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Proteomic analysis of rubber trees uncovers a systemic response to white root rot disease

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Abstract

White root rot disease caused by *Rigidoporus microporus* (Sw.) Overeem is a disastrous root disease found in rubber trees (*Hevea brasiliensis*). It significantly reduces natural rubber production and triggers plant death. In the early stages of infection, the aboveground parts of the diseased plant are still healthy. However, by the time that disease symptoms are apparent, it is too late for the plant to recover. Thus, this study aims to understand the systemic response of rubber trees during root infection by using 2D-PAGE coupled with LC-MS/MS. The root system of rubber tree clone RRIM600 was inoculated with *R. microporus* for 50 days and the stems were then collected for analysis. The results indicate that fungal infection of underground rubber tree parts can trigger changes in the proteome profile of asymptomatic aboveground parts. Fifteen protein spots were found to be differentially expressed between pathogen-inoculated and mock-inoculated plants. Nine spots were significantly changed after infection ($p < 0.05$). Small heat shock proteins were the major group of stress-related proteins that were significantly down-regulated after infection. Moreover, the hydrogen cyanide releasing enzymes, antioxidant enzymes and photosynthesis associated proteins were down-regulated in the stems of infected trees. The down-regulation of several proteins that are involved in the stress defense response contributed to white root rot disease susceptibility of the RRIM600 clone. This research contributes to a better understanding of the mechanisms behind rubber tree systemic responses to white root rot disease, and the candidate proteins that may be useful in rubber trees breeding programs.

Keywords: *Hevea brasiliensis*; Proteome; *Rigidoporus microporus*; Small heat shock proteins; white root rot.
Abbreviations: 2D-PAGE_two-dimensional polyacrylamide gel electrophoresis; emPAI_exponentially modified protein abundance index; LC-MS/MS_ liquid chromatography-tandem mass spectrometry; pI_isoelectric point.

Introduction

Among the more than 2,500 plant species that produce rubber latex, the rubber tree, or *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg., is the only major commercial source for natural rubber production. Natural rubber (*cis*-1,4-polyisoprene) possesses special properties, including high elasticity, efficient heat dispersion and abrasion resistance, that cannot be replaced by synthetic rubber (Hayashi, 2009). Recent data from 2021 revealed that Thailand is currently the largest natural rubber producer, followed by Indonesia, Côte d'Ivoire, Vietnam and Malaysia (www.trademap.org). However, a major problem in rubber plantations is infection by pathogens, leading to the reduction of wood and natural rubber productivity. A wide range of pathogens can attack the leaves and stems, as well as the roots of rubber trees, and cause several diseases such as powdery mildew, black stripe and white root rot disease (Mazlan et al., 2019; Wastie, 1975).

White root rot disease, caused by the soil-borne fungus *Rigidoporus microporus* (Sw.) Overeem is the most destructive root disease in Asian and African rubber tree plantations (Mohammed et al., 2014). Besides rubber trees, this disease can infect other tropical and subtropical plants such as cassava, cacao, avocado, obeche, teak, tea, coffee, cinnamon, and pineapple. White root rot disease was first recorded in Malaysia by H.N. Ridley in 1904, and it then dispersed throughout equatorial forests, especially in high rainfall areas. In Thailand, the southern region is the most suitable area for growing rubber trees due to the fertile soil and high rates of precipitation. However, these growth conditions are also favorable to white root rot pathogen invasion. Moreover, the most popular rubber tree clone in

the region, RRIM600, is susceptible to white root rot disease (Wattanasilakorn et al., 2012). Thus, this disease is a major cause of wood and latex yield loss in rubber trees in Thailand.

Rigidoporus microporus hyphae can grow several meters in soil without wood debris until encountering and attaching to suitable hosts. They colonize and invade roots by secreting lignin-degrading enzymes, including laccase and manganese peroxidase (Galliano et al., 1991; Nandris et al., 1987). In the early stages of infection, disease symptoms cannot be noticed from the aboveground parts of the rubber tree. By the time white rhizomorphs are present at the tree base, the root system is already destroyed and the plant faces water and nutrient deficiency. Subsequent symptoms include small canopy size, yellow leaves, and reduced latex yield. Infection progresses until the death of the plant, with reddish-brown fruiting bodies of *R. microporus* appearing on the decaying tree (Omorusi, 2012).

There has been no effective strategy or marker for early detection of white root rot disease until recently. Molecular techniques are the most extensively used tools for understanding plant-pathogen interactions and observing plant defense responses. A previous study on the interaction between rubber trees and *R. microporus* found that several genes are involved in cell wall modification, signal transduction, and pathogenesis-related (PR) proteins were differentially expressed in the necrotic tissue of different rubber clones (Oghenekaro, 2016). In addition, *R. microporus* inoculation on stems altered PR protein expression in the leaves of both susceptible and tolerant rubber tree clones (Woraathasin et al., 2017). This evidence suggests that *R. microporus* infection could induce systemic acquired resistance (SAR) in rubber trees. Therefore, roots infected with this pathogen might induce systemic responses in aboveground parts of rubber trees.

