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RECOMBINANT PROTEIN FOLIAR APPLICATION ACTIVATES SYSTEMIC ACQUIRED RESISTANCE AND INCREASES TOLERANCE AGAINST PAPAYA DIEBACK DISEASE

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ABSTRACT

Similar to animals, plants possess 'immune memory' in response to invading pathogens that lead to enhanced defense reaction following pathogen exposure. Systemic acquired resistance (SAR) is a well-characterized type of plant immunity and is associated with coordinated expression of a set of pathogenesis-related (PR) genes and proteins also known as SAR markers. Induction of SAR in plants was shown to be initiated by group of chemicals and biological compounds known as SAR inducers that can be used for the management of important plant diseases. Elucidation and characterization of potential SAR inducers as potential elicitors that can protect papaya from the papaya dieback disease pathogen were carried out using HRPX protein, which was produced as a recombinant protein in an *Escherichia coli* system. Disease severity analysis in a glasshouse experiment indicated lower disease infection rates in the HRPX-treated plants than in water-treated plants. Selected SAR-associated defense gene expression was also shown to increase in treated plants, via quantitative real-time PCR analysis, confirming enhanced disease response through SAR activation. In this report, the selected recombinant protein was shown to activate the SAR mechanism in papaya for increased tolerance against papaya dieback disease, which was proven via physiological and molecular analysis.

Contribution/Originality: This study documents the process utilized to produce *Erwinia mallotivora* HRPX recombinant protein using a heterologous *E. coli* recombinant system. The recombinant protein produced was later tested and assessed for its ability to act as a systemic acquired resistance (SAR) inducer to increase papaya tolerance against papaya dieback disease.

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1. INTRODUCTION

Carica papaya – widely known as papaya – is a tropical plant that is highly associated with its appealing fruit and medicinal properties arising from various parts of the plant. Papaya remains one of the most popular species in the Caricaceae family, part of the angiosperm group within the kingdom Plantae (Evans & Ballen, 2012). Papaya was once widely grown in Malaysia. Malaysian consumers prefer the fruit for daily consumption while the leaves, which contain considerable levels of flavonoids, alkaloids, and phenolic compounds, are used as a remedy for fevers, dengue fever, cardiovascular diseases, cancer, malaria, asthma, ulcers, eczema, hypoglycemia, and hyperlipidemia (Anuar, Zahari, Taib, & Rahman, 2008; Aziz, Kassim, Kasim, Haque, & Rahman, 2015; Dharmarathna, Wickramasinghe, Waduge, Rajapakse, & Kularatne, 2013; Otsuki et al., 2010). Even though the demand for papaya is high, production in Malaysia is severely threatened by the emergence of diseases caused by bacteria, viruses, phytoplasma and fungi (Rabu & Mat, 2005). Since 2006, the phytopathogen *Erwinia mallotivora* has led to a decline of the papaya industry in Malaysia despite being the second-highest exporter globally in 2004, just behind Mexico, with a total production of 58,149 MT covering more than 21% of the global papaya trade market (Anon, 2009). Commercial varieties, including Sekaki, Solo, and Eksotika, are very susceptible to the disease, thus making it harder for the papaya industry in Malaysia to be revitalized and attain its former prominence.

Papaya dieback disease in Malaysia is caused by *E. mallotivora*, a flagellar, biotrophic phytopathogenic bacterium (Noriha, Hamidun, & Indu, 2011). The disease has led to devastating effects in term of yield loss and national incomes for both domestic and international trade. Infected papaya fruit exhibits lesions and spots on the leaves and crown, eventually accompanied by water damage to the stem (Gardan, Christen, Achouak, & Prior, 2004). Fruit quality is also affected by defoliation and spots and blemishes that downgrade its quality, rendering it unsuitable for consumption and export. Eventually the infected plant will succumb to the disease, resulting in death (Mohd Taha et al., 2019).

