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# iTRAQ Proteins Analysis of Early Infected Papaya Plants with Papaya Dieback Pathogen

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**ABSTRACT**

Papaya dieback disease is characterized by the greasy water-soaked lesions and spots on leaves and crowns. Defoliation and blemished of the papaya fruits are also being observed as a result of infection by *Erwinia mallotivora*. In an attempt to understand the molecular mechanisms leading to the bacteria pathogenesis and the papaya plant response to infection in a compatible reaction, protein profiling during 24 hours post infection was studied using iTRAQ mass spectrometry analysis. The bacterium was sprayed into wounded leaves of a susceptible papaya cultivar (*Eksotika 1*) and proteome analysis was performed. The comparison of protein patterns of the treated and the control plants were carried out by labelling the control sample with iTRAQ 8 plex reagent 113 and inoculated samples with the iTRAQ 8 plex reagent 115 which were then analysed by peptide mass fingerprinting and identified by searches in public databases. Biochemical changes occurring in infected tissues were observed. Among the differentially expressed proteins were enolase, maturase K, superoxide dismutase, ascorbate peroxidase, phosphoribulokinase, CT114 and a hypothetical protein with unknown function.

## INTRODUCTION

The Papaya fruit is of considerable economic importance in Malaysia. Papaya industry in Malaysia faces a major threat from bacteria pathogens that cause rapid decline in production. The pathogens are affecting popular Malaysian export varieties like Eksotika, Solo and Sekaki [1]. A disease known as papaya dieback caused by the Gram-negative bacteria, *Erwinia mallativora* [2] has been proclaimed under the 1976 plant quarantine act for any infected plants to be destroyed immediately. Similar to other plant bacterial pathogens, these bacteria enter its host through stomatal openings and wounds [3]. Papaya dieback disease attacks the entire parts of papaya plants including shoot, leaf, frond, bar and also the fruit with typical symptoms of greasy water-soaked lesions and spots on leaves and fruit [4]. Papaya plant diseases with similar symptoms have also been reported in Java in 1930's [5], Mariana Islands [6], US Virgin Island [3], Venezuela [7] and in the Carribbean's[8].

Quantitative Mass Spectrometry (MS)-based proteomics is an emerging technique in the field of plant proteomics. They have made tremendous progress in revealing the function of genes in genomes especially when combined with other functional omics platforms [9,10]. One of them, a novel, MS-based approach known as Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) has been developed for relative quantification of proteins that relies on the differentiation of primary amino groups in intact proteins. Development of the iTRAQ technology provides quantitative information for the elucidation of protein markers and differential protein expression studies [11]. It has been used to quantitatively compare the protein expression between two or more samples [12]. The isobaric mass design of the iTRAQ reagents enables differentially labelled proteins to appear as a single peak during the MS/MS analysis which can be then be quantified based on the peak area of the reporter ions.

Papaya, like any other plants is subjected to numerous biotic stresses such as pathogen invasion and diseases. In a compatible host-pathogen interaction, the pathogen is successful in its invasion strategy and manages to colonize the plant. However, during the initial invasion, plants will still give out defensive response which involves metabolic and physical alteration such as lignifications, suberization, callose deposition and phytoalexin production to restrict the pathogen entry [13]. For better understanding of the disease, knowledge on the underlying mechanism of the papaya dieback disease and also plant response towards the disease is crucial in management and future studies of the disease. In this research, compatible reaction of papaya in response to *E. mallotivora* attack was carried out using proteomic technology. Study of plant leaf proteins which were induced after 24 hours infection with this pathogen was carried out. The main objective of this study was to understand the molecular mechanisms in the infected papaya. Changes in the gene expression were anticipated as the abundance of the proteins was modified by the *E. mallotivora* infection. Analysis of the proteome observed after the infection could contribute to better understanding of the gene functions in both the plant and the pathogen.

