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THESIS

CHARACTERIZATION AND EXPRESSION STUDY OF *ETHYLENE RESPONSE SENSOR 1* (*DEN-ERS1*) IN *DENDROBIUM* SPP.

MONTHATHIP THONGKUM

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Full length of *Ethylene Response Sensor1* (*Den-ERS1*) was isolated from 2 *Dendrobium* cultivars; 'Khao Sanan' and 'Pompadour'. The *Den-ERS1* from 'Khao Sanan' and 'Pompadour' cDNA sequences were 2,299 and 2,307 nucleotides in length which encode 621 and 622 amino acid, respectively. Genome organization of *Den-ERS1* consists of 3 exons (exon I, II and III) and 2 introns (intron I and II). The presence of *cis* acting elements within putative promoter region and introns was determined using bioinformatics approach including PLACE and PlantCARE. Core promoter, TATA and CAT box was found at position -57 and -74 from transcription start site. Important ethylene related elements including ethylene response elements (ERE; ATTCAAA) in 5' flanking region and wound-responsive element (WUN; AAATTTCCT) in intron II were found. Southern analysis indicated a single copy of *Den-ERS1* in 'Khao Sanan' and 'Pompadour'. The presence of *Den-ERS1* transcript in orchid organs as well as during flower development and flower senescence both natural and induced was investigated using Northern analysis. *Den-ERS1* was found differentially expressed in every orchid organ. Within flower parts, *Den-ERS1* transcripts were found highly accumulated in lip and column. The transcripts were accumulated in young bud (B3) and decreased as flower development progressed and increased again at full bloom (OF4). During flower senescence, the expression was high at early senescent stages and decreased towards the end of senescence. Pollination and emasculation induced flower senescence in both 'Khao Sanan' and 'Pompadour' and reduction in *Den-ERS1* expression.

Student's signature Thesis Advisor's signature ___ / ___ / ___

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Monthathip Thongkum
March 2010

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LIST OF ABBREVIATIONS

ACC = 1-Aminocyclopropane–1-carboxylic acid
AVG = 1-aminoethoxyvinylglicine
AOA = aminooxyacetic acid
bp = base pairs
ºC = degree Celsius
cm = centimeter
CTR = constitutive triple response
dH_2O = distilled water
DEPC = diethyl pyrocarbonate
DNA = deoxyribonucleic acid
DNase = deoxyribonuclease
dNTPs = deoxynucleotide triphosphate (s)
E. coli = *Escherichia coli*
EDTA = ethylene diamine tetraacetic acid
EIL = ethylene insensitive 3-like
EIN = ethylene insensitive
ERF = ethylene response factor
ERS = ethylene response sensor
ETR = ethylene triple response
g = gram
h = hour
HCl = hydrogen chloride
IPTG = isopropyl-beta-D-thylogalactopyranosine
kb = kilobase
KCl = potassium chloride
kDa = kilodalton
l = liter
M = molar
MAPK = mitogen activated protein kinase
mg = milligram

LIST OF ABBREVIATIONS (Continued)

min = minute (s)
μg = microgram
μl = microlitre
μmol = micromolar
ml = milliliter
mM = millimolar
mmol = millimole
mRNA = messenger ribonucleic acid
MW = molecular weight
NaCl = sodium chloride
NaOH = sodium hydroxide
ng = nanogram
nm = nanometer
OD = optical density
PCR = Polymerase chain reaction
pmol = piccomole
RNA = ribonucleic acid
RNase = ribonuclease
rpm = rotations per minute
RT = room temperature
SDS = sodium dodecyl sulphate
sec = second
TAE = tris-acetate EDTA
UV = ultraviolet
X-gal = 5-bromo-4-chloro-3-indoyl-beta-D-galactopyranosine

CHARACTERIZATION AND EXPRESSION STUDY OF *ETHYLENE RESPONSE SENSOR 1* (*DEN-ERS1*) IN *Dendrobium* spp.

INTRODUCTION

Ethylene is an important hormone which plays important roles in plant physiological processes including fruit ripening and flower senescence. The level of ethylene production is depended on plant tissue types and developmental stages (Guo and Ecker, 2004). It is also found increasing during plant responses to pathogens and environmental stresses (Cheong *et al*., 2002). This small and gaseous molecule functions as a plant signal through ethylene signal transduction pathway. This pathway is a complex and comprising of several protein molecules. Among them, ethylene receptors are found directly interact with ethylene with copper ion as a cofactor (Rodriguez *et al*., 1999; Hirayama *et al*., 1999; Woeste *et al*., 2000). Ethylene receptors were isolated and characterized from many plant species including rose (Muller *et al*., 2000), carnation (Shibuya *et al*., 2002), delphinium (Tanase and Ichimura, 2006) and gladiolus (Arora *et al*., 2006). The effect of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene receptor in extending vase life such as carnation (Hassan and Gerzson, 2002), Chrysanthemum (Serek *et al.,* 1998; Hassan and Gerzson, 2002), *Rosa hybrid* (Serek et al 1994; Muller *et al*., 2000; Ma *et al.,* 2006) and *Dendrobium* 'Karen' (Uthaichay *et al.,* 2007) highlighted an important role of ethylene and ethylene receptor in flower senescence.

Orchid is one of important cut flowers in Thailand. With its beauty, attractive color and relatively long vase life, orchid is commonly cultivated and used as cut flowers. During and after harvest, flowers is cut, packed, transported, stored and displayed. These processes are often caused damage and/or physiological changes to orchid and consequently induced ethylene production. An individual flower is found discolored, drooped and eventually dropped. Such incidents are flower senescent symptoms that reduced orchid flower value and its shelf life. In order to delay/reduce these unwanted incidents, investigation into ethylene signaling is needed.

In this study, *Ethylene Response Sensor 1* (*Den-ERS1*) was isolated and characterized from two *Dendrobium* commercial cultivars, ‘Khao Sanan’ and ‘Pompadour’. The presence of its transcript in orchid organs as well as during flower development and flower senescence both natural and induced was investigated using Northern analysis.

OBJECTIVES

1. To isolate and characterize *Ethylene Response Sensor 1* (*Den-ERS1*) from *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’

2. To determine the expression of *Ethylene Response Sensor 1* (*Den-ERS1*) in various organs of *Dendrobium* orchid

3. To determine the expression of *Ethylene Response Sensor 1* (*Den-ERS1*) during flower development and senescence

LITERATURE REVIEW

1. Ethylene

Ethylene, a gaseous plant hormone, is a simple molecule composing of two atoms of carbon and four atoms of hydrogen (C_2H_4). Ethylene is involved in growth and development processes ranging from germination of seed to senescence of various organs and plant responses to environmental stresses (Arshad and Frankenberger, 2002). Although ethylene is produced by many plant organs, the level of production is depended on plant species, cultivar, tissue type, and developmental stages (Kumar *et al*., 1998; Ma *et al*., 2002). Moreover, ethylene is produced during plant responses to environmental and biological stresses (Morgan and Drew, 1997).

Although plants response to ethylene in various ways, the triple response is well documented. After ethylene treatment, plants which are grown in the dark will develop apical hook tightening, loss of gravitropism and radical stem expansion (Guzman and Ecker, 1990). Such responses give plant advantages during germination in protecting seedling apex and young leaves during soil emergence (Moussatche, 2004). The triple response is very useful for genetic studies and has been exploited in order to search for mutants that show deviant behavior in the presence or absence of ethylene (Guzman and Ecker, 1990). The mutant screens yield two classes of mutants: those are insensitive to the presence of ethylene and do not show the triple response, and those that show a constitutive triple response in the absence of ethylene (Kieber *et al*., 1993; Kende and Zeevart, 1997).

2. Ethylene Biosynthesis

Ethylene biosynthetic pathway is begun with the conversion of L-methionine (L-Met) to S-adenosylmethionine (Ado-met or SAM) by SAM synthetase. SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase with pyridoxal phosphate as a co-factor. In the last step, ACC is converted into ethylene by

ACC oxidase (Yang and Hoffman, 1984) (Figure 1). ACC synthase is commonly regarded as a rate-limiting step (McClellan and Chang, 2008).

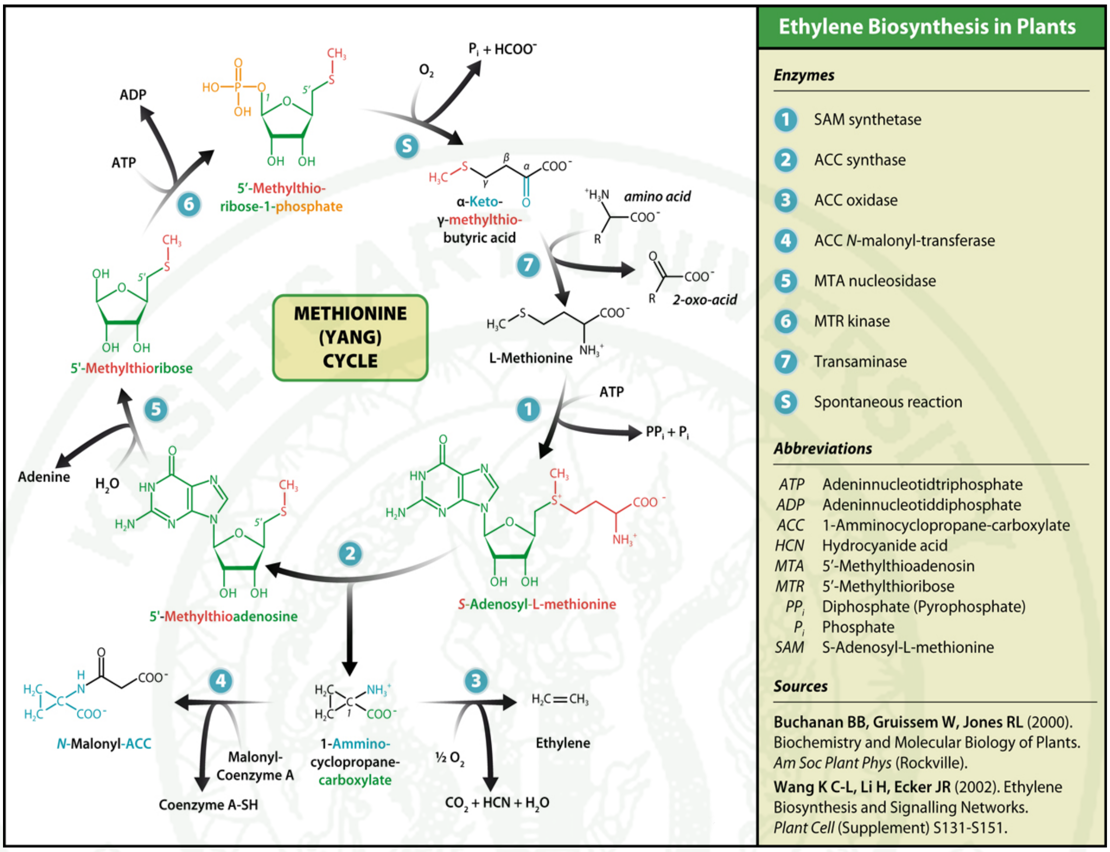


Figure 1 The ethylene biosynthetic pathway of higher plants

Source: Buchanan *et al*., 2000

Ethylene is distributed via intercellular spaces and moved through symplast (inner side of the plasma membrane) from cells to cells (Bleecker and Kende, 2000) (Figure 2). Long- distance distribution is achieved by releasing ACC into the vascular tissue where it is moved to site of action. ACC is then converted into ethylene (Sauter, 2003).

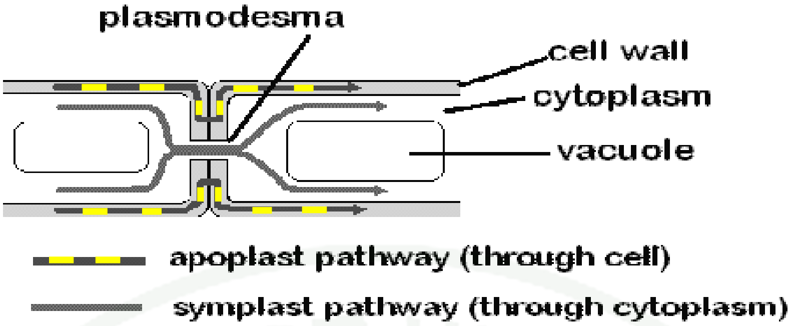


Figure 2 Diagram of the apoplast and symplast pathways through plant cells
Source: Jackacon (2007)

3. Ethylene signal transduction

Plant responses to ethylene can be divided into three steps; perception of ethylene, transduction of ethylene signal and synthesis of ethylene responded protein. Upon arrival, ethylene is perceived by ethylene receptors and transferred through elaborated signal transferring pathway called ethylene signal transduction pathway (Figure 3). Like many plant signal transduction pathways, ethylene signal transduction pathway is based on protein phosphorylation. Ethylene binds to disulfide-link dimers of ethylene receptors. Copper cofactor in this interaction is provided by RAN1, an ATPase copper transporter homolog (Hirayama *et al*., 1999; Woeste *et al*., 2000). Ethylene receptors are located at endoplasmic reticulum associated membrane and show high similarity to bacterial two-component regulators. Recently, RTE/GR, a novel protein, was found positively regulating *At-ERS1* in *Arabidopsis* (Zhou *et al*., 2007). In the absence of ethylene a putative MAPKK kinase, constitutive Triple Response 1 (CTR1), negatively regulates down stream components such as Ethylene Insensitive 2 (EIN2). When binds to ethylene, ethylene receptors are able to interact with CTR1 allowing downstream signaling to proceed. EIN2 is also reported to play important roles in auxin, cytokinin and abscisic acid signal transduction (Wang *et al*., 2007). This could be a cross talk between plant hormone pathways (Cara and Giovannoni, 2008). EIN2 regulates transcription regulators, EIN3/EILs, locating in nucleus. EIN3 activates ethylene responses by binding to the EIN3-binding site (EBS) in the promoter of ERF1 (Solano *et al*., 1998). ERF1 encodes a transcriptional activator that binds to the GCC-box in the promoters of several ethylene-responsive genes. A key regulatory step in the pathway is the degradation of EIN3 and EIL1 by the 26S proteasome-dependent pathway, mediated by an SCFEBF1/2 E3 ligase complex containing F-Box proteins EBF1 and EBF2 (Guo *et al*., 2003; Gagne *et al*., 2004; Lee *et al*., 2006; Binder *et al*., 2007). EIN 3 is stabilized through phosphorylation at specific site by MPK3/6 which is regulated by MKK9 (Yoo *et al*., 2008).

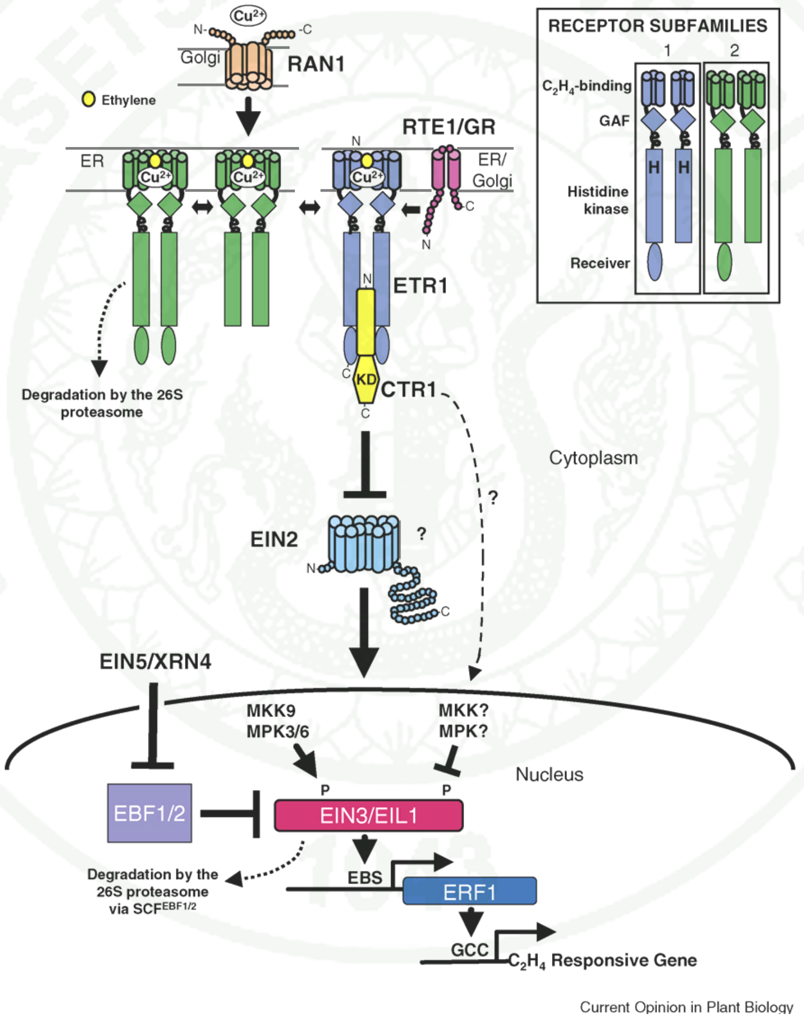


Figure 3 Model of the ethylene signal transduction pathway

Source: Kendrick and Chang (2008)

4. Ethylene receptor and its interaction with ethylene

Ethylene receptors were the first plant hormone receptor to be identified and characterized (Chen *et al*., 2002). Studies in bean, *Arabidopsis* and melon suggested that ethylene receptors locating at membrane of endoplasmic reticulum (Evans *et al*., 1982a, 1982b; Chen *et al*., 2002; Ma *et al*., 2006; Grefen *et al*., 2007). However, tobacco ethylene receptors were found in plasma membranes (Xie *et al*., 2003) and small amounts were reported in Golgi bodies (Chen *et al*., 2002).

Five ethylene receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) were identified from *Arabidopsis* (Hua *et al*., 1995; Hua and Meyerowitz, 1998; Sakai *et al*., 1998). All of which display high ethylene binding affinity (Hall *et al*., 2000; Schaller and Bleecker 1995; Schaller *et al*., 1995). While six ethylene receptors (*LeETR1-LeETR6*) were reported in tomato (Lashbrook *et al*., 1998; Wilkinson *et al*., 1995; Tieman and Klee, 1999), five receptors were reported having ethylene binding affinity. Although numbers of ethylene receptors are varied from species to species, ethylene receptors contain common structure features including ethylene binding domain, GAF domain, kinase domain and receiver domain. Ethylene binding domain locating at N-terminus consists of putative transmembrane amino acids (Hall *et al*., 2000). The interaction of ethylene and ethylene receptors requires copper ion as a cofactor and resulting in conformational change (Woeste *et al*., 2000). Binder (2007) hypothesized ethylene receptor signal state model (Figure 4). In the absence of ethylene (State 1), ethylene binding domain is depicted as a homodimer with each monomer containing three transmembrane helices. These include regions important for copper/ethylene binding, turning off the receptor, and maintaining the receptor in the signaling state. When ethylene binds, there is an intermediate conformation of ethylene binding domain where the receptor is still transmitting signal (State 2). This is predicted to be an unstable conformation. While ethylene is bound, it is in equilibrium with State 3 where ethylene is bound and signal transmission is turned off.

Depending of the numbers of ethylene binding domains, ethylene receptors can be divided into three subfamilies; subfamily I, II and III (Figure 5). Subfamily I have three ethylene binding domains while subfamily II receptor have four domains. Subfamily III receptors are identified exclusively from cyanobacteria (Mount and Chang, 2002). Qu *et al.* (2007) suggested that ETR1 and ERS1 (subfamily I) played greater roles in regulation of ethylene signaling as compared to members in subfamily II. The predominant roles might result from enhanced ability to stimulate the kinase activity of CTR1.

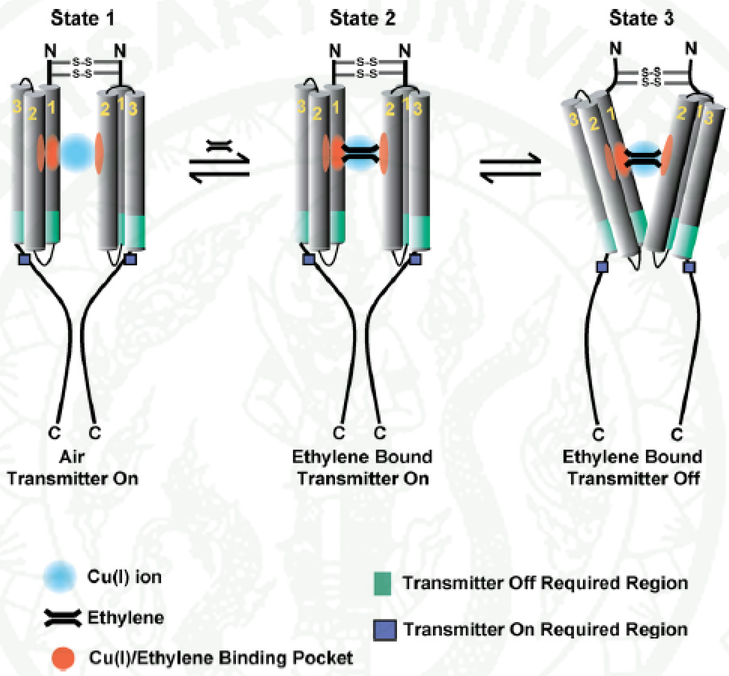


Figure 4 Ethylene receptor signal state model

Source: Binder (2007)

GAF domain is a cGMP binding site and predominately mediates non-covalent and reversible receptor association *in vivo* (Ho *et al*., 2000; Grefen *et al*, 2007). Kinase domain contains five sub-domains that define the catalytic core of histidine kinases including conserved histidine (H), asparagine (N), phenylalanine (F) and glycine (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. The receiver domain is the only domain in ethylene receptor, which not found in every ethylene receptors. It locates at C-terminus and contains conserved aspartate (D) that can be phosphorylated (Kevin, 2002).

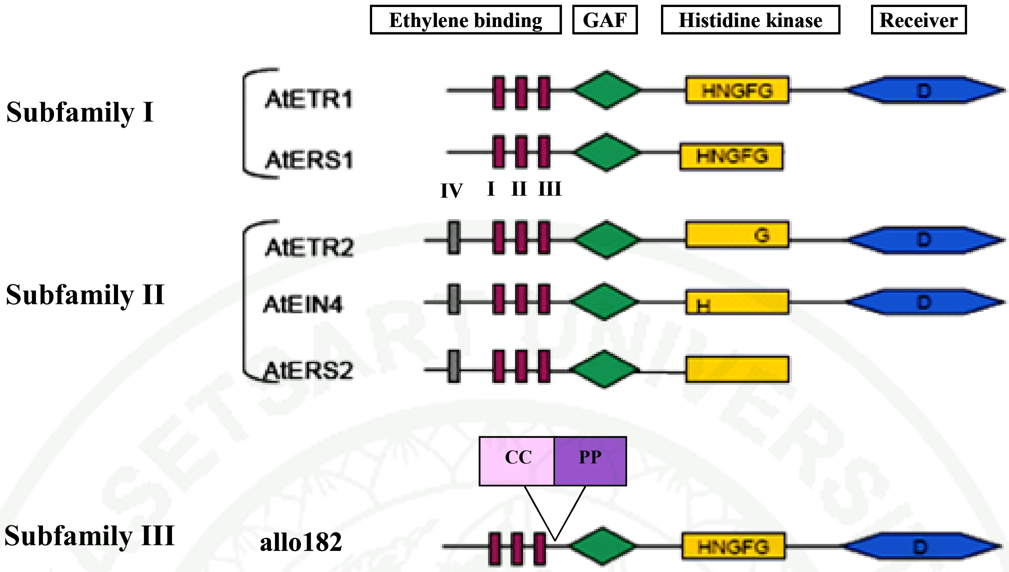


Figure 5 Ethylene receptors structure found in *Arabidopsis* (subfamily I-II) and cyanobacteria (subfamily III) contains coiled-coil domain (CC) and PAS/PAC domain (PP).

Source: Lori (2003); Mount and Chang (2002)

5. Ethylene receptors and their roles in flower development

Flower development is a determine process where floral primodia arise on the flanks of the apical meristem in place of leaves. The primodia continues to enlarge and eventually form mature flower. The transition from vegetative to reproductive growth is a major phase change in flowering plants (angiosperms). In Arabidopsis, plant hormone signaling is known to regulate suites of morphogenic processes and its role in flowering-time control is emerged as one key-controlling step (Sheen and Kay, 2004).

The role of ethylene in inhibiting growth of plant organs including leaf, root and flower under flavor conditions is well documented (Pierik *et al.*, 2006). Among plant regulators, DELLA, a family of five negative regulator proteins, play important roles in controlling flowering and flower development. Achard et al (2007) reported that ethylene delayed floral transition by modulating DELLA protein activity via CTR dependent ethylene response pathway. Ethylene also regulates levels of active GA, which play important role in promoting flowering through DELLA and the actions of EIN3, EIL1 and CTR1 (Li and Guo, 2007; Yoo *et al.,* 2009). Interaction between GA-GA receptor and DELLA results in degradation by proteosome pathway (Gomi and Matsuoka, 2003; Sun and Gubler, 2004). One of DELLA proteins, RGA, also regulated flower development related genes such as aquaporin-like genes, dehydrins, and Pro oxidase genes (Hou *et al.,* 2008).

At cellular level, the changes during flower development involve cell elongation and expansion. Aquaporins, protein channels transporting water across biological membranes are reported involving in many plant developmental processes including cell expansion, organ movement and elongation (Tyerman *et al.,* 2002). In rose (*Rosa hybrida* 'Samatha') ethylene treatment resulted in significant reduction in petal size, inhibition in expansion of petal abaxial subepidermal cells and decrease in petal water content. In *Rh-PIP2* silenced flower, petal expansion was severed inhibited and showed anatomical features similar to that of ethylene treated flower. Further investigation showed that ethylene reduced *Rh-PIP2* expression in petal (Ma *et al.,* 2008)

6. An overview of flower senescence

Flower senescence is the last phase of flower development. It involves two major steps; cell death in petal and mobilization of food reserves to other parts of plants (Rubinstein, 2000). Events associated with petal cell death include membrane changes, biochemical and molecular changes, loss of cellular protein, losses of nucleic acids, carbohydrate and cell wall alteration (Rubinstein , 2000; Hopkins *et al.,* 2007) (Figure 6). Autophagy is found responsible for the degradation of cellular components in the vacuole during petal senescence. The indicators of autophagy include vesicles and cytoplasmic material inside the vacuole, an increase in vacuolar size and a loss of cytoplasmic volume and organelle content. The tonoplast then collapses and cell death occurs (Bassham, 2009).