Holistic approaches have been taken by the Malaysian government and researchers to curb the widespread transmission of this disease (Abu Bakar et al., 2017). During the early spread of this disease, infected trees were destroyed and crop rotation practices implemented (Chan & Baharuddin, 2010). Interim measures were introduced, such as systematic farming; changes in the management and operation of farms, including limiting vehicular access to farmland, cleaning of farm equipment, and farm workers' clothing; and monitoring of drainage and drainage systems' processes (Ahmad et al., 2014; Mohd Taha et al., 2019). Daily supervision and the process of eradication of suspected disease on the farm were also carried out regularly. Advancement in disease management strategies, supported by plant-pathogen interaction studies, has advanced alternative crop protection technology by utilizing the host systemic acquired resistance (SAR) mechanism to control phytopathogen attack (Dangl, Horvath, & Staskawicz, 2013). Accumulation in the plant of the hormone salicylic acid (SA) is closely linked with SAR induction (Gao, Zhu, Kachroo, & Kachroo, 2015). These measures are supported by research on the usage of chemicals and synthetic chemicals such as SA and jasmonate, and of virulent, avirulent, and non-pathogenic microbes (Birkenbihl, Liu, & Somssich, 2017). The introduction of SAR is closely connected with increased oxidative cell burst, changes in cell wall composition, de novo synthesis of compounds like phytoalexin, and increased expression of PR proteins (Fu & Dong, 2013). Plants treated with various SAR inducers have been demonstrated to exhibit increased tolerance, and sometimes increased resistance, with observation of subsequent pathogen challenge resulting in a stronger elicitation of the plant defense mechanism (Bektas & Eulgem, 2015; Jung, Tschaplinski, Wang, Glazebrook, & Greenberg, 2009). The application of such SAR inducers has paved the way for plant disease control and management and, specifically, as an alternative crop protection strategy in managing papaya dieback disease.

Type III secretion systems are widely used by phytopathogens to deliver effectors into their host cells to cause disease (Alfano & Collmer, 2004; Chen et al., 2017; Göhre & Robatzek, 2008). *Natriuretic peptide receptor 1 (NPR1)* is essential in plants for the expression of PR genes involved in SAR activation and defense of the plant against invasion and attack, through increased expression of PR genes (Cao, Glazebrook, Clarke, Volko, & Dong, 1997; Kim & Delaney, 2002). As a key transcriptional regulator of SA signaling, NPR1 is important in the induction of host systemic immunity. Overexpression of a rice *NPR1* homolog led to constitutive activation of the defense response and hypersensitivity to light (Chern, Fitzgerald, Canlas, Navarre, & Ronald, 2005). Type III effectors have been shown to interact with *NPR1* to destabilize host immunity and thus have the potential as target molecules to stimulate the signaling molecule for plant immunity, together with defense onset and commencement (Chen et al., 2017; Jones & Dangl, 2006).

Our previous study indicated that HRPX, an *E. mallotivora* Type III effector, was upregulated in in vitro cultures of *E. mallotivora* stimulating the expression of pathogenicity (*hrp*) and virulence proteins of the pathogen. This finding was later confirmed via real-time quantitative polymerase chain reaction (q-PCR) analysis (Abu Bakar et al., 2017). Thus, the present study was conducted to investigate the effectiveness of a selected *E. mallotivora* Type III effector, HRPX, produced as a recombinant protein to aid in the control of papaya dieback disease. We aimed to determine its effectiveness as a SAR inducer and understand the molecular events underlying the SAR process regarding the induced resistance process.

2. MATERIALS AND METHODS

2.1. Plants, Plasmids, Bacterial Strains, and Growth Conditions

Carica papaya (Eksotika I) seedlings were grown in polybags filled with mixed compost. The plants were grown in a glasshouse at 27°C with a 12-h day/night cycle. Plasmid pGEM-T (Promega) and pET32b (Novagen) were respectively used as cloning and expression vector. The *E. coli* strains Top10 (Invitrogen, USA) and BL21 (DE3)

were used as cloning and expression host, respectively. Both *E. coli* strains were grown in LB broth medium supplied with 100 µg/ml of ampicillin at 37°C. *E. mallotivora* was cultured in LB broth at 28°C.

