# การบ่งชี้ชนิดของโปรตีนที่มีการแสดงออกแตกต่างกันในต้นยางพาราที่ติดเชื้อโรครากขาว

# Identification of Differentially Expressed Proteins in Response to White Root Rot Disease in Rubber Tree

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## บทคัดย่อ

โรครากขาวในยางพาราซึ่งเกิดจากเชื้อราในดินชนิด *Rigidoporus microporus* เป็นปัญหาสำคัญของประเทศผู้ผลิตยางทั่วโลกรวมถึงประเทศไทย ปัจจุบันยังไม่มีวิธีควบคุมโรคที่มีประสิทธิภาพและยังไม่มีวิธีการตรวจสอบโรคในระยะเริ่มแรก การศึกษาผลกระทบของเชื้อราต่อการแสดงออกของโปรตีนในต้นยางช่วยให้สามารถพัฒนาวิธีการตรวจสอบโรคได้ งานวิจัยนี้ทำการวิเคราะห์โปรตีนทั้งระบบเพื่อศึกษาการเปลี่ยนแปลงของโปรตีนในต้นยางที่เป็นโรค โดยวิเคราะห์โปรตีนที่มีการแสดงออกแตกต่างกันในลำต้นหลังจากทำการปลูกเชื้อเป็นเวลา 50 วัน พบว่าโปรตีนกลุ่มใหญ่ที่แสดงออกแตกต่างกันทำหน้าที่เกี่ยวกับการสร้างพลังงาน การสังเคราะห์แสง การเปลี่ยนแปลงของผนังเซลล์ และการตอบสนองต่อความเครียด ข้อมูลที่ได้ทำให้เข้าใจถึงปฏิสัมพันธ์ระหว่างเชื้อรากับต้นยางและอาจใช้เป็นแนวทางสำหรับการพัฒนาวิธีการตรวจสอบโรครากขาวต่อไป

## Abstract

White root rot disease caused by soil born fungus, *Rigidoporous microporus* is one of the important problems in rubber producing countries including Thailand. To date, there is no effective method for disease control and also early disease detection. Understanding of how the pathogen affects the protein expression in rubber tree may assist in the development of an early detection protocol. Here, proteomics analysis was performed to investigate proteome changes in infected rubber tree. The differentially expressed proteins (DEPs) in stem were identified at 50 days post inoculation. A large portion of DEPs are known to function in energy metabolism, photosynthesis, cell wall synthesis/modification and stress responses. The obtained data provide information for understanding the molecular mechanism involved in rubber tree- *R. microporus* interaction and also application clues for white root rot disease diagnosis.

**Keywords**: Proteomics, *Rigidoporus microporus*, rubber tree, white root rot disease

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## Literature Review

Rubber tree (*Hevea brasiliensis*) is a perennial plant that produces natural rubber. Presently, commercial source of natural rubber mainly comes from rubber tree. Thailand is the world's leader in natural rubber production. In 2017, production of natural rubber in Thailand is about 4.84 million tons (Special Affairs Group Bureau of Policy and Planning Office of the Permanent Secretary for Interior, 2017). In general, there are many factors influencing rubber production including biotic and abiotic stresses. According to biotic stresses, fungi are one of the most important pathogens. White root rot is one of the important rubber tree diseases caused by soil borne fungus, *Rigidoporus microporus* (Polyporales, Basidiomycota) syn. *Rigidoporus lignosus* (Tangonan et al., 2008). The disease effects are most likely associated with growth and reduced latex yield. It can result in extensive death of the trees and occasionally losses of a whole stand. White root rot disease was shown as a major problem for farmers in a smallholdings survey in Thailand especially in the Southern part (Kaewchai, 2013). In 2008 - 2010, it was found that 95% of root diseases found in rubber tree plantation in the South of Thailand were identified as white root rot disease (Rojsujit et al., 2011). Early detection of this root disease is difficult because of insidious nature of the white root rot infection. Most of trees bearing the visible symptoms could not be treated or recovered. Early disease detection and prevention in plantation are imperative. To do this efficiently, understanding of molecular mechanisms underlying plant immunity is required. However, until now, there is a little information about the molecular mechanisms of rubber tree after infection by this pathogenic fungus. In recent years, the application of proteomic approaches as a tool for global expression analysis has allowed the high throughput analysis of protein expression. The techniques have been used to explore a number of proteins of plant diseases caused by abiotic and biotic stresses including pathogenic fungi (Havanapan et al., 2016; Trupiano et al., 2012; Zhang et al., 2018).

In this research, bottom-up proteomic techniques was used to study differential protein expression with the objective of looking for white root rot disease related proteins in rubber tree that might be useful for the tree diagnosis. The reprogrammed proteins of disease trees are involved in several biological processes including establishing a defense response. Therefore, this work should help us understand the basic processes during the rubber tree-*R. microporus* interaction and may also contribute to improve resistance breeding toward this pathogen in the future.