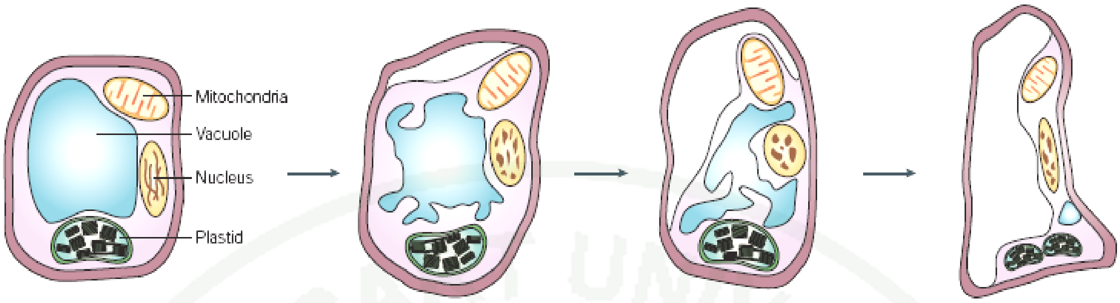


Figure 6 Morphological of programmed cell death in plants. Chromatin condensation and DNA cleavage into 50-kb fragments were observed before the apparent disruption of the vacuole, which takes places during the late stages of cell death. Blebbing of the vacuole and plasma membranes, and late destruction of organelles were also observed. At the final stage, the plasma membrane collapses and separates from the cell wall ending with the leakage of the dead cell's content into the apoplast.

Source: Lam (2004)

7. Induced senescence

Pollination-induced flower senescence is observed mainly among species in which flower longevity is sensitive to exogenous ethylene, and therefore the effects of pollination on flower senescence are likely mediated by endogenous ethylene. During a compatible pollination, the stigma communicates via a transmissible signal to the ovary and petals that a successful pollination has occurred (O'Neill *et al.,* 1993; Larsen et al, 1995). This signal results in a series of post-pollination events, which vary between species, but may include ovule maturation, ovary growth, pigmentation changes, wilting (senescence) or abscission of the petals. These are collectively called senescent symptoms (Fitting, 1909). This type of senescence is therefore induced. In flowers such as orchid with several florets in an inflorescence, pollination results in synchronized senescence.

Flower emasculation is a process to remove androecium (male reproductive organs) in the process of cross-pollination. Emasculation is reported to stimulate flower senescence in plant species including *Cymbidium* (Woltering, 1989), petunia (Woltering *et al.,* 1997), *Dianthus* (Woltering *et al.,* 1993; Sato *et al.,* 2000), tomato (Atanassova and Georgive, 2000) and *Oncidium* 'Gower ramsey' (Huang *et al.,* 2007). In orchid emasculation is a process to remove pollinia and pollinia cap (anther cap) (Woltering, 1989). In *Cymbidium*, emasculation led to rapid coloration of the lip and advanced wilting of the petal, sepal and early peak of ethylene production in lip and column (Woltering, 1989; Woltering *et al.,* 1997).

8. The roles of ethylene signaling in flower senescence

The senescence in flowers is mediated in part by changes in endogenous hormone levels and hormone sensitivity. Transgenic plants with altered hormone biosynthesis or perception are being used to study the hormonal regulation of senescence and the interactions between these signaling pathways. Floral senescence in many species is accelerated by the plant hormone ethylene such as daylily (Lay-Yee *et al*., 1992), tulip (Celikel and van Doorn, 1995) and sandersonia (Eason and de Vre, 1995). In these flowers, petal senescence is associated with an increase in endogenous ethylene production. Ethylene production has been found to increase during senescence in ethylene sensitive flower such as carnation (Shibuya *et al.,* 2000, 2002) and *Delphinium* (Tanase and Ichimura, 2006). Interference with the biosynthesis of ethylene in ornamental plants such as carnation and orchid through chemicals AVG (1-aminoethoxyvinylglycine) and AOA (aminooxyacetic acid), both inhibitors to ACC synthase, successfully reduced ethylene production and essentially extended their vase life (Brown and Mayak, 1981; Serek and Andersen, 1993; Woltering and Harkema, 1994; Son *et al.,* 1994; Harkema *et al.,* 1987).

Ethylene signal is perceived by ethylene receptors therefore blocking ethylene effects at the receptor level is likely to be more effective as it will protect against both endogenous and exogenous ethylene (Serek and Reid, 1993). 1-Methylcyclopropene

(1-MCP), a cyclic olefine, is found to irreversibly bind to ethylene receptors and prevents ethylene from inducing a conformational change (Sisler and Blankenship, 1996) and subsequently adopted for commercial use (Blankenship and Dole, 2003). 1-MCP applied at a concentration as low as 20 nL L^{-1} could improve longevity of carnation (Porat *et al*., 1995). The spectacular effect of 1-methylcyclopropene (1-MCP) has been well documented in a range of ornamental species in preventing petal senescence and abscission (Serek *et al*., 1994; Serek and Sisler, 2001).

ETR1 homologues have been isolated from diverse flower species including geranium (Dervinis *et al*., 2000), rose (Muller *et al*., 2000), carnation (Shibuya *et al*., 2002; Narumi *et al*., 2005), *Delphinium* (Kuroda *et al*., 2003; Tanase and Ichimura, 2006), gladiolus (Arora *et al*., 2006) and *Oncidium* (Huang *et al*., 2007). Interestingly, only one group of receptor, ERS1, was identified in member of Orchidaceae such as *Oncidium* (Huang *et al.,* 2007) and *Phalaenopsis* (Chang, 2002). Ma *et al*. (2006) reported that the expression of two ethylene receptor genes (*Rh-ETR1* and *Rh-ETR3*) and two CTR (*Rh-CTR1* and *Rh-CTR1*) genes may regulate flower opening in cut rose cv. Samantha. Tanase and Ichimura (2006) suggested that increase of *Dl-ERS1-3* and *Dl-ERS2* transcript level may involve in *Delphinium* sepal senescence but may not determine the ethylene response of the abscission. In carnation, Shibuya *et al*. (2002) demonstrated that *DC-ERS2* and *DC-ETR1* are responsible for ethylene perception and their expression is regulated in tissue-specific manner and independently of ethylene perception in flowers during senescence. In *Phalaenopsis equestris, PeERS* was differentially expressed in orchid organs (root, leaf and flower). Relatively high amounts were detected in old leaf in comparison with other organ, suggesting that *PeERS* may involve in the organ senescence (Chang, 2002). Huang *et al*. (2007) reported the differential expression of *OgERS1* in different organs and the pollinia cap dislodgment alleviated the mRNA levels of ethylene receptor initiated by exogenous ethylene treatment. Jones (2008) reported that transgenic petunia with *etr1-1* mutant did not exhibit pollination-induced corolla senescence. Comparative studies of the molecular and biochemical changes occurring following pollination of Wild type and *etr1-1* petunia flowers were able to identify components of the pollination signaling pathways which were regulated by ethylene.

Dendrobium orchid is the most commercially important cut flower in Thailand. *Dendrobium* 'Khao Sanan' and 'Pompadour' are popular hybrid species for commercial and widely used as parental for breeding. However, little is known about ethylene perception in orchid genus *Dendrobium* flower. Therefore, the objective of this study is to clone and determine expression of *Ethylene Response Sensor 1* (*Den-ERS1*) in *Dendrobium* organ and during flower development and senescence.

MATERIALS AND METHODS

1. Plant Materials

Two cultivars of *Dendrobium* spp. including ‘Pompadour’ and ‘Khao Sanan’ were grown and maintained with regular fertilization in a greenhouse at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. Their organs (root, leaves, shoot, flower and peduncle) were used for genomic DNA and total RNA extraction. In this study flower development stages were divided as followed; flower bud (B1-B3), open floret (OF1-OF4) and 3 stages of senescence flower (S1-S4) (Figure 7).

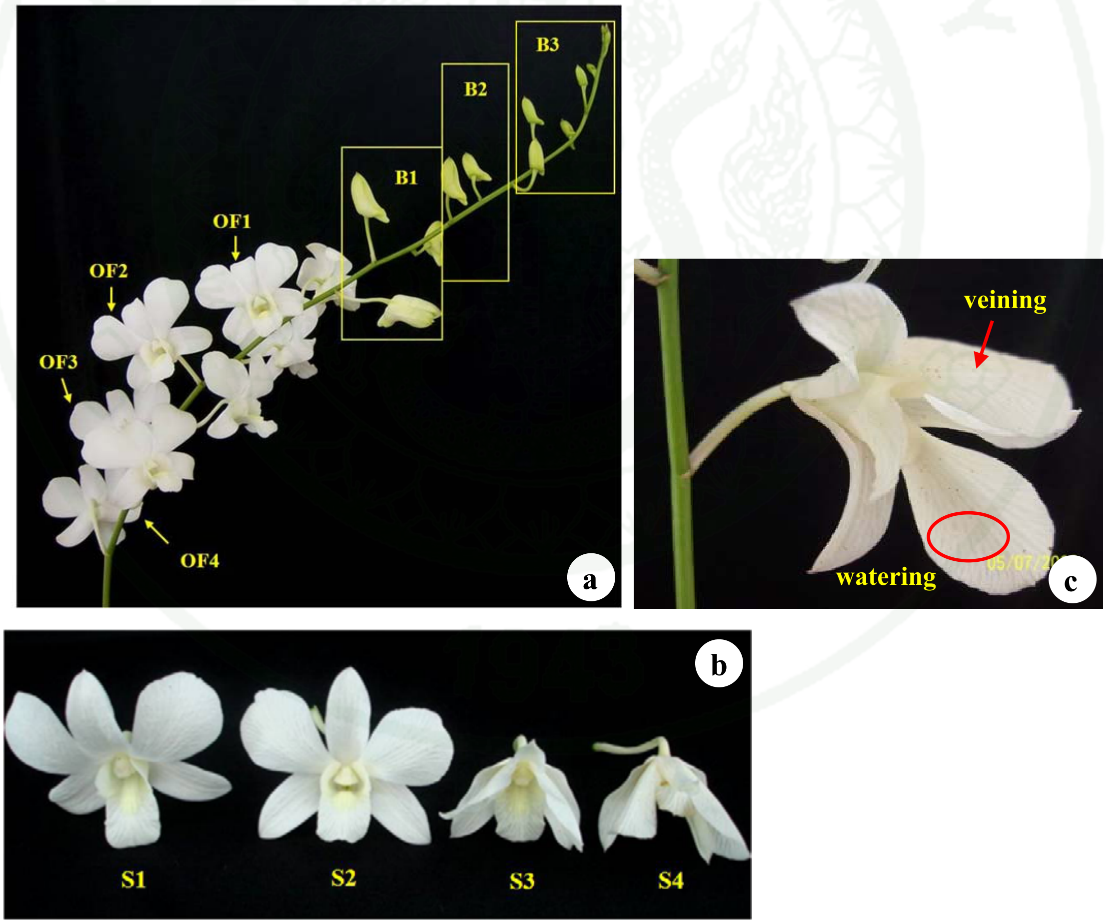


Figure 7 *Dendrobium* orchid flower during flower development (a) flower senescence (b) stages and flower senescence symptom (c)

2. Induction of flower senescence

2.1 Emasculation

Pollinia and pollinia cap were removed from full bloom florets of *Dendrobium*. Each inflorescence stem was cut at a 45° angle, 12 cm from the basal end of the first open floret, and held in a 15-mL centrifuge tube containing 10 mL of distilled water. Inflorescences were held under natural light conditions at ambient temperature (25°C) and relative humidity (70 ± 2% RH) during the period of study.

2.2 Pollination

Full bloom florets were hand-pollinated by placing the pollinia on the stigma Pollinia and pollinia cap were also removed. Each inflorescence stem was cut at a 45° angle, 12 cm from the basal end of the first open floret, and held in a 15-mL centrifuge tube containing 10 mL of distilled water. Inflorescences were held under natural light conditions at ambient temperature (25°C) and relative humidity (70 ± 2% RH) during the period of study.

3. Ethylene measurement

Detached flowers held in 10 ml vial containing 8 mL of distilled water were placed in an airtight plastic box and kept at 25 °C for 2 h. Gas samples (1 mL) were taken from the headspace and injected to gas chromatography (Shimadzu GC-8A, Kyoto, Japan) equipped with an alumina separating column and a flame ionization detector. The experiment was done in four replications.

4. Plant genomic DNA extraction

Orchid young leaves were ground into fine powder in liquid nitrogen. Four hundred microliters of TLES buffer (0.1M Tris-HCl, 0.1M LiCl, 0.01M EDTA, 1% SDS) containing 2% sodium sulfite and equal volume of phenol were added. The suspension was mixed by vortexing for 5 min. The content was separated by centrifugation (Eppendorf, Germany) at 12,000 rpm for 10 min. Upper phase was transferred to a new microcentrifuge tube and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and mixed by vortexing for 5 min. The two phases were separated by centrifugation at 12,000 rpm for 10 min at 4 °C and upper phase was transferred to a new microcentrifuge tube. An equal volume of cold isopropanol was added and mixed by inverting. The DNA was precipitated by centrifugation at 12,000 rpm for 15 min at 4 °C. The pellet was washed with 70% ethanol, dried under room temperature and resuspended in 100 µl of dH_2O containing 20 µg/µl of *RNase* A. The DNA was incubated for 30 min at 37 °C to digest the RNA and stored at -20 °C for long term or 4 °C for short term use. The concentration and purity of DNA was determined using spectrophotometer (Ultrospec® 500/1100) at 260 and 280 nm and agarose gel electrophoresis (Bio-Rad, U.S.A.).

5. Total RNA extraction

Orchid tissue was ground in liquid nitrogen to fine powder and extracted with TLES buffer (0.1 M Tris-HCl, 0.1 M LiCl, 0.01 M EDTA, 1% SDS) containing 2% sodium sulfite and equal volume of phenol and mixed by votexing 10 min. The content was separated by centrifugation for 15 min at 12,000 rpm. The supernatant was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The two phases were separated by centrifugation at 12,000 rpm for 10 min at 4 °C and upper phase was transferred to a new microcentrifuge tube containing an equal volume of 4 M LiCl and incubated at 4 °C overnight. The RNA was precipitated by centrifugation at 12,000 rpm for 20 min at 4 °C. The pellet was resuspended in *RNase*-free water and incubated at 37 °C for 30 min. The RNA was precipitated again with 0.1 volume of 2.5 M NaOAc (pH 5.8), 2.5 volumes of 100% EtOH and kept at -80 °C overnight. The RNA was precipitated by centrifugation at 12,000 rpm for 20 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 *RNase*-free water. The concentration and purity of total RNA were determined using 1% agarose gel electrophoresis and spectrophotometer (Ultrospec® 500/1100) at 260 and 280 nm.

6. Reverse-Transcription-polymerase chain reaction (RT-PCR) for *Den-ERS1* isolation

6.1 *DNase* I treatment

One microgram of total RNA was *DNase* treated by adding of 1 µl of 10X *DNase* buffer, 1 µl of *DNase* I (10 U/µl) (Fermentas) and *RNase*-free water to total volume of 10 µl. The reaction was incubated at 37 °C for 30 min. It was terminated by adding 1 µl of 0.1 M EDTA (pH 8.0) and incubated at 65 °C for 10 min. The *DNase* I treated RNA was used as template in RT reaction.

6.2 Reverse Transcription (RT)

One microliter of 30 pmol reverse specific primer (ERSG1-3'G1R) was added into the *DNase* treated RNA and brought into final volume of 11 µl with *RNase*-free water. The reaction was heated at 70 °C for 5 min and quenched on ice. Four microliters of 5X *M-MuLV* RT buffer (Fermentas), 2 µl of 10mM dNTPs, 0.5 µl of RNase inhibitor (40 U/µl) (Fermentas), and 1.5 µl of DEPC-treated water were added to the mix. It was incubated at 37 °C for 5 min and 1 µl of *M-MuLV* reverse transcriptase (200U/µl) (Fermentas) was added. The reaction was incubated at 42 °C for 1 h and then stopped by incubating at 70 °C for 10 min.

6.3 Reverse Transcription-Polymerase chain reaction (RT-PCR)

One microliter of cDNA from reverse transcription reaction was amplified by adding into PCR tube containing 30 pmol of primer each following: ERSG1-F2 (5' TGA(C/T)TGCATTGA(A/C)CCACAATG3'), ERSG1-F7 (AGGATGGCAGCTTCG AGTTAGAG3'), ERSG1-F5(r1) (5' ATCTGTGCTTGTACACTCGAA3'), ERSG1-R7 (5'ATCGCTTGGTATAGGGTGGAACT3'), 5 µl of 10X *Taq* buffer with $(NH_4)_2SO_4$, 4 µl of 25 mm $MgCl_2$, 1 µl of 10 mm dNTPs, 0.5 µl of *Taq* DNA polymerase (5U/µl) (Fermentas). The mix was adjusted to final volume of 50 µl. In addition, nested reverse primers were also used separately with primer ETR1-R3

(5’(A/C)CTG(A/G) (A/C)GG(A/G)ATCAAAACCAT3’) and ERSG1-3’G1R (5’ TTTTTCTAATCATTTTTTGGCTTAT3’) under identical condition. The PCR condition was as followed; 94 °C for 3 min, follow by 35 cycle of denaturing at 94 °C for 30 sec, annealing at 50 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. PCR assembly, and amplified by using ERSG1-ATG (5’ATGGAAGGCTGTGACTGCATT3’) and ERSG1-TGA (5’TTCGAGTGTACAA GCACAGAT3’),

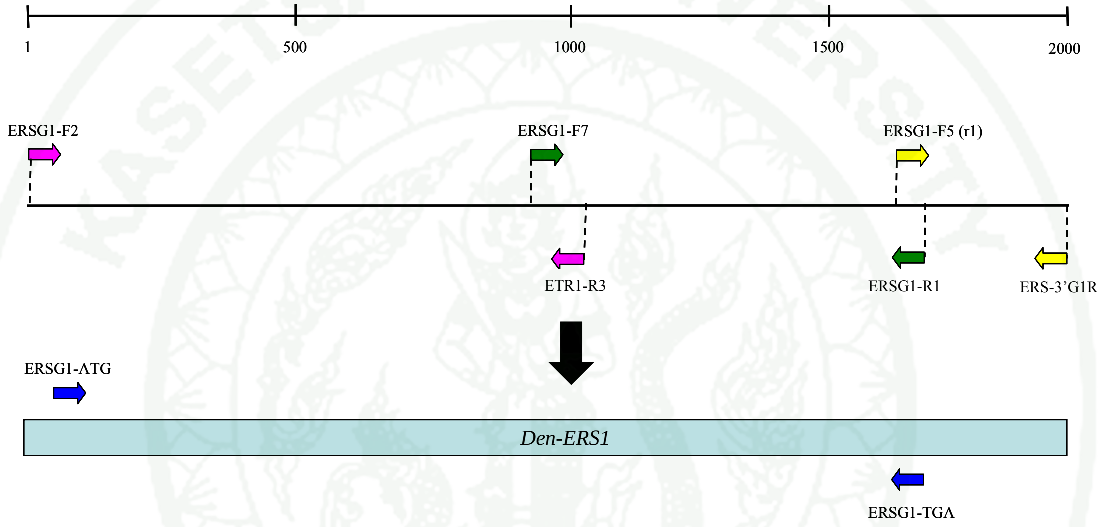


Figure 8 Represent diagram of primer pairs used for *Den-ERS1* isolation

7. *Den-ERS1* 5’Flanking Region Isolation

Den-ERS1 5’flanking region isolation was done using ligation mediated PCR with adapter primer (AP1 5’ TCCTAATACGACTCACTATAGGGC 3’) from Cottage, *et al.* (2001). This strategy was comprised of 3 steps 1) restriction digestion, 2) adaptor annealing and ligation and 3) PCR amplification. Reverse primers comprised of ERSG1-R9 (5’GCAGTCACAGCCTTCCAT3’), ERSG1-R10 (5’ ACCAAACTGTATAAGCAC3’), ERSG1-R11 (5’CAGATCAGGAATTATGTG3’) and ERSG1-R12 (5’ATTGCTAGCTACGTAACGTA3’) (Figure 9).

7.1 Restriction Digestion

Ten micrograms of genomic DNA were restriction digested overnight at 37 °C, with 50 units of *XbaI* (Fermentas) in a final volume of 100 µl. The enzymes were heat inactivated for 10 min at 65 °C. The digested DNA was precipitated by adding of 0.1 volume of 3 M sodium acetate (pH 5.4) and 2 volumes of absolute ethanol. The mixture was vortexed, and centrifuged at 12,000 rpm for 15 min. The pellet was washed with 70% ethanol, centrifuged at 12,000 rpm for 10 min, air dried and resuspended in 20 µl of dH_2O.

7.2 Adapter Annealing and Ligation

Oligonucleotides ADAPL (5’ CTAATACGACTCACTATAGGGCTCGA GCGGCCGCCCGGGCAGGT3’) and ADSPS (5’ P-ACCTGCCC-H_2N 3’) were used as adaptor following Cottage *et al*., (2001). The nucleotides were resuspended in dH_2O at a concentration of 100 pmol/ µl. Twenty microliters of each adaptor was pipetted into a 0.5 ml microcentrifuge tube and overlaid with mineral oil. The adaptors were heated for 2 min at 100 °C and the solution was allowed to cool for 1 h at RT. The annealed adaptors were decanted from under oil and stored at -20 °C. Ten microliters of the digested genomic restriction was ligated to 1 µl of the annealed adaptors with 2 µl of T4 DNA ligase (3 units/ml; Fermentas). The ligation was incubated overnight at 16 °C and heat inactivated for 10 min at 65 °C. This ligation mix was called the adaptor library.

7.3. PCR Amplification

Den-ERS1 5’flanking region was isolated from the adapter library by ligation-mediated amplification. The PCR reaction mixture consisted of 5 µl of adaptor library, 5 µl of 10X PCR buffer (10 mM Tris, pH 8.8, 50 mM KCl and 0.1% Triton X-100), 2 mM $MgCl_2$, 2 mM of dNTPs and 1U of *Taq* polymerase (Fermentas), 10 pmol/µl of the reverse gene specific primer and the adaptor primer AP1, was adjusted to a final volume of 50 µl with dH_2O. The cycle of the amplification was as followed; 94 °C 5 min 1 cycle and 94 °C 1 min, 55 °C 1 min and 72 °C 2 min for 35 cycles and followed by 1 cycle of 72 °C 10 min. The second PCR products were determined by agarose gel electrophoresis.

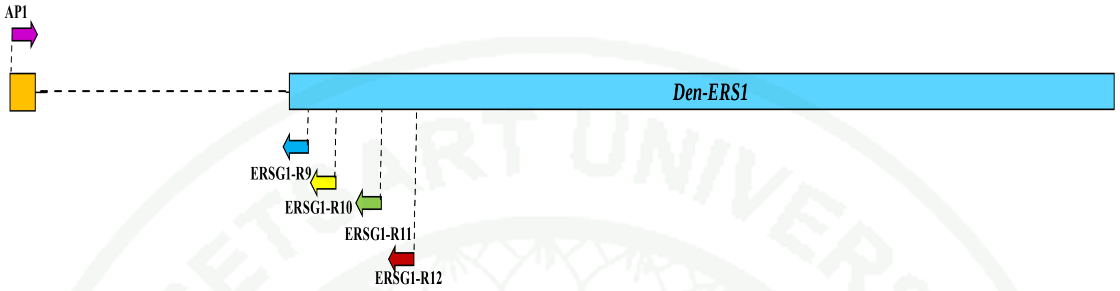


Figure 9 Represent diagram of primer pairs used for *Den-ERS1* 5'flanking region isolation

8. Intron amplification

One hundred nanograms of genomic DNA were amplified by PCR technique. Primers in this experiment were shown in table 1. The PCR reaction consisted of 5 µl of 10X *Taq* buffer with $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl 30 pmol of primers, 0.25 µl of *Taq* DNA polymerase (5U/µl) (RBC). The mix was adjusted to final volume of 50 µl. The PCR condition was as followed; 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

Table 1 List of primers using in PCR for *Den-ERS1* 5’flanking region isolation

Primer	Sequence (5’→ 3’)
Forward primer	
ERSG1-ATG	ATGGAAGGCTGTGACTGCATT
ERSG1-F3	ATTGTTCATGACGTACGTACGT
ERSG1-F7	GTTCGTAGATGCAGTTGGCCA
ERSG1-F4(r3)	CTCTTCACTAAATTTGCGCATACC
ERSG1-F5(r1)	ATCTGTGCTTGTACACTCGAA
Reverse primer	
ERSG1-R6	CGTTGACGTTGTGAGTCGTGAC
ERSG1-R7	ACGTGAGATGCGTAGTGATTGCA
ERSG1-R3	GGTTTGCGCAAATTTAGTGAAGAG
ERSG1-3’G1R	TTTTTCTAATCATTTTTTGGCTTAT

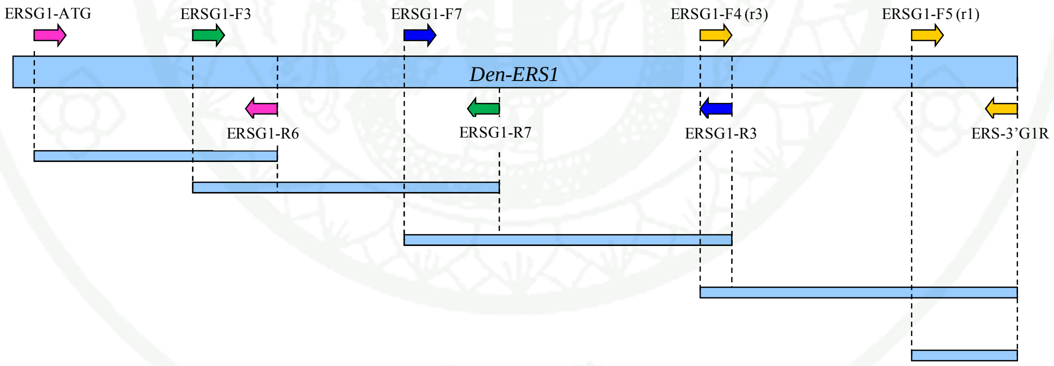


Figure 10 Represent diagram of primer pairs used for *Den-ERS1* intron isolation

9. DNA Cloning

The PCR product was directly ligated into pGEM-T easy vector (Promega) essentially as recommended by the manufacturer. The reaction mix contained 5µl of 2X rapid ligation buffer, 1 µl of pDrive vector (50 ng/µl), and 1-4 µl of PCR product.

The reaction mix was made up to 10 µl with dH_2O, spun down and incubated at 16 °C overnight. Ten microliters of the ligation mix was added to 100 µl of *Escherichia coli* strain DH5α competent cells and the mixture was incubated on ice for 30 min. The competent cells were then heat shocked for 90 sec at 42 °C and placed on ice for 2 min. The eight hundred microliters of 2xYT medium (16 g/l bacto- tryptone, 10 g/l bacto-yeast extract and 5g/l NaCl) was added into competent cells and incubated in a shaking incubator for 60 min at 37 °C. The cell suspension was plated out on 2XYT agar containing IPTG (100 mM), X-gal (20 mg/ml) and amplicilin (100 µg/ml) and incubated overnight at 37 °C. The recombinant clones were identified by blue/white colony selection.

