การวิเคราะห์การแสดงออกของยีนและการผลิตรีคอมบิแนนต์ดีเฟนซินของข้าว *Oryza sativa* L.

นางสาวกมลวรรณ วีรวานิช

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GENE EXPRESSION ANALYSIS AND RECOMBINANT DEFENSINS PRODUCTION OF RICE *Oryza sativa* L.

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
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เปปไทด์ต้านจุลชีพเป็นเปปไทด์สายสั้น ๆ ที่โดยทั่วไปประกอบด้วยกรดอะมิโนน้อยกว่า 100 ตัว โดยมีกรดอะมิโนชิสเทอีนเป็นจำนวนมาก เปปไทด์เหล่านี้มีคุณสมบัติการต้านเชื้อแบบไม่จำเพาะต่อทั้งแบคทีเรียและรา ด้วยกลไกการเข้าทำลายเยื่อหุ้มเซลล์ของจุลชีพ ดีเฟนซินเป็นหนึ่งในกลุ่มเปปไทด์ต้านจุลชีพในพืช ที่สามารถพบได้ในข้าว *Oryza sativa* L. ก่อนหน้านี้ ดีเฟนซิน 7 และดีเฟนซิน 8 จากข้าวที่ผลิตในแบคทีเรียสามารถออกฤทธิ์ยับยั้งการเจริญของจุลชีพก่อโรคพืช ในงานวิจัยนี้จึงโคลนยีนทั้งสองและวิเคราะห์รูปแบบการแสดงออกโดยใช้ quantitative RT-PCR ในข้าวหอมมะลิไทย *Oryza sativa* L. ssp. *indica* cv. KDML105 แล้วนำมาเปรียบเทียบผลกับข้าวญี่ปุ่น *Oryza sativa* L. ssp. *japonica* แม้ว่าลำดับนิวคลีโอไทด์ของทั้งสองยีนในข้าวสองสายพันธุ์เหมือนกัน 100 เปอร์เซ็นต์ และมีรูปแบบการแสดงออกที่เหมือนกันในทุกช่วงอายุ แต่ระดับการแสดงออกในแต่ละอวัยวะแตกต่างกัน เมื่อใบข้าวมีการติดเชื้อของ *Xanthomonas oryzae* pv. *oryzae* ยีนดีเฟนซินทั้งสองจะมีการเพิ่มระดับการแสดงออกในวันที่ 8 หลังจากการติดเชื้อ แสดงถึงความเกี่ยวข้องกับการต้านเชื้อจุลชีพก่อโรคของยีนดีเฟนซิน นอกจากนี้ ผลจากการวิเคราะห์ *in silico* แสดงให้เห็นว่าความเครียดที่เกิดจากสิ่งไม่มีชีวิต ได้แก่ ความแห้ง ความเย็น การดูดซึมน้ำ การขาดออกซิเจน และ การขาดน้ำ ส่งผลกระทบต่อระดับการแสดงออกของยีนทั้งสอง เมื่อดีเฟนซิน 7 และ 8 ถูกเชื่อมต่อกับ GFP สามารถพบโปรตีนได้ในส่วนภายนอกเซลล์ อย่างไรก็ตามรีคอมบิแนนต์ดีเฟนซินไม่สามารถตรวจพบได้จากส่วนของอาหารเหลวที่ใช้เลี้ยงรากลอยของ *Nicotiana benthamiana* และเซลล์แขวนลอย BY-2 ของยาสูบที่มีการแสดงออกของยีนดีเฟนซิน ผู้วิจัยจึงได้ทำให้เกิดการแสดงออกเพียงชั่วคราวของดีเฟนซินในใบ *N. benthamiana* โดยใช้เทคนิค agroinfiltration และทดสอบฤทธิ์ต้านเชื้อจุลชีพของโปรตีนนี้ในพืช แม้ว่าโปรตีนที่ผลิตได้จากใบจะมีปริมาณต่ำแต่รีคอมบิแนนต์ดีเฟนซินทั้งสองมีฤทธิ์ในการยับยั้งเชื้อก่อโรคพืช *Xanthomonas campestris* pv. *glycines* ในเซลล์พืชได้ จากผลการทดลองแสดงให้เห็นว่าเปปไทด์เหล่านี้อาจถูกนำมาใช้ประโยชน์ในการควบคุมโรคพืชและ *N. benthamiana* สามารถใช้เป็นเจ้าบ้านทางเลือกในการคัดเลือกเปปไทด์ต้านจุลชีพอื่น ๆ ได้

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KAMONWAN WEERAWANICH: GENE EXPRESSION ANALYSIS AND RECOMBINANT DEFENSINS PRODUCTION OF RICE *Oryza sativa* L.. ADVISOR: ASST. PROF. SUPAART SIRIKANTARAMAS, Ph.D., CO-ADVISOR: ASST. PROF. WARANYOO PHOOLCHAROEN, Ph.D., 89 pp.

Antimicrobial peptides (AMPs) are small cysteine-rich peptides which generally contain less than 100 amino acid residues. They have a non-specific antimicrobial activity against bacteria and fungi by invading their cell membranes. Defensin (DEF) represents a group of AMPs in plants including rice *Oryza sativa* L. Previously, rice DEF7 (OsDEF7) and rice DEF8 (OsDEF8) were heterologously produced in bacteria exhibiting a growth inhibitory effect against several phytopathogens. In this study, both DEFs were cloned and analysed for gene expression patterns using quantitative RT-PCR in Thai jasmine rice, *Oryza sativa* L. ssp. *indica* cv. KDML105 comparing to the results of those Japanese rice *Oryza sativa* L. ssp. *japonica*. Although the nucleotide sequences of both genes in the two cultivars show 100% identity and similar gene expression pattern at the examined developmental stages was observed, their expression levels were shown to be different between organs. Upon *Xanthomonas oryzae* pv. *oryzae* infection in rice leaves, both *OsDEF*s showed high upregulation at 8 days post-infection suggesting their involvement in pathogen defense. In addition, *in silico* analysis also demonstrated that the expression levels of these two *OsDEF*s were affected by abiotic stress including drought, cold, imbibition, anoxia, and dehydration. Using GFP fusion approach, OsDEFs were demonstrated to localize to the extracellular compartment. However, the recombinant OsDEFs cannot be detected in culture medium when expressed in the hairy roots of *Nicotiana benthamiana* and tobacco BY-2 suspension cells. Subsequently, transiently expression of these peptides in *N. benthamiana* leaves was performed by agroinfiltration and *in planta* inhibitory activity of these proteins was tested. Even though the low levels of these proteins were detected in the leaves, both recombinant proteins exhibited *in planta* inhibitory activity against phytopatogens, *Xanthomonas campestris* pv. *glycines*. These results suggested that these peptides might be exploited further for disease control and *N. benthamiana* could be used as an alternative host for screening other antimicrobial peptides.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
		Co-Advisor's Signature	.....
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CHAPTER I
INTRODUCTION

1.1 Statement of problem

Thailand is an agricultural country that exports agricultural products to many countries all over the world. Rice (*Oryza sativa* L.) is one of the top exported products generating a great amount of income. However, rice cultivation has been facing many problems affecting the grain yield. One of the important issues is pathogen invasion causing rice diseases. Many farmers use chemical to deal with the problem but this can potentially affect health of both farmers and consumers, and the environment. There are many rice diseases caused by microbial infection such as brown spot disease infected by *Helminthosporium oryzae*, Fusarium wilt infected by *Fusarium oxysporum,* rice blast infected by *Magnaporthe grisea*, and bacterial blight infected by *Xanthomonas oryzae.* Alternative way that might solve this problem is to introduce antimicrobial peptide (AMP) to disease control. AMP is small and naturally peptides which play role as host defense peptides against microbial invasion. Natural AMP are found in both prokaryotic and eukaryotic organisms (Montesinos, 2007). Although plant AMP has been studied less than that from animals, many studies have confirmed that AMP overexpression showed significantly improved microbial resistance in plants (Carlson *et al*., 2006; Hammami *et al*., 2009b). From previous studies, Silverstein *et al*. (2007) have identified 825 and 598 genes encoding putative AMP in *Arabidopsis thaliana* and *O. sativa*, respectively. These high numbers of genes open an opportunity to discover potent peptides to be used in controlling diseases. However, most of those rice AMP has not been functionally characterized. Therefore, our group has been attempting to explore those rice AMP. Previously, we performed *in silico* analyses to screen potent rice AMP and reported that the recombinant rice AMP, namely OsDEF7 and OsDEF8, produced in *Escherichia coli* exhibit antimicrobial activity against

phytopathogens such as *X. oryzae* and *F. oxysporum* (Tantong *et al*., 2016). Therefore, in this thesis, the alternative system for these peptide productions changing from bacteria to plants since plant system provides cost-effective production, low cost for purification, and exhibit high scale-up capacity (Holaskova *et al*., 2015) were explored. In addition, gene expression profiles and subcellular localization of these peptides were performed to provide additional *in vivo* functions of both peptides.

1.2 Antimicrobial peptide (AMP)

In plants, the first-line of defense to prevent their cells from microbes is their physical barrier as known as cell walls composed of cellulose, lignin, cutin, and polysaccharides. Plants also produce secondary metabolites for protecting themselves from microbial pathogens and predators. Moreover, plants have their own small peptides that eliminate microbial invasion without requiring adaptive immunity (Kulkarni, 2006). This is known as AMP which is a small cysteine-rich peptide generally having less than 100 amino acids. Thus, AMPs are peptides that have ability to protect host cells from infection of any microbes, but do not include enzymes eliminating microbes (Maroti *et al*., 2011). It has a non-specific antimicrobial activity against bacteria and fungi by invading their cell membranes (Jenssen *et al*., 2006). It shows activity over other antibiotics because AMP targets to the cell membrane of pathogens making it harder for the AMP target to develop resistance.

Plant AMP has a function to protect plant from bacteria and fungi. AMP from *Oudneya africana* seed exhibited antibacterial activity against *E. coli, Listeria monocytogenes, Bacillus subtilis, Pseudomonas aeruginosa*, *Enterococcus hirae Staphylococcus aureus* and also yeast *Candida albicans* (Hammami *et al*., 2009b). In addition, there are many studies reported that AMP helps protecting plants from virus and insects (Castro and Fontes, 2005; Padovan *et al*., 2010). Because of the antimicrobial activity of AMP, transgenic plants overexpressing AMP were constructed. Metchnikowin peptide from *Drosophila melanogaster* was overexpressed in barley

showing antifungal activity against powdery mildew (Rahnamaeian and Vilcinskas, 2012). An AMP from *Pinus monticola* was overexpressed in canola exhibiting antifungal activity against *Leptosphaeria maculans*, *Sclerotinia Sclerotiorum* and *Alternaria brassicae* (Verma *et al*., 2012). Transgenic rice overexpressing either cecropin B from *Bombyx mori* (Sharma *et al*., 2000) or Np3, Np5 from *Fenneropenaeus chinensis* exhibited antibacterial activity against *X. oryzae* (Wang *et al*., 2011).

1.2.1 Types and structures of plant AMP

In plants, a specific group of defense-related proteins are cysteine rich peptides (CRPs) that are specifically represented in the plant kingdom. The arrangement and the number of cysteine residues in the primary structure distinguish AMP groups. Those cysteine residues form different patterns of disulfide bridges in secondary structure that can be classified into seven groups including defensin (DEF), thionin (THION), lipid transfer protein (LTP), hevein-type peptides, knottin-type peptide, cyclotide, and snakin (Hammami *et al*., 2009a) (Figure 1). Most of these peptides contain eight cysteine residues that form four disulfide bonds, which are responsible for stabilizing their three-dimensional structures and the folding of proteins (Carvalho and Gomes, 2009).

1.2.1.1 Defensin (DEF)

Normally, plant DEF contains 45-54 amino acids in the primary structure which consists of a basic mature peptide and an N-terminal acidic signal peptide. DEFs have been reported to display the antimicrobial activities against bacteria, fungi, yeast, and also insects. Previously, they are known as γ-thionins with an approximately size of 5 to 10 kDa. In general, DEFs consist of six to eight cysteines and conserve in almost all plants. These three dimensional DEFs are stabilized by four disulfide bridges. Their structures are quite resemble to each other by forming disulfide bond from two positions of cysteine for connecting three β-sheets with one α-helix. These disulfide bonds help stabilizing the protein structure (Tavares *et al*., 2008).

1.2.1.2 Thionin (THION)

THION is a short peptide around 45–48 amino acids with four to eight of cysteine residues. THIONs have been classified into three groups, α-, β-, and ϫ-THIONs by the pattern of disulfide bridges and β-sheet differently. Nevertheless, three-dimensional structures of α- and β-THIONs are similar. It has been found that their C-terminal domains are important for antimicrobial activity, as well as basic amino acids; histidine arginine and lysine (Stec, 2006).

1.2.1.3 Lipid transfer protein (LTP)

It is a basic peptide which contains 70 to 93 amino acids with four disulfide bridges forming by eight cysteine residues. There are two families of LTPs; LTP1 and LTP2 (Kader, 1996). Some LTPs have been confirmed for their three-dimensional structures demonstrating a flexible hydrophobic tunnel within the molecule that could bind to lipids at different sizes (Lerche *et al*., 1997; Charvolin *et al*., 1999) suggesting their associated function with transportation of lipids and sphingolipids via the cell membrane. LTP proteins are also involved in microbial resistance (Tavares *et al*., 2008).

1.2.1.4 Hevein-type peptide

This type of peptide is a small chitin-binding peptide which is structurally similar to hevein that is the peptide isolated from rubber latex (*Hevea brasiliensis*). These peptides form four disulfide bridges from eight cysteine residues. These AMPs are effective fungicides or plant defensive peptides against pests and microorganisms (Tavares *et al.,* 2008; Odintsova *et al.,* 2009) since the main composition of the cell walls of fungi and invertebrates is chitin.

1.2.1.5 Knottin-type peptide

This peptide type is formed by the inhibitor cystine knot (ICK) and a triple-stranded β-sheet stabilizing by the three disulfide bonds (Nawrot *et al*., 2014). The intramolecular bonds of knottins known as cystine knots which have multiple

biological functions in plants against any pathogens. There are a few peptides of this group exhibiting α-amylase inhibitor activity (Chagolla-Lopez *et al*., 1994; Gao *et al*., 2001). Knottins are formed as proproteins containing an N-terminus signal peptide that directs protein for secretion (De Bolle *et al*., 1996). Moreover, the knottin have been isolated from plants; PAFP-S from *Phytolacca americana* and Mj-AMP1 from *Mirabilis jalapa* L. exhibiting antifungal activities (Cammue *et al*., 1992; Gao *et al*., 2001). Besides, PA1b peptide from garden pea plays role as insecticides by inhibition of vacuolar ATPase (Chouabe *et al*., 2011).

1.2.1.6 Cyclotide

This group of AMP was firstly found in the coffee-family plant *Oldenlandia affinis* (Rubiaceae) (Gran, 1973). They are ribosomally synthesized CRPs that are cyclized head-to-tail backbone. They are small peptides approximately 30 amino acids with three disulfide bonds forming by six conserved cysteine residues. Their hydrophobic face locating on different regions of the peptide surface might affect the membrane disruption of pathogens (Kamimori *et al.,* 2005).

1.2.1.7 Snakin

Snakin is a basic AMP consisting of approximately 60 amino acid residues. These peptides form six disulfide bridges from twelve cysteine residues. Their amino acid sequences are similar to the members of gibberellic acid stimulated in *Arabidopsis* (GASA) family and the gibberellic acid stimulated transcript (GAST) family from tomato (Segura *et al*., 1999; Berrocal-Lobo *et al*., 2002). Moreover, plant hormones can control most of these genes suggesting their involvement in hormonal signaling pathways regulating hormonal levels and responses (Nahirñak *et al.,* 2012).