Proteomics is becoming a powerful tool enabling the study of defense mechanisms during plant-pathogen interactions. Among the various approaches, 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) used in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) can detect quantitative global protein changes that are involved in plant response (Fang et al., 2015). 2D-PAGE has been used in the analysis of several plant species and their interactions with pathogenic fungi, including apples with *Collectotrichum gloeosporioides* (Rockenbach et al., 2015), grapevine with *Botrytis cinerea* (Dadakova et al., 2015), alfalfa with *Fusarium proliferatum* (Cong et al., 2017), wheat with *Blumeria graminis* f. sp. *tritici* (Bgt) (Li et al., 2017), and cabbage with *Plasmodiophora brassicae* (Moon et al., 2020). These previous studies reveal the potency of this proteome technique.

In this study, 2D-PAGE coupled with LC-MS/MS was applied to observe the systemic response induced in rubber tree (RRIM600) stems after *R. microporus* root infection. This proteome analysis provides data for understanding the molecular mechanism of rubber trees during a systemic response to white root rot disease.

Results and Discussion

Proteome profile of rubber tree stems following R. microporus infection

In order to understand plant systemic responses during white root rot disease infection, the protein profiles of rubber tree stems collected 50 day post-infection with *R. microporus* strain NK6 were compared with those of 50-day mock-inoculated samples. Protein samples from 4 replications of each treatment were pooled and analyzed by 2D-PAGE (Fig 1). Protein spots of interest (i.e., that differed in intensity by at least 1.2-fold) were excised from gels and identified by LC-MS/MS. In this study, 167 spots were matched between *R. microporus*-inoculated and mock-inoculated trees. Fifteen spots were classified as differentially expressed proteins (DEPs) following pathogen invasion (Table 1). Among these, fourteen protein spots were found to be down-regulated, and eight spots showed a significant reduction ($p < 0.05$), while just one spot, corresponding to oxygen-evolving enhancer protein 1 (spot 14), was found to be 1.7-fold significantly up-regulated (Fig 2). Interestingly, five spots identified as small heat shock proteins (sHSPs) were substantially down-regulated in *R. microporus* inoculated trees (Table 1, Fig 2). Differential expression of sHSPs was found in several plants after infected by fungal pathogens (Acosta-Muñiz et al., 2012; Wang et al., 2006). Moreover, proteins associated with stress response, carbohydrate metabolic pathway and photosynthesis were also down-regulated in the fungal-inoculated sample (Table 1). Similarly, some proteins involved in repair, defense, and primary metabolism were down-regulated when rubber tree was infected by aggressive *R. microporus*, indicating plant weakness in defense (Siddiqui et al., 2017). The transcription of the defense related gene, phenylalanine ammonia lyase (*HbPAL*) in young leaves was sharply increased in white root rot disease tolerant clone but relatively unresponsive in the susceptible clones after infection (Sangsil et al., 2016). Comparison of gene and protein expression profiles between *R. microporus*-inoculated and mock-inoculated rubber trees provides information on how the trees respond to this fungal pathogen, suggesting the criterion for disease tolerance.

Small heat shock proteins

Heat shock proteins (HSPs) were first discovered in fruit flies (*Drosophila melanogaster*) exposed to heat by Ritossa (1962). After that, these proteins were studied in many organisms including bacteria, animals and plants. HSPs function as molecular chaperones that are involved in protein folding. They are grouped into 5 classes based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and small HSPs with monomeric masses of 12-42 kDa. Plants express these proteins in cytosol, endoplasmic reticulum, mitochondria, and chloroplast (Al-Whaibi, 2011; Banerjee and Roychoudhury, 2018; Park and Seo, 2015).

Small heat shock proteins (sHSPs) are abundant in plant cells. These proteins are divided into 11 subclasses depending on their amino acid sequence similarity and subcellular localization. There are at least seven subclasses localized in cytosol (CI, CII, CIII, CIV, CV, CVI, and CVII), two subclasses are targeted to mitochondria (MI and MII) and at least one subclass each in the endoplasmic reticulum, plastids, and peroxisomes. Although amino acid sequences of sHSPs show high variation, all of them exhibit the same conserved C-terminal α-crystallin domain and compact β-sheet sandwich structure (Ma et al., 2006; Scharf et al., 2001; Waters et al., 2008). These proteins are expressed under both normal and stress conditions by acting as co-chaperones without energy utilization in order to avoid irreversible protein aggregation and insolubilization. While sHSPs cannot directly refold proteins, they can bind with denatured, unfolded and unstable proteins, which then allow high molecular weight HSPs to refold the proteins into their native forms (Mogk et al., 2003; Montfort et al., 2002). Several studies have confirmed that sHSPs are involved in

plant heat tolerance (Chen et al., 2014; Feng et al., 2019; Kim et al., 2012).

Interestingly, five protein spots (spots 3-7, Fig. 1, Table 1) that were identified as sHSPs were significantly down-regulated ($p < 0.05$) in rubber tree stems after *R. microporus* infection (Fig 2). Among them, spots 4 and 5 were predicted to be sHSP17.3 from rubber trees with different accession numbers (XP_021675560.1 and XP_02168446.1, respectively). These two spots showed identical pI values but slightly different molecular masses (Fig 1). This may be due to differences in the number of amino acids (Fig 3) or post-translational processing. Spots 6 and 7 were predicted to be sHSP17.9 of accession numbers XP_021655193.1 and XP_021671162.1, respectively, as they exhibited almost identical amino acid sequences (Fig 4) and theoretical MW/pI values. Thus, the variation in pI values observed in this experiment might be the result of protein modification by phosphorylation (Haslbeck and Vierling, 2015).

