 68 °C for 5 min. The PCR products were electrophoresed through 1 % agarose gel and stained with ethidium bromide.

Table 1 Primers used for cDNA synthesis and PCR amplification of DNA fragments, used to determine mRNA expression of β-glu and β-actin genes.

Primers	Primer sequences (5' to 3')	References
HbPR2-F16	TATGGAATGCAAGGCAACAA	Acc. No. DQ989337
HbPR2-R210	GTTTGCATTGG AAGGATTGG	
β-actin upstream	TCCATAATGAAGTG TGATGT	Kobayashi *et al*. 2000
β-actin downstream	GGACCTGACTCGTCATACTC	

Protein analysis

Leaf samples were ground with protein extraction buffer (homogenized buffer: 100 mM Tris-HCl pH 6.8, 4 % SDS, 12 % 2-mercaptoethanol), using 0.1 g sample per 1 ml homogenized buffer, and the tissue samples were then centrifuged at 10,000 rpm. The aqueous phase was collected, added into sample buffer (125 mM Tris-HCl, pH 6.8, 4 % SDS, 8 % 2-mercaptoethanol, 20 % glycerol, 0.02 % Bromphenol Blue), and heated to 95 °C to denature the proteins. The extracted protein samples were separated in 10 % NuPAGE® Tris-Acetate Mini Gels (Life technologies, Carlsbad, CA, USA) in NuPAGE® MOPs SDS running buffer (novex© by Life technologies, Carlsbad, CA, USA). The gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA) for 30 min, then washed with distilled water overnight. Finally, each gel was dried in vacuum and photographed. A protein ladder for 10 - 250 kDa was used as a calibration marker (BioLabs, New England).

Results

Effects of *Phytophthora* infection on *Hevea* clones RRIT 251 and RRIM 600

In order to test the responses of the *Hevea* clones RRIT 251 and RRIM 600, they were inoculated with *P. palmivora*. Development of necrotic lesions on the Para rubber leaves was used as indicator that the pathogen had successfully invaded plant tissues. Necrotic lesions started to develop on the Para rubber leaves and spread further from the inoculation points within 24 hpi. The dark paths appearing around the inoculation point were considered to be infected areas in the leaves. The number of infected plants was counted, and expansion of the necrotic lesions was observed. The necrotic lesions were sequentially observed at 24, 48, and 72 hpi. The *P. palmivora* aggressively invaded the leaves of the Para rubber clone RRIM 600 at 24 hpi, and was distributed to most parts of the leaves by 72 hpi (**Figure 1**). The percentage infected by inoculation (PI) was calculated to compare the resistances of the clones. For the RRIM 600 clone, infections were 100 % successful while, for the RRIT 251 clone, the PI was only 33.34 % (10 infected leaves/30 leaves). Furthermore, the necrotic lesions caused by *P. palmivora* in the RRIM 600 clones grew faster than in the RRIT 251 clones.

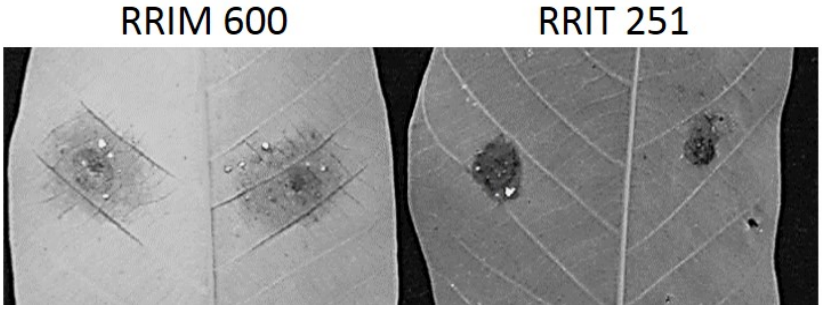

Figure 1 Necrotic lesions in *Hevea* clones RRIM 600 and RRIT 251 caused by *Phytophthora palmivora* infection 72 h post inoculations on leaves.

