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Functional characterisation and evaluation of *Erwinia mallotivora* bifunctional chorismate mutase via TargeTron® gene knockout system

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Abstract

Erwinia mallotivora is the causal agent of papaya dieback disease, a devastating diseases affecting papaya production in Malaysia. Preliminary omics studies had successfully uncovered the key effectors of *Erwinia mallotivora's* (Em's) pathogenicity, where chorismate mutase (CM) was among the most prominent effectors in *E. mallotivora*. An in-depth in silico characterisation and functional analysis of the CM was carried out. Sequence analysis revealed that *E. mallotivora*'s CM protein contained two catalytic motifs; chorismate mutase type II (CM 2) and prephenate dehydratase (PDT) and a regulatory domain (ACT), where no signal peptide was detected in its sequence. Based on this characterisation, it is confirmed that our targeted CM is a non-secreted bifunctional chorismate mutase/prephenate dehydratase (Cmp). Sequence homology search showed that EmCmp is highly conserved (>80% identity) with various bifunctional Cmp from several *Erwinia* and *Pantoea* species (n ≥60). To confirm its role in Em virulency, a Cmp knockout mutant of Em (*EmΔCmp)* was generated via TargeTron® Gene Knockout system. The *EmΔCmp* strain significantly loss its virulency to papaya seedlings as compared to the wild *E. mallotivora* strain in the inoculation assay, revealing the evidence of EmCmp function and importance as the target gene for future research towards disease management strategy in papaya.

Keywords: *Erwinia mallotivora, papaya, chorismate mutase, knock out*

Introduction

Whether there are susceptible or resistant to plant phytopathogenic diseases, plants are prone to pathogen attacks that affect global food security and plant ecology. There are myriads of pests and pathogens that can be among other insects, fungi, bacteria or viruses. In response, plants develop several defence mechanisms to survive many potential aggressions from these biotic threats efficiently. The threat of emerging pathogens causing plant disease occurs within a particular ecology and ecosystem, either naturally or through the introduction to their plant host by the individual due to climate changes and the evolutionary evolvement of a pathogen (Nejat et al. 2017). Sustainable management of pests and diseases to ensure uninterrupted agricultural productivity and food supply to the world's populations is vital to sustaining agriculture production (Anderson et al. 2016). Control of these plant pathogens against the backdrop of human-population growth and climate change is thus essential for improving food security.

Papaya (*Carica papaya* L.) used to be among the top economically significant tropical fruit grown for local consumption and export in Malaysia; covering 6.9% of the global export market in 2016. This comes before the devastating disease affecting the Malaysian papaya industry. Papaya dieback disease is triggered by *Erwinia mallotivora*, a phytopathogen (Maktar et al. 2008; Mohd Taha et al. 2019). *Erwinia mallotivora (*Em*)* is a part of the Enterobacteriaceae family, a gram negative bacterium that causes dieback disease in papaya. One of the prominent characteristics of *E. mallotivora* infection is the appearance of black spots and water soaked lesions on the papaya fruits, leaves and stems. It causes papaya dieback disease in *Carica papaya* plants through the secretion of several virulence effectors, although the exact mechanism of the pathogen's persistent infection is still under investigation (Redzuan et al. 2014; Juri et al. 2020). Disease management strategies which includes the pathogen sequencing, pathogen detection, genetic engineering strategy to produce plant resistance to the pathogen, transcriptomic and proteomic analysis of the

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papaya defence response to the *E. mallotivora* infection together with mutagenesis of selected *E. mallotivora* virulence factors were carried out with the aim to study this pathogen and control the disease to sustain the economic value of the important papaya crop (Sekeli et al. 2018; Juri et al. 2020; Tamizi et al. 2020; Abu Bakar et al. 2021;).

Differential gene expression studies showed several virulence factors that serve as a key factor in *E. mallotivora* adaptive and persistent mechanism of infection for its prolonged infection in papaya plants. One of the putative virulence factors detected to be highly expressed is the chorismate mutase. Chorismate mutase (CM) is a critical enzyme in the shikimate pathway responsible for the synthesis of aromatic amino acids. This enzyme catalyses chorismate to prephenate, which is an intermediate molecule for the biosynthesis of phenylalanine and tyrosine (Kast et al. 2000). Many bacterial CM are bifunctional enzymes that are fused to a prephenate dehydratase (PDT) for phenylalanine biosynthesis (Huccetogullari et al. 2019), a prephenate dehydrogenase for Tyrosine biosynthesis, or to 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase. Besides these prokaryotic bifunctional enzymes, monofunctional CMs occur in prokaryotes as well as in fungi, plants and nematode worms (He et al. 2021; Helmstaedt et al. 2001). Interestingly, chorismate mutase was also characterised as a part of virulence factors in several phytopathogens, such as in *Burkholderia glumae*, the causative agent of bacterial panicle blight in rice (Karki and Ham, 2014). The secretome analysis from *Xanthomonas citri* subsp. citri, a citrus canker pathogen, revealed chorismate mutase as a potential virulence factor (Ferreira et al. 2016). In *Xanthomonas*, this enzyme was identified as one of the seven unique conserved secreted virulence factors involved in immune dysfunction in plant pathogenic (Assis et al. 2017). In *Acidovorax citrulli,* chorismate mutase *was* not only involved in phenylalanine biosynthesis but also involved in virulence and other mechanisms, including biofilm formation, twitching motility, and stress tolerance (Kim et al. 2020).