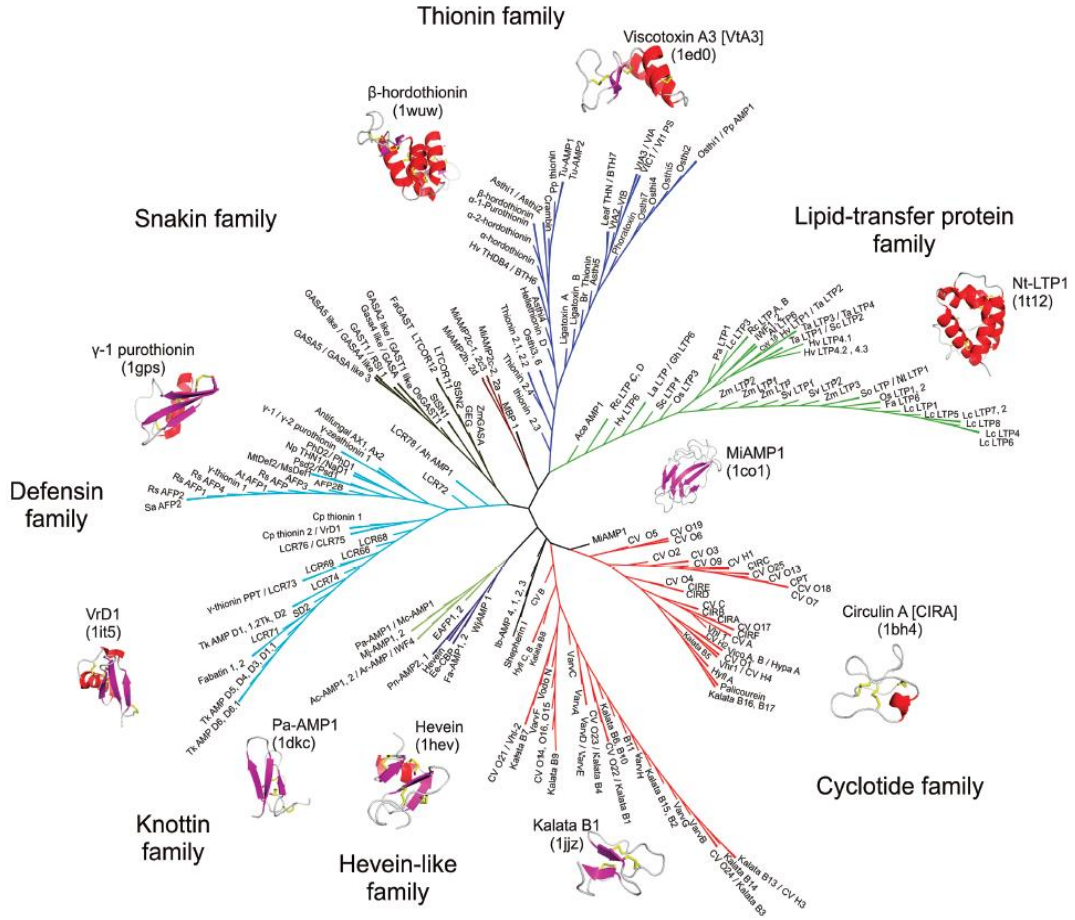

Figure 1. Phylogenetic tree of plant AMP groups (Taken from Hammami *et al.*, 2009a)

1.2.2 Mechanism of AMP

There are several models explaining the mechanisms of AMP interacting with membranes of microbial. Many AMPs have a potential to fold into amphipathic alpha-helices with hydrophobic and hydrophilic sides. The hydrophobic side groups of these peptides bind to the hydrophobic lipidic core of the bilayer, leading to three different possible outcomes; barrel-stave model (Figure 2), carpet model (Figure 3), and toroidal model (Figure 4) (Giuliani *et al*., 2008).

1.2.2.1 Barrel-stave model

In this model, peptides first get assembled in the surface of the membrane and then α-helical peptides bind to the membrane. The hydrophobic site of peptide binds with the hydrophobic lipid core of the membrane bilayer and the hydrophilic peptide parts forming the interior pores on the membrane.

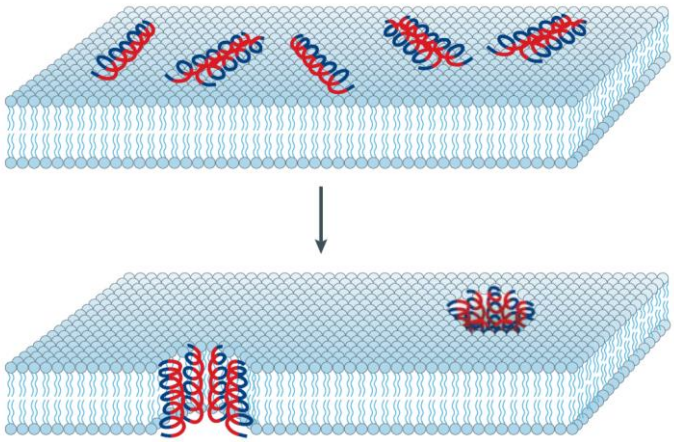

Figure 2. The barrel-stave model of mechanism of AMP (Taken from Brogden, 2005). The hydrophilic and hydrophobic regions of the peptides are shown in red and blue, respectively.

1.2.2.2 Carpet model

In this model, peptides invade the microbial membrane by covering the lipid bilayer with an extensive layer of peptides. Firstly, peptides bind to the surface of lipid membrane so that their hydrophilic groups face with water molecules and hydrophobic groups of peptides bind with the bilayer. When the high concentration of peptides is present on the surface of phospholipids, membrane permeation is triggered. Lipid membranes are then disintegrated leading to micellization and pore formation.

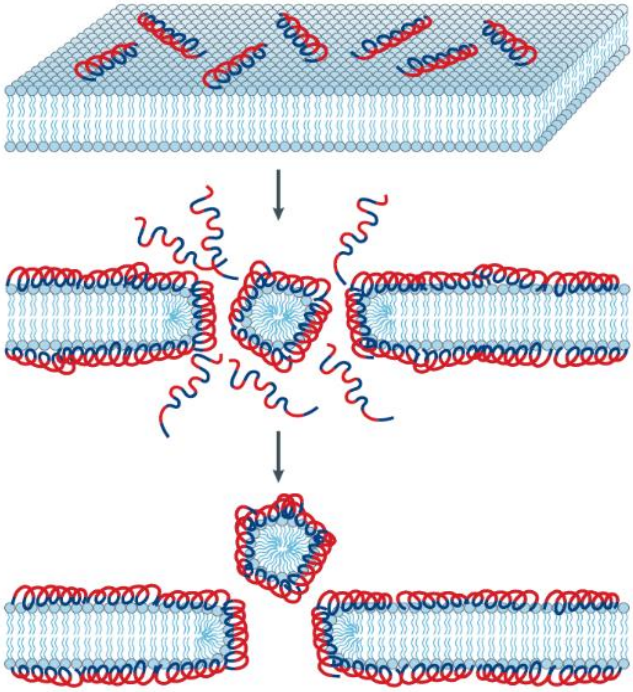

Figure 3. The carpet model model of mechanism of AMPs (Taken from Brogden, 2005). The hydrophilic and hydrophobic regions of the peptides are shown in red and blue, respectively.

1.2.2.3 Toroidal model

In this model, the helix peptides bind to membrane and insert themselves into the membrane bilayer and induce lipid monolayers to bend continuously through the pore so that the water core is lined by both the lipid head groups and the inserted peptides. To form a toroidal pore, the polar faces of the peptides are associated with the polar head groups of the lipids.

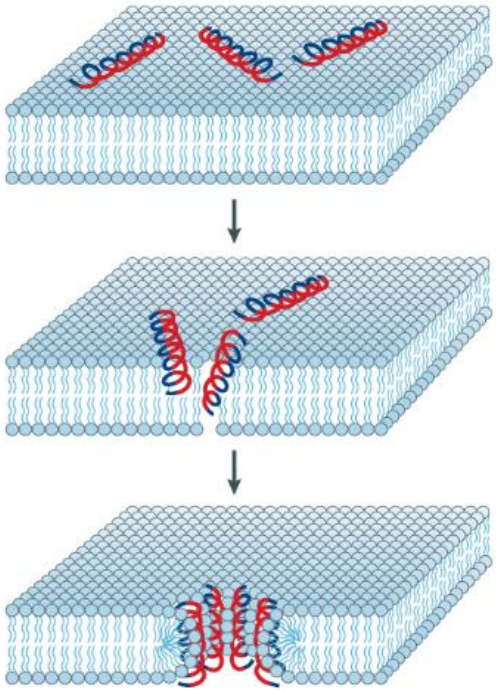

Figure 4. The toroidal model of mechanism of AMP (Taken from Brogden, 2005). The hydrophilic and hydrophobic regions of the peptides are shown in red and blue, respectively.

1.2.3 Subcellular localization of AMP

Understanding where peptides localize subcellularly would make one speculating their possible *in vivo* function(s). Plant AMP has been reported to be

localized to different parts of the cells depending on their signal/transit peptides (Table 1) suggesting their various biological functions. However, most of them are extracellular proteins strongly suggesting their involvement during pathogen attack.

Table 1. Subcellular localization of plant AMPs

Plant	AMP	Subcellular localization	Reference
Dahlia merckii	Dm-AMP1	extracellular	Jha *et al*., 2009
Medicago sativa	MsDef1	extracellular	Terras *et al*., 1995
Medicago sativa	alfAFP	extracellular	Gao *et al*., 2000
Nicotiana alata	NaD1	vacuole	Lay *et al*., 2003
Oryza sativa	OsGASR1	extracellular or the cell wall	Furukawa *et al*., 2006
Oryza sativa	OsGASR2	extracellular or the cell wall	Furukawa *et al*., 2006
Raphanus sativus	Rs-AFP2	cell membrane	Jha and Chattoo, 2010
Solanum tuberosum	SN1	cell membrane	Nahirnak *et al*., 2012
Triticum aestivum	TaGASR1	cell membrane or cytosol	Zhang *et al*., 2016
Vitis vinifera	VvAMP1	extracellular	de Beer and Vivier, 2008

1.2.4 Defensin (DEF)

Plant DEFs are a plant AMP family of small basic peptide. The first plant DEF was isolated from barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). Initailly, this AMP group was classified as a subgroup of the thionin family, called the ϫ-thionins (Colilla *et al*., 1990). After that, they were renamed this group of proteins as "plant DEF" because of functional and structural similarities with mammalian and insect DEF (Terras *et al*., 1995). Usually, most plant DEFs were isolated from seeds (Broekaert *et al*., 1995) but It is now clear that plant DEFs can express in all tissues of the plant

including pollen, leaves, flowers, fruits , roots, shoots, cotyledons, and barks (de Oliveira Carvalho and Gomes, 2009). The mature peptide of plant DEF is basic with a pI of approximately 9 and molecular mass between 5 and 7 kDa. There are conserved eight cysteine residues which form four disulfide bonds for the stabilization of the three-dimensional structure. Plant DEFs show conserved structure in different plants. Despite their usual fold, the overall of sequence identity between these DEFs is normally less than 35 % and they also differ in their activities (eg, *NaD1* from *Nicotiana alata* and *RsAFP1R* from *Aphanus sativus*) exhibit antifungal activities, *VrD2* from *Vigna radiata* inhibits α-amylase, and Brazzein from *Pentadiplandra brazzeana* one is a sweet tasting protein (Figure 5) (Van der Weerden *et al*., 2013).

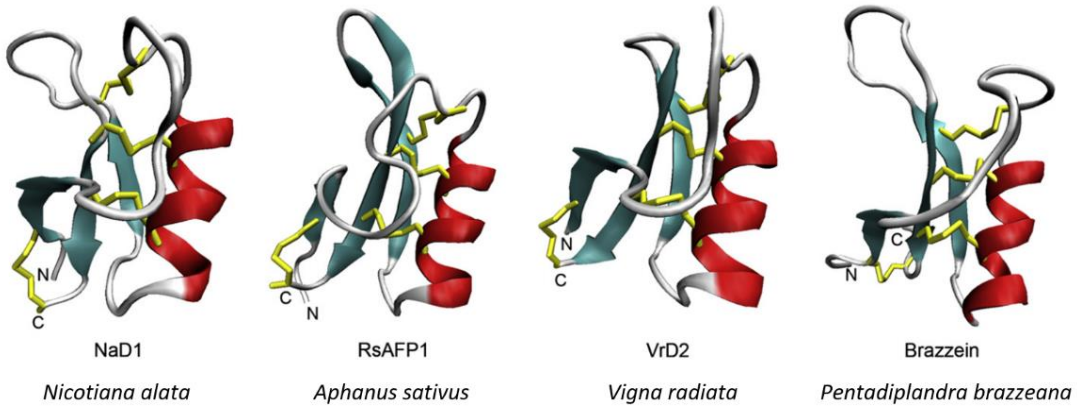

Figure 5. Conserved structures of plant DEFs. A comparison of the three-dimensional structure of DEFs from different plants. All peptides share a conserved triple-stranded an α-helix (red) to β-sheet (cyan) tethered by three disulfide bonds (yellow) with a fourth disulfide bond joining the N- and C-termini (Taken from Van der Weerden *et al*., 2013).

Over the last decade, transgenic plants overexpressing plant DEFs have been shown to increase resistance against several fungal and bacterial diseases indicating their *in planta* potential to act as defense peptides (Table 2). However, a direct *in vivo* role of plant DEFs in defense response has not yet been established (De coninck *et al*., 2012). Heterologous expression of peptides in transgenic plants are mostly used for increased crop yield and quality and improved cost-efficient crop production. (Holaskova *et al*., 2015).

Table 2. Overview of transgenic plants overexpressing a plant DEF and their resulting resistance/tolerance (Modified from De Coninck *et al*., 2013)

Defensin name	Transformed plant	Increased resistance/tolerance	Reference
AtPDF1.1	*Arabidopsis thaliana*	*Cercospora beticola*	De Coninck *et al*., 2010
BsD1	*Nicotiana tabacum*	*Phytophthora parasitica*	Park *et al*., 2002
BjD	*Arachis hypogaea*	*Pheaoisariopsis personata,*	Anuradha *et al*., 2008
		Cercospora arachidicola	
	Nicotiana tabacum	*Fusarium moniliforme,*	
		Phytophthora parasitica	
BrD1	*Oryza sativa*	*Nilaparvata lugens*	Choi *et al*., 2009
DmAMP1	*Carica papaya*	*Phytophthora palmivora*	Zhu *et al*., 2007
	Oryza sativa	*Magnaporthe oryzae, Rhizoctonia solani*	Jha *et al*., 2009
	Solanum melongena	*Botrytis cinerea, Verticillium albo-atrum*	Turrini *et al*., 2004a
MsDEF1/alfAFP	*Solanum lycopersicum*	*Fusarium oxysporum* f.sp. *lycopersici*	Abdallah *et al*., 2010
	Solanum tuberosum	*Verticillium dahliae*	Gao *et al*., 2000
NmDef02	*Solanum tuberosum*	*Phytophthora infestans, Alternaria solani*	Portieles *et al*., 2010
	Nicotiana tabacum	*Phytophthora parasitica* var. *nicotianae,*	
		Peronospora hyoscyami f.sp. *tabacina*	
Ovd	*Brassica napus*	*Sclerotinia sclerotiorum*	Wu *et al*., 2009
DRR230	*Brassica napus*	*Leptosphaeria maculans*	Wang *et al*., 1999
RsAFP2	*Nicotiana tabacum*	*Alternaria longipes*	Terras *et al*., 1995
	Oryza sativa	*Magnaporthe oryzae, Rhizoctonia solani*	Jha and Chattoo, 2010
	Solanum lycopersicum	*Fusarium oxysporum* f.sp. *lycopersici,*	Kostov *et al*., 2009
		Botrytis cinerea	
	Triticum aestivum	*Fusarium graminearum, Rhizoctonia cerealis*	Li *et al*., 2011
DEF2	*Solanum lycopersicum*	*Botrytis cinerea*, reduced seed setting,	Stotz *et al*., 2009b
		pollen viability, growth changes	
WT1/WjAMP1	*Colocynthis citrullus*	*Alternaria solani, Fusarium oxysporum*	Ntui *et al*., 2010
	Oryza sativa	*Magnaporthe grisea*	Kanzaki *et al*., 2002
	Phalaenopsis orchid	*Erwinia carotovora*	Sjahril *et al*., 2006
	Solanum tuberosum	*Botrytis cinerea*	Khan *et al*., 2006
ZmDEF1	*Nicotiana tabacum*	*Phytophthora parasitica*	Wang *et al*., 2011

Not only plant DEFs are involved in defense response but also they have been reported to be induced by other stresses including cold (Koike *et al*., 2002; Carvalho *et al.*, 2006), wounding (Pervieux *et al*., 2004; Bahramnejad *et al.*, 2009), and drought stresses (Do *et al.*, 2004). Moreover, they also play roles in plant growth and development. Previous work reported that tomato DEF, *DEF2,* expresses during early developing flowers. DEFs at the mRNA, peptide and post-translational processing levels are differentially regulated in developing flowers. Constitutive overexpression or antisense suppression of *DEF2* affect reduced seed production and pollen viability (Stotz *et al*., 2009). Interestingly, many plant DEFs and DEF-like peptides were found to be expressed in the female gametophyte cells of *A. thaliana* (Punwani *et al*., 2007; Wuest *et al*., 2010), *Z. mays* (Cordts *et al*., 2001; Amien *et al*., 2010) and *Torenia fournieri* (Okuda *et al*., 2009). For example, DEF *ZmES4* from *Z. mays* is found to be expressed in the synergid cells (Amien et al., 2010). Nevertheless, plant defensin-like peptides also have been expressed in the male gametophyte. They are involved in the self-incompatibility system, developed by plants to prevent self-fertilization in *Brassicaceae* (Higashiyama, 2010; Marshall *et al*., 2011).

Regarding the structures of DEFs, both monomeric and multimeric forms of DEFs have been reported. Purified protein extracts from the leaves of a DEF2-overexpressing transgenic tomato in a monomer form inhibit the tip growth of *B. cinerea* (Stotz *et al*., 2009). Dimerization of NaD1, a potent antifungal plant DEF from *Nicotiana alata* flowers, enhances its fungal cell killing when comparing with monomeric protein. This studies identified Lys4 as an important residues for the formation of NaD1 dimer. The site-directed mutagenesis of Lys4 reduces the dimer formation. Importantly, the reduced ability of the Lys4 mutant to dimerize involves in decreased antifungal activity (Lay *et al*., 2012). Terras *et al*. (1992) demonstrated that non-reduced radish DEFs Rs-AFP1 and Rs-AFP2 had molecular masses of 15 kDa (trimer) and/or 20 kDa (tetramer) using SDS-PAGE analysis. This was in contrast to the reduced and S-pyridylethylated

derivatives of both peptides which appeared in single bands with a molecular weight around 5 kDa. Similar demonstration for multimers of other plant DEFs, namely Ah-AMP1, Dm-AMP1, Dm-AMP2, Ct-AMP1, and Hs-AFP1 was also reported (Osborn *et al*., 1995).