10. Plasmid preparation by alkaline lysis

The recombinant clones were grown overnight in 3 ml of 2XYT containing 100 µg/ml of ampicillin at 37 °C and shaken at 250 rpm. Recombinant plasmids were purified by a modified method of Sambrook *et al*. (1989). One and a half milliliter of cell suspension was centrifuged at 8,000 rpm for 1 min and the supernatant was discarded. The pellet was resuspended in 100 µl of chilled solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) mixed by vortexing and incubated on ice for 5 min. Two hundred microliters of freshly prepared solution II (200 mN NaOH, 1% SDS) was added, and the content mixed by inverting. The solution was then neutralized by adding 150 µl of ice-cold solution III (3 M potassium acetate, 5 M glacial acetic acid), mixed by vortexing for 1 min and placed on ice for 5 min. An equal volume of chloroform was added. The mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. The upper phase was transferred into a new tube. The recombinant plasmids DNA was precipitated with 2.5 volumes of chilled absolute ethanol and centrifuged at 12,000 rpm for 10 min. The pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. The pellet were air dried, resuspended in 20 µl with dH_2O containing 10 µg/ml *RNase*A, incubated for 30 min at 37 °C and stored at -20 °C.

11. DNA sequencing

The purified recombinant plasmid DNA (100-500 ng) was prepared using an ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) as described by the manufacturer and automates sequenced using SP6 and T7 DNA polymerase as primers.

12. Sequence analysis

Sequences were checked for their authenticity and assembled using Seqman (Lasergene® version 7.2). The chomotographs fo each sequence were also used to confirm nucleotide sequence. The complete sequence was compared with other sequences in GenBank database using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Ethylene receptor sequences from Genbank database were retrieved and compared with *Den-ERS1* using MegAlign (Lasergene® version 7.2) and ClustalW (http://www.ebi.ac.uk /Tools/clustalw2/).

13. Southern blot analysis

Thirty micrograms of orchid genomic DNA were digested with 100 U of restriction endonuclease (*Eco*RV, *Sac*I and *Xho*I) at 37 °C in the total volume of 300 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 12,000 rpm for 15 min at 4 °C. The pellet was washed with 500 µl of 70% ethanol, air dried and resuspended in 25 µl of dH_2O. The digested DNA was separated on 1% agarose gel containing 1X TAE (0.8 mM Tris, 0.1 % glacial acetic acid (V/V), 0.04 mM EDTA, pH 8.0) at 60 V for 2 h. Plasmid containing an open reading frame (ORF) of *Den-ERS1* of ‘Pompadour’ with 1,924 bp in size was used as a positive control. The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min and neutralized by shaking the gel in 0.4 N NaOH for 15 min at room temperature. The DNA was transferred to positively charge nylon membrane (Roche). The membrane was prehybridized in 15 ml of standard hybridization buffer (5X SSC, 0.1% N-laurylsacosine, 0.02% SDS, 1% blocking reagent) at 65 °C for 1 h and hybridized overnight with 0.4 µg/ ml of *Den-ERS1* ‘Pompadour’ ethylene binding domain probe with 600 bp in size. After hybridization, the membrane was washed with 2XSSC/ 0.1% SDS for 5 min twice and 0.1XSSC/ 0.1% SDS at 65 °C for 15 min twice. The blocking buffer was added onto the membrane and shaken at room temperature for 45 min. Anti-DIG was added to the membrane (1 µl of anti-DIG: 20,000 µl of blocking buffer) and shaken for 30 min. The membrane was washed twice in washing buffer (1X maleic acid pH 7.5/ 0.3% (V/V) Tween 20) for 15 min. The membrane was transferred to the detection buffer (0.1M Tris-HCl pH 9.5/ 0.1M NaCl) and shaken gently for 5 min. Finally, CDP star solution (800 µl of detection buffer + 4 µl CDP star) was added and the membrane was exposed to X-ray film.

14. Northern blot analysis

Twenty five micrograms of total RNA were used for agarose/formaldehyde electrophoresis at 60 volt for 150 min. The 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 120 °C for 30 min. The membrane was prehybridized with 15 ml of high SDS concentration hybridization buffer (7% SDS , 50% formamide, 5XSSC, 0.1% N-lauroylsarcosine, 2% blocking solution, 50 mM sodium phosphate, pH 7.0) at 50 °C for 1 h and hybridized overnight with 0.4 ug/ml of specific probe in high SDS concentration hybridization buffer. After hybridization, the membrane was washed with 2XSSC/ 0.1% SDS for 5 min twice and 0.1XSSC/ 0.1% SDS at 65 °C for 15 min twice. The blocking buffer was added to the membrane and shaken at room temperature for 45 min, Anti-DIG was added to the membrane (1 µl of anti-DIG: 20,000 µl of blocking buffer) and shaken for 30 min. The membrane was washed twice in washing buffer (1X maleic acid pH 7.5/0.3% (V/V) Tween 20) for 15 min. The membrane was transferred to the detection buffer (0.1M Tris-HCl pH 9.5/ 0.1M NaCl) and shaken gently for 5 min. Finally, CDP star solution (800 µl of detection buffer + 4 µl CDP star) was added and the membrane was exposed to X-ray film.

RESULTS

1. Isolation of *Ethylene response sensor 1* from *Dendrobium* orchid (*Den-ERS1*)

1.1 Orchid Genomic DNA Extraction

Orchid genomic DNA was extracted from orchid young leaves. The quality and concentration of orchid genomic DNA was determined using 1 % agarose gel electrophoresis and spectrophotometer at 260 and 280 nm. Agarose gel electrophoresis showed orchid genomic DNA from 2 cultivars; ‘Khao Sanan’ and ‘Pompadour’ (Figure 11). Orchid DNA were used to amplify 5’ untranslated region (5' UTR) and Southern blot analysis.

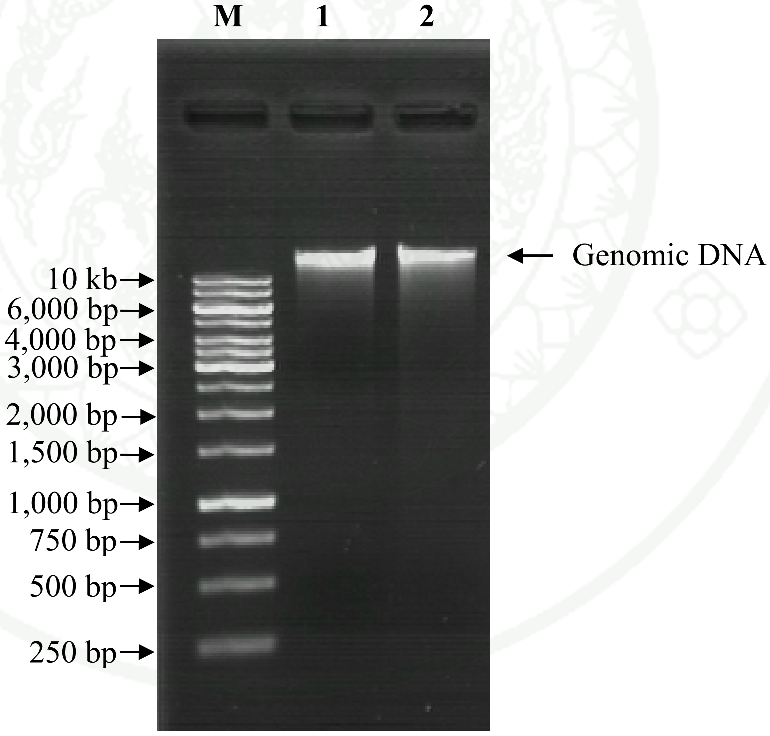


Figure 11 Agarose gel electrophoresis of orchid genomic DNA from ‘Khao Sanan’ (lane 1) and ‘Pompadour’ (lane 2) varieties. The electrophoresis was performed at 90 V for 1 h and agarose was stained for 15 min with ethidium bromide. 1 kb DNA Ladder (M) was shown on the left.

1.2 Orchid RNA extraction

Orchid RNA was extracted from orchid flower. The quality and concentration of orchid RNA was determined using 1 % agarose gel electrophoresis and spectrophotometer. Agarose gel electrophoresis showed orchid RNA from 2 cultivars; 'Khao Sanan' and 'Pompadour' (Figure 12). Orchid RNA were used to amplified open reading frame (ORF), 3' untranslated region (3' UTR) and Northern blot analysis.

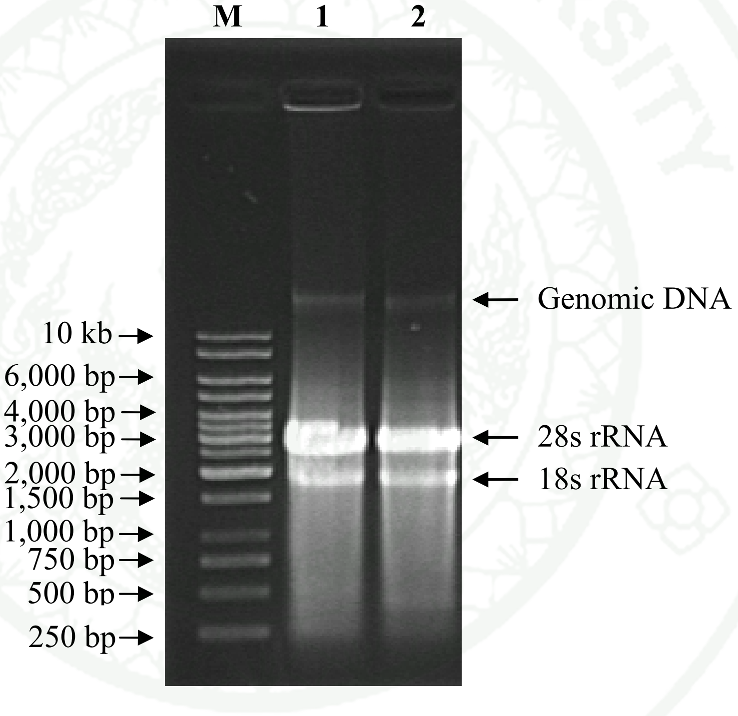


Figure 12 Agarose gel electrophoresis of orchid RNA from 'Khao Sanan' and 'Pompadour' varieties. The electrophoresis was performed at 90 V for 1 h and agarose was stained for 15 min with ethidium bromide. 1 kb DNA Ladder (M) was shown on the left.

1.3 Amplification of *Den-ERS1*

1.3.1 Amplification of *Den-ERS1* open reading frame (ORF)

Orchid RNA of ‘Khao Sanan’ and ‘Pompadour’ varieties was used to amplify *Den-ERS1* open reading frame (ORF) region using RT-PCR with specific primers (ERSG1-ATG and ERSG1-R1). A PCR product with 1924 bp in size was amplified and cloned into pGEM T-easy cloning vector (Promega) (Figure 13).

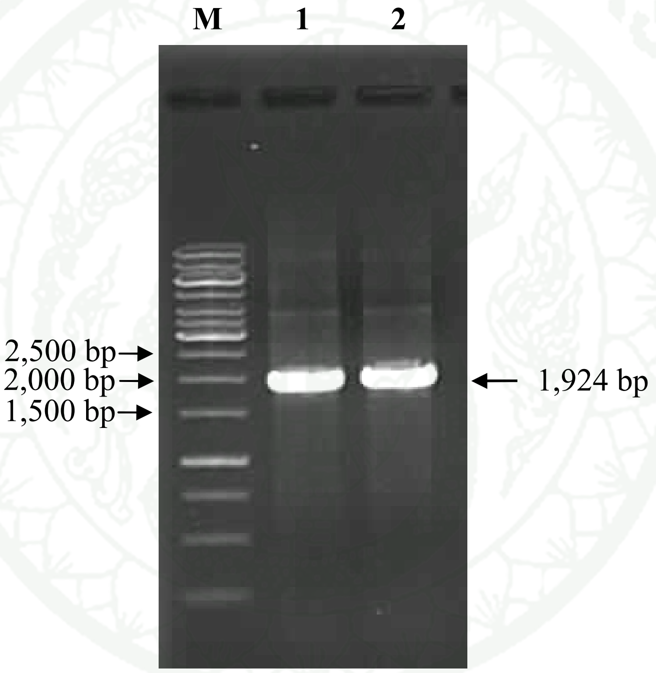


Figure 13 Agarose gel electrophoresis of PCR products of *Den-ERS1* open reading frame (ORF) of ‘Khao Sanan’ (lane 1) and ‘Pompadour’ varieties (lane 2). One kb ladder molecular weight marker (M) was shown on the left.

1.3.2 Amplification of *Den-ERS1* 3' untranslated region (3' UTR)

Den-ERS1 3' untranslated region (3' UTR) region was amplified using RT-PCR with specific primers (ERSG1-F4r3 and ERS 3’G1R). A PCR product with 639 bp in size was amplified and cloned into pGEM T-easy cloning vector (Promega) (Figure 14).

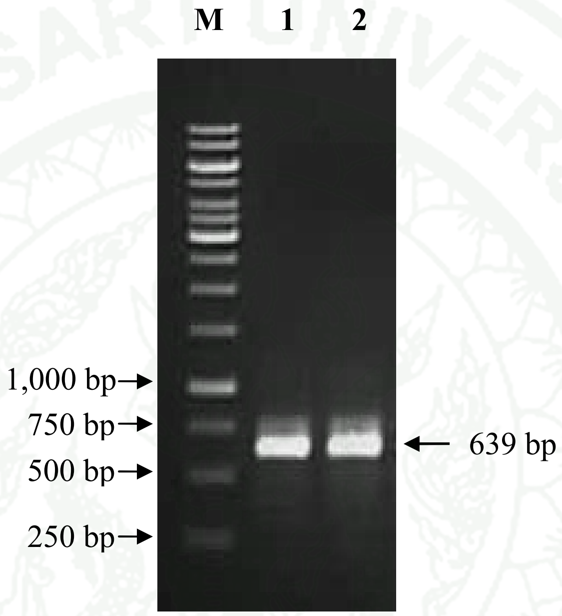


Figure 14 Agarose gel electrophoresis of PCR products of *Den-ERS1* 3’ untranslated region (3’UTR) of ‘Khao Sanan’ (lane 1) and ‘Pompadour’ (lane 2). One kb ladder molecular weight marker (M) was shown on the left.

1.3.3 Amplification of *Den-ERS1* 5' untranslated region (5' UTR) and putative promoter region

Orchid genomic DNA from *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’ were digested with a restriction enzyme *Xba*I. Digested genomic DNA was ligated into adapter ADAPL/ADSPL to create an adapter ligated library. The adaptor ligated library was amplified with adaptor forward primer AP1 and ERSG1-R9, R10, R11 and R12 reverse primers. The result was shown in Figure 15. All PCR products had expected size. The PCR product size 895 bp in ‘Khao Sanan’ and 890 bp in ‘Pompadour’ varieties of primer AP1 and ERSG1-R10 (Figure 15; lane 2 and 7) were selected and cloned in to pGEM T-easy (Promaga).

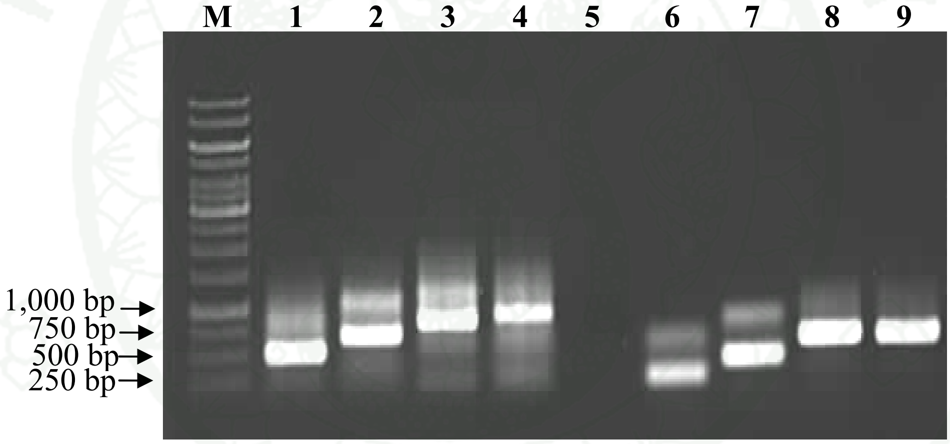


Figure 15 The ligation-mediated PCR of ‘Khao Sanan’ (lane 1-4) and ‘Pompadour’ (lane 6-9) genomic DNA digested with *Xba*I. The 5’UTR and promoter region were amplified with nested adaptor forward primer AP1 and *Den-ERS1* specific reverse primers: R9 (lane 1; 6), R10 (lane2; 7), R10 (lane 3; 8) and R12 (lane4; 9). Lane 5 is a blank lane. One kb ladder molecular weight marker (M) was shown on the left.

1.3.4 Amplification of *Den-ERS1* intron

The presence of intron in genomic DNA sequence of *Den-ERS1* was determined by genomic DNA amplification. Primers were designed to cover the entire gene and their locations were based on the location of intron reported in other plant ERS1 including rice (AY043031), Arabidopsis (U21952) and maize (AY359578). The locations of primers were shown in Figure 16. As a control, a plasmid (*Den-ERS1* ORF) containing entire *Den-ERF1* Open Reading Frame obtained from cDNA was also amplified. The results of amplification were shown in Figure 16a and b. All except a set of primers (ERSG1-F7 and ERSG1-R3) gave identical size of PCR product DNA fragment from both *Dendrobium* orchid DNA and plasmid control. Amplification with ERSG1-F7 and ERSG1-R3 primers gave a 700 bp PCR product from genomic DNA while 466 bp product was amplified from the plasmid control. In order to confirm the presence of intron, all PCR products from genomic DNA amplification were cloned into pGEM T-easy cloning vector and automate sequenced.

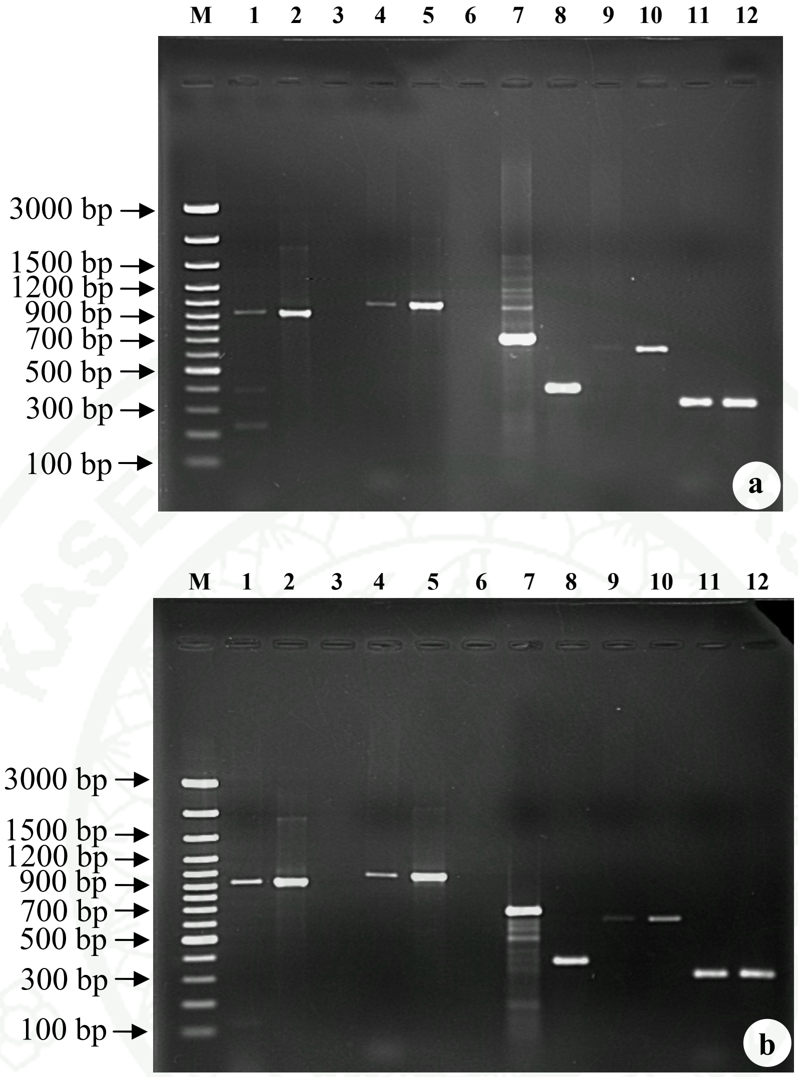


Figure 16 DNA amplification of intron from *Dendrobium* genomic DNA from 'Khao Sanan' (a) and 'Pompadour' (b). Lane 3 and 6 were blank lanes. Lane 1, 4, 7, 9 and 11 were amplified from *Dendrobium* genomic DNA. Lane 2, 5, 8, 10 and 12 were amplification of the plasmid control. Lane 1 and 2 using ERSG1-ATG and ERSG1-R6 primers, lane 4 and 5 using ERSG1-F3 and ERSG1-R7 primers, lane 7 and 8 using ERSG1-F7 and ERSG1-R3 primers, lane 9 and 10 using ERSG1-F4(R3) and ERSG1-3'G1R primers, lane 11 and 12 using F5(R1) and 3'G1R primers. The 100 bp molecular weight marker (M) was shown on the left.

2. Characterization of *Den-ERS1*

2.1 *Den-ERS1* cDNA sequences

Ethylene Response Sensor 1 (*Den-ERS1*), cDNA were completely cloned and sequenced from *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’ The complete *Den-ERS1* from ‘Khao Sanan’ cDNA sequence was 2,299 nucleotides in length and the open reading frame (ORF) encoded 621 amino acids (Figure 17). The complete *Den-ERS1* from ‘Pompadour’ cDNA sequence was 2,307 nucleotides in length and the open reading flame (ORF) encoded 622 amino acids (Figure 18) Both of Den-ERS1 putative protein had predicted molecular weight of 70 kDa.

The size of *Den-ERS1* 5’UTR from *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’ were 70 and 81 bp, respectively. 3’UTR were 359 and 360 bp, respectively. DNA sequence comparison of coding region, 5’UTR and 3’UTR were shown in Table 2. *Den-ERS1* coding region showed high similarity to *ERS1* gene from other plant species at 99.9-67.6%. *Den-ERS1* 5’UTR showed high similarity to *ERS1* gene from to *Phalaenopsis* cv. ‘True Lady’ at 93.8% and showed some similarity to 5’UTR of *ERS1* gene from other plant species at 79.7-24.2%. *Den-ERS1* 3’UTR showed some similarity of *ERS1* gene from other plant species at 57.0-68.3%.

Figure 17 Nucleotide sequence and the deduced amino acid sequence of putative ethylene responsive sensor 1 *Den-ERS1* from ‘Khao Sanan’ variety . Numbers on the right refer to nucleotide residues. The translation start site is underlined. The asterisk denotes the stop codon.

```
TTTCTTCTTT TGCTTTTATT TGTATAAAGG GATGTAGGAG GAAGATAGCA GGTACAGCAG TTCCTTAAAA  70

ATGGAAGGCT GTGACTGCAT TGAGCCACAA TGGCCTGCTG ATGAGCTTTT AGTGAAGTAT CAATATATCT  140
 M  E  G   C  D  C  I   E  P  Q   W  P  A   D  E  L   L  V  K  Y   Q  Y  I
CTGATTTCTT TATTGCCCTT GCCTACTTCT CCATTCCACT GGAGCTAATA ATTTATTTCG TGAAGAAGTC  210
S  D  F  F   I  A  L   A  Y  F   S  I  P  L   E  L  I   I  Y  F   V  K  K  S
TTCATTTTTC CCATATAGAT GGGTGCTTAT ACAGTTTGGT GCTTTCATTG TCCTTTGTGG AGCAACCCAC  280
  S  F  F   P  Y  R   W  V  L  I   Q  F  G   A  F  I   V  L  C   G  A  T  H
TTGATAAACC TGTGGACATT CACCATGCAC TCAAGGACAC TTGCTATAGT AATGACTGTA GCAAAAGTTT  350
 L  I  N   L  W  T  F   T  M  H   S  R  T   L  A  I   V  M  T  V   A  K  V
CAACTGCGGT TGTGTCATGT GCAACAGCCT TGATGCTTGT TCACATAATT CCTGATCTGT TAAGTGTAAA  420
S  T  A  V   V  S  C   A  T  A   L  M  L  V   H  I  I   P  D  L   L  S  V  K
AACAAGGGAG CTTTTTCTGA GGAACAAAGC TGAAGAACTT GATAGAGAGA TGGGTCTTAT ACGCACACAG  490
  T  R  E   L  F  L   R  N  K  A   E  E  L   D  R  E   M  G  L   I  R  T  Q
GAAGAAACAG GGAGGCATGT GAGGATGCTC ACTCATGAAA TTCGAAGTAC ACTTGACAGA CACACTATAC  560
 E  E  T   G  R  H  V   R  M  L   T  H  E   I  R  S   T  L  D  R   H  T  I
TAAAGACCAC TCTTGTTGAA CTTGGGAGGA CTCTAGATTT GGCAGAATGT GCTTTATGGA TGCCTTCACG  630
L  K  T  T   L  V  E   L  G  R   T  L  D  L   A  E  C   A  L  W   M  P  S  R
GACTGGGCTC AACCTTCAGC TTTCACATAC TCTAAGCAAC CAAATTCCTG TTGGATCTGT TGTTTCTACT  700
  T  G  L   N  L  Q   L  S  H  T   L  S  N   Q  I  P   V  G  S   V  V  S  T
AACCTTCCTA TTGTTAATCA AGTTTTTAAT AGCAGTCGTG CAGTTAGAAT TCCACATACA TGTCCGCTTG  770
 N  L  P   I  V  N  Q   V  F  N   S  S  R   A  V  R   I  P  H  T   C  P  L
CAAGGTTTCA ACATCAGACA GGAAGATATG TACCACCAGA GGTGGTTGCA GTCCGAGTGC CACTCTTACA  840
A  R  F  Q   H  Q  T   G  R  Y   V  P  P  E   V  V  A   V  R  V   P  L  L  H
TCTATCAAAT TTCCAAATAA ATGATTGGCC TGAGCTGTCT GCTAAAAGTT TTGCTGTGAT GGTCTTGATG  910
  L  S  N   F  Q  I   N  D  W  P   E  L  S   A  K  S   F  A  V   M  V  L  M
CTACCATCAG ATAGTGCAAG GAAATGGCAT GTTTATGAAC TAGAGCTTGT TGAGGTAGTT GCAGATCAGG  980
 L  P  S   D  S  A  R   K  W  H   V  Y  E   L  E  L   V  E  V  V   A  D  Q
TTGCAGTTGC TCTTTCACAT GCTGCCATCT TGGAGGAATC CATGCGGGCA CGAGATCTCC TCTTGGATCA  1050
V  A  V  A   L  S  H   A  A  I   L  E  E  S   M  R  A   R  D  L   L  L  D  Q
GAATGTTGCT TTAGATTTAG CACGACAGGA GGCAGAGATG GCCATTCGTG CACGCAATGA TTTTTTAGCT  1120
  N  V  A   L  D  L   A  R  Q  E   A  E  M   A  I  R   A  R  N   D  F  L  A
GTCATGAACC ATGAGATGCG GACTCCCATG CATGCAATCA TTGCCCTCTC CTCCCTGCTT CTTGAAACTG  1190
 V  M  N   H  E  M  R   T  P  M   H  A  I   I  A  L   S  S  L  L   L  E  T
AACTGACTCC AGAGCAACGT TTGATGGTAG AAACCATCTT AAAGAGTAGT AACTTGCTAG CAACCCTAAT  1260
E  L  T  P   E  Q  R   L  M  V   E  T  I  L   K  S  S   N  L  L   A  T  L  I
CAATGATGTT TTAGACCTTT CTAAGCTTGA GGATGGCAGC TTCGAGTTAG AGGCCACAGT TTTCAATCTT  1330
  N  D  V   L  D  L   S  K  L  E   D  G  S   F  E  L   E  A  T   V  F  N  L
CATACTGTCT TCAGAGAGGT CGTAAATTTG ATAAAGCCAA TAGCGGCTGT CAAAAAGTTG TCAGTGTTCG  1400
 H  T  V   F  R  E  V   V  N  L   I  K  P   I  A  A   V  K  K  L   S  V  F
TGTCTCTTTC TCCGGACTTG CCATCACTTG CCATTGGAGA TGAGAAACGG CTTATACAAA CTATGCTTAA  1470
V  S  L  S   P  D  L   P  S  L   A  I  G  D   E  K  R   L  I  Q   T  M  L  N
TGTTGTTGGC AATGCTGTTA AGTTTACAAA GGAGGGTAGT ATATCTATTA CTGCGACTAT TGCAAAATCC  1540
  V  V  G   N  A  V   K  F  T  K   E  G  S   I  S  I   T  A  T   I  A  K  S
GATTCCTTGA GAGATTCGCG AGACCCAGAG TTCCACCCTA TACCAAGCGA TGGGTATTTC TATTTACGAG  1610
 D  S  L   R  D  S  R   D  P  E   F  H  P   I  P  S   D  G  Y  F   Y  L  R
TACAGGTTAA AGACACCGGT TGCGGAATAA GTCCACTGGA GTTACCACGC CTCTTCACTA AATTTGCGCA  1680
V  Q  V  K   D  T  G   C  G  I   S  P  L  E   L  P  R   L  F  T   K  F  A  H
TACCCAGAAT GGTTCTTACA AAGGCTACAC GGGCTCTGGA CTTGGGCTTG CCATTTGCAA GAGATTTGTA  1750
  T  Q  N   G  S  Y   K  G  Y  T   G  S  G   L  G  L   A  I  C   K  R  F  V
AACCTCATGA AAGGACGCAT TTGGCTCGAA AGTGAAGGTA TTGGCAAAGG TTGCACCACC ATTTTCATTG  1820
 N  L  M   K  G  R  I   W  L  E   S  E  G   I  G  K   G  C  T  T   I  F  I
TGAAGCTGGG CATCAGTGAA GATCCCACTC TTCGGTATCA GCAAAAGTTA TTGCCCCCAA TCCCCAAGGA  1890
V  K  L  G   I  S  E   D  P  T   L  R  Y  Q   Q  K  L   L  P  P   I  P  K  D
TGAGAAGAAT TCGATCCCAT CGAAGATTCG GCATCAGAGA AGCTTGTAGA TTGAAGCCAA GGCGAGCATT  1960
  E  K  N   S  I  P   S  K  I  R   H  Q  R   S  L  *
TCGAAGCCGT TGGATCTGTG CTTGTACACT CGAAGCTAAG GTCAACGTGA AGTGCAAGTA AAAGAAGCCA  2030
TTTTCTATCA TTCCAGACTT CCAGATAGCA GAGCTTGAAC TGAAAACTGT AAGTGAAGTA TTACAAATTG  2100
GTTACTTATC CGTTACAATT CATTATGAGT TAGTACTAAT GAGAGAGTTA TCAAATCTGA AAGAGTGGTG  2170
CTCAGTATTC ACTTAGCTTA CATGGAGTTA TGTTGTCACT ACACAATTTA TATGATAATC TTTTATTTTC  2240
TAACGAGGAC AGGAGATTGA TCCTGCTGAT TAATAAGCCA AAAAATGATT AGAAAAAAA
```