2.2. Isolation and Cloning of HRPX

Total RNA from overnight culture (28°C) of *E. mallotivora* was extracted using a total RNA extraction kit (Qiagen, USA) following the manufacturer's protocol, followed by cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad, USA). PCR was performed using a pre-designed, specific HRPX primer pair and other PCR components from Promega (USA). The primer sequences were HRPX forward, 5'-GGATCCGATGCAATTTCCGAGCGACAT-3' and HRPX reverse, 5'-AAGCTTTCATTGTACGATTCCAAATC-3', with an expected amplicon size of ~1.5 kbp. The *Bam*HI and *Hin*DIII sites were added to facilitate the cloning processes. The total 20 µL reaction mixture contained 2 µL of 10 × PCR buffer, 2 µL of MgCl₂ (20 mM), 0.5 µL of each dNTP (2.5 mM), 1 µL of DNA, 0.6 µL of each primer, and 1 µL of Pfu DNA polymerase (5 units/µL). The program used was an initial incubation time of 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C for annealing, and 30 s at 72°C for extension, followed by a cycle of final elongation for 7 min at 72°C.

The PCR product was visualized by 1% agarose gel electrophoresis. The band was excised from the gel, purified, and cloned into pGEM-T Easy vector (Promega) according to the manufacturer's protocol, followed by transformation into competent TOP10 *E. coli* via the heat shock method. Transformed cells were plated on LB medium supplemented with 100 µg/µL ampicillin and 20 µg/mL X-gel. White colonies were selected for colony PCR and plasmids of positive clones were extracted, double digested with *Bam*HI and to confirm the presence of the insert (HRPX). The digested HRPX fragment was subcloned into the pET32b (Novagen) expression vector with 6× His tags downstream and transformed into a competent BL21 *E. coli* expression strain. Transformed cells were plated on LB medium supplemented with 100 µg/µL ampicillin.

2.3. Expression and Purification of HRPX Recombinant Protein

A colony of pET32b/HRPX-transformed BL21 was cultured in 5.0 mL of LB broth medium supplemented with 100 µg/µL ampicillin overnight at 37°C. The culture was transferred into 400 mL of LB broth medium containing 100 µg/mL ampicillin and was grown at 37°C to OD₆₀₀ 0.5. To induce expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to the cell culture and was grown for a further 6 h before harvesting by centrifugation at 2,500 × g for 15 min.

The bacterial pellet was resuspended and lysed with 10 mL of Bugbuster reagent (Novogen) supplemented with a tablet of protease inhibitor (Thermo Fisher, USA). The lysed cell suspension was centrifuged at 20,000 × g for 30 min at 4°C. The pellet was resuspended in 10 mL of inclusion bodies (0.5M Tris pH 7.4, 75 mM NaCl, and 10% glycerol) supplemented with 0.5% triton and was rotated for 15 min at 4°C before centrifuging at 20,000 × g for 30 min at 4°C. The pellet was then resuspended in 10 mL of solubilization buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 8 M urea) supplemented with a tablet of protease inhibitor and was rotated overnight at 4°C. Then, the protein suspension was centrifuged at 3,500 × g for 15 min at 4°C and the supernatant was collected for purification using a Ni-NTA column (His tag protein purification) by the Acta Prime Chromatography System. The purified HRPX protein was quantified following the Bradford method (Quick Start™ Bradford Protein Assay, BioRad) using bovine serum albumin as standard (Bradford, 1976).

To visualize the purified protein, approximately 20 µL was mobilized on two sets of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with polyacrylamide concentration of 12 and 5% for separation and stacking gel, respectively. One set of the gel was imaged using Coomassie Brilliant Blue R250 while the other was presented for Western Blot analysis. Briefly, the protein was transferred to a polyvinylidene fluoride membrane. The membrane was blocked with blocking buffer before probing with an anti-His antibody, with gentle agitation for 2 h at 37°C. After three washes with 1× Tris Buffered Saline with Tween 20 (TBST), the membrane was further incubated with anti-mouse IgG alkaline phosphatase conjugate for 2 h at 37°C. The membrane was washed again with 1× TBST, followed by incubation with the substrate solution (BCIP/NBT) until the bands appeared.

