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Expression of defense-related genes in papaya seedling infected with *Erwinia mallotivora* using real-time PCR

(Ekspresi gen berkaitan ketahanan dalam anak pokok betik yang dijangkiti *Erwinia mallotivora* menggunakan PCR masa nyata)

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Keywords: *Carica papaya*, papaya dieback disease, defense-related genes, gene expression, real-time PCR

Abstract

Papaya is an important fruit crop in Malaysia for the domestic and export markets. The papaya industry is currently facing a major threat from the papaya dieback disease for which no effective control measure is known to prevent further spreading of the disease. The causal agent of the disease, *Erwinia mallotivora* was only identified lately. Hence, to understand the molecular mechanism underlying the host-pathogen interaction, leaves of papaya seedling were infected with a suspension of *E. mallotivora* at the concentration of 10^6 cfu and subsequently, the expression profiles of four identified potential defense-related genes namely *peroxidase*, *aquaporin*, *leucine-rich protein* and *zinc finger protein* were analysed using quantitative real-time PCR. The expression profiles were captured at seven different time points post-infection at 0, 4, 8, 12, 24, 48 and 72 h respectively. Among the four defense-related genes analysed, *peroxidase*, *zinc finger protein* and *leucine-rich protein* genes were found to be up-regulated at early stage of infection when compared to control. Meanwhile the expression of the *aquaporin* gene was found to be significantly up-regulated at later stage in response to *E. mallotivora* infection.

Introduction

Papaya is an important tropical fruit in the international market and the global production was reported to be 11.22 million metric tonnes in 2010 (FAOSTAT 2012). Malaysia plays a major role in contributing to the global papaya production and becomes one of the major exporters of papaya. However, the papaya industry is currently facing a major threat from papaya dieback disease. This is a highly destructive disease which can cause damage to papaya tree and fruit yield loss was up to 100% (Roshidi 2010). There is no effective control of the disease and the only solution is to demolish the infected papaya farm to prevent further spreading of the disease.

It takes years to identify the causal agent of this disease until recently *Erwinia* sp., *E. papayae* (Maktar et al. 2008) and *Erwinia mallotivora* (Noriha et al. 2011) were reported to cause the outbreak of papaya dieback disease. Further analysis had been carried out by Noriha et al. (2011) to confirm *E. mallotivora* as the main causal agent of the disease in Peninsular Malaysia.

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Better understanding of the host-pathogen interaction and pathogenic mechanism are essential before any strategies can be devised to control the disease. Furthermore, understanding the molecular mechanism underlying the host-pathogen interaction is crucial in identifying potential defence-related genes which can assist in the development of resistant varieties.

Previously, gene expression study of host-pathogen interaction in papaya dieback disease had been conducted using SAGE technology (Khairun et al. 2010). Gene expression profiles and the list of genes that showed transcriptional changes due to the infection were obtained with the short tag sequence generated. However, there is a need to further validate these genes and identify potential candidates that are specifically involved in the defense mechanism. The present study attempted to quantitatively validate using real-time PCR four defense-related genes, namely *peroxidase*, *aquaporin*, *leucine-rich protein* and *zinc finger protein*, which are ranged in the top ten of the list.

Materials and methods

Plant materials and pathogen inoculation

A total of 12 *Carica papaya* var. Eksotika seedlings (4 months old) purchased from MARDI Seed Unit were used in the study. There were three seedlings in each treatment. For the control, three seedlings were treated with autoclaved double distilled water (ddH_2O) and for the treatment, three seedlings were treated with suspension cultures of overnight grown *E. mallotivora* at a concentration of 10^6 colony forming unit (cfu) (OD_{600}~0.5). Leaves of the papaya seedling were pricked using a needle, subsequently inoculated with 100 µl of autoclaved ddH_2O for control or suspension bacteria culture for infected sample. Another set of papaya seedlings consisted of six seedlings was inoculated for papaya dieback disease symptom observation. Three seedlings were inoculated on the leaves and three seedlings were inoculated on the stems.