The development of knock out mutants is important in determining the function of genes that play major roles during plant-pathogen pathogenesis. This is a key factor towards future prevention and treatment strategies for plant phytopathogen. Accordingly, mobile group II introns that exist in about 25% of bacterial genomes were exploited in gram-positive as well as gram-negative bacteria for the construction of knockout mutants in a recombination-independent fashion (Key and Fisher 2017). Group II introns are the main component of the TargeTron® system that has been used as the channel to modify targeted genes through gene insertions. TargeTron® recognise DNA target sites via RNA-DNA base-pairing interactions and site-specific binding reverse transcriptase. TargeTron®, particularly has been shown to be able to engineer difficult microorganisms like cellulolytic bacteria for functional application and knockout study.

Here, bifunctional *E. mallotivora*'s CM protein was targeted to be further characterised in our study as it was discovered to be highly expressed in media cultures of *E.mallotivora* that encourage virulence proteins expression. As part of future management studies, the functional characterisation of this protein was conducted using TargeTron® system and sequence characterisation via bioinformatic tools.

Materials and method

Schematic presentation of the activities for the functional characterisation and evaluation of *Erwinia mallotivora* bifunctional chorismate mutase via TargeTron® gene knockout system is shown in *Figure 1*.

Sequence analysis of EmCmp

The nucleotide and protein sequence of EmCmp was obtained from our previous transcriptomic sequencing and profiling study of *E.mallotivora* (Juri et al. 2020). BLASTp tools were used for the identification of the homologous sequences and structural analysis of EmCmp sequence in the NCBI non-redundant sequence database (http://www.ncbi.nlm.nih.gov/).

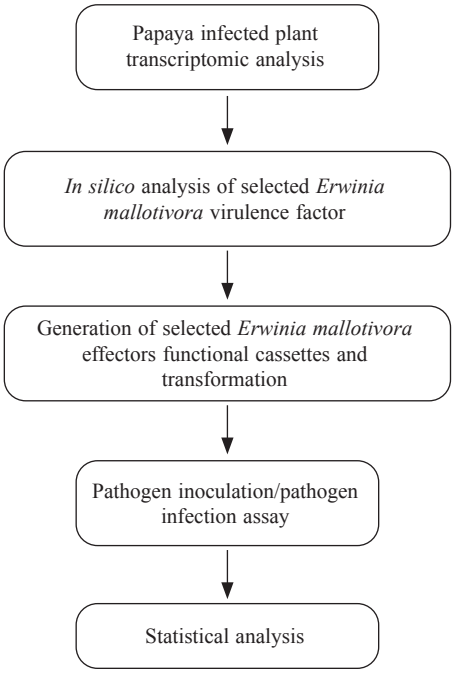


Figure 1. Schematic presentation of activities carried out for the functional characterisation and evaluation of Erwinia mallotivora bifunctional chorismate mutase via TargeTron® gene knockout system

Domain and protein 3D model prediction

Identification of the protein domain was carried out using the Motif finder server (http://www.genome.jp/tools/motif/). The 3D structure prediction of EmCM was made using SWISS-Model Workspace (https://swissmodel.expasy.org/) using the most well-matched template. Determination of the predicted structure was by the QMEAN program in the ExPASy server of SWISS-Model Workspace.

Subcellular localisation and transmembrane topology prediction

The TBpred and Gneg-mPLoc server was used to predict the Subcellular localisation of *EmChoMut*. The prediction for the presence of N-terminal signal peptides and transmembrane helices of EmCM was carried out using the Phobius server (http://phobius.sbc.su.se/).