Similar to our results, proteomic analysis of avocado (*Persea americana*) roots infected with a soil-borne pathogen causing root rot disease, *Phytophthora cinnamomic*, showed down-regulation of sHSP17.3 (Acosta-Muñiz et al., 2012). Moreover, the proteome profile of non-infected area in Austrian pine (*Pinus nigra*) inoculated with *Sphaeropsis* shoot blight and canker disease pathogens (*S. sapinea* and *Diplodia scrobiculata*) showed both up- and down-regulation of several sHSPs at 26 days after inoculation. This research also supported the role of sHSPs in systemic-induced responses in host plants (Wang et al., 2006). In addition, previous research studies have suggested that the differential expression of some sHSPs in plants affects disease resistance. In *Arabidopsis*, overexpression of the soybean co-chaperone GmHsp22.4 resulted in a considerable drop in the number of root-knot worms (*Meloidogyne javanica*) inside the plant, whereas the knocked-out line showed an increase in nematode populations (Hishinuma-Silva et al., 2020). Similarly, overexpressing and silencing *OsHsp*18.0 in rice enhanced *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resistance and susceptibility, respectively (Kuang et al., 2017).

From the above evidence, it appears that plant sHSPs are involved in protein stability and cell homeostasis, which enhances defense mechanisms during pathogen infection. Consequently, the down-regulation of several sHSPs in *R. microporus*-infected trees could be linked to the susceptibility of the RRIM600 rubber clone to white root rot disease.

Photosynthesis-related proteins

Photosynthesis is affected by numerous factors. Plants might enhance or reduce this process in order to gain more energy for self-protection. Photosynthesis occurs not only in leaves, but also in green stems, which enables sufficient food supply and growth promotion (Bloemen et al., 2016). Ribulose bisphosphate carboxylase oxygenase, or rubisco, is the carbon dioxide (CO_2) fixing enzyme that is essential in the Calvin cycle. Another important group of proteins in photosynthesis are the oxygen-evolving enhancer proteins (OEEs), which are involved in oxygen evolving activity and water splitting in the photosystem II (PSII) complex (Murakami et al., 2002; Yi et al., 2006). OEEs consist of three subunits, OEE1 (33 kDa), OEE2 (23 kDa) and OEE3 (16 kDa). OEE1 was indicated to be the most important protein for oxygen evolution and PSII stability in the mangrove (*Bruguiera gymnorrhiza*) (Sugihara et al., 2000).

In this study, two proteins, oxygen-evolving enhancer protein 1 (OEE1), chloroplastic (XP_021671274.1) and rubisco large chain (A0A1W6FAV4), were found to be significantly down-regulated ($p < 0.05$) in *R. microporus*-infected trees by 2.8- and 1.2-fold, respectively (spots 13 and 15, Fig 1, Fig 2, Table 1). Root diseases can interrupt water and nutrient absorption and transportation to the shoot, which can cause photosynthesis reduction as well as growth limitation and yield loss. In accordance with our study, the photosynthetic capacity of wheat and *Eucalyptus nitens* was reduced when infected with the root rot pathogens *Pythium irregulare* and *Armillaria luteobubalina*, respectively (Agustini et al., 2015; Aldahadha et al., 2012). Moreover, pine trees (*P. nigra*) inoculated with two fungal pathogens (*S. sapinea* and *D. scrobiculata*) exhibited reductions in rubisco, OEE1 and OEE2 through a systemic response, which indicated photosynthetic suppression (Wang et al., 2006). In addition, plant pathogens that attacked leaf parts also interfered with the photosynthesis process. Proteomic analysis of wheat exhibited down-regulation of rubisco caused by powdery mildew (*B. graminis* f. sp. *tritici*), which also corresponds to the down-regulation of this enzyme in rice-*Rhizoctonia solani* interactions (Li et al., 2017; Prathi et al., 2018). Strawberry leaf proteome analysis revealed the up-regulation of rubisco large chain at 24 hours post inoculation, but this protein was then obviously down-regulated at 48 and 72 hours after inoculation by *Colletotrichum fragariae*, an anthracnose pathogen (Fang et al., 2012).

Interestingly, the differential expression of both OEE1 and OEE2 was found in fusarium head blight resistant and susceptible lines of wheat. Both OEE1 and OEE2 were significantly upregulated only in the resistant line, suggesting their important role in maintaining PSII activity when infected with *Fusarium graminearum* (Zhang et al., 2013). Soybean rubisco and OEEs were involved in nonhost resistance (NHR) in soybean against *Bipolaris maydis*. In particular, OEE1 was upregulated 11-fold in stem and 2.5-fold in leaf, which suggested that soybean photosynthesis was reprogrammed under *B. maydis* stress conditions (Dong et al., 2015). Given the findings of previous studies, it is not unexpected that photosynthesis-related proteins are down-regulated in the *R. microporus*-infected RRIM 600 rubber trees, which is one of the susceptible clones.