## MATERIALS AND METHODOLOGY

### Plant material, bacterial inoculation and sampling

*Carica papaya* (Eksotika I) [14] seeds were germinated in the greenhouse in flats containing potting soil. Four-week-old papaya seedlings were divided into two groups of three-plant each to serve as experimental replicates. Plants were grown in a greenhouse at Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Malaysia.

*E. mallotivora* strains were grown at 28ºC in nutrient broth to OD600 of 1, harvested by centrifugation, and resuspended in the same media at a density of $10^7$ cfu/ml. Bacterial were sprayed onto leaves at the volume of 10 ml per plant after initial wounding of the plants. Plants that had gone through the same wounding process but sprayed with the same volume of media without the bacteria were used as control. Leaf samples were collected from the plants representing the infected and control 24 hours post infection. The leaf tissues were frozen in liquid nitrogen in the field immediately following collection and stored at -80 ºC until analysis. The plants were kept for papaya dieback symptom observation until all infected plants reached stage 5; the plant death.

### Protein extraction and quantification

Total proteins were extracted from papaya leaves harvested at 24 hpi (hours post infiltration). The leaf samples were ground in liquid nitrogen and proteins were precipitated with 10% trichloroacetic acid (TCA) in acetone. Precipitated proteins were collected by centrifugation at 13000g for 10 min at 4 ºC. The pellet was washed 3 times with ice cold 80% acetone, air dried at room temperature and resuspended in resolubilisation buffer (7 M urea, 2 M thiourea and 4% 3-[(3- Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] for 30 minutes with vigorous vortexing and sonications. The concentration of all protein extracts was estimated using Bradford assay. Briefly, 5 mL of each protein sample and BSA standards were mixed with 20% (v/v) Bradford reagent (Bio-Rad, Hercules, CA, USA). The quantity of solubilized protein was measured at 595 nm absorbance using spectrophotometer. 20ug of proteins were loaded onto 12% SDS PAGE for visualization. The quantified samples were later freeze dried using VirTis freeze dryer.

### Quantitative mass spectrometry analysis using iTRAQ reagent

For iTRAQ analysis, proteins were dissolved in iTRAQ dissolution buffer and precipitated using ITSI Bio's ToPREP kit to remove iTRAQ interfering chemicals in the sample. Quantification of proteins was carried out using Bradford protein assay technique. 100 ug of peptides from each sample was labeled with 2 plexiTRAQ reagents according to the manufacturer's instruction (Applied Biosystems Inc., Foster City, CA). Proteins extracted from the infected papaya were labelled with iTRAQ 8 plex reagent 115 while the uninfected controls were labelled with iTRAQ 8 plex reagents 113. All the labelled proteins were reduced and alkylated and digested using trypsin as the enzyme.
Labeled peptides from the two samples were combined into one tube and cleaned and fractionated by strong cation exchange (SCX) chromatography. The SCX was used to separate the peptides in the first dimension. The iTRAQ-labeled peptides were then eluted from the SCX column using 225mM and 450mM Ammonium acetate. Samples were loaded onto a PicoFrit C18 nanospray column (New Objective) using a Thermo Scientific Surveyor Autosampler and later into the LTQ XL mass spectrometer (Thermo Scientific) for MS: MS analysis. Ion spectra were collected in an information-dependent acquisition (IDA) mode. The IDA mode settings included continuous cycles of one full-scan TOF MS from *m/z* 400 to 1,100 (1.5 sec) plus four product ion scans from *m/z* 50 to 2,000 (3 sec each). Precursor m/z values were selected from a peak list automatically generated by Analyst QS software (Applied Biosystems) from the TOF MS scan during acquisition, starting with the most intense ion.

Raw data files obtained were searched using Proteome Discoverer 1.3 (Thermo Scientific) and the SEQUEST algorithm against the most recent species-specific database for plant downloaded from NCBI. Trypsin was the selected enzyme allowing for up to two missed cleavages per peptide. Proteins were identified when two or more unique peptides had X-correlation scores greater than 1.5, 2.0, and 2.5 for respective charge states of +1, +2, and +3. ProteinPilot™ 2.0 software (Applied Biosystems) was used for peptide matching, protein identification, and relative protein quantitation. The relative abundance of each peptide was determined by ProteinPilot™ using the peak areas of signature ions from the iTRAQ-labeled peptides.