## Materials and Methods

### Plant material and pathogen inoculation

White root rot disease causing fungus, *Rigidoporus microporus* strain SK42 was isolated from living roots of rubber trees in the south of Thailand, where the disease symptoms were observed. Isolation and pathogenicity test were carried out as described by Kaewchai et al. (2009). *R.*

*microporus* strain SK42 was grown on sorghum seeds and used in the study. Sorghum seeds (100 g) were well washed with water and autoclaved at 121°C for 20 min. The seeds in each flask were inoculated with 5 discs of 0.5 cm diameter from fungal culture on PDA medium and incubated at 30°C for 14 days. Budded rubber trees (cultivar RRIM 600, which is widely grown in the South of Thailand) at an age of 8 months were used in this experiment. The fungal culture was inoculated next to the root system of the rubber tree. Control plants were mock inoculated with free fungal culture material. The experiment was conducted in triplicates. The stem samples were collected at 50 dpi (days post inoculation) for protein extraction. At 50 dpi, the trees did not show the symptom aboveground but the roots were completely infected as shown in Figure 1.


**Figure 1** Infection and symptom of *Rigidoporus microporus* on rubber tree root at 50 dpi. Control: mock inoculated root, SK42: *R. microporus SK42* infected root.

**GeLC-MS/MS based proteomic analysis**

Total stem protein was extracted by the modified method of Hurkman and Tanaka (1986). Three biological replicates from control and 50 dpi were analyzed. Proteins were firstly separated using one dimension electrophoresis (1DE, 12.5% SDS-PAGE) and then sequentially cut horizontally into 15 pieces. Proteins in each gel piece were in-gel digested with trypsin and analyzed using nano LC–MS/MS. The samples were subjected to an Ultimate 3000 nano-LC system (Dionex, Surrey, UK) coupled with a micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) followed the method described by Danpaiboon et al. (2014). Mascot database search was used to analyze all MS/MS samples and was set up to search against Swiss-Prot database. UniProtKB (https://www.uniprot.org/help/uniprotkb) and Blast2GO (https://www.blast2go.com/) were used for the annotation and functional classification of identified proteins. Exponentially Modified Protein Abundance Index (emPAI), a label free approach, was served for protein quantification.

## Results and Discussion

To obtain the global view of stem proteome changes and to identify differentially expressed proteins of rubber tree cultivar RRIM 600 infected with *R. microporus*, a pathogen of white root rot disease, GeLC-MS/MS based proteomic approach was used. The differentially expressed proteins in stem were identified at 50 dpi (days post inoculation). The protein profile of stem from control and infected trees ranged in masses from >10 to 100 kDa as shown in Figure 2. The SDS-PAGE gels containing proteins were then cut horizontally into 15 slices, in-gel digested with trypsin and analyzed using nano LC–MS/MS. For quantitative analysis, a minimum threshold of 2-fold change in protein abundance was applied to filter the dataset. All the differentially expressed proteins were placed into 12 functional categories based on biological process (Figure 3A-3B). The largest group of up-regulated proteins in infected tree is related to cellular process while the largest group of down-regulated proteins is related to response to stimulus. This differentially expressed stem proteins after root pathogen infection suggests that the defense responses are sent via a series of signaling molecules from root to the aboveground tissues of the tree. This has been also shown in several studies where responses triggered belowground could be transported aboveground and vice versa for the better defense strategies in plants (Bezemer and van Dam, 2005; Jeffery Daim et al., 2015).

The up-regulated proteins were involved in energy metabolism (ATP synthase subunit alpha and beta, Aconitate hydratase), photosynthesis (Chlorophyll a-b binding protein, photosystem II CP47 chlorophyll apoprotein) and cell wall synthesis/modification (sucrose synthase, tubulin alpha chain) (Table 1). ATP synthase subunit alpha was the highest up-regulated protein, which involved in catabolic reaction of ATP to provide energy for plant metabolism. Photosynthesis at branch and stem level have been shown to play a key role in maintaining plant carbon economy and hydraulic function under critical conditions such as drought stress (Bloemen et al., 2016). Particularly, white root rot pathogen can increase drought stress while infecting the roots (Gori et al., 2013). This might explain why Chlorophyll a-b binding protein and photosystem II CP47 chlorophyll apoprotein were up-regulated in stem of infected rubber tree. Cell wall synthesis/modifying proteins help plants adjust to environmental changes by regulating growth and controlling the entry of pathogens. Regulation of cell wall protein activity results in growth modulation during drought for adaptation to water deficit. In addition, products released during wall modification can trigger plant defense signaling (Sasidharan et al., 2011).