1.3 Rice DEFs (*OsDEF7* and *OsDEF8*)

There are almost 600 putative genes encoding AMP, predicted from the cysteine-rich characteristic of translated peptides in rice (Silverstein *et al*., 2007). However, the possible activities/functions of most rice AMP has not been determined. Our group used bioinformatics tools, such as gene coexpression network analysis (Lee *et al*., 2009) and gene expression pattern analysis (Jain *et al*., 2007) to identify and characterize rice DEFs. Coexpression network analysis employs gene expression profiles from publicly available microarray data and is based on the assumption that coexpressed genes participate in the same biosynthetic pathways/biological functions. Then, out of 59 rice DEFs annotated in the genome database, we chose two genes which are *OsDEF7* (LOC_Os02g41904) and *OsDEF8* (LOC_Os03g03810). This is because these two genes show higher gene expression *in planta* than the other rice DEFs and also are coexpressed with pathogen-responsive genes (Figure 6). Our group heterologously produced rice OsDEF7 and OsDEF8 from Japanese rice (*Oryza sativa* L. ssp. *Japonica* cv. Nipponbare) in bacteria *E. coli* using glutathione S-transferase (GST) fusion protein. After cleaving GST, the recombinant peptides showed a growth inhibitory effect against bacteria; *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and *Erwinia carotovora* with minimum inhibitory concentration (MIC) 0.6 to 63 μg/ml and slightly active against the phytopathogenic fungi *Fusarium oxysporum* and *Helminthosporium oryzae.* The calculated molecular weights of mature OsDEF7 and OsDEF8 were 5.6 and 6.0 kDa, respectively, but OsDEF7 and OsDEF8 were showed

single 11 and 12 kDa bands, respectively, in SDS-PAGE after purification. We proposed that OsDEF7 and OsDEF8 form homodimeric peptides (Tantong *et al*., 2016).

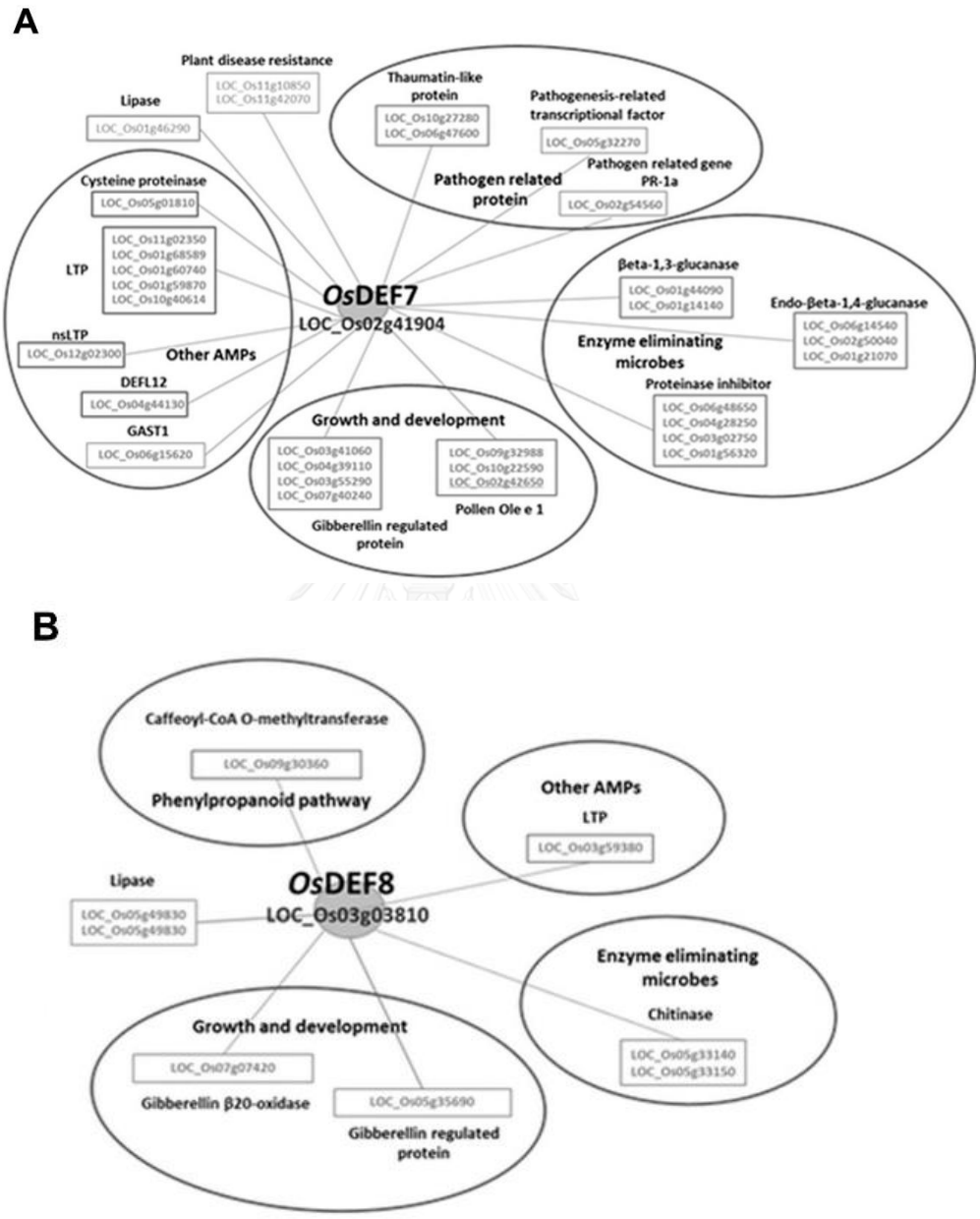

Figure 6. Gene coexpression network analysis of *OsDEF7* (A) and *OsDEF8* (B) (Taken from Tantong *et al*., 2016).

1.4 Plant-based recombinant protein production

Plant-based system provides cost-effective production, low purification cost, and high scale-up capacity (Holaskova *et al*., 2015). The major advantages of plant-based system are high quality, yield product and homogeneity. Proper folding, glycosylation, and disulfide bond formation which are important for protein activity, can also be achieved (Ma *et al*., 2003; Ramessar *et al*., 2008). When plant-based expression systems are compared with bacterial expression platforms, bacterial expression systems are more desirable because yeast and bacteria are easy to maintain and transform while plant-based systems require more effort, cost, and time. Nevertheless, once accepted, plant-based systems offer high-capacity scale-up, cost-efficient production, fast, easy, and low-cost storage and purification (Holaskova *et al*., 2015). Thus, plants might be a suitable alternative system for production of AMP without the requirement for controlled expression while bacterial expression systems of an AMP needs controlled or induced transcription and/or fusion to protein or a carrier peptide. Moreover, plant-based systems are considered to be safe because plants are independent of endotoxins and human or animal pathogens, and there is almost no danger of product contamination (Daniell *et al*., 2001; Fischer *et al*., 2004; Magnusdottir *et al*., 2013). There are different system to be used in this study. Each system is reviewed here.

1.4.1 Suspension cultures of plant cells

Undifferentiated cultured cells of plants are easy to manipulate. Plant cells suspension cultures are composed of comparatively homogeneous suspensions of quickly dividing cells established by transferring of callus into liquid media. Cell suspension culture of transgenic plant could also be prepared by direct transformation (Xu *et al*., 2011). The most popular plant suspension cells for recombinant production in suspension cultures is tobacco (*N. tabacum*), because these cells are multiply

rapidly and easy to maintain and transform. Nevertheless, there are other plant species have been used for establishment of cell suspension cultures including soybean, tomato, carrot, and rice (Xu *et al*., 2011). Transgenic rice cell cultures was used for expression of human lysozyme (Huang *et al*., 2002). The carrot root cell suspension culture is a plant-based system that have been reported to produce first commercially available therapeutic protein (Shaaltiel *et al*., 2007). This cell suspension culture was produced recombinant glucocerebrosidase protein, formulated as Elelyso™ (Protalix Biotherapeutics Inc., Israel), has been approved by the US Food and Drug Administration (FDA). The recombinant glucocerebrosidase was used for enzyme replacement therapy of Gaucher's disease (Aviezer *et al*., 2009; Grabowski *et al*., 2014). Production of glucocerebrosidase in carrot cell suspensions also provides pathogen-free manufacturing and cost-efficient compared to produce in mammalian cell or ovary cells of Chinese hamster.

1.4.2 Hairy root cultures

This systems are widely used for biotechnological applications such as synthesis of phytochemicals, therapeutic protein production, and biotransformation of exogenous substrates (Banerjee *et al*., 2012; Ono and Tian, 2011). Hairy root cultures are generated by the *Agrobacterium rhizogenes* infection into plant cells. This is a stable incorporation of genetic material into the host plant cell genome. Normally, after transformation two to four weeks, hairy roots grow in a highly branched with many lateral roots (Pavlov *et al*., 2002; Sevón and Oksman-Caldentey, 2002). Hairy root cultures can be used for rapid evaluation of protein activity as well as yield and efficiency of protein production in plant-based systems. Hairy root cultures can be used to display a defensive role of AMP in disease-susceptible tissues of plants. Hairy roots of transgenic potato overexpressing a levamisole-mimetic synthetic peptide (LEV-I-7.1) were investigated the effects against nematodes *Globodera pallida*. The

transgenic hairy roots exhibited around 50% reduction in the number of obligate root parasites (Liu *et al*., 2005).

1.4.3 Transient expression

From all of plant-based expression systems, transient expression by *Agrobacterium* or viral infection attracts much attention because of short time scale for production and development, high yields obtained, and expression levels. Gene expression starts within one day to a few weeks depending on the hosts, products, and the expression vectors employed. Nowadays, infiltration into leaf tissues with a suspension of *Agrobacterium tumefaciens* harboring the gene of interest in an expression vector represents the most commonly used for testing new constructs. Usually, *N. benthamiana* leaves were used for transient expression (Yang *et al*., 2000; Sparkes *et al*., 2006). Moreover, subcellular localization and production of a plant AMP was demonstrated in a transient expression system using a fluorescent tag. Western blot analysis of extracted proteins from *N. benthamiana* leaves clearly demonstrated the presence of the recombinant AMP product in protein bodies derived from ER (Company *et al*., 2014).

1.5 *In planta* activity of plant AMP

Although *in vitro* assays of AMP is quite easier than tests of *in planta* assays, there are also limitations. Activity of a protein in a transformed plant can be significantly different from *in vitro* performance; therefore, *in vitro* data can provide some direction on AMP selection but must be considered with prudence. It has also been reported that plant AMP is more effective *in planta* than *in vitro* (Cary *et al*., 2000; Visser *et al*., 2012). This is because AMP might require post-translational processing to achieve the best functional conformation. *In vitro* experiment may provide an initial screen to determine candidate AMP but it might also considerably

underestimate activity of peptide sequences requiring post-translational modifications. In addition, highly amphipathic AMPs are more effective *in planta* than *in vitro* and there is an evidence that AMP *in vitro* form large aggregates that hinder function, whereas *in planta*, AMP likely exist as discrete molecules or much smaller aggregates (Stover *et al*., 2013).

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Agarose gel electrophoresis: Mupid®-exU (Advance Co., LTD, Japan)

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Autopipette (Gilson, France)

Balance: PB303-L (Mettler Toledo, USA)

Benchtop Centrifuge Mikro 120 (Hettich, Germany)

Biospectrometer (Eppendorf, Germany)

Blotting equipment (Biorad, USA)

CFX96 Touch Real-Time PCR (Biorad, USA)

Cooled Incubator/climatic chamber KBF720 (Binder, Germany)

Digital Balance (Mettler Toledo, USA)

Fridge and Freezer (Electrolux, Sweden)

Gel Documentation: Gel Doc™ (Syngene, England)

Incubator: Memmert (Wisconsin Oven Distributors, LLC., USA)

Incubator shaker (Kuhner shaker, Switzerland)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific, UK)

Laminar flow: Bio Clean Bench (SANYO, Japan)

Magnetic stirrer and heater: C-MAG HS7 (IKA®, Germany)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

MicroPulser electroporator (Bio-Rad, USA)

Microwave (Sharp, Thailand)

Mini Centrifuge (Bio-Rad, USA)

Mixer mill: MM400 (Retsch®, Germany)

Power supply: PowerPac^{TM} Basic (Bio-Rad, USA)

Refrigerated centrifuge: Legend XTR (Thermo Scientific, USA)

Refrigerated centrifuge: Universal 320R (Hettich, Switzerland)

Rotary shaker (IKA Labortechnik, Germany)

SDS-gel electrophoresis: AE-6530 (ATTO, Japan)

Thermal Cycler: T100TM Thermal Cycle (Bio-Rad, USA) 392935047216

ThermoE Cooling & Heating Block CHB-A4-2415 (Bioer Technology, China)

ThermoMixer C (Eppendorf, Germany)

pH meter (Mettler Toledo, USA)

Vortex (Scientific industries, USA)

2.1.2 Chemicals

$(NH_4)_6{\cdot}Mo7O_24{\cdot}4H_2O$ (Ajax, New Zealand)

2, 4-Dichlorophenoxyacetic acid (Sigma, USA)

2-(N-morpholino)ethanesulfonic acid (Bio Basic, USA)

5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt (Thermo Scientific, USA)

Absolute ethanol (Carlo Erba Reagenti, Italy)

Acetosyringone (PhytoTechnology Laboratories, USA)

Acrylamide (ACROS, Belgium)

Agar powder (Himedia, India)

Agarose (Bioline, UK)

Ammonium nitrate (Ajax, New Zealand)

Ammonium persulfate: $(NH_4)_2S_2O_8$ (Sigma, USA)

Beta-mercaptoethanol (Sigma, USA)

Bis-Acrylamide (Sigma, USA)

Boric acid (Bio Basic, USA)

Bromophenol blue (Carlo Erba Reagenti, Italy)

Calcium chloride (Carlo Erba Reagenti, Italy)

Citric acid (Bio Basic, USA)

Coomassie brilliant blue R-250 (Bio Basic, USA)

Copper (II) sulfate heptahydrate (Ajax, New Zealand)

dATP, dCTP, dGTP, and dTTP (Fermentas, USA)

Disodium phosphate (Ajax, New Zealand)

Dithiothreitol: DTT (Bio Basic, USA)

EDTA (Bio Basic, USA)

Gel red nucleic acid gel stain (Biotium, USA)

Glacial acetic acid (Merck, Germany)

Glycerol (Ajax, New Zealand)

Glycine (Bio Basic, USA)

Heiter (Kao, Thailand)

Hydrochloric acid (Merck, Germany)

Imidazole (Bio Basic, USA)

Iron (III) chloride hexahydrate (Ajax, New Zealand)

Kelcogel Gellan Gum (CP kelco, USA)

Magnesium chloride (Ajax, New Zealand)

Magnesium chloride hexahydrate (Ajax, New Zealand)

Magnesium sulfate haxahydrate (Ajax, New Zealand)

Manganese (II) chloride tetrahydrate (Ajax, New Zealand)

Methanol (Merck, Germany)

Monosodium phosphate (Ajax, New Zealand)

Murashige and Skoog basal salt mixture (PhytoTechnology Laboratories, USA)

Murashige and skoog vitamins (PhytoTechnology Laboratories, USA)

Nitro-blue tetrazolium chloride (Thermo Scientific, USA)

Peptone (Himedia, India)

Potassium chloride (Ajax, New Zealand)

Potassium dihydrogen phosphate (Ajax, New Zealand)

Potassium hydroxide (Ajax, New Zealand)

Potassium sulfate (Ajax, New Zealand)

Skim milk (Himedia, India)

Sodium chloride (Ajax, New Zealand)

Sodium dihydrogen phosphite (Ajax, New Zealand)

Sodium dodecyl sulfate (Bio Basic, USA)

Sodium hydroxide (Ajax, New Zealand)

Sucrose (Carlo Erba Reagenti, Italy)

Sulfuric acid (Merck, Germany)

TEMED (Invitrogen, USA)

Thiamine hydrochloride (Bio Basic, USA)

Tricine (Bio Basic, USA)

Tris (Bio Basic, USA)

Triton™ X-100 (Sigma, USA)

Tween (Fluka, Switzerland)

Yeast extract (Himedia, India)

Zinc sulfate heptahydrate (Ajax, New Zealand)

2.1.3 Enzymes

Gateway® LR Clonase® Enzyme II mix (Thermo Scientific, USA)

iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA)

Phusion® High-Fidelity DNA polymerase (New England Biolabs, USA)

RNase-Free DNase set (Qiagen, Germany)

SsoFast EvaGreen Supermix (Bio-Rad, USA)

Taq DNA polymerase (New England Biolabs, UK)

2.1.4 Plasmids

CloneJET PCR Cloning Kit (Thermo Scientific, USA)

pCR™8/GW/TOPO® TA Cloning Kit (Thermo Scientific, USA)

pEAQ-HT-DEST3 (pEAQ3) (Peyret and Lomonossoff, 2013)

pEAQ-HT-DEST1 (pEAQ1) (Peyret and Lomonossoff, 2013)

pGBW2 (Nakagawa et al., 2007)

pGWB5 (Nakagawa et al., 2007)

2.1.5 Molecular kits

Bio-Rad protein assay (Bio-Rad, USA)

Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)

His SpinTrap™ Spin Columns and Kit (GE Healthcare, UK)