Infection by *Phytophthora* induced expression of β-1,3-glucanase gene

Total RNA was isolated from the uninfected Para rubber leaves (control samples) and tissues around necrotic zones of the infected leaves at 72 hpi. The negative control samples had been wounded and "infected" with distilled water. The RNA samples were amplified by RT-PCR for the β-glu gene. The β-glu genes and the housekeeping β-actin gene were co-amplified using gene specific primers. The β-actin gene was used as an internal control to verify the RNA load in each well, and its PCR product band did not vary with the treatments (**Figure 2**, lower panel). Gene expression of β-glu and β-actin in the inoculated leaves and the un-inoculated leaves (control) is shown in **Figure 2**. The expression of β-glu differed between un-inoculated and inoculated plants ,with no detectable constitutive gene expression in the control, while in *P. palmivora* infected tissues, the specific PCR product band was detected (**Figure 2**, above panel). The expression levels did not differ between the clones. Moreover, there was no difference between wounded and unwounded leaves without *P. palmivora* infection (data not shown).

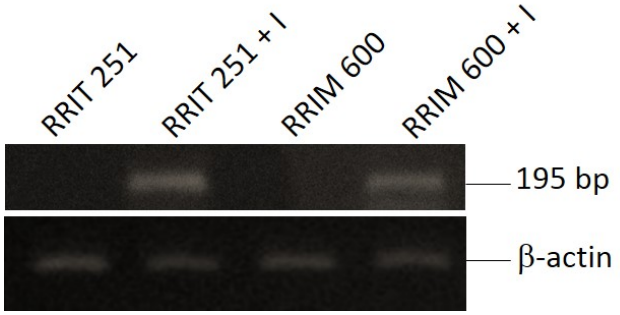

Figure 2 Expression of β-1,3-glucanase gene in *Hevea* clones RRIT 251 and RRIM600. The specific band (195 bp) was observed only with *Phytophthora* inoculation (RRIT 251+I and RRIM 600+I) at 3 days post inoculation, while not present without *Phytophthora* inoculation (RRIT 251 and RRIM 600). β-actin was used as internal control.

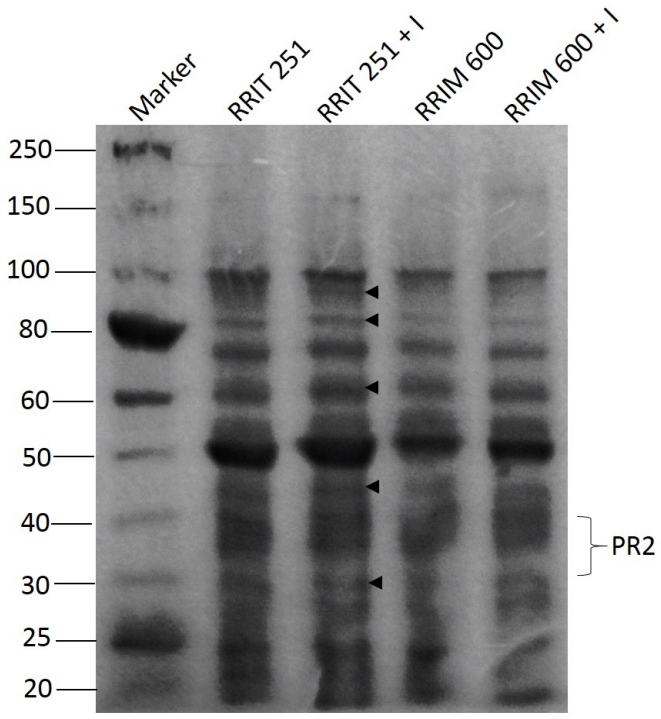

Figure 3 Accumulation of β-1,3-glucanase (PR2) in *Hevea* clones RRIT 251 and RRIM 600. The cases after inoculation with *Phytophthora* (RRIT 251+I and RRIM 600+I) are compared to RRIT 251 and RRIM 600, without inoculation that served as a control.

Accumulation of β-1,3-glucanase

In order to evaluate the protein products, total proteins were extracted from both healthy leaves and tissues around necrotic paths from both Para rubber clones. The protein samples were then subjected to denatured SDS-PAGE to determine the amount of β-glu gene expression products with calibrating protein ladder markers (10 - 250 kDa). SDS-PAGE analysis of crude proteins had the same banding patterns for the clones RRIT 251 and RRIM 600, with and without inoculation (**Figure 3**), for at least up to 72 h after inoculation. Variety specific proteins of molecular masses of approximately 30, 45, 60, 80 and 90 kDa were found, with higher expression in RRIT 251 than in RRIM 600 (**Figure 3**, arrows). The estimated molecular weight of β-1,3-glucanase was in a range of 30 - 40 kDa. Up to 72 hpi, this enzyme accumulated slightly more in *P. palmivora* inoculated leaves (RRIT 251+I and RRIM 600+I) than in non-inoculated leaves (**Figure 3**).