Samples collection

Treated leaves were collected at seven different time points post-infection at 0, 4, 8, 12, 24, 48 and 72 h. Approximately 2 cm^2 of the inoculated leaves from three seedlings in each treatment were excised and pooled as one sample for each time point. There was no sample collection done on the set of papaya seedlings inoculated for papaya dieback disease symptom observation.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of control and inoculated leaf samples using RNAzol reagent (Molecular Research Centre, Ohio, USA) according to the protocol supplied by the manufacturer. The total RNA obtained was visualised in a 1% (w/v) agarose gel and quantified using a NanoDrop™ Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA). Subsequently, a total of 1 µg total RNA was used to synthesize cDNA by using Quantitect Reverse Transcriptase Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The cDNA synthesized was stored at –20 °C for further use.

Primers

Specific primers were designed using Primer Premier 5.0 program for *peroxidase*, *aquaporin*, *leucine-rich protein* and *zinc finger protein* genes based on the full length sequence obtained from public domain, Phytozome v5.0: *Carica papaya*. Primer sequences of two identified housekeeping genes, *actin* (FJ696416.1) and *40sRP*, were obtained from Waznul Adly et al. (2010). All primers (*Table 1*) were synthesized by AITBIOTECH (Singapore).

Table 1. Primer sequences of defense-related and housekeeping genes used in real-time PCR analysis

Target gene	Forward primer (5'-3')	Reverse primer (3'-5')	Product size (bp)
Defense-related gene			
peroxidase	TACTATTGGAACCGCAGGATGTG	GGGAGGAATGATGGGTTTATGG	102
aquaporin	GGGCTCACTCGGTATAATCGC	CAGTAAACCCAGTTGTCGGTAAAG	156
leucine-rich protein	CGGCGCAGCTTTCATCAG	CTTGGCGAACCTTCCCTTG	158
zinc finger protein	CGGCAGAAGAAGATTCAGGAGA	TGCGGTGTTCCATCCAAAGA	149
Housekeeping gene			
actin	TTCCACTATGTTCCCTGGTATT	TCCTATCCAGACGCTGTATTTC	119
40sRP	TGGCAAAGCCTACAAAGACTATCA	AGGAATGGGAAGGGAGGGAGAT	78

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR was performed in 96-well optical reaction plate using a Step-One Plus Real-Time PCR system (Applied Biosystems, California, USA). The assay was carried out in a final volume of 20 µl that contained 1x Power SYBR® Green Master Mix (Applied Biosystems, California, USA), 900 nM of forward and reverse primers, and 100 ng of cDNA template. Negative control used autoclaved double distilled water to replace the cDNA template. All PCR reactions were performed in triplicates. The amplification program consisted of 1 cycle of 95 °C for 10 min (pre-incubation), followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension). Subsequently, a melting curve analysis was done to determine the specificity of the reaction by incubating the reaction at 95 °C for 15 s, annealing at 60 °C for 1 min and followed by slowly heating at 0.3 °C/sec to 95 °C while continuously monitoring the fluorescence signal.

Determination of PCR efficiency

Reverse transcription was performed with 1 µg total RNA as template using Quantitect Reverse Transcriptase Kit (Qiagen, Hilden, Germany). Then, the reverse transcription mixture was subjected to 5-fold serial dilution and the diluted mixture was used to performed quantitative PCR to generate a standard curve for determination of PCR efficiency, precision and dynamic range. As stated in Rasmussen (2001), efficiency was calculated using the slope of the log-linear phase by the formula: $E = 10^{(-1/slope)} - 1$. A good PCR efficiency should range between 90 – 110% with slope between –3.1 to –3.6 (Rasmussen 2001; Tichopad et al. 2003). Melt curve analysis was also performed to determine the specificity of each primer.