Presto™ Mini Plasmid kit (Geneaid, Taiwan)

RNeasy® Plant Mini kit (Qiagen, Germany)

2.1.6 Miscellaneous

100 bp DNA Ladder (New England Biolabs, USA)

Gel Loading Dye, Purple (6X), no SDS (New England Biolabs, USA)

Spectra™ Multicolor Low Range Protein Ladder (Thermo Scientific, USA)

2.1.7 Antibiotics

Ampicillin (Bio Basic, USA)

Carbenicillin (Bio Basic, USA)

Cefotaxime (Bio Basic, USA)

Gentamycin (Bio Basic, USA)

Hygromycin B (Bio Basic, USA)

Kanamycin (Bio Basic, USA)

Rifampicin (Bio Basic, USA)

Spectinomycin (Bio Basic, USA)

Streptomycin (Bio Basic, USA)

2.1.8 Antibody

Pierce His tag mouse mAB (Thermo Scientific, USA)

Goat anti-mouse IgG secondary antibody (Thermo Scientific, USA)

2.1.9 Bacterial strains

Agrobacterium rhizogenes strain ATCC 43057

Agrobacterium tumefaciens strain GV3101

Escherichia coli DH5α

Xanthomonas campestris pv.*glycines* (TISTR 786)

Xanthomonas oryzae pv. *oryzae* (DOAC 4-1570)

2.1.10 Plant materials

Thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML105)

Nicotiana benthamiana

Tobacco BY-2 cells

2.1.11 Softwares and database

ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

ExPaSy Bioinformatics Resource Portal (http://au.expasy.org/tools/ protparam.html)

GENVESTIGATOR (https://genevestigator.com/gv/)

NCBI (http://www.ncbi.nlm.nih.gov/)

Phytozome (http://www.phytozome.net/)

PrediSi PREDIction of SIgnal peptides (http://www.predisi.de/)

WOLF PSORT (http://www.genscript.com/psort/wolf_psort.html)

2.2 Methods

2.2.1 Gene expression patterns and nucleotide sequencing of *OsDEF7* and *OsDEF8* in Thai jasmine rice

2.2.1.1 Gene expression patterns of *OsDEF7* and *OsDEF8* in normal condition

2.2.1.1.1 Rice cultivation

Thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML105) seeds were germinated in DI water at room temperature without light 2-3 days until coleoptiles appeared. DI water was changed every day. Germinated seeds were then transferred to a plastic net placing on floating plastic tray with 0.5x Yoshida's nutrient solution (Yoshida *et al*., 1976) and put in a growth chamber under the condition, 60% relative humidity, 25 °C, and light intensity of 6000 lux with a 16 h photoperiod. Half- strength Yoshida's solution was added to maintain the solution level. After one week, rice plants were moved into separate glass bottle supplemented with Yoshida's solution. The cultivation continues in the growth chamber and rice samples were collected at 5 days (germinating seed), 2 weeks (seedling), and 1 month (mature). Flowers were also collected. Rice leaves and roots were collected separately. Samples were wrapped in aluminum foil and frozen in liquid nitrogen. Frozen tissues were then ground into a fine powder using a mixer mill (Retsch, MM400) at 30 Hz for 30 s.

2.2.1.1.2 RNA extraction and cDNA synthesis

Approximately 100 mg of ground sample was used for RNA extraction. Total RNA was isolated from the ground frozen rice tissue samples at different developmental stages and organs using the RNeasy® Plant Mini kit (Qiagen, USA). Total RNAs were treated with RNase-Free DNase for 10 min at room temperature. The cDNAs were synthesized by iScript cDNA synthesis kit (Bio-Rad, USA) using 1 µg RNA as the template.

2.2.1.1.3 Determination of DNA and RNA concentration

DNA or RNA concentrations were determined using spectrophotometry. Their concentrations can be calculated by absorbance at 260 nm (A260) of the solution. DNA solution has the concentration of 50 µg/ml when A260 is equal to 1 while RNA solution is 40 µg/ml.

2.2.1.1.4 Gene expression analysis by real-time quantitative RT-PCR

The quantitative RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, USA). Each PCR reaction contained 5 µl of EvaGreen Supermix, 2 µl of the diluted cDNA (20 ng RNA), 2 µl of nuclease free water and 10 µM of each primer in a total reaction volume of 8 µl. The primers used to amplify *OsDEF7* were def7RT-F (5′-GTGAGCAGCAACAACTGCG-3′) and def7RT-R (5′-CGACGAGCAATGCGACTG-3′), and that used to amplify *OsDEF8* were def8RT-F (5′-TGATCGATGAACCAGCAGCTA-3′) and def8RT-R (5′-GGATGGATGGTGGATGCAC-3′). Real-time PCR reactions were performed in a CFX96™ Real-Time PCR (BIO-RAD, USA). PCR reactions were performed under the following conditions: denaturation at 95 °C for 30 sec, 40 cycles of denaturation (95 °C for 5 sec); and annealing and extension (58 °C for 10 sec), denaturation at 95 °C for 15 sec, and melt curve construction starting from 65 °C for 15 sec (0.5 °C increment until 95 °C). Plate read was conducted every cycle at the end of annealing and extension step, and after each increment for melting curve. To determine efficiency of the primer

pair, real-time PCR was performed with a serial dilution of template concentration. CT values of at least five cDNA concentrations were plotted on x-axis against log cDNA dilution on y-axis to create standard curve. PCR efficiency was calculated from: E = 10-1/slope, which E is the efficiency. Percent PCR efficiency should be within 90-110%. All real-time PCRs were done in three technical replicates. Relative gene expression levels were calculated by comparative CT method (Schmittgen and Livak, 2008) using *OsEF*-1α as a reference gene. The primers used to amplify *OsEF*-1α were OsEF1α-F (5′-ATGGTTGTGGAGACCTTC-3′) and OsEF1α-R (5′-TCACCTTGGCACCGGTTG-3′). Fold change of gene expression between two samples is equal to 2-ΔΔCT that ΔΔCT = (CT gene of interest – CT *OsEF*-1α) sample A - (CT gene of interest - CT *OsEF*-1α) sample B. At least three biological replicates were used in every quantitative RT-PCR experiment. These expression pattern in Thai jasmine rice was compared to the Genevestigator microarray database of Japanese rice (Hruz, 2008).

2.2.1.2 Gene expression analysis of *OsDEF7* and *OsDEF8* in an infected condition

X. oryzae pv. *oryzae* was cultured in nutrient broth at 28°C with 250 rpm shaking for 48 h. The cultures were diluted with nutrient broth to an appropriate optical density at 600 nm (OD_{600}) of 0.2. Then, two weeks old rice leaves were cut out around 1 inch from the tip and dipped into the suspension culture of *X. oryzae* pv. *oryzae* for 1 min. For, the control set, rice leaves were dipped in autoclaved nutrient broth. Infected rice and control rice plants were left for 6 h, 4 days, and 8 days. The treated leaves, two inches from the cut edge, were collected. Then, RNA was extracted from the samples. The cDNAs were synthesized. The quantitative RT-PCR was performed as previously described.

2.2.1.3 Nucleotide sequencing of *OsDEF7* and *OsDEF8* of Thai jasmine rice

2.2.1.3.1 Amplification of *OsDEF7* and *OsDEF8* from cDNA

OsDEF7 and *OsDEF8* were amplified using cDNA from seedling leaf. The primers used to amplify *OsDEF7* were def7-F (5′-ATGGCTCCGTCT CGTCGCATG -3′) and def7-R (5′- CTAGCAGACCTTCTTGCAGAAG -3′), and that used to amplify *OsDEF8* were def8-F (5′- ATG GAGGCTTCACGCAAGGTGTT -3′) and def8-R (5′- TCAGGGGCAGGGCTTGGTGC -3′). PCR amplifications were performed with Phusion®High-Fidelity DNA polymerase (New England Biolabs, USA). The PCR conditions of 50 µl amplification reaction were consisted of 1x phusion buffer, 200 µM dNTP, 0.5 µM each primer, 1 µl cDNA and Phusion DNA polymerase 1.0 units/50 µl PCR . The PCR thermal cycling conditions were 98°C for 30 s, 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 15 s, and then a final extension at 72°C 5 min.

2.2.1.3.2 Agarose gel electrophoresis

The PCR products were analyzed by using 1% agarose gel electrophoresis. Agarose powder was dissolved in 1X TBE buffer (Tris-HCl, Boric acid, EDTA), boiled the solution. The gel was poured into tray and the plastic comb was placed in the gel. After the gel was completely set, PCR products were mixed with 5X loading dye DNA which mix with gelred nucleic acid gel stain and loaded each well. The size was determined by comparing with DNA Marker 100 bp. Electrophoresis was performed at 100 mV 25 min. DNA fragment was detected by gel documentation under UV transilluminator.

2.2.1.3.3 Cloning of *OsDEF7* and *OsDEF8* to ColneJET vector

The PCR products were ligated into CloneJET PCR Cloning Kit (Thermo Scientific, USA). The 3 µl of ligation reaction was transformed into *E. coli* DH5α by electroporation at 1.5 kV. The culture was recovered at 37 °C for 1 h. The recombinant plasmid was

selected in 50 µg/ml ampicillin LB agar plate. Colony PCR was performed for selecting of transformed *E. coli.* The transformed colony was cultured in 3 ml of LB containing 50 µg/ml ampicillin in the temperature-controlled shaker at 37°C, 250 rpm overnight. The recombinant plasmid was extracted using Presto™ mini plasmid kit (Geneaid, Taiwan) and sent to Bio-basic, Canada for sequencing analysis. After that, the nucleotide sequences of *OsDEF7* and *OsDEF8* in Thai jasmine rice were compared with the nucleotide sequences of Japanese rice from phytozome database (http://www.phytozome.net/).

2.2.1.4 The Genevestigator tool analysis

Genevestigator microarray database (https://genevestigator.com/gv/) (Hruz, 2008) was used to analyze the expression levels of *OsDEF7* and *OsDEF8* in Japanese rice at different developmental stages and organs. The abiotic stress including drought, cold, imbibition, anoxia, and dehydration of *OsDEF7* and *OsDEF8* in japonica, amaroo, and indica rice was also examined.

2.2.2 Construction and transformation of plant expression vector

2.2.2.1 Codon optimization for *N. benthamiana*

*OsDEF*s were codon-optimized for *N. benthamiana* using codon optimization tool (https://www.idtdna.com/CodonOpt). The synthetic codon-optimized *OsDEF7* or *OsDEF8* gene (gBlocks® Gene Fragments, Integrated DNA Technologies, Singapore) which was included 6xHis-tag and a peptide, SEKDEL, as ER retention motif at the downstream.

2.2.2.2 Amplification of *OsDEF7* and *OsDEF8*

The pUC18 containing either *OsDEF7* or *OsDEF8* was used as templates for destination vector pEAQ-HT-DEST3 (pEAQ3) (Peyret and Lomonossoff, 2013), pGWB2 and pGWB5 (Nakagawa *et al*., 2007). All vectors used in this study are listed in Table 3.

The following primers were designed to exclude the stop codon for pEAQ3 and pGWB5: def7-F (5'-ATGGCTCCGTCTCGTCGCATG-3'), def7nostopcodon-R (5'-GCAGACCTT CTTGCAGAAGCA -3'), def8-F (5'-ATGGAGGCTTCACGCAAGGTGTT-3'), and def8 nostopcodon -R (5'-GGGGCAGGGCTTGGTGCA-3'). The primers were designed including stop codon for pGWB2: def7-F (5′-ATGGCTCCGTCT CGTCGCATG -3′), def7-R (5′-CTAGCAGACCTTCTTGCAGAAG -3′), (5′- ATGGAGGCTTCACGCAAGGTGTT -3′) and def8-R (5′- TCAGGGGCAGGGCTTGGTGC -3′). Opt*OsDEF*-His-SEKDEL gene synthetic were used as a template for destination vector pEAQ-HT-DEST1 (pEAQ1) (Peyret and Lomonossoff, 2013). The primer which used to amplify opt*OsDEF7*-His-SEKDEL were def7sekdel-F (5′-ATGGCACCTTCACGAAGGAT-3′) and sekdel-R (5′-TTCAGAAAAGGACGAATTGTAG -3′), and that used to amplify opt*OsDEF8*-His-SEKDEL were def8sekdel-F (5′- ATGGAAGCCAG TCGTAAAGTG -3′) and sekdel-R (5′-TTCAGAAAAGGACGAATTGTAG -3′) PCR amplifications were performed with Phusion®High-Fidelity DNA polymerase (New England Biolabs, USA). The 50 µl of PCR reactions were consisted of 1x Phusion buffer, 200 µM of dNTP, 0.5 µM of each primer, 1 µl of cDNA and Phusion DNA polymerase 1.0 units/50 µl PCR. The PCR thermal cycling conditions were 98°C for 30 s, 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 15 s, and then a final extension at 72°C 5 min. Then, agarose gel electrophoresis was performed to confirm the size of PCR products

2.2.2.3 Cloning vector ligation

PCR product was purified using Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). Then, 3' A overhang was added to the purified PCR product. The reaction consists of 1 µl of 10 mM dNTP, Taq 1 µl of polymerase, 1 µl of 10x Taq buffer, and 7 µl of purified PCR product and were incubated at 72 °C for 10 min. Subsequently, the A overhanged-PCR product was ligated into pCR®8/GW/TOPO®TA cloning kit (Thermo Scientific, USA).The ligation reaction mixture with 1 µl PCR product, 0.5 µl salt solution, 0.5 µl pCR™8/GW/TOPO® TA vector and 1 µl water was incubated at 22-23 °C for 5 min. The 3 µl of ligation reaction was transformed into *E. coli* DH5α by electroporation.

The recombinant plasmid was selected in LB agar plate containing 50 µg/ml spectinomycin. Colony PCR was performed for selecting of transformed *E. coli*. The transformed colony was cultured in 3 ml of LB containing 50 µg/ml spectinomycin in the temperature-controlled shaker at 37°C, 250 rpm overnight. The recombinant plasmid was extracted using Presto™ mini plasmid kit (Geneaid, Taiwan). Sequencing analyses were performed by Bio-basic, Canada.

2.2.2.4 LR recombination of pEAQ3-*OsDEF*, pEAQ1-opt*OsDEF*-His-SEKDEL, pGWB2-*OsDEF*, and pGWB5-*OsDEF*

OsDEF7 and *OsDEF8* in the expression vectors were generated using Gateway cloning technology. Either *OsDEF7* or *OsDEF8* coding region in pCR™8/GW/TOPO® TA vector was recombined into the pGWB2 destination vector, or the C-terminal GFP fusions destination vector, pGWB5, or the pEAQ1 vector, or the C-terminal His-tag fusions destination vector, pEAQ3) using LR Clonase® Enzyme II mix (Thermo Scientific, USA). The reaction was consisted of 1–7 µl entry clone (50–150 ng), 1 µl destination vector (150 ng/µl), TE buffer pH 8.0 to 8 µl and 2 µl of LR Clonase, and was incubated at 25°C for 1 h. The 3 µl of ligation reaction was transformed into *E. coli* DH5α by electroporation. The recombinant plasmid was cultured in a LB selection agar medium with 50 µg/ml kanamycin and hygromycin B for pGWB2, pGWB5 and 50 µg/ml kanamycin for pEAQ1, pEAQ3. Colony PCR was performed for selecting of transformed *E. coli*. The transformed colony was cultured. The recombinant plasmid was extracted using Presto™ mini plasmid kit (Geneaid, Taiwan).

Table 3. Summary of the vectors used in this study.

vector	purpose
CloneJET	Cloning vector for sequencing
pCR™8/GW/TOPO® TA	Cloning vector for LR recombination
pEAQ-HT-DEST3 (pEAQ3)	Expression vector containing His-tag used for BY2 transformation and transient expression in *N. benthamiana* leaf
pEAQ-HT-DEST1 (pEAQ1)	Expression vector used for transient expression in *N. benthamiana* leaf
pGBW2	Expression vector used for hairy root transformation
pGWB5	Expression vector containing GFP tag used for subcellular localization

2.2.2.5 *Agrobacterium* transformation

All constructed expression vectors containing either *OsDEF7* or *OsDEF8* were transformed into *A. tumefaciens* by electroporation at 1.5 kV. For only the constructed pGWB2 vector were transformed into *A. rhizogenes.* After transformation, *Agrobacterium* culture was recovered at 28 ℃ for 1.5 h. The transformed *A. tumefaciens* cell was selected in LB agar medium with 50 μg/ml of kanamycin, hygromycin B, gentamycin, and rifampicin for pGWB2, pGWB5 and 50 μg/ml kanamycin, gentamycin, and rifampicin for pEAQ1, pEAQ3. The transformed *A. rhizogenes* cell was selected in 50 μg/ml kanamycin and hygromycin B LB agar medium for pGWB2.

2.2.3 Subcellular localization

2.2.3.1 *N. benthamiana* cultivation for infiltration

N. benthamiana seeds were germinated in small pots with peat moss and grown under controlled condition 25 ℃, and light intensity of 4000 lux with a 16 h photoperiod for one and a half months. The plants were watered 3 times per week.

2.2.3.2 Tobacco BY-2 cells cultivation

Tobacco BY-2 cell were obtained from RIKEN BRC, Japan. The plant cells were cultured on Murashige and Skoog salt mixture medium containing 3% sucrose, 0.2 mg/ml KH_2PO_4 and 0.2 µg/ml 2, 4-dichlorophenoxyacetic acid in dark at 25 °C. Plant cultures on agar plate were subcultured once a month and the culture in liquid medium were subcultured every week with 120 rpm shaking on orbital shaker.