Figure 18 Nucleotide sequence and the deduced amino acid sequence of putative ethylene responsive sensor 1 *Den-ERS1* from ‘Pompadour’ variety . Numbers on the right refer to nucleotide residues. The translation start site is underlined. The asterisk denotes the stop codon.

```
TCAGTGATAT ATTTCTTCTT TTGCTTTTAT TTGTATAAAG GGATGTAGGA GGAAGATAGC AGGTACAGCA  70

GTTCCTTAAA AATGGAAGGC TGTGACTGCA TTGAGCCACA ATGGCCTGCT GATGAGCTTT TAGTGAAGTA  140
             M  E  G   C  D  C   I  E  P  Q   W  P  A   D  E  L   L  V  K  Y
TCAATATATC TCTGATTTCT TTATTGCCCT TGCCTACTTC TCCATTCCAC TGGAGCTAAT TTATTTCGTG  210
  Q  Y  I   S  D  F   F  I  A  L   A  Y  F   S  I  P   L  E  L  I   Y  F  V
AAGAAGTCTT CATTTTTCCC ATATAGATGG GTGCTTATAC AGTTTGGTGC CTTCATTGTC CTTTGTGGAG  280
 K  K  S   S  F  F  P   Y  R  W   V  L  I   Q  F  G  A   F  I  V   L  C  G
CAACCCACTT GATAAACCTG TGGACATTTA CCATGCACTC AAGGACACTT GCTATAGTAA TGACTGTAGC  350
A  T  H  L   I  N  L   W  T  F   T  M  H  S   R  T  L   A  I  V   M  T  V  A
AAAAGTTTCA ACTGCGGTTG TGTCATGTGC AACAGCCTTG ATGCTTGTTC ACATAATTCC TGATCTGTTA  420
  K  V  S   T  A  V   V  S  C  A   T  A  L   M  L  V   H  I  I  P   D  L  L
AGTGTAAAAA CAAGGGAGCT TTTTCTGAGG AACAAAGCTG AAGAACTTGA TAGAGAGATG GGTCTTATAC  490
 S  V  K   T  R  E  L   F  L  R   N  K  A   E  E  L  D   R  E  M   G  L  I
GCACACAGGA AGAAACAGGG AGGCATGTGA GGATGCTCAC TCATGAAATT CGAAGTACAC TTGACAGACA  560
R  T  Q  E   E  T  G   R  H  V   R  M  L  T   H  E  I   R  S  T   L  D  R  H
CACTATACTA AAGACCACTC TTGTTGAACT TGGGAGGACT CTAGATTTGG CAGAATGTGC TTTATGGATG  630
  T  I  L   K  T  T   L  V  E  L   G  R  T   L  D  L   A  E  C  A   L  W  M
CCGTCACGGA CTGGGCTCGA CCTTCAACTT TCACATACTC TAAGCAACCA AATTCCTGTT GGAACTGTTG  700
 P  S  R   T  G  L  D   L  Q  L   S  H  T   L  S  N  Q   I  P  V   G  T  V
GTTCTACTAA CCTTCCTATT GTAAACCAAG TTTTTAATAG CAGTCGTGCA GTTAGAAATC CCCATACATG  770
G  S  T  N   L  P  I   V  N  Q   V  F  N  S   S  R  A   V  R  N   P  H  T  C
TCCGCTTGCA AGGTTCCAAC CTCAGACAGG AAGATATGTA CCACCAGAGG GGGTTGCAGG CCGAGTGCCA  840
  P  L  A   R  F  Q   P  Q  T  G   R  Y  V   P  P  E   G  V  A  G   R  V  P
CTCTTACATC TATCAAATTT CCAAATAAAT GATTGGCCTG AGCTGGCTGC TAAAAGTTTT GCTGTGATGG  910
 L  L  H   L  S  N  F   Q  I  N   D  W  P   E  L  A  A   K  S  F   A  V  M
TCTTGATGCT ACCATCATAT AGTGCAAGGA AATGGCATGT TTATGAACTA GAGCTTGTTG AGGTAGTTGC  980
V  L  M  L   P  S  Y   S  A  R   K  W  H  V   Y  E  L   E  L  V   E  V  V  A
AGATCAGGTT GCAGTTGCTC TTTCACATGC TGCCATCTTG GAGGAATCCA TGCGGGCACG AGATCTCCTC  1050
  D  Q  V   A  V  A   L  S  H  A   A  I  L   E  E  S   M  R  A  R   D  L  L
CTGGATCAGA ATGTTGCTTT AGATTTAGCA CGACAGGAGG CAGAGATGGC CATTCGTGCA CGCAATGATT  1120
 L  D  Q   N  V  A  L   D  L  A   R  Q  E   A  E  M  A   I  R  A   R  N  D
TTTTAGCTGT CATGAACCAT GAGATGCGGA CTCCCATGCA TGCAATCATT GCCCTCTCCT CCCTGCTTCT  1190
F  L  A  V   M  N  H   E  M  R   T  P  M  H   A  I  I   A  L  S   S  L  L  L
TGAAACTGAA CTGACTCCAG AGCAACGTTT GATGGTAGAA ACCATCTTAA AGAATAGTAA CTTGCTAGCA  1260
  E  T  E   L  T  P   E  Q  R  L   M  V  E   T  I  L   K  N  S  N   L  L  A
ACCCTAATCA ATGATGTTTT AGACCTTTCT AAGCTTGAGG ATGGCAGCTT CGAGTTAGAG CCACAGTTTT  1330
 T  L  I   N  D  V  L   D  L  S   K  L  E   D  G  S  F   E  L  E   P  Q  F
CAATCTTTCA TACTGTCTTC AGAGAGGTCG TAAATTTGAT AAAGCCAATA GCGGCTGTCA AAAAGTTGTC  1400
S  I  F  H   T  V  F   R  E  V   V  N  L  I   K  P  I   A  A  V   K  K  L  S
AGTGTTCGTG TCTCTTTCTC CGGACTTGCC ATCACTTGCC ATTGGAGATG AGAAACGGCT TATACAAACT  1470
  V  F  V   S  L  S   P  D  L  P   S  L  A   I  G  D   E  K  R  L   I  Q  T
ATGCTTAATG TTGTTGGCAA TGCTGTTAAG TTTACAAAGG AGGGTAGTAT ATCTATTACT GCGACTATTG  1540
 M  L  N   V  V  G  N   A  V  K   F  T  K   E  G  S  I   S  I  T   A  T  I
CAAAATCCGA TTCCTTGAGA GATTCGCGAG ACCCAGAGTT CCACCCTATA CCAAGCGATG GGTATTTCTA  1610
A  K  S  D   S  L  R   D  S  R   D  P  E  F   H  P  I   P  S  D   G  Y  F  Y
TTTACGAGTA CAGGTTAAAG ACACCGGTTG CGGAATAAGT CCACTGGAGT TACCACGCCT CTTCACTAAA  1680
  L  R  V   Q  V  K   D  T  G  C   G  I  S   P  L  E   L  P  R  L   F  T  K
TTTGCGCATA CCCAGAATGG TTCTAACAAA GGCTACACGG GCTCTGGACT TGGGCTTGCC ATTTGCAAGA  1750
 F  A  H   T  Q  N  G   S  N  K   G  Y  T   G  S  G  L   G  L  A   I  C  K
GATTTGTAAA CCTCATGAAA GGACGCATTT GGCTTGAAAG TGAAGGTATT GGCAAAGGTT GCACCACCAT  1820
R  F  V  N   L  M  K   G  R  I   W  L  E  S   E  G  I   G  K  G   C  T  T  I
TTTCATTGTG AAGCTGGGCA TCAGTGAAGA TCCCACTCTT CGGTATCAGC AAAAGTTATT GCCCCCAATC  1890
  F  I  V   K  L  G   I  S  E  D   P  T  L   R  Y  Q   Q  K  L  L   P  P  I
CCCAAGGATG AGAAGAATTC GATCCCATCG AAGATTCGGC ATCAGAGAAG CTTGTAGATT GAAGCCAAGG  1960
 P  K  D   E  K  N  S   I  P  S   K  I  R   H  Q  R  S   L  *
CGAGCATTTC GAAGCCGTTG GATCTGTGCT TGTACACTCG AAGCTAAGGTC AACGTGAAGT GCAAGTAAA  2030
AGAAGCCATT TTCTATCATT CCAGACTTCC AGATAGCAGA GCTTGAACTGA AAACTGTAAG TGAAGTATT  2100
ACAAATTGGT TACTTATCCG TTACAATTCA TTATGAGTTA GTACTAATGAG AGAGTTATCA AATCTGAAA  2170
GAGTGGTGTT CAGTATTCAC TTAGCTTACA TGGAGTTATG TTGTCACTACA CAATTTATAT GATAATCTT  2240
TTATTTTCTA ACGAGGACAG GAGATTGATC CTGCTGATTA ATAAGCCAAAA AATGATTAGA AAAAAA     2307
```

Table 2 Percent identity of nucleotide sequence ethylene response sensor 1 (*Den-ERS1*) from 'Khao Sanan' (ks) and 'Pompadour' (mp) variety comparing with ERS1 genes from other plant species. Clustal W ananlysis was done using the MegAlign program (Lasergene® version 7.2)

Gene	Accession number	Source	(% identity)		
			Coding region	Non-coding region	
				5' UTR	3' UTR
Den-ERS1(ks)	FJ628419	*Dendrobium* 'Khao Sanan'	100	100	100
Den-ERS1	AY746972	*Dendrobium* 'Sonia Bom17'	99.9	73.9	98.9
Den-ERS1(mp)	FJ628420	*Dendrobium* 'Pompadour'	98.9	84.3	99.7
Ph-ERS1	AF113541	*Phalaenopsis* cv. 'KC butterfly'	91.6	79.7	78.9
OgERS1	AF276233	*Oncidium* cv. 'Gower Ramsey'	91.5	67.1	77.3
Ph-ETR1	AF055894	*Phalaenopsis* cv. 'True Lady'	91.4	93.8	78.3
Ph-ERS	AJ563284	*Phalaenopsis equestris*	91.4	-	-
Gl-ERS1	AB180247	*Gladiolus* hybrid cultivar 'Traveler'	79.1	-	-
MaERS1	AB266315	*Musa acuminata*	78.5	24.2	29.7
Dl-ERS1-1	AB055429	*Delphinium* 'MagicFountains dark blue'	74.2	30.3	47.1
Dl-ERS1-3	AB201245	*Delphinium* x *belladonna*	73.8	30.3	46.4
OsERS	AF013979	*Oryza sativa* Indica Group	73.6	38.6	37.3
SaERS	EU851574	*Saccharum officinarum*	73.3	ND	ND
Zm-ERS1	AY359578	*Zea mays*	73.1	ND	ND
AcERS1	EU170627	*Actinidia deliciosa*	72.9	29.2	41.1
Li-ERS1	DQ408428	*Lilium formosanum* x *Lilium longiflorum*	72.7	31.7	34.0
Pa-ERS1	AB015497	*Passiflora edulis*	71.7	37.9	40.2
Vi-ERS1	AF098272	*Vigna radiata*	71.2	30.0	37.6
PyERS1	AF386515	*Pyrus pyrifolia*	70.4	28.6	38
Pe-ERS1	DQ154118	*Vigna radiata*	68.9	36.2	28.8
AtERS1	NM_129658	*Arabidopsis thaliana*	68.0	44.3	32.1
Ch-ERS1	AF547624	*Chrysanthemum* x *morifolium*	67.6	39.3	32.6

2.2 Non-coding regions of *Den-ERS1*

Non-coding regions including putative promoter region, 5’ untranslated region (5’UTR) and introns were isolated and characterized from genomic DNA of *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’. Putative promoter sequences of *Den-ERS1* from both ‘Khao Sanan’ and ‘Pompadour’ varieties were 325 and 323 nucleotides in length, respectively (Figure 19a and b).

The sizes of *Den-ERS1* intron I and II from both ‘Khao Sanan’ and ‘Pompadour’ varieties were 131 and 181 bp respectively. Splice sites of intron 5’ **GU**…………**AG** 3’ were found in both introns (Figure 19a and b).

-325 **TCGAG CGGCCGCCCG GGCAGGTCTA GGGAACCTTC CCCCGAACAA**

-280 **TTTTAATAAG GCTAATTTGC TGATAGTCAT CACTTTTGTC TATCACATTT TCATTCTGTT CCTTGGCAAA**

-210 **AATTAACATA GCTTAGATAA TTTACTTAGC AACATTTTGA GGGTTTTTGC GTTGTAATTT AGTTATTTAT**

-140 **CCAACTTTCA TGTAATTTTC CATGCTTGAG CCTACATCTT CTGTTGTAAT GTGTAGTTTG TTGATTGTTA**

-70 **TTTCAAAATT ATTTTCCTTG TGAGGATGAT GAAATGCACT ATCTTCTTTG CTTCTCCTTT CAGCGATTTA**

+1 *TTTCTTCTTT TGCTTTTATT TGTATAAAGG GATGTAGGAG GAAGATAGCA GGTACAGCAG TTCCTTAAAA*

(a)

-323 **TCG AGCGGCCGCC CGGGCAGGTC TAGAGAACCT TCCCCCGAAC**

-280 **AATTTTAATA ATGCCAATTT GCTGATAGTC ATCACTTTTG TCTATCACAT TTTCATTCTG TTCCTTGGCA**

-210 **AAAATTAACA TAGCTTAGAT AATTTACTTA GCAACATTTG AGGGTTTTTG CGTTGTAATT TAGTTGTTTA**

-140 **TCCAACTTTC ATGTAATTTT CCATGCTTGA GGCTACATCT TCTGTTGTAA TGTGTAGTTT GTTGATTGTT**

-70 **ATTTCAAAAT TATGGTCCTT GTGAGGATGA TGAAATGCAC TATTTCTTTG CTTCTCCTTT CAGTGATATA**

+1 *TCAGTGATAT ATTTCTTCTT TTGCTTTTAT TTGTATAAAG GGATGTAGGA GGAAGATAGC AGGTACAGCA*

(b)

Figure 19 Nucleotide sequence of orchid *Den-ERS1* 5’ flanking region (bold) and 5’ untranslated region (Italic). The flanking region were -325 bp in ‘Khao Sanan’ (a) -323 bp in ‘Pompadour’ (b).

1349

|

.... ACAGTTTTCA ATCTTCATAC TGTCTTCAGA GAG**GTAGATG TGTTATACTT**

TTTTCATACT GTCTTCAGCC ACAGTTATGT TTATACTTAT ATTTTTTCTT TTGGCAAATT TAATTTTCTA

1477

|

CAAGTTTTGG TTAGCTGATT ATTTTTTTAT GTTATAACAT AGGTCGTAAA TTTGATAAAG CCAATAGCGG

CTGTCAAAAA GTTGTCAGTG TTCGTGTCTC TTTCTCCGGA CTTGCCATCA CTTGCCATTG GAGATGAGAA

ACGGCTTATA CAAACTATGC TTAATGTTGT TGGCAATGCT GTTAAGTTTA CAAAGGAGGG TAGTATATCT

ATTACTGCGA CTATTGCAAA ATCCGATTCC TTGAGAGATT CGCGAGACCC AGAGTTCCAC CCTATACCAA

1745

|

GCGATGGGTA TTTCTATTTA CGAGTACAG**G TAACTTGATG AACCGGAACT GTATTTAGAA CTCTAATAGT**

TCTTCAATTA TTCTTAGCAG ATTTAAGCGA GGACTTAAAA ATTGATTTGA CATGTTTAGA GCATATGGAA

1925

|

ATTTCCTGAT TTGAAGGTTC TTTTACAAAT TTGTGTAGAA TAACTATATA TACTTCTTTT TTTGGTACAG

GTTAAAGACA CCGGTTGCGG AATAAGTCCA ....

(a)

1357

|

.... AGTTTTCAAT CTTTCATACT GTCTTCAGAG AG**GTAGATGT GTTTATACTT**

TTTTCATACT GTCTTCAGCC ACAGTTATGT TTATACTTAT ATTTTTTTCC TTTGGCAAAT CTAATTTTCT

1487

|

ACAAGTTTTG GTTAGCTGAT TATTTTTTTA TGTTATAATA TAGGTCGTAA ATTTGATAAA GCCAATAGCG

GCTGTCAAAA AGTTGTCAGT GTTCGTGTCT CTTTCTCCGG ACTTGCCATC ACTTGCCATT GGAGATGAGA

AACGGCTTAT ACAAACTATG CTTAATGTTG TTGGCAATGC TGTTAAGTTT ACAAAGGAGG GTAGTATATC

TATTACTGCG ACTATTGCAA AATCCGATTC CTTGAGAGAT TCGCGAGACC CAGAGTTCCA CCCTATACCA

1755

|

AGCGATGGGT ATTTCTATTT ACGAGTACAG **GTAACTTGAT GACCCGGAAC TGTATTTACA ACTCTGATAG**

TTCTTCAATT ATTCTTAGCA GATTTAAGCG AGGACTTAAA AATTGATTTG ACATGTTTAG AGCATATGGA

AATTTCCTGA TTTGAAGGTT CTTTTACAAA TTTGTGTAGA GTAACTATAT ATACTTCTTT TTTTGGTACA

1935

|

GGTTAAAGAC ACCGGTTGCG GAATAAGTCC ....

(b)

Figure 20 Nucleotide sequence of orchid *Den-ERS1* intron I and II (bold). The length of intron I and intron II were 131 bp and 181 bp, in both of 'Khao Sanan' (a) and 'Pompadour' (b).

2.3 Genome organization of *Den-ERS1*

Genome organization of *Den-ERS1* consists of 3 exons (exon I, II and III) and 2 introns (intron I and II). The sizes of exon I, II and III from ‘Khao Sanan’ are 1,273, 267 and 324 bp, respectively. The sizes of exon I, II and III from ‘Pompadour’ are 1,276, 267 and 324 bp, respectively. The sizes of intron I from ‘Khao Sanan’ and ‘Pompadour’ II are 129 and 131 respectively, Introns II are identical, 181 bp. (Figure 20a and b). The *Den-ERS1* genome organization was compared with those of *Oryza sativa* Indica ERS1 (*OsERS*, accession number AY043031) and Arabidopsis ERS1 (*AtERS1*, accession number U21952) (Figure 21). *Den-ERS1* lacks the first intron presented in both sequences. The size of exon I from *Den-ERS1* is equivalent to combine size of exon I and II in both *OsERS* and *AtERS1*. The size of *Den-ERS1* exon II is equivalent to those of exon III from *OsERS* and *AtERS1*. Approximately half of *Den-ERS1* exon III (exonIIIa) is equivalent to exon IV of *OsERS* and *AtERS1*. The size of *Den-ERS1* exon IIIb was 197 bp comparing with 240 and 171 bp of *OsERS* exon V and *AtERS1* exon V.

The size of *Den-ERS1* intron I and II were 131 and 181 bp respectively. There is no comparable size of any intron of *OsERS* and *AtERS1* to those of *Den-ERS1* introns.

DNA sequence comparison of exons and introns was shown in Table 3 and 4. *Den-ERS1* exon I showed high similarity to exon I and II of *OsERS* and *AtERS1* at 70.5-77.0%. *Den-ERS1* exon II showed some similarity to exon III of *OsERS* and *AtERS1* at 64.4-68.0%. *Den-ERS1* exon IIIa showed some similarity to exon IV of *OsERS* and *AtERS1* at 57.0-68.3%. *Den-ERS1* exon IIIb showed some similarity to exon V of *OsERS* and *AtERS1* at 56.6-58.6% (Table 4).

DNA sequence comparison of *Den-ERS1* intron I with *OsERS* intron II and *AtERS1* intron II showed some similarity at 38.7-51.8%. *Den-ERS1* intron II showed some similarity at 31.2-50.7% (Table 3).

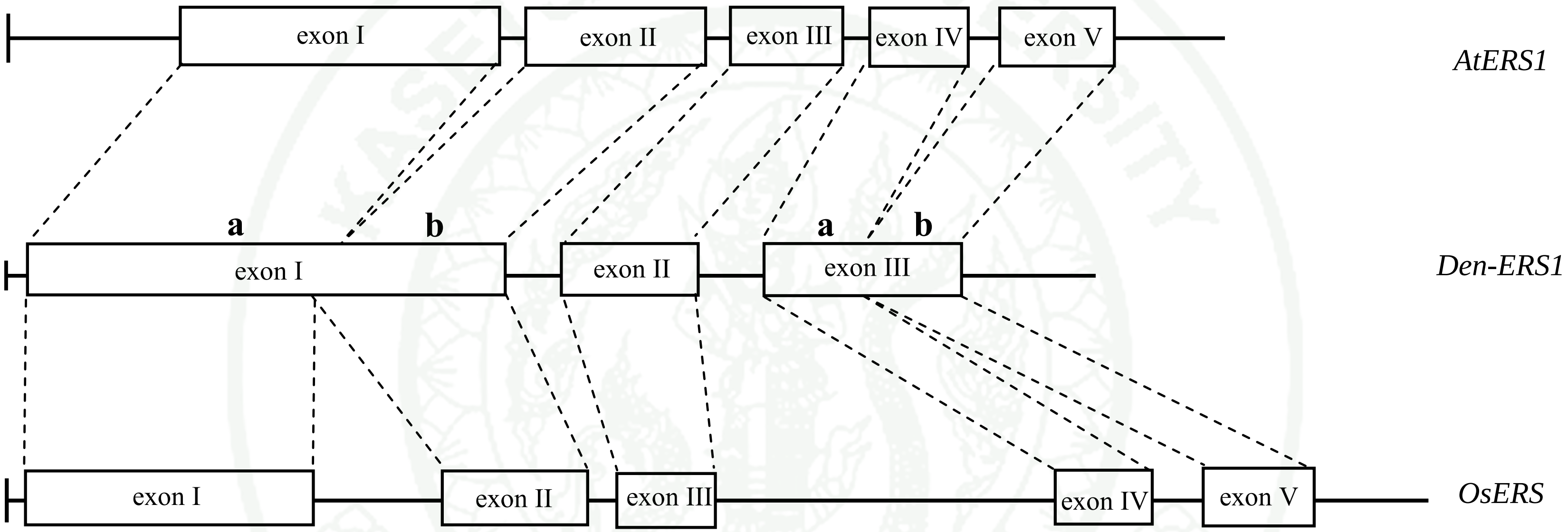


Figure 21 Schematic comparison of *Dendrobium* ethylene responsive sensor 1 (*Den-ERS1*) genome organization. The species and corresponding accession numbers are as follows; *Dendrobium* 'Pompadour' (*Den-ERS1*, FJ628420), *Oryza sativa* Indica Group (*OsERS*, AY043031) and *Arabidopsis thaliana* (*AtERS1*, U21952)

Table 3 Percent identity of *Den-ERS1* ‘Khao Sanan’ (Ks) and ‘Pompadour’ (Mp) intron I and II compared with intron II and III of Arabidopsis *ERS1* (*AtERS1*, accession number U21952) and *Oryza sativa* Indica *ERS1* (*OsERS*, accession number AY043031).

	Percent identity			
	AtERS1 Intron II	*OsERS* Intron II	*AtERS1* Intron III	*OsERS* Intron III
Ks-intron I	25.0	33.0	-	-
Mp-intron I	25.0	31.8	-	-
Ks-intron II	-	-	26.9	29.3
Mp-intron II	-	-	28.2	29.3

Table 4 Percent identity of *Den-ERS1* ‘Khao Sanan’ (Ks) and ‘Pompadour’ (Mp) exon compared with of Arabidopsis *ERS1* (*AtERS1*, accession number U21952) and *Oryza sativa* Indica *ERS1* (*OsERS*, accession number AY043031).

