EXPRESSION DETERMINATION OF ETHYLENE SIGNAL TRANSDUCTION GENES IN KHAW DOK MALI 105 (KDML105) RICE DURING RICE TUNGRO BACILLIFORM VIRUS (RTBV) INFECTION

INTRODUCTION

Rice (*Oryza sativa* indica) is the most important cereal crop of Thailand. It is not only a main staple food but also a major export crop. Although there are many varieties of rice, only few are cultivated with consumer acceptance. Khaw Dok Mali 105 (KDML 105) is a premium rice variety famous of its aroma and cooking quality. Therefore, it is major rice exporting of Thailand which has market shares in many areas of the world. However, this variety is particularly susceptible to several diseases including ragged stunt, blast, and tungro. Among them, tungro disease causes severe reduction in rice yield.

Many studies in plant-pathogen interacti on indicated that diseases are results of an interaction between plant and pathogen. During infection, signaling cascades are activated leading to defense mechanism, which trigger cellular response. With sufficient speed, these responses are generally effective in preventing pathogen multiplication. Several phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene act as signal molecules. However ethylene, a simple readily diffusible hormone, has been well studied and plays a key role in regulating developmental processes and mediating responses to abiotic and biotic stresses. Many studies have been proposed that the ethylene pathway primarily regulates resistance to pathogens, as mutants defective in ethylene biosynthesis or signaling show increased susceptibility to pathognes such as *Xanthomonas campestris*, *Pseudomonas syringae* and *Fusarium oxysporum* (Steven et al., 1998). Therefore, ethylene has been a target for studying resistance mechanisms in the last decades.

Although, rice tungro disease is a major problem in rice, there are few studies related to signaling in the plant. Most studies focus on the pathogen and resistant gene in host. Rice Tungro Bacilliform Virus (RTBV) is a good model to study the viral

infection into plant without insect vector. Using *Agrobacterium* clone containing RTBV, the virus can be introduced into plant without insect vector. The molecular interaction between virus and plant can be studied directly. The study related to pathogen signaling protein in rice is not only important for academic purpose but also vital to understand the magnitude of respond and specificity in rice before and during pathogen contact.

Consequently, there is considerable interest in understanding the signal transduction networks that control the activation of defense responses in plants. In this study, we intend to identify plant genes that have crucial roles in regulating the activation of defense responses. We are interested genes in ethylene perception family, which composed of series of histidine kinases, important components for signaling system, MAPK kinase cascades, EIN2/EIL and ethylene responsive factor (ERF) genes. In this study, we focus on detecting of the response of rice (*Oryza sativa indica*) to tungro disease through the determination of signaling in rice before and during the contact with rice tungro pathogen using *Agrobacterium* inoculation technique.

LITERATURE REVIEW

1. Rice (*Oryza sativa*)

1.1 Classification

Rice is one of the oldest cultivated food plants. It is an annual cereal like wheat, maize and sorghum. It belongs to the family *Gramineae* (grasses), known today as *Poaceae* in genus *Oryza*, which is comprised of 23 species. Within genus *Oryza*, two (*O. sativa* and *O. glaberrima*) are cultivated species *Oryza sativa* originated in Asia is the most cultivated species found throughout the entire world. *Oryza glaberrima* originated in West Africa and found only in that part of the world (Center for cooperation international of research agronomic development, 2002).

There are 3 main varieties rice growing in Asia including Indica, Japonica, and Javanica. Indica, is a long-grain rice and cultivated in South-east Asia, India and Pakistan. Japonica, a small round grain variety, is mainly grown in Northern China, Japan and Korea. Javanica is only grown in Indonesia.

1.2 Cultivation in Thailand

Rice (*Oryza sativa L.*) is the most important crop of Thailand. Production of rice is for both local consumption and export. Thailand has suitable climate such as light condition, temperature and humidity for rice cultivation. Therefore, rice is cultivated in many parts of Thailand including Northern, Southern, Central and Southeastern regions on the sum of 60 million Rai of growing area, with paddy output about 22-23 million tons per year. About 25% of rice land are irrigated, while the rest is still rain-fed rice (Office of Agricultural Economic, 2003).

There are many varieties of cultivated rice in Thailand such as Chainat, Suphanburi, Klongluang, Go Ko, Patumthani, and Khaw Dok Mali 105 (KDML 105). Among them, KDML is a premium rice variety famous of its aroma fragrance and

cooking quality. Therefore, it is major rice exporting of Thailand, which has gained market shares in many areas of the world. In 2003, the EU imported a total of 167,552 tons of white rice, with Thai rice accounting for 72,127 tons or 43.05% of the total imports (Oryza Thailand Rice Market Report, 2003).

Table 1 Quantity of the world rice export

(Unit: 1000 metric tons)

Country	2002	2003	2004	Oct /2005
Thailand	7,245	7,552	10,137	7,500
India	6,650	4,421	3,172	4,500
Viet Nam	3,245	3,795	4,295	4,800
U.S.A.	3,295	3,834	3,090	3,550
China	1,963	2,583	880	750
Burma	1,002	388	130	150
Total	23,400	22,573	21,704	21,250

Source: The Rice Exporter Association (2005)

Although, Thailand is the largest rice exporter, there are several problems constraining rice yield and quality including farm management, soil fertility, pests, as well as by diseases. Among many constraints limiting rice production, diseases cause huge loss in yield.

2. Pathogens

Thailand is in a tropical area where the climate is hot and humid. Such conditions encourage the multiplication and infection of many rice pathogens such as bacteria, fungi, and viruses (see Table 2). Diseases can occur in all stages of rice cultivation. The severity of disease depends on the virulent of pathogen, rice variety, environmental condition and age of plant.

Table 2 Examples of common diseases in rice

Type of pathogen	Disease	Causative agent
Fungi	Blast	*Pyricularia oryzae*
	Brown leaf spot	*Helminthosporium oryzae*
	Narrow brown leaf spot	*Cercospora oryzae*
	Bakanae disease	*Gibberella fujijuroi*
	Sheath rot	*Acrocylindrium oryzae*
	Sheath blight	*Thanatephorus cucumeris*
	False smut	*Ustileginoidea virens*
Bacteria	Bacteria leaf blight	*Xanthomonas oryzae*
	Bacteria leaf streak	*Xanthomonas translucens*
Virus	Yellow orange leaf	*Rice Tungro Bacilliform Virus*
	Ragged stunt	*Rice Ragged Stunt Virus*

Source: Anonymous (2003)

Of all diseases, tungro causes severely reduce rice yield. Tungro disease occurs in South, Southeastern Asia, and Southern China with estimated annual crop loss of $680 million. Its significance has become increasingly since mid-1960s as a consequence of planting susceptible varieties. In Thailand, the first outbreak of rice tungro disease occurred in 1964 and successive outbreaks transmitted by the green leafhopper increased in severity each year. The productivity of the rice crop in Thailand has since decreased by 50-70% of the year 1964 yield. This disease is caused by Rice Tungro Bacilliform Virus (RTBV) (Ahmed, 2001).

2.1 Rice Tungro Bacilliform Virus (RTBV)

Rice tungro disease or yellow orange leaf disease is caused by Rice Tungro Bacilliform Virus (RTBV) and Rice Tungro Spherical Virus (RTSV). Their viral carrier is a green leafhopper, *Nephotettix virescens*. Symptoms of tungro disease in

rice plants vary according to the age of the plant, rice variety, and virus strains. If rice is co-infected by these two types of virus, it will show typical severe symptoms of yellow-orange leaf discoloration, plant stunting and reduced yield. On the other hand, if rice is infected only with RTBV, it shows milder symptom. In contrast, rice plants are symptom less if they are infected only with RTSV. Transmission of the tungro disease will succeed if rice is infected first by RTSV, followed by RTBV. This suggests that RTSV require helper proteins from RTBV to show the severe form of the disease (Jones et al., 1991, Etienne et al., 2000).

Rice Tungro Bacilliform Virus (RTBV) packages its 8 kb genome as double-stranded DNA that has two discontinuities, one in each strand. It has a bacilliform structure with width and length of 38 nm x 200 nm. This virus is classified in the genus *Badnavirus,* family *Caulimoviridae*. Its nucleic acid is replicated by reverse transcription (Marmey et al., 2005).

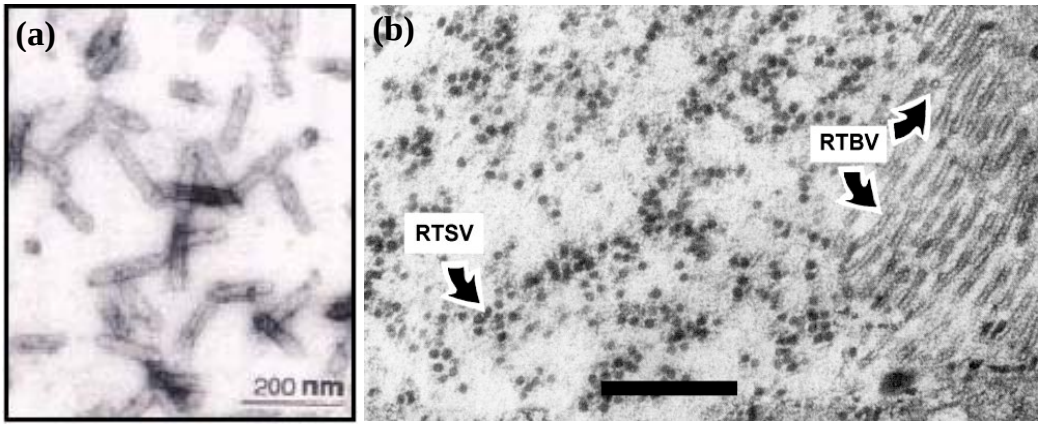


Figure 1 (a) Electron micrograph of RTBV particles

(b) Thin section of rice plant, cultivar TN1, infected with RTBV and RTSV, showing particles of both viruses in the cytoplasm.

Source: Sta et al. (1993), Osmat and Chancellor (2001)

Neither RTBV nor its DNA can be transmitted to host plants by mechanical inoculation but requires an insect vector. As a consequence, it was difficult to study this virus. In 1991, Indranil and colleagues showed that a full-length clone of RTBV

DNA was infectious when delivered into rice plants using *Agrobacterium* (Sta et al., 1999). The molecular interaction between virus and plant can be studied directly.

As many years has gone by, plant viruses are still forming a major problem in the cultivation of many vegetable and crops throughout the world. Generally, these pathogens have been controlled using conventional techniques such as crop rotation, cross-protection, chemical control of their vectors, and breeding for resistance. The development of appropriate methods to manage tungro was constrained for long time by a limitation of knowledge. Although, the International Rice Research Institute (IRRI) in Philippine succeeded in their national breeding program in developing resistant varieties, resistance to tungro disease has proven only partial. The resistant rice varieties are only resistant to the vector but not to the viruses (Azzam et al., 2002). Although, many reports are studied about and resistant genes in host, very few focus on plant-pathogen interaction. Therefore, a novel insight in the natural defense mechanisms of plants to pathogens is provided an interesting option to form the basis knowledge for development of viral disease management.

3. Plant-pathogen interaction

Although, plants are exploited as sources of food and shelter by a wide range of pathogens, they have evolved a sophisticated defense response to deal with this pressure, based on a combination of constitutive and inducible responses. The interaction between plants and microbial pathogens are specific, complex, and dynamic. Invasion by the pathogen triggers recognition, and response in the plant leading to signaling cascades and the up or down regulation of numerous genes involved in the interaction that in turn may lead to adaptation or evasion by the pathogen (John et al., 2000).

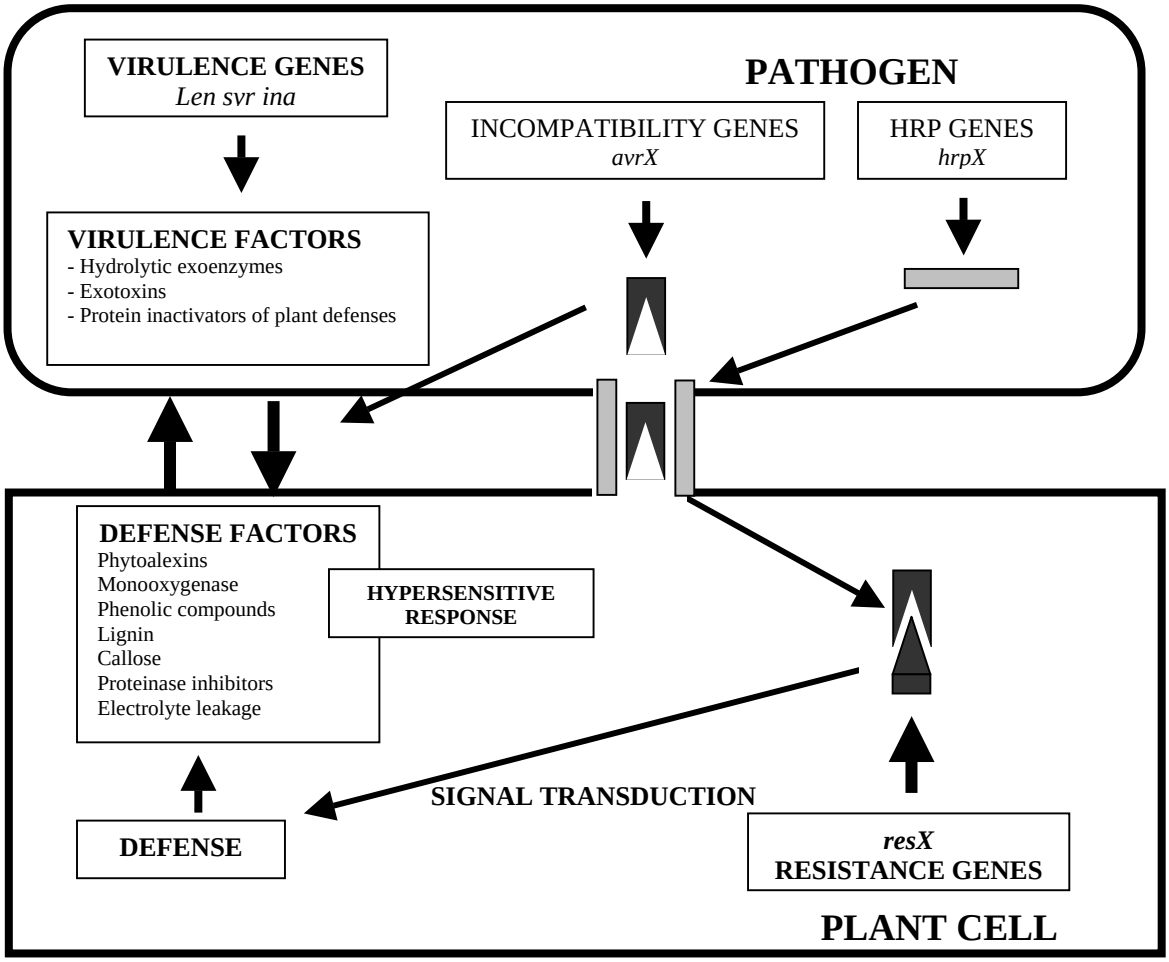


Figure 2 Simplified schematic representation of the elicitor receptor model in plant-bacterial interaction. The virulence genes and factors in the pathogen are confronted with the defense genes and factors in the plant host cell. Incompatibilty genes in the bacterial pathgen encode avirulence factors (*avrX*) which are secreted by a type III secretion system controlled by the hrp gene (*hrpX*). The avirulence factors are detected by the resistance genes (*resX*) or factors in the plant cell which trigger the hypersensitive reaction and defense.
Source: Montesinos et al. (2002)

In figure 2, when plants are challenged by a pathogen infection, early local defense reactions such as hypersensitive response (HR) are activated in order to counteract the pathogen attack. This local response often triggers defense response throughout the plant, a phenomenon known as systemic acquired resistance (SAR). The HR and SAR depend on interaction between a resistance (*R*) gene product in the

plant and corresponding phytopathogen avirulence (*Avr*) gene product. It has been predicted that phytopathogen *Avr* products function as ligands and host *R* products function as receptors in an interaction leading plant to detect the presence of pathogens through recognition signaling compounds, called elicitors. The complexes of elicitor binding on receptor resulting in signal relayed through the signal transduction pathway, that resulted in activation of defense mechanism such as generation of β-chitinase, phytoalexin, and pathogenesis related (PR) protein (Conrath et al., 2002). Therefore, the defense strategy is based on pathogen recognition, cell-to-cell communication and signal transduction (Pontier et al., 1998). This strategy was demonstrated that the molecular events constituting critical steps of plant-pathogen interactions seem to involve the perception in the plant and signal transduction that lead to defense responses.