Cyanogenesis-related proteins

Rubber trees accumulate cyanogenic glucosides (CGs) which can break down to release toxic hydrogen cyanide (HCN). In rubber cyanogenesis, CGs are synthesized from amino acid precursors including valine and isoleucine, which are then are stored in vacuoles separate from HCN-releasing enzymes to prevent toxic effects. CGs play an important role in plant defense responses against herbivores and microbes, as well as also being carbon and nitrogen sources (Kongsawadworakul et al., 2009; Osbourn, 1996). During catabolism, CGs are cleaved to release a sugar moiety and α-hydroxynitrile by β-glucosidase. The α-hydroxynitrile is then converted to a ketone/aldehyde and HCN, either spontaneously or by the action of α-hydroxynitrile lyase (Du Fall and Solomon, 2011). HCN can function in plant defense mechanisms or be transformed into non-toxic nitrogen sources.

β-glucosidase and hydroxynitrile lyase activities have been shown to rapidly increase following mechanical injury of rubber tree leaves (Kadow et al., 2012). High amounts of HCN are toxic not only to plant enemies, but to the plants themselves, and can inhibit CO_2 fixation during photosynthesis.

Table 1. List of differentially expressed proteins in rubber tree stems after *R. microporus* NK6 inoculation

Spot no.	UniProt Accession no.	Protein name	Score	Sequence coverage (%)	emPAI	Theoretical MW/pI (kDa)	Experimental MW/pI (kDa)	Intensity change (Fold)
Antioxidant								
1	A0A6A6LDB2	Superoxide dismutase [Cu-Zn]	1052	40	5.06	15.4/5.6	16.0/6.0	-1.3
2	Q8GZP1	L-ascorbate peroxidase	16960	70	20.89	27.5/5.8	28.0/6.0	-1.3
Stress response								
3	A0A6A6MV90	SHSP domain-containing protein/ homologous to 22.0 kDa class IV heat shock protein-like (XP_021636451.1)	865	47	2.06	22.3/6.6	25.0/6.5	-2.1
4	A0A6A6N0X9	SHSP domain-containing protein/ homologous to 17.3 kDa class I heat shock protein-like (XP_021675560.1)	9462	58	57.85	21.5/6.4	20.0/6.2	-2.3
5	A0A6A6MHQ2	SHSP domain-containing protein/ homologous to 17.3 kDa class I heat shock protein-like (XP_021682446.1)	2048	57	15.64	16.5/5.5	19.0/6.2	-1.9
6	A0A6A6LTE0	SHSP domain-containing protein/ homologous to 17.9 kDa class II heat shock protein-like (XP_021655193.1)	1117	56	9.54	15.6/5.6	17.0/5.8	-2.3
7	A0A6A6LTF4	SHSP domain-containing protein/ homologous to 17.9 kDa class II heat shock protein-like (XP_021671162.1)	1261	57	19.59	15.2/5.4	17.0/5.5	-2.1
Isoflavonoid biosynthesis								
8	A0A6A6NHG1	NmrA domain-containing protein/ homologous to isoflavone reductase-like protein (XP_021644235.1)	13949	49	14.87	33.2/5.5	34.0/5.7	-1.3
Calcium ion binding								
9	A0A6A6LY76	Uncharacterized protein/ homologous to calmodulin-7 (XP_009140 755.1)	557	47	4.23	16.8/4.1	16.0/4.1	-1.4
Cyanogenesis								
10	P52704	(S)-hydroxynitrile lyase	4034	50	10.66	29.5/5.2	30.0/5.4	-1.5
11	A0A6A6LCZ1	AB hydrolase-1 domain-containing protein/ homologous to (S)-hydroxynitrile lyase (XP_021647581.1)	3669	50	8.42	29.5/5.2	30.0/5.5	-2.6
12	Q84L69	P66 protein/ homologous to beta glucosidase (ABL01537.1)	5697	32	5.52	61.5/6.1	66.0/5.8	-1.2
Photosynthesis								
13	A0A6A6L164	Uncharacterized protein/ homologous to oxygen-evolving enhancer protein 1, chloroplastic (XP_021671274.1)	10370	63	40.93	35.6/6.7	33.0/5.2	-2.8
14	A0A6A6L164	Uncharacterized protein/ homologous to oxygen-evolving enhancer protein 1, chloroplastic (XP_021671274.1)	12053	61	25.88	35.6/6.7	33.0/5.3	+1.7
15	A0A1W6FAV4	Ribulose bisphosphate carboxylase large chain	13781	56	46.44	53.8/6.1	54.0/6.3	-1.2