	Percent identity							
	AtERS1 Exon I+II	*OsERS* Exon I+II	*AtERS1* Exon III	*OsERS* Exon III	*AtERS1* Exon IV	*OsERS* Exon IV	*AtERS1* Exon V	*OsERS* Exon V
Ks-exon I	66.3	74.7	-	-	-	-	-	-
Mp-exon I	65.0	73.5	-	-	-	-	-	-
Ks-exon II	-	-	58.4	63.7	-	-	-	-
Mp-exon II	-	-	58.1	63.3	-	-	-	-
Ks-exon IIIa	-	-	-	-	50.8	64.1	-	-
Mp-exon IIIa	-	-	-	-	50.8	64.1	-	-
Ks-exon IIIb	-	-	-	-	-	-	39.0	43.9
Mp-exon IIIb	-	-	-	-	-	-	39.0	43.7

2.4 Cis-acting element analysis

The presence of cis acting elements within putative promoter region, 5' untranslated regions (5'UTR) and introns was determined using bioinformatics approach. Public databases including PLACE and PlantCARE were used and the results were shown in Table 5, 6 and 7. Core promoters, TATA and CAT box were found at position -57 and -74 from transcription start site.

Within putative promoter region (Table 5), important elements including transcription activation (MYBST1; GGATA), hormone response elements; ethylene (ERE; ATTCAAA), gibberrellin (WRKY71OS; TGAC and TATCCAOSAMY; TATCCA) and jasmonate (GCCcore; GCCGCC) were found.

In intron I (Table 6), transcriptional activator elements with abscisic acid signaling MYB1ST (GGATA) were found, MYB2CONSENSUSAT (TAACGG) and MYBATRD22 (CTAACCA). The PYRIMIDINEBOXHVEPB1 (TTTTTTCC) is cis- and trans- acting element regulated by abscisic acid and gibberrellin.

CAREOSREP1 (CAACTC) and WRKY71OS (TGAC) (+11 and +90) were found in intron II (Table 7) involving in gibberellin regulatory element. Other elements found including CATATGGMSAUR (CATAGE), WBOXATNPR1 (TTGAC) and WUN (AAATTTCCT) were auxin response, salicylic regulatory element and wound-responsive element, respectively

Table 5 C*is*-acting elements found once in *Den-ERS1* 5'flanking region * Location from +1 transcription start site, (+) sense strand, (-) = antisense strand

Factors or site names	Sequences	Frequency /location*	Description		
			Gene	**Plant species**	**Function**
TATA-box (Tjaden *et al.*, 1995)	TTATTT	1/+57	Glutamine synthetase gene (GS)	Pea (*Pisum sativum*)	core promoter element around -30 of transcription start
CAATBOX1 (Shirsat *et al.*, 1989)	CAAT	1/+74	Legumin A-type gene (*legA*)	Pea (*Pisum sativum*)	promoter consensus sequence
ERELEE4 (Itzhaki *et al.*, 1994)	ATTTCAAA	1/+64	glutathione-S-transferase (*GST1*) gene	Tomato (*Lycopersicon esculentum*)	ethylene responsive element
WRKY71OS (Zhang *et al.*, 2004)	TGAC	1/+252	Amylase gene (*Amy32b*)	rice (*Oryza sativa*)	gibberrellin-responsive element
TATCCAOSAMY (Chen *et al.*, 2006)	TATCCA	1/+148	Alpha-amylase gene (*Amy*)	rice (*Oryza sativa*)	gibberrellin-responsive element
GCCCORE (Brown *et al.*, 2003)	GCCGCC	1/+301	Plant defensin gene (*PDF1.2*), thionin gene (*Thi2.1*) and pathogenesis-related type4 (*PR4*)	*Arabidopsis thaliana*	jasmonate-responsive element

Table 6 C*is*-acting elements found once in *Den-ERS1* intron I. * Location from +1 transcription start site, (+) = sense strand, (-) antisense strand

Factors or site names	Sequences	Frequency /location*	Description		
			Gene	**Plant species**	**Function**
MYB1AT (Abe *et al*., 2003)	WAACCA	1/-99	Dehydration-resposive gene (*rd22*)	*Arabidopsis thaliana*	abscisic acid signaling transcriptional activators
MYB2CONSENSUSAT (Abe *et al*., 2003)	YAACKG	1/-42	Dehydration-resposive protein binds (*rd22BP1*)	*Arabidopsis thaliana*	abscisic acid signaling transcriptional activators
MYBATRD22 (Abe *et al*., 1997)	CTAACCA	1/-99	Dehydration-resposive gene (*rd22*)	*Arabidopsis thaliana*	drought and abscisic acid regulated gene
PYRIMIDINEBOXHVEPB1 (Cercos *et al*., 1999)	TTTTTTC	1/+63	Cysteine proteinase gene, (*EPB-1*)	Barley (*Hordeum vulgare*)	cis- and trans- acting element regulated by abscisic acid and gibberrellins

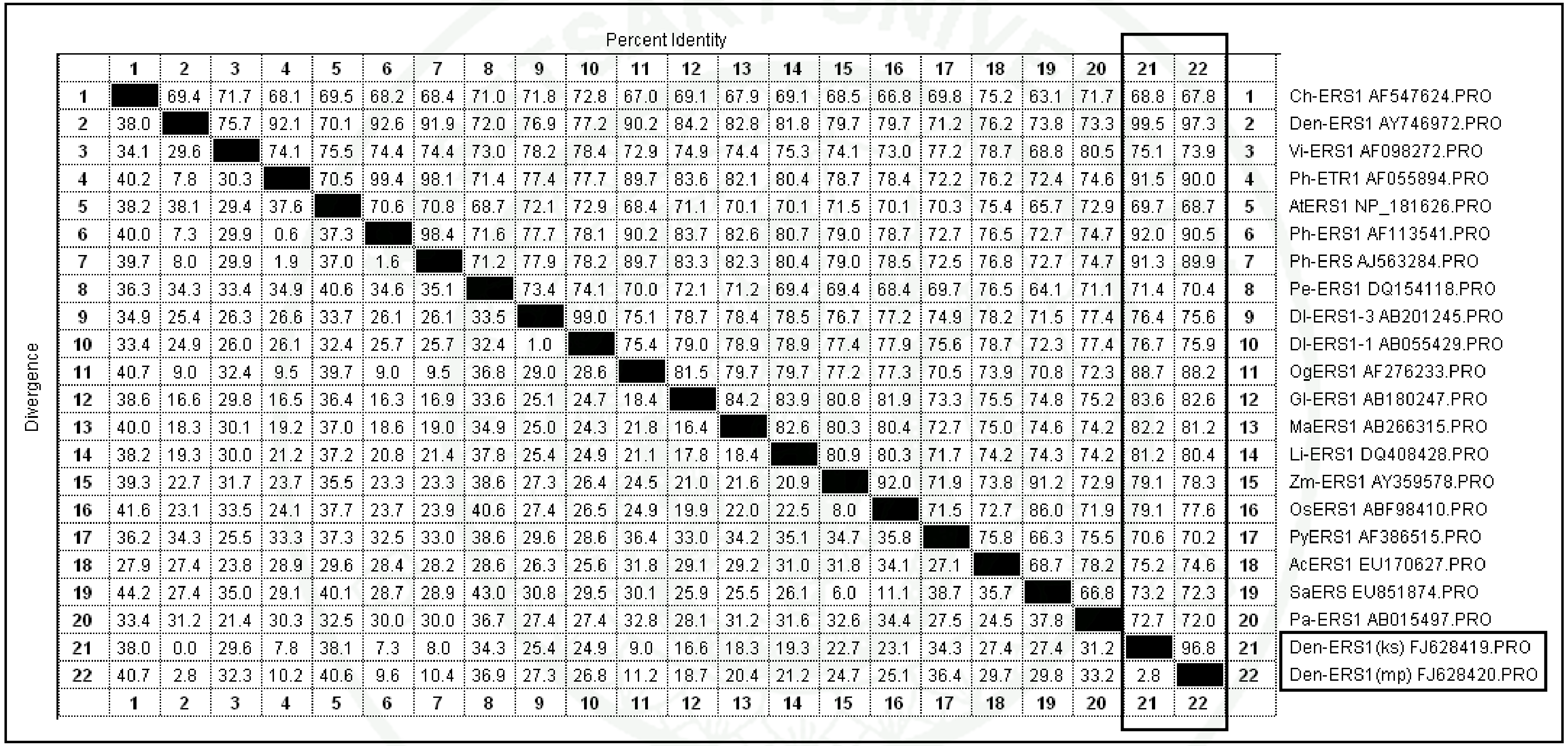
Table 7 *Cis*-acting elements found once *Den-ERS1* intron II. * Location from +1 transcription start site, (+) = sense strand, (-) = antisense strand

Factors or site names	Sequences	Frequency /location*	Description		
			Gene	**Plant species**	**Function**
WUN (Pastuglia *et al.*, 1997)	AAATTTCCT	1/+110	*S* gene receptor family	*Brassica oleracea*	wound-responsive element
CATATGGMSAUR (Xu *et al.*, 1997)	CATAGE	1/+103	SAUR (Small Auxin-Up RNA) 15A gene promoter	Soybean (*Glycine max*)	auxin response
CPBCSPOR (Fusada *et al*., 2005)	TATTAG	1/-34	NADPH-protochlorophyllide reductase gene	Cucumber (*Cucumis sativus* L.)	Cytokinin-enhanced protein binding
WBOXATNPR1 (Chen *et al*., 2002)	TTGAC	1/+88	Nitrogen permease reactivator gene (*NPR1*)	*Arabidopsis thaliana*	activated by WRKY18 which is a transcriptional repressor of the salicylic acid, response to environmental stress and plant defense reponse
WRKY71OS (Zhang *et al*., 2004)	TGAC	1/+89	Amylase gene (*Amy32b*)	rice (*Oryza sativa*)	gibberrellin-responsive element

3. Den-ERS1 putative protein

3.1 Den-ERS1 amino acid sequence alignment

The amino acid alignment between Den-ERS1 from ‘Khao Sanan’ accession number FJ628420 (GenBank) and Den-ERS1 from ‘Pompadour’ accession number FJ628419 (GenBank) indicated that both ethylene receptors shared high amino acid sequence identity at 96.8%. Amino acid sequences of Den-ERS1 from ‘Khao Sanan’ and Den-ERS1 from ‘Pompadour’ were also compared with ERS1 from other plant species. The deduced amino acid sequences of Den-ERS1(ks) showed 99.5%, 91.3%, 88.7%, 69.7% similarity to *Dendrobium* Sonia ‘Bom17’ (Den-ERS1, AY746972) *Phalaenopsis equestis (*Ph-ERS, AJ563284), *Oncidium* cv. 'Gower Ramsey' (OgERS1, AF276235) and *Arabidopsis thaliana* (AtERS1, NP_181626) (Table 8). Phylogenetic tree generated from the ethylene response sensor gene families from various plant species demonstrated that Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’ were closely related to ERS1 from genus Orchidaceae (Figure 22). ERS1 from monocot plants were also found in separate sub-clusters to ERS1 from dicot plants. Interestingly, AtERS1 had high degree of difference to other ERS1 from dicot plants. The species and corresponding accession numbers used in the comparison were as follows; *Dendrobium* ‘Khao Sanan’ (Den-ERS1(ks), FJ628419), *Dendrobium* ‘Sonia Bom17’ (Den-ERS1, AY746972), *Dendrobium* ‘Pompadour’ (Den-ERS1(mp), FJ628420), *Phalaenopsis* cv. ‘KC butterfly’ (Ph-ERS1, AF113541), *Oncidium* cv. ‘Gower Ramsey’ (OgERS1, AF276233), *Phalaenopsis* cv. ‘True Lady’ (Ph-ETR1, AF055894), *Phalaenopsis equestris* (Ph-ERS, AJ563284), *Gladiolus* hybrid cultivar 'Traveler' (Gl-ERS1, AB180247), *Musa acuminata* (MaERS1, AB266315), *Delphinium* 'MagicFountains dark blue' (Dl-ERS1-1, AB055429), *Delphinium* x *belladonna* (Dl-ERS1-3, AB201245), *Oryza sativa* Indica Group (OsERS, AF013979), *Saccharum officinarum* (SaERS, EU851574), *Zea mays* (Zm-ERS1, AY359578), *Actinidia deliciosa* (AcERS1, EU170627), *Lilium formosanum* x *Lilium longiflorum* (Li-ERS1, DQ408428), *Passiflora edulis* (Pa-ERS1, AB015497), *Vigna radiate* (Vi-ERS1, AF098272), *Pyrus pyrifolia* (*PyERS1*, AF386515), *Vigna* *radiata* (Pe-ERS1, DQ154118), *Arabidopsis thaliana* (AtERS1, NM_129658) and *Chrysanthemum* x *morifolium* (Ch-ERS1, AF547624).



Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1	■	69.4	71.7	68.1	69.5	68.2	68.4	71.0	71.8	72.8	67.0	69.1	67.9	69.1	68.5	66.8	69.8	75.2	63.1	71.7	68.8	67.8	1	Ch-ERS1 AF547624.PRO
2	38.0	■	75.7	92.1	70.1	92.6	91.9	72.0	76.9	77.2	90.2	84.2	82.8	81.8	79.7	79.7	71.2	76.2	73.8	73.3	99.5	97.3	2	Den-ERS1 AY746972.PRO
3	34.1	29.6	■	74.1	75.5	74.4	74.4	73.0	78.2	78.4	72.9	74.9	74.4	75.3	74.1	73.0	77.2	78.7	68.8	80.5	75.1	73.9	3	Vi-ERS1 AF098272.PRO
4	40.2	7.8	30.3	■	70.5	99.4	98.1	71.4	77.4	77.7	89.7	83.6	82.1	80.4	78.7	78.4	72.2	76.2	72.4	74.6	91.5	90.0	4	Ph-ETR1 AF055894.PRO
5	38.2	38.1	29.4	37.6	■	70.6	70.8	68.7	72.1	72.9	68.4	71.1	70.1	70.1	71.5	70.1	70.3	75.4	65.7	72.9	69.7	68.7	5	AtERS1 NP_181626.PRO
6	40.0	7.3	29.9	0.6	37.3	■	98.4	71.6	77.7	78.1	90.2	83.7	82.6	80.7	79.0	78.7	72.7	76.5	72.7	74.7	92.0	90.5	6	Ph-ERS1 AF113541.PRO
7	39.7	8.0	29.9	1.9	37.0	1.6	■	71.2	77.9	78.2	89.7	83.3	82.3	80.4	79.0	78.5	72.5	76.8	72.7	74.7	91.3	89.9	7	Ph-ERS AJ563284.PRO
8	36.3	34.3	33.4	34.9	40.6	34.6	35.1	■	73.4	74.1	70.0	72.1	71.2	69.4	69.4	68.4	69.7	76.5	64.1	71.1	71.4	70.4	8	Pe-ERS1 DQ154118.PRO
9	34.9	25.4	26.3	26.6	33.7	26.1	26.1	33.5	■	99.0	75.1	78.7	78.4	78.5	76.7	77.2	74.9	78.2	71.5	77.4	76.4	75.6	9	Dl-ERS1-3 AB201245.PRO
10	33.4	24.9	26.0	26.1	32.4	25.7	25.7	32.4	1.0	■	75.4	79.0	78.9	78.9	77.4	77.9	75.6	78.7	72.3	77.4	76.7	75.9	10	Dl-ERS1-1 AB055429.PRO
11	40.7	9.0	32.4	9.5	39.7	9.0	9.5	36.8	29.0	28.6	■	81.5	79.7	79.7	77.2	77.3	70.5	73.9	70.8	72.3	88.7	88.2	11	OgERS1 AF276233.PRO
12	38.6	16.6	29.8	16.5	36.4	16.3	16.9	33.6	25.1	24.7	18.4	■	84.2	83.9	80.8	81.9	73.3	75.5	74.8	75.2	83.6	82.6	12	Gl-ERS1 AB180247.PRO
13	40.0	18.3	30.1	19.2	37.0	18.6	19.0	34.9	25.0	24.3	21.8	16.4	■	82.6	80.3	80.4	72.7	75.0	74.6	74.2	82.2	81.2	13	MaERS1 AB266315.PRO
14	38.2	19.3	30.0	21.2	37.2	20.8	21.4	37.8	25.4	24.9	21.1	17.8	18.4	■	80.9	80.3	71.7	74.2	74.3	74.2	81.2	80.4	14	Li-ERS1 DQ408428.PRO
15	39.3	22.7	31.7	23.7	35.5	23.3	23.3	38.6	27.3	26.4	24.5	21.0	21.6	20.9	■	92.0	71.9	73.8	91.2	72.9	79.1	78.3	15	Zm-ERS1 AY359578.PRO
16	41.6	23.1	33.5	24.1	37.7	23.7	23.9	40.6	27.4	26.5	24.9	19.9	22.0	22.5	8.0	■	71.5	72.7	86.0	71.9	79.1	77.6	16	OsERS1 ABF98410.PRO
17	36.2	34.3	25.5	33.3	37.3	32.5	33.0	38.6	29.6	28.6	36.4	33.0	34.2	35.1	34.7	35.8	■	75.8	66.3	75.5	70.6	70.2	17	PyERS1 AF386515.PRO
18	27.9	27.4	23.8	28.9	29.6	28.4	28.2	28.6	26.3	25.6	31.8	29.1	29.2	31.0	31.8	34.1	27.1	■	68.7	78.2	75.2	74.6	18	AcERS1 EU170627.PRO
19	44.2	27.4	35.0	29.1	40.1	28.7	28.9	43.0	30.8	29.5	30.1	25.9	25.5	26.1	6.0	11.1	38.7	35.7	■	66.8	73.2	72.3	19	SaERS EU851874.PRO
20	33.4	31.2	21.4	30.3	32.5	30.0	30.0	36.7	27.4	27.4	32.8	28.1	31.2	31.6	32.6	34.4	27.5	24.5	37.8	■	72.7	72.0	20	Pa-ERS1 AB015497.PRO
21	38.0	0.0	29.6	7.8	38.1	7.3	8.0	34.3	25.4	24.9	9.0	16.6	18.3	19.3	22.7	23.1	34.3	27.4	27.4	31.2	■	96.8	21	Den-ERS1(ks) FJ628419.PRO
22	40.7	2.8	32.3	10.2	40.6	9.6	10.4	36.9	27.3	26.8	11.2	18.7	20.4	21.2	24.7	25.1	36.4	29.7	29.8	33.2	2.8	■	22	Den-ERS1(mp) FJ628420.PRO
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

Table 8 Percent identity of deduce amino acid sequence of ERS1 from different species. Den-ERS1 from ‘Khao Sanan’ Den-ERS1 (ks) and ‘Pompadour’ Den-ERS1 (mp) were shown in box. Cluster analysis was done using the MegAlign program (Lasergene® version 7.2).

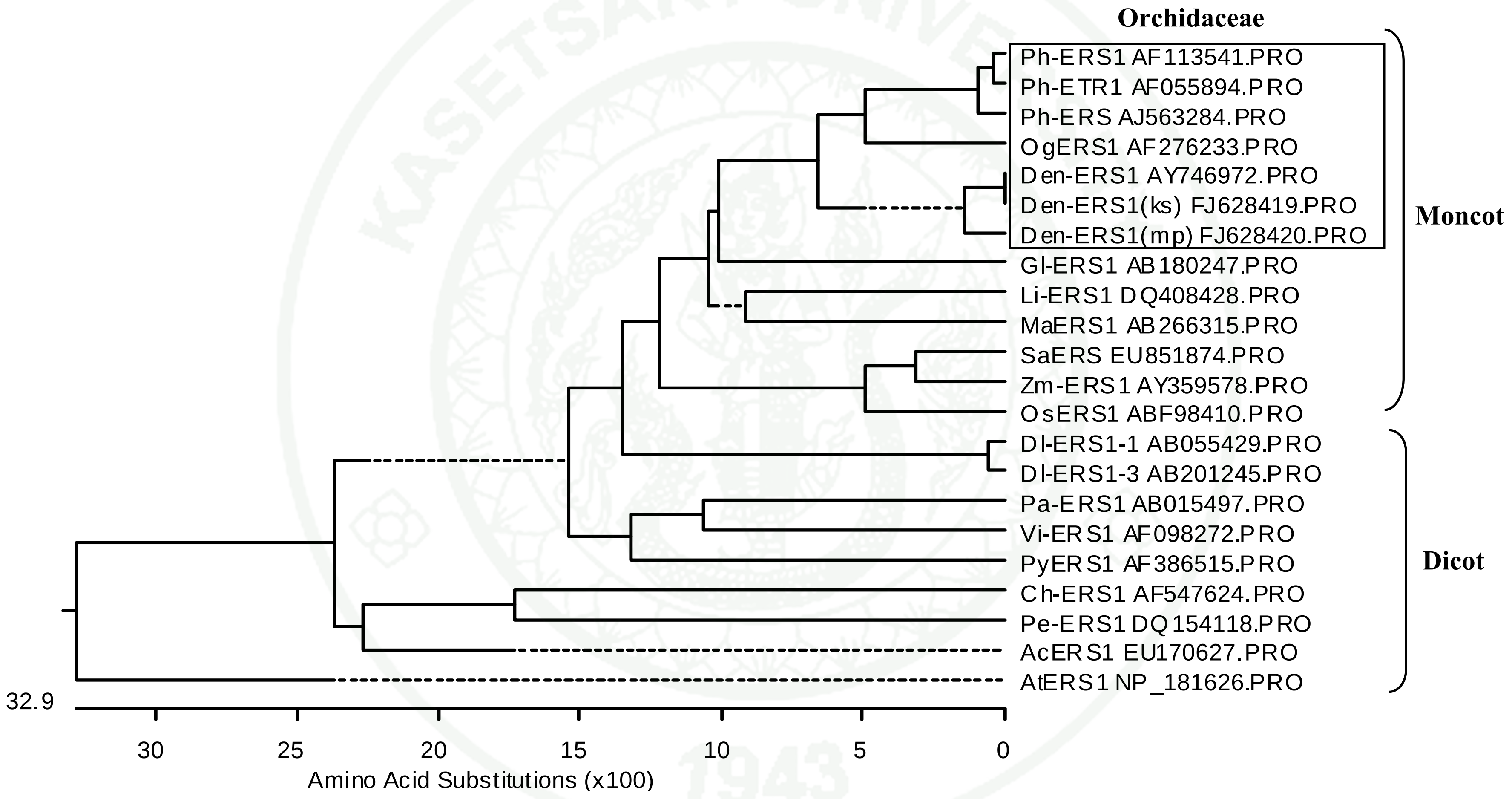


Figure 22 Phylogenetic relationship of the deduced amino acid sequences of ethylene receptor family genes from different plant species. Amino acid sequence analysis was done using MegAlign program (Lasergene® version 7.2).

3.2 Protein analysis

3.2.1 Identification of conserved domain in Den-ERS1

Amino acid sequences of Ethylene Response Sensor 1 (*Den-ERS1*), from ‘Khao Sanan’ and ‘Pompadour’ were aligned with ERS1 from of two genus of Orchidaceae (*Phalaenopsis*, *Oncidium*) and *Arabidopsis* using Clustal W. Three domains including transmembrane domains (ethylene binding domains), GAF domain and histidine domain were found in Den-ERS1 putative protein from ‘Khao Sanan’ and ‘Pompadour’ (Figure 23). Both Den-ERS1(ks) and Den-ERS1(mp) consisted of three ethylene binding domains therefore classified as a member of subfamily I. An additional isoleucine (I) was found at position 40 from start codon, methionine, in Den-ERS1 from ‘Pompadour’ in ethylene binding domain I. The amino acid sequence of three ethylene binding domains between *Phalaenopsis* (*Ph-ERS*, AJ563284), *Oncidium* (*OgERS1*, AF276233) and *Arabidopsis* (*AtERS1*, NM_129658) showed highly similarity. Five highly conserved motifs (H, N, G1, F and G2) proposed to be important for histidine kinase activity are present in both Den-ERS1(ks) and Den-ERS1(mp). At C-terminus, conserved amino acid sequence specific in *Phalaenopsis* and *Oncidium* ERS1---RAGQAEADPFGLK--- were found while this area were not present in amino acid sequence at C-terminus of Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’. Moreover, these areas were found distinct in each plant species. The role of this conserved sequence is currently unknown.