4. Signal transduction

Living organisms have a diverse and sophisticated signaling to recognize and respond to their environmental conditions. Responses to environmental signals are generally initiated by inducing conformational changes in target receptors. The implicit information in these structural changes is propagated, amplified and translated into various forms through a series of events through signal transduction (Bleecker et al., 1999). Many study indicated that, disease resistance in plants often involves highly specific recognition of an invading pathogen. Recognition triggers signal transduction cascades that activate plant defense responses. Defense responses can include production of antimicrobial enzymes and compounds, induction of an apoptotic programmed cell death response, and induction of a systemic immune response that causes subsequent infections to be met with a more vigorous defense response (Baker et al., 1997).

The key to understanding plant defense responses lies in the elucidation of the signaling pathways involved in their regulation. A large amount of research has identified several important defense signaling pathways regulated by phytohormones, such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene

(ET). As shown in figure 3, there is ample evidence for positive crosstalk among salicylic acid, jasmonic acid, ethylene and other phytohormonal signals as effectors of defense gene expression. Studies on biotic and abiotic stress signaling in plant demonstrate points of convergence in the signaling circuitry. Often, ethylene pathway is manifest in common molecular or metabolic responses and perhaps more important in plant phenotype that is functionally adaptive to a particular stress. Among of phytohormone, the history of ethylene research is a venerable one, it is a small readily diffusible phytohormone and serves as an important signaling molecule in plants, both in regulating developmental processes such as seed germination, fruit ripening, abscission, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, flower opening, senescence, pollination, fruit ripening and degreening, and mediating responses to environmental stimuli (Jane, 2001). Plants under stress from both abiotic and biotic stresses usually stimulate ethylene synthesis, which is perceived by ethylene receptors and triggers cellular responses further downstream (Kim et al., 2003). Furthermore, these hormones regulate a wide range of defense-related genes, making them likely candidates as signals that coordinate plant response to pathogens (Penninckx et al. 1998).

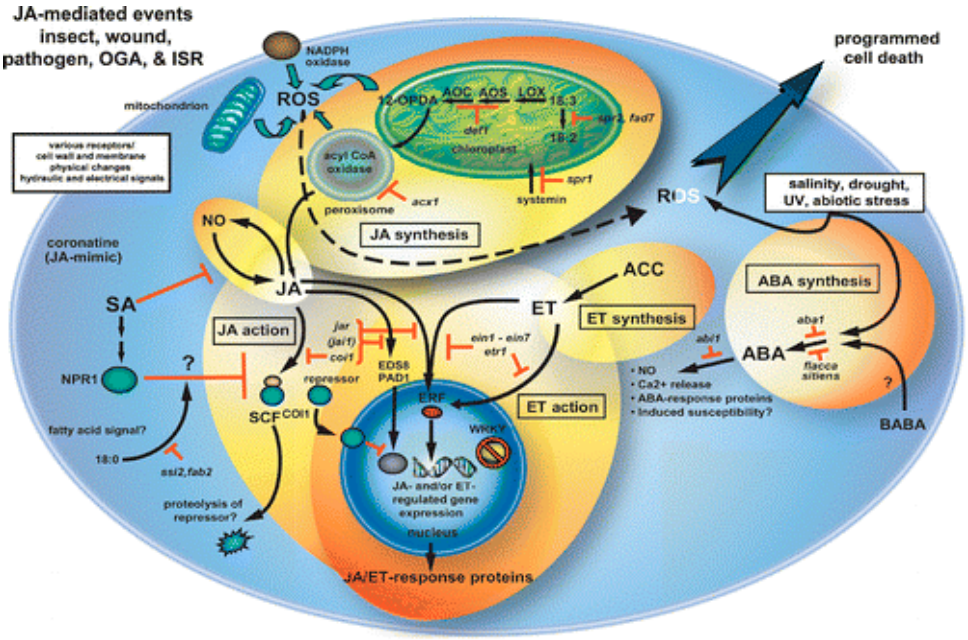


Figure 3 Signal crosstalk between Synthesis and action of SA, JA, ABA and ET pathway in response to environment.
Source: Richard (2005)

4.1 Ethylene signal transduction

The ethylene biosynthetic pathway has been well characterized in the past 40 years and its production is tightly regulated both by internal signals and environmental stimuli during plant development (Kevin et al., 2002). While much is known about the regulation of ethylene biosynthesis, the issue of convergence in ethylene signaling pathways that invoke adaptation to changes in an organisms has captured the attention of many biologists in the last decade. This interest spans diverse levels of inquiry, from molecular and cellular studies of the perception and transduction of external signals to ecological analyses of their interactions.

Many studies have been proposed that the ethylene pathway primarily regulates resistance to pathogens, as mutants defective in ethylene biosynthesis or signaling show increased susceptibility to pathogens such as *Xanthomonas campestris*, *Pseudomonas syringae* and *Fusarium oxysporum* (Steven et al., 1998). In addition, ethylene may also activate plant defense related processes such as the production of phytoalexins, pathogenesis-related (PR) proteins, the induction of the phenylpropanoid pathway, and cell wall alterations (Lund et al., 1998, Aloni et al., 1998). During the attacks by pathogens or pests, ethylene is formed. Mutants with either production or the perception of ethylene increase susceptibility to several pathogens and pests (Corne et al., 2001). Furthermore, the application of exogenous ethylene was found to induce resistance or susceptibility depending on the plant pathogen interaction studies. Similar effects have been observed upon application of inhibitors of ethylene perception or biosynthesis. For instance, several ethylene insensitive mutants of *Arabidopsis* have been reported to exhibit enhanced disease susceptibility to *Botrytis cinerea* (Thomma et al., 1999) and *Pseudomonas syringae* in tomato indicating that ethylene dependent defenses contribute to basal resistance against these pathogens (Pieterse et al., 1998). A similar phenomenon was observed in soybean mutants with reduced sensitivity to ethylene. Severe symptoms were developed in response to infection by the fungal pathogens *Septoria glycines* and *Rhizoctomia solani* (Hoffman et al., 1999). Therefore, ethylene has been a target for studying resistance mechanisms in the last decades.

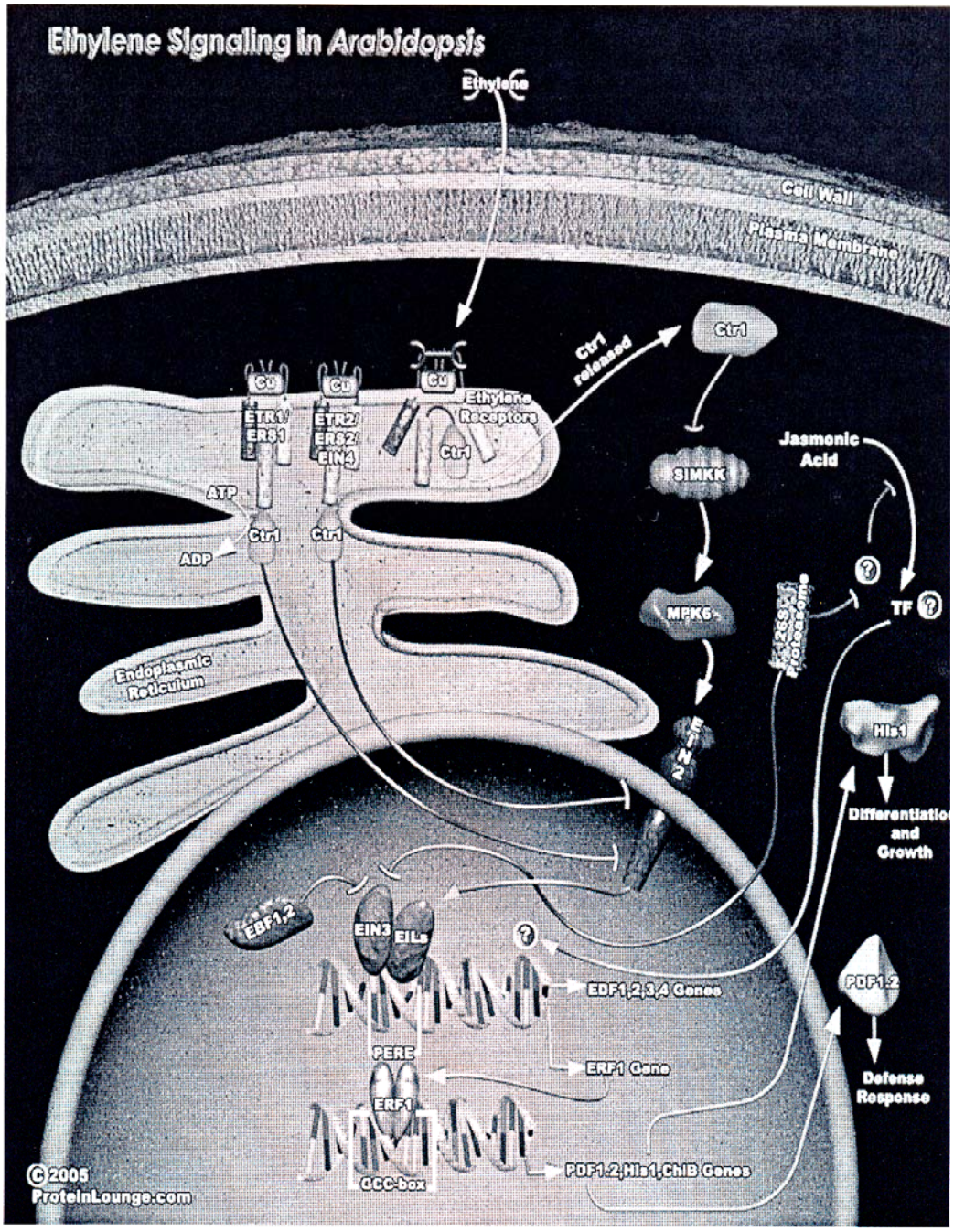


Figure 4 A current view of ethylene signal transduction pathway.
Source: Anonymous (2005)

Ethylene signal transduction, as revealed by studies in *Arabidopsis*, provides an interesting example of how information-processing systems have evolved in plants. In figure 4, the ethylene signal is perceived by a family of receptors, which located on ER membrane. Binding of ethylene to members of the receptor family (represented by

ETR1) is mediated by a single copper ion (Cu). Ethylene negatively regulates the signal transduction pathway upon binding the receptor through a conformational change in CTR1 that reduces its kinase activity. In the other hand, when there is no ethylene, CTR1 is active and can repress downstream ethylene responses. Upon inactivation of CTR1, SIMKK is relieved from inhibition and activates ethylene signaling through a cascade to downstream components including EIN2 and EIN3/EILs. EIN3/EILs initiate a transcription factor cascade through activation of Ethylene Responsive Factors (ERF), which can bind to a primary ethylene responsive element in the promoters of ethylene responsive genes (Lori, 2003). Many of ethylene responsive (ERF) proteins are known to regulate gene expression through interaction with a cis-element called the GCC-box, which is found in several ethylene-responsive genes such as *PLANT DEFENSIN 1.2* (*PDF1.2)* and *HOOKLESS1* (*HLS1*). These genes encode effector proteins that are needed to execute a wide variety of ethylene responses (Lohrmann et al., 2002).

4.1.1 Ethylene receptors

For ethylene perception, ethylene is perceived by five member of ethylene receptors including ETR1, ERS1, ETR2, ERS2 and EIN4 that resemble to bacterial two-component system. All members contain an N-terminal membrane-associated sensor domain that shows high affinity ethylene binding when expressed in yeast (Ildoo et al., 2002). The ethylene receptor proteins can be structurally separated into three domains including sensor domain, histidine kinase domain and response regulator domain. The sensor domain can be further subdivided into the amino terminal ethylene binding subdomain and a second subdomain referred as GAF. The functional significance of the GAF region has yet to be elucidated. However, it must play some role in signal transduction as it joins the ligand binding portion of the receptor to the proposed signal transmission domain and is one of the most highly conerved domains within the ethylene receptor family.

Two-component signaling systems, consisting of a histidine protein kinase that senses a signal input and a response regulator that mediates the output, are ancient and

evolutionarily conserved signaling mechanisms in prokaryotes and eukaryotes. The histidine kinase domain contains five subdomains that define the catalytic core of histidine kinases including H, N, F, G1 and G2. The residues thought to be essential for histidine kinase activity are conserved in ETR1 and ERS1, but are not completely conserved in ETR2, ERS2, and EIN4 (Chen et al., 2005). Conserved histidine residue and are activated via trans-autophosphorylation by the catalytic domain of the histidine kinase. They subsequently transfer the phosphoryl group to the aspartate acceptor residue of a response regulator domain. Based on these distinguishing features and overall sequence similarity, the receptor family can be divided into two subfamilies based on structural similarities. The ETR1-like subfamily, containing ETR1 and ERS1, is characterized by having three hydrophobic subdomain. Whereas, the ETR2-like subfamily, including ETR2, EIN4, and ERS2, show an additional hydrophobic extension at N-terminus and carry degenerate histidine kinase domains that lack one or more elements considered necessary for catalytic activity (Figure 5) (Etheridge et al., 2005).

The response regulator domain has sequence identity to the output portion of bacterial two-component systems and contains an aspartate (D) that can be phosphorylated in bacterial protein. Similar to bacterial histidine kinase, response regulator domain is only found in certain members of plant receptor family including ETR1, ETR2 and EIN4.

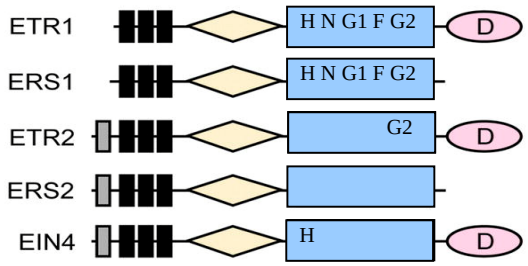


Figure 5 The *Arabidopsis* ethylene receptor family.
Source: Scheller and Kieber (2002)

4.1.2 Ethylene responsive factors

In transcriptional level, a number of families of transcription factors have been implicated in plant stress responses because their expression is induced or repressed under different stress conditions.