Data analysis

Comparative C_P method or known as $\Delta\Delta C_P$ (Livak and Schmittgen 2001) was performed to analyse the expression data. The analysis was carried out based on housekeeping genes as endogenous control to determine the quantity of target in a sample relative to the quantity of target in a reference sample. Normalisation was done using the geometric mean of multiple housekeeping genes in normalisation factor calculation as described by Vandesompele et al. (2002). Data analysis was done using StepOne™ Software Version 2.2 (Applied Biosystems, California, USA) which included statistical analysis to determine variation and standard error of the gene expression. Subsequently, fold change expression was determined by performing a pair-wise comparison with infected samples to the respective water treated control at the same time points.

Results and discussion

Infection of Erwinia mallotivora on 4-month-old papaya seedlings

Discolouration of infected leaves were observed 3 days post-innoculation and disease symptom of dark brown lesions developed at the inoculated part as early as 5 days post-inoculation. The lesions extended along the vein of infected leaf and became necrotic (*Plate 1A*). The leaves turned yellow and dropped off at later stage. However, necrotic lesions were not observed at the infected stem at the beginning. Water soak lesions were firstly visible at the inoculated part after 6 days inoculation (*Plate 1B*). As the disease advanced, the water soak regions began to dry out and shrink after 10 days inoculation. The shrinkage caused the stem to bend as a dieback symptom which led to plant destruction at the later stage of infection (*Plate 1C*).

Similar development of disease symptoms were observed on infected papaya plants in the field. The bacteria invaded the plants through open wounds, colonised and moved through the plant vascular system, which explained the disease symptom development along leaf veins and stem (Noriha et al. 2011). This was in agreement with the observation made on invasion and migration of green fluorescent protein-labelled *E. amylovora* in apple leaves by Bogs and Geider (1999). Invading pathogen was visualised to travel along the xylem vessels and subsequently penetrated into the parenchyma intercellular spaces. The bacteria attacked and colonised the vascular system to exploit plant nutrients for their survival and multiplication. These resulted in blocking of the transport system of nutrient and water which eventually lead to plant death (Oh and Beer 2005).

Sensitivity and specificity of the primer sets

PCR amplification efficiency was determined by producing standard curves for each primer set. All standard curves obtained showed PCR efficiency achieved 92.1 – 102.7% for each primer set tested and the coefficient of determination (R^2) ranged from 0.997 – 0.999. The results obtained indicated that the designed primer sets were sensitive and accurate for the qRT-PCR (*Figure 1*). In addition, a single peak melting profile of each primer sets obtained from melting curve analysis of the PCR product revealed the specificity of the designed primers (*Figure 2*).

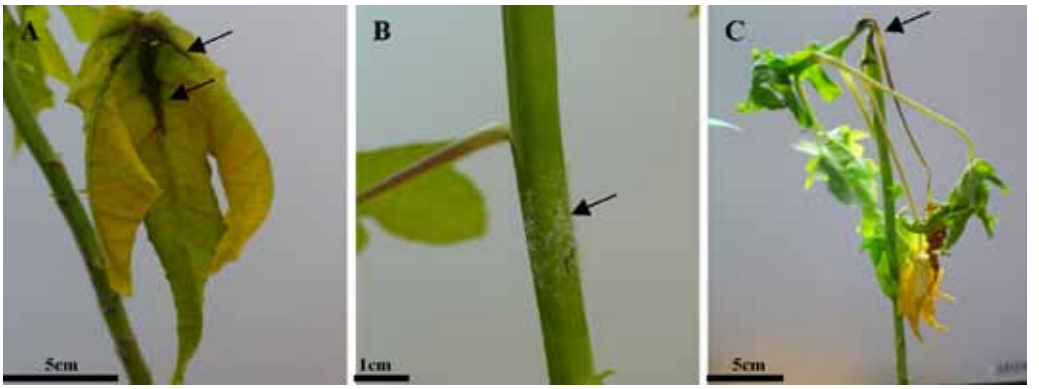

Plate 1. Papaya dieback disease symptoms observed on papaya seedlings infected with Erwinia mallotivora, (A) Necrotic lesions developed along the vein of an infected leaf, (B) Water soak lesions observed on an infected stem and (C) Infected areas shrank and caused the stem to bend, leading to destruction of the infected papaya plant