2.2.3.3 Agroinfiltration in *N. benthamiana* leaves

A single colony of recombinant *A. tumefaciens* harboring either of pGWB5-*OsDEFs* was cultured in LB medium containing 50 µg/ml kanamycin, 50 µg/ml hygromycin B, 50 µg/ml gentamycin, and 50 µg/ml rifampicin and then grown for 2 nights at 28°C with shaking at 250 rpm. The cultures were harvested by centrifugation at 5000 × g for 15 min, and the pellet was resuspended in 10 mM MMA buffer (MES-$MgCl_2$-acetosyringone) to an appropriate optical density at 600 nm (OD_{600}) of 1 and was incubated at room temperature for 2-3 h. The cells were used for the co-infiltration of 1:1 ratio with *A. tumefaciens* containing p19, silencing suppressor (Lindbo, 2007) which cultured in LB medium containing 50 µg/ml kanamycin, 50 µg/ml gentamycin, and 50 µg/ml rifampicin into the abaxial of six week-old plants, using a needleless 1-ml syringe. The infiltrated plants were left for five days. Pieces of the infiltrated leaves were sampled from the infected area and observed under the confocal laser scanning microscopy (Olympus FluoView FV1000). GFP was excited at 488 nm and the emission filter wavelengths were 497 to 526 nm for GFP.

2.2.3.4 Transient transformation of BY-2 cells

Single colony of *A. tumefaciens* harbouring gene of interest was cultured in LB medium containing proper antibiotic then grown for 2 nights at 28°C with shaking at 250 rpm. Agrobacterium culture was harvested and resuspended in MS salt mixture medium to an appropriate optical density at 600 nm (OD_{600}) of 1. The *A. tumefaciens*

harbouring gene of interest culture 100 µl and 100 µl of *A. tumefaciens* containing p19 was co-incubated with 5 ml of 3 days BY-2 cell which was subcultured in MS salt mixture liquid medium and then add 20 mM acetosyringone 5 µl. The co-incubated culture was sat at 25°C in dark. After 5 days, the cultures were harvested by centrifugation at 12000 × g for 15 min. The supernatant was observed under the UV light.

2.2.4 In plant production of recombinant OsDEFs

2.2.4.1 Hairy root

2.2.4.1.1 *N. benthamiana* cultivation in sterile condition

N. benthamiana seeds were washed with 70% alcohol for 1 min with shaking and then removed. Subsequently, they were washed in a mixture of 200 µl Heiter, 1 ml sterile water, and 2 µl triton-x100 with shaking at 40 rpm for 10 min. Then, seeds were washed with sterile water 3-5 times and dried them on filter paper. Sterile seeds were grown in MS vitamin agar medium containing ½ MS vitamin, 2% glucose, and 0.1% kelcogel in glass bottle at 25 °C, and light intensity of 4000 lux with a 16 h photoperiod for one and a half months.

2.2.4.1.2 Generating hairy root

Single colony of the transformed *A. rhizogenes* cell was cultured in LB medium with antibiotic and then grown for 2 nights at 28°C with shaking at 250 rpm. Agrobacterium culture was harvested and resuspended in MS vitamin medium to an appropriate optical density at 600 nm (OD_{600}) of 1. The leaf of *N. benthamiana* was cut into 1-1.5 square and transfer to 20 ml MS liquid medium in plate. Subsequently, 100 µl of *A. rhizogenes* harboring gene of interest was added into plate and incubated at 28 °C in dark for 2 night. After that, infected leaf was washed with MS medium 3 times for eliminating Agrobacterium and then transferred onto MS agar containing 500

µg/ml of cefotaxime. The transgenic root from *N. benthamiana* leaf was selected in MS agar containing 50 µg/ml kanamycin and 50 µg/ml hygromycin B. The transgenic root that was capable of growing in MS selection medium was transferred to MS liquid medium.

2.2.4.2 BY-2 cells

2.2.4.2.1 BY-2 cells transformation

Five ml of three days old BY-2 cells was transferred into a culture dish, then incubated at 25 ℃ with 100 µl of *A. tumefaciens* (OD_{600}=1) harboring either of transgene expression constructs, pEAQ3-*OsDEFs*, and 5 µl 20 mM acetosyringone in the dark. After 48 h of cocultivation, the cultures were washed with MS salt mixture medium for three times and plated on MS salt mixture agar medium containing 50 µg/ml kanamycin and 500 µg/ml carbenicillin. After 2-3 weeks, the transformed BY-2 cell were transferred onto fresh solid MS medium salt mixture plate containing 50 µg/ml kanamycin for selection. The positive clone was inoculated into liquid medium containing 50 µg/ml kanamycin for one week with 120 rpm shaking.

2.2.4.2.2 Protein detection

The supernatants of cell suspension culture were collected by centrifugation at 5000 ×g for 15 min. Proteins were separated by 12% SDS polyacrylamide gel electrophoresis at a constant 20 mA per gel for 1.30 h in tricine buffer and stained with coomassie brilliant blue. For Western blot analysis, gel and filter papers were incubated with a transfer buffer (25 mM Tris, 150 mM glycine and 20% methanol). PVDF membrane was activated with absolute methanol. Gel, membrane, and filter paper were placed on Wet/Tank Blotting System (Bio-RAD) at a constant 350 mA for 30 min. After that 10% of skim milk was blocked in 1x Tris-buffered saline (TBS) and 0.05 % (v/v) tween 20 (TBST) at room temperature with shaking for one h before stored at 4 °C. After washing out the blocking solution using TBST 3 times for 10 min each, PVDF

membrane was incubated with primary antibody (Pierce his tag mouse mAB diluted) at 1:2000 dilution in TBST for 1.30 h at room temperature with 40 rpm shaking. Then, the membrane was washed three times and incubated with secondary antibody (alkaline phosphatase conjugated goat anti-mouse) at 1:1000 dilution in TBST for 1 h at room temperature with 40 rpm shaking. After that, membrane was washed three times with TBST before the protein detection by color development using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is used in a conjunction with nitroblue tetrazolium (Thermo Scientific, USA) for the colorimetric detection of alkaline phosphatase activity.

2.2.4.3 *N. benthamiana* leaves

A. tumefaciens harboring either pEAQ3-OsDEFs or pEAQ1-OsDEF-His-SEKDELs was infiltrated into of six week-old plants same as mentioned in 2.2.3.3 without co-infiltration with p19. After 5 days, infiltrated leaves were collected and frozen by liquid nitrogen. Frozen leaves of 0.1 mg fresh weight were ground into a fine powder using a mixer mill and extracted in 1 ml lysis buffer (10 mM Tris-HCl pH 6.2, 50 mM KCl, 6 mM MgCl2, 0.4 M NaCl, 1% (v/v) Triton X-100, and 10 mM EDTA), centrifuged at 15,000 g for 15 min at 4 ℃ and then purified using His SpinTrap (GE Healthcare, UK). Purified protein was separated and performed western blot analysis as previously described in 2.2.4.2.2.

2.2.5 *In planta* inhibitory activity

A. tumefaciens harboring either pEAQ3-OsDEFs or pEAQ1-OsDEF-His-SEKDELs adjusted the OD_{600} of 2 was co-infiltrated at 1:1 ratio with *X. campestris,* which was cultured in nutrient broth at 28 ℃ 250 rpm, adjusted the OD_{600} of 0.4 into the abaxial of six week-old plants same as mentioned in 2.2.3.3. The infiltrated plants were left

for 7-9 days and observed the disease symptoms of *N. benthamiana* leaf comparing with control in same leaf.

CHAPTER III

RESULTS

3.1 Nucleotide analysis of *OsDEF7* and *OsDEF8* in Thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML105)

Both *OsDEF*s of Thai jasmine rice were cloned into CloneJET vector for comparing nucleotide sequences with that of Japanese rice from phytozome database (http://www.phytozome.net/). The nucleotide sequences of both genes in these two subspecies showed 100% identity (Figure 7) (Figure 8).

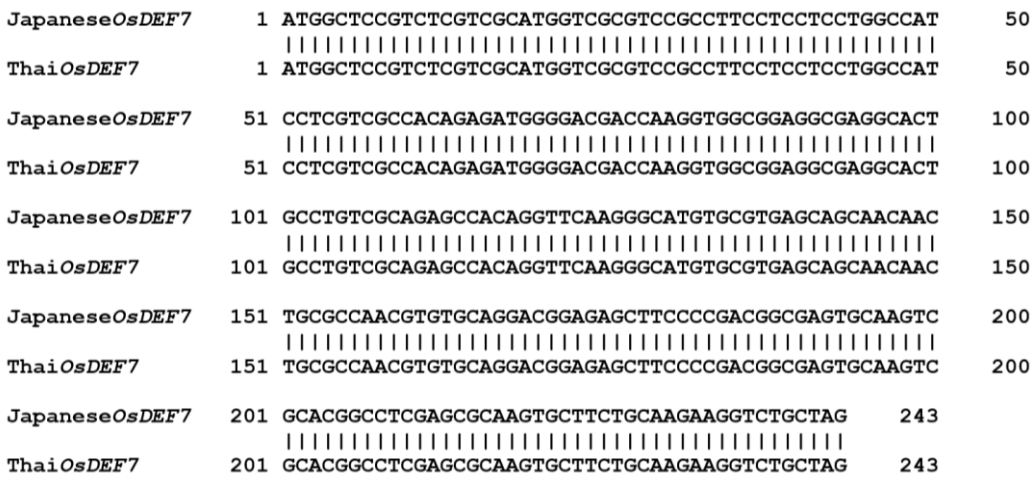

Figure 7. Nucleotide sequence alignment of *OsDEF7* from *Oryza sativa* L. ssp. *indica* cv. KDML105 and *Oryza sativa* L. ssp. *japonica* cv. Nipponbare

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Japanese OsDEF8      1 ATGGAGGCTTCACGCAAGGTGTTCTCGGCCATGCTTCTCATGGTGCTGCT     50
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Thai OsDEF8          1 ATGGAGGCTTCACGCAAGGTGTTCTCGGCCATGCTTCTCATGGTGCTGCT     50

Japanese OsDEF8     51 GCTTGCAGCCACTGGTGAGATGGGCGGGCCGGTGATGGTGGCGGAGGCTC    100
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Thai OsDEF8          51 GCTTGCAGCCACTGGTGAGATGGGCGGGCCGGTGATGGTGGCGGAGGCTC    100

Japanese OsDEF8    101 GGACGTGCGAGTCGCAGAGCCACCGGTTCAAGGGCCCGTGCGCCCGCAAG    150
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Thai OsDEF8         101 GGACGTGCGAGTCGCAGAGCCACCGGTTCAAGGGCCCGTGCGCCCGCAAG    150

Japanese OsDEF8    151 GCGAACTGCGCCAGCGTATGCAACACGGAGGGCTTCCCCGACGGCTACTG    200
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
Thai OsDEF8         151 GCGAACTGCGCCAGCGTATGCAACACGGAGGGCTTCCCCGACGGCTACTG    200