In *Arabidopsis* genome, 124 genes encode putative ERF proteins that form a superfamily of genes (Riechmann et al., 2000). In figure 6, within the large group of transcription factor, there are numerous ERF proteins which can divided into four class based on their structural organization and considering the transctiptional activities of ERFs. The ERF class I consist of nuclear localization signal at C-terminal end. While class II ERF family, nuclear localization signal is lining on ERF domain and composed of EAR motif at C-terminal end. The ERF class IV characterization by a conserved N-terminal MCGGAII/L motif which function has not been establish (Barthelemy et al., 2003).

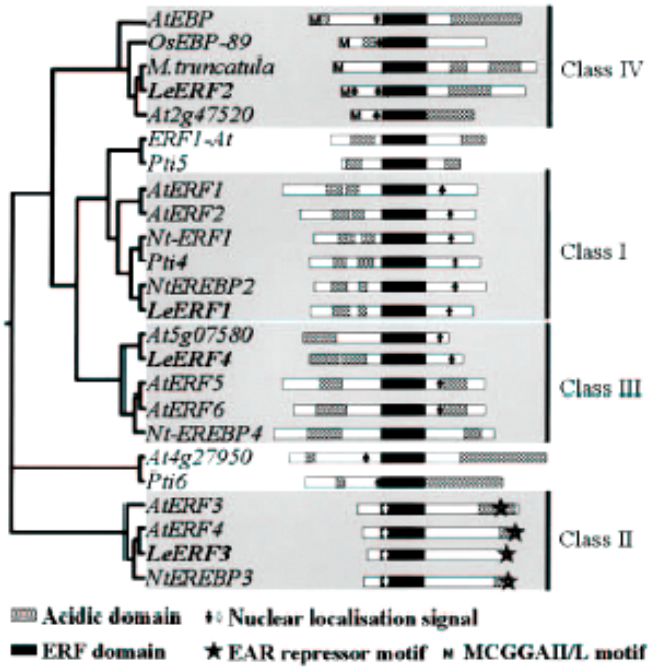


Figure 6 Phylogenetic tree of homologues Ethylene Responsive Factor (ERF) class I to IV.

Source: Barthelemy et al. (2003)

The ERF proteins identified to date function as transcription factors that are responsive to signals induced by extracellular stress. They are involved in the induction of gene expression by stress factors, such as pathogens, cold and by components of stress signal transduction pathways such as ethylene, abscisic acid and jasmonic acid (Fujimoto et al., 2000).

Several plant transcription factors play roles in defense gene regulation, such as ERF transcription factors, which is a crucial part of the plant response to pathogen stress by regulating the expression of pathogenesis related (PR) genes (Guo et al., 2004). Many ERF proteins are known to regulate gene expression through interaction with a cis-element called the GCC box. A number of plant promoter elements that can respond to diverse environmental stimuli have been identified including the GCC box, an ethylene responsive element initially found in several pathogenesis related (PR) promoters genes (Fujimoto et al., 2000). Moreover, ERF genes have also been found to be involved in defense responses in other plants.

In tomato and tobacco, ERF genes are induced after infection by many of pathogen such as *Pseudomonas syringae* (Thara et al., 1999), tobacco mosaic virus (Horvath et al., 2000), and *Cladosporium fulvum* (Durrant et al., 2000). Significantly, several ERF transcription factors that confer enhanced disease resistance when overexpressed, such as *ERF1*, and *Pti4* are transcriptionally regulated by pathogens, ethylene, and jasmonic acid (Fujimoto et al., 2000). Plants that overexpression *Ethylene Responsive Factor 1* (*ERF1*) show an increased resistance to several necrotrophs (Berrocal et al., 2002). In addition, work with transgenic plants also supports the implication of ethylene in resistance against different pathogens. Constitutive expression of *Ethylene Responsive Factor 1* (*ERF1*) increases *Arabidopsis* resistance to B*acillus cinerea* and *Pseudomonas cucumerina* (Berrocal et al., 2002). It is important to consider that the ERF familly is composed of a high number of genes in *Arabidopsis*, therefore, other members of this family could participate in the regulation of these defense-related genes. In fact, another member of the ERF family such as ERF2, was induced rapidly after ethylene and jasmonate treatment. In this study, we focus on the way to detect the response of rice (*Oryza*

sativa) to tungro disease through the determination of signaling in rice before and during the contact with rice tungro pathogen. We will investigate the expression level and timing of ethylene signal transduction genes both in ethylene perception and transduction. The outcome of this study will provide the basic knowledge of how rice plants transduce the signal in response to pathogen infection.

MATERIALS AND METHODS

Materials

1. General Solutions

- Agarose gel loading buffer (6X): 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol
- Agarose/formaldehyde gel: 1%agarose, 1X MOPS and 2% formaldehyde
- Amplicilin 100 mg/ml filter sterilized
- Chloroform: isoamyl alcohol (24:1)
- CTAB/NaCl solution: 10% CTAB, 0.7 M NaCl
- CTAB precipitation buffer: 1% (w/v) CTAB, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0
- Denaturing solution: 0.5 M NaOH, 1.5 M NaCl
- Detection buffer: 0.1 M Tris-Cl, 0.1 M NaCl, pH 9.5
- 0.01% Diethyl pyrocarbonate (DEPC) in dH_2O
- DNA extraction buffer: 2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA , pH 8.0
- 70% ethanol
- Formamide
- High salt TE buffer: 10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, pH 8.0, 1 M NaCl
- High SDS hybridization buffer: 7% SDS , 50% formamide, 5XSSC, 0.1% N-lauroylsarcosine, 2% blocking solution, 50 mM sodium phosphate, pH 7.0
- High stringency wash solution: 2XSSC, 0.1% SDS
- Kanamycin 50 mg/ml

- LB: 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl.
- LBA: 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl and 1.5% bacto-agar
- 10 M LiCl stock solution
- Low stringency wash solution: 0.5XSSC, 0.1% SDS
- Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl
- 10XMOP: 200 mM MOPS buffer, 50 mM Sodium acetate, 20 mM EDTA
- Neutralization buffer: 1 M Tris, pH 8.0, 1.5 M NaCl
- Phenol:chloroform:isoamyl alcohol (25:24:1)
- RF1: 100 mM KCl, 50 mM $MnCl_2.4H_2O$, 10 mM $CaCl_2$, 15% glycerol
- RF2: 10 mM MOPs, 10 mM KCl, 75 mM $CaCl_2.2\ H_2O$, 15% glycerol
- Rifampicin 50 mg/ml
- RNA extraction buffer: 1 M Tris-HCl pH 9.0, 1% SDS
- RNase (DNase free) prepared by dissolving 100 mg of RNaseA in 10 ml dH_2O, boiled for 20 min and stored at –20°C
- 10X SDS (w/v) stock solution
- Sodium acetate: 3 M sodium acetate, pH 5.4
- SOB media: 2% becto-tryptone, 0.5% becto-yeast extract, 0.05% NaCl, 25 mM KCl
- Solution I: 50 mM glucose, 25 mM Tris-HCl. PH 8.0, 10 mM EDTA, pH 8.0
- Solution II: 0.2 N NaOH, 1% SDS
- Solution III: 3 M potassium acetate, 5 M galcial acetic acid
- Standard hybridization buffer: 5X SSC, 0.1% N-laurylsacosine, 0.02% SDS, 1% blocking reagent
- SSTE buffer: 1 M NaCl, 0.5% SDS, 10 mM Tris, 1 mM EDTA, pH 8.0

- 50X TAE electrophoresis buffer: 40 mM Tris, 5.17% glacial acetic acid v/v), 2 mM EDTA, pH 8.0
- TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0
- Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20
- X-gal: 2% in dimethylformamide
- 0.5X washing solution: 0.5X SSC, 0.1% SDS
- 2X washing solution: 2X SSC, 0.1% SDS
- 2XYT: 1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl
- 2XYT agar: 1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl, 1.5% agar powder

2. General Equipment

- Centrifuge (Centution 8000 Series)
- Centrifuge (Jouan MR 231)
- Centrifuge (Lannet Spectrafuge 16M)
- Centrifuge (Sigma 4K 10)
- Digital Gel Documentation and Analysis System (SYNGENE)
- Gel electrophoresis set (BIO-RAD wide mini sub^{TM} cell)
- Gel electrophoresis set (SCIE-PLAS)
- Gel electrophoresis set (I-Mupid, Cosmo Bio)
- Spectrophotometer (Ultrospec$^{®}$ 500/1100)
- PCR (Perkin Elmer Cetus of Hybaid Thermal Cycles)
- PCR (PTC-200 DNA Engine)
- PCR (Thermo Hybaid PxE Thermo Cycler)

Methods

1. Plant Materials

Two week old of *Oryza sativa* variety Khaw Dok Mali 105 (KDML 105) was selected for this experiment. Rice was grown in Netted House at Kasetsart University, Kamphaengsean Campus, Nakhon Pathom in the condition at 33.8°C and 57% humidity on day and 29.2°C and 72% humidity on night.

2. Genomic DNA Extraction

Rice leaves were used for genomic DNA extraction using CTAB method (Ausubel et al., 1992). One gram of leaf tissue was frozen in liquid nitrogen and ground to fine powder. Seven volumes of CTAB were added and mixed by vortexting. The suspension was incubated at 65°C for 30 min. The content was separated by centrifugation for 5 min at 12,000 rpm. The supernatant was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The phases were separated by centrifugation at 12,000 rpm. The aqueous phase was transferred to a new tube and added 150 μl of CTAB/NaCl. Solution was mixed by vortexting and extracted again with an equal volume of chloroform: isoamyl alcohol (24:1). The aqueous phase was transferred to a new tube and added 600 μl of CTAB precipitation buffer. DNA was precipitated by centrifugation for 10 min at 8,000 rpm. The pellet was dissolved in 150 μl of high salt TE. An equal volume of isopropanol was added and kept at -20°C for 1 hr. The DNA was precipitated by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol, dried under room temperature and resuspended in 40 μl of TE buffer containing 20 μg/μl of RNase A. The concentration and purity of DNA was determined by spectrophotometer (Ultrospec® 500/1100) at 260 and 280 nm. The DNA was incubated at 55°C for 15 min. It was stored at -20°C for long-term or 4°C for short-term use.

3. *Agrobacterium* inoculation

3.1 Agroinoculation

An infectious clone of RTBVCN (Chainat isolated) (kindly provided by Dr. Nathwong: BIOTEC, THAILAND) was introduced into two week olds rice plants by agroinoculation technique. Ten microliters of *Agrobacterium* (LBA4301) clone of RTBV suspension were plated on 2X-YT media containing 50 μg/ml of kanamycin and 50 μg/ml of rifampicin as selective agent. *Agrobacterium* cultures without RTBV construct were plated on 2X-YT containing only Rifampicin (50 ug/ml) and incubated at 28°C for 48 hr. The *Agrobacterium* clones were scraped from the media surface and resuspended in deionized water. Ten microliters of *Agrobacterium* suspension (10^{10} C.F.U/ml) were injected at the base of a stem of a two-week old rice plant using 1 milliliter epidemic syringe. The leaves of RTBVCN infected rice were collected at various times after infection and used for total RNA extraction. Control plant was injected with either 10 μl of distilled water or 10 μl of *Agrobacterium* culture to determine the effect of wounding and *Agrobacterium* infection, respectively.

3.2 Detection of viral DNA in rice plants

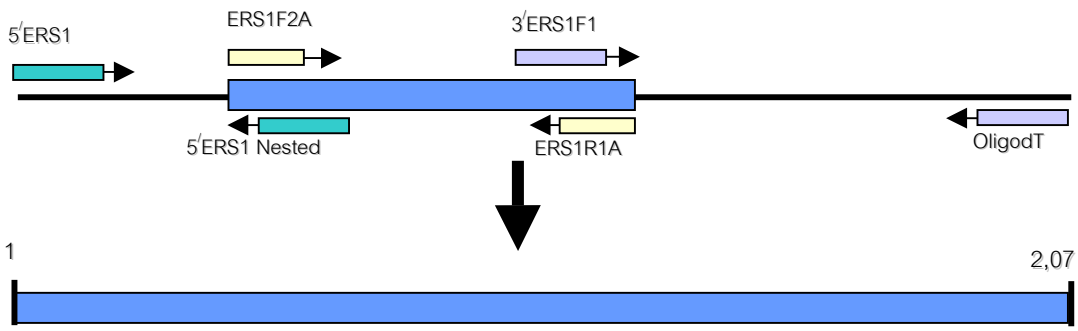
The presence of RTBVCN genome in inoculated rice was determined by PCR analysis. Small pieces of infected leaves were ground in liquid nitrogen to powder. Twenty microliters of 2 M NaOH was added. The mixture was centrifuged. One microliter of upper phase was transferred into 500 μl of 1 M Tris-HCl, pH9.0 and used as template for PCR. The reaction mixture contained 1X PCR buffer, 10 mM each of deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase, and RTBV specific primer (kindly provided by Dr. Nathwong, BIOTEC, THAILAND). PCR was performed under to the following condition: 94°C for 4 min and 35 cycles of 94° C for 1 min, 50° C for 1 min, and 72° C for 1.30 min.

4. RNA Extraction

The RNA was extracted from rice leaves at various times after RTBV infection using seed extraction method (Naito et al., 1994). Rice tissues was ground in liquid nitrogen to fine powder and transferred into an appendrof tube containing 300 µl of RNA extraction buffer and stirred for 3 min at RT. The content was separated by centrifugation at 10,000 rpm for 5 min. The supernatant was transferred to a new tube and extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The nucleic acid was precipitated using of 0.1 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. The solution was incubated at -80°C for 10 min before centrifugation at 12,000 rpm for 10 min at 4°C. The pellet was resuspended in 100 µl of DEPC treated water and centrifuged at 12,000 rpm for 10 min. The aqueous phase was transferred to a new tube containing an equal volume of 4 M LiCl and incubated at -80°C for 1 hr. The RNA was precipitated by centrifugation at 12,000 rpm for 20 min at –4°C. The pellet was collected and added 1 ml of 2 M LiCl before centrifugation at 12,000 rpm for 5 min at -4°C. The pellet was washed with 70% ethanol and dried under room temperature. The RNA pellet was resuspended in 40 µl of TE buffer RNase free and stored at –80°C. The concentration and purity of total RNA were determined using spectrophotometer (Ultrospec® 500/1100) at 260 and 280 nm.

5. Identification of ethylene receptor genes from rice (*Oryza sativa*)

(A) Os-ERS1



(B) Os-ERS2

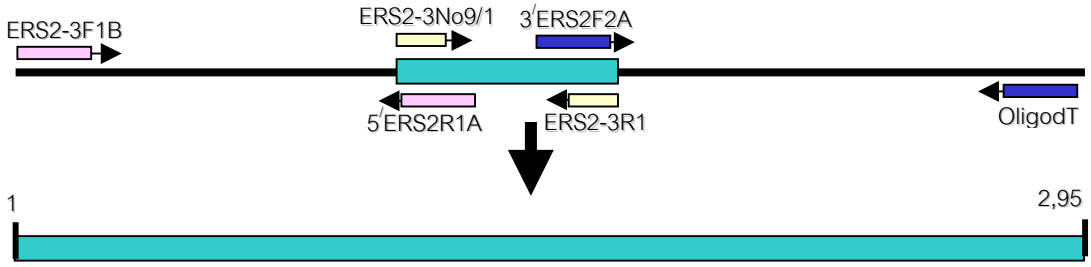


Figure 7 Represent diagram of primer pairs used for Os-ERS1 and Os-ERS2 isolation.