2.4. Plant Treatment

At 4 months of growth, papaya seedlings were sprayed with HRPX protein formulation three times at 1-week intervals. For control, plants were treated with water. The experimental design adopted a complete randomized design with ten replicates for each sprayed treatment. Inoculation of *E. mallotivora* in papaya plants was conducted 1 week after the final formulation treatment. *E. mallotivora* was cultured in LB broth and grown at 28°C in an incubator shaker to OD₆₀₀ 1.0. About 10 mL of *E. mallotivora* culture at a concentration of 1 × 10⁶ colony-forming units (CFU) was injected into all seedlings at the level of the third node from the shoot.

2.5. Disease Index and Statistical Analysis

Evaluation of the progression of *E. mallotivora* infection was made through disease severity (DS) statistical analysis, with severity evaluated using a 5-point scale: 0 = symptomless, 1 = leaf vein blackening, 2 = leaf vein blackening + slight wilting, 3 = leaf stalk wilting, 4 = stem blackening, and 5 = plant death. Scoring was applied from days 3 to 21 post inoculation at 3-day intervals. The disease severity index (DSI) was computed based on a previous study (Abu Bakar, Mohd Zulfadli, Juri, & Johari, 2018). The experiments were repeated three times, and the

data were analyzed using analysis of variance followed by comparison means using Duncan multiple range tests (Samsuddin et al., 2018).

2.6. Leaf Sampling and Expression Profiling of PR Genes

Sampling of leaves was conducted on day 20 after the first application of HRPX foliar treatment. All plant samples were immediately immersed in liquid nitrogen and stored at -80°C until further analysis. Leaf tissues were ground to a fine powder and extraction of total RNA was conducted using the GeneJET Plant RNA Purification kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Removal of genomic DNA contamination was performed using DNase I (Thermo Fisher Scientific, USA).

Two micrograms of RNA was transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's protocol. Synthesized cDNA was used as template for real-time PCR for four reported PR genes in papaya (*NPR*, *PR1B*, *PR1D*, and *Osmotin*) (Norliza, Rafidah, & Mohd, 2016) and two housekeeping genes (*actin* and *40SRNP*) to normalize expression fold.

A list of the primers used as given in Table 1. Real-time PCR was performed using a 96-well-plate BIO-RAD CFX96 Real-time PCR System thermo cycler (Bio-Rad, Berkeley, CA, USA). The PCR reaction per well contained 2 μg of first-strand cDNA, 0.4 μM forward and reverse primers, and 1 \times master mix of SensiFAST SYBR No-Rox Kit (Bioline, UK). For negative control, sterile distilled water was added to the master mix rather than cDNA. Ten microliters of PCR reaction components were transferred to the wells, in triplicate. The PCR condition was set to 95°C for 2 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

Table 1. List of primers used.

Gene	Forward primer	Reverse primer	Size (bp)
Osmotin	TCTACTTGCCAAGCTCCAAA	GGGCAATTGTTCTTACTTGA	132
PR1d	CTCGGTTTCGTTTAGGGTGTG	TGGATTATTGGGAACTTTTATTGA	155
PR1b	CGTCCTTTTGGTTTCTGGGA	TTTTTGTTCGTTACTCACACATGA	151
NPR	CGCCTTTCAGAATCCAAGAG	ATCATGTTCCAGACGTGCAA	155
Actin	TTCCACTATGTTCCCTGGTATT	TCCTATCCAGACGCTGTATTTTC	119
40SRNP	TGGCAAAGCCTACAAAGACTATCA	AGGAATGGGAAGGGAGGGAGAT	78

3. RESULTS AND DISCUSSION

3.1. In Silico Characterization of HRPX

A previous study showed that the Type III Secretion System plays a major role during *E. mallotivora* pathogenesis (Juri et al., 2020). Hairpin or hrp proteins are part of the Gram-negative plant-pathogenic Type III Secretion System and have been shown to induce resistance in plants against various pathogens (Fontanilla, Montes, & De, 2005; Kim, Jeon, Oh, Moon, & Hwang, 2004; Liu et al., 2010).

Increased expression of *E. mallotivora* HRPX genes was shown during infection, which was further validated using real-time analysis (Abu Bakar et al., 2017; Juri et al., 2020). This gene was hypothesized to be a candidate gene for induction of SAR during subsequent infection. Thus research was initiated to further characterize the HRPX gene from *E. mallotivora*, which involved *in silico* characterization and protein function in induction of SAR for the management of papaya dieback disease.

