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Sequence Analysis of Ethylene Response Sensor Gene Isolated from *Vanda* Miss Joaquim Flower

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ABSTRACT

A specific ethylene receptor protein is a major key for ethylene perception into plant tissues. Thus, the Ethylene Response Sensor (*ERS*) gene was isolated from the ethylene-sensitive flowers of *Vanda* Miss Joaquim and its deduced ethylene response sensor (ERS) was aligned with three other reported orchid ERS genes to identify the specific divergence in orchid ethylene receptor proteins. The results from multiple alignment showed that the amino acid sequence of the *Vanda* ERS was 95% identical to *Phalaenopsis* ERS (the ethylene-sensitive species), but shared 90–91% identity with the ERS isolated from two ethylene-insensitive orchid species, *Dendrobium* hybrid Khao Sanan and *Oncidium* Gower Ramsey. The most conserved regions were located at the N-terminus of the polypeptide being three transmembrane hydrophobic regions; however, the most variable regions, particularly for the *Dendrobium* ERS and *Oncidium* ERS, were located in the ATP-binding site of the histidine kinase domain and the amino acid sequence of the downstream histidine kinase domain at the C-terminus. The findings suggest the sequence similarity in the ATP-binding site can be used to distinguish the closely related ERS proteins. In considering the level of *ERS* gene expression, there was an abundance of *ERS* transcript accumulation in fully opened *Vanda* Miss Joaquim flowers (particularly in the column and the lip followed by the perianth tissue) compared with root and leaf tissues.

Keywords: ethylene receptor, Vanda orchid, flower senescence

INTRODUCTION

Ethylene perception in plant tissue requires specific ethylene receptors localized in the endoplasmic reticulum membrane and a signal transduction pathway by phosphorylation to coordinate downstream responses via a unique mitogen-activated protein kinase (MAPK) cascade (Hall *et al*., 2007). When ethylene binds to the receptors, the receptor conformation changes and inhibits the action of receptor complexes between the ethylene receptors and constitutive triple response1 (CTR1) kinase which is a Raf-like MAPK kinase kinase and acts as a negative regulator of the ethylene responses to the downstream signaling component, ethylene insensitive2 (EIN2) kinase (Kieber *et al*., 1993; Huang *et al*., 2003; Ouaked *et al*., 2003). EIN2 is an endoplasmic reticulum membrane-localized protein that is cleaved to allow its C-term domain to transport to the nucleus when it is dephosphorylated in response to ethylene (Qiao *et al*., 2012). Then, the EIN2 signal is sent into the nucleus to activate a number of transcription factors including EIN3

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and ethylene insensitive-like (EIL). The EIN3/EIL activates a set of transcription factors called ethylene response factors (ERFs) or ethylene response element-binding proteins (EREBPs) to regulate the expression of target genes and induce the expression of other transcription factors in response to ethylene (Guo and Ecker, 2004).

In higher plants, five ethylene receptor genes—two ethylene responses (*ETR1* and *ETR2*), two ethylene response sensors (*ERS1* and *ERS2*) and one ethylene insensitive nucleolus membrane protein (*EIN4*)—have been successfully cloned from *Arabidopsis thaliana*. All of them shared amino acid similar to two bacterial components of the histidine kinases (Chang *et al.*, 1993; Hua *et al.*, 1995; Hua *et al.*, 1998; Sakai *et al.*, 1998). Consequently, they were classified into two subfamilies based on their protein structures. The N-terminal regions of the proteins in subfamily I, such as ETR1 and ERS1, contained three putative transmembrane subdomains and the C-terminus contained five conserved residues of the histidine kinase domains (Chang *et al.*, 1993). However, the N-terminal region at the first transmembrane subdomain of ETR2, EIN4 and ERS2 (members of subfamily II) lacked two conserved cystein residues whose function was still unclear (Chang *et al.*, 1993; Hua *et al.*, 1995).

The ethylene receptor gene of tobacco (*Nicotiana tabacum*), which is responsive to salt stress, has been defined for four hydrophobic regions of a GAF domain defined below (Aravind and Ponting, 1997), two kinase domains involved in the transmission of the signal to downstream components and a receiver domain (Zhang *et al.*, 2001). The GAF-binding domain which is named after some of the proteins in which it is found—cGMP-specific phosphodiesterases, adenylyl cyclases and a formate hydrogen lyase transcriptional activator—plays regulatory roles in catalytic activity via the binding of ligands such as nucleotides and small molecules (Aravind and Ponting, 1997). Recently, GAF has been identified in more than 7,400 proteins in a wide variety of signal transduction pathways in various tissues and organisms (Schultz, 2009). The ethylene receptor ETR1 from *Arabidopsis* contains a single GAF domain homologous to GAF in adenylate cyclase, histidine kinase, cGMP-specific phosphodiesterase, protein phosphatase and phosphotransferase proteins from various organisms (Aravind and Ponting, 1997). The latter two kinase domains were later defined as two subdomains—namely, the ATP-binding subdomain and the putative His phosphorylation subdomain (Hua *et al.*, 2006).