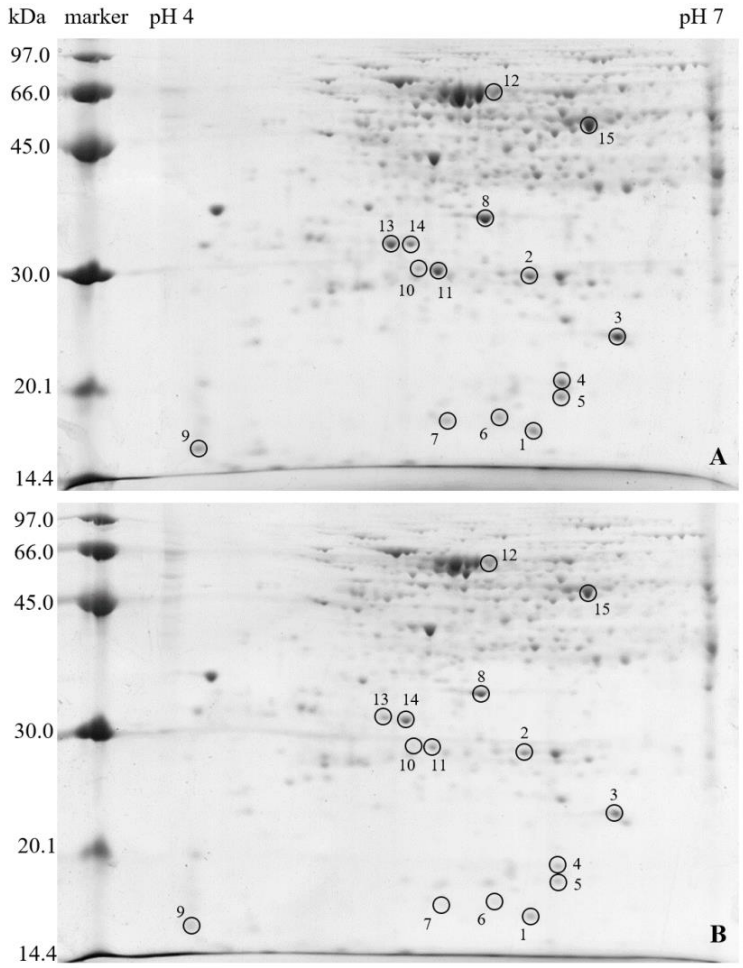

Fig 1. Representative 2D-PAGE images of proteins from rubber tree stems at 50 dpi. **A,** mock-inoculation; **B,** *R. microporus* NK6-inoculation.

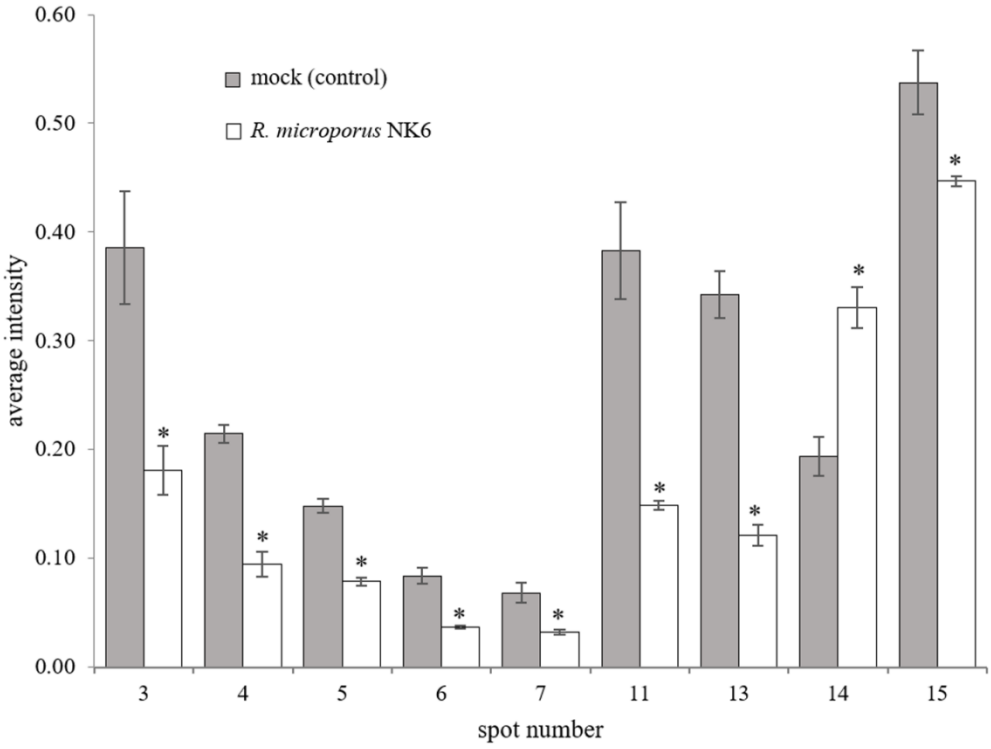

Fig 2. Proteins differentially expressed in rubber tree stems after mock- and *R. microporus* NK6-inoculation. Relative protein abundance was quantified from the average intensity of 2D-PAGE spots based on three replications. * $P < 0.05$.

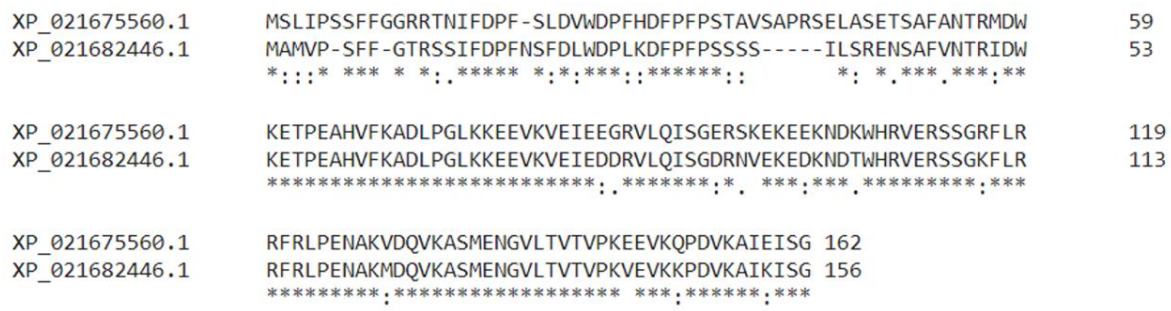

Fig 3. Amino acid sequence alignment of homologous sHSP17.3 in rubber trees. Spots 4 and 5 were homologous to sHSP17.3 of accession no. XP_021675560.1 and XP_021682446.1, respectively. Asterisks (*) indicate perfect alignment, colons (:) indicate strong similarity, dots (.) indicate weak similarity and gaps indicate a poor match.