Japanese OsDEF8    201 CCACGGCGTCCGCCGCCGCTGCATGTGCACCAAGCCCTGCCCCTGA    246
                       |||||||||||||||||||||||||||||||||||||||||||||
Thai OsDEF8         201 CCACGGCGTCCGCCGCCGCTGCATGTGCACCAAGCCCTGCCCCTGA    246
```

Figure 8. Nucleotide sequence alignment of *OsDEF8* from *Oryza sativa* L. ssp. *indica* cv. KDML105 and *Oryza sativa* L. ssp. *japonica* cv. Nipponbare

3.2 *In silico* analysis

3.2.1 Expression level of *OsDEF*s in Japanese rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare)

The Genevestigator microarray database of Japanese rice cultivar Nipponbare provides the expression level of *OsDEF*s in different developmental stages (e.g., germinating seed, seedling, mature, and flowering) and also organs (e.g., root and leaf) (Figure 9). *OsDEF7* was highly expressed in the germinating stage whereas flower was the organ which genes were most expressed for *OsDEF8*. Between organs at seedling stage, *OsDEF7* showed equally expression in root and leaf while root was the organ that showed higher expression than the leaf.

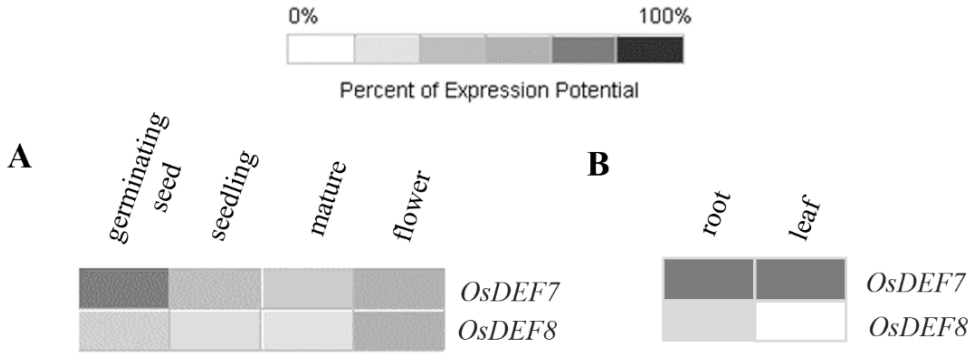
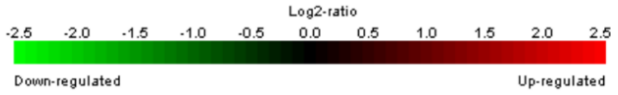


Figure 9. The expression levels of *OsDEF7* and *OsDEF8* in *Oryza sativa* L. ssp. *japonica* at different developmental stages; germinating seed, seedling (leaf and root), mature, and flower (A) and between organs; root and leaf of seedling (B) from the Genevestigator microarray database (https://www.genevestigator.ethz.ch)

3.2.2 Expression level of *OsDEF*s under abiotic stress

We also investigated the expression of these two genes under abiotic stress using the Genevestigator tool. *OsDEF7* and *OsDEF8* expression levels were influenced by several stress conditions (Figure 10). Particularly, *OsDEF7* was upregulated when treated under imbibition and anoxia conditions whereas *OsDEF8* was upregulated under drought, cold, and dehydration treatments.



Stress	Tissue	Cultivar	*OsDEF7*	*OsDEF8*	Experiment ID
drought	flag leaf	japonica			OS-00068
cold	seedling leaf	japonica			OS-00103
imbibition	embryo	japonica			OS-00108
anoxia	embryo	amaroo			OS-00051
dehydration	seedling	indica			OS-00008

Figure 10. The expression levels of *OsDEF7* and *OsDEF8* in abiotic stress by the following stress: drought, cold, imbibition, anoxia, and dehydration from the Genevestigator microarray database (https://www.genevestigator.ethz.ch).

3.3 Gene expression patterns of *OsDEF7* and *OsDEF8* in Thai jasmine rice using quantitative RT-PCR

3.3.1 Under normal condition

The level of gene expression of both DEFs in Thai jasmine rice was compared at the different stages including germination, seedling, mature until flowering. The results from quantitative RT-PCR showed that *OsDEF7* was highly expressed in the germinating stage whereas inflorescence was the organ in which *OsDEF8* was most expressed (Figure 11). These findings were similar to the patterns found in the Genevestigator database showing the data from Japanese rice (Figure 9). Noticeably, almost all tissues tested, *OsDEF7* was higher expressed than *OsDEF8.* Although similar gene expression pattern in developmental stages was found between Thai and Japanese rice, the gene expression pattern of *OsDEF7* in roots and leaves was different. *OsDEF7* in Japanese rice exhibited equally expression in roots and leaves but, in Thai rice, *OsDEF7* expressed much higher in roots more than leaves in both seedling and mature stages.

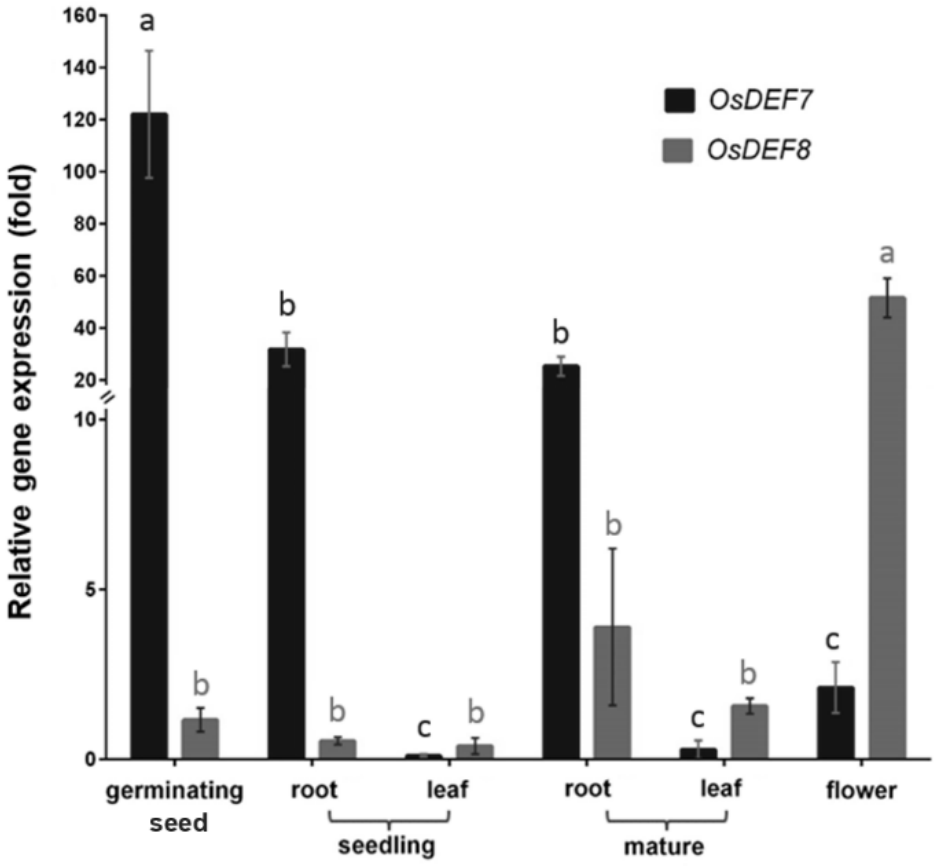


Figure 11. Relative gene expression of *OsDEF7* and *OsDEF8* in Thai jasmine rice from quantitative RT-PCR which were normalized to the standard EF1α gene and were calculated to relatively compare with the germinating stage of *OsDEF8*. Each value represents the mean ± SD (n = 3) with different letters indicate significant differences ($P < 0.05$) for each gene.

3.3.2 Infection with *X. oryzae* pv. *oryzae*

Thai jasmine rice was infected by *X. oryzae* pv. *oryzae* causing bacterial blight disease and collected at 6 hpi, 4 dpi, and 8 dpi. The disease symptom of the infected rice and the control were shown in Figure 12. The results from quantitative RT-PCR showed that *OsDEF7* and *OsDEF8* were upregulated upon infection with a high upregulation at 8 dpi (Figure 13). At the early infection time, *OsDEF8* was upregulated much quicker than *OsDEF7* at 4 dpi. Eventually, at 8 dpi, *OsDEF7* was expressed higher than the *OsDEF8*. The induction of both genes upon *X. oryzae* pv. *oryzae* infection suggests their involvement in pathogen defense.

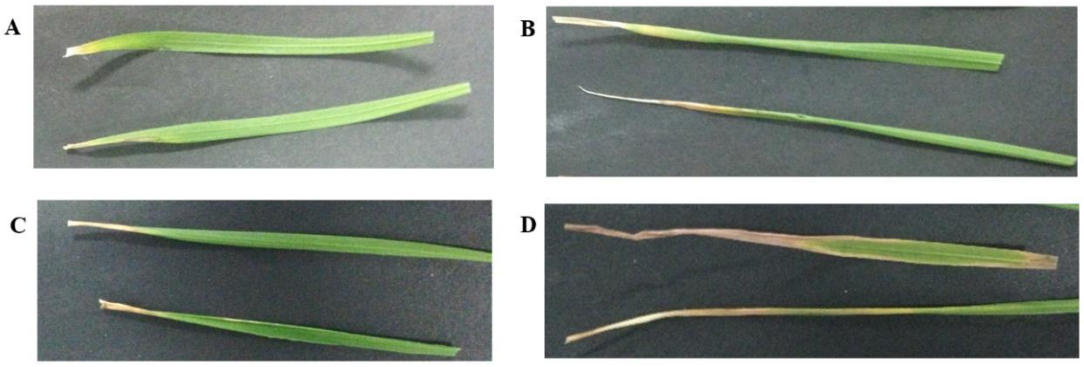


Figure 12. The leaves of Thai jasmine rice infected by *X. oryzae* pv. *oryzae*. Control leaves at 4 days post-infection (dpi) (A), and 8 dpi (C). Infected leaves at 4 dpi (B), and 8 dpi (D).

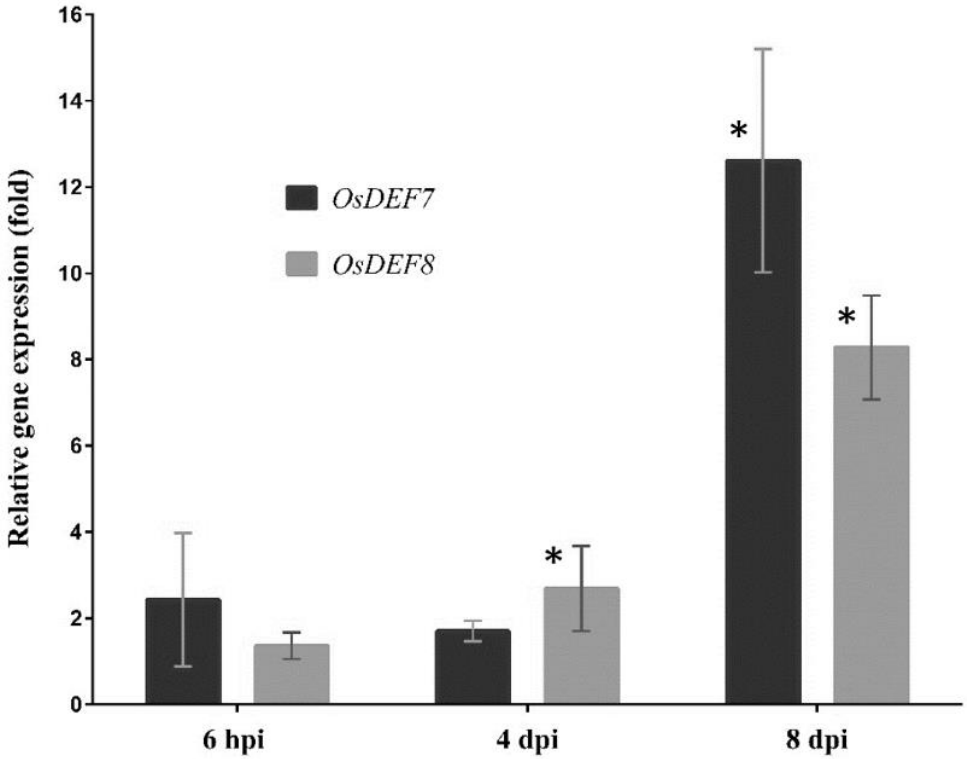


Figure 13. Relative gene expression of *OsDEF7* and *OsDEF8* in Thai jasmine rice after infected by *X. oryzae* pv. *oryzae*. determined by quantitative RT-PCR. Gene expression data were normalized to the standard *EF1α* gene and were calculated to relatively compare with the controls at each time point. Each value represents the mean ± SD (n = 3) with the asterisks indicate significant differences ($P < 0.05$) compared to their respective controls. (hpi, hours post-infection; dpi, days post-infection)

3.4 Subcellular localization

According to the prediction of protein subcellular localization using WoLF PSORT (Horton et al., 2007; http://wolfpsort.org), OsDEF7 and OsDEF8 were predicted to be localized to the extracellular compartment. To confirm the localization of both OsDEFs in plant, GFP was fused to the peptide's C terminus in a binary vector under 35S promoter. After that, either OsDEF7::GFP or OsDEF8::GFP fusion protein was

transiently expressed in the leaves of *N. benthamiana* and tobacco BY2-cell by agrobacterium-mediated gene transfer. For *N. benthamiana* leaf, at five days after infiltration, leaf cells were analyzed using confocal laser scanning microscopy. Most cells showed GFP fluorescence only in the periphery of the cell (Figure 14), indicating either extracellular or membrane localization of the fusion proteins compared with GFP control which localized in nucleus and periphery of the cell (Salinas *et al*., 2006; Bartetzko *et al*., 2009). In addition, we did not find any membrane-anchore sequences in both DEFs.

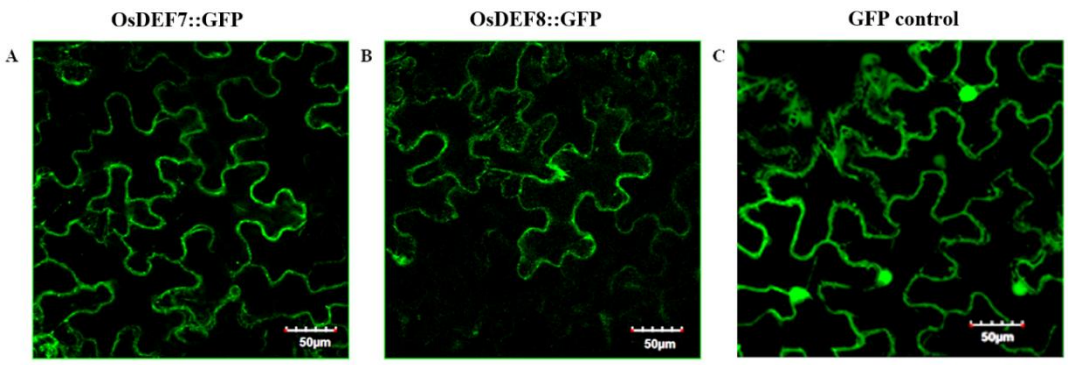


Figure 14. Confocal images showing fluorescence signals from *Agrobacterium* infiltrated leaf epidermal cells. *N. benthamiana* leaves were agroinfiltrated with (A) OsDEF7::GFP, (B) OsDEF8::GFP, and (C) GFP control. Scale bars represent 50 µm.

To demonstrate if the peptides are localized to cell membrane/cell wall or secreted, transient expression in BY2-cell was performed. OsDEF7::GFP and OsDEF8::GFP co-incubated with P19, which is a suppressor for RNAi, showed higher fluorescence in the culture medium under UV light than OsDEF::GFP without P19 while GFP control did not show fluorescence in the culture medium (Figure 15). These results strongly confirm that both OsDEFs are secreted into the extracellular compartment and P19 helps for higher protein production.

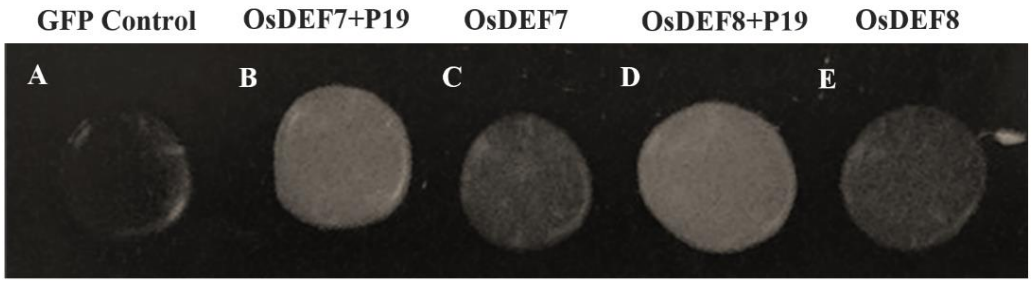


Figure 15. Fluorescence observed in the culture medium of transiently transformed BY2-cell with (A) 35S:GFP, (B) 35S:OsDEF7::GFP co-infected with 35S:P19, (C) 35S:OsDEF7, (D) 35S:OsDEF8::GFP co-infected with 35S:P19, and (E) 35S:OsDEF8

3.5 Production of recombinant rice AMPs in different system of plant tissues

3.5.1 Hairy roots

To produce the recombinant DEFs in plants, *OsDEF7* or *OsDEF8* were separately cloned into the pGWB2 expression vector which has the CaMV 35S promoter. We firstly evaluate the production from *N. benthamiana* hairy roots. *A. rhizogenes* harboring the gene of interest was infected into leaf of *N. benthamiana* for generating transgenic root. After 1-2 month(s), transformed roots were generated (Figure 16A) and transferred into a selection medium plate (Figure 16B). Finally, the selected hairy roots were cultured in the liquid medium (Figure 16C). According to the previous subcellular localization experiment, we expected to detect OsDEF proteins in the culture medium. Unfortunately, DEFs cannot be detected on SDS-PAGE analysis (data not show). Subsequently, RNAs of transgenic roots were extracted and cDNA was synthesized for investigation of the RNA expression. Although we cannot detected protein on SDS-PAGE, the expression of mRNA can be detected (Figure 17), suggesting that the transgenic roots expressed the genes.

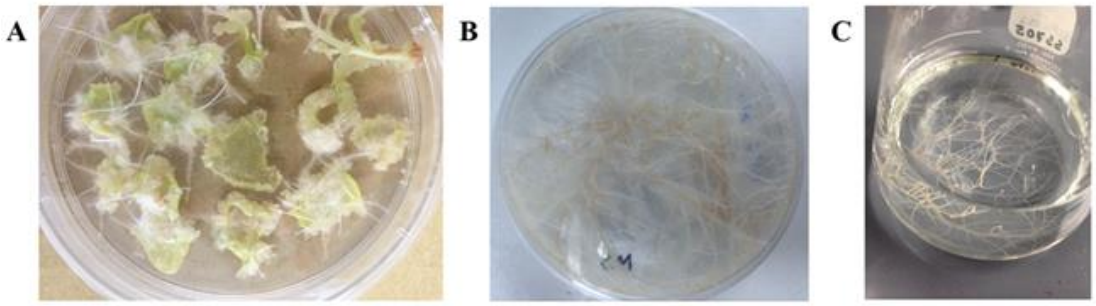


Figure 16. (A) The hairy root generated from *N. benthamiana* leaves on MS agar containing 500 µg/ml of cefotaxime. (B) The transgenic hairy root on a selection MS agar plate containing 50 µg/ml kanamycin and 50 µg/ml hygromycin B. (C) The transgenic hairy root cultured in the MS liquid medium

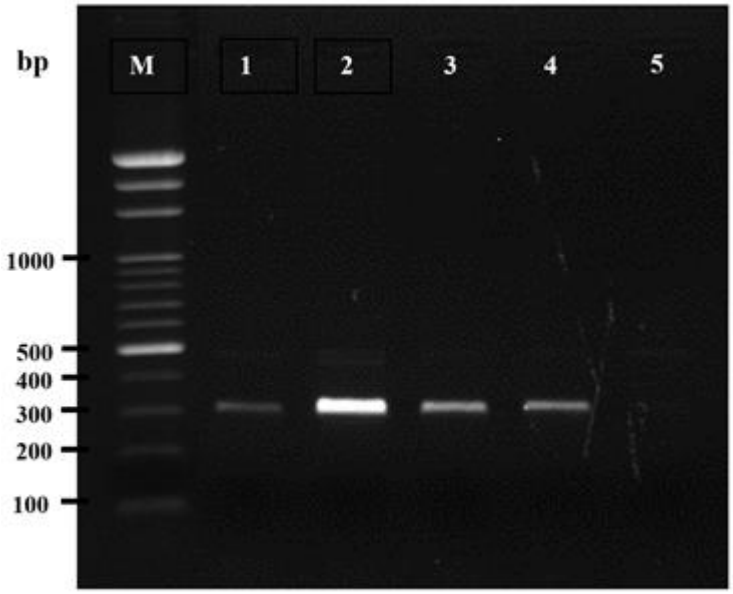


Figure 17. Gel electrophoresis results of PCR amplification of *OsDEF* genes from cDNAs of respective transgenic hairy roots. Gene products were visualised on 1 % agarose gel. Lane M: 100 bp DNA ladder marker; Lane 1: pGWB2-*OsDEF7* line 1; Lane 2: pGWB2-*OsDEF7* line 2; Lane 3: pGWB2-*OsDEF8* line 1; Lane 4: pGWB2-*OsDEF8* line 2; Lane 5: negative control

3.5.2 BY-2 cells

Since the peptide production from the transgenic hairy roots was unsuccessful, the peptide production from BY-2 suspension cells was performed. Either *OsDEF7* or *OsDEF8* was cloned into the pEAQ3 expression vector containing His-tag. *A. tumefaciens* harboring the gene of interest was transformed into BY-2 cell. After 1-2 month(s), transformed BY-2 cells were grown and transferred into selection medium plates (Figure 18A). Then, the transgenic BY-2 was cultured in the liquid medium containing a suitable antibiotic. (Figure 18B). We expected to detect OsDEF proteins in the culture medium according to our subcellular localization. Unfortunately, DEFs cannot be detected by Western blot analysis. Subsequently, RNA of transformed BY-2

cell was extracted and synthesized cDNA for investigation of the RNA expression. Although we could not detect any protein, the expression of mRNA can be detected (Figure 19) as same as that of the transgenic hairy root.

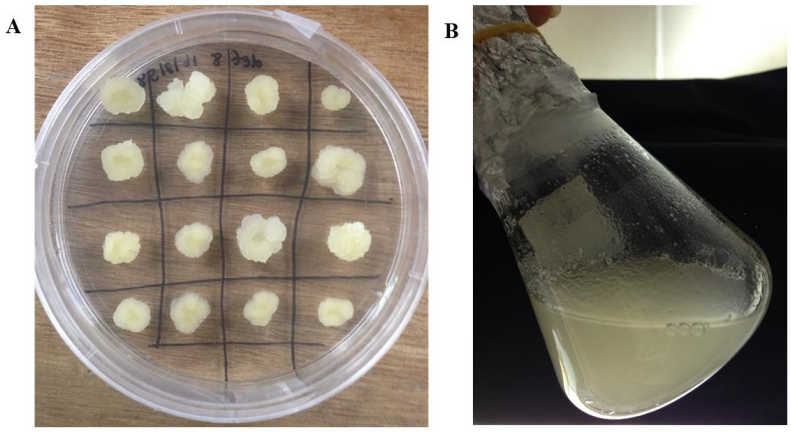


Figure 18. (A) Transgenic BY-2 cell on a selection medium plate (2 weeks after transfer to the plate) (B) Transgenic BY-2 in liquid medium at day 7 after inoculation to the liquid medium.

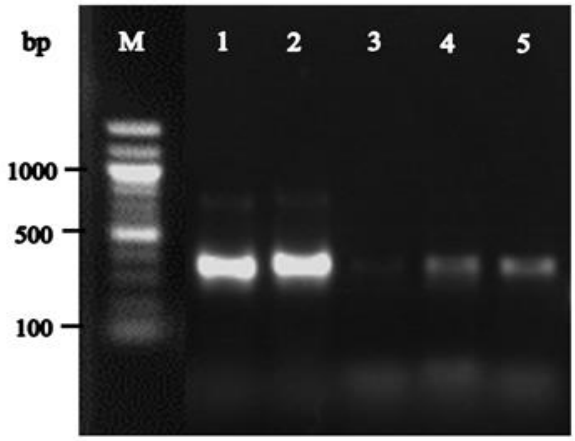


Figure 19. Gel electrophoresis results of PCR amplification of *OsDEF* genes from cDNAs of respective transgenic BY-2 cell. Gene products were visualised on 1% agarose gel. Lane M: DNA ladder; Lane 1: pEAQ3-*OsDEF*7 line 1; Lane 2: pEAQ3-*OsDEF*7 line 2; Lane 3: negative control; Lane 4: pEAQ3-*OsDEF8* line 1; Lane 5: pEAQ3-*OsDEF8* line 2

3.5.3 *N. benthamiana* leaves

To confirm the transient expression of recombinant DEFs, the infiltration of *N. benthamiana* leaves was performed. *A. tumefaciens* harboring either pEAQ3-*OsDEF7* or pEAQ3-*OsDEF8* was infiltrated into leaves and then crude proteins were extracted from leaves. Though, the OsDEFs could not be detected by western blot analysis. Since DEF has a strong cationic charge, we thought that DEFs might form strong interactions with the cell wall compartment. To relocalize OsDEFs subcellularly, we designed another construct targeting the OsDEFs to the ER compartment; pEAQ1-OsDEF-His-SEKDEL. Finally, we were able to detect the recombinant peptides which showed the bands at the expected size of ~ 9 kDa (Figure 20). However, the unknown bands of ~17 kDa were also detected using His-tag antibody in crude protein extracts and purified protein fractions, even also in the GFP infiltrated leaves.

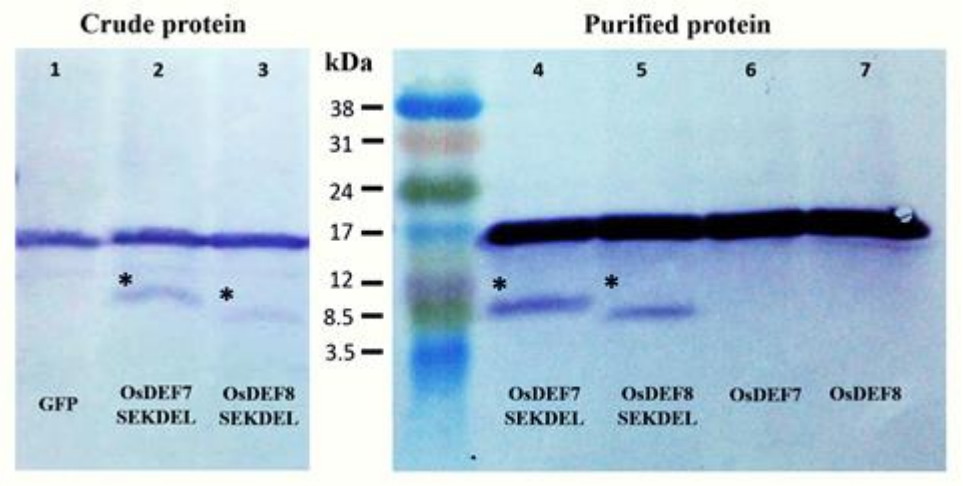


Figure 20. Western blot analysis of crude and purified protein from *N. benthamiana* leaves after 5 days infiltration. Total soluble protein was extracted from leaves 0.1 g/ml fresh weight in Tris-HCl buffer. For purified protein, 2 ml of crude protein was purified using His Spin Trap and loaded 20 µl of purified protein (lane 4-7) or crude protein (lane 1-3) per lane. Proteins were separated by 12% SDS-PAGE before transfer to PVDF membrane. Recombinant proteins were detected using Pierce His tag mouse primary antibody diluted 1:2000 followed by the alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody diluted 1:1000. The signal was detected by BCIP/NBT.

3.6 *In planta* antimicrobial activity of recombinant OsDEF7 and OsDEF8

Although *in vitro* antimicrobial activity from recombinant OsDEF7 and OsDEF8 produced from *E. coli* was confirmed. Here, we performed *in planta* inhibitory activity test. *A. tumefaciens* harboring either pEAQ3-OsDEFs or pEAQ1-OsDEFs-SEKDEL were co-infiltrated with *X. campestris* into *N. benthamiana leaf*. After 9 days, both secreted-OsDEFs and ER-localized OsDEFs exhibited the disease resistance comparing with both controls which were co-infiltrated *X. campestris* and *A. tumefaciens* with either empty vector or non-transformed (Figure 21).

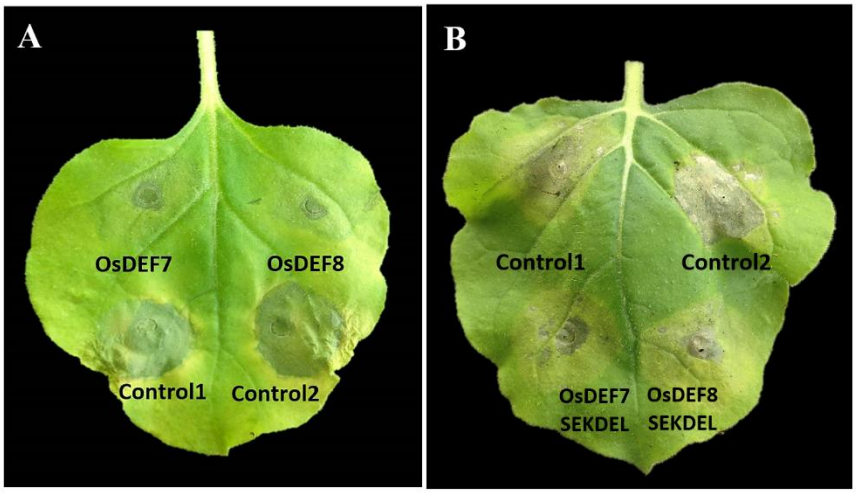


Figure 21. *A. tumefaciens* harboring (A) pEAQ3-*OsDEF7*, pEAQ3-*OsDEF8* (B) pEAQ1-*OsDEF7*-SEKDEL, pEAQ1-*OsDEF8*-SEKDEL, blank pEAQ3 vector (control 1) or none (control 2) only was co-infiltrated with a ratio of 1:1 with *X. campestris* into *N. benthamiana* leaf. This photo was taken at 9 days after infiltration.

CHAPTER IV
DISCUSSION

DEFs are AMPs that are part of the innate immune system, contributing to the first line of defense against invading pathogens. Post-translational modifications such as disulfide bond formation and folding are critical for maintaining AMP structure and biological activity. These modifications can be performed properly by plant-based systems (Holaskova *et al*., 2015). Although our group successfully produced the recombinant OsDEF7 and OsDEF8 in *E. coli* and the proteins exhibited antimicrobial activities, detailed characterizations and alternative production system have never been reported. In this thesis, I performed characterization of these genes and investigated their expression in plant-based systems.

OsDEF7 and *OsDEF8* show subspecies-specific expression pattern