Most researchers (Guo and Ecker, 2004) have reported intensive study of ethylene receptor genes from arabidopsis, but the current study was interested in ethylene receptor genes from flowers and in particular, orchid flowers. A few ethylene receptor genes—namely, *ERS* genes belonging to subfamily I—have been isolated from orchid species, for example, *Phalaenopsis* (Do *et al.*, 1999), *Oncidium* (Huang *et al.*, 2007) and *Dendrobium* orchids (Suwanagul *et al.*, 2008; Thongkum *et al.*, 2009). The sequences and gene organizations of the homologous orchid *ERS1* were highly conserved (Lashbrook *et al.*, 1998; Suwanagul *et al.*, 2008). However, there are no data available for the ethylene receptor gene isolated from the most ethylene-sensitive species, *Vanda* Miss Joaquim, the national flower of Singapore. As the flowers of *Vanda* Miss Joaquim produce ethylene at a high rate and are very sensitive to ethylene, the removal of the pollinia causes severe color fading or the destruction of anthocyanin within 24 hr due to the release of endogenous ethylene (Akamine, 1963). The production of ethylene in *Vanda* Miss Joaquim flowers could reach the peak level of 3.442 nL.hr^{-1} per gram of flower at 32 hr after emasculation (Goh *et al.*, 1985). Therefore, the current study aimed to clone, sequence and analyze the ethylene receptor gene response to endogenous ethylene in the *Vanda* Miss Joaquim flower. In addition, the ethylene receptor amino acid sequences of *Vanda* Miss Joaquim were compared to ethylene receptor amino acid sequences of other orchid species that were both sensitive and insensitive. The findings will be important in suggesting the key divergent amino acid residues for distinctive ERSs from sensitive and insensitive orchid species.

MATERIALS AND METHODS

Total RNA extraction

Vanda Miss Joaquim plants (*Papilionanthe* Miss Joaquim, *Papilionanthe hookeriana* × *Papilionanthe teres*) were purchased from an orchid nursery, Pathum Thani, Thailand. The total RNA of *Vanda* was extracted from 200 mg of opened flower, leaf and root tissues by the lithium chloride precipitation method of Lievens *et al*. (1997).

Reverse transcriptase polymerase chain reaction

Total RNA (1 µg) was reverse transcribed to cDNA by Ready-To-Go You-Prime First-Strand Beads (GE Healthcare; Chalfont St Giles, UK) using two primers—namely, 5'ERS-R (5'-CCG CAT CTC ATG GTT CAT GAC-3') and 3'ERS-R (5'-CGA GTG TAC AAG CAC AGA TCC A-3')—according to the manufacturer's instructions. The 5′-end and the 3′-end *Vanda* cDNAs were obtained by amplifying the cDNA with two pairs of primers, the first pair being 5'ERS-F (5'-AAC CAT GGA AGG CTG -3') and 5'ERS-R and the second pair being 3'ERS-F (5'-GCT GTC ATG AAC CAT GAG ATG CGG AC-3') and 3'ERS-R. The reaction mixture (25 µL) consisted of 2.5 µL of 10× polymerase chain reaction (PCR) buffer, 7.5 µM of each primer (forward and reverse primers), 0.5 µL of i-Taq^{TM} DNA polymerase (iNtRON Biotechnology; Seongnam, Korea) and 50 ng of cDNA template. The generated DNA fragments were accomplished using the following conditions: denaturing for 5 min at 95 °C followed by 10 cycles of amplification with 30 s of denaturing at 95 °C, 1 min of annealing at 50 °C, 2 min of extension at 72 °C followed by 20 cycles of amplification with 30 s of denaturing at 95 °C, 1 min of annealing at 55 °C, 2 min of extension at 72 °C and a final extra extension step of 10 min at 72 °C at cycle completion. The PCR products were ligated into pGEM®-T Easy vector (Promega Co.; Medison, WI, USA) and then were transformed into *Escherichia coli* XL1-blue strain and the target DNA (pERSJ27) confirmed to be *ERS* by DNA sequencing. The full-length cDNA sequence data of the genes from the current study have been deposited at Genbank with GenBank ID: HQ540309 and AEE69544 for its nucleotide and deduced amino acid, respectively.

Primary structure analysis

The sequences of four orchid ERSs—*Phalaenopsis equestris* (GenBank accession number identification (ID:) CAD91247), *Oncidium* Gower Ramsey (GenBank ID: AAQ14309), *Dendrobium* Khao Sanan (GenBank ID: ACN22272), *Vanda* Miss Joaquim ERS (GenBank ID: AEE69544), and *Petunia hybrida* (GenBank ID: AAZ81984)—were retrieved from the protein database of NCBI (http://www.ncbi.nlm.nih.gov/). The sequences of ERSs were aligned using CLUSTALW (Thompson *et al.*, 1994) and the phylogenetic tree of plant ERS amino acid sequences was produced with the MEGA package version 5.1 and the neighbor-joining method (Tamura *et al.*, 2011).