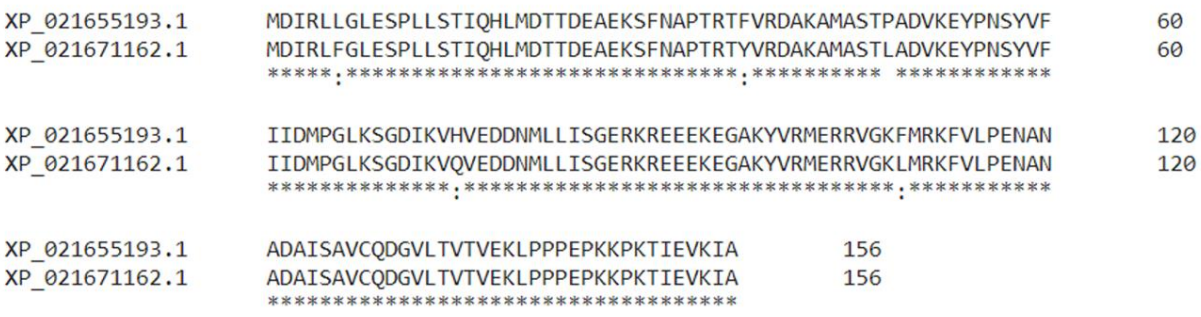

Fig 4. Amino acid sequence alignment of homologous sHSP17.9 in rubber trees. Spots 6 and 7 were homologous to sHSP17.9 of accession no. XP_021655193.1 and XP_021671162.1, respectively. Asterisks (*) indicate perfect alignment, colons (:) indicate strong similarity, dots (.) indicate weak similarity and gaps indicate a poor match.

resulted in no carbon skeletons and defensive metabolites (Lieberei et al., 1996). Moreover, HCN does not appear to be effective against all pathogens. For instance, highly cyanogenic rubber clones were susceptible to the South American Leaf Blight (SALB) disease caused by the cyanide-tolerated fungus *Microcyclus ulei* (Lieberei et al., 1989). Moreover, HCN does not appear to be effective against all pathogens. For instance, highly cyanogenic rubber clones were susceptible to the South American Leaf Blight (SALB) disease caused by the cyanide-tolerated fungus *Microcyclus ulei*. Similarly, in lima beans (*Phaseolus lunatus*), high cyanogenic accessions were resistant to herbivores but susceptible to *C. gloeosporioides* (Ballhorn et al., 2010).

In this study, spots 10 and 11 were identified as the (S)-hydroxynitrile lyase of accession numbers P52704 and XP_021647581.1, respectively. Spot 11 was significantly down-regulated (2.6-fold) in infected trees (Table 1, Fig 1, Fig 2). Moreover, spot 12, which is homologous to β-glucosidase (ABL01537.1), was down-regulated by 1.2-fold (Fig 1, Table 1). The down-regulation of cyanogenesis-involving enzymes after *R. microporus* infection may lead to the reduction of CG breakdown and HCN liberation. The relationship between cyanogenesis and defense mechanisms against fungal pathogens in rubber trees should be further investigated.

Proteins related to reactive oxygen species scavengers

Production of reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) play an important role during the interaction between plant hosts and pathogens. The roles of ROS are regulated by maintaining their threshold levels in the presence of several enzymatic and nonenzymatic antioxidants found in plant tissues (Kaur et al., 2016). This delicate balance of ROS generation and scavenging is an effective strategy for combating the effects of pathogen attack in plants (Wang et al., 2019).

In this study, two differentially expressed proteins were identified as ROS scavenging enzymes. Cu-Zn superoxide dismutase (spot 1) and L-ascorbate peroxidase (spot 2) were down-regulated (Fig 1, Table 1) after *R. microporus* NK6 infection. Superoxide dismutases (SODs) catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Essentially, in plants, there are three groups of SODs based on the metals in their active sites: copper and zinc (Cu,Zn-SODs), manganese (Mn-SODs) and iron (Fe-SODs). In this study, Cu,Zn-SOD (A0A6A6LDB2) was 1.3-fold down-regulated (spot 1, Fig 1, Table 1) in the stems of rubber trees 50 days post-infection with *R. microporus*. In accordance with our result, reductions of SOD content in plants under pathogen infection were reported in patchouli and barley after being infected with *Ralstonia solanacearum* and *Fusarium culmorum*, respectively (Harrach et al., 2013; Xie et al., 2017). Furthermore, the destruction of root systems by wood-decaying fungi can increase water deficit in plants. As shown in peas and wheat, SOD activity in leaves was significantly reduced during drought stress (Alexieva et al., 2001). In addition, L-ascorbate peroxidase (L-APX) with accession number Q8GZP1 was found to be 1.3-fold down-regulated in *R. microporus*-infected rubber trees (spot 2, Fig 1, Table 1). Activity of this enzyme, as well as protein expression, were found to be decreased in other plant-

pathogen interactions, such as interactions between tomatoes-*B. cinerea* and wheat-*B. graminis* f. sp. *tritici* (Kuzniak and Sklodowska, 2005; Li et al., 2017). Furthermore, the proteome profile of grapevine leaves (*Vitis vinifera*) under long-term drought stress revealed the down-regulation of L-APX (Krol and Weidner, 2017). A decrease in APX gene expression or enzyme activity might imply that plants cannot overcome the oxidative burst, and thus fail to defend themselves against stresses. Hence, the down-regulation of Cu,Zn-SOD and L-APX in our study might be related to the effect of root pathogen-infection, as such infection might cause drought stress in rubber trees 50 days post-infection.