Discussion

It has been well established that plants, when challenged by phytopathogens like fungi, bacteria, or viruses, often respond with the synthesis of a specific group of proteins called PR proteins. Among these, β-1,3-glucanase (PR2) plays an important role in the defense against several pathogens [6-9]. In this study, we investigated the expression of β-glu in response to *P. palmivora* infection, and expansion of necrotic lesions on Para rubber leaves in clones RRIT 251 and RRIM 600. With the inoculation method used, the RRIT 251 clone had better resistance to infection than the RRIM 600 clone. RT-PCR analysis revealed that both clones expressed the β-glu gene after *P. palmivora* inoculation. Denatured SDS-PAGE

demonstrated that β-1,3-glucanase protein accumulated in both clones, with or without *P. palmivora* inoculation.

The β-glu gene is usually expressed at low levels in plant, but when plants are infected by phytopathogenic fungi, β-1,3-glucanase enzyme concentration increases dramatically. In some plants, the mRNA of tomato acidic β-1,3-glucanase is overexpressed in leaves infected with *Cladosporium fulvum* [22]. In the current study, the specific 195 bp band indicated β-glu gene overexpression from infection by *P. palmivora* in both the RRIT 251 and RRIM 600 clones of *H. brasiliensis,* relative to normal expression in un-inoculated plants. However, the induction of β-1,3-glucanase by pathogens can vary between clones of a plant species. For instance, when the production of β-1,3-glucanase upon *Corynespora cassiicola* infection was compared in different clones of *H. brasiliensis*, significantly different enzyme activity was observed between clones during the infection period [23]. In the current study, the separation of proteins by SDS-PAGE to basic proteins revealed no obvious differences between the clones, or between healthy and *P. palmivora* infected cases. However, proteins of molecular masses 30, 40, 60, 80, and 90 kDa were found more abundantly in RRIT 251 than in RRIM 600, although the protein functions are not known. The clones may differ in gene expression after pathogen infection, and may also differ in protein expression. The enzyme activity of β-1,3-glucanase and the expression of other proteins were not determined in this study.

We have shown that infection by *P. palmivora* leads to overexpression of the β-glu gene in Para rubber clones RRIT 251 and RRIM 600. The spreading of necrotic lesions induced by *P. palmivora* infection differed between the clones. Potential causes for the difference in resistance to infection include: (i) the different physiological properties of the clones, and (ii) the presence of other PR proteins, which were not measured and might have contributed to the resistance of RRIT 251 clone. The resistance against *Phytophthora* may not depend on a single gene's expression. In several studies, transgenic plants overexpressed genes in the PR1, PR2, PR3 and PR5 families that mediated host plant resistance to phytopathogenic fungi. The co-expression of multiple proteins in transgenic plants is more effective than the expression of a single gene for antifungal activity [24]. The β-1,3-glucanase gene, or chitinase gene, has been transferred to a number of plant species in studies of resistance against various pathogens. These transgenic plants have enhanced resistance against fungal diseases, or delayed symptom development, compared with control plants [25-27]. For instance, tobacco class I β-1,3-glucanase and chitinase genes transferred to tomatoes were expressed, and increased the tolerance of infection by *Fusarium oxysporum* f.sp. *lycopersici* [27].

The expression of β-glu increases after infection with various pathogens in several host plants, including barley [28], maize [29], pepper and soybean [30], wheat [31], chickpea [32] and peach [33]. This protein expression is not only induced by pathogen infection, but also by other factors like salicylic acid, which induces the accumulation of β-glu mRNA in tobacco [34,35], similar to abscisic acid in tobacco [36-38].

Conclusions

The role of β-glu in defense mechanisms against leaf fall disease in *Hevea* is supported by the overexpression of β-glu induced by *P. palmivora* infection in 2 *Hevea* clones. The RRIT 251 clones had better resistance to infection than the RRIM 600 clones, which may be associated with the rapid induction and prolonged expression of β-glu genes or other genes. In addition to molecular response to pathogen infection, other factors, including physiological barriers, may differ between these clones, affecting resistance. The overexpression of β-glu in this study was observed in 195 bp products. This study did observe, but did not clarify, a difference in host-plant resistance between the clones. Such clarification might be obtainable by cloning of full genes, more extensive expression observations of β-glu (PR2) and other genes such as chitinase (PR3), and measurements of enzyme activities.

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