The domain analysis of *E. mallotivora*'s HRPX protein sequence (ORF) showed that this hrp protein has a signal transduction histidine kinase motif, located at 240-483 of the c-terminal end of its amino acid sequence (Figure 1). Meanwhile, a BLASTp homology search in Genbank nonredundant databases revealed that this protein has the highest identity (80-99.8%) with PAS domain-containing sensor histidine kinase and PAS domain S-box protein from several *Pantoea* and *Erwinia* species (Table 2).

PAS domain-containing sensor histidine kinase is present in archaea, eubacteria, and eukarya. It is part of a two-component system (TCS), functioning as a protein kinase that phosphorylates a target protein in response to various signals. In bacteria, TCSs are pairs of proteins with a modular structure comprising a sensing (input) domain at the N-terminal, and a C-terminal conserved kinase core (transmitter) domain that interacts with a response regulator protein (RR), where the RR protein is usually a transcription factor. Activation of the RR triggers specific downstream responses (Merighi, Majerczak, Stover, & Coplin, 2003).

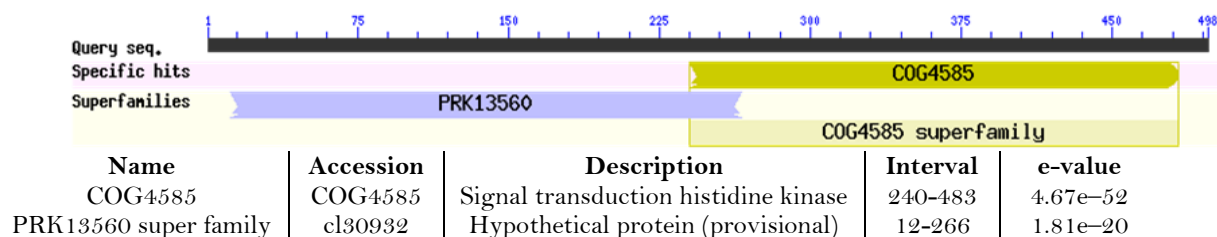


Figure 1. Domain hits.

Table 2. BLASTp analysis of *E. mallotivora* HRPX.

Description	Query cover (%)	e-value	Per. ident. (%)	Accession no.
PAS domain-containing sensor histidine kinase (<i>E. mallotivora</i>)	79	0.0	100.00	WP_034936536.1
PAS domain-containing sensor histidine kinase (<i>Pantoea</i> sp. VS1)	79	0.0	99.80	WP_088518176.1
PAS domain S-box protein (<i>Erwinia psidii</i>)	79	0.0	91.57	WP_124231880.1
PAS domain S-box protein (<i>Pantoea agglomerans</i>)	79	0.0	90.36	WP_158150184.1
PAS domain S-box protein (<i>Pantoea vagans</i>)	79	0.0	90.08	WP_061060958.1
PAS domain S-box protein (<i>Erwinia tracheiphila</i>)	79	0.0	86.37	AXF77209.1
PAS domain-containing sensor histidine kinase (<i>E. tracheiphila</i>)	79	0.0	86.17	WP_016191016.1
HRPX <i>P. agglomerans</i> pv. <i>gypsophilae</i>	77	0.0	84.98	AAF76212.1
PAS domain S-box protein (<i>Pantoea</i> sp. <i>paga</i>)	79	0.0	83.84	WP_143991671.1
PAS domain-containing sensor histidine kinase (<i>Erwinia amylovora</i>)	79	0.0	81.17	WP_004155353.1
PAS domain-containing sensor histidine kinase (<i>Erwinia tasmaniensis</i>)	79	0.0	81.38	WP_012440278.1
PAS domain-containing sensor histidine kinase (<i>Erwinia piriflorinigrans</i>)	79	0.0	80.97	WP_023653749.1
PAS domain-containing sensor histidine kinase (<i>Erwinia pyrifoliae</i>)	79	0.0	81.17	WP_012669312.1
PAS domain S-box protein (<i>Erwinia</i> sp. Ejp617)	79	0.0	80.97	WP_014543259.1
PAS domain S-box protein (<i>Pantoea</i> sp. PNA 03-3)	77	0.0	82.19	WP_110268456.1