Isoflavonoid biosynthesis-related proteins

Isoflavone reductase (IFR) is an important enzyme in the isoflavonoid phytoalexin biosynthesis pathway. Induction of the isoflavone biosynthesis genes, including IFRs, is associated with resistance to plant diseases such as stem and root rot in soybean and bacterial blight in common bean (Cheng et al., 2015; Cox et al., 2021; Bi et al., 2022). In particular, in soybean, it was indicated that induction of the isoflavone pathway is associated with common bacterial blight-resistance (Cox et al., 2021). In this study, one protein spot (spot 8, Fig 1, Table 1) was similar to the isoflavone reductase-like (IRL) in accession number XP_021644235.1. It was 1.3-fold down-regulated in *R. microporus*-infected trees. Normally, plant IFR or IRL are promoted during biotic and abiotic stress in order to eliminate pathogens and to protect the plant from injury (Kim et al., 2003; Potenza et al., 2001). IRL also enhances the oxidative stress tolerance that usually occurs during pathogen attack. Overexpression of the *IRL* gene (*OsIRL*) in rice resulted in less damage caused by ROS in chloroplast (Kim et al., 2010). Likewise, soybeans in which *GmIFR* was overexpressed exhibited significantly lower levels of ROS than the non-transgenic line after being infected with *Phytophthora sojae*. Moreover, this transgenic soybean exhibited increased production of glyceollins and isoflavonoid phytoalexin, which might help in ROS-scavenging and pathogen elimination (Cheng et al., 2015).

In contrast, this study reveals that the systemic response of a susceptible rubber tree clone, RRIM600, after white root rot pathogen infection showed down-regulation of the IRL protein in stems, leading to defense failure. Similarly, a study examining a susceptible line of alfalfa that was infected with leaf blight disease (*Mycosphaerella pinodes*) revealed that *IFR* transcript and medicarpin levels were suppressed by a fungal suppressor (Toyoda et al., 2013). Further studies should investigate the *R. microporus* effector to better comprehend disease pathogenicity.

Calcium binding-related proteins

Calmodulin (CaM) is an important Ca^{2+} sensor that converts calcium signals into cellular responses by interacting with a wide range of proteins (Ranty et al., 2006). CaM proteins contain four EF-hands motifs that are able to selectively bind a single Ca^{2+} ion that decodes Ca^{2+} signals into downstream effectors, modulating a range of cellular processes including gene regulation and stress responses (Ghorbel et al., 2021). Many studies have demonstrated that CaM plays a role in plant defense against pathogens (Yu et al., 2018; Lu et al., 2019; Wöhner and Emeriewen, 2019).

In this study, a 1.4-fold down-regulated protein in infected rubber trees was found to be homologous to calmodulin-7 (spot 9, Fig 1, Table 1). Correspondingly, *CaM13*-silenced tobacco was susceptible to bacterial (*R. solanacearum*) and fungal (*R. solani* and *Pythium aphanidermatum*) pathogens when compared to the wild type (Takabatake et al., 2007). Another study found that soybeans in which *GmCaM4* was overexpressed revealed higher resistance to the fungal pathogens *Alternaria tenuissima* and *Phomopsis longicolla* (Rao et al., 2014). Moreover, disease symptoms caused by *X. campestris* in pepper (*Capsicum annuum*) were decreased in a *CaCaM1*-overexpressed line (Choi et al., 2009). In addition, CaM involved in salicylic acid (SA) production, which is a critical hormone in plant immune systems, have been shown to positively and negatively regulate genes in the SA biosynthesis pathway (Du et al., 2009; Wang et al., 2009). Therefore, the down-regulation of calmodulin-7 in this study might be linked to the susceptibility of rubber tree clone RRIM600 to white root rot disease.

Materials and methods

Pathogen inoculation and sample collection

Rigidoporus microporus NK6, a virulent strain, was collected from the diseased roots of a rubber tree in Southern Thailand. Isolation and pathogenicity tests were conducted following previous work (Kaewchai et al., 2009). The pure fungus was cultured on potato dextrose agar (PDA) medium, allowing fungal mycelia to cover the entire PDA surface. Then, *R. microporus* NK6 mycelia on PDA were cut into 0.5-cm diameter discs and inoculated in Erlenmeyer flasks containing 100 g of sterile sorghum seeds. Each flask was inoculated with 5 pathogen-covered PDA discs and incubated at 30°C for 14 days. Fungus-free PDA discs were also prepared using the same process to create mock culture material.