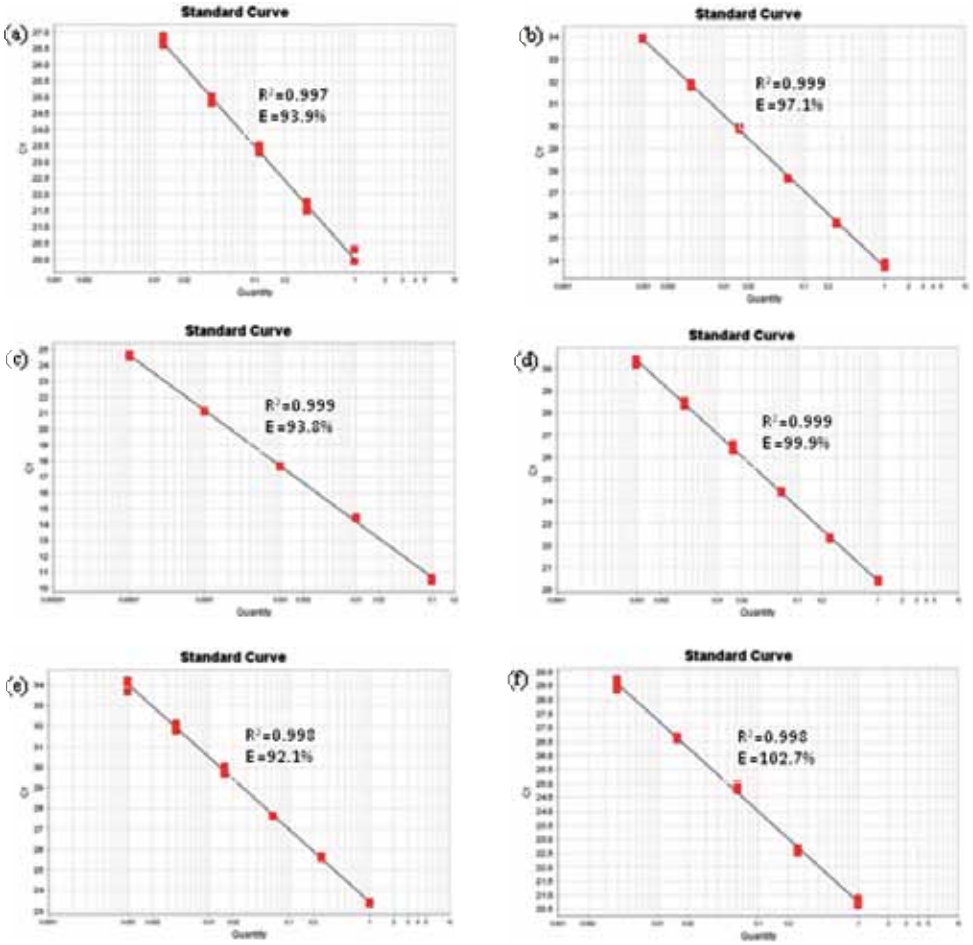

Figure 1. Standard curve generated for house-keeping genes, (a) 40sRP, (b) actin and defense related genes, (c) peroxidase, (d) zinc finger protein, (e) leucine-rich protein and (f) aquaporin to determine the PCR efficiency

Defense-related gene expression profile in infected papaya seedling

Gene expression profiles obtained from qRT-PCR analysis revealed that defense-related genes were regulated in infected papaya seedlings at different time points after infection with *E. mallotivora* compared to control. Among the four defense-related genes tested, three genes showed up-regulation at early stage of infection and one gene showed up-regulation at later stage (*Figure 3*). *Peroxidase*, *zinc finger protein* and *leucine-rich protein* genes were found up-regulated at the early stage of infection and were greatly induced immediately after the infection. Inversely, up-regulation of *aquaporin* gene was observed only after 1 day of infection.