3.2. Cloning, Expression, and Purification of Recombinant HRPX

A specific primer was designed to amplify HRPX from *E. mallotivora* (Figure 2A). The amplified HRPX gene was cloned into pGEM-T Easy vector and the presence of the HRPX in the vector was confirmed by colony PCR (Figure 2B). The integrity of the recombinant vector pGEM-T/HRPX was confirmed by double digestion using restriction enzymes *Bam*HI and *Hin*DIII (Figure 2C). The digested HRPX fragment was then successfully subcloned into the expression vector pET32b (Novagen), where the identity and orientation of HRPX in the construct was confirmed by sequencing. Cloned HRPX gene sequencing showed 100% homology with the reference sequence. Expression of the HRPX gene in the pET-20b expression vector was induced in the presence of IPTG. Proteins in the supernatant and cell lysate pellet were analyzed using SDS-PAGE (data not shown), with the majority of the expressed protein being detected in the pellet of cell lysate. The recombinant protein was successfully purified using a Ni-NTA column (Figure 3), having a molecular weight of approximately 50 kDa. Approximately 50–100 mg of pure recombinant protein is normally obtained from every 2-l culture of LB broth expression culture, induced with 0.5 mM IPTG, 37°C for 2 h. The amount of pure HRPX protein obtained was sufficient for the foliar application treatment of papaya seedlings to determine the effect of SAR inducement for increased tolerance to papaya dieback disease.

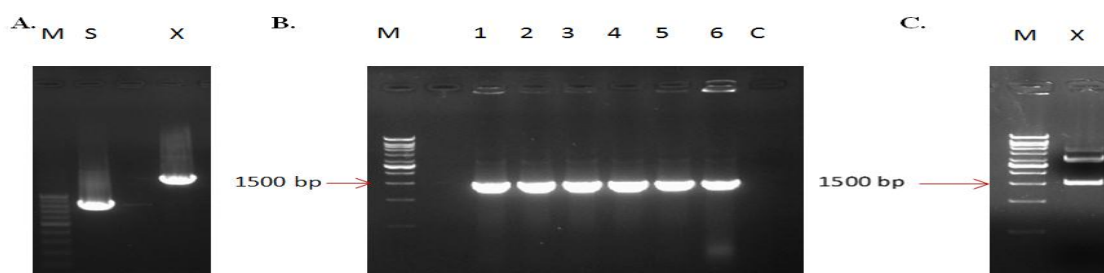
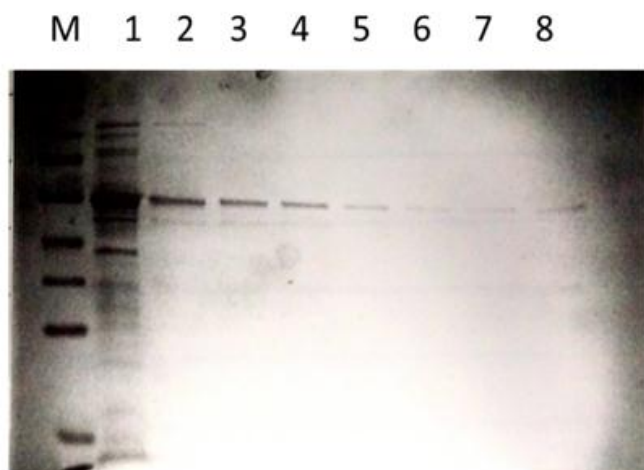


Figure 2. (A) Amplification of HRPX gene via PCR. A single gene was amplified from the PCR reaction. The band was excised, purified, and cloned into pGEM-T Easy vector. (B) Colony PCR. A single band was detected at the size expected for HRPX. M: 1-kb marker; 1–6: colonies; C: control (PCR reaction without colony). (C) The plasmid from the transformed colony was digested with the restriction enzymes *Bam*HI and *Hin*DIII, to determine the presence of the insert. M: 1-kb marker; X: HRPX.