Several down-regulated proteins during *R. microporus* infection were related to stress responses (calmodulin, 14-3-3 like protein, L-ascorbate peroxidase, glyceraldehyde-3-phosphate dehydrogenase) (Table 2). Calmodulins function in the regulation of plant development and stress responses by converting calcium signals into transcriptional responses, protein phosphorylation or

metabolic changes. Calmodulins showed differential expression and localization in plants under stress (Perochon et al., 2011). 14-3-3 proteins act as phosphosensors, binding phosphorylated client proteins and modulating their functions. They are currently implicated in plant signal transduction processes, including those controlling metabolism, hormone signaling, cell division, and responses to abiotic and biotic stimuli (Lozano-Durán and Robatzek, 2015).

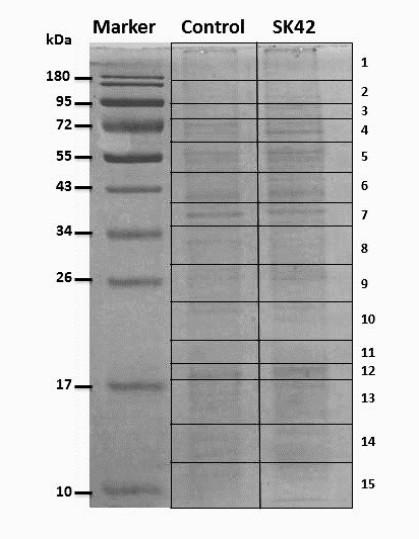


**Figure 2** One-dimensional protein profile of the rubber tree stem. The grids indicate how the gel bands (1-15) were horizontally cut for nano LC–MS/MS analysis. Control: mock inoculated tree, SK42: *R. microporus* SK42 infected tree. Marker: Protein molecular weight maker (kDa).

Decrease of ascorbate peroxidase, a hydrogen peroxide-scavenging enzyme, may lead to an accumulation of hydrogen peroxide in rubber tree (Table 2). Apart from its toxicity, hydrogen peroxide plays a key role in signaling leading to strengthening of other defense-related genes. The differential expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may indicate some stresses in rubber tree. Besides its key role in glycolysis, GAPDH is a multifunction protein especially in plant abiotic stress response including drought or osmotic stresses (Zeng et al., 2016).

## Conclusion

The proteome analysis of rubber tree infected with *R. microporus* showed the differential protein expression in stem. Especially the up-regulation of proteins in energy metabolism, photosynthesis and cell wall synthesis/modification indicates rubber tree effort to defend from the fungal invasion and its related stress. However, weak defense was also demonstrated from the down-regulation of many defense related proteins. The validation of gene expression should be further investigated in the future to confirm about the differential protein expression in response to *R. microporus* infection.