Significant up-regulation of *peroxidase* gene compared to control started at 0 h after infection and achieved the maximum level at 8 h after infection (*Figure 3A*). The expression level of *peroxidase* gene in infected sample achieved 6.5 fold and decreased to 2.5 fold compared to control at 8 h and 12 h after infection respectively. This pattern suggested that early response of the gene happened when pathogen encountered the plant tissue. However, expression of the gene was not sustained

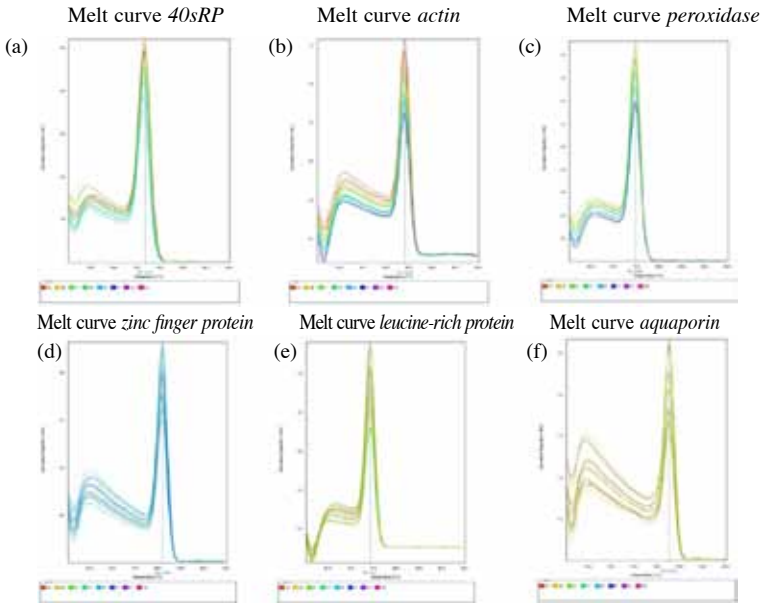

Figure 2. Single peak observed in melting curve analysis of PCR product for house-keeping genes, (a) 40sRP, (b) actin and defense related genes, (c) peroxidase, (d) zinc finger protein, (e) leucine-rich protein and (f) aquaporin are specific to amplify the particular genes