A.



B.

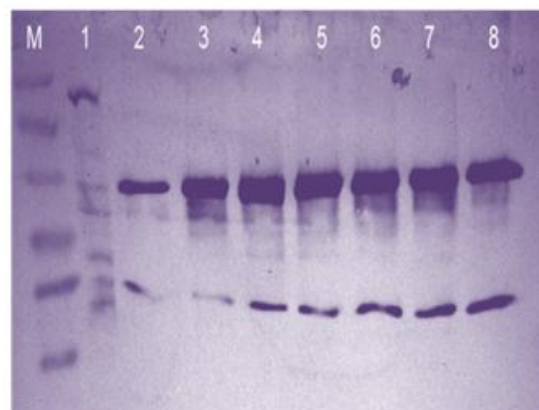


Figure 3. (A) SDS-PAGE and (B) Western blot showing the purity of HRPX protein after purification using a Ni-NTA affinity column. M: 1-KB marker; 1: HRPX before purification; 2–8: fractions collected after purification.

3.3. Pathogen Inoculation, Plant Disease Severity Assessment, and Profiling of SAR-responsive Genes

To investigate the capability of HRPX recombinant protein in enhancing papaya resistance against dieback pathogen, both the HRPX-sprayed and control plants were injected with approximately 10 mL of *E. mallotivora* culture at a concentration of 1×10^6 CFU, at the third node from the shoot. Overall, although both control and HRPX-sprayed plants were infected, from days 3–13 reduced disease symptoms were shown by the latter compared to the former. By day 13, control plants were totally infected and died (Figure 4).

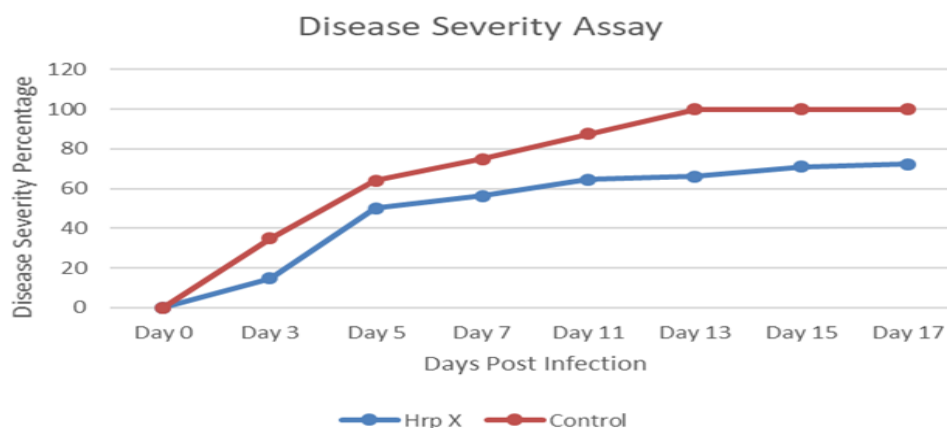


Figure 4. Disease severity analysis for assessment of the effect of recombinant HRPX treatment on papaya plants.

To investigate the effect of HRPX protein on the expression levels of defense-related genes, HRPX-sprayed and control leaves were harvested/collected and their expression was analyzed via real-time PCR. Leaf sampling was conducted one day after the third application of HRPX foliar spray treatment, when RNA was extracted (Figure 5A). The selected genes were *NPR*, *PR1B*, *Osmotin*, and *PR1D*. Overall, profiling analysis showed that all four genes were upregulated in response to HRPX treatment as compared to control (Figure 5B). The highest expression was recorded for *PR1D*, with ~13-fold expression in HRPX-treated plants compared to control. *PR1B* and *NPR* also showed significant increase in expression (~2- to 2.8-fold) in HRPX-treated plants, whereas an insignificant increase was seen for *Osmotin*, with ~1.7-fold expression. *NPR*, *PR1B*, *Osmotin*, and *PR1D* are pathogenesis-related genes previously reported as potential SAR markers (Abu Bakar et al., 2018). The activation of SAR markers suggested that HRPX recombinant protein had induced papaya plant resistance via a SAR mechanism. With regard to DS scoring, Figure 4, and the profiling of SAR-responsive genes, this result suggested that HRPX activates the SAR response against papaya dieback pathogen but is unable to enhance plant survival since it is a regulatory protein that does not directly interact with the host. It has been reported that in several necrogenic bacteria, including *Erwinia* and *Pantoea*, HRPX is one of two key regulatory genes (*HRPX1*) that are required/vital for activation of the expression of *hrp* operons, which will finally result in activation of promoters containing *hrp* boxes (e.g., the hairpin water-soaking *wts* and *hrp* secretion genes) (Merighi et al., 2003; Zhao, Wang, Nakka, Sundin, & Korban, 2009).