Degenerated PCR primers of ERS1-4 5′ AAAACHACWCTTGTKGAG 3′, ERS1-9 5′ CATTTCATGRTTCAKDAC 3′, ERS2-3 5′ CAGGTAGCGGTCGCATTG TC 3′ and ERS2-10 5′ CCAGCTCAAGACTCCCAT 3′ were designed from ERS consensus sequences. One microgram of KDML105 rice genomic DNA was used as template for PCR amplification. Nucleotide sequences of Os-ERS1 and Os-ERS2 were cloned into pDrive vector (QIAGEN) and determined using DNA sequencer (Applied Biosystem). The nucleotide sequences were used to design specific primer to amplify full length cDNA sequence of Os-ERS1 and Os-ERS2.

The full length cDNA sequence of Os-ERS1 and Os-ERS2 were isolated using RT-PCR technique. For the first strand cDNA synthesis, three micrograms of total RNA from rice leave were used in reverse transcription reaction containing of 30 pmole of ethylene receptor reverse primer ERS1-R1A 5′CGCTATCAGATGGAAGC ATC 3′ or ERS2-3 R1 5′ CCAGCTCAAGACTCCCAT 3′, 1 mM of each dNTPs and 1 μl of formamide. The reaction was heated at 65°C for 5 min and quenched on –80°C 100% ethanol. Then reverse transcription mix containing of 5X First-Strand buffer, 0.1 M DDT, 20 U of RNase OUT™ and 200 U of superscript™ III (Invitrogen) were added. The reaction was incubated at 42°C for 50 min. The cDNA was used for amplification.

Thirty picomoles of ethylene receptor specific primer including, ERS1-F2A 5′-GGCTTTGTGTCCGTGCG-3′, ERS1-R1A 5′-CGCTATCAGATGGAAGCATC-3′

and ERS2-3 No.9/1 5′-CAGGTAGCGGTCGCATTGTC-3′, ERS2-3R1 5′-CCAGCTCAAGACTCCCAT-3′, were used for PCR amplification. The cycle of PCR reaction for amplification of Os-ERS1 was performed under the following conditions: 94°C for 4 min and 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min and followed by 1 cycle of 72°C 10 min. The cycle of PCR reaction for amplification of Os-ERS2 was as followed: 94°C for 4 min and 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and followed by 1 cycle of 72°C 10 min.

5′ and 3′ RACE method was carried out to generate the full length cDNA sequence. The sequence of gene specific primer was as follows: 5′ERS1 (sense: 5′ TA AACATTGGGTATGGATG 3′), 5′ERS1 Nested (antisense: 5′ AGCAAGTCAGGGATA ATATG 3′), ERS2-3F1-B (sense: 5′ GGATGGATCATGTGATTGC 3′), 5′ERS2R1-A (antisense: 5′ TTTTGCTCCATTAGTAG 3′), 3′ ERS1-F1(sense:5′ CGAGCAATCGTT TCTTT 3′), 3′ERS2-F2-A (antisense: 5′ AGTGGCAGTGGATTG 3′) and Oligo dT (antisense: 5′ TTTTTTTTTTTTTTTTTTTTV 3′). Nucleotide sequences of ERS1 and ERS2 were cloned to pDrive vector (QIAGEN) and determined using DNA sequencer (Amplied Biosystem). The sequences were analyzed using BLAST program (NCBI) and ClustalW (EBI). The phylogenetic tree was constructed using the MegAlign program (DNAStar).

6. Purification of PCR product

PCR products were adjusted to final volume of 100 μl with distilled water and 0.1 vol. of 3 M sodium acetate, 2.5 vol. of absolute ethanol, and 1 μl of glycogen (20 mg/ml) were added. The mixture was incubated at -20°C for 30 min. The DNA was precipitated by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol and air-dried at room temperature. The pellet was resuspended in 10 μl of distilled water and stored at -20°C.

7. Ligation of DNA Fragments

The sticky end PCR products were ligated into pDrive vectors using a QIAGEN®PCR Cloning Kit essentially as described by the manufacturer. The ligation reactions were prepared in the 0.5 ml thin wall microcentrifuge tube. The reaction mix contained 0.5 μl of pDrive cloning vector (10 ng/ul), 4 μl of purified PCR-product, and 2.5 μl of ligation master mix in the total volume of 10 microliters. The reaction was incubated overnight at 16°C and heated at 65°C for 10 min to inactivate the reaction. The ligation product was used directly in transformation reaction or stored at -20°C until use.

8. Competent Cell Preparation

One milliliter from 10 ml overnight culture of *E. coli* DH5α cell was incubated in 250 ml of SOB media at 37°C to an OD_{600} of 0.2-0.4. The culture was incubated on ice for 15 min and the cells were collected by centrifugation at 3,500 rpm for 15 min at 4°C. The supernatant was removed and the pellet was resuspended in 33 ml of chilled RF1. The cells were placed on ice for 15 min before collecting the pellet by centrifugation at 3,500 rpm for 15 min at 4°C. The cells were resuspended in 4 ml of chilled RF2 and aliquoted 100 μl in each microcentrifuge tube. The competent cells were stored at -80°C.

9. Transformation of Recombinant Plasmid

Four microliters of ligated product were added to 100 μl of chilled competent cell and incubated on ice for 30 min. Cells were heat shocked at 42°C for 45 s and incubated on ice for 2 min. Eight hundred microliters of LB media were added and incubated at 37°C with shaking at 250 rpm for 1 hr. The cells were collected by centrifugation at 8,000 rpm for 2 min and resuspended in 300 μl of LB media. The cell suspension were spread on LB agar containing X-gal (0.06%), IPTG (29 μg/ml), and ampicilin (50 μg/ml) and incubated overnight at 37°C. Recombinant clones were

identified by blue/white screening. The white colonies were picked up and cultured overnight in 2.5 ml of LB containing 50 μg/ml of ampicilin at 37°C with shaking at 250 rpm.

10. Small Scale Isolation of Plasmid DNA, Mini-Prep

Plasmid DNA was extracted by a modification method of Sambrook et al. (1989). Recombinant clone was grown overnight at 37°C with vigorous shaking in three ml of LB containing 50 μg/ml of ampicilin. One and a half milliliters of cell suspension was aliquoted and collected by centrifugation for 30 s at 10,000 rpm. The pellet was resuspended in 100 μl of Solution I and mixed vigorously. Two hundred microliters of freshly prepared solution II was added and mixed gently by inverting 2-3 times. The solution was neutralized by adding 150 μl of solution III. The content was extracted with an equal volume of chloroform and centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to a new tube containing two volume of 100% ethanol and centrifuged at 12,000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol and dried at room temperature. The pellet was resuspended in 20 μl of TE buffer containing 10 μg/ml of RNase A and incubated at 55°C for 15 min.

11. Electrophoresis of nucleic acid

DNA was separated in 1-1.5% agarose gels containing TAE buffer. Gels were stained with ethidium bromide (0.5 mg/ml) and visualized on UV transluminator (SYNGENE).

12. Southern analysis

12.1 DNA Digestion

Twenty micrograms of genomic DNA were digested with 50 U of restriction endonuclease (*Bam*HI, *Hind*III and *Msp*I) at 37° C in the total volume of

150 μl. The digested DNA was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase was precipitated by 2.5 volume of absolute ethanol and 1 μl of glycogen (20 mg/ml) and centrifuged at 14,000 rpm for 15 min at 4° C. The pellet was washed with 500 μl of 70% ethanol and dried. The pellet was resuspended in 25 μl of dH_2O. The digested DNA was separated on 1% agarose gel containing 1X TAE at 60 V for 2 hr. The gel was stained with ethidium bromide.

12.2 Probe preparation

All ethylene receptor and ethylene responsive factor probes were prepared by direct labeling of amplification product with Dioxygenin-11-uridine-5′-triphosphate (DIG-11-UTP) using PCR DIG labeling mix (Roche). Four micrograms of ethylene receptor and ethylene responsive factor cloned (Os-ETR1, Os-ETR2 and four groups of ethylene responsive factor cloned were kindly by Dr. Burns, BIOTEC THAILAND) were digested with 3 U of *Mlu*I (Fermentas) and *Xho*I (Fermentas) in ten microliters reaction mix. Digested clone were electrophorated on 1% agarose at 60 V for 50 min. The piece of ethylene receptor or ethylene responsive factor cloned were eluted from agarose gel using DNA purification kit (Fermentas) and used as template for probe preparation.

The PCR reaction mix contained 5 μl of 10X PCR buffer, 5 μl of PCR DIG labeling mix, 10 ng of plasmid DNA, 10 pmol of each deoxy ribonucleotide, 30 pmol of each primer and 1 U of *Taq* DNA polymerase (Finnzyme). The PCR cycle for ERS1 probe amplification was as followed; 94°C 4 min 1cycle, 94° C 1 min 59° C 1 min and 72° C 1 min 35 cycles and followed by 72° C 10 min. The cycle of PCR reaction for ERS2 probe is; 94°C 4 min 1cycle, 94° C 1 min 55° C 1 min and 72° C 1 min 35 cycles and followed by 72° C 10 min. In hybridization reaction, the probes were used at the concentration of 0.4 μg/ml. All probes were denatured by boiling for 10 min and quenched on ice for 5 min before used.

12.3 Southern blot hybridization

The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min. The DNA was denatured by shaking the gel twice in denaturing solution for 15 min at RT and neutralized by shaking the gel twice in neutralization buffer for 15 min at RT. The DNA was transferred to nylon membrane positively charge (Roche) and baked at 80° C for 2 h. The membrane was prehybridized in 15 ml of standard hybridization buffer at 65° C for 1 h and hybridized overnight with 0.4 μg/ml of ERS specific probes (The probe was diluted in standard hybridization buffer). The membrane was washed by shaking with 2X washing solution at RT for 10 min and washed twice with 0.5X washing solution at 60° C for 15 min. The membrane was soaked with washing solution and shaken for 10 min at RT.

12.4 Detection of DIG-Labeled Nucleic Acids

The membrane was agitated in 1X blocking solution (Roche) for 45 min and incubated in antibody solution (Anti-Digoxigenin-AP was diluted in 1X blocking solution at 1:10,000) for 30 min. The membrane was washed twice with washing buffer for 15 min at RT and incubated in detection buffer for 3 min. The membrane was transferred to new plastic bag containing chemiluminescent substrate CDP-Star™ (Roche) diluted in detection buffer at 1:200 and incubated in the dark for 15 min. Semi-dry membrane was placed in a plastic bag and sealed. The membrane was exposed to the X-ray film for detection of chemiluminescent signal.

13. Northern analysis

13.1 Agrorose/Formaldehyde gel

Total RNA was separated by agarose/formaldehyde gel electrophoresis by the method described by Sagerstrom and Sive (1996). An equal volume of RNA loading buffer were added into RNA sample and heated to 65° C for 15 min. The

content was chilled on ice for 5 min, then one microliter of ethidium bromide was added and electrophoresed at 60 V for 2 hr.

13.2 Northern blot hybridization

Twenty micrograms of total RNA were used for electrophoresis on agarose/formaldehyde gel. The RNA in agarose/formaldehyde gel was incubated twice in 20X SSC for 15 min. The RNA was transferred to Nylon membrane (Roche) using capillary with 20X SSC overnight and baked at 80°C for 2 hr. The membrane was prehybridized with 15 ml of high SDS concentration hybridization buffer at 50°C for 1 hr and hybridized overnight with 0.4 ug/ml of specific probe (The probe was diluted in high SDS concentration hybridization buffer). The membrane was washed by shaking twice with 2X washing solution at RT for 15 min and 0.5X washing solution at 60°C for 15 min, respectively. The membrane was soaked with washing solution and shaken at RT for 10 min.

13.3 Detection of DIG-Labeled Nucleic Acids

The membrane was agitated in 15 ml of 1X blocking solution (Roche) for 45 min and incubated for 30 min with 15 ml of Anti-DIG solution at dilution 1:10000. The membrane was washed twice by shaking with washing buffer for 15 min at RT and incubated in detection buffer for 3 min. The membrane was transferred to new plastic bag containing chemiluminescent substrate CDP-Star^{TM} (Roche) diluted in detection buffer at 1:200 for 15 min. Semi-dry membrane was placed in a plastic bag and sealed. The membrane was exposed to the X-ray film for detection of chemiluminescent signal.

RESULTS

1. Cloning, Sequencing and Characterization of *Os-ERS1* and *Os-ERS2*

Os-ERS1 and Os-ERS2 cDNA were completely cloned and sequenced from Khaw Dok Mali 105 rice using RT-PCR technique. 5′-RACE and 3′-RACE strategies were employed to obtain the full-length cDNA sequences. The complete Os-ERS1 cDNA sequence was 2,072 nucleotides in lengths and the open reading flame (ORF) encoded 636 amino acids (Figure 8). The complete Os-ERS2 cDNA sequence was 2,950 nucleotides in lengths and the open reading flame (ORF) encoded 635 amino acids (Figure 9).

The sequence alignment of Os-ERS1 between KDML105 and japonica variety (accession number AF013979) indicated that both ethylene receptors shared high nucleotide and amino acid sequence identity at 99.6% and 98.6% (Figure 10, 11 and 12), respectively. Similarity, comparison of nucleotide and amino acid sequence of Os-ERS2 from KDML105 with japonica variety (accession number AF460181) showed a high sequence similarity at 99.2% and 99.4% (Figure 13, 14 and 15), respectively. Phylogenetic tree generated from the ethylene receptor gene families from rice and various plant species demonstrated that Os-ERS1 and Os-ERS2 from KDML105 are more closely related to ERS1 and ERS2 from japonica rice ethylene receptor (Figure 16 and 17). The monocot Os-ERS1 and Os-ERS2 were in separate sub-clusters to dicot plants. Interestingly, At-ERS2 has shown to separate from other plants. Although there is limited number of ethylene receptor genes isolated from monocotyledonous species so far, the results illustrate that there is a presence of a sub-cluster containing ethylene receptor members from monocot species.