### RNA isolation from plants and quantitative real time analysis

Young leaf samples were collected from a pool of three plants to normalize variations among individual plants. Total RNA was prepared from pooled leaves of using Qiagen Plant RNA extraction kit following the manufacturer's instruction. Contaminating genomic DNAs were treated using the DNA-free kit (Ambion), the concentration and purity of RNA samples were determined by UV absorbance spectrophotometry. RNA integrity was also checked using agarose gel electrophoresis. Primers of selected pathogenesis genes were designed using Primer 3

software. Sequences were obtained from public databases. Actin and 40sRP were used as housekeeping controls. Primer sequences are available upon request. PCR was performed with Sensi Fast SYBR Green qPCR Supermix (Bioline) using 0.5 mM each primer on a Step One Plus Real Time qPCR System (Applied Biosystems). Cycling profile was 95◦C, 2 min; 40 cycles of 95◦C, 60◦C for 15s; followed by denaturation for melting curve analysis.

## RESULTS AND DISCUSSIONS

Susceptible papaya plants were inoculated with *Erwinia mallotivora* to identify plant pathogen-induced proteins. Although the Eksotika variety is susceptible to the papaya dieback pathogen, these plants also produce a broad-spectrum defense response known as Pathogen-Associated Molecular Pattern Triggered Immunities (PAMPs) [15]. Bacterial *E. mallotivora* strains were sprayed onto the leaves and after three days, the plants started to show early symptoms of papaya dieback disease (**Fig.** 1). To understand the susceptible papaya responses to *E. mallotivora*, we performed a quantitative proteomic study of plant leaf proteins induced after the infection of this pathogen. Proteomic technique iTRAQ were used to analyze the differential expression of proteins after 24 hours of infection to describe global gene expression changes on the papaya plants protein during the onset of the papaya dieback disease. This differential protein expression was characterized quantitatively

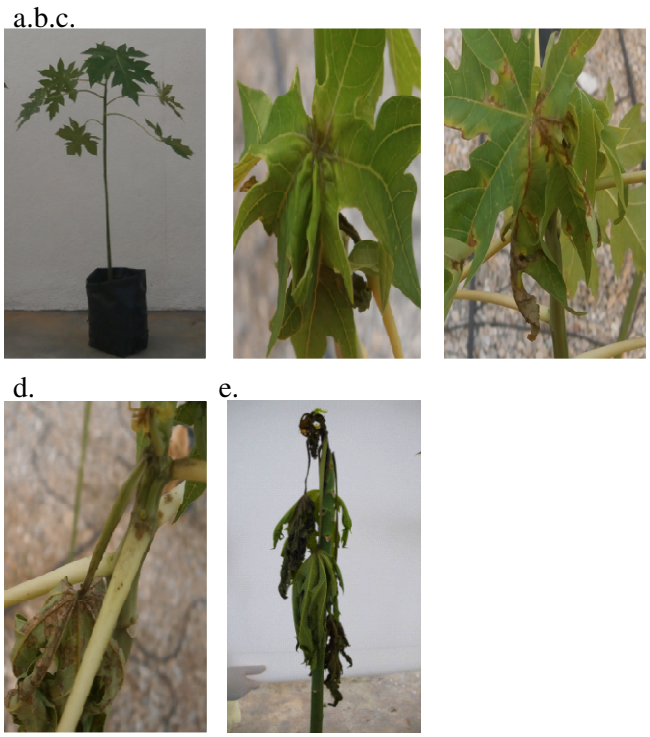


**Fig**.1. Disease progression of Papaya Dieback infected planted post days after infection. The disease starts with symptomless stage (a). After 3-6 days of infection, infected papaya leaves veins will start to blacken (b), followed by slight wilting of the leaves (c). The papaya stem will also start to wilt (d) and at last the plant will succumb to the disease resulting in plant death (e).

