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ANALYSES OF PUTATIVE GENES INVOLVED IN THE DEFENSE MECHANISM OF GRACILARIA CHANGII IN RESPONSE TO AGARASE AND AGAROLYTIC BACTERIA TREATMENTS

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By

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August 2014

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Seaweed *Gracilaria* is the main source of agar worldwide. Information on the induced defense mechanisms of seaweed is scarce, particularly in the aspect of seaweed-microbe interactions. A set of differentially expressed genes from *G. changii* in response to agarase were generated from a previous study through next generation sequencing of the seaweed transcriptomes. In this study, *G. changii* was treated with both agarase (which generates the microbe-induced molecular patterns) and a marine agarolytic bacteria, ABS1 isolated from degrading seaweed. The result from the previous study (agarase treatment) was verified and temporal gene expression of candidate genes at 1, 6 and 24 hours post-treatment (hpt) in response to both agarase and bacteria treatments was profiled by quantitative reverse-transcription real-time PCR (qRT-PCR). A total of 20 out of 22 candidates verified have the same gene expression patterns as the next generation sequencing result, demonstrating a 90.9% positive correlation between the two analyses. Four candidates encoding plasma membrane calcium-transporting ATPase (*GcPMCA*), vanadium bromoperoxidase type 1 (*GcVBPO1*), 3-phosphoshikimate 1-carbpxyvinyltransferase (*GcEPSP*), and 12-oxophytodienoate reductase (*GcOPR*) showed more than 2-fold up-regulation compared to that of control samples upon agarase treatment, implying their importance in defense response. The gene encoding vanadium bromoperoxidase type 2 (*GcVBPO2*) showed more than 2-fold up-regulation compared to that of the control sample upon bacteria treatment. *GcVBPO1* and *GcVBPO2* displayed different expression profiles in response to the two treatments, indicating the existence of more than one signaling pathways in the transcriptional regulation of vanadium bromoperoxidase. The gene expression of 16 and 10 candidates were further profiled in agarase and bacteria treated samples at different time points, respectively. Most candidates were up-regulated at 1 hpt compared to that of the control sample at the same time point, indicating a rapid modulation of transcription in *G. changii* upon agarase treatment. The gene expression of these candidates displayed different expression profiles in bacteria treated samples. *GcEPSP* and *GcVBPO2*, were found to have the highest fold change when treated by agarase and agarolytic bacteria respectively, at 1 hpt. Temporal gene expression profile of candidates upon agarase and bacteria treatments in *G.*

changii indicated the occurrence of calcium signaling (at early stage), synthesis of tyrosine and phenylalanine through 4-hydroxyphenylpyruvate pathway, production of chorismate and jasmonic acid, increase of bromoperoxidation and heme peroxidation, and repression of photosynthesis upon pathogenic invasion. In conclusion, this study has provided further understanding on the gene expression of putative defense genes in *G. changii*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS GEN ANGGAPAN YANG TERLIBAT DALAM MEKANISME PERTAHANAN *GRACILARIA CHANGII* TERHADAP RAWATAN AGARASE DAN BAKTERIA YANG MENDEGRADASI AGAR