Figure 23 Amino acid alignment of ethylene receptor gene, *Dendrobium* 'Khao Sanan' (Den-ERS1ks, FJ628419), *Dendrobium* 'Pompadour' (Den-ERS1mp, FJ628420), *Phalaenopsis equestris* (Ph-ERS, AJ563284), *Oncidium* cv. 'Gower Ramsey' (OgERS1, AF276233) and *Arabidopsis thaliana* (AtERS1, NM_129658). Sequences were aligned by the ClustalW. Conserved motifs are indicated above the alignment vertical bars indicate putative regions of three N-terminal transmembrane regions and five sequence motifs for histidine protein kinase domain (H, N, G1, F, and G2).

```
                                      I                            II
                          __________________           __________________
Den-ERS1ks MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFGAFIVLCGATHL 70
Den-ERS1mp MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLELIIYFVKKSSFFPYRWVLIQFGAFIVLCGATHL 71
OgERS1     MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFGAFIVLCGATHL 70
Ph-ERS     MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFGAFIVLCGATHL 70
AtERS1     MESCDCFETHVNQDDLLVKYQYISDALIALAYFSIPLEL-IYFVQKSAFFPYKWVLMQFGAFIILCGATHF 70
           **.***:*.:   *:********** :*************:**:****:***:******:******:

                          III
           __          ______________________________
Den-ERS1ks INLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLRNKAEELDREMGLIRTQEE 141
Den-ERS1mp INLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLRNKAEELDREMGLIRTQEE 142
OgERS1     INLWTFTMHSRTVAIVMTVAKVSTAGVSCATALMLGHIIPDLVSVKTRELFLRNKAEELDREMGLIRTQEE 141
Ph-ERS     INLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLRNKAEELDREMGLIRTQEE 141
AtERS1     INLWMFFMHSKAVAIVMTIAKVSCAVVSCATALMLVHIIPDLLSVKNRELFLKKKADELDREMGLILTQEE 141
           **** * ***:::*****:**** * ********* ******:***.*****::**:********* ****

Den-ERS1ks TGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAECALWMPSRTGLDLQLSHTLSNQIPVGTVGSTNLP 212
Den-ERS1mp TGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAECALWMPSRTGLNLQLSHTLSNQIPVGSVVSTNLP 213
OgERS1     TGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAECALWMPLRTGLHLQLSHTLNNKIPVGSVVSINLP 212
Ph-ERS     TGRHVRMLTHEIRSTLDRHTILRTTLVELGRTLDLVECALWMPSRTGLNLQLSHTLNNQIPVGSVVSINLP 212
AtERS1     TGRHVRMLTHGIRRTLDRHTILRTTLVELGKTLCLEECALWMPSQSGLYLQLSHTLSHKIQVGSSVPINLP 212
           ********** ** ********:*******:** * ******* ::** *******.::* **: .  ***

Den-ERS1ks IVNQVFNSSRAVRNPHTCPLARFQPQTGRYVPPEGVAGRVPLLHLSNFQINDWPELAAKSFAVMVLMLPSY 283
Den-ERS1mp IVNQVFNSSRAVRIPHTCPLARFQHQTGRYVPPEVVAVRVPLLHLSNFQINDWPELSAKSFAVMVLMLPSD 284
OgERS1     IVNQIFNSSRAGRIPNTCPLARFQPYTGRYVPQEVVAVRVPLLHLSNFQINDWPELSPKSFAVLVLMLPSD 283
Ph-ERS     VVNQVFNSSCAVRIPHTCPLARFQPHTGRYVPPEVVAVRVPLLHLSNFQINDWPELSAKNFAVMVLMLPSD 283
AtERS1     IINELFNSAQAMHIPHSCPLAKIGPPVGRYSPPEVVSVRVPLLHLSNFQGSDWSDLSGKGYAIMVLILPTD 283
           ::*::***: * : *::****::   .*** * * *: *********** .**.:*: *.:*::**:**:

                                                                          ▼
Den-ERS1ks SARKWHVYELELVEVVADQVAVALSHAAILEESMRARDLLLDQNVALDLARQEAEMAIRARNDFLAVMNHE 354
Den-ERS1mp SARKWHVYELELVEVVADQVAVALSHAAILEESMRARDLLLDQNVALDLARQEAEMAIRARNDFLAVMNHE 355
OgERS1     SARKWHAYELELVEVVADQVAVALSHAAILEESMRARDLLMDQNVALDLARREAEMAIRARNDFLAVMNHE 354
Ph-ERS     SARKWHVYELELVEVVADQVAVALSHAAILEESMRARDQLMDQNVALDLARREAEMAIRARNDFLAVMNHE 354
AtERS1     GARKWRDHELELVENVADQVAVALSHAAILEESMHARDQLMEQNFALDKARQEAEMAVHARNDFLAVMNHE 354
           .****: :****** *******************:*** *::**.*** **:*****::************

Den-ERS1ks MRTPMHAIIALSSLLLETELTPEQRLMVETILKNSNLLATLINDVLDLSKLEDGSFELEPQFSIFHTVFRE 425
Den-ERS1mp MRTPMHAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEATVFNLHTVFRE 426
OgERS1     MRTPMHAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEATIFNLHTVFRE 425
Ph-ERS     MRTPMHAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEVTVFNLHTVFRE 425
AtERS1     MRTPMHAIISLSSLLLETELSPEQRVMIETILKSSNLVATLISDVLDLSRLEDGSLLLENEPFSLQAIFEE 425
           *********:**********:****:*:*****.***:****.******:*****: **     ::::*.*

                                               ▼
Den-ERS1ks VVNLIKPIAAVKKLSVFVSLSPDLPSLAIGDEKRLIQTMLNVVGNAVKFTKEGSISITATIAKSDSLRDSR 496
Den-ERS1mp VVNLIKPIAAVKKLSVFVSLSPDLPSLAIGDEKRLIQTMLNVVGNAVKFTKEGSISITATIAKSDSLRDSR 497
OgERS1     AVNLIKPIAAVKKLSLFVSLAPDLPSCAIGDEKRLLQIMLNVVGNAVKFTKEGTIFITVNIAKPDSLRD-- 494
Ph-ERS     VVNLIKPIAAVKKLSLIVFLSPDLPSCAVGDEKRLMQTMLNVVGNAVKFTKEGSISITASIAKPDSLRDPR 496
AtERS1     VISLIKPIASVKKLSTNLILSADLPTYAIGDEKRLMQTILNIMGNAVKFTKEGYISIIASIMKPESLQELP 496
           .:.******:*****  : *:.***: *:******:* :**::********** * * ..* *.:**::

                                 ▼            ▼                ▼
Den-ERS1ks DPEFHPIPSDGYFYLRVQVKDTGCGISPLELPRLFTKFAHTQNGSNKGYTGSGLGLAICKRFVNLMKGRIW 503
Den-ERS1mp DPEFHPIPSDGYFYLRVQVKDTGCGISPLELPRLFTKFAHTQNGSYKGYTGSGLGLAICKRFVNLMKGRIW 504
OgERS1     -PDFYPAPTDGHFYLRVQVKDTGCGISPQELPHLFTKFAHTQNGSDKGYSGSGLGLAICKRFVNLMKGHIW 501
Ph-ERS     DPEFYPIPSDGHFYLRVQIKDTGCGISPQELPHLFTKFAHAQNGSDKGYNGSGLGLAICKRFVNIMKGHIW 503
AtERS1     SPEFFPVLSDSHFYLCVQVKDTGCGIHTQDIPLLFTKFVQPRTGTQRNHSGGGLGLALCKRFVGLMGGYMW 503
            *:*.*  :*.:*** **:******* . ::* *****.:.:.*: :.:.*.*****:*****.:* * :*

Den-ERS1ks LESEGIGKGCTTIFIVKLGISEDPTLRYQQKLLPPI-------------PKDEKNSIPSKIRHQRSL 621
Den-ERS1mp LESEGIGKGCTTIFIVKLGISEDPTLRYQQKLLPPI-------------PKDEKNSIPSKIRHQRSL 622
OgERS1     LESEGIGKGCTTIFIVKLGMSEDPTLRYQQKLLPPIRAGQAEADPFGLKPAALKDELMPKIRYQRSL 631
Ph-ERS     PESEGIGKGCTTIFIVKLGISEDPAHRFQHKLLPPIRAGQSEADAFGSKPTPT-DLIPLKNRYQRSL 633
AtERS1     IESEGLEKGCTASFIIRLGICNGPSSSSGSMALHLA--------------------AKSQTRPWNW- 613
           ****: ****: **::***:.:.*:     *                          : *  .
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3.2.2 Secondary structures

Protein structure analysis of Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’ was done using program Protean (Lasergene® version 7.2). The surface probability of Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’ was compared with *Dendrobium* Sonia ‘Bom17’ (Den-ERS1, AY746972) *Phalaenopsis equestis* (Ph-ERS, AJ563284), *Oncidium* cv. 'Gower Ramsey' (OgERS1, AF276235) and *Arabidopsis thaliana* (AtERS1, NP_181626). The area of ethylene binding domains between amino acid 28-113 showed hydrophobic and trans-membrane probability. The overall structure was similar in all five putative proteins except at C-terminus (from amino acid 600 towards the end). The C-terminus of Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’ showed high surface probability comparing with other ERS1 protein (Figure 24).

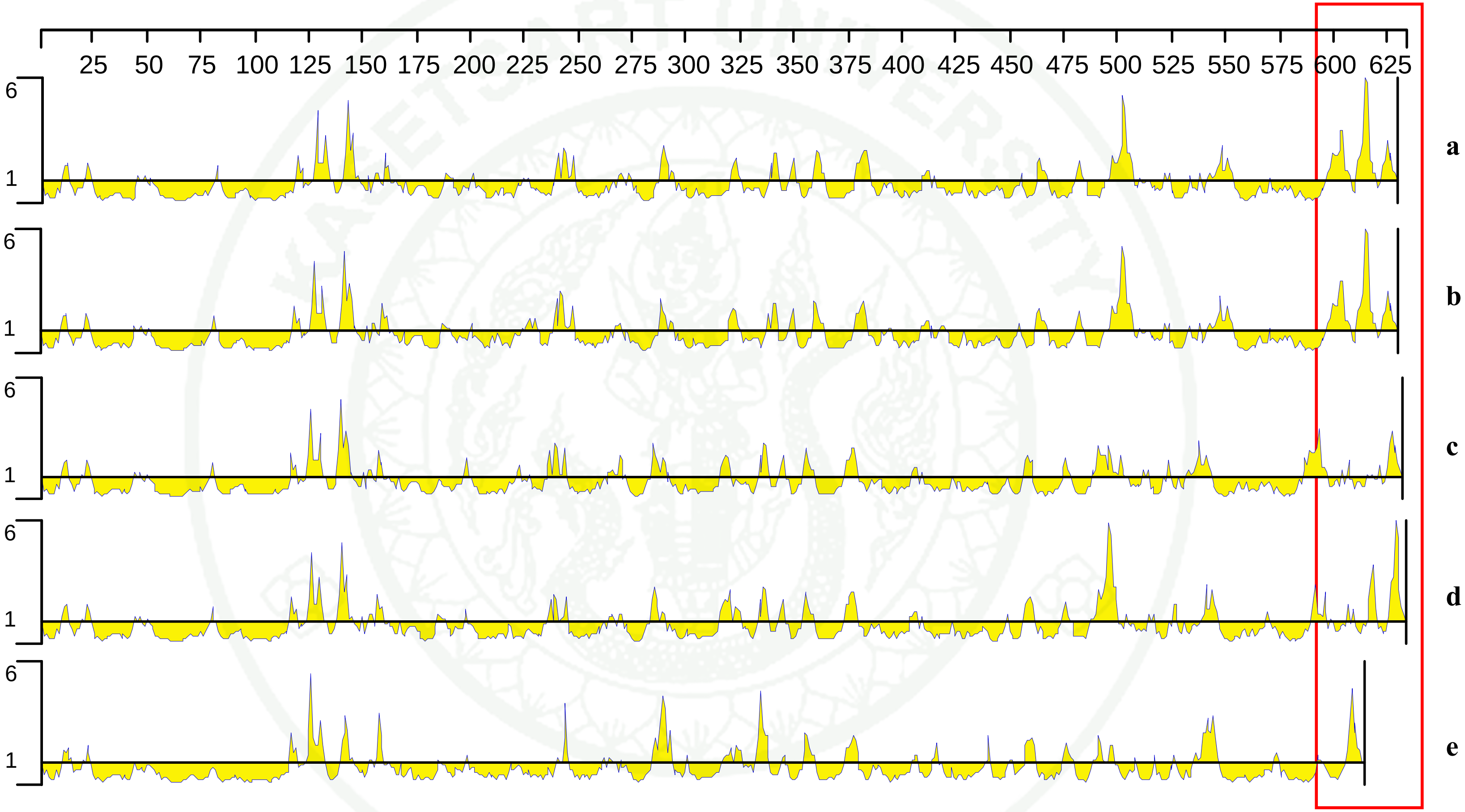
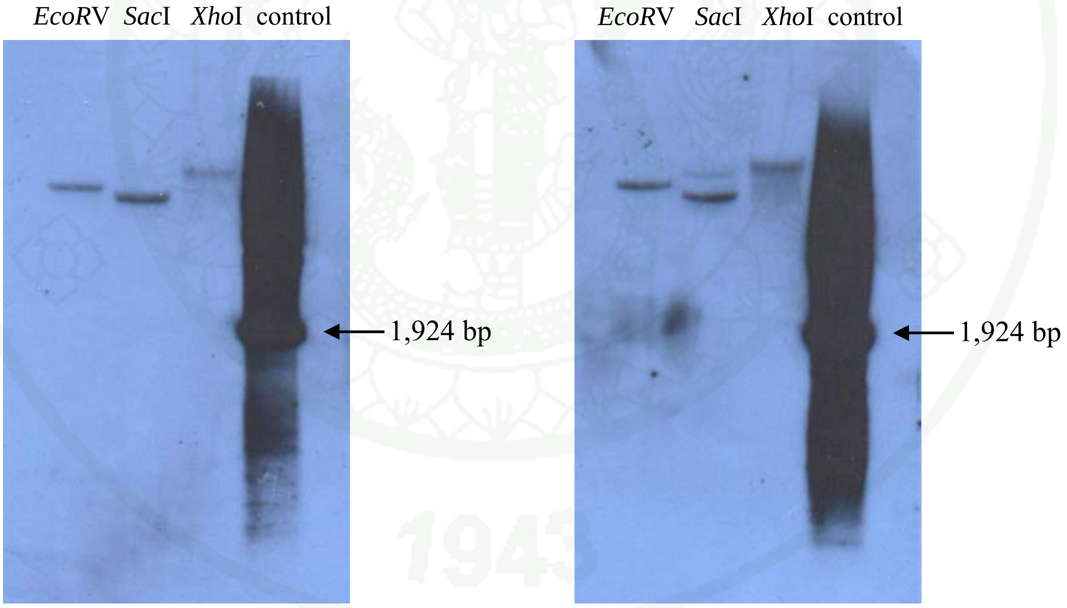


Figure 24 Protein analysis of surface probability of Den-ERS1 from 'Khao Sanan' (a), Den-ERS1 from 'Pompadour' (b), *Oncidium* Gower Ramsey (OgERS1) (c), *Phalaenopsis equestis (*Ph-ERS) (d) and *Arabidopsis thaliana* (AtERS1) (e) by Protean Program (Lasergene® version 7.2)

4. Determination of *Den-ERS1* copy number

High quality of orchid genomic DNA was digested with *Eco*RV, *Sac*I, and *Xho*I restriction endonuclease. The completely digested orchid genomic DNA was separated on 1% agarose gel. The copy number of *Den-ERS*1 in two *Dendrobium* 'Khao Sanan' and 'Pompadour' was determined by Southern analysis. Using ethylene binding domains of *Den-ERS1* 'Pompadour' as a probe, a single band was observed in *Dendrobium* 'Khao Sanan' and 'Pompadour' genomic DNA digested with *Eco*RV and *Xho*I. Using *Sac*I, a band with storng signal was observed in 'Pompadour' genomic DNA (Figure 25). The size of positive bands was identical in both 'Khao Sanan' and 'Pompadour'. The results suggested that the presence of single copy of *Den-ERS1* in both 'Khao Sanan' and 'Pompadour' possibility of another ethylene receptor gene in 'Pompadour'.



(a) **(b)**

Figure 25 Genomic Southern analysis of *Den-ERS1* 'Khao Sanan' (a) and 'Pompadour' (b) 30 µg total genomic DNA was digested with *Eco*RV, *Sac*I, and *Xho*I, electrophoresed and hybridized with *Den-ERS1* 'Pompadour' DIG labeled probe. *Den-ERS1* 'Pompadour' (ORF) PCR product was used as a positive control.

5. Ethylene Signal Transduction Genes in *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’

5.1 *Den-ERS1* transcripts in orchid organs

The expression of *Den-ERS1* in orchid organs was investigated using Northern analysis. Orchid plant consists of leaves, shoot, root, peduncle, flower bud and open floret (Figure 26). *Den-ERS1* transcripts were detected in every orchid organ. There was no detectable difference in *Den-ERS1* expression during leaf development in both ‘Khao Sanan’ and ‘Pompadour’. The expression of *Den-ERS1* in open floret from ‘Pompadour’ was noticeably less than in other organs.

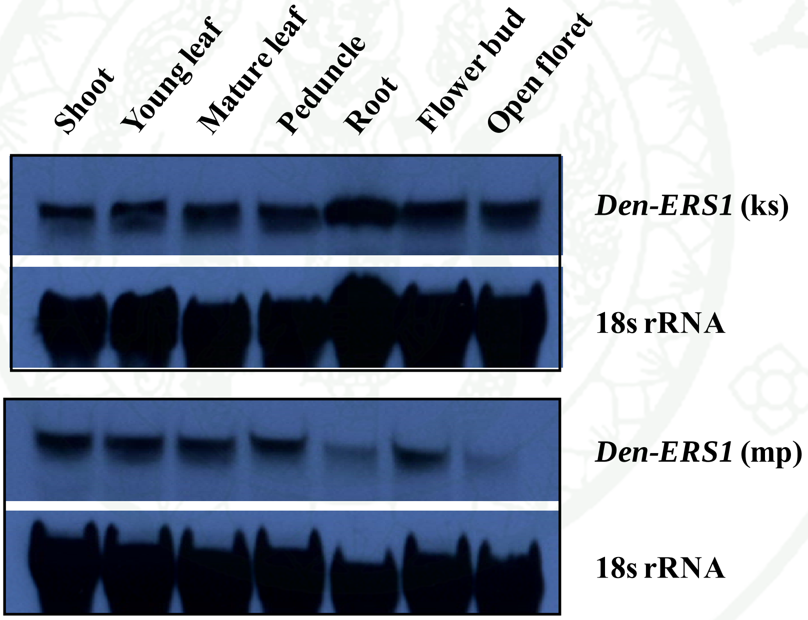


Figure 26 *Den-ERS1* expression in orchid organs including shoot, leaf (young and mature), peduncle, root, flower bud and open floret from two *Dendrobium* cultivars ‘Khao Sanan’ (ks) and ‘Pompadour’ (mp). Each sample contained 25 µg of total RNA. Endogenous *Den-ERS1* DIG-label probe were used for samples in each cultivars. *18S rRNA* DIG-label probe was used as positive control.

5.2 *Den-ERS1* transcripts in flower components

Dendrobium floret comprises of several components including sepal, petal, lip, column, ovary, pedicel (Figure 27a). Pollinia and pollinia cap is included in column. In this study, the presence of *Den-ERS1* transcripts in two stages of flowers; flower bud (B2) and open floret (OF1) from two *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’ were investigated (Figure 27b). In both varieties, *Den-ERS1* transcripts were accumulated more in flower bud than in open floret. *Den-ERS1* transcripts were found accumulated largely in lip and column. In ‘Khao Sanan’, large amounts of *Den-ERS1* transcripts were also detected in petal. Small amounts were detected in sepal, ovary and pedicel. In open floret, similar expression pattern was displayed except the high level of *Den-ERS1* expression in ovary.

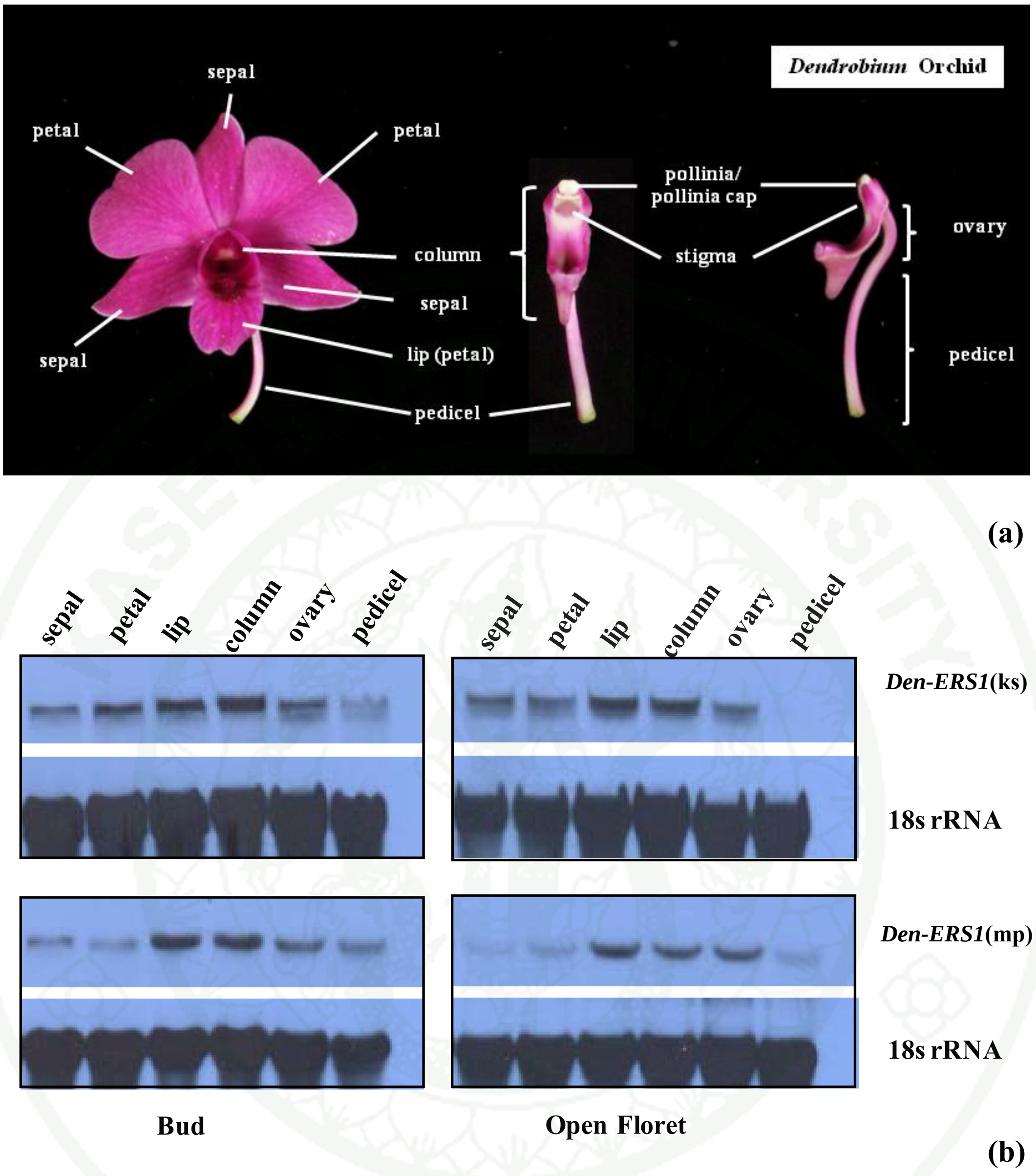


Figure 27 Orchid flower components (a) and *Den-ERS1* expression in *Dendrobium* ‘Khao Sanan’ and ‘Pompadour’ expression (b). Each sample contained 25 µg of total RNA. Endogenous *Den-ERS1* DIG-label probe was used for samples in each cultivars. *18S rRNA* DIG-label probe was used as positive control.

5.3 *Den-ERS1* transcripts accumulation during flower development and senescent stages

Den-ERS1 expression was detected in every stages of flower development from young bud (B3) to senescent floret (S3) (Figure 28). *Den-ERS1* transcripts were accumulated in young bud (B3) and decreased as flower development progressed. *Den-ER1* transcripts were increased again at full bloom (OF4). During flower senescence, the expression was high at early senescent stages (S1 and 2) and decreased towards the end of senescence (S3). *Den-ER1* expression pattern was similar in both 'Khao Sanan' and 'Pompadour'. In 'Khao Sanan' ethylene production in various stages of flower was low in B1-OF4 and increase in ethylene production in senescent stage (S1-S4) until reaching the peak in S3 stage (Figure 29). Total RNA from S4 stage was too severely degraded for expression study. It is therefore not included.

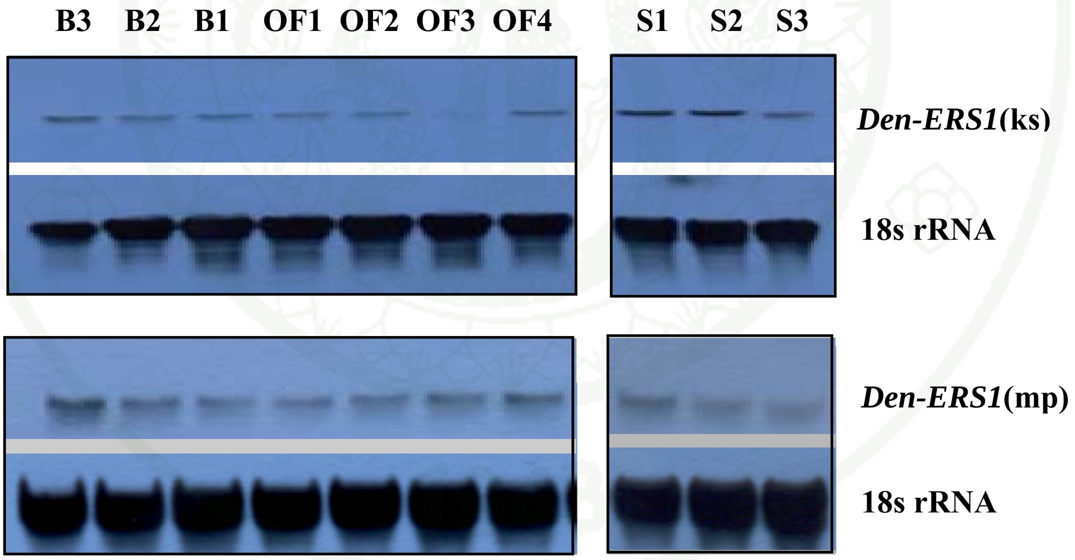


Figure 28 *Den-ERS1* expression in various stages of flower development and flower senescence. Total RNA was isolated from in *Dendrobium* 'Khao Sanan' and 'Pompadour' expression. Each sample contained 25 µg of total RNA. Endogenous *Den-ERS1* DIG-label probe were used for samples in each cultivars. *18S rRNA* DIG-label probe was used as positive control.

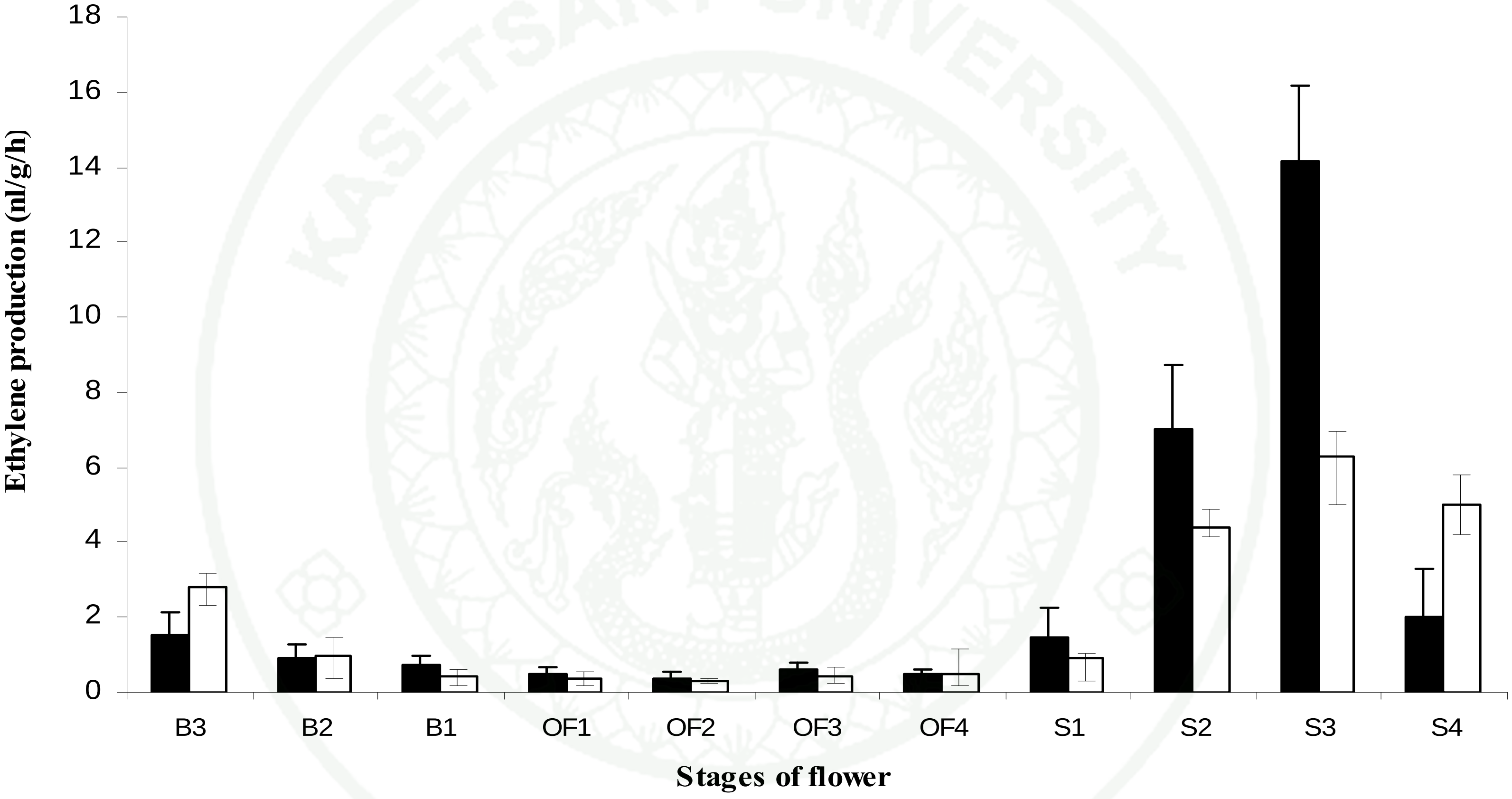


Figure 29 Ethylene production of 'Khao Sanan' (■) and 'Pompadour' (□) flowers at different stages of flower development (B3-OF4) and senescent stages (S1-S4). The experiments were done in 4 replications

5.4 *Den-ERS1* expression during induced senescence

Induction of flower senescence by pollination and emasculation in *Dendrobium* ‘Khao Sanan’ (Figure 30) and ‘Pompadour’ (Figure 31) were shown. Senescent symptoms were started 24 h (1 d) after pollination (Figure 34) and 2 days after emasculation. The symptom characters with drooping, veining, watering and yellowing were shown.

5.4.1 Emasculation

The expression of *Den-ERS1* was observed in emasculated florets over 7 day period. The results were compared with the un-emasculated open florets. In un-emasculated florets of ‘Khao Sanan’, *Den-ERS1* expression level was stable from day 1-6 and increased in day 7 (Figure 32a). In emasculated florets, the expression level was stable from day 1-4 and decreased afterwards (Figure 32b). In un-emasculated open florets of ‘Pompadour’, *Den-ERS1* expression level was peaked at day 4 and declined afterwards (Figure 32c). In emasculated floret, the expression was stable until day 6. The amounts of *Den-ERS1* transcripts were greatly reduced on day 7 (Figure 32d).

Ethylene production in un-emasculated open floret was low from day 1-7 in both ‘Khao Sanan’ and ‘Pompadour’. In emasculated florets from both varieties showed the increase in ethylene production until reaching the peak on day 5. Ethylene production was two times higher in ‘Pompadour’ than that of ‘Khao Sanan’ (Figure 33).

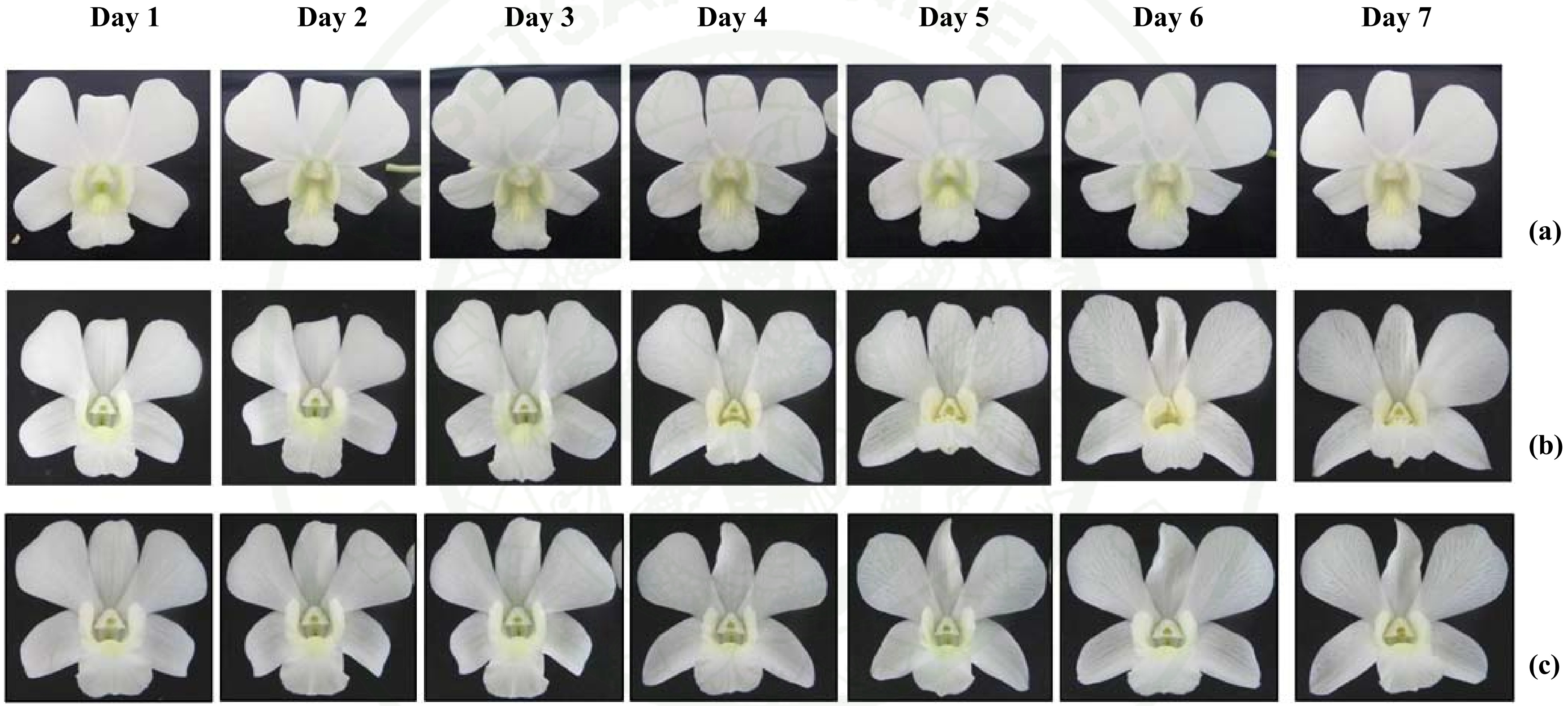


Figure 30 Senescence symptom of *Dendrobium* 'Khao Sanan' during induced senescence control (a) emasculated (b) and pollinated (c) on day 1 to day 7

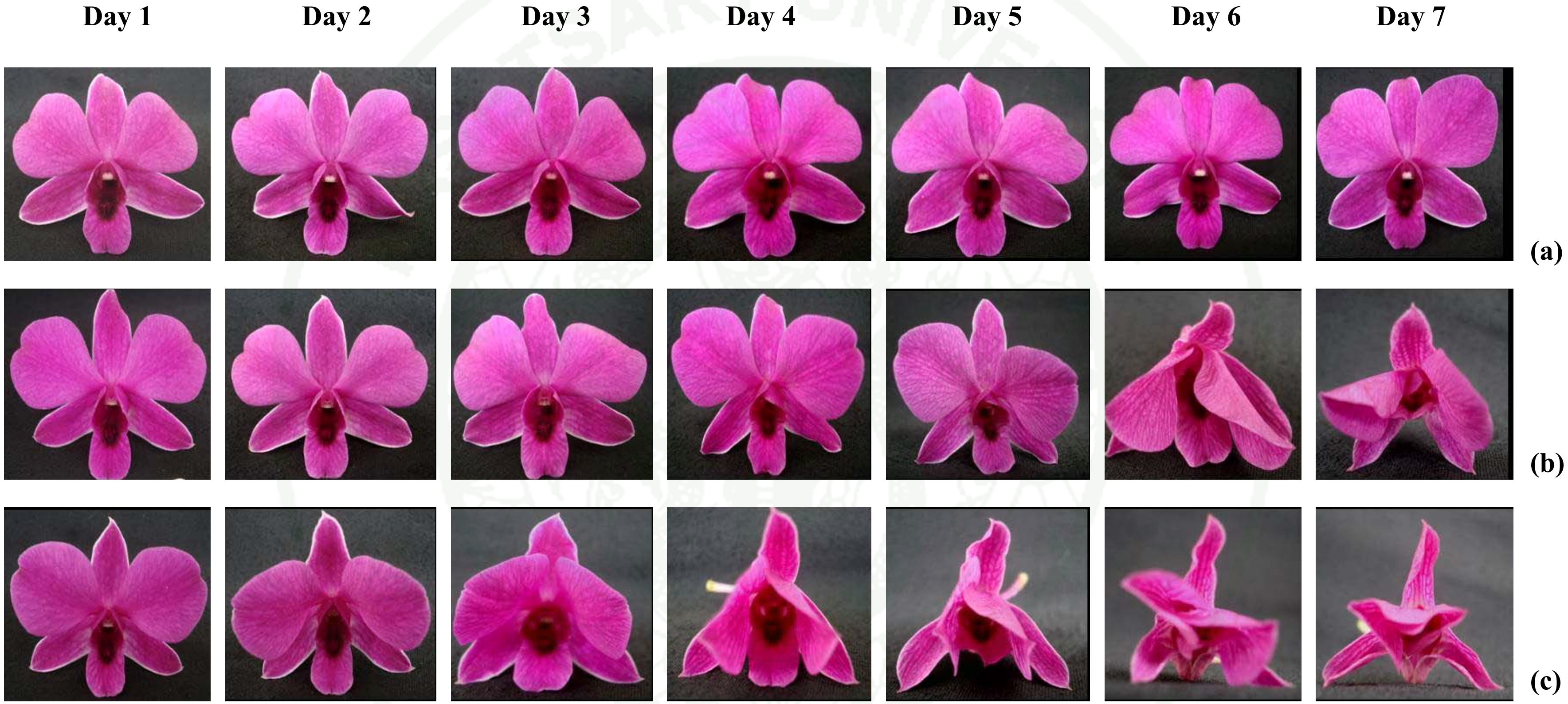


Figure 31 Senescence symptom of *Dendrobium* ‘Pompadour’ during induced senescence control (a) emasculated (b) and pollinated (c) on day 1 to day 7

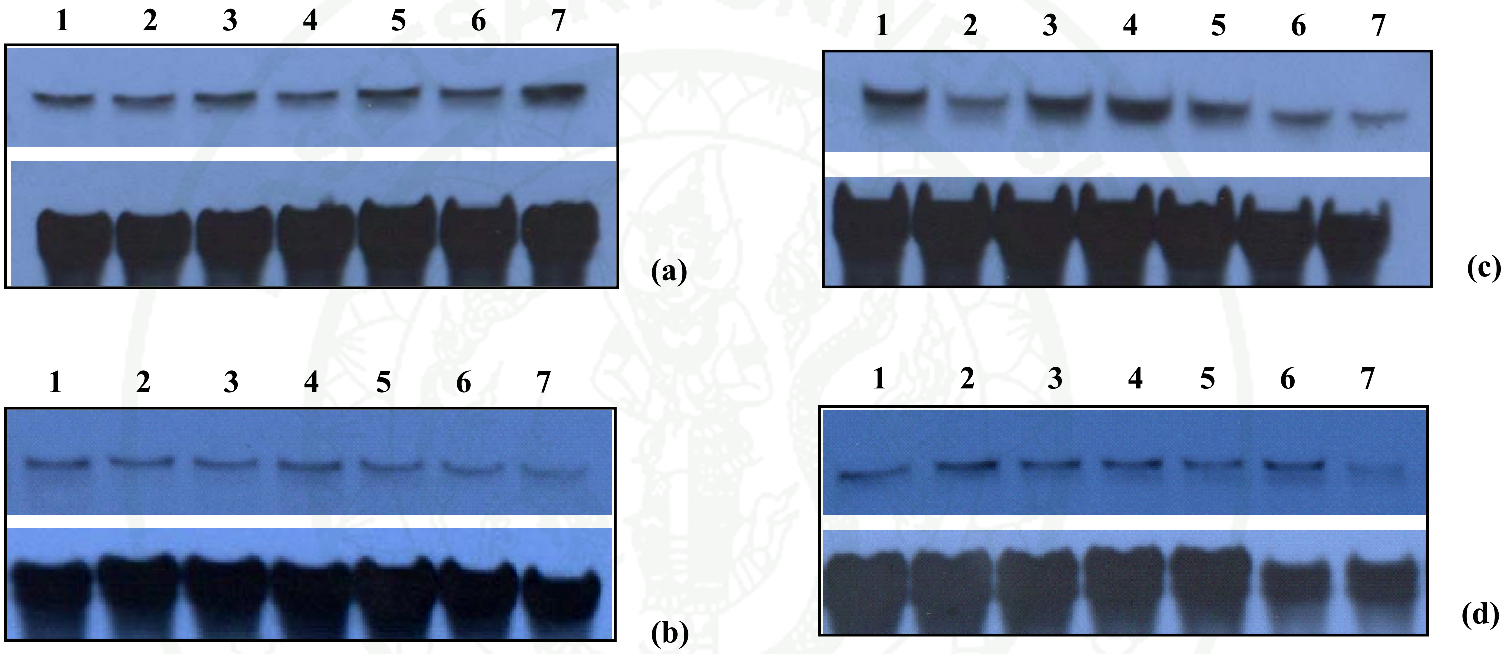


Figure 32 Expression of *Den-ERS1* from ‘Khao Sanan’ (a and b) and ‘Pompadour’ (c and d) during 7 day period after emasculation. Total RNA was isolated from un-emasculated open florets (a) and (c) and emasculated open florets (b) and (d). Each sample contained 25 µg of total RNA. Endogenous *Den-ERS1* DIG-label probe were used for samples in each cultivars. 18S RNA DIG-label probe was used as positive control. The numbers in (b) and (d) represented day after emasculation (DAE). The numbers in (a) and (c) represented 7 day period in un-emasculation.

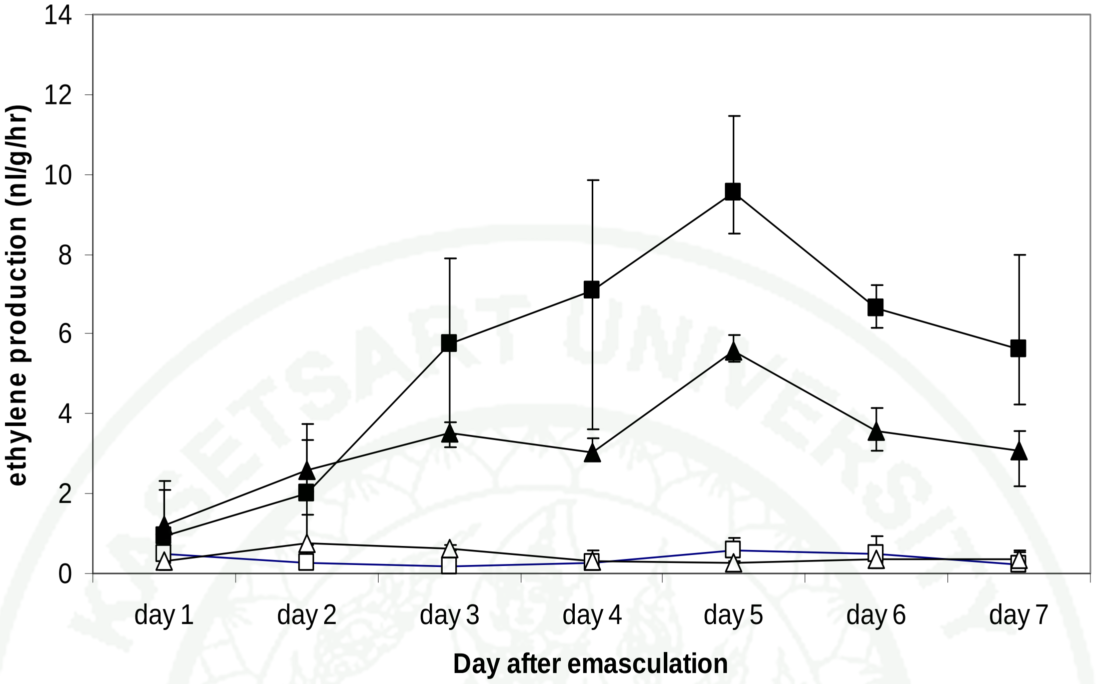


Figure 33 Ethylene production of emasculated *Dendrobium* ‘Pompadour’ (■), ‘Khao Sanan’ (▲) and unemasculated ‘Pompadour’ (□), ‘Khao Sanan’ (△). The experiments were done in 4 replications.

5.4.2 Pollination

The expression of *Den-ERS1* was observed in pollinated florets over 7 day period. The results were compared with the un-pollinated open florets. In un-pollinated open florets of 'Khao Sanan', *Den-ERS1* expression level was stable from day 1-6 and increased in day 7 (Figure 35a). In pollinated florets, the expression level was very low in day 1 and increased from day 2-6 and decreased on day7 (Figure 35b). In un-pollinated open florets of 'Pompadour', *Den-ERS1* expression level was peaked at day 4 and declined afterwards (Figure 35c). In pollinated floret, the expression level was very low in day 1 and 2 and increased from day 3-6 and decreased on day7 (Figure 35d).

Ethylene production in un-pollinated flower was low from day 1-7 in both 'Khao Sanan' and 'Pompadour'. In day 1, ethylene production of pollinated florets was increased 34 fold from un-pollinated florets in 'Pompadour' and 'Khao Sanan'. This elevation occurred at the same time period of post-pollinated symptom (drooping) (Figure 34). Although the ethylene production was declined after day 1, the production in both varieties was still significantly higher than that of un-pollinated florets (Figure 36).

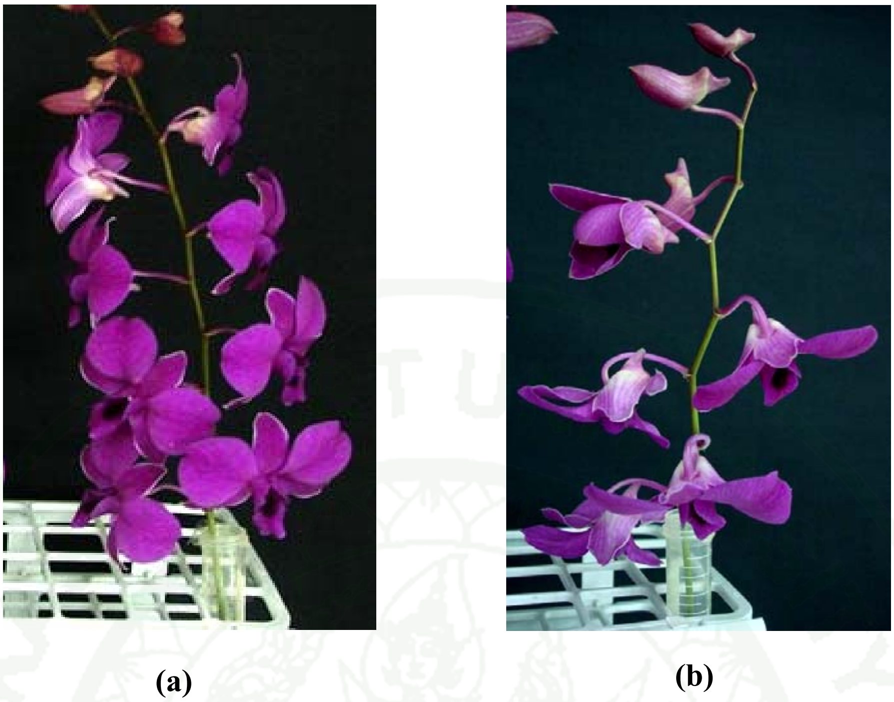

(a) (b)

Figure 34 Post-pollination symptom of *Dendrobium* ‘Pompadour’ occurred in 24 h: un-pollinated florets (a) and pollinated floret (b).

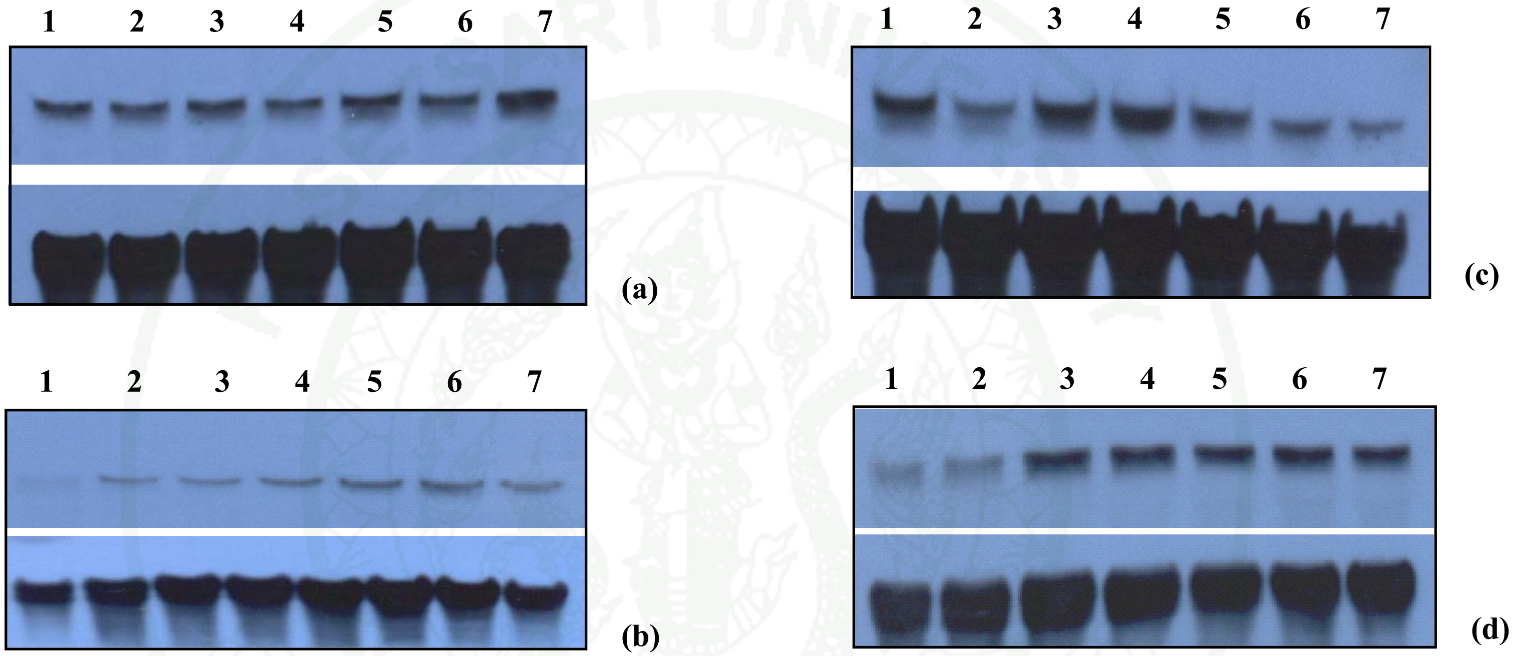


Figure 35 Expression of *Den-ERS1* from 'Khao Sanan' (a and b) and 'Pompadour' (c and d) during 7 day period after pollination. Total RNA was isolated from un-pollinated open florets (a) and (c) and pollinated open florets (b) and (d). Each sample contained 25 µg of total RNA. Endogenous *Den-ERS1* DIG-label probe were used for samples in each cultivars. 18S RNA DIG-label probe was used as positive control. . The numbers in (b) and (d) represented day after pollination (DAP). The numbers in (a) and (c) represented 7 day period in un-pollination.

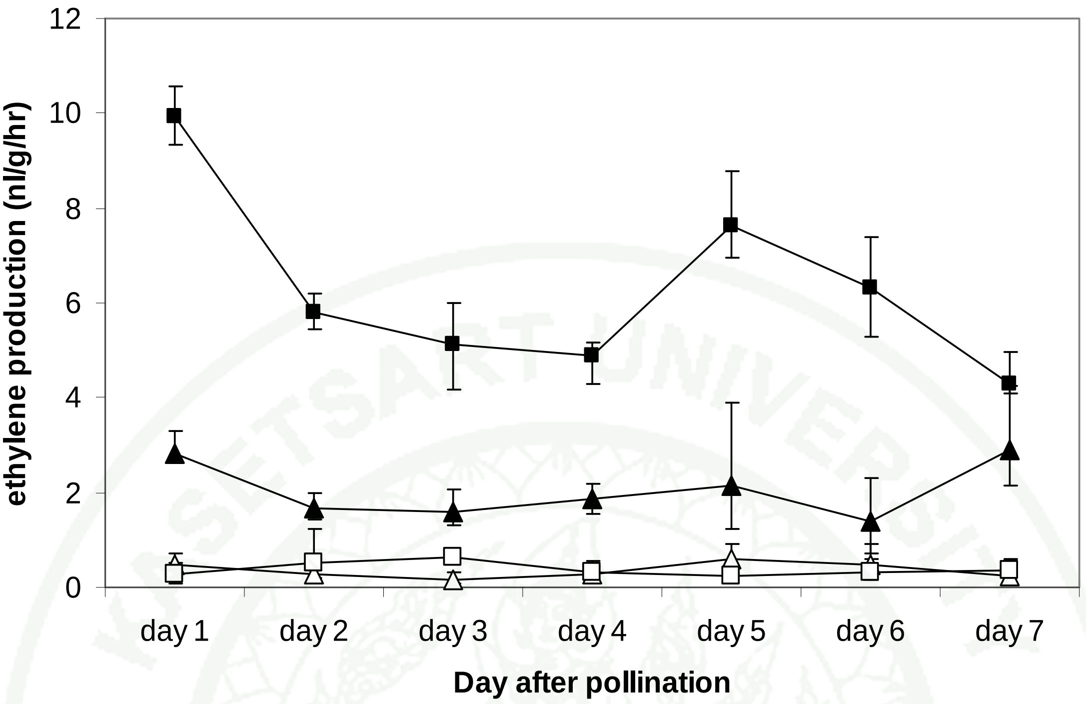


Figure 36 Ethylene production of pollinated florets *Dendrobium* ‘Pompadour’ (■), ‘Khao Sanan’ (▲) and un-pollinated open florets ‘Pompadour’ (□), ‘Khao Sanan’(△). The experiments were done in 4 replications.

DISCUSSION

1. Isolation of Ethylene Response Sensor 1 (*ERS1*) and promoter region from *Dendrobium* hybrid

In this study Ethylene Response Sensor1 (*Den-ERS1*) was isolated from 2 *Dendrobium* cultivars; 'Khao Sanan' and 'Pompadour'. cDNA sequences were cloned from total RNA using RT-PCR and 3'RACE. The putative promoter region and 5'untranslated region (5'UTR) was cloned from genomic DNA by ligation mediated PCR. Orchid flowers particularly 'Pompadour' variety contains high anthocyanin and phenolic compound contents which inhibit PCR reaction (Jose and Usha, 2000). Our nucleic acid extraction gives high quality of genomic DNA and total RNA by removing unwanted substances with sodium sulfite (Singh *et al*., 2002).

Sequence comparison indicated high sequence homology in both nucleotide and amino acid levels (98-100%) among ERS1 from various *Dendrobium* varieties including 'Khao Sanan' and 'Pompadour' in this study and Bom #17 (Suwanagul *et al*, 2007). The sequence also showed high sequence homology to other monocot ERS1 including rice, sugarcane and maize. This is the first study where the genome organization of *Den-ERS1* was also determined. It is found that the genome organization of *Den-ERS1* was distinct to other reported ERS1. Genome organization of *Den-ERS1* consists of 3 exons (exon I, II and III) and 2 introns (intron I and II). Other reported ERS1 genes including rice (AY043031), maize (AY359578), sugarcane (EU851874) and Arabidopsis (U21952) commonly consist of 5 exons and 4 introns. Generally, exons which can be translated into protein are highly conserved while intron sequence and length can be variable. Recently, however, introns were found playing important roles in gene regulation and tissue specific. *Cis* acting elements within introns which play important roles in gene regulation are almost exclusively associated with the first or second intron (Farrell and Bassett, 2007).