The proteomic experiment began with sample preparation and protein extraction from the selected samples and this presents a critical step for obtaining reliable results. Protein extraction however can be particularly challenging especially when dealing with plant proteins as plants samples have been known to cause problems due to the level of phenolic compounds during the protein extraction process [16]. For this study, a modified TCA acetone method was used to obtain papaya protein with relatively low amount of contaminating proteins. After 24 hpi (hours post infiltration), papaya leaf protein extracts were prepared by TCA acetone protein extraction and precipitation technique. The concentration of all protein extracts was estimated using Bradford assay [17]. For 100 mg starting materials the amount of total proteins obtained using this method ranges normally from 100 to150 µg in total.

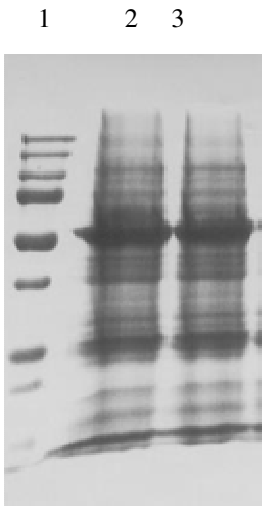


**Fig.** 2. 20 µg of proteins from the treated (Lane 3) and control samples (Lane 2) were run on a 12% SDS PAGE and stained using Coomassie Briliant Blue. Lane1 is protein markers.

**Fig.** 2 represents the 20 µg of proteins from the treated and control samples run on a 10% SDS PAGE before being sent for iTRAQ analysis. No significant differences were observed between the infected and control plants based on the SDS PAGE. This was expected as SDS PAGE doesn't have the resolution and ability to separate, identify or quantified differently expressed proteins.

Precipitated proteins were dissolved in iTRAQ , digested using the trypsin enzyme and and the control sample was labeled with iTRAQ 8 plex reagents 113 while the treated sampled was labeled with iTRAQ 8 plex reagent 115. The samples were cleaned and fractionated by cation exchange chromatography and loaded into the mass spectrometer to obtain peptide peaks for identification and quantification. Identification of proteins were based on the nearest sequenced homologue alignment results and was only considered statistically significant at $p<0.05$. **Fig.** 3 represents the example of MS/MS spectrum identified from the study. More than 700 proteins were identified out of more than 550 proteins that had quantitative iTRAQ ratios. Based on the quantification analysis carried out, the treated samples with protein ratio with more than 1.5 were considered up regulated

while proteins with ratio less than 0.67 were considered down regulated. Gene ontology classifications were assigned to the proteins and the proteins were categorized as having functions in defence response, signal transduction and signalling, metabolism, protein destination and storage, and protein synthesis. **Table** 1 highlights some of the proteins with significant differential protein expression between the infected samples and the control. Enolase, maturase K and hypothetical proteins were among the proteins with highest ratio between infected and control. Other upregulated proteins were: cysteine protease, MADS–box protein, zinc finger protein, ascorbate peroxidase, cytochrome P450, glutathione S transferase, C43, a hypothetical protein and WRKY transcription factor. Ribulosebiphosphate carboxylase, rubiscoactivase, phosphoribulokinase and CT114 however are found to be down regulated more than 4 fold in treated samples.