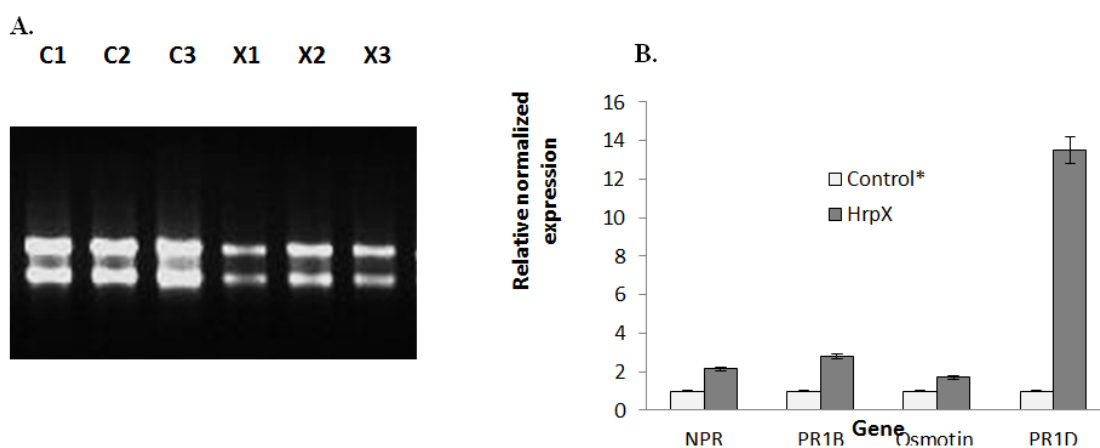


Figure 5. (A) RNA extracted from control (C1–C3) and treated (X1–X3) plants. (B) Expression analysis of papaya defense-related genes in control plants (non-sprayed) and recombinant HRPX foliar-sprayed plants.

The usage of recombinant protein to induce a hypersensitive response and SAR in tobacco was reported by Shen et al. (2019). In their research, a novel effector from *Bacillus subtilis* BU412, which was produced as recombinant protein, was shown to increase the production of reactive oxygen species (H_2O_2 and O_2^-) along with that of tobacco plant defense enzymes. Similar findings were recorded by Fontanilla et al. (2005) when tomato plants were sprayed with Harpin (Ea). Plants infected by *Phytophthora infestans* developed higher tolerance to the pathogen after subsequent foliar application of the protein (Fontanilla, Montes, & De, 2005).

Thus, for our future research, the expression conditions for HRPX will be optimized and the parameters determined for increased resistance against papaya dieback disease using recombinant HRPX. Concurrently, we will also assess the underlying mechanisms of HRPX stimulation in plant defense responses against papaya dieback disease for future application of the protein in the management of this disease.

4. CONCLUSIONS

Erwinia mallotivora HRPX was analyzed via *in silico* analysis, cloned, and expressed in an *E. coli* system and investigated to determine its ability to induce SAR in the management of papaya dieback disease. Foliar application of the HRPX recombinant protein was tested to evaluate its effectiveness in inducing SAR in papaya for enhanced disease resistance to the papaya dieback pathogen. Disease progression data recorded for disease severity assay indicate that plants thus treated were more tolerant to *E. mallotivora* infection, with reduced disease symptoms compared to control plants. Thus, HRPX has the potential for use as part of SAR inducer formulation in the fight to revitalize the Malaysian papaya industry against the notorious papaya dieback disease.

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