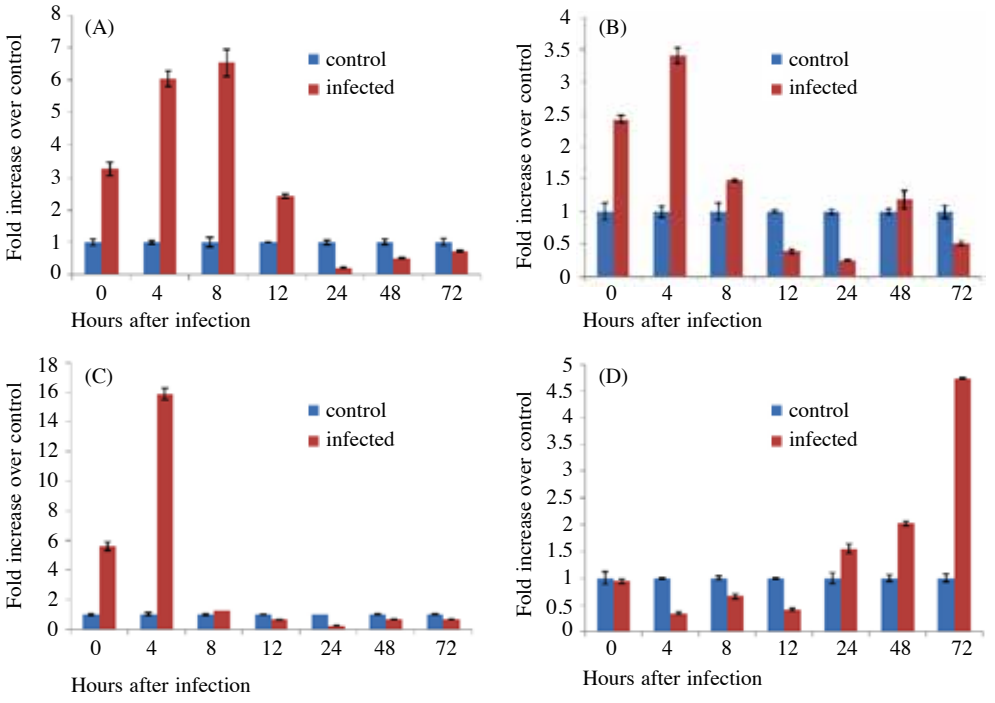

Figure 3. Expression of the potential defense-related gene in papaya leaves infected with Erwinia mallotivora and control leaves treated with autoclaved double distilled water: (A) peroxidase, (B) zinc finger protein, (C) leucine-rich protein and (D) aquaporin genes

throughout the infection as it gradually decreased and became down-regulated after 24 h compared to control. Similarly, up-regulation of *peroxidase* gene was observed in sunflower as early as 2 h after infection by *Alternaria helianthi* (Anjana et al. 2007) and in apple flower at early stage when infected by *E. amylovora* (Sarowar et al. 2011).

In this study, we demonstrated that regulation of *peroxidase* was one of the early events responding to the host-pathogen interaction (Cook et al. 1995; Harrison et al. 1995) since this gene was highly induced once infection occurs and the expression level decreased to normal after 2 days. This is in accordance to *peroxidase* roles as a key enzyme in primary response during plant pathogen interaction (Blilou et al. 2000). On top of that, we also presume that the highly expressed gene at the early stage is responsible for inducing further defense responses from the plants, such as generation of oxidative response in plant during pathogen attack.

Peroxidase gene is from a multigene family which exhibits diverse expression pattern and having different roles in plant defense responses (Hiraga et al. 2001; Passardi et al. 2004, 2005). Specific function and role of the *peroxidase* gene analysed in this study in response to the *E. mallotivora* infection still remained unclear and need to be further validated in future.

Similarly, expression of *zinc finger protein* gene was induced immediately in response to *E. mallotivora* inoculation. Up-regulation of the gene expression was observed 0 – 8 h after infection (*Figure 3B*). Maximum level of expression was reached at approximately 3.5 folds compared to control at 4 h after infection and started to decline after 8 h post-infection. This was similar with the finding reported by Sarowar et al. (2011) on the expression profiles of genes regulated by the inoculation of *E. amylovora* in apple flower. *Zinc finger protein* gene was found up-regulated at the early stage of inoculation and suspected to be involved in early defense responses in apple blossom. Oh et al. (2005) demonstrated that *zinc finger protein* plays a crucial role in the activation of the pathogen defense response in plants. On top of that, *zinc finger protein* which is one of the largest family of transcription factor in plants, was reported to be involved in regulation and signal transduction of main biological processes which include regulating pathogen responses (Takatsuji 1998) and development of programmed cell death (Ciftci-Yilmaz and Mittler 2008).

In this study, the infected plants showed rapid response towards the pathogen infection by up-regulating *zinc finger protein* gene expression at the early stage to promote and activate other defense responses to counter pathogen attack. However, phenomenon of programmed cell death was not observed in papaya when infected with *E. mallotivora*. Development of disease symptom started with necrotic lesions at inoculated points followed by wilting of whole leaves. This indicated that formation of the necrotic lesions was not sufficient to prevent migration of pathogen from the invaded points to other areas of the leaves.

Leucine-rich protein gene expression was found to be up-regulated at the early stage of infection and achieved the highest level at 4 h after infection with 16 fold over the control (*Figure 3C*). Surprisingly, the expression level declined drastically to normal level after 8 h of infection. *Leucine-rich protein* gene was shown to play an essential role at the early stage of plant defense mechanism. The gene was induced during plant-pathogen interactions, however, it has no direct inhibitory effect against the pathogen (Shanmugam 2005). Induction of the gene at the beginning stage of infection is to take part in signal transduction event for activating other defense genes in initiating further defense mechanism in plants (Shanmugam 2005; Jacques et al. 2006).