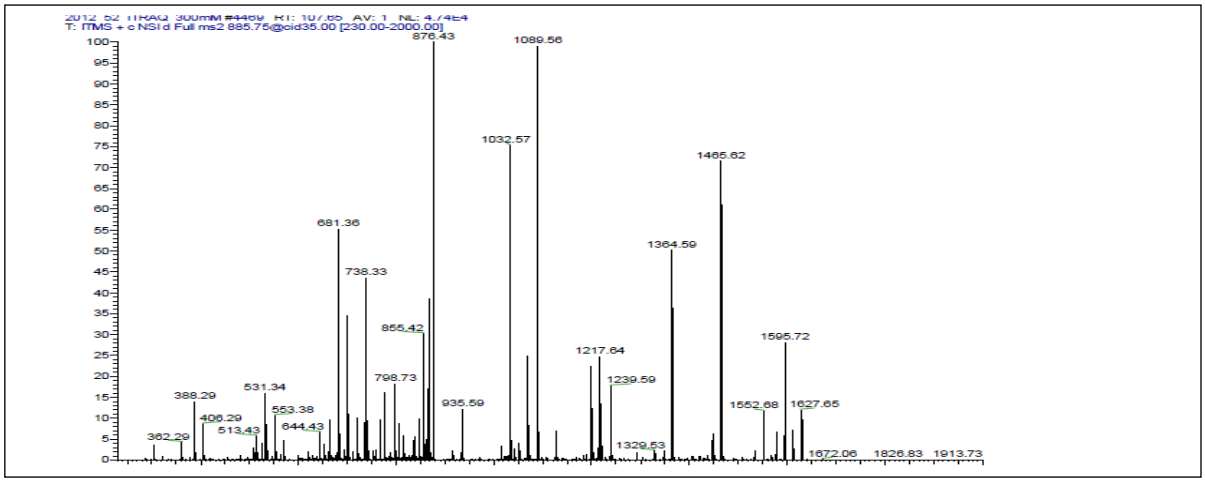


**Fig.** 3. Peptide spectrum representing a part of Enolase protein after alignments of the predicted molecular weight of the peptide sequences with corresponding homologous peptides from the database.

**Table** 1: Examples of proteins with elevated expression after 24 hpi by the papaya dieback pathogens.

| **Protein** | **Similarity** | **Biological process** | **Ratio Infected: Control** | **Predicted MW (kDa)** |
|---|---|---|---|---|
| Glutathione S transferase | Arabidopsis thaliana | Metabolic process/ Disease Response/ Defence | 1.504 | 25 |
| WRKY transcription factor | Medicago truncatula | Regulatory | 0.866 | 43.4 |
| Endo 1,4 glucanase | *Ricinus communis* | Defense | 0.569 | 54.3 |
| CT114 | Lycopersicon peruvianum) | Metabolic Process | 0.244 | 23.2 |
| Phosphoribulokinase | | Metabolic Process | 0.264 | 45.0 |
| Ribulose 1,5-biphosphate (RUBISCO) carboxylase | Nicotiana tabacum | Metabolic Process | 0.540 | 25.9 |
| Rubisco activase | Nicotiana tabacum | Metabolic Process | 0.673 | 15.9 |
| Chlorophyll a-b binding protein | Phaseolus vulgaris | Metabolic Process | 0.481 | 13.1 |
| Maturase K | *Manlyua chejuensis* | Metabolic Process | 0.287 | 59.6 |
| Enolase | Oryzae sativa | Metabolic Process | 2.287 | 45.9 |
| Bel1 homeotic protein | *Ricinus communis* | Metabolic Regulation | 7,658 | 47.8 |
| Subunit 2 catalase | Gossypium hirsitum | Antioxidant/ Response to stress | 1.657 | 56.9 |
| Superoxide dismutase | Carica Papaya | Antioxidant/ Response to stress/Defense | 1.531 | 6.05 |
| Ascorbate Peroxidase | Musa acuminata | Antioxidant/ Response to stress//Defense | 1.578 | 22.2 |
| Cysteine Protease Inhibitor | Populus balsamifera | Defense | 1.699 | 25.5 |
| Cystein Protease | *Carica Papaya* | Metabolic Process/Defense | 1.712 | 51.4 |
| Cytochrome P 450 | *Ricinus communis* | Response to stress | 1.631 | 34.1 |