Generation of EmCmp knockout mutant (ΔEmCmp)

To validate the importance of Cmp during pathogenesis, the generation of the EmCmp knockout mutant (*ΔEmCmp*) was carried out with the TargeTron® Knockout system. Recombinant DNA technology protocols were followed according to Sambrook et al. (1989). Polymerase chain reaction (PCR) was performed with Hot start Taq DNA polymerase (Qiagen, Hilden, Germany). TargeTron® algorithm (Sigma) design accessible at http://www.sigmagenosys.com/targetron/ was used to obtain potential insertion sites. The combination of gene-specific and intron-specific primers named IBS, EBS1d and EBS2 (*Figure 5*) was used to synthesise PCR fragments to generate functional cassettes as directed by the TargeTron® manual (Velázquez et al. 2029). The primers were synthesised by First Base (Malaysia).

A 350 bp product with XhoI and BsrGI sites was obtained. Restriction enzymes *Xho*I and *BsrG*I (New England Biolabs) were used to digest the PCR product being ligated into the pACD4K-C linear vector and transformed into *E.coli* DH5α strain. Transformants were selected on LB agar plates containing 50 µg/ml kanamycin. The selection of potential target site and generation of *ΔEmCmp* functional cassettes were carried out using PCR and sequencing analysis before the generation of the targeted *ΔEmCmp* in Em.

Erwinia mallotivora competent cells for electroporation were generated based on a modified method by Abdelhamed et al. (2013) To develop a *ΔEmCmp* strain, the *E. mallotivora* was cultured at 28 °C until its growth reached 1.0 at 600 nm. Centrifugation of the bacteria was carried out at 12,000 g for 2 min before washing with 0.5 M sucrose. The pellet was resuspended in 0.5 M sucrose. One microgram of the pAR1219 and the plasmid construct for *ΔEmCmp* were electroporated into *E. mallotivora* cells using a Bio-Rad micropulse (Bio-Rad) at 2.5 kV. The transformed bacteria were plated on LB agar plates containing kanamycin at 30 °C.

Evaluation of ΔEmCmp via disease severity assay

To determine the mutant activity, both the wild and knockout mutant (*ΔEmCmp*) of *E. mallotivora* strains were grown in LB broth at 28 °C incubator shaker until reaching OD600 of 1.0. The papaya plants were grown and maintained in MARDI. The plants were planted individually in pots. The mutants screening was carried out using 4 month old seedlings of *Carica papaya* (Eksotika I). Three biological replicates were used for each control and treatment (Tamizi et al. 2020). Inoculation of both strains was carried out via injection method. Around 5 mL of *E. mallotivora* culture was injected at the area of 15 cm from the shoot area of the stem at the concentration of 1 x 10^8 cfu. Sterile water-injected plants were included as a control. Data on disease symptoms and progression were monitored until day 20 and extended to day 30 after the inoculation (dpi) for the mutants. The evaluation for disease severity uses the 5-stage scales, which is; 0 = symptomless, 1 = leaf vein blackening, 2 = leaf vein blackening + slightly wilting, 3 = leaf stalk wilting, 4 = stem blackening and 5 = plant died. The disease severity index (DSI) was computed following previous study by Abu Bakar et al. 2018. The data were analysed for significant differences via SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA) after three rounds of experiments.

Results and discussion

TargeTron® technology which immobilises mobile group II introns to recognise target DNA sites through RNA-DNA base-pairing and interactions, is a feasible, flexible and efficient tool for targeted mutagenesis and gene disruption of microorganisms. TargeTron® is also a gene editing technique apart from the CRISPR-Cas system.

Previously, our research has shown bifunctional Cmp as one of the important *E. mallotivora* effectors during the onset of papaya dieback disease (Abu Bakar et al. 2017 and Juri et al. 2020). Chorismate mutase activity was shown to be essential in bacteria, fungi and plants, where several of these enzymes have been well investigated and characterised. Although the function of this protein in most organisms is similar, the sequence and overall structure of chorismate mutase proteins that catalysed this reaction can be varied in different species and organisms (Westfall et al. 2014). Thus, it is empirical to characterise the sequence via *in silico* sequence characterisation.

IBS1/2	AAAAAAGCTTATAATTATCCTTAGAATACACAGGCGTGCGCCCAGATAGGGTG
EBS1/delta	CAGATTGTACAAATGTGGTGATAACAGATAAGTCACAGGCTGAAACTTACCTTTCTTTGT
EBS2	TGAACGCAAGTTTCTAATTTCGATTTATTCTCGATAGAGGAAAGTGTCT

Figure 2. Primers for ΔEmChoMut used in an overlap PCR reaction

In silico analysis of EmCmp

The EmCmp gene sequence was 1,122 bp long and encoded 386 amino acids. The homology search of EmCmp protein sequence showed a close and high identity hit (≥80%) with more than 60 bifunctional chorismate mutase/prephenate dehydratase sequences of other *Erwinia* and *Pantoea* species (*Table 1*), which both of the bacteria species are under enterobacteria family. The highest identity was recorded in *Erwinia psidii* (90%), *Erwinia endophytica* (86%) and *Pantoea sp. BAV 3049* (85%). This indicates that CM protein sequence is highly conserved, especially among the accounted *Erwinia* and *Pantoea* species.