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Rumpai laut *Gracilaria* merupakan sumber agar yang utama di dunia. Maklumat mengenai mekanisme pertahanan teraruh rumpai laut adalah terhad, terutamanya dalam aspek interaksi rumpai laut dengan mikroorganisma. Satu set calon gen yang mempunyai ekpresi yang berbeza dalam *G. changii* yang bertindak balas terhadap agarase telah dijana daripada kajian terdahulu melalui penjujukan generasi baru ke atas transkriptom rumpai laut. Dalam kajian ini, *G. changii* dirawat dengan agarase (yang menjana corak molekul yang teraruh di mikrob) dan bakteria marin yang mendegradasi agar, ABS1 yang diasingkan daripada rumpai laut yang mereput. Keputusan daripada kajian terdahulu (rawatan agarase) telah disahkan dan ekspresi calon-calon pada 1, 6, dan 24 jam selepas rawatan agarase dan bakteria telah diprofil dengan kaedah kuantitatif tindak balas berantai polymeras masa nyata (qRT-PCR). Sebanyak 20 daripada 22 calon yang disahkan mempunyai corak ekspresi gen yang sama dengan keputusan penjujukan generasi baru, menunjukkan 90.9% korelasi positif antara kedua-dua analisis tersebut. Empat calon mengekodkan 'plasma membrane calcium-transporting ATPase' (*GcPMCA*), 'vanadium bromoperoxidase type 1' (*GcVBPO1*), '3-phosphoshikimate 1-carbpxyvinyltransferase' (*GcEPSP*), dan '12-oxophytodienoate reductase' (*GcOPR*) menunjukkan peningkatan ekspresi gen yang lebih daripada 2 kali ganda berbanding dengan sampel kawalan dalam rawatan agarase, membayangkan kepentingan mereka dalam tindak balas pertahanan. Gen yang mengekodkan 'vanadium bromoperoxidase type 2' (*GcVBPO2*) menunjukkan peningkatan ekspresi gen yang lebih daripada 2 kali ganda berbanding dengan sampel kawalan dalam rawatan bakteria. *GcVBPO1* dan *GcVBPO2* memaparkan profil ekspresi yang berbeza di bawah dua rawatan tersebut, mencadangkan kewujudan lebih daripada satu laluan isyarat dalam pengawalan transkripsi 'vanadium bromoperoxidase'. Ekpresi 16 calon dari rawatan agarase and 10 calon dari rawatan bakteria pada masa yang berbeza telah diprofil dengan lebih lanjut. Kebanyakan calon menunjukkan peningkatan ekspresi gen pada 1 jam selepas rawatan berbanding dengan sampel kawalan pada masa yang sama, mencadangkan pengawalan yang cepat dalam transkripsi *G. changii* di bawah rawatan agarase. Ekspresi gen calon-calon tersebut memaparkan profil ekspresi yang berbeza di bawah rawatan bakteria. *GcEPSP* dan *GcVBPO* masing-masing didapati

mencatatkan peningkatan ekspresi yang paling tinggi dalam rawatan agarase dan rawatan bakteria, pada 1 jam selepas rawatan. Keputusan profil ekpresi calon-calon dalam rawatan agarase dan bakteria pada *G. changii* pada masa yang berbeza menunjukkan berlakunya isyarat kalsium (pada peringkat awal), sintesis tirosina dan fenilalanina melalui laluan 4-hidroksifenilpiruvat, penghasilan korismat dan asid jasmonik, peningkatan proses pengoksidaan bromo dan heme, dan penindasan fotosintesis di bawah serangan patogen. Kesimpulannya, kajian ini telah meningkatkan pemahaman terhadap ekspresi gen yang dijangka terlibat dalam pertahanan *G. changii*.

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I certify that a Thesis Examination Committee has met on 28.08.2014 to conduct the final examination of Lim Ee Leen on her thesis entitled "Analyses of Putative Genes Involved in the Defense Mechanism of *Gracilaria changii* in Response to Agarase and Agarolytic Bacteria Treatments" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

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LIST OF ABBREVIATIONS

AA	ascorbate
AAA	aromatic amino acid
AO	ascorbate oxidase
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Cq	quantification cycle value
DAHP	3-deoxy-d-arabino-heptulosonate- 7-phosphate
dATP	deoxyadenosine triphosphate
DEPC	diethylpyrocarbonate
DHA	dehydroascorbate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPI	diphenylene iodonium
DSP	dual-specific protein phosphatase
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EPSP	5-enolpyruvylshikimate 3-phosphate synthase
ERCA	endoplasmic reticulum calcium-transporting ATPase
hpt	hour post treatment
IPTG	isopropyl-beta-D-thiogalactopyranoside
JA	jasmonic acid
LB	Luria-Bertani
LOX	lipoxygenase
LPLAT	lysophospholipid acyltransferase
MAMP	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
MEGA	molecular evolutionary genetics analysis
MeJA	methyl jasmonate

MIMP	microbe-induced molecular pattern
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
OD	optical density
p.p.t.	parts per thousand
PCR	polymerase chain reaction
Phe	phenylalanine
PMCA	plasma membrane calcium transporting ATPase
PUFA	polyunsaturated fatty acids
PUFA	polyunsaturated fatty acid
qRT-PCR	quantitative reverse-transcription real-time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SSW	synthetic sea salt water
TAE	Tris/ acetate/ EDTA
Trp	tryptophan
Tyr	tyrosine
VBPO	vanadium bromoperoxidase
VHOC	volatile halogenated organic compound
VHPO	vanadium-dependent haloperoxidase

CHAPTER 1

INTRODUCTION

"Seaweeds" (or macroalgae) are macroscopic, multicellular marine algae that usually dwell on the coastal region of oceans (Lobban and Harrison, 1996; Khan *et al*., 2009). They are photosynthetic but the absence of roots, leafy shoots, flowers, and vascular tissues distinguishes them from other marine plants (Diaz-Pulido and McCook, 2008). Generally, seaweeds are categorized into three groups, viz. red algae (Rhodophyte), green algae (Chlorophyte) and brown algae (Phaeophyte), primarily based on the composition of their photosynthetic pigments (Khan *et al*., 2009).