Based on the differential protein analysis, changes induced by *Erwinia mallotivora* infection are proving to be all around and include those associated with plant metabolism and general plant defense responses [18]. One of the significant responses demonstrated by the plants is the up regulation of genes that are associated to photosynthesis. Decreased in expression of chloroplast chlorophyll a/b binding protein, Rubisco biphosphate carboxylase and Rubisco activase were observed. Rubisco biphosphate carboxylase and Rubisco activase which are enzymes that play major roles in Calvin cycle. Rubisco activase has the effect to elevate ATP hydrolyzing activity and increased the inhibitor sugar phosphate diassociation. As a result, carbon dioxide concentration will be limited [19]. Maturase K is also found to be down regulated along with the other photosynthesis related proteins in this study. Maturase K is a chloroplast transcript processing enzyme that acts as post transcriptional splicing factor which can increase the plant photosynthetic machinery. Acting as splicing factor for plant group II introns from premature RNA, maturase K have been shown to accumulate in response to pathogen attack [20]. Maturase K presumptive role is likely to keep the plant cells alive under conditions of increased stress. Proteins related to photosynthesis have been shown to be down regulated in response to plant infection by *P. syringae*[21], *Albugo candida*[22],*Blumeria gramininis*[23] and abutilon mosaic virus [24]. An explanation for the decline of the photosynthesis rate in infected plants might be due to the switching off of the mechanism in order to initiate the defence process [25].

Enolase proteins increased and are among the highest protein expressed during papaya infection with *Erwinia mallotivora*. This protein was also shown to be highly transcribed in the stem tissues of *Olea europaea* plants during infection with *Pseudomonas savastanoi*[26]. Agrobacterium tumefaciens induced tumors in *Beta vulgaris* also increased the enolase enzyme activity up to 2,77 fold [27]. Enolase are found in any organisms that are capable of glycolisis or fermentation as a part of the pathway. It is responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Enolase is found to be highly induced in plants during pathogen attack and in plant response to abiotic stress [18,28]. Pandey et al. [29], in their search to characterize the molecular mechanisms of cold tolerance in *Borytis cinerea*, identified enolase as a transcriptional regulator. Given its high expression during *Erwnia mallotivora* attack, this protein might also function as transcriptional regulator protein and has the potential to serve as marker to detect the presence of the *Erwinia mallotivora* in plant tissues

Peroxidase protein is well known in the roles of enhanced resistance to plant biotic stress. They belong to a large family of enzymes that are able to oxidize different substrates in the presence of $H_2O_2$. Expression of peroxidase leads to the activation of plant cell death and defence mechanism [30]. The overproduction of reactive oxygen species (ROS) is one of the first responses of plant cells in response to biotic and abiotic stimuli. The mechanism which is also known as oxidative burst can inhibit pathogen multiplication due to the highly toxic environment produced by the process [31]. Peroxidase are also involved in auxin metablolism and lignifications process which provides cell wall cross linking activity through the reduction of $H_2O_2$ and transfer of electrons to lignin precursors, auxin and secondary metabolites molecules. However, during the study peroxidase expression is found to be moderately expressed. Interestingly, ascorbate peroxidase and catalase, proteins that are involved in the detoxification of active oxygen species and protecting plant cells against ROS toxic effect in plants are overexpressed. Plant cells are toxic to the accumulation of high levels of ROS. Studies have shown that ascorbate peroxidase and catalase act as major ROS scavenging mechanisms of plants which are involved in controlling ROS overproduction [32,33].The over expression of these scavengers after 24 hpi are expected as a detoxification mechanism from excess ROS after *Erwinia mallotivora* infection.

Cysteine proteases is a part of defence system against pathogen in plant that acts as activator of program cell death. In plant, program cell death are regulated between both cystein proteases and cysteine protease inhbitors group of proteins [34]. Cysteine protease inhbitor has been suggested to be involved in response to biotic and abiotic stresses as they are highly expressed during harsh condition such as cold, drought, salt stress, oxidant stress and wounds. Cysteine protease inhbitor activation is also a part of defence response by plant when attack by pathogens. They act through the regulation of digestive proteases by forming a complex either on the active site or on the allosteric site with the target protein [35]. Several important cysteine protease inhbitor have been characterized from different plant species and engineered into crop plants for protections against pest and pathogens. Based on the iTRAQ analysis the expression of cysteine proteases and cysteine protease inhbitors are increased during the early infection of susceptible papaya with *Erwinia mallotivora* as a part of the papaya defence mechanism against the dieback patogen.