Another advantage of *in silico* analysis and molecular modelling studies of a targeted gene sequence is the ability to predict the catalytic mechanism of EmCmp based on the sequence characterisation. Insights into this enzyme were further carried out using structural analyses and molecular modelling studies. Analysis of the EmCmp protein sequence has classified this protein under the "bifunctional P-protein, chorismite mutase/prephenate dehydratase" family. This protein family plays a central role in phenylalanine biosynthesis that contains two catalytic motifs named chorismate mutase type II (CM 2) and prephenate dehydratase (PDT), and additional of a regulatory domain (ACT) (*Figure 3*). Each motif was detected once, with CM 2 detected from residues 1 to 92, PDT from residues 105-285 and ACT from residues 299-376. Also known as P-protein, the CM 2 (EC: 5.4.99.5) domain catalyses the chorismate to prephenate, while the PDT domain is involved in the process of prephenate to phenylpyruvate. Phenylpyruvate is subsequently transaminated via aromatic amino acid aminotransferase to phenylalanine. This enzyme is allosterically (negatively) regulated by the concentration of these amino acids (tyrosine, tryptophan and phenylalanine) (Liu et al. 2015)

Based on the signal peptide predictor (Phobius; http://phobius.sbc.su.se/), EmCmp contains no signal peptide. Thus, the protein is not destined toward the secretory pathway. No transmembrane segment was also detected for this protein as predicted by the prediction of transmembrane helices in proteins software (TMHMM Server v. 2.0; http://www.cbs.dtu.dk/services/TMHMM/) and supported by the gene ontology function as involved in the biological process of chorismate metabolic process (GO:0046417) and L-phenylalanine biosynthetic process (GO:0009094) with the molecular activity of prephenate dehydratase (GO:0004664) and chorismate mutase activity (GO:0004106).

Based on the presence of the three types of domains and un-involvement in the secretory pathway, our target EmCmp is confirmed to belong to non-secreted CM (CM-*nonsec*). A genomic study on pathogenic species of the Xanthomonadaceae family identified two CM genes, named non-secreted CM (CM-*nonsec*) and secreted CM (CM-*sec*). CM-*non*ces has three domains: an N-terminal chorismate mutase (CM), internal prephenate dehydratase (PDT) and a C-terminal Phe regulatory (ACT) and is responsible for aromatic amino acid synthesis for bacteria metabolism. Whereas the CM-*sec* has only a CM domain and a signal peptide and is secreted into host plants. (Assis et al. 2017).

The 3D model prediction of EmCmp was conducted using SWISS-MODEL (https://swissmodel.expasy.org/). The Cristal structure of L-Phe inhibited prephenate dehydratase from *Chlorobium tepidium* TLS was used as a template to generate the 3D model. The ligand-binding site is labelled with green colour while the active sites are in purple (*Figure 4*). The orange colour is the motif of CM 2, while the yellow is the PDT motif.

Construction and molecular validation of *ΔEmCmp* strain

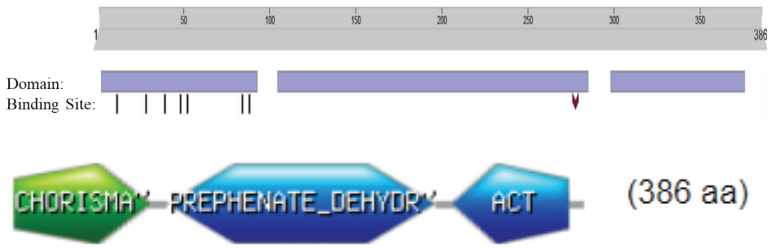
E. mallotivora is a gram-negative bacterium of the Enterobacteriaceae family that is responsible for the outbreak of papaya dieback disease in Malaysia. One of the striking features of *E. mallotivora* infection is the greasy, water-soaked lesions and spot-on leaves. The mechanism of persistent infection and the reasons for the host immune system fails to fight the pathogen are not well understood. One hypothesis is that differential gene expression is an important adaptive mechanism used by *E. mallotivora* to support its continued survival in the papaya plant. One way to test this hypothesis is by performing mutational analysis.