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TTAACATTGG  GTATGGATGG  ATGTGACTGC  ATCGAGCCAC  TATGGCCAAC  TGATGAGCTC  CTCATCAAGT   70
              M  D  G     C  D  C     I  E  P     L  W  P  T     D  E  L     L  I  K
ATCAGTACAT  CTCGGACTTC  TTCATAGCCC  TCGCATATTT  CTCGATCCCG  TTGGAACTAA  TTTATTTCGT  140
Y  Q  Y  I     S  D  F     F  I  A     L  A  Y  F     S  I  P     L  E  L     I  Y  F  V
GAAGAAGTCA  TCCTTCTTCC  CATACAGATG  GGTTTTGATC  CAGTTTGGTG  CATTTATAGT  CCTATGTGGA  210
  K  K  S     S  F  F     P  Y  R  W     V  L  I     Q  F  G     A  F  I  V     L  C  G
GCGACTCATC  TCATAAGCCT  GTGGACTTTC  ACCACACACA  CAAAGACTGT  TGCTATGGTC  ATGACAGTCG  280
 A  T  H     L  I  S  L     W  T  F     T  T  H     T  K  T  V     A  M  V     M  T  V
CAAAGGTTTC  AACGGCTGTT  GTGTCCTGTG  CGACAGCTTT  GATGCTTGTA  CATATTATCC  CTGACTTGCT  350
A  K  V  S     T  A  V     V  S  C     A  T  A  L     M  L  V     H  I  I     P  D  L  L
GAGCGTGAAA  ACAAGGGAAT  TGTTTCTGAA  GAATAAAGCT  GAACAGCTTG  ATAGGGAGAT  GGGCTTGATT  420
  S  V  K     T  R  E     L  F  L  K     N  K  A     E  Q  L     D  R  E  M     G  L  I
AGGACACAGG  AAGAGACTGG  GAGGCATGTT  AGGATGCTTA  CCCATGAAAT  CAGAAGCACT  CTTGATAGAC  490
 R  T  Q     E  E  T  G     R  H  V     R  M  L     T  H  E  I     R  S  T     L  D  R
ATACGATTTT  GAAGACTACA  CTTGTTGAGC  TAGGAGGGAC  CTTGGGCCTG  GAAGAATGTG  CCCTATGGAT  560
H  T  I  L     K  T  T     L  V  E     L  G  G  T     L  G  L     E  E  C     A  L  W  M
GCCATCAAGA  AGCGGTTCAA  GTCTTCAGCT  TTCTCATACT  CTCCGCCACC  AGATTACTGT  TGGGTCAACT  630
  P  S  R     S  G  S     S  L  Q  L     S  H  T     L  R  H     Q  I  T  V     G  S  T
GTATCAATTA  ATCTTCCTGT  TGTCAATCAA  GTGTTCAGTA  GCAACAGAGC  AATTATAATA  CCCCACACAT  700
 V  S  I     N  L  P  V     V  N  Q     V  F  S     S  N  R  A     I  I  I     P  H  T
CTCCTTTGGC  ACGGATCCGA  CCTCTTGCAG  GGAGATATGT  TCCACCAGAA  GTGGCTGCAG  TGCGAGTTCC  770
S  P  L  A     R  I  R     P  L  A     G  R  Y  V     P  P  E     V  A  A     V  R  V  P
TCTTCTACAT  CTTTCAAACT  TTCAAATAAA  TGATTGGCCA  GAGCTTTCAG  CAAAGAGCTA  TGCAATCATG  840
  L  L  H     L  S  N     F  Q  I  N     D  W  P     E  L  S     A  K  S  Y     A  I  M
GTTCTGATGC  TTCCATCTGA  TAGCGCAAGA  AAATGGCATG  TGCATGAGTT  GGAGCTTGTT  GAGGTCGTGG  910
 V  L  M     L  P  S  D     S  A  R     K  W  H     V  H  E  L     E  L  V     E  V  V
CTGATCAGGT  TGCAGTTGCA  CTTTCTCATG  CAGCTATTCT  TGAAGAGTCC  ATGCGTGCGC  GTGATCTGCT  980
A  D  Q  V     A  V  A     L  S  H     A  A  I  L     E  E  S     M  R  A     R  D  L  L
AATGGAGCAA  AATGTTGCTC  TGGATTTAGC  TCGACGTGAG  GCTGAAATGG  CTATCCGTGC  TCGTAATGAT 1050
  M  E  Q     N  V  A     L  D  L  A     R  R  E     A  E  M     A  I  R  A     R  N  D
TTCCTGGCTG  TTATGAATCA  TGAAATGAGA  ACACCAATGA  ATGCAATAAT  AGCCCTTTCG  TCCTTACTTT 1120
 F  L  A     V  M  N  H     E  M  R     T  P  M     N  A  I  I     A  L  S     S  L  L
TGGAAACCGA  ACTTACTCCT  GAGCAGCGCT  TGATGGTGGA  AACAGTCTTG  AAAAGCAGCA  ATCTTTTAGC 1190
L  E  T  E     L  T  P     E  Q  R     L  M  V  E     T  V  L     K  S  S     N  L  L  A
AACACTCATC  AATGATGTGT  TAGATCTTTC  CAAACTTGAG  GATGGTAGCC  TTGAATTGGA  GATTAAAGCA 1260
  T  L  I     N  D  V     L  D  L  S     K  L  E     D  G  S     L  E  L  E     I  K  A
TTCAATCTTC  ATGCTGTTTT  CAAGGAGGTA  ATGAGTTTCA  TCAAGCCAAT  CGCAGCCATC  AAGAGACTGT 1330
 F  N  L     H  A  V  F     K  E  V     M  S  F     I  K  P  I     A  A  I     K  R  L
CTGTGTCAGT  TATGTTGGCA  CCAGACTTGC  CATTATGTGC  CATCGGTGAT  GAAAAGAGGC  TAATGCAAAC 1400
S  V  S  V     M  L  A     P  D  L     P  L  C  A     I  G  D     E  K  R     L  M  Q  T
TATTTTGAAT  ATCTCTGGCA  ATGCTGTTAA  GTTTACAAAG  GAGGGCCACA  TCACACTTGT  AGCTTCTGTT 1470
  I  L  N     I  S  G     N  A  V  K     F  T  K     E  G  H     I  T  L  V     A  S  V
GTGAAGGCTG  ACTCTTTAAG  AGAATTCAGA  ACCCCAGATT  TTCACCCAAC  TGCAAGCGAT  GACAATTTTT 1540
 V  K  A     D  S  L  R     E  F  R     T  P  D     F  H  P  T     A  S  D     D  N  F
ATTTGAAAGT  TCAGATTAAA  GATACGGGCT  GTGGCATTAG  CCCTCAGGAT  CTACCTCAGG  TATTTACAAA 1610
Y  L  K  V     Q  I  K     D  T  G     C  G  I  S     P  Q  D     L  P  Q     V  F  T  K
GTTTCCTCAA  TCTCAGCCTG  GAGGAAACAG  GGGATACAGT  GGTAGTGGTC  TTGGCCTTGC  CATTTGCAAG 1680
  F  P  Q     S  Q  P     G  G  N  R     G  Y  S     G  S  G     L  G  L  A     I  C  K
AGGTTTGTTA  CTTTGATGGG  AGGGCACATC  TGGTTGGATA  GCGAAGGAAC  AGGAAGAGGC  TGCACAGTAA 1750
 R  F  V     T  L  M  G     G  H  I     W  L  D     S  E  G  T     G  R  G     C  T  V
CGTTTGTCAT  CCAGCTTGGC  ATATGCGATA  ATACGAATGC  ATATCAGCAG  AAGCTGATTC  CTCTAGTCTG 1820
T  F  V  I     Q  L  G     I  C  D     N  T  N  A     Y  Q  Q     K  L  I     P  L  V  W
GCCGAGCAGT  GGGGATGCTG  ATTTTGTTGG  TCCGGTGCCA  AATGCTCCTA  ATGAAGAGAA  AGGTCAGGCT 1890
  P  S  S     G  D  A     D  F  V  G     P  V  P     N  A  P     N  E  E  K     G  Q  A
TCTCTGAATC  TCGGTATCAG  AGAAGTATAT  TGAGCTACTG  TAAATTGATC  GACGGCATTG  TATCAAGTAG 1960
 S  L  N     L  G  I  R     E  V  Y     *
GGCCTGGTTA  GTTTGCCTCT  AATTTTGTGC  CTAGCCTTGT  GATAGCTGGA  ATATATTTGT  ACAAATAATG

TACAGCAGAT  GGGACTGCAT  AATGCAGCTA  TGCACCTGGG  AT
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Figure 8 Nucleotide sequence and the deduced amino acid sequence of putative ethylene responsive sensor 1 (Os-ERS1) from KDML105 variety rice. Numbers on the right refer to nucleotide residues. The translation start site is underlined. The asterisk denotes the stop codon.

```
ATGGATGGAT  CATGTGATTG  CATTGAGCCG  CTCTGGCAGG  CGGGTGATCT  CCTTGTAAAG  TATCAGTACA    1
 M  D  G     S  C  D  C     I  E  P     L  W  Q     A  G  D  L     L  V  K     Y  Q  Y
TATCTGACTT  CTTTATTGCG  CTCGCTTACT  TCTCGATCCC  TTTGGAGCTC  ATATATTTCG  TTAAGAAGTC   70
I  S  D  F     F  I  A     L  A  Y     F  S  I  P     L  E  L     I  Y  F     V  K  K  S
AGCATTCTTC  CCTTACCGAT  GGGTGCTTAT  ACAATTCGGC  GCATTCATTG  TTCTTTGTGG  GGCAACCCAC  140
  A  F  F     P  Y  R     W  V  L  I     Q  F  G     A  F  I     V  L  C  G     A  T  H
CTGATAAATT  TGTGGACTTT  TGCCATATAT  ACCAAGAGTA  TAGCTGTGGT  ACTGACAGTG  GCGAAAGCAG  280
 L  I  N     L  W  T  F     A  I  Y     T  K  S     I  A  V  V     L  T  V     A  K  A
CGACAGCGGT  TGTTTCGTGC  ATCACAGCTT  TGATGCTTGT  GCATATAATT  CCTGATTTGT  TGAATGTGAA  350
A  T  A  V     V  S  C     I  T  A     L  M  L  V     H  I  I     P  D  L     L  N  V  K
GTTGAGAGAG  AGATTTCTGA  AGGATAAGGC  TGATGAGCTT  GATAGAGAGA  TGGGGATTAT  AAGAACACAA  420
  L  R  E     R  F  L     K  D  K  A     D  E  L     D  R  E     M  G  I  I     R  T  Q
GAGGAGACAG  GAAGACATGT  CCACATGCTG  ACCCATGAGA  TAAGAAGCAC  ACTTGACAGG  CACACCATTC  490
 E  E  T     G  R  H  V     H  M  L     T  H  E     I  R  S  T     L  D  R     H  T  I
TGCGAACTAC  GCTCGTCGAG  CTGGGAAGGA  CTCTTGCTTT  AGCGGAGTGT  GCCCTGTGGA  TGCCAACACG  560
L  R  T  T     L  V  E     L  G  R     T  L  A  L     A  E  C     A  L  W     M  P  T  R
CTCTGGATCG  GCCCTTCAGC  TCTCTCATAC  GATATATAAC  AGCGCAGCAA  TTGGATCAGT  TGTTCCTATC  630
  S  G  S     A  L  Q     L  S  H  T     I  Y  N     S  A  A     I  G  S  V     V  P  I
AACCTTCCCA  TTGTCAGTAA  GGTTTTTAAT  AGTAACCGTG  TAGTAAAAAT  TCCGCATACC  TCCCCGTTAG  700
 N  L  P     I  V  S  K     V  F  N     S  N  R     V  V  K  I     P  H  T     S  P  L
CTTCGATAAC  GGCTGACAAA  AGCAGGTATG  TGCCACCAGA  GGTTGTGGCT  ATCCGTGTTC  CACTGCTGCA  770
A  S  I  T     A  D  K     S  R  Y     V  P  P  E     V  V  A     I  R  V     P  L  L  H
CCTTACGAAT  TTCCAAATAA  ATGATTGGCC  TGAGCTATCT  GCAAAAAGCT  TTGCAGTAAT  GGTTTTGATG  840
  L  T  N     F  Q  I     N  D  W  P     E  L  S     A  K  S     F  A  V  M     V  L  M
CTCCCTCCAG  ACAGTGCAAG  AGAATGGCGG  CCGCATGAAC  GGGAGCTTGT  TGAAGTCGTC  GCTGATCAGG  910
 L  P  P     D  S  A  R     E  W  R     P  H  E     R  E  L  V     E  V  V     A  D  Q
TAGCGGTCGC  ATTGTCTCAT  GCTGCCATTT  TGGAAGAGTC  CATGCGGGCC  CGTGATCTAC  TAATGGAGCA  980
V  A  V  A     L  S  H     A  A  I     L  E  E  S     M  R  A     R  D  L     L  M  E  Q
AAACATTGCT  CTTGATGCAG  CACGGCGGGA  GGCAGAAATG  GCTATTTGTG  CCTGTAATGA  TTTTCTTGCT 1050
  N  I  A     L  D  A     A  R  R  E     A  E  M     A  I  C     A  C  N  D     F  L  A
GTAATGAACC  ATGAAATGCG  GACTCCTATG  CGAGCAATCG  TTTCTTTGTC  CTCTCTCCTT  TTAGAAACAA 1120
 V  M  N     H  E  M  R     T  P  M     R  A  I     V  S  L  S     S  L  L     L  E  T
ATCTTAGTGC  TGAACAACGC  CTAATGGTTG  AGACTATACT  AAAGAGTAGC  GATCTTCTGG  CAACTCTTAC 1190
N  L  S  A     E  Q  R     L  M  V     E  T  I  L     K  S  S     D  L  L     A  T  L  T
GAATGATGTT  TTGGACGTTT  CAAAGCTTGA  GAATGGGAGT  CTTGAGCTGG  AAATTGCACC  TTTTAATTTG 1260
  N  D  V     L  D  V     S  K  L  E     N  G  S     L  E  L     E  I  A  P     F  N  L
CATTCCACCT  TTACAGATGT  GGTTAATTTG  ATTAAGCCAG  TAGCAGCGTG  CAAGAGGCTC  TCGGTTATGG 1330
 H  S  T     F  T  D  V     V  N  L     I  K  P     V  A  A  C     K  R  L     S  V  M
TCACTTTGGC  ACCAGAGTTA  CCTCTACATG  CTATTGGTGA  TCAAAAGCGA  TTGATGCAAA  TAATCCTAAA 1400
V  T  L  A     P  E  L     P  L  H     A  I  G  D     Q  K  R     L  M  Q     I  I  L  N
TGTTGCCGGG  AACTCCATTA  AGTTCACAAA  GGAGGGTCAT  GTTTCGATTA  CAGCTTCTAT  GGCCAGACCA 1470
  V  A  G     N  S  I     K  F  T  K     E  G  H     V  S  I     T  A  S  M     A  R  P
GATGCTTTGA  GAGGTCCACA  TGAACCTGAC  TACCATCCAG  TTGTCTCTGA  TGGATTCTTT  TACCTGGCTG 1540
 D  A  L     R  G  P  H     E  P  D     Y  H  P     V  V  S  D     G  F  F     Y  L  A
TTCAGGTAAA  AGACACAGGC  TGCGGAATCA  GCCCTCAGGA  TATGCCCCAC  ACATTCAGAA  AGTTTGCACA 1610
V  Q  V  K     D  T  G     C  G  I     S  P  Q  D     M  P  H     T  F  R     K  F  A  H
CCCTGAAAAC  GCAGGCAAAT  GGAACAGTGG  CAGTGGATTG  GGGCTGGCCC  TTTCCAGAAG  ATTTGTCAGT 1680
  P  E  N     A  G  K     W  N  S  G     S  G  L     G  L  A     L  S  R  R     F  V  S
CTAATGGAAG  GTAACATCTG  GCTCGAGAGC  GAAGGCGTCG  GGAAGGGCTG  TACCGCGATG  TTCTTTGTGA 1750
 L  M  E     G  N  I  W     L  E  S     E  G  V     G  K  G  C     T  A  M     F  F  V
AACTTGGCAT  GCCTGAGAAA  CCAAATGCAA  ACCTCCGAAG  AATGGCACCG  CACCCTCTAC  AGCCAAACCA 1820
K  L  G  M     P  E  K     P  N  A     N  L  R  R     M  A  P     H  P  L     Q  P  N  Q
AGGAGCTGGA  GGCCCTGATG  CCCTCAGTAT  ATCCATAATG  GACAGCAACC  CGAGGGTTCC  TCGGGTTCGC 1890
  G  A  G     G  P  D     A  L  S  I     S  I  M     D  S  N     P  R  V  P     R  V  R
TACCAATCAA  GCGTATGAGA  AGCACCGATA  AACCATCGCC  ATGCCAAGGC  AGAGCATCCG  ACCCAGTTTT 1960
 Y  Q  S     S  V  *
GCCATCACAG  TTTACAGCTC  CATTTGCAGC  TCTCTTTTTG  ATGTATGGAT  GGTAGATGTT  GTTCTTGTCC 2030
ATCAGTGGAG  GTACCTTGGG  CATGCTGGGA  TAAGTTTCAT  TGGCATTCCT  GAATCTTGAT  TTCAGTTGTG 2100
GGGGTGATTG  GGGGGTCTTA  AGTGGTGACA  ATTGGGAAAG  CTGATGTAGT  ATGCTGTTGT  CTGCACGAGT 2170
CTGAAGTTAC  TGGGAAGTGC  CAATTTACTG  CAAGTGACAG  TGAACATGAA  GGTCTATGGC  CATCTTACAT 2240
CTCTGATACC  CTGAGCTATA  GAAGAGCTTG  GACGAACGTG  TTTATTTTTC  TTACTTTTTT  CCCTAGATTA 2310
GGGCTACAAC  CTGGAAGGGA  TCGATCTACT  AGCTGATTGC  AGTATCATTT  GTACTTTTTG  TTTTTTTTCT 2380
NAAATAAGAG  TATCAGTGGN  AANTGTAAGT  GTTACTGCAG  TGATAANAAG  AACAAAAAAA  TAAAACAAA  2449
```