Cis acting element analysis indicated the presence of ethylene responsive element (ATTTCAAA) in promoter region of *Den-ERS1*. This element was found in promoter region of ethylene respond genes such as glutathione-*S*-transferase gene (*GST1*) senescence-regulated gene in carnation (Itzhaki *et al*., 1994). GCC box or GCCcore (GCCGCC) plays a key role in conferring jasmonate responsiveness to a *PDF1.2* gene from Arabidopsis promoter (Brown *et al*., 2003) was found in promoter region of *Den-ERS1*. The authors suggested that ethylene response factors also appear to play important roles in regulating jasmonate-responsive gene expression, possibly via interaction with the GCC-box. WUN (AAATTTCCT) described as a wound-responsive element in *Brassica oleracea*, was found (Pastuglia *et al*., 1997). Hormone related elements found in *Den-ERS1* regulatory region including gibberrellin-responsive element i.e WRKY71OS (TGAC) (Zhang *et al*., 2004), TATCCAOSAMY (TATCCA) (Chen *et al*., 2006), abscisic acid signaling element i.e. MYB1AT (WAACCA), MYB2CONSENSUSAT (YAACKG), MYBATRD22 (CTAACCA) (Abe *et al*., 1997; Abe *et al.,* 2003), an auxin response element i.e. CATATGGMSAUR (CATAGE) (Xu *et al*., 1997) and repressor of the salicylic acid, responding to environmental stress and plant defense response WBOXATNPR1 (TTGAC) (Chen *et al*., 2002) were also found.

Southern analysis indicated a single of *Den-ERS1* in genome of both ‘Khao Sanan’ and ‘Pompadour’. Another ethylene receptor gene was also proposed in ‘Pompadour’. The presence of multiple copies was also reported in *Oncidium* ‘Gower Ramsey’ (*OgERS1*) and chrysanthemum (*DgERS1*) (Huang *et al*., 2007; Nauumi *et al*., 2005). Multiple copies of genes could affect regulation of gene expression and subsequently numbers of transcripts.

2. Characterization of Ethylene Response Sensor 1 (Den-ERS1)

Amino acid sequences of Den-ERS1 from ‘Khao Sanan’ and ‘Pompadour’ were analyzed for their functional domains. There are three domains including three ethylene binding domains, GAF domain and histidine kinase domain. The ethylene binding domain and histidine kinase domain are involved in affinity to ethylene binding and efficiency to transmit the signal to downstream components, while the function of the GAF region is a cGMP binding site and predominately mediates non-covalent and reversible receptor association *in vivo* (Ho *et al*., 2000; Grefen *et al*, 2007). These domains are highly conserved. They are nearly identical in 3 *ERS1* genes reported from *Dendrobium, Oncidium* and *Phalaenopsis (*Suwanagul *et al*., 2007, Huang *et al*., 2007 and Chang, 2002).

Amino acid sequences of Den-ERS1 from 'Khao Sanan' and 'Pompadour' were found at C-terminus lacking 13 amino acids was found in *Phalaenopsis* (*Ph-ERS*) and *Oncidium* (*OgERS1*) between amino acid 603-604. This distinct pattern is located outside the conserved regions of histidine kinase domain. It unlikely involves in ethylene binding activity and yet to be studied.

3. Expression study of *Den-ERS1* during flower development

Den-ERS1 was found expressed in every organ including root, shoot, leaf, flower, and peduncle. *Den-ERS1* expression was not affected by developmental stages of leaf, which is a vegetative organ. The expression of *Den-ERS1*during flower development (reproductive organ) was investigated and found to be at similar level throughout flower development process. Flower buds and open florets were dissected and *Den-ERS1*transcripts were found highly accumulated in lips and column (containing male reproductive organ and part of female reproductive organ; Figure 26a). Similarly, *OgERS1* transcripts were found in every organ including root, pseudobulbs, leaves, flower bud and fully open flower in *Oncidium* orchid. The highest expression of *OgERS1* transcripts were also found at bud stage and in fully opened flowers in flower development (Huang *et al.,* 2007).

The increase in *Den-ERS1* transcripts was found associated with beginning of flower senescence. The transcripts were declined sharply toward the end of senescence. In carnation, *Dc-ERS2* showed a decreasing trend toward the last stage natural senescence (Shibuya *et al.,* 2002). In ethylene sensitive 'Seiko-no-makoto' chrysanthemum, *DG-ERS1* mRNA was present in a large amount in the petals and leaves, especially in stage-3 leaves, on day 0 (at the full-opening stage of flower) and its levels decreased markedly in both tissues with the lapse of time in air (Narumi et al. 2005). The authors suggested that reduction in *ERS1* transcripts was correlated with an increase in ethylene sensitivity in chrysanthemum during flower senescence. Ethylene production in *Dendrobium* 'Khao Sanan' and 'Pompadour' orchid flower was low during flower developmental stages (B3-OF4). Ethylene production was high at the beginning of flower senescence and sharply declined towards the end of senescence process. Ethylene production has been found to increase during senescence in ethylene sensitive flower such as carnation (Shibuya *et al*., 2000) and *Rosa hybrid* (Serek *et al* 1994; Muller *et al.,* 2000). Interestingly, the effect of ethylene on gene expression was varied among flower parts. Ethylene receptors have been shown to negatively regulate the ethylene signal transduction pathway in Arabidopsis (Hua and Mayerowitz, 1998). Knocking out ethylene receptors causes a constitutive ethylene response in mutant plants. From this negative control model, it was hypothesized that plant response to ethylene is negatively correlated with the number of receptors (Klee and Tieman, 2002, Tanase and Ichimura, 2006). In this study, the amounts of receptors were indirectly measured though its transcripts. Our results suggested that the reduction of *Den-ERS1* transcripts also caused ethylene sensitive in *Dendrobium* 'Khao Sanan' and 'Pompadour'.

4. ***Den-ERS1* expression during induced senescence**

Pollination is a process that pollen is transferred in plants and enabling fertilization of male gamete and ovule hence sexual reproduction occurs. Pollination initiates changes in flowers including ethylene production, epinasty, colour fading, abcission, ovary growth and senescence (O'Neil and Nadeau, 1997; van Doorn, 1997). Studies in ethylene sensitive plant species including carnation, petunia and *Dendrobium hybrid* indicated that there was sharp increase in ethylene production from styles, ovaries, receptacles and petals after pollination (Jones and Woodson, 1999; Larsen *et al.,* 1995). Bui and O'Neil (1998) reported that ethylene production in stigma and style caused subsequent release of ethylene from other floral organs. Pollination was also induced premature petal and sepal senescence in *Dendrobium* 'Pompadour' (Ketsa and Rugkong, 1999). Ketsa and Rungkong (1999) reported that pollination induced ethylene climacteric, respiratory climacteric, epinasty and increased in fresh weight without alteration of anthocyanin content in *Dendrobium* ‘Pompadour’. Ketsa and Rugkong (2000) further reported the peak of ethylene in pollinated flower at 9 h after pollination at 1.5 fold higher than un-pollinated flower. Similarly, the elevation of ethylene production after pollination was observed in this experiment with both ‘Khao Sanan’ and ‘Pompadour’ concomitant with senescence symptom induced by pollination. Ethylene production on day 1 of ‘Khao Sanan’ and ‘Pompadour’ was 9.92 and 2.81 nl/g/h indicating that ‘Pompadour’ produced 3.5 fold more ethylene than ‘Khao Sanan’. This high level of ethylene production in post pollination increased ethylene sensitive and also correlation of senescent symptoms particularly in ‘Pompadour’ (Borochov and Woodson, 1989; Jones, 2002).

Expression studies indicated a delay in *Den-ERS1* expression in pollinated flower both cvs. comparing with un-pollinated flower. The role of ethylene signaling in induced flower senescence was investigated using transgenic petunia. The delay in flower senescence and increase in ethylene production after pollination was reported in transgenic petunias with *At-ETR1* mutant (Wilkinson et al, 1997). This indicated that ethylene signaling is highly conserved and plays major role in flower senescence and transgenic petunia was responded to pollination. In addition it also suggested that flower consisted of mechanism of feedback control of ethylene synthesis postulated to be either through suppression of ethylene synthetic genes or induction of ACC-conjugating gene expression (Wilkinson *et al*., 1997). Although there are marked difference in the symptoms of induced and natural flower senescence, the studies in transgenic petunia indicated that both types of senescence was accompanied by similar hallmarks of senescence including DNA fragmentation, increase in protease and nuclease activities, reduced protein level (Jones, 2008, Jones *et al*., 2005, Langston *et al*., 2005). The level of ethylene sensitivity as indicated by flower senescence after pollination was reported to be varied among transgenic *ETR1* petunia lines as seen in two varieties investigated in this study (Shibuya *et al*., 2004).

In this study, the induction of flower senescence by emasculation was also investigated in ‘Khao Sanan’ and ‘Pompadour’. The results indicated that ethylene production was induced and peaked at day 5 in both varieties. The level of ethylene production was 9 and 5 fold higher than un-emasculated flower from ‘Pompadour’ and ‘Khao Sanan’, respectively. The senescent symptom was visible on day 2. In contrary to pollination, emasculation induced the reduction in *Den-ERS1* expression towards the late stage of flower senescence. Huang *et al.* (2007) investigated the effects of emasculation on Oncidium flower and reported the elevation of ethylene production and the rise of *OgERS1* expression and declined towards the end of flower senescence with the overall decrease in *OgERS1* expression comparing to un-emasculated flower. In *Delphinium* flower, stamen removal was also increased the sepal abscission and ethylene production (Ichimura *et al*., 2009).

The evidence from this study and other reports suggested that ethylene and ethylene signaling through *Den-ERS1* played important roles in induced flower senescence by pollination and emasculation.

CONCLUSIONS

Study on ethylene response sensor 1(*Den-ERS1*) gene in two cultivars of *Dendrobium hybrid* ‘Khao Sanan’ and ‘Pompadour’ can be summarized as following:

1. *Den-ERS1* was completely cloned, sequenced and characterized from two cultivars of *Dendrobium* ‘Pompadour’ and ‘Khao Sanan’. Their nucleotide and amino acid sequences were nearly identical. The genome organization of *Den-ERS1* was distinct from other reported plant *ERS1* with 3 exons and 2 introns.

2. Untranslated regions, 5’flanking regions, intron I and intron II of *Den-ERS1* were cloned, sequenced and characterized. Two ethylene related elements, ERE (ATTTCAAA) and WUN (AAATTTCCT) were identified from 5’flanking region and intron II, respectively.

3. Putative Den-ERS1 protein contains 3 ethylene-binding domains (transmembrane), GAF domain and histidine kinase domain. All conserved amino acid in each domain were found.

4. C-terminus of Den-ERS1 amino acid sequence of consists of a short peptide, which displays species specificity among plant species.

5. Generally, the expression of *Den-ERS1* was followed similar pattern in both ‘Pompadour’ and ‘Khao Sanan’.

6. The expression of *Den-ERS1* was detected in every orchid organs including shoot, root, leaf (young and mature), peduncle, flower bud and open floret.

7. There was little change in *Den-ERS1* expression level during flower development. However, there was a sharp increase in *Den-ERS1* at the beginning of flower senescence and decrease towards the end of flower senescence. Concomitantly, ethylene production also follows the similar pattern.

8. Pollination and emasculation induced flower senescence, ethylene production and reduced *Den-ERS1* expression.

9. The results suggested that *Den-ERS1* might play important roles in natural and induced senescence in *Dendrobium* orchid.

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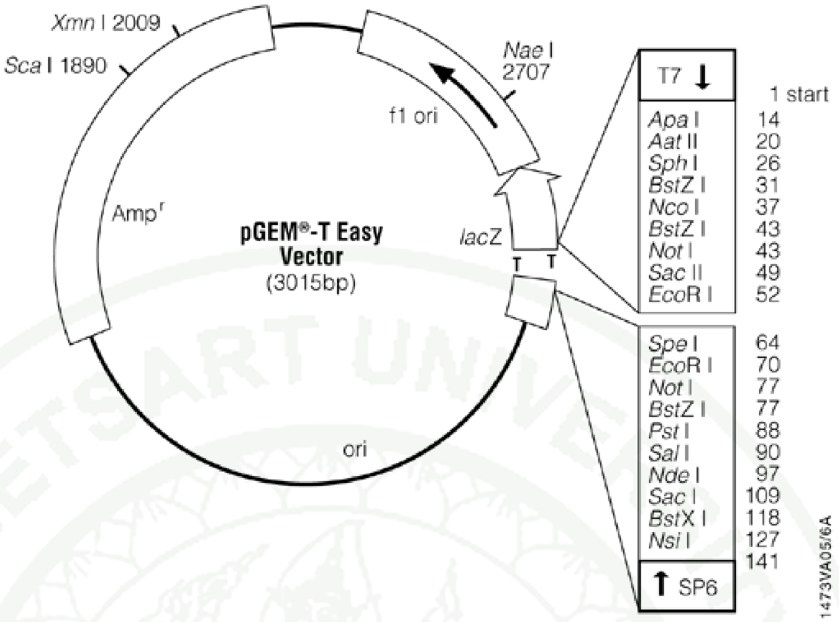
APPENDICES

Appendix A

pGEM-T Easy cloning vector



Appendix Figure 1 pGEM-T Easy vector (Promega)

Appendix B

Identification of *Den-ERS1 cis*-acting element and introns

Appendix Table 1 The presence of *cis*-acting elements in *Den-ERS1* 5'flanking region using PLACE Database

Factor or Site Name		Location	Strand	Signal Sequence	SITE
AACACOREOSGLUB1	site	240	(-)	AACAAAC	S000353
ANAERO3CONSENSUS	site	72	(+)	TCATCAC	S000479
ARR1AT	site	246	(+)	NGATT	S000454
BOXLCOREDCPAL	site	30	(+)	ACCWWCC	S000492
CAATBOX1	site	43	(+)	CAAT	S000028
CAATBOX1	site	58	(+)	CAAT	S000028
CAATBOX1	site	248	(-)	CAAT	S000028
CACTFTPPCA1	site	76	(+)	YACT	S000449
CACTFTPPCA1	site	291	(+)	YACT	S000449
CACTFTPPCA1	site	138	(+)	YACT	S000449
CACTFTPPCA1	site	315	(-)	YACT	S000449
CARGCW8GAT	site	258	(+)	CWWWWWWWWG	S000431
CARGCW8GAT	site	258	(-)	CWWWWWWWWG	S000431
CCAATBOX1	site	57	(+)	CCAAT	S000030
DOFCOREZM	site	78	(-)	AAAG	S000265
DOFCOREZM	site	189	(-)	AAAG	S000265
DOFCOREZM	site	299	(-)	AAAG	S000265
DOFCOREZM	site	310	(-)	AAAG	S000265
EBOXBNNAPA	site	148	(+)	CANNTG	S000144
EBOXBNNAPA	site	148	(-)	CANNTG	S000144
ERELEE4	site	254	(+)	AWTTCAAA	S000037
GATABOX	site	67	(+)	GATA	S000039
GATABOX	site	131	(+)	GATA	S000039
GATABOX	site	318	(+)	GATA	S000039
GATABOX	site	86	(-)	GATA	S000039
GATABOX	site	182	(-)	GATA	S000039
GCCCORE	site	8	(+)	GCCGCC	S000430
GT1CONSENSUS	site	131	(+)	GRWAAW	S000198
GT1CONSENSUS	site	92	(-)	GRWAAW	S000198
GT1CONSENSUS	site	199	(-)	GRWAAW	S000198
GT1CONSENSUS	site	180	(-)	GRWAAW	S000198
GT1CONSENSUS	site	200	(-)	GRWAAW	S000198
GTGANTG10	site	274	(+)	GTGA	S000378
GTGANTG10	site	316	(+)	GTGA	S000378
GTGANTG10	site	75	(-)	GTGA	S000378
GTGANTG10	site	88	(-)	GTGA	S000378
IBOXCORE	site	131	(+)	GATAA	S000199
IBOXCORE	site	181	(-)	GATAA	S000199
INRNTPSADB	site	95	(+)	YTCANTYY	S000395
MYBCORE	site	225	(+)	CNGTTR	S000176
MYBST1	site	182	(-)	GGATA	S000180
MYCCONSENSUSAT	site	148	(+)	CANNTG	S000407
MYCCONSENSUSAT	site	148	(-)	CANNTG	S000407
NAPINMOTIFBN	site	233	(-)	TACACAT	S000070
POLASIG3	site	50	(+)	AATAAT	S000088
POLLEN1LELAT52	site	296	(-)	AGAAA	S000245
RAV1AAT	site	145	(+)	CAACA	S000314
RAV1AAT	site	226	(-)	CAACA	S000314
RAV1AAT	site	243	(-)	CAACA	S000314
SEF4MOTIFGM7S	site	157	(+)	RTTTTTR	S000103
SEF4MOTIFGM7S	site	112	(-)	RTTTTTR	S000103
SREATMSD	site	181	(+)	TTATCC	S000470
TATABOX5	site	252	(+)	TTATTT	S000203
TATCCAOSAMY	site	182	(+)	TATCCA	S000403
WBOXHVISO1	site	70	(-)	TGACT	S000442
WBOXNTERF3	site	70	(-)	TGACY	S000457
WRKY71OS	site	71	(-)	TGAC	S000447

Appendix Table 2 The presence of *cis*-acting elements in *Den-ERS1* intron I using PLACE Database

Factor or Site Name		Location	Strand	Signal Sequence	SITE
ARR1AT	site	107	(+)	NGATT	S000454
ARR1AT	site	78	(-)	NGATT	S000454
CACTFTPPCA1	site	16	(+)	YACT	S000449
CACTFTPPCA1	site	27	(+)	YACT	S000449
CACTFTPPCA1	site	54	(+)	YACT	S000449
DOFCOREZM	site	18	(-)	AAAG	S000265
DOFCOREZM	site	70	(-)	AAAG	S000265
EECCRCAH1	site	75	(-)	GANTTNC	S000494
GT1CONSENSUS	site	84	(-)	GRWAAW	S000198
GT1CONSENSUS	site	20	(-)	GRWAAW	S000198
GT1CONSENSUS	site	64	(-)	GRWAAW	S000198
GT1CONSENSUS	site	65	(-)	GRWAAW	S000198
GT1GMSCAM4	site	20	(-)	GAAAAA	S000453
GT1GMSCAM4	site	64	(-)	GAAAAA	S000453
MARTBOX	site	57	(+)	TTWTWTTWTT	S000067
MARTBOX	site	110	(+)	TTWTWTTWTT	S000067
MYB1AT	site	99	(-)	WAACCA	S000408
MYB2AT	site	42	(-)	TAACTG	S000177
MYB2CONSENSUSAT	site	42	(-)	YAACKG	S000409
MYBATRD22	site	99	(-)	CTAACCA	S000175
MYBCORE	site	42	(+)	CNGTTR	S000176
POLASIG3	site	109	(-)	AATAAT	S000088
POLLEN1LELAT52	site	86	(-)	AGAAA	S000245
PYRIMIDINEBOXHVEPB1	site	63	(+)	TTTTTTCC	S000298
REALPHALGLHCB21	site	98	(-)	AACCAA	S000362
ROOTMOTIFTAPOX1	site	59	(+)	ATATT	S000098
ROOTMOTIFTAPOX1	site	127	(-)	ATATT	S000098
SORLIP1AT	site	38	(+)	GCCAC	S000482
TATABOX5	site	110	(+)	TTATTT	S000203

Appendix Table 3 The presence of *cis*-acting elements in *Den-ERS1* intron II using PLACE Database

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     Factor or Site Name         Loc.(Str.)       Signal Sequence             SITE #
____________________________________________________________________________________
-10PEHVPSBD               site     50  (+)  TATTCT                             S000392
-10PEHVPSBD               site    148  (-)  TATTCT                             S000392
-300ELEMENT               site    131  (-)  TGHAAARK                           S000122
ARR1AT                    site     60  (+)  NGATT                              S000454
ARR1AT                    site     84  (+)  NGATT                              S000454
ARR1AT                    site    118  (+)  NGATT                              S000454
BIHD1OS                   site     89  (-)  TGTCA                              S000498
CAATBOX1                  site     46  (+)  CAAT                               S000028
CAATBOX1                  site     82  (-)  CAAT                               S000028
CACTFTPPCA1               site    162  (+)  YACT                               S000449
CATATGGMSAUR              site    103  (+)  CATATG                             S000370
CATATGGMSAUR              site    103  (-)  CATATG                             S000370
CPBCSPOR                  site     34  (-)  TATTAG                             S000491
CURECORECR                site    176  (+)  GTAC                               S000493
CURECORECR                site    176  (-)  GTAC                               S000493
DOFCOREZM                 site    131  (-)  AAAG                               S000265
DOFCOREZM                 site    167  (-)  AAAG                               S000265
EBOXBNNAPA                site    103  (+)  CANNTG                             S000144
EBOXBNNAPA                site    103  (-)  CANNTG                             S000144
GT1CONSENSUS              site    108  (+)  GRWAAW                             S000198
GT1CONSENSUS              site    112  (-)  GRWAAW                             S000198
MYCCONSENSUSAT            site    103  (+)  CANNTG                             S000407
MYCCONSENSUSAT            site    103  (-)  CANNTG                             S000407
POLASIG3                  site     48  (-)  AATAAT                             S000088
SEF4MOTIFGM7S             site     77  (-)  RTTTTTR                            S000103
WBOXATNPR1                site     88  (+)  TTGAC                              S000390
WRKY71OS                  site     89  (+)  TGAC                               S000447
```