Recently, a novel gene editing method called 'TargeTron® Gene Knockout System' that is based on the modified group II intron insertion strategy with the aid of site-specific binding reverse transcriptases was developed (Liu et al. 2015). Around 25% of bacterial genomes have been shown to have mobile group II introns. This mobile group II introns jump between genes through a retrotransposition mechanism mobile with the aid of an intron-encoded protein (IEP) possessing RNA maturase, endonuclease, and reverse transcriptase activity. They function by inserting directly into a DNA target site and later with the help of the associated intron-encoded protein, which will then reverse transcribed them (Cerisy et al. 2019). This method appears to effectively create mutations in a wide range of gram-positive and gram-negative bacterial organisms.

As the TargeTron® system has been successfully employed in a variety of gram-positive, we hypothesised that this technology should allow for targeted, selectable gene inactivation for the CM gene. The construction of *ΔEmCmp* was carried out using TargeTron® Gene Knockout System. Identification of binding Lltr group II intron site within the EmCmp gene sequence for the generation of mutated PCR products was carried out using the algorithm located at Sigma Aldrich TargeTron® website (www.sigmaaldrich.com/targetron). Intron target recognition is achieved by the insertion of the intron (labelled as EBS2, EBS1 and δ) into the target gene between the IBS1 and 'δ' sites (*Figure 5*). The assembly of the PCR fragment in splicing by overlap PCR reaction was carried out using the generated primers identified from the

Table 1. Summary of BLASTp Analysis of *Erwinia mallotivora* chorismate mutase sequence

Scientific name	Max score	Total score	Query cover	E value	Per. ident	Acc. Len	Accession
Erwinia mallotivora	800	800	100%	0	100	386	WP_034936009.1
Erwinia mallotivora	793	793	100%	0	98.7	386	WP_261641554.1
*Erwinia psidi*i	718	718	99%	0	90.1	386	WP_124234252.1
Erwinia psidii	716	716	99%	0	89.84	386	WP_267443847.1
*Erwinia endophytic*a	665	665	99%	0	86.2	386	WP_152322184.1
Pantoea sp. BAV 3049	682	682	99%	0	85.68	386	WP_158784118.1
Erwinia sp. ErVv1	679	679	98%	0	85.6	386	WP_067707880.1
Pantoea sp. IMH	681	681	98%	0	85.34	386	WP_024965495.1
Erwinia sp. 198	685	685	99%	0	85.16	386	WP_125287477.1
Erwiniaceae	679	679	99%	0	85.16	386	WP_105593994.1
Erwinia persicina	685	685	99%	0	84.9	386	WP_253459013.1
Pantoea coffeiphila	678	678	99%	0	84.9	386	WP_205066126.1
Erwinia tracheiphila	680	680	98%	0	84.82	386	WP_233478874.1
Pantoea sp. CCBC3-3-1	678	678	98%	0	84.82	386	WP_147199474.1
Erwinia tracheiphila	678	678	98%	0	84.55	386	WP_016191810.1
Erwinia typographi	674	674	100%	0	84.46	386	WP_034892835.1
Erwinia phyllosphaerae	681	681	99%	0	84.11	386	WP_246761903.1
Erwinia sp.	678	678	99%	0	84.11	386	HBV38522.1
Erwinia	680	680	100%	0	83.94	386	WP_053143930.1
Erwinia billingiae	678	678	100%	0	83.68	386	WP_013203479.1
Erwinia sp. JUb26	670	670	99%	0	83.59	386	WP_123330822.1
Erwinia tasmaniensis	665	665	99%	0	83.07	386	WP_012442355.1
Erwinia persicina	660	660	99%	0	82.81	386	WP_191934033.1
Pantoea agglomerans	660	660	99%	0	82.81	386	WP_200643251.1
Erwinia sp. J316	662	662	100%	0	82.64	386	WP_154753684.1
Erwinia sp. J780	662	662	100%	0	82.64	386	WP_156286983.1
Erwinia piriflorinigrans	660	660	99%	0	82.55	386	WP_023654088.1
Erwinia	658	658	99%	0	82.55	386	WP_133845974.1
Erwinia amylovora	660	660	99%	0	82.29	386	WP_004155958.1
Erwinia iniecta	656	656	99%	0	82.29	386	WP_052900053.1
Erwinia amylovora	658	658	99%	0	82.03	386	WP_099289506.1
Erwinia persicina	658	658	99%	0	82.03	386	WP_137268988.1
Erwinia amylovora	657	657	99%	0	82.03	386	WP_168395032.1
Erwinia persicina	657	657	99%	0	82.03	386	WP_062744150.1
Erwinia	655	655	99%	0	82.03	386	WP_012669018.1
Erwinia amylovora	659	659	99%	0	81.77	386	WP_168426715.1
Erwinia amylovora	658	658	99%	0	81.77	386	WP_004168823.1
Erwinia toletana	657	657	99%	0	81.77	386	WP_017803679.1
Erwinia	655	655	99%	0	81.77	386	WP_099753848.1
Erwinia pyrifoliae	653	653	99%	0	81.77	386	WP_104945026.1
unclassified *Erwinia*	655	655	99%	0	81.51	386	WP_056232263.1
Winslowiella arboricola	655	655	99%	0	81.51	386	WP_267142472.1
bacteria symbiont BFo1 *of Frankliniella occidentalis*	653	653	99%	0	81.51	386	KMV69141.1
Erwinia sp. 9145	647	647	98%	0	81.41	386	WP_034916508.1
Erwinia oleae	645	645	98%	0	81.15	386	WP_034947885.1