Rhodophytes are characteristically red in colour, which is caused by the red pigment phycoerythrin. They are the major source of agar and carrageenans (Chapman, 2013; Lopez-Bautista, 2010). The second largest genus of Rhodophyta is *Gracilaria*. A total of 20 species of *Gracilaria* have been recorded for Malaysia, and one of them is *Gracilaria changii* (Yow *et al.*, 2011). *G. changii* is an edible seaweed that is rich in omega fatty acid and various other nutrients essential for health (Norziah and Ching, 2000). It is also the main source of high-quality agar and agarose with good gel strength (Armisen, 1995; Phang *et al*., 1996). As the utilization for agar in various industries increases, expansion in the cultivation of *Gracilaria* is expected to meet the increasing demand. In light of the great commercial value of *Gracilaria*, diseases that threaten their cultivation are of paramount concern.

Seaweed is constantly being challenged by microorganisms such as viruses, bacteria, fungi and etc. (Potin *et al*., 2002; Cosse *et al*., 2008). Many bacteria that rely on seaweeds as a source of nutrients are able to enzymatically decompose seaweed cell wall. Their pathogenic attacks cause wounds and diseases on seaweed, generating symptoms such as rot, bleaching, lesion, and malformation (Weinberger, 2007). To overcome the attack of these pathogens, seaweeds have evolved a variety of defense mechanisms. Continuous synthesis of new cell wall and chemical deterrents are part of the defensive tactics of seaweeds against pathogens (Cosse *et al*., 2008; Weinberg and Potin, 2010).

Besides constitutive defense mechanisms, recent studies reveal defense of seaweed that varies with time or environmental factors, indicating the existence of induced defense mechanisms (Weinberger and Potin, 2010). This mode of defense mechanism is initiated by recognition of pathogenic invasion through the perception of pathogenic origin elicitors or host endogenous elicitors such as oligosaccharides (Weinberger *et al*., 1999). Endogenous elicitors of host are the cell wall fragments released during enzymatic attacks of pathogens (e.g. agarase). Upon recognition of the attack, induced defense mechanisms evoke a series of responses such as oxidative burst, emission of volatile halogenated organic compounds (VHOCs), production of oxylipins, synthesis of inducible secondary metabolites and etc. (Potin, 2008).

Information on the induced defense mechanisms of seaweed is scarce compared to terrestrial plants (Cosse *et al*., 2008). The majority of investigations on seaweed defense mechanisms focused on seaweed-herbivore interactions, while the seaweed-microbe interactions have been neglected (Weinberger, 2007). Furthermore, the seaweed-pathogen interactions have hardly been studied beyond the phenomenology of infection; the regulation of defense at molecular level remains largely unexplored (Weinberg and Potin, 2010). Hence, there is an urgent need to investigate the gene regulation involved in the induced defense mechanisms of seaweed in response to pathogenic invasion. A set of differentially expressed candidates from *G. changii* in response to agarase were generated from a previous study through next generation sequencing of the seaweed transcriptomes (unpublished data). In this study, the expression of those candidates in *G. changii* upon both agarase and agarolytic bacteria treatment was analyzed with quantitative reverse-transcription real-time PCR (qRT-PCR).

The objectives of this study were:

1) To verify the gene expression of candidate defense genes from *Gracilaria changii* in response to agarase treatment.
2) To compare the gene expression of candidate genes in response to agarase and agarolytic bacteria treatment.
3) To profile the gene expression of selected genes in response to agarase and agarolytic bacteria treatment at different time points.

The results from this study may provide further understanding on the defense mechanisms of seaweed in response to cell-wall degrading enzymes and agarolytic bacteria invasion. This may in turn facilitate prevention and action against bacterial disease in seaweeds.

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