Gene expression analysis by qualitative real-time polymerase chain reaction

To evaluate the *ERS* transcripts in the various tissues of *Vanda* Miss Joaquim, qualitative real-time PCR (qPCR) was performed according to Peirson *et al.* (2003), with *5.8S rRNA* being used as the reference gene. One microgram samples of total RNA from separate samples of the root, leaf, fully opened flower, lip, perianth and column of *Vanda* Miss Joaquim were reverse transcribed to cDNA with two primers—namely, RT-ACO(R) 5′−GCATCCATAAAGCACATTCTG−3′ and 5.8S rRNA(R) (5′-GCT TGA AGC CCA GGC AGA CG-3′). Using the SuperScript III One-Step Reverse Transcriptase Polymerase Chain

Reaction (RT-PCR) System with a Platinum® *Taq* DNA Polymerase kit (Invitrogen; Carlsbad, CA, USA), the 380 bp of *ERS* and 198 bp *5.8S rRNA* were together generated by two pairs of primers, with the first being RT−ERS(F) (5′−GTTTGGTGCCTTCATTGTTCTT−3′) and RT-ERS(R) for *ERS* and the second pair being 5.8S rRNA(F) (5′-ATG ACT CTC GAC AAT GGA TTT-3′) and 5.8S rRNA(R). Meanwhile, the standard curve of *ERS* gene copy numbers was constructed from five serial dilutions with final concentrations of 2.97×10^6, 1.39×10^5, 6.96×10^4, 2.37×10^4 and 2.97×10^3 copies of the cloned *ERS* gene in pERSJ27. Each orchid sample reaction containing 0.6 μL of cDNA template along with 7.5 μM primers in a final reaction volume of 10 μL was set up in triplicate to ensure the reproducibility of the results. The real-time PCRs were accomplished using the following conditions: denaturing for 5 min at 94 °C followed by 35 cycles of amplification with 20 s of denaturing at 95 °C, 15 s of annealing at 57 °C and 30 s of extension at 72 °C by Eppendorf Mastercycle® ep realplex real-time PCR (Eppendorf International; Hamburg-Eppendorf, Germany). At the end of the PCR run, a melting curve was generated and analyzed using the following conditions: denaturing for 15 s at 95 °C, 15 s at 60 °C and 15 s at 95 °C. The amounts of SYBR® green were examined by the realplex 1.5 software. The gene copy number in each sample was evaluated using Avogadro’s number (1 mol = 6.22×10^{23} molecules) according to de Bievere and Peiser (1992) based on the known molecular weight of the pERSJ27. The mean and standard deviation of the copy number of the genes were calculated. Statistical analysis at the 95% significance level was determined using one-way analysis of variance, and multiple comparisons were analyzed using Duncan’s multiple range test (Freund and Wilson, 2003).

RESULTS

Sequence characterization and gene organization analysis

A full-length clone of the *Vanda* ethylene receptor gene was constructed from two RT-PCR fragments—the 1,068 bp-5' region and 909 bp-3' region—of *Vanda* cDNAs generated from the total RNA of *Vanda* Miss Joaquim flowers at the fully opened stage. After searching for its homologous sequences to reported ethylene receptor genes in the database, the cDNA sequences of the *Vanda* ethylene receptor gene were 1,953 bp long containing one open reading frame of 1,899 bp predicted to encode a protein of 633 residues with an M_r (molecular weight of the protein) of 71.1 kDa. The cloned *Vanda* ethylene receptor gene was submitted to the GenBank database (ID: HQ540309) for the ethylene response sensor nucleotide [*Papilionanthe hookeriana* × *Papilionanthe teres*] with GenBank ID:AEE69544 for its deduced amino acid. Apart from the highly conserved sequence among orchid ERSs (90–95% amino acid identity), *Vanda* ERS shared 81% amino acid identity to the ERS of gladiolus (*Gladiolus* hybrid cultivar) with GenBank ID: BAD20704 and banana (*Musa acuminata* AAA Group) with GenBank ID: AAQ13533.

The predicted ERS protein sequence contained three putative functional regions, including a transmembrane hydrophobic region (residues 26–104), a GAF domain (residues 158–335) and two histidine kinase domains (residues 349–589) as shown in Figures 1A and 1B. The transmembrane region contained three putative transmembrane domains, designated I-III. The histidine-kinase-like region contained two domains, the first being histidine kinase A (HisKA), the phosphoacceptor, located at residues 343–408 and the second being the HATPase-like domain, which was the ATP binding site, located at residues 455–586. However, the C-terminus of the *Vanda* ERS polypeptide lacked a receiver domain. Consequently, *Vanda* ERS was classified as a member of ethylene receptor subfamily I.