Prosite		Domain	Binding site
CHORISMATE_MUT_2 (PS51168)	Chorismate mutase	1 – 92	11, 28, 39, 48, 52, 84, 88
PREPHENATE_DEHYDR_3 (PS51171)	Prephenate dehydratase	105 – 285	278
ACT (PS51671)	ACT	299 – 376	-

Figure 3. Motif of Erwinia mallotivora chorismate mutase based on analysis conducted using Motif finder; http://www.genome.jp/tools/motif/)

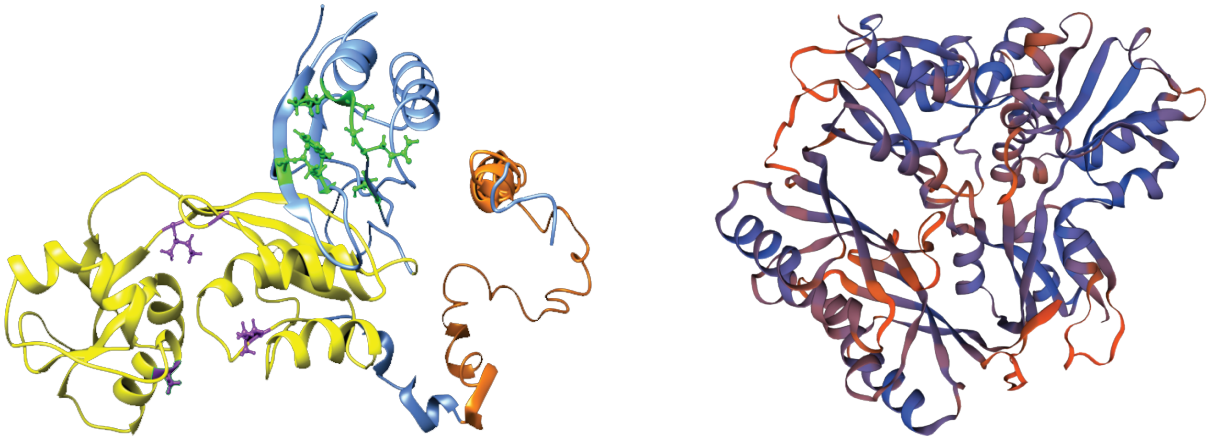


Figure 4. 3D Model of Erwinia mallotivora chorismate mutase using I-TASSER software (I-TASSER; https://zhanglab.ccmb.med.umich.edu/I-TASSER/)

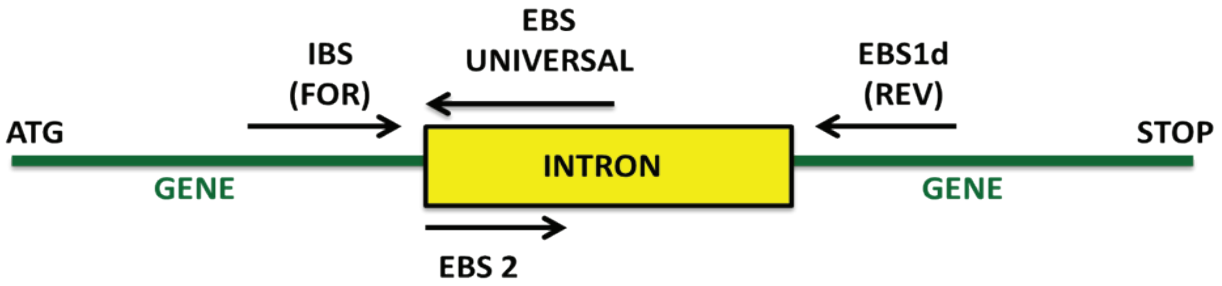


Figure 5. The schematic presentations of the production of EmCmp functional cassettes for targeted gene disruption and splicing by overlap extension PCR

analysis. The production of EmCmp functional cassettes for targeted gene disruption and splicing by overlap extension PCR was carried out, as shown in *Figure 5*. The PCR utilising the primers in *Figure 2* to mutate intron at several positions generated a PCR fragment of 350 bp in size with *Hind*III and *BsrG*I restriction enzymes at the 5' and 3' UTR region. Plasmids containing the targeted fragments were amplified and digested with *Hind*III and *BsrG*I restriction enzymes and cloned into the pACD4K-C linear vector to generate a construct for the targeted *ΔEmCmp*.