Activation of network of transcription factors involved in defense response is one of the mechanisms employed by plants in response to pathogen attack. One of the proteins involved is the WRKY transcription factor. iTRAQ quantification data obtained showed up regulation of a putative WRKY transcription factor during the *Erwinia mallotivora* infection. As a transcription factor, WRKY is involved in various stresses response and have been shown to regulate jasmonate and salicylic disease response pathway in plant [36]. Activation of this WRKY transcription factor after pathogen triggered immunity (PTI) is triggered by pathogen associated molecule patterns (PAMPS) which act as the plant first tier resistance system [37].

Upon pathogen attack, plants are known to increase plant defense genes such as WRKY transcription factor and proteins known as pathogenesis-related proteins (PR) [38,39]. There about 13 groups of PR proteins which includes PR-1, ß-1,3-glucanases (PR-2), chitinases (PR-3), PR-4 and osmotin (PR-5) [40]. However based on the iTRAQ protein quantification results, very limited amount of these pathogenesis related protein expression were observed. To confirm the induction of these defense genes, primers were designed for several known pathogenesis related proteins from papaya and used in the Quantitaive Real Time analysis. Initial quantitative real time PCR were carried using cDNA of inoculated plants with the pathogen after 24 hours infection and control plants inoculated with the same media minus the bacteria. The analysis showed that all the pathogenesis related genes tested shown similar or slightly higher fold of expression (less than 2 fold). These however were considered not highly significant when compared between the infected and the control (**Fig.** 4). These data support the minimal expression of these

proteins as shown by the iTRAQ data obtained. This may also indicate that the expression of the selected pathogenesis related genes are not high enough to protect the papaya from the pathogen attack.

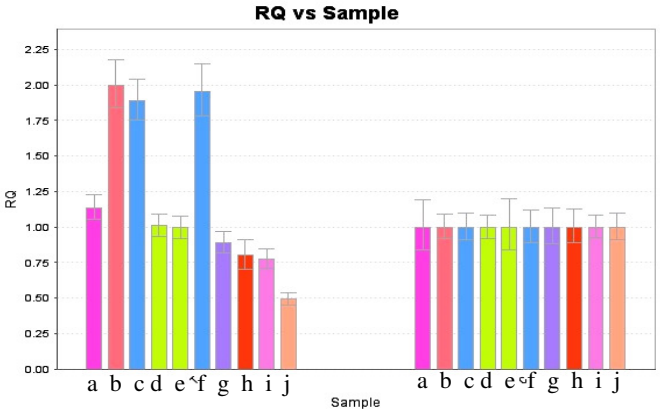


**Fig.** 4. Real time PCR analysis of selected pathogenesis related genes protein in 24 hpi Eksotika papaya. The genes tested were: a) Chitinase, b) Peroxidase Precursor, c) Chitinase Class III, d) Ascorbate oxidase, e) Osmotin, f) Peroxidase, g) PRI_c, h) PR1_a, i) PR1_b, j) PR1_d.

## CONCLUSION

By using aiTRAQ proteomic approach, a series of proteins, displaying differential expression patterns 24 hours after inoculation during the course of infection, were identified. Some of them were related to carbon metabolism and photosynthesis, implying an adaptive change in physiology, plant defence/stress response, signal transduction, amino acid transport, cell wall structure, protein metabolism, and other functions.However the identification of pathogenesis related proteins after inoculation is showed insignificant changes in profiles observed during the proteomic study. Validation using Real Time Quantitative PCR supported the findings. Further validation of important genes obtained from this study needs to be validated. This study has paved the way to dissect the expression network associated with susceptible papaya host response to *Erwinia mallotivora* infection. For future studies, further research will be continue to discover, confirm and monitor the actual players (genes, proteins and metabolites) that are involved in mediating specific cellular processes in the papaya upon infection with the papaya dieback pathogen.

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