To further characterize *OsDEF7* and *OsDEF8*, additional *in silico* analysis was performed using the databases in which most information were from *Oryza sativa* L. ssp. *japonica* cv. Nipponbare. The *in silico* analyses showed that the expressions of *OsDEF*s are most highly expressed in seed and inflorescence while the common target organ, leaf, for phytopathogen infection showed very low expression (Tantong *et al*., 2016). Some DEFs are also supposed to play a role in protection of the vulnerable germinating seed against pathogen. For example, RsAFP1 and RsAFP2 from radish seeds are preferentially released during seed germination after disruption of the seed coat and the amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is suppressed (Terras *et al*., 1995). The results from quantitative RT-PCR analysis in Thai jasmine rice also showed highly express in seed and inflorescence. Although *OsDEF7* and *OsDEF8* showed similar gene expression patterns in developmental stages with the database while it was different between

organs root and leaf. These findings suggest a difference in gene regulation between subspecies.

OsDEF7 and _OsDEF8_ are involved in pathogen response

Although *OsDEF7* and *OsDEF8* showed relatively low expression in the leaf, they were upregulated upon infection with *X. oryzae* pv. *oryzae* when compared to the controls. Previously, *in silico* analysis showed that *OsDEF7* and *OsDEF8* were also upregulated when infected with rice blast fungus, *M. oryzae* at 48 hpi and rice bacterial blight, *X. oryzae* pv. *oryzae* at 24 hpi (Tantong *et al*., 2016). However, quantitative RT-PCR results in this thesis demonstrated that both genes were upregulated at very late infection stage (after 4 dpi). Taken together with the low expression of *OsDEF*s in the leaves and the late upregulation, this might be a reason that rice plants are susceptible to *X. oryzae* pv. *oryzae* and also other pathogens. If there is any process to induce the expression of antimicrobial OsDEFs much quicker, it might be possible that rice plant will be tolerant to the pathogens. It has been reported in many plants such as pea, tobacco, *Arabidopsis*, and spruce that *DEF*s gene are also upregulated upon pathogen infection (Lay and Anderson, 2005).

***In silico* expression analysis hints the involvement of _OsDEF7_ and _OsDEF8_ in various functions**

DEFs might be involved in other functions. Since in this study using the Genevestigator tool, we showed that the expression levels of these two *OsDEF* genes were affected by abiotic stress including drought, cold, imbibition, anoxia, and dehydration. This observation suggested that OsDEFs might be involved in other processes and was supported by several reports. A DEF, *Tad1*, isolated from the crown tissues of winter wheat (*Triticum aestivum*) was induced during cold acclimation (Koike *et al*., 2002). Strong accumulation of *CADEF1* mRNA in pepper leaves was activated in

response to wounding, high salinity and drought (Do *et al*., 2004). Moreover, tomato DEF *DEF2* is expressed during early flower development. DEF mRNA abundance, peptide expression and processing are differentially regulated in developing flowers. Antisense suppression or constitutive overexpression of DEF2 reduces pollen viability and seed production (Stotz *et al*., 2009). In *Zea mays*, the DEF *ZmES4* is demonstrated to be expressed in the synergid cells and shown to be involved in pollen tube bursting by activating the potassium channel *KZM1* (Amien *et al*., 2010). Furthermore, we have previously found using *in silico* analysis that *OsDEF*7 and *OsDEF*8 are coexpressed with gibberellin-regulated protein (Tantong *et al*., 2016). The question is whether or not these hormones/small molecules play a role in the expression of AMP when plants defend themselves against disease. There is an evidence showing that plant hormones indeed regulate the expression of AMP. A tomato DEF, tgas118, seems to be regulated by gibberellins throughout flower development (Van den Heuvel *et al*., 2001). However, molecular details how DEF works have not been completely understood.

OsDEF7 and OsDEF8 are secreted to extracellular compartment

Subcellular localization of OsDEF strongly supports the involvement in pathogen response since they are secreted to extracellular that could interact with pathogen first. Secretory proteins have to pass through endoplasmic reticulum for correct folding and assembly of proteins (Vitale and Denecke, 1999). Expression of AMPs in the apoplast is preferred because most pathogenic microorganisms are present in the extracellular space during the early stages of infection. OsDEFs localized in the apoplastic region of the plant tissue could interact with the microbial membrane leading to membrane destabilization, and reduced proliferation of pathogen in the plant tissue, thus imparting enhanced disease resistance to phytopathogens (Jha *et al*., 2009). Moreover, the result from transient BY-2 cell showed that P19 could help for transient protein expression. Previous studies showed that p19 of *Cymbidium ringspot*

virus (CymRSV) inhibits RNA silencing via its small RNA-binding activity *in vivo*. Small RNAs bound by p19 *in planta* are bona fide double-stranded siRNAs and they are silencing competent in the in vitro RNA-silencing system. P19 also suppresses RNA silencing in the heterologous *Drosophila in vitro* system by preventing siRNA incorporation into RISC (Lakatos e*t al*., 2004). Hence, these results from transient expression *N. benthamiana* leaf and transient BY-2 cell strongly confirm that both OsDEFs are secreted into extracellular compartment and P19 helps for higher protein production.

OsDEF7 and OsDEF8 are successfully produced in *N. benthamiana* leaves

OsDEF7 and OsDEF8 were clearly demonstrated to be localized in the extracellular compartment. However, they could not be detected in the medium of transformed hairy roots and BY-2 cell cultures, even though the expression of mRNA was found. This might be because the low level of protein expression and/or there is a strong interaction with cell wall component. How OsDEF::GFP can be secreted but OsDEF could not. It might be explained that OsDEF::GFP could not form a strong interaction with cell wall component since GFP is a large protein (~27 kDa) and OsDEFs are just only 6 kDa. Therefore, any interactions that retain OsDEFs to the cells could be disrupted. Subsequently, I tried another plant system, *N. benthamiana* leaves, to express both peptides. After protein purification, I still cannot detect the OsDEF proteins. From previous study, it is suggested that cationic AMPs tend to be difficult to detect and isolate from complex plant tissue (Company *et al*., 2014). They, therefore, expressed synthetic linear AMP BP100, a short cecropin A-melittin hybrid AMP, with the addition of SEKDEL (ER retention signal) at C-terminal of which preserved its antimicrobial activity and provided easier detection (Company *et al*., 2014). Then, I optimized codon of *OsDEF* for *N. benthamiana* and added His-tag together with SEKDEL peptide. The result form Western blot analysis showed that both OsDEFs can

be detected in monomer forms. This is in contrast to our previous recombinant OsDEF productions in *E. coli*, that OsDEF7 and OsDEF8 formed homodimers around 15 kDa (Tantong *et al*., 2016). Even though the ER-localized OsDEFs were detected as monomers, it cannot be ruled out that that the secreted OsDEFs would also form monomers. Nevertheless, Stover *et al*. (2013) suggested that proteins in *in vitro* usually form large aggregates that could hinder their functions, whereas *in planta*, AMPs likely exist as discrete molecules or much smaller aggregates. This might explain how OsDEF7 and OsDEF8 produced *in planta* form monomers. It has been found that most DEFs are produced as monomers (Kovalskaya and Hammond, 2009; De Beer and Vivier, 2011). However, a previous study reported that dimerization of NaD1, a DEF from *N. alata* flowers, enhances its antifungal activity when compared to the monomeric protein (Lay *et al*., 2012). Therefore, it can be conclude that different DEFs might form differently either as monomers or dimers, possibly depending on their primary amino acid sequences. Definitely, their overall structures would affect antimicrobial activity.

Although the purification of ER-localized OsDEFs has been performed using polyhistidine affinity tag, unfortunately, there was an unexpected band around 17 kDa which is clearly a protein from *N. benthamiana* leaf that can bind with His-tag antibody. To improve the purification, gel filtration chromatography might be used to separate OsDEFs from the unknown protein. Nevertheless, the purified proteins yielded in a low concentration and they might not be sufficient to perform *in vitro* activity testing so *in planta* activity testing was preferred. Since the previous works reported that *X. campestris* and *X oryzae* are considered as non-adaptive pathogens for *N. benthamiana* but they could induce a hypersensitive response in this plant (Metz *et al*., 2005; Gonzalez *et al*., 2007). I demonstrated that both OsDEFs show *in planta* inhibition against *X. campestris* in *N. benthamiana* leaves. It has been reported that many AMPs are more effective *in planta* than *in vitro* (Cary *et al*., 2000; Visser *et al*., 2012). These observations suggest that AMP may require post-translational processing

to achieve functional conformation and to contribute to a better activity of a peptide (Holaskova *et al*., 2015). Also, Stover *et al*. (2013) suggested a higher activity of AMP *in planta* because of an ability to form smaller AMP aggregates, in contrast to larger aggregates formed *in vitro* that would interfere their inhibitory functions. Therefore, transgenic plants overexpressing OsDEFs could develop resistance against pathogen infection.

Various reports on DEFs demonstrated the heterologous DEF production in diverse hosts, such as bacteria, yeasts, and plants, mainly for research purposes. Among bacteria, *E. coli* is the most commonly used for DEF expression. *Arabidopsis halleri* foliar DEF (Marquès *et al*., 2009), *Tephrosia villosa* DEF (Vijayan *et al*., 2008) and DEF from sweet potato (Huang *et al*., 2008; Kovalskaya *et al*., 2009) have been expressed using this heterologous system that benefits from a wealth of commercially available strains and plasmids, besides short generation times. Plant DEFs expression in yeast has mainly been performed using the yeasts *Saccharomyces cerevisiae* or *Pichia pastoris* (Cabral *et al*., 2003; Kant *et al*., 2009). Heterologous DEF expression has also been tested in plants (Jha *et al*., 2009; Anuradha *et al*., 2008; Abdallah *et al*., 2010). From this study, OsDEF7 and OsDEF8 were successfully expressed in plant-based systems but they were produced in low concentrations and difficult to extract and purify when comparing with the expression in *E. coli* (Tantong *et al*., 2016). There are a few suggestions for production improvement of OsDEF7 and OsDEF8. Since we used His-tag for purification and got the unexpected band on Western blot so if there is a DEF antibody might can solve this problem. Even though we cannot get highly purified peptides, it is possible that we can use crude protein of OsDEF from transient expression to test *in vitro* activity but we have to make sure that crude proteins contain no *Agrobacterium* after the infiltration. In Addition, the expression vector could be changed for a higher protein production, although the vector used in this study was claimed for a very high protein production (Sainsbury *et al*., 2009; Peyret and

Lomonossoff, 2013). Generating transgenic plants might also be a proper way for expression of OsDEF7 and OsDEF8 because there are many previous studies successfully expressing DEFs in transgenic plants for example; overexpression of DmAMP1 in *O. sativa* exhibited antimicrobial activities against *M. oryzae* and *Rhizoctonia solani* (Jha *et al*., 2009), the transgene BjD in *N. tabacum* could resist *Fusarium moniliforme* and *Phytophthora parasitica* pv. *nicotianae* (Anuradha *et al*., 2008). In term of application, transient expression in *N. benthamiana* leaf might be suitable for screening other AMPs because of short-time period expression. Furthermore, Wu *et al*. (2011) expressed Trx-Ace-AMP1 from onion in *E. coli.* Trx-Ace-AMP1 protein was spreaded together with a pathogen *A. solani* causing early blight disease onto tomato leaves with a cotton swab. One week after inoculation, leaves treated with Trx-Ace-AMP1 had less disease symptoms compared with control. If the production of OsDEF7 and OsDEF8 were improved to achieve high concentration of protein, we might be able to use them to spray onto the crop field for increasing resistance against phytopathogens.

CHAPTER V
CONCLUSIONS

In this study, the alternative system for OsDEF7 and OsDEF8 productions changing from bacteria to plants was evaluated. Gene expression profiles and subcellular localization of these peptides were also performed to provide additional *in vivo* functions of both peptides.

The nucleotide sequences of Thai jasmine rice *Oryza sativa* L. ssp. *indica* cv. KDML105 and Japanese rice *Oryza sativa* L. ssp. *japonica* cv. Nipponbare showed 100% identity. Although the similar gene expression patterns at developmental stages were found between, the OsDEF7 expression levels in roots and leaves were shown to be different suggesting a difference in gene regulation between subspecies. In addition, the infected Thai jasmine rice by *X. oryzae* pv. *oryzae* showed the upregulations of OsDEF7 and OsDEF8 suggesting the involvement in pathogen response. Moreover, *in silico* gene expression analyses using Genevestigator tool confirm that the expression levels of these two *OsDEF* genes were affected by abiotic stress including drought, cold, imbibition, anoxia, and dehydration.

For recombinant peptide production, both OsDEFs were shown to be localized to the extracellular compartment using GFP fusion approach. Even though OsDEFs cannot be detected in the medium of transformed hairy root of *N. benthamiana* and tobacco BY-2 cell culture, the expression of mRNA was found suggesting that OsDEFs might be produced but not secreted. Nevertheless, the ER-retained OsDEFs in *N. benthamiana* leaves was successfully produced at low concentrations but both secreted OsDEFs and ER-retained OsDEFs exhibit the disease resistance *in planta* when infected with *X. campestris.* These results provide information to develop an alternative host producing these secreted AMPs.

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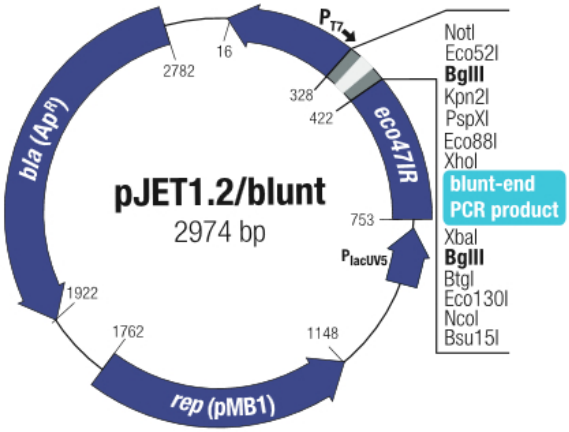
APPENDIX

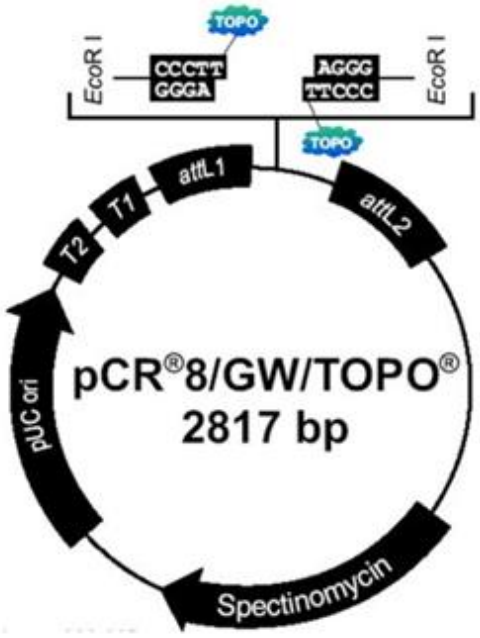
Appendix A

Vector maps

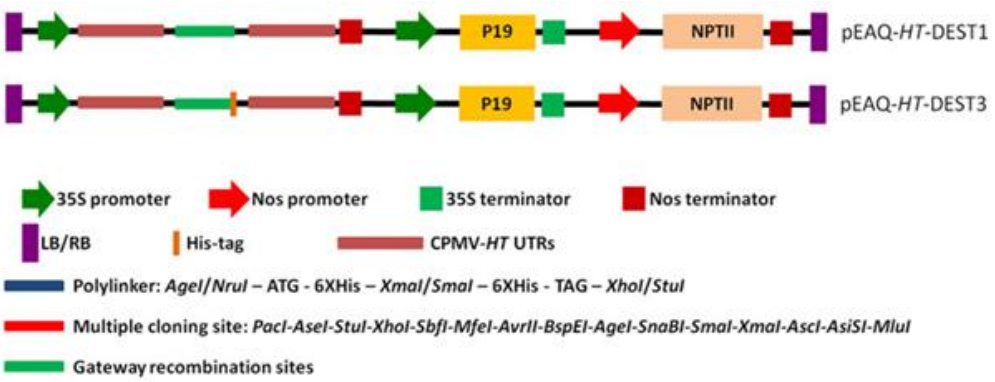
CloneJET PCR Cloning Kit (taken from www.lifetechnologies.com)



pCR™8/GW/TOPO® TA Cloning Kit (taken from www.thermofisher.com)

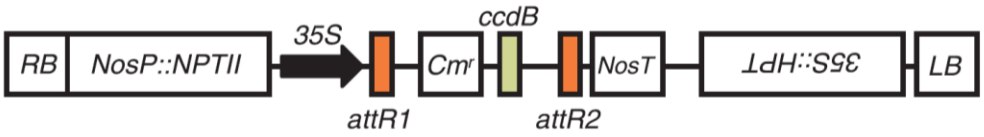


pEAQ-HT-DEST1 (pEAQ1) and pEAQ-HT-DEST3 (pEAQ3) (taken from Peyret and Lomonossoff, 2013)



pGBW2 and pGWB5 (taken from Nakagawa *et al*., 2007)

pGBW2



pGWB5

Appendix B

The nucleotide sequence alignment