Grafted RRIM600 rubber tree seedlings (8 months old) were used in this experiment. The fungal culture was inoculated at the bottom of a tree pot. Control plants were mock inoculated with free fungal culture material. The experiment was conducted using four replicates. In order to study the systemic response of rubber trees during root infection, stem samples were collected at 50 days post inoculation (dpi), kept in liquid nitrogen and stored at -80 °C for protein analysis. This plant material preparation was conducted in an isolated area of Department of Earth Science, Faculty of Natural Resources, Prince of Songkla University. The potentially hazardous biological agents were be properly disposed at the end of experimentation.

Protein extraction

Total proteins were extracted using a modified phenol-based method (Hurkman and Tanaka 1986). Three grams of stem samples were ground in liquid nitrogen with mortar and pestle. The ground sample was mixed with 5 ml of extraction buffer (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose) and 5 ml of phenol buffer saturated with Tris-HCl pH 8.8. Then, the mixture was transferred to a 1.5-ml centrifuge tube and centrifuged at 4 °C at 11,000 xg for 30 min. The phenol phase was transferred to a new centrifuge tube and precipitated in 1 ml of precipitation solution (0.1 M ammonium acetate in 100% methanol) at -80 °C for at least 2 h. Then, the sample was centrifuged at 4 °C at 11,000 xg for 30 min and the precipitation solution was discarded. The pellet was washed twice with ice-cold washing solution I (0.1 M ammonium acetate in methanol containing 10 mM DTT) and once with ice-cold washing solution II (80% acetone containing 10 mM

DTT). The protein pellet was allowed to air dry and resuspended in the optimal volume of rehydration buffer (7 M urea, 2 M thiourea, 30 mM DTT, 4% CHAPS). The protein concentration was quantified with the Bio-Rad protein assay (Bradford, 1976) using a UV-VIS spectrophotometer at 595 nm of light absorbance. Rehydrated protein samples were kept at -80 °C for further analysis.

***Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE*)**

Proteins from the 4 replicates were pooled by mixing equal amounts (100 µg) of the protein samples and then decontaminated with the 2-D Clean-Up Kit (GE Healthcare, USA). For the first dimension, 7-cm linear IPG strips of pH 4-7 (GE Healthcare, USA) were incubated overnight with 125 µl of sample solution [60 µg of proteins, (0.5% (v/v) IPG buffer, 40 mM DTT, 1.2% DeStreak, and rehydration buffer] at room temperature. Isoelectric focusing was performed with the Ettan IPGphor 3 IEF system (GE Healthcare, USA). The focused strips were equilibrated once with equilibrium buffer [6 M Urea, 75 mM Tris-HCl, 30% (w/v) glycerol, 2% (w/v) SDS, 0.002% Bromophenol blue] containing 50 mg DTT, and once with equilibrium buffer containing 125 mg Iodoacetamide (IAA). For the second dimension, 12.5% polyacrylamide gels (8 x 9 x 0.1 cm) were used to separate the focused proteins at a constant voltage of 100 V and 20 mA/gel in running buffer [0.025 M Tris-HCl pH 8.8, 0.192 M glycine, and 0.1% (w/v) of SDS]. The gels were then stained with Coomassie Brilliant Blue R-250 and imaged by an image scanner with Labscan software (GE Healthcare, USA).

Protein pattern analysis and LC-MS/MS

The protein patterns from 2D-PAGE were analyzed using Image Master 2D platinum version 6.0 (GE Healthcare, USA). The protein spots were autonomically matched and their volume and intensity were quantified in the software. Differentially expressed protein spots that showed ratios greater than 1.2-fold in intensity were manually selected and sent for protein identification via liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (TripleTOF 6600+, Sciex, USA) at Salaya Central Instrument Facility, Mahidol University, Thailand. Protein spot samples were digested by trypsin and subjected to LC-MS/MS analysis. Amino acid sequences of peptide fragments were matched using the *H. brasiliensis* protein sequence database in UniProt. Proteins containing the same molecular weights and pI values as our 2D-PAGE gel results and with the highest scores and emPAI values were designated as potentially identified proteins. Uncharacterized proteins were searched against homologous proteins from the NCBI database. These proteins were annotated for their biological processes, molecular function, and cellular components based on Gene Ontology information (UniProt).

Statistical analysis

Spot intensities were used to determine the relative concentration of proteins in the experimental samples. The spot intensities from three different gels of the same experimental variables (4 replications) can be combined to give a refined estimate of the intensity for the matched spots by treatment. Statistical analysis was conducted using PASW Statistics 18 software (Mahidol University, Thailand). For differentially-expressed proteins, the average intensity of 2D-PAGE spots from *R. microporus*-inoculated and mock-inoculated rubber tree stems were compared using independent t-tests. For all results we present means and standard errors.

Conclusions

In this work, the stem proteome was investigated in order to understand rubber tree (RRIM600) systemic responses at 50 days after white root rot pathogen (*R. microporus* NK6) inoculation. Although disease symptoms were not evident in aboveground plant parts, 2D-PAGE and LC-MS/MS analysis revealed that many small heat shock proteins were significantly decreased in stems after root infection, as well as photosynthesis-related proteins and cyanogenesis-related enzymes. Antioxidant enzymes, an isoflavonoid biosynthesis-related protein, and a calcium-binding protein were all down-regulated as well. This investigation provides support for the systemic response of aboveground plant parts during root disease invasion, and provides notable information for future research on biological markers for rubber tree breeding programs.

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