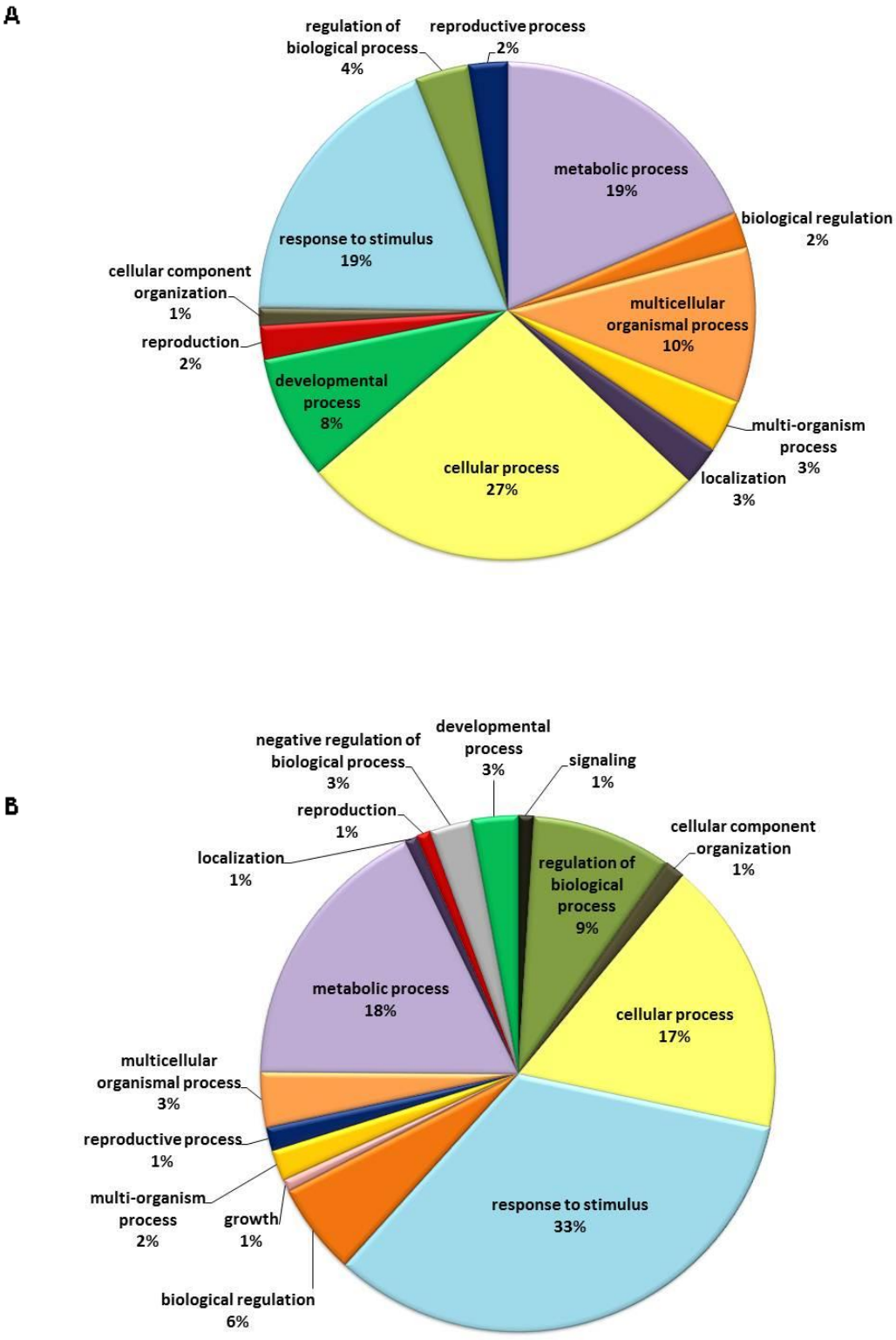


**Figure 3** Classification of proteins up-regulated (A) and down-regulated (B) in infected rubber tree according to their biological process

**Table 1** List of the top 10 up-regulated proteins in infected rubber trees

| Protein Name | Swiss-Prot Accession | Peptide Sequence | Putative biological function |
|---|---|---|---|
| ATP synthase subunit alpha | ATPAM_OENBI | VVDALGVPIDGR | Energy metabolism |
| Sucrose synthase | SUSY_MEDSA | LLPDAVGTTCGQRLEK | Sucrose metabolism / Cell wall synthesis |
| Tubulin alpha chain | TBA_DAUCA | CGINYQAPTVVPGGDLAK | Cell wall modification |
| Chlorophyll a-b binding protein 1 | CB21_MAIZE | AASTMAISSTAMAGTPIKVGSFGEGR | Photosynthesis |
| ADP, ATP carrier protein 1 | ADT1_WHEAT | MTQNLGISVPIMSPSPMFANAPPEK | ADP, ATP transport |
| ATP synthase subunit beta | ATPB_COFAR | TVLIMELINNIAKAHGGVSVFGGVGER | Energy metabolism |
| Histone H2B | H2B10_ARATH | AMGIMNSFINDIFEK | Nucleosome assembly |
| Photosystem II CP47 chlorophyll apoprotein | PSBB_POPDE | LAFYDYIGNNPAK | Photosynthesis |
| Aconitate hydratase | ACOC_SOLTU | TSLAPGSGVVTK | Energy metabolism |
| V-type proton ATPase catalytic subunit A | VATA_CITUN | VSGPVVIADGMNGAAMYELVR | Proton translocation |

**Table 2** List of the top 10 down-regulated proteins in infected rubber trees

| Protein Name | Swiss-Prot Accession | Peptide Sequence | Putative biological function |
|---|---|---|---|
| Histone H4 | H4_ARATH | IFLENVIRDAVTYTEHAR | Nucleosome assembly |
| Calmodulin | CALM_WHEAT | EADVDGDGQINYEEFVK | Stress response |
| Ribulose bisphosphate carboxylase large chain | RBL_DENCL | GGLDFTKDDENVNSQPFMR | Photosynthesis |
| Cytochrome c | CYC_PASSA | TKCAQCHTVELGAGHK | Respiration chain |
| Major latex allergen Hev b 5 | ALL5_HEVBR | NETPEVTKAEETK | - |
| 10 kDa chaperonin | CH10_ARATH | EGDTVLLPEYGGTQVK | Stress response |
| Profilin | PROF_PRUPE | KSTLALLIGIYDEPMTPGQCNMIVER | Actin cytoskeleton organization |
| Glyceraldehyde-3-phosphate dehydrogenase | G3PD_MAIZE | AASFNIIPSSTGAAK | Glycolysis/Stress response |
| 14-3-3-like protein | 14331_ARATH | LAEQAERYEEMVEFMEK | Stress response |
| L-ascorbate peroxidase 5 | APX5_ARATH | LAWHDAGTYDAK | Stress responses |

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