It was also suggested that *leucine-rich protein* plays a crucial role in plant innate immunity system involving the recognition of pathogen virulence protein and signal transduction in plant defense mechanism (Feys et al. 2005; Federici et al. 2006). Hence, the expression profile of *leucine-rich protein* gene obtained from the study is in an agreement with these arguments. High expression level of the *leucine-rich protein* gene is needed for the plants to recognise and detect pathogen virulence protein once the pathogen encounters plant tissue during the inoculation process.

In general, *aquaporin* functions in plant cells to facilitate water transport and control water status (Chrispeels and Maurel 1994). On top of that, *aquaporin* has been reported to play an important role in maintaining water balance at the cell level in defense responses toward salt and drought stresses (Abdeeva et al. 2008). However, Aharon et al. (2003) stated that the role of the gene in retaining water balance during stress is complex. Over expression of plasma membrane *aquaporin* gene in tobacco improved plant growth under favourable growth condition; in contrast, it showed lethal effect during stress condition.

In the event of *E. mallotivora* infection, bacterial attacked and colonised the vascular system leading to the collapse of nutrient and water transport system (Noriha et. al. 2011). As observed, disease symptoms of leaf discolouration started on day 3 and lesions developed on day 5 after infection. We believe the induction of the gene occurred while water balance was interrupted even before disease symptom was observed. This explained the expression of *aquaporin* gene (*Figure 3D*) was only up-regulated approximately 2 folds at 24 h after infection compared to control and reached greatest level (4.5 folds) at 72 h after infection. However, correlation of *aquaporin* gene with water soak lesion symptom and pathogen defense responses need to be exploited. Even though the gene was induced, it may not be directly involved with the plant defense ability, but promotes plant defense response that involves integration of signal transduction pathway.

Conclusion

The pathogen induced defense-related gene expression in papaya seedlings during the early and later stage of *E. mallotivora* infection suggested that there are specific roles that are played by the genes involved in the plant defense mechanism. Even though the level of *peroxidase*, *zinc finger protein, leucine-rich protein and aquaporin* gene expression level was quantified using qRT-PCR, the regulation of gene expression needs to be determined. In addition, there is still a need to carry out functional validation to identify the specific defense-related genes for the purpose of development of resistant papaya varieties.

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Abstrak

Betik ialah satu tanaman buah-buahan yang penting di Malaysia untuk pasaran tempatan dan juga eksport. Industri betik kini mengalami ancaman utama daripada penyakit mati rosot yang masih tidak mempunyai kaedah pengawalan berkesan untuk menghalang penyebaran penyakit ini. Ejen penyebab penyakit ini, *Erwinia mallotivora* juga baru dikenal pasti kebelakangan ini. Oleh itu, untuk memahami mekanisme secara molekul yang terlibat dalam interaksi antara hos-patogen, daun anak benih betik telah dijangkiti dengan *E. mallotivora* pada kepekatan 10^6 cfu dan seterusnya, profil ekspresi empat gen berkaitan ketahanan yang dikenal pasti berpotensi iaitu *peroxidase*, *aquaporin*, *leucine-rich protein* dan *zinc finger protein* telah dianalisis menggunakan kuantitatif PCR masa nyata. Profil ekspresi dianalisis pada tujuh masa berlainan iaitu, 0, 4, 8, 12, 24, 48 dan 72 jam lepas infeksi. Antara empat gen berkaitan ketahanan yang dianalisis, gen *peroxidase*, *zinc finger protein* dan *leucine-rich protein* didapati meningkat pada peringkat awal infeksi berbanding dengan kawalan. Sementara itu, ekspresi gen *aquaporin* didapati meningkat secara signifikan pada peringkat akhir sebagai gerak balas terhadap infeksi *E. mallotivora*.

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