In nature, the T7 RNA polymerase was not expressed in the *E. mallotivora*. In order to express the intron and interrupt the chromosomal genes in Em, the plasmid pAR1219 containing the T7 promoter under the control of the IPTG inducible lac UV5 promoter was controlled and transformed into the *E. mallotivora* strain. This will allow the mutation of the CM gene in the genome of the bacteria. The effector's functional cassette carrying the mutated CM gene was successfully transformed into the *Erwinia mallotivora*-pAR1219 strains. The selection of positive transformants with mutations was carried out using kanamycin selection plates whereby positive *ΔEmCmp* were detectable after 48 hours of growth.

Further validation of the intron insertions was carried out using PCR analysis. Analysis of genomic DNA

isolated from the transformed colonies carried out through PCR analysis of gene-specific and intron-specific primers verified the presence of the targeted mutation in the pathogen. Verification using specific primers for the pathogen was also carried out to confirm the *ΔEmCmp* mutants. *E. mallotivora* HrpN, Isochorismate mutase and HrpS genes were detected in the *ΔEmCmp* mutants. Further proof of the mutants was carried out by DNA sequencing (data not shown).

Analysis of *E. mallotivora* mutants using *E. mallotivora* mutant's specific primers was conducted to verify and confirm that the *ΔEmCmp* mutants were targeted in *E. mallotivora* strains. The targeted primers were designed based on the TargeTron® linker and generated 350 bp bands in positives colonies that have been retargeted by the intron as depicted in *Figure 6*.

Confirmation of chorismate mutase involvement in Em pathogenicity

Cmp deletion mutant of *E. mallotivora* (*ΔEmCmp*) was successfully generated via TargeTron® Gene Knockout System. To evaluate and as proof of evidence of the contribution of Cmp in *E. mallotivora* pathogenicity, wild-type *E. mallotivora* and *ΔEmCmp* strain was inoculated to papaya seedlings.

The progression of disease severity of both *E. mallotivora* strain infections was represented in *Table 2*. The symptom on wild-type *E. mallotivora* -infected plants started to appear on day 3 of post-inoculation. The leaf vein started to blacken at day 6 and by day 9, the leaf was shown to be further blackened and slightly wilting. On day 12, the leaf stalk started to wilt and the stem started to blacken on day 16. The progression of the disease continued until day 20, when at this stage, the plants were shown to be severely infected and ultimately died.

Interestingly in the study using the *ΔEmCmp*, the results differ from the papaya infected with wild-type *E. mallotivora*. No symptoms were observed until day 15 of post inoculation. The leaf vein started to blacken with slight wilting from day 20 until day 25. However, on day 30, the leaves with symptoms started to fall off the tree, and only healthy leaves remained. In the end, all the trees survive from infection. The Cmp knockout mutation clearly led to reduced *E. mallotivora* virulence in infected papaya. This result confirmed the model for functional studies to determine important *E. mallotivora* virulence genes and established the importance of Cmp for *E. mallotivora* pathogenicity.

It can be postulated that the mutation of Cmp gene leads to disruption of the *E. mallotivora* cell membrane and wall integrity as well wall formation. The comparative proteomics analysis conducted between wild with the mutated Cmp *Acidovorax citrulli* (Ac) identified that an abundance of proteins involved in cell wall/membrane/envelop biogenesis was altered. In addition, proteins classified in group U (intracellular trafficking and secretion) were detected only in AcDcmpAc. These results implicate that impaired CmpAc in Ac may alter membrane integrity and/or secretion systems. Therefore, it can be postulated that the abnormal cell membranes may be weakened and are easily broken during the protein extraction steps, and hence, proteins related to cell wall/membrane/envelop biogenesis and intracellular trafficking and secretion were abundantly found in the comparative proteomic analysis. Similar to *S. pneumonia* (Qin et al. 2013), it is also hypothesised that the weakened cell membrane/wall may be responsible for the higher biofilm formation observed in AcDcmpAc compared with other strains.