Figure 9 Nucleotide sequence and the deduced amino acid sequence of putative ethylene responsive sensor 2 (Os-ERS2) from KDML105 variety rice. Numbers on the right refer to nucleotide residues. The translation start site is underlined. The asterisk denotes the stop codon.

```
ERS1-KDML105      MDGCDCIEPLWPTDELLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLIQFGA  60
ERS1-Japonica     MDGCDCIEPLWPTDELLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLIQFGA  60
                  ************************************************************

ERS1-KDML105      FIVLCGATHLISLWTFTTHTKTVAMVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTREL 120
ERS1-Japonica     FIVLCGATHLINLWTFTTHTKTVAMVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTREL 120
                  ***********.************************************************

ERS1-KDML105      FLKNKAEQLDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGGTLGLEECA 180
ERS1-Japonica     FLKNKAEQLDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGGTLGLEECA 180
                  ************************************************************

ERS1-KDML105      LWMPSRSGSSLQLSHTLRHQITVGSTVSINLPVVNQVFSSNRAIIIPHTSPLARIRPLAG 240
ERS1-Japonica     LWMPSRSGSSLQLSHTLRHQITVGSTVSINLPVVNQVFSSNRAIIIPHTSPLARIRPLAG 240
                  ************************************************************

ERS1-KDML105      RYVPPEVAAVRVPLLHLSNFQINDWPELSAKSYAIMVLMLPSDSARKWHVHELELVEVVA 300
ERS1-Japonica     RYVPPEVAAVRVPLLHLSNFQINDWPELSAKSYAIMVLMLPSDSARKWHVHELELVEVVA 300
                  ************************************************************

ERS1-KDML105      DQVAVALSHAAILEESMRARDLLMEQNVALDLARREAEMAIRARNDFLAVMNHEMRTPMN 360
ERS1-Japonica     DQVAVALSHAAILEESMRARDLLMEQNVALDLARREAEMAIRARNDFLAVMNHEMRTPMN 360
                  ************************************************************

ERS1-KDML105      AIIALSSLLLETELTPEQRLMVETVLKSSNLLATLINDVLDLSKLEDGSLELEIKAFNLH 420
ERS1-Japonica     AIIALSSLLLETELTPEQRLMVETVLKSSNLLATLINDVLDLSKLEDGSLELEIKAFNLH 420
                  ************************************************************

ERS1-KDML105      AVFKEVMSFIKPIAAIKRLSVSVMLAPDLPLCAIGDEKRLMQTILNISGNAVKFTKEGHI 480
ERS1-Japonica     AVFKEVMSFIKPIAAIKRLSVSVMLAPDLPLCAIGDEKRLMQTILNISGNAVKFTKEGHI 480
                  ************************************************************

ERS1-KDML105      TLVASVVKADSLREFRTPDFHPTASDDNFYLKVQIKDTGCGISPQDLPQVFTKFPQSQPG 540
ERS1-Japonica     TLVASVVKADSLREFRTPDFHPTASDDNFYLKVQIKDTGCGISPQDLPQVFTKFPQSQPG 540
                  ************************************************************

ERS1-KDML105      GNRGYSGSGLGLAICKRFVTLMGGHIWLDSEGTGRGCTVTFVIQLGICDNTNAYQQKLIP 600
ERS1-Japonica     GNRGYSGSGLGLAICKRFVTLMGGHIWLDSEGTGRGCTVTFVIQLGICDNTNAYQQKLIP 600
                  ************************************************************

ERS1-KDML105      LVWPSSGDADFVGPVPNAPNEEKGQASLNLGIREVY 636
ERS1-Japonica     LVWPSSGDADFVGPVPNAPNEEKGQASLKSRYQRSI 636
                  ****************************:   :.
```

Figure 10 Amino acid sequence alignment of Os-ERS1 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF013979) using ClustalW program.

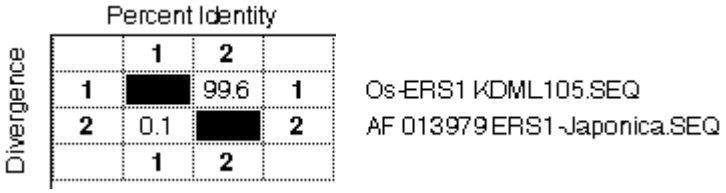

Figure 11 The nucleotide sequence distance of Os-ERS1 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF013979). Cluster analysis was done using MegAlign program (DNASTAR).