```
Original seq      1   ATGGCTCCGTCTCGTCGCATGGTCGCGTCCGCCTTCCTCCTCCTGGCC   48
                      |||||.||.||.||..|.||||||.||....||.|||.|.||||||.|||
Optimized seq     1   ATGGCACCTTCACGAAGGATGGTTGCTAGTGCTTTCTTGCTCCTCGCC   48
                        M  A  P  S  R  R  M  V  A  S  A  F  L  L  L  A

Original seq      49  ATCCTCGTCGCCACAGAGATGGGGACGACCAAGGTGGCGGAGGCGAGG   96
                      ||.||.||.||.||.||||||||.|||||.|||||.||.||||||..|.
Optimized seq     49  ATACTGGTAGCAACCGAGATGGGTACGACTAAGGTCGCAGAGGCCCGT   96
                        I  L  V  A  T  E  M  G  T  T  K  V  A  E  A  R

Original seq      97  CACTGCCTGTCGCAGAGCCACAGGTTCAAGGGCATGTGCGTGAGCAGC   144
                      |||||.|||...|||..|||.||.||||||||.|||||.||.||...|
Optimized seq     97  CACTGTCTGAGCCAGTCCCATAGATTCAAGGGTATGTGTGTTAGTTCC   144
                        H  C  L  S  Q  S  H  R  F  K  G  M  C  V  S  S

Original seq      145 AACAACTGCGCCAACGTGTGCAGGACGGAGAGCTTCCCCGACGGCGAG   192
                      ||.||.||||||.||||||||||||.||.||||||.||||||.||.||||||.
Optimized seq     145 AATAATTGCGCTAACGTGTGCAGAACTGAGAGTTTCCCAGATGGCGAA   192
                        N  N  C  A  N  V  C  R  T  E  S  F  P  D  G  E

Original seq      193 TGCAAGTCGCACGGCCTCGAGCGCAAGTGCTTCTGCAAGAAGGTCTGC   240
                      |||||.||.||.||.||.||..|.||||||||||||.||.||||||.|||
Optimized seq     193 TGCAAATCTCATGGGCTGGAAAGAAAGTGCTTCTGTAAAAAGGTATGC   240
                        C  K  S  H  G  L  E  R  K  C  F  C  K  K  V  C

Original seq      241 TAG-----------------------------------          243
                      *
Optimized seq     241 CACCATCATCACCACCATTCAGAAAAGGACGAATTGTAG          279
                        H  H  H  H  H  H  S  E  K  D  E  L  *
```


The nucleotide sequence alignment of the original sequence and codon-optimized *OsDEF7* for *N. benthamiana* including 6xHis-tag and SEKDEL ER retention at the C-terminal.

```
Original seq      1    ATGGAGGCTTCACGCAAGGTGTTCTCGGCCATGCTTCTCATGGTGCTG    48
                       ||||||.||....||.||.|||||||...|||||||.|.||.||||||..|.
Optimized seq     1    ATGGAAGCCAGTCGTAAAGTGTTCAGCGCCATGTTACTGATGGTTTTA    48
                        M  E  A  S  R  K  V  F  S  A  M  L  L  M  V  L

Original seq      49   CTGCTTGCAGCCACTGGTGAGATGGGCGGGCCGGTGATGGTGGCGGAG    96
                       ||.||.||.||||||.||.||||||||.||.||.||||||||.||.||.
Optimized seq     49   CTCCTCGCCGCCACAGGAGAGATGGGAGGTCCCGTGATGGTAGCTGAA    96
                        L  L  A  A  T  G  E  M  G  G  P  V  M  V  A  E

Original seq      97   GCTCGGACGTGCGAGTCGCAGAGCCACCGGTTCAAGGGCCCGTGCGCC    144
                       ||..|.||.||||||||.||...|||.|.||||||||||.||.|||
Optimized seq     97   GCCAGAACATGCGAGTCTCAATCACACAGATTCAAGGGCCCTTGTGCC    144
                        A  R  T  C  E  S  Q  S  H  R  F  K  G  P  C  A

Original seq      145  CGCAAGGCGAACTGCGCCAGCGTATGCAACACGGAGGGCTTCCCCGAC    192
                       ||.||||||.||||||.||...|||.||.||.||.||.||.||||||.||.
Optimized seq     145  CGTAAGGCAAACTGTGCATCCGTTTGTAATACTGAAGGATTCCCTGAT    192
                        R  K  A  N  C  A  S  V  C  N  T  E  G  F  P  D

Original seq      193  GGCTACTGCCACGGCGTCCGCCGCCGCTGCATGTGCACCAAGCCCTGC    240
                       ||||||.||||||||.||.||.||.||.||.||||||.||||||.||||||.
Optimized seq     193  GGCTATTGCCACGGTGTGCGTCGTCGTTGTATGTGTACCAAACCCTGT    240
                        G  Y  C  H  G  V  R  R  R  C  M  C  T  K  P  C

Original seq      241  CCCTGA-------------------------------------         246
                       ||. *
Optimized seq     241  CCTCACCATCATCACCACCATTCAGAAAAGGACGAATTGTAG          282
                        P  H  H  H  H  H  H  S  E  K  D  E  L  *
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The nucleotide sequence alignment of the original sequence and codon-optimized *OsDEF8* for *N. benthamiana* including 6xHis-tag and SEKDEL ER retention at the C-terminal.

Appendix C

Preparation of polyacrylamide gel electrophoresis

1. Stock reagents

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjust pH to 6.8 with conc. HCl and adjust volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.15 g

Adjust pH to 8.8 with conc. HCl and adjust volume to 100 ml with distilled water

10% (w/v) SDS solution

SDS 10 g

Add distilled water to the final volume of 100 ml

10% Ammonium persulfate ($(NH_4)_2S_2O_8$)

Ammonium persulfate 1 g

Add distilled water to the final volume of 10 ml

2. SDS-PAGE

12% Separating gel, 8ml

30% Acrylamide solution	3.2 ml
1.5 M Tris-HCl pH 8.8	2 ml
Distilled water	2.6 ml
10% SDS solution	80 µl
10% $(NH_4)_2S_2O_8$	80 µl

TEMED	5 µl

5% Stacking gel, 5 ml

30% Acrylamide solution	1 µl
1 M Tris-HCl pH 6.8	1.25 µl
Distilled water	2.6 ml
10% SDS solution	50 µl
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	4 µl

5X Sample buffer, 10 ml

1 M Tris-HCl pH 6.8	0.6 ml
50% Glycerol	5 ml
2-Mercaptoethanol	0.5 ml
10% SDS solution	2 ml
1% Bromophenol blue	1 ml
Distilled water	0.9 ml

One part of sample was mixed with four parts of protein sample.

10X Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	121.14 g
Tricine	179.20 g
SDS	10 g

Adjust volume with distilled water to 1 litre (No pH adjustment is required, final pH should be 8.3), One part of electrophoresis buffer was dissolved in nine parts of distilled water.

APPENDIX D

Preparation of Western blot reagents

Stock reagents

10X Tris buffer saline (TBS) pH 7.6, 1 litre

Tris (hydroxymethyl)-aminomethane	60.5 g
NaCl	87.6 g

Adjust to pH 7.6 and adjust to final volume of 1 litre

One part of TBS buffer was mixed with nine parts of distilled water.

10X Tris glycine buffer (TG) pH 8.3, 1 litre

Tris (hydroxymethyl)-aminomethane	30 g
Glycine	144 g

(Final pH should be 8.3)

One part of electrophoresis buffer was mixed with nine parts of distilled water.

APPENDIX E

Preparation of agarose gel electrophoresis buffer

10X Tris boric EDTA (TBE) buffer, pH 8.3

Tris (hydroxymethyl)-aminomethane	108 g
Boric acid	55 g
EDTA	9.3 g

Adjust pH to 8.3 and adjust volume to 1 litre

APPENDIX F

Preparation of Yoshida's stock solution

Element	Reagent (AR grade)	Preparation (g/100 ml)	
N	NH_4NO_3	9.14	
P	$NaH_2PO_4 \cdot 2H_2O$	4.03	
K	K_2SO_4	7.14	
Ca	$CaCl_2$	8.86	
Mg	$MgSO_4 \cdot 7H_2O$	32.4	
Mn	$MnCl_2 \cdot 4H_2O$	150 mg	Dissolve separately; then combine with 5 ml of concentrated H_2SO_4. Make up to 100 ml with distilled water.
Mo	$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_4O$	7.4 mg	
B	H_3BO_3	93.4 mg	
Zn	$ZnSO_4 \cdot 7H_2O$	3.5 mg	
Cu	$CuSO_4 \cdot 5H_2O$	3.1 mg	
Fe	$FeCl_3 \cdot 6H_2O$	770 mg	
	Citric acid (monohydrate)	1190 mg	

VITA

Miss Kamonwan Weerawanich was born on January 13th, 1992. She graduated with the Second-Class Honors Bachelor's degree from the Department of Biochemistry, Faculty of Science, Chulalongkorn University. Then, she continued studying Master degree in Biochemistry and Molecular Biology program, Faculty of Science at Chulalongkorn University.

She participated in many scientific conferences during those years. She got outstanding poster presentation award in the 5th International Biochemistry and Molecular Biology Conference 2016 at Songkhla, Thailand and her proceeding was published in the title of "Cloning, gene expression, and subcellular localization analysis of rice DEF 7 and 8 from Oryza sativa L. cv. KDML 105". She also joined the 28th Annual Meeting of the Thai Society for Biotechnology and International Conference at Chiang Mai, Thailand and the Biological Sciences Graduate Congress (BSGC) at the University of Malaya, Kuala Lumpur, Malaysia. Moreover, she participated in in silico analysis and in planta antimicrobial activity of other rice antimicrobial peptides. She is one of the authors in the article that has been published in Peptides (2016), 84, 7-16, entitled "Two novel antimicrobial defensins from rice identified by gene coexpression network analyses" The other two publications, "Heterologous expression and antimicrobial activity of OsGASR3 from rice (Oryza sativa L.)" and "In silico analyses of rice thionin genes and the antimicrobial activity of OsTHION15 against phytopathogens" that she co-authored are now under review.