Appendix Table 4 The presence of *cis*-acting elements in *Den-ERS1* 5'flanking region using PlantCARE Database

Box I

Site Name	Organism	Position	Strand	Matrix score.	sequence
Box I	Pisum sativum	254	+	7	TTTCAAA

Function light responsive element

CAAT-box

Site Name	Organism	Position	Strand	Matrix score.	sequence
CAAT-box	Glycine max	42	+	5	CAATT
CAAT-box	Brassica rapa	148	-	5	CAAAT
CAAT-box	Glycine max	57	+	5	CAATT
CAAT-box	Hordeum vulgare	247	-	4	CAAT
CAAT-box	Arabidopsis thaliana	56	+	5	CCAAT
CAAT-box	Brassica rapa	59	-	5	CAAAT

Function common cis-acting element in promoter and enhancer regions

ERE

Site Name	Organism	Position	Strand	Matrix score.	sequence
ERE	Dianthus caryophyllus	253	+	8	ATTTCAAA

Function ethylene-responsive element

GARE-motif

Site Name	Organism	Position	Strand	Matrix score.	sequence
GARE-motif	Brassica oleracea	223	+	7	TCTGTTG

Function gibberellin-responsive element

GC-motif

Site Name	Organism	Position	Strand	Matrix score.	sequence
GC-motif	Zea mays	34	+	6	CCCCCG

Function enhancer-like element involved in anoxic specific inducibility

Skn-1_motif

Site Name	Organism	Position	Strand	Matrix score.	sequence
Skn-1_motif	Oryza sativa	70	+	5	GTCAT

Function cis-acting regulatory element required for endosperm expression

Sp1

Site Name	Organism	Position	Strand	Matrix score.	sequence
Sp1	Oryza sativa	8	-	6	GGGCGG

Function light responsive element

TATA-box

Site Name	Organism	Position	Strand	Matrix score.	sequence
TATA-box	Lycopersicon esculentum	45	+	5	TTTTA
TATA-box	Arabidopsis thaliana	319	+	4	TATA
TATA-box	Glycine max	48	+	5	TAATA

Function core promoter element around -30 of transcription start

Appendix Table 5 The presence of *cis*-acting elements in *Den-ERS1* intron I using PlantCARE Database

CAAT-box

Site Name	Organism	Position	Strand	Matrix score.	sequence
CAAT-box	Brassica rapa	71	+	5	CAAAT

Function common cis-acting element in promoter and enhancer regions

MBS

Site Name	Organism	Position	Strand	Matrix score.	sequence
MBS	Arabidopsis thaliana	38	-	6	TAACTG

Function MYB binding site involved in drought-inducibility

TATA-box

Site Name	Organism	Position	Strand	Matrix score.	sequence
TATA-box	Arabidopsis thaliana	9	-	5	TATAA
TATA-box	Arabidopsis thaliana	54	+	4	TATA
TATA-box	Avena sativa	48	+	12	TATATTTATATTT
TATA-box	Arabidopsis thaliana	118	-	5	TATAA
TATA-box	Arabidopsis thaliana	46	-	11	TATAAATATAAA
TATA-box	Lycopersicon esculentum	111	+	5	TTTTA
TATA-box	Arabidopsis thaliana	53	-	5	TATAA
TATA-box	Arabidopsis thaliana	119	+	4	TATA
TATA-box	Arabidopsis thaliana	10	+	4	TATA
TATA-box	Arabidopsis thaliana	47	-	5	TATAA

Function core promoter element around -30 of transcription start

TC-rich repeats

Site Name	Organism	Position	Strand	Matrix score.	sequence
TC-rich repeats	Nicotiana tabacum	79	+	9	ATTTTCTTCA

Function cis-acting element involved in defense and stress responsiveness

Appendix Table 6 The presence of *cis*-acting elements in *Den-ERS1* intron II using PlantCARE Database

CAAT-box

Site Name	**Organism**	**Position**	**Strand**	**Matrix score.**	**sequence**
CAAT-box	Glycine max	45	+	5	CAATT
CAAT-box	Brassica rapa	119	-	5	CAAAT
CAAT-box	Hordeum vulgare	81	-	4	CAAT
CAAT-box	Brassica rapa	139	-	5	CAAAT
CAAT-box	Glycine max	80	-	5	CAATT
CAAT-box	Brassica rapa	136	+	5	CAAAT
CAAT-box	Brassica rapa	85	-	5	CAAAT

Function common cis-acting element in promoter and enhancer regions

TATA-box

Site Name	**Organism**	**Position**	**Strand**	**Matrix score.**	**sequence**
TATA-box	Glycine max	34	+	5	TAATA
TATA-box	Brassica napus	156	+	6	ATATAT
TATA-box	Lycopersicon esculentum	131	+	5	TTTTA
TATA-box	Arabidopsis thaliana	159	+	4	TATA
TATA-box	Lycopersicon esculentum	76	-	5	TTTTA
TATA-box	Arabidopsis thaliana	157	+	4	TATA
TATA-box	Arabidopsis thaliana	155	+	8	TATATATA

Function core promoter element around -30 of transcription start

WUN-motif

Site Name	**Organism**	**Position**	**Strand**	**Matrix score.**	**sequence**
WUN-motif	Brassica oleracea	109	+	9	AAATTTCCT

Function wound-responsive element

CIRRICULUM VITAE

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AWARDS:

- Consolation prize from 7th national horticultural congress 2008, Oral Presentation in topic ‘A rapid and simple method for total nucleic acid isolation from high polyphenol and polysaccharide plants’