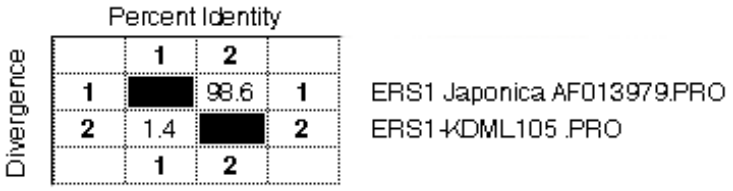

Figure 12 The amino acid sequence distance of Os-ERS1 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF013979). Cluster analysis was done using MegAlign program (DNASTAR).

```
Os-ERS2-KDML105    MDGSCDCIEPLWQAGDLLVKYQYISDFFIALAYFSIPLELIYFVKKSAFFPYRWVLIQFG  60
Os-ERS2-Japonica   MDGSCDCIEPLWQADDLLVKYQYISDFFIALAYFSIPLELIYFVKKSAFFPYRWVLIQFG  60
                   **************.*********************************************

Os-ERS2-KDML105    AFIVLCGATHLINLWTFAIYTKSIAVVLTVAKAATAVVSCITALMLVHIIPDLLNVKLRE 120
Os-ERS2-Japonica   AFIVLCGATHLINLWTFAIYTKTIAVVLTVAKAATAVVSCITALMLVHIIPDLLNVKLRE 120
                   **********************:*********************************** 

Os-ERS2-KDML105    RFLKDKADELDREMGIIRTQEETGRHVHMLTHEIRSTLDRHTILRTTLVELGRTLALAEC 180
Os-ERS2-Japonica   RFLKDKADELDREMGIIRTQEETGRHVHMLTHEIRSTLDRHTILRTTLVELGRTLALAEC 180
                     ************************************************************

Os-ERS2-KDML105    ALWMPTRSGSALQLSHTIYNSAAIGSVVPINLPIVSKVFNSNRVVKIPHTSPLASITADK 240
Os-ERS2-Japonica   ALWMPTRSGSALQLSHTIYNSAAIGSVVPINLPIVSKVFNSNRVVKIPHTSPLASITADK 240
                     ************************************************************

Os-ERS2-KDML105    SRYVPPEVVAIRVPLLHLTNFQINDWPELSAKSFAVMVLMLPPDSAREWRPHERELVEVV 300
Os-ERS2-Japonica   SRYVPPEVVAIRVPLLHLTNFQINDWPELSAKSFAVMVLMLPPDSAREWRPHERELVEVV 300
                   ************************************************************

Os-ERS2-KDML105    ADQVAVALSHAAILEESMRARDLLMEQNIALDAARREAEMAICACNDFLAVMNHEMRTPM 360
Os-ERS2-Japonica   ADQVAVALSHAAILEESMRARDLLMEQNIALDAARREAEMAICACNDFLAVMNHEMRTPM 360
                   ************************************************************

Os-ERS2-KDML105    RAIVSLSSLLLETNLSAEQRLMVETILKSSDLLATLTNDVLDVSKLENGSLELEIAPFNL 420
Os-ERS2-Japonica   RAIVSLSSLLLETNLSAEQRLMVETILKSSDLLATLTNDVLDVSKLENGSLELEIAPFNL 420
                   ************************************************************

Os-ERS2-KDML105    HSTFTDVVNLIKPVAACKRLSVMVTLAPELPLHAIGDQKRLMQIILNVAGNSIKFTKEGH 480
Os-ERS2-Japonica   HSTFTDVVNLIKPVAACKRLSVMVTLAPELPLHAIGDQKRLMQIILNVAGNSIKFTKEGH 480
                   ************************************************************

Os-ERS2-KDML105    VSITASMARPDALRGPHEPDYHPVVSDGFFYLAVQVKDTGCGISPQDMPHTFRKFAHPEN 540
Os-ERS2-Japonica   VSITASMARPHALRGPHEPDYHPVVSDGFLYLAVQVKDTGCGISPQDMPHTFRKFAHPEN 540
                   **********.******************:****************************** 

Os-ERS2-KDML105    AGKWNSGSGLGLALSRRFVSLMEGNIWLESEGVGKGCTAMFFVKLGMPEKPNANLRRMAP 600
Os-ERS2-Japonica   AGKWNSGSGLGLALSRRFVSLMEGNIWLESEGVGKGCTAMFFVKLGMPEKPNANLRRMAP 600
                   ************************************************************

Os-ERS2-KDML105    HPLQPNQGAGGPDALSISIMDSNPRVPRVRYQSSV 635
Os-ERS2-Japonica   HPLQPNQGAGGPDALSISIMDSNPRVPRVRYQSSV 635
                   ********************************** 
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Figure 13 Amino acid sequence alignment of Os-ERS2 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF460181) using ClustalW program.

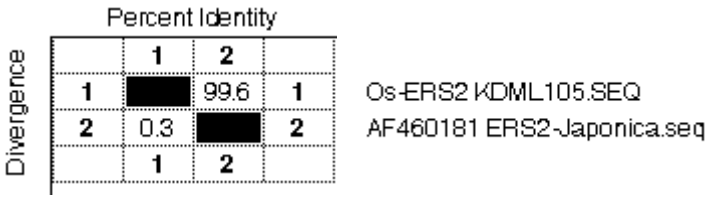

Figure 14 The nucleotide sequence distances of Os-ERS2 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF460181). Cluster analysis was done using MegAlign program (DNASTAR).

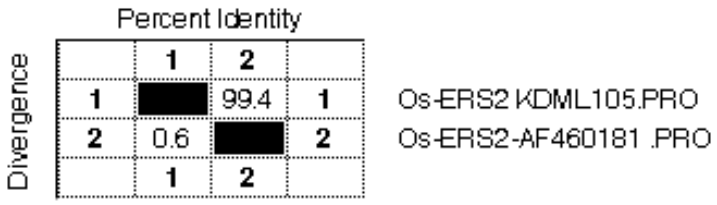

Figure 15 The amino acid sequence distances of Os-ERS2 from KDML105 variety rice compared with *Oryza sativa* japonica variety (Accession number AF460181). Cluster analysis was done using MegAlign program (DNASTAR).

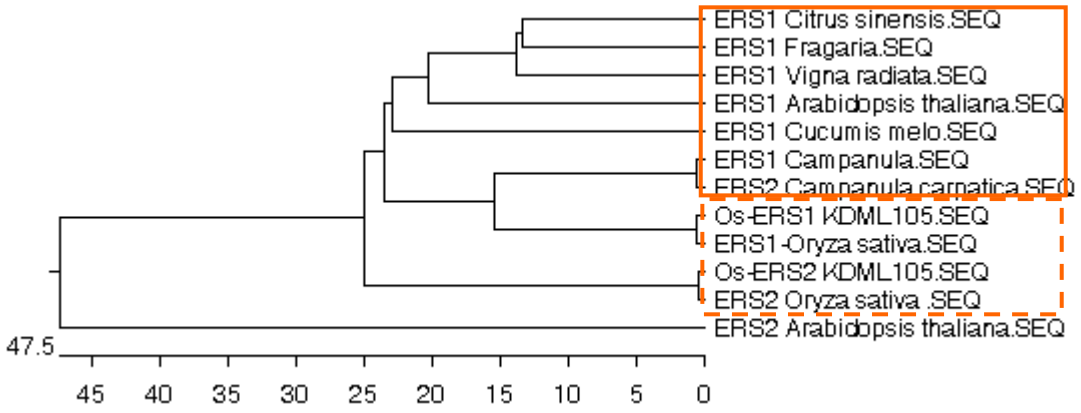

Figure 16 Phylogenetic tree of Os-ERS1 and Os-ERS2 nucleotide sequences from rice and other plants. Cluster analysis was done using the MegAlign program (Lasegene). The species and corresponding accession number are as follow: *Oryza japonica* (AF013979 and AF460181), *Arabidopsis thaliana* (NM_129658 and NM_100312), *Cucumis melo* (AF037368), *Fragaria x ananassa* (AJ297512), *Citrus sinensis* (AF092088), *Vigna radiata* (AF098270) and *Campanula carpatica* (AF 413668 and AF413669).

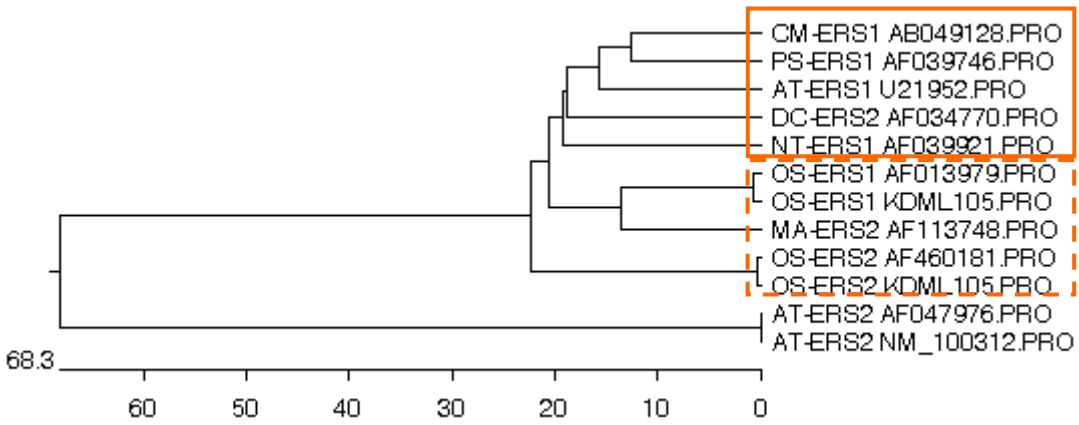

Figure 17 A phylogenetic tree generated with amino acid sequence of ethylene receptor genes from various species. The accession number that appeared in Genbank database are listed below:*Arabidopsis thaliana*: AT-ERS1 (U21952), AT-ERS2 (NM_100312), AT-ERS2 (AF047976), *Oryza sativa*: OS-ERS1 (AF013979), OS-ERS2 (AF460181), *Dianthus caryophyllus*: DC-ERS2 (AF034770), *Cucumis melo*: CM-ERS (AB049128), *Pisum sativum*: PS-ERS1 (AF039746), *Musa acuminata*: MA-ERS2 (AF113748), *Nicotiana tabacum*: NT-ERS1 (AF039921).

2. Determination of Os-ERS1 and Os-ERS2 Genes Copy Number

Genomic DNA was extracted from young leaf tissue of KDML105 rice using CTAB method (Figure 18). The concentration and purity of DNA were determined at 260 and 280 nm. *Bam*HI, *Hind*III and *Msp*I restriction endonuclease were used to digest rice genomic DNA. Rice genomic completely digested by each of restiction endonuclease was separated on 1% agarose gel as seen in figure 19. The copy number of Os-ERS1 and Os-ERS2 in rice genome was determined by Southern analysis. Using Os-ERS1 probe, a single band of approximately at 3 kb and 9 kb was observed in *Bam*HI and *Hind*III lanes, respectively (Figure 20a). When the Southern blot was probe with Os-ERS2 specific probe, the *Msp*I lane shown single band of approximately 3 kb (Figure 20b).

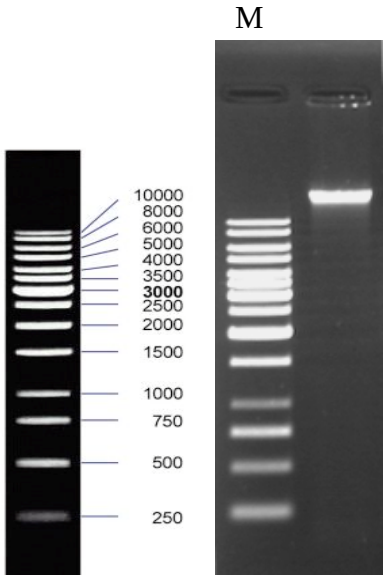

Figure 18 Ethidium bromide stained 1% agarose gel of KDML105 rice genomic DNA following an electrophoresis at 60 V for 50 min. Molecular weight marker (M) is shown on the left.

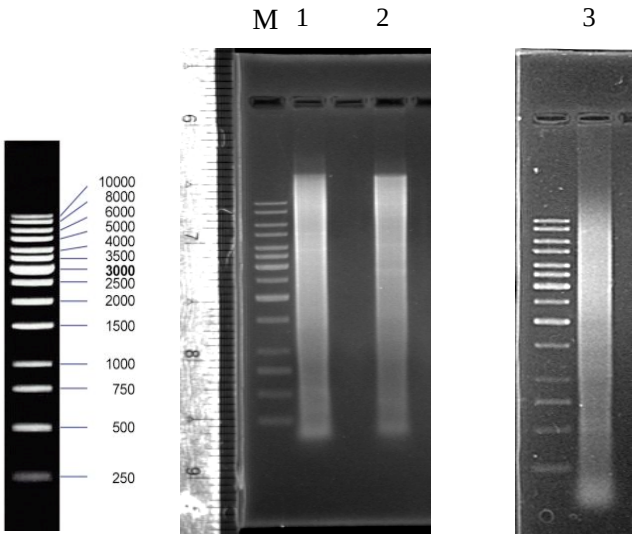

Figure 19 Ethidium bromide stained 1% agarose gel of KDML105 rice genomic DNA following the electrophoresis at 60 V for 1.5 hrs. M: 1 kb ladder molecular weight marker, Lane 1: KDML 105 rice genomic DNA digested with *Bam*HI, Lane 2: : KDML 105 rice genomic DNA digested with *Hind*III and Lane 3: KDML 105 rice genomic DNA digested with *Msp*I.

(a) (b)

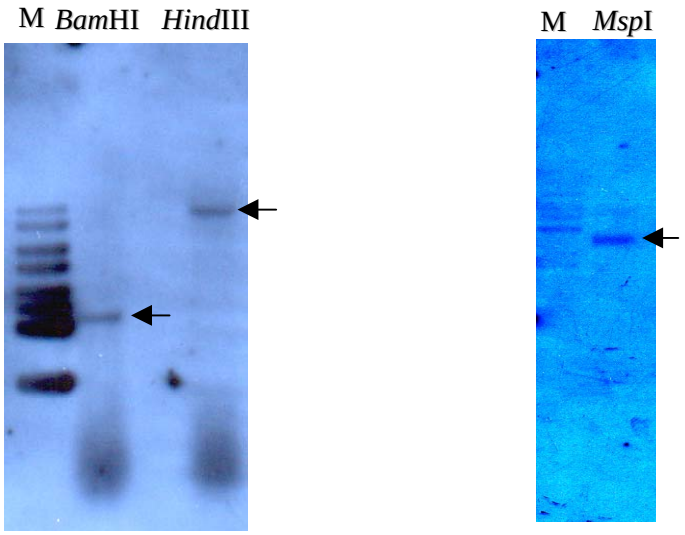

Figure 20 Southern analysis of Os-ERS1 and Os-ERS2 genes in *Oryza sativa* indica, Khaw Dok Mali 105 variety. (a) Genomic DNA of KDML105 rice was digested with *Bam*HI and *Hind*III and probed with Os-ERS1 specific probe. (b) Genomic DNA of KDML105 rice was digested with *Msp*I and probed with Os-ERS2 specific probe.

3. Identification of Conserved Domains in Os-ERS1 and Os-ERS2 Putative Proteins

Amino acid sequences of Os-ERS1 and Os-ERS2 from Khaw Dok Mali 105 (KDML105) rice were aligned using rpsblast (NCBI). Result indicated that, there are three domains including transmembrane domain (ethylene binding domain), GAF domain and histidine domain presenting on Os-ERS1 and Os-ERS2 proteins (Figure 20). The conserved amino acid sequences of transmembrane domain comparing in among of two varieties rice and *Arabisopsis* was shown in figure 22. In KDML105 and japonica variety, only three hydrophobic segments (I-III) were found. The amino acid sequence on those three segments between KDML105 and japonica rice was highly similarity. The forth hydrophobic segment (IV) was presented only in ERS2 of Arabidopsis (At-ERS2).

The amino acid sequences alignment of putative histidine protein kinase domains indicated that, five highly conserved motifs (H, N, G1, F and G2) are present in both Os-ERS1 and Os-ERS2 of KDML105 and japonica variety (Figure 22), which are proposed to be important for histidine kinase activity of the two-component regulator (Chen et al., 2005). Structure analysis showed that, conserved leusine (L) residue no H motif (consensus sequence, --HE---PL) presenting in both ERS1 and ERS2 japonica variety, was substituted to be methionine (M) on Os-ERS1 and Os-ERS2 of KDML105 rice. Furthermore, the arginine (A) conserved residue on N-motif (consensus sequence, --Q---N—NA) was substituted with serine (S). The consensus proline (P) amino acid sequence on F motif (consensus sequence, -F-PF) of both KDML105 and japonica variety were substituted to be lysine (K) and arginine (R), respectively. The region containing sequence (R/KXmNKXnD), motif implicated in cGMP binding domain (Yew Seng et al., 2000), originally identified as GAF domain is presented in both Os-ERS1 and Os-ERS2 receptor isoforms (Figure 24). Os-ERS2 protein structure, the position of asparagine (N), a polar amino acid, on cGMP binding site was substituted to be a hydrophobic leusine (L) residue. Moreover, Os-ERS1 and Os-ERS2 are missing the response regulator domain, which has sequence identity to the output portion of bacterial two component systems.

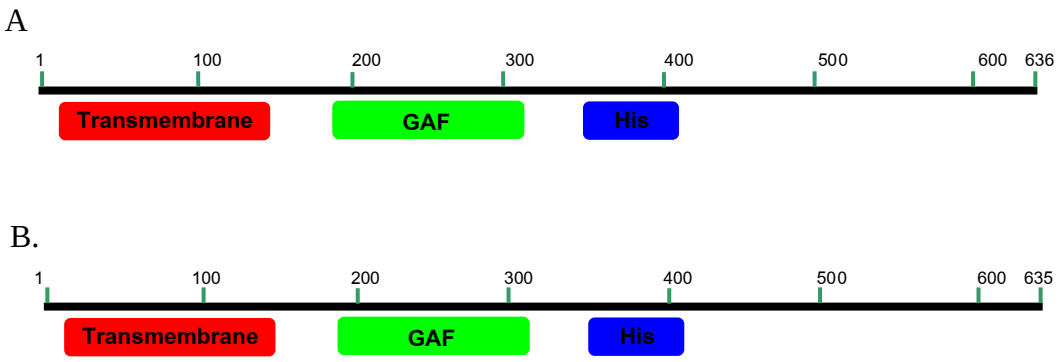

Figure 21 Represent diagram of conserved domain of Os-ERS1 (A) and Os-ERS2 (B) protein in *Oryza sativa indica* variety KDML105 using rpsblast. The black bar represent polypeptide chain of ethylene responsive sensor protein. The red, green and blue box refer to conserved domain within the amino peptide. Transmembrane doamin (red box): these domain is high affinity to bind ethylene molecules. GAF domain (green box): this domain present in phytochromes and cGMP-specific phosphodiesterases. His KA (blue box): His Kinase A domain; these domains contain a conserved His residue and are activated via trans-autophosphorylation by the catalytic domain of the histidine kinase in two component signaling system. They subsequently transfer the phosphoryl group to the Asp acceptor residue of a response regulator protein.

```
Os-ERS1-Japonica  ------------------------------MDG-CDCIEP-LWPTDELLIKYQYISDFF    27
Os-ERS1-KDML105   ------------------------------MDG-CDCIEP-LWPTDELLIKYQYISDFF    27
Os-ERS2-Japonica  ------------------------------MDGSCDCIEP-LWQADDLLVKYQYISDFF    28
Os-ERS2-KDML105   ------------------------------MDGSCDCIEP-LWQAGDLLVKYQYISDFF    28
At-ERS1           ------------------------------MES-CDCFET-HVNQDDLLVKYQYISDAL    27
At-ERS2           --MLKTLLVQWLVFFFFFLIGSVVTAAEDDGSLSLCNCDDEDSLFSYETILNSQKVGDFL    58
                          IV

Os-ERS1-Japonica  IALAYFSIPLELIYFVKKS-SFFPYRWVLIQFGAFIVLCGATHLINLWTFTTHTKTVAMV    86
Os-ERS1-KDML105   IALAYFSIPLELIYFVKKS-SFFPYRWVLIQFGAFIVLCGATHLISLWTFTTHTKTVAMV    86
Os-ERS2-Japonica  IALAYFSIPLELIYFVKKS-AFFPYRWVLIQFGAFIVLCGATHLINLWTFAIYTKTIAVV    87
Os-ERS2-KDML105   IALAYFSIPLELIYFVKKS-AFFPYRWVLIQFGAFIVLCGATHLINLWTFAIYTKSIAVV    87
At-ERS1           IALAYFSIPLELIYFVQKS-AFFPYKWVLMQFGAFIILCGATHFINLWMFFMHSKAVAIV    86
At-ERS2           IAIAYFSIPIELVYFVSRTNVPSPYNWVVCEFIAFIVLCGMTHLLAGFTYGPHWPWVMTA   118
                          I                        II

Os-ERS1-Japonica  MTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAEQLDREMGLIRTQEETGRHV   146
Os-ERS1-KDML105   MTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAEQLDREMGLIRTQEETGRHV   146
Os-ERS2-Japonica  LTVAKAATAVVSCITALMLVHIIPDLLNVKLRERFLKDKADELDREMGIIRTQEETGRHV   147
Os-ERS2-KDML105   LTVAKAATAVVSCITALMLVHIIPDLLNVKLRERFLKDKADELDREMGIIRTQEETGRHV   147
At-ERS1           MTIAKVSCAVVSCATALMLVHIIPDLLSVKNRELFLKKKADELDREMGLILTQEETGRHV   146
At-ERS2           VTVFKMLTGIVSFLTALSLVTLLPLLLKAKVREFMLSKKTRELDREVGIIMKQTETSLHV   178
                          III

Os-ERS1-Japonica  RMLTHEIRSTLDRHTILKTTLVELGGTLGLEECALWMPSRSGSSLQLSHTLRHQIT----   200
Os-ERS1-KDML105   RMLTHEIRSTLDRHTILKTTLVELGGTLGLEECALWMPSRSGSSLQLSHTLRHQIT----   200
Os-ERS2-Japonica  HMLTHEIRSTLDRHTILRTTLVELGRTLALAECALWMPTRSGSALQLSHTIYNSAA----   201
Os-ERS2-KDML105   HMLTHEIRSTLDRHTILRTTLVELGRTLALAECALWMPTRSGSALQLSHTIYNSAA----   201
At-ERS1           RMLTHGIRRTLDRHTILRTTLVELGKTLCLEECALWMPSQSGLYLQLSHTLSHKIQ----   200
At-ERS2           RMLTTKIRTSLDRHTILYTTLVELSKTLGLKNCAVWIPNEIKTEMNLTHELRPRIDDENE   238
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Figure 22 Alignment of the putative transmembrane domains (ethylene biding domain) between *Oryza sativa* and *Arabidopsis thaliana*. The four hydrophobic segments (I to IV) are underlined (Jian Hua et al., 1998).

Figure 23 Alignment of the putative histidine protein kinase domains. The five consensus motifs (H, N, G1, F, and G2) found in bacterial histidine protein kinases are indicated on the top of the sequences. Open triangle symbol are indicate nonpolar residues, filled diamonds indicate polar residues. The asterisk signs indicate basic residues, minus signs indicate acidic residues and plus sign indicate the position with more than 50% conservation in bacterial histidine kinase (Jian Hua et al., 1998).