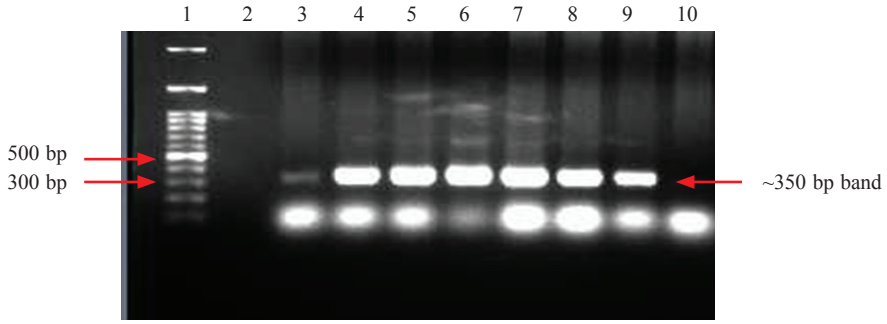
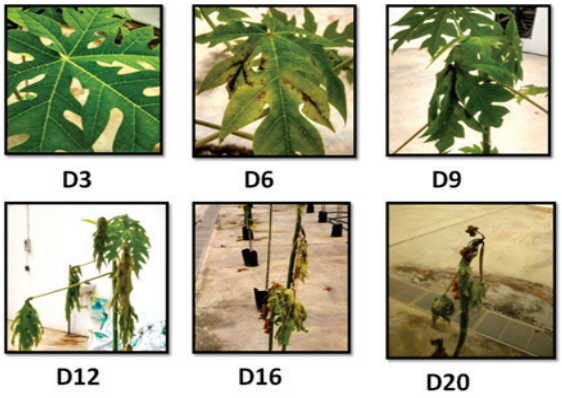
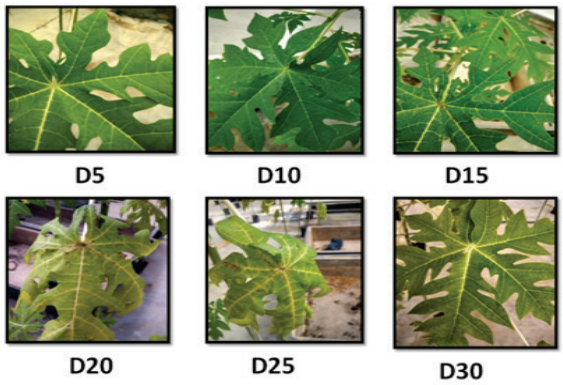


Figure 6. Analysis of Erwinia mallotivora mutants using TargeTron® Construct Specific Primers. The targeted primers were designed based on the TargeTron® linker and generated 350 bp bands in positives colonies (lane 3-9). Lane 1 depicts 100bp marker, Lane 10 is the negative control (PCR reaction without any sample).

Table 2. The dieback disease severity scoring in papaya seedlings inoculated with wild and CM knockout mutant (Δ*EmCM*) of *E. mallotivora*. (Stage 0 = symtompless, Stage 1 = leaf vein blackening, Stage 2 = leaf vein blackening + slightly wilting, Stage 3 = leaf stalk wilting, Stage 4 = stem blackening and Stage 5 = plant died).

Strain of *Erwinia mallotivora*	Scoring of infection						
	Day-3	Day-6	Day-9	Day-12	Day-16	Day-20	Day-30
Wild type	1	2	2	3	4	5	5
							
Knockout mutant (Δ*EmCM*)	0	0	0	0	1	2	0
							

Conclusion

This present study has explored the structural properties of the chorismate mutase gene identified from *E. mallotivora* the pathogen for papaya dieback disease. Here, we have generated the *in silico* information of this gene based on its sequence. Chorismate mutase genes were characterised using bioinformatic tools and were shown to contain all elements of the chorismate mutase motif. Further studies to characterise the genes and their function were carried out via functional mutation. Modified group II intron was successfully used to create mutations in the *E. mallotivora* chorismate mutase gene using the TargeTron® gene knockout system. In support of driving the expression of the modified group II introns in *E. mallotivora*, the plasmid expressing T7 polymerase was inserted upstream to the transcription start site. The constructs were then evaluated by transformation into *E. mallotivora*. Transformants with mutations were identified by the antibiotic selection, PCR analysis and validated via sequencing.

From our observation, plants with wild-type *E. mallotivora* strain infection exhibited increased symptoms of the infection for the next subsequent days which ended by causing death to the papaya plant. Meanwhile, the papaya plant infected with mutant knockout strains infection showed different polar symptoms from wild type infection. Even though infection symptoms appear to be somewhat late but, in the end, all the trees survive from infection. The findings of this study suggest that the genes (knockout strain) are made inoperative. Symptoms of the knockout mutant might be due to the different function each gene carry and how it affects the defence response mechanism. The outcomes of this project will have significant implications in defining the pathogenesis of *E. mallotivora*.

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