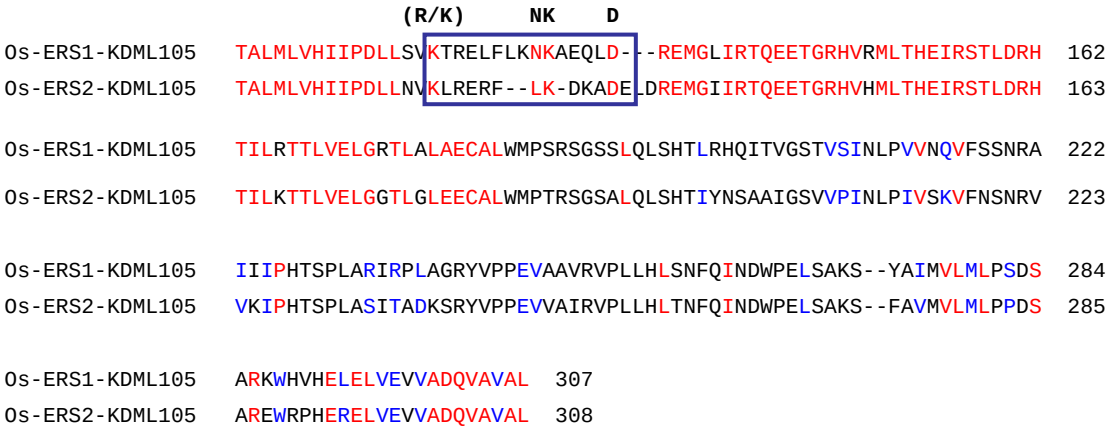

Figure 24 Alignment of N-terminal domain of Os-ERS1 and Os-ERS2 protein in *Oryza sativa* indica, KDML105 variety using rpsblast. The GAF domains of cGMP-regulated phosphodiesterases and some adenylyl cyclases contain a (R/K)X(m)NKX (n)D motif implicated in cGMP binding domain signature is indicated in the box (Yew Seng et al., 2000).

4. Determination of Ethylene Signal Transduction Genes in Khaw Dok Mali 105 Rice During Rice Tungro Bacilliform Virus (RTBV) Infection

In order to understand the signaling system of ethylene in Khaw Dok Mali 105 (KDML105) rice during Rice Tungro Bacilliform Virus (RTBVCN) infection, RNA gel blot was performed to monitor the expression of four ethylene receptor and four ethylene responsive factor genes at various time after the infection. Agroinoculation technique was used to introduce RTBVCN clone into rice plant. *Agrobacterium* without infectious clone of RTBVCN and water steriled were used as control of this technique.

For ethylene perception (Figure 25), Os-ERS1, Os-ERS2, Os-ETR1 and Os-ETR2 were investigated. Before the inoculation, transcription level of Os-ERS1, Os-ETR1 and Os-ETR2 were detected at low level or none at all. Then the expression of those three genes were elevated after the RTBVCN infection, while the expression of Os-ERS2 is constitutively expressed. Interestingly, the patterns of Os-ERS1 and Os-ETR2 were shown to be rhythmic pattern which reach the highest level at 6 and 4 hr after the infection. The mRNA expression of Os-ETR1 was slightly induced by RTBVCN inoculation and remained to be hold. Furthermore, a little effect of *Agrobacterium* and water injection was observed.

In nuclear event (Figure 26), transcription level of four ethylene responsive factor (ERF) genes that act as transcriptional factor controlling ethylene responsive gene were investigated. The result was revealed that, Os-ERFG1 is expressed in non-infected rice and injection effect was also observed only in this group, whereas the expression of another group of ethylene responsive factor (Os-ERFG2, Os-ERFG3 and Os-ERFG4) were detected at low level or none at all in both non-infected and water injected rice. Interestingly, four isoforms of Os-ERFG1 were found in among of RTBVCN, *Agrobacterium* and water injection, while only a single isoform was observed in non-infected rice. The isoform presenting in non-infected rice was found to be a mainly isoform. Moreover, it is interesting to note that among of RTBVCN, *Agrobacterium* and water injection were resulted in a detectable increase of another

two smaller isoform of Os-ERFG1 transcript within 30 min. Then the transcription level of biggest isoform of group 1 responsive factor was observed within 1 and 2 hr after *Agrobacterium* and RTBVCN inoculation, respectively. Another group of ethylene responsive factor was also activated by RTBVCN inoculation which significantly difference in their patterns. The rhythmic pattern was found on Os-ERFG3 and Os-ERFG4 which elevated to reach highest level at 1 and 2 hr and was further down-regulated at 12 hr after RTBV inoculation, respectively. Moreover, the expression of Os-ERFG2 is shown to slightly induce and raised to highest level at 12 hr post-inoculation. Interestingly, *Agrobacterium* inoculation induced a strong increase in Os-ERFG2 expression within 30 min after *Agrobacterium* inoculation and the level of expression remained to be increase after a day of infection, while the expression was shown to suppress in RTBV inoculated rice. Effect of *Agrobacterium* inoculation was also observed in among of Os-ERFG1, Os-ERFG3 and Os-ERFG4. In Agroinoculated rice, the transcriptional pattern of Os-ERFG2, Os-ERFG3 and Os-ERFG4 was shown to slightly induce and rhythmic pattern was not found.

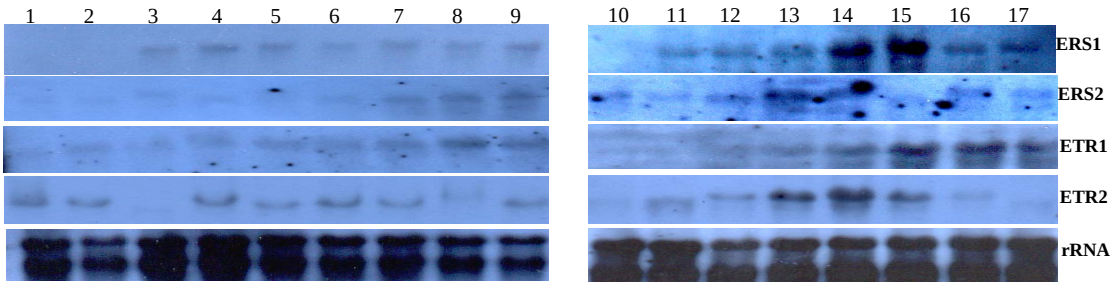

Figure 2 Northern analysis of ethylene receptor family in KDML105 during pathogen infection. Twentyfive micrograms of total RNA were used for RNA gel blot analysis and hybridized with Os-ERS1, Os-ERS2, Os-ETR1, Os-ETR2 and rRNA probes. Lane1 and 2: rice sample at 4 and 24 hr after injected with dH_2O, Lane 3, 4, 5, 6, 7, 8 and 9: rice at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and 24 hr after infected with *Agrobacterium*, Lane 10: Control (Non-infected rice), Lane 11, 12, 13, 14, 15, 16 and 17: rice at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and 24 hr after infected with infectious clone of Rice Tungro Bacilliform Virus Chainate isolate (RTBVCN).

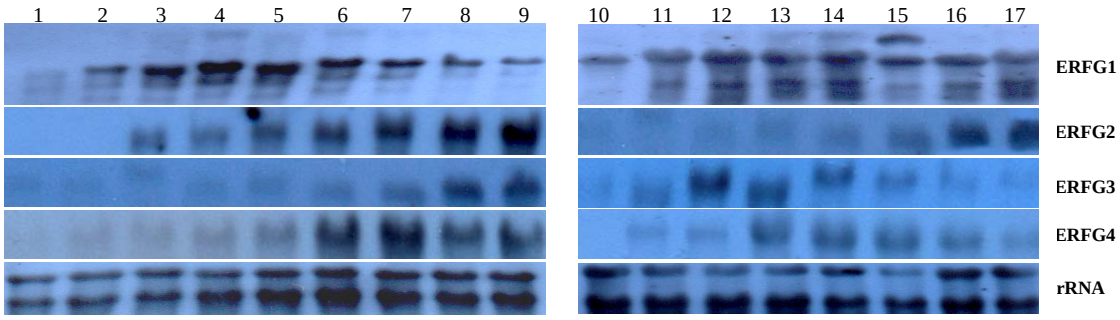

Figure 26 Northern analysis of ethylene responsive factor (ERF) family in KDML105 during pathogen infection. Twentyfive micrograms of total RNA were used for RNA gel blot analysis and hybridized with Os-ERFG1, Os-ERFG2, Os-ERFG3, Os-ERFG4 and rRNA probes. Lane1 and 2: rice sample at 4 and 24 hr after injected with dH_2O, Lane 3, 4, 5, 6, 7, 8 and 9: rice at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and 24 hr after infected with *Agrobacterium*, Lane 10: Control (Non-infected rice), Lane 11, 12, 13, 14, 15, 16 and 17: rice at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and 24 hr after infected with infectious clone of Rice Tungro Bacilliform Virus Chainat isolate (RTBVCN).

DISCUSSION

In this study, putative ethylene signal transduction genes including Ethylene Responsive Sensor 1 (Os-ERS1) and Ethylene Responsive Sensor (Os-ERS2) were isolated from Khaw Dok Mali 105 (KDML105) rice. The deduced amino acid sequences of Os-ERS1 and Os-ERS2 have high similarity. This suggested functional homologue of Os-ERS1 and Os-ERS2. When their amino acid sequences were compared with other plant ERSs, the phylogenic tree indicated that monocot Os-ERS1 and Os-ERS2 were in separate sub-clusters to dicot plants and At-ERS2 was in separate cluster to other plant ERSs. Moreover, Os-ERS1 and Os-ERS2 were in separate sub-cluster. It remains to be seen that the pattern will be held when more sequences are added into the tree. Additionally, phylogenic relationships of Os-ERS1 and Os-ERS2 and some ERS proteins from *Arabidopsis* indicated that Os-ERS1 was closely related to At-ERS1, while Os-ERS2 was significantly difference to At-ERS2.

Amino acid sequence of Os-ERS1 and Os-ERS2 were analyzed for their functional domains. There are three domains including transmembrane, histidine kinase and GAF domains presenting in Os-ERS1 and Os-ERS2. The transmembrane and histidine kinase are involved in affinity to ethylene binding and efficiency to transmit the signal to downstream components, while the function of the GAF region is not known (Chen et al., 2005). However, it must play some role in signal transduction as it joins the ligand binding portion of the receptor to the proposed signal transmission domain and is one of the most highly conserved domains within the ethylene receptor family. In Arabidopsis, hydrophobic segment IV of transmembrane domain was found on At-ERS2, whereas it is not presented on Os-ERS1 and Os-ERS2 of both KDML105 and japonica variety. This extra domain might affect ethylene affinity. Moreover, substitution of amino acid residue on H and F motif of histidine kinase region in KDML105 probably involved in the conformational of both Os-ERS1 and Os-ERS2 which subsequently affected their affinity and efficiency to receive and transducer the signal into the cell. Qu and Schaller (2004) reported that a site-directed mutation eliminated histidine kinase activity in ETR1. However, the mutation had a modest effect upon the ability of the receptor to repress ethylene responses.

In order to monitor the dynamic of ethylene signaling pathway in response to Rice Tungro Bacilliform Virus (RTBVCN) infection, Northern analysis technique was performed. In this study, the expression of four putative ethylene receptor genes and four groups of ethylene responsive factors (ERFs) were investigated. The four ethylene receptors including Os-ERS1, Os-ERS2, Os-ETR1 and Os-ETR2 were determined during various time after RTBV inoculation. Comparison to non-infected rice, transctiption of Os-ERS1, Os-ETR1 and Os-ETR2 were strongly enhanced by RTBV inoculation, whereas Os-ERS2 had been constitutively expressed. The expression of Os-ERS1 and Os-ETR2 demonstrated a distinct rhythmic pattern which reached the highest level at 6 and 4 hr after the inoculation by RTBVCN, respectively. The Os-ETR1 expression was slightly induced overtime. Moreover, effect of *Agrobacterium* inoculation and wounding was observed at low level. Three out of four (Os-ERS1, Os-ETR1 and Os-ETR2) ethylene receptor genes were activated in response to RTBV inoculation. Only Os-ERS2 was not responded and the expression pattern was different. These may reflect their functional differences.

Northern analysis revealed that transcriptional levels of all four groups of ethylene responsive factors were elevated by RTBVCN inoculation. Interestingly, four isoforms of Os-ERFG1 were induced by both RTBVCN and *Agrobacterium* inoculation, while only one isoform was presented in non-infected rice. This result is supported by several publications that ERFG1 is induced after the infection of many pathogens (Berrocal et al., 2002; Chen et al., 2002). It was also demonstrated that constitutive expression of ERFG1 enhances the resistance of Arabidopsis plants to several fungi (Berrocal et al., 2002). Because the infected rice was eventually developed RTBV symptoms, the level of ERFG1 expression was likely insufficient or the timing of expression was too late for the development of its defense.

Os-ERFG2 transcription is strongly enhanced by infection with *Agrobacterium* alone and the expression showed accelerated kinetics, whereas the expression is showed to suppress by RTBV inoculation. This might indicated that the transcriptional response is triggered by a specific pathogen factors. The expression of Os-ERFG3 and Os-ERFG4 were induced by RTBVCN inoculation and demonstrated rhythmic

pattern. This pattern was not found in *Agrobacterium* inoculation excepted in Os-ERFG1. When compared the rhythmic pattern of expression between the receptor (Os-ERS1 and Os-ETR2) and ethylene responsive factor (Os-ERFG1, Os-ERFG3 and Os-ERFG4). We found that the highest peak of rhythmic pattern of ethylene responsive family was detected at 2-4 hr after the RTBVCN inoculation which faster than ethylene receptor that reached the highest level at 4-6 hr. These results suggested that the highest level of expression of ethylene responsive factor genes were resulted the increasing of ethylene production which may be elevated during the early stage of pathogen challenge. After the reduction of ethylene production, plants may enhance the synthesizing of ethylene receptor to de-sensitizing the ethylene signal transduction. Because the receptors are negative regulators of the pathway, an increase in the number of receptors could result de-sensitizing of the pathway (Hau and Medyerowitz, 1998).

It is clear from this study that several ethylene receptors and responsive factors involving in rice responses to viral infection. Although it seems redundant and complicate, studies in plants and Drosophila indicated that overlapping sets of synergistic and antagonistic physiological responses are likely serve purposes of monitoring and integrating multitude of inputs to reach the action at cellular and whole-organism levels (Burack and Shaw, 2000; Lohrmann and Harter, 2002). A recent study demonstrated the ethylene and cytokinin signal integration in *Arabidopsis* through a response regulator, *Arabidopsis* response regulator 2 (ARR2) (Hass et al., 2004). Further studies into plant response to viral pathogen are urgently needed to understand for better manage and control the plant diseases.

CONCLUSION

1. Nucleotide and amino acid sequence of both Os-ERS1 and Os-ERS2 from Oryza sativa indica Khaw Dok Mali 105 variety share high degree similarity to ERS1 and ERS2 from Oryza sativa japonica variety.

2. There is only single copy of both Os-ERS1 and Os-ERS2 present in genome of Khaw Dok Mali 105 rice which lied on chromosome number 3 and 5, respectively.

3. There are three domains including transmembrane, histidine and GAF domain found in Os-ERS1 and Os-ERS2 protein.

4. Os-ERS1, Os-ETR1 and Os-ETR2 elevated by RTBV inoculation and may involve in viral perception.

5. ERFs are involved in rice response to viral infection which complicated responses.

6. In this study, the result provides the basic information of possible association of ethylene signaling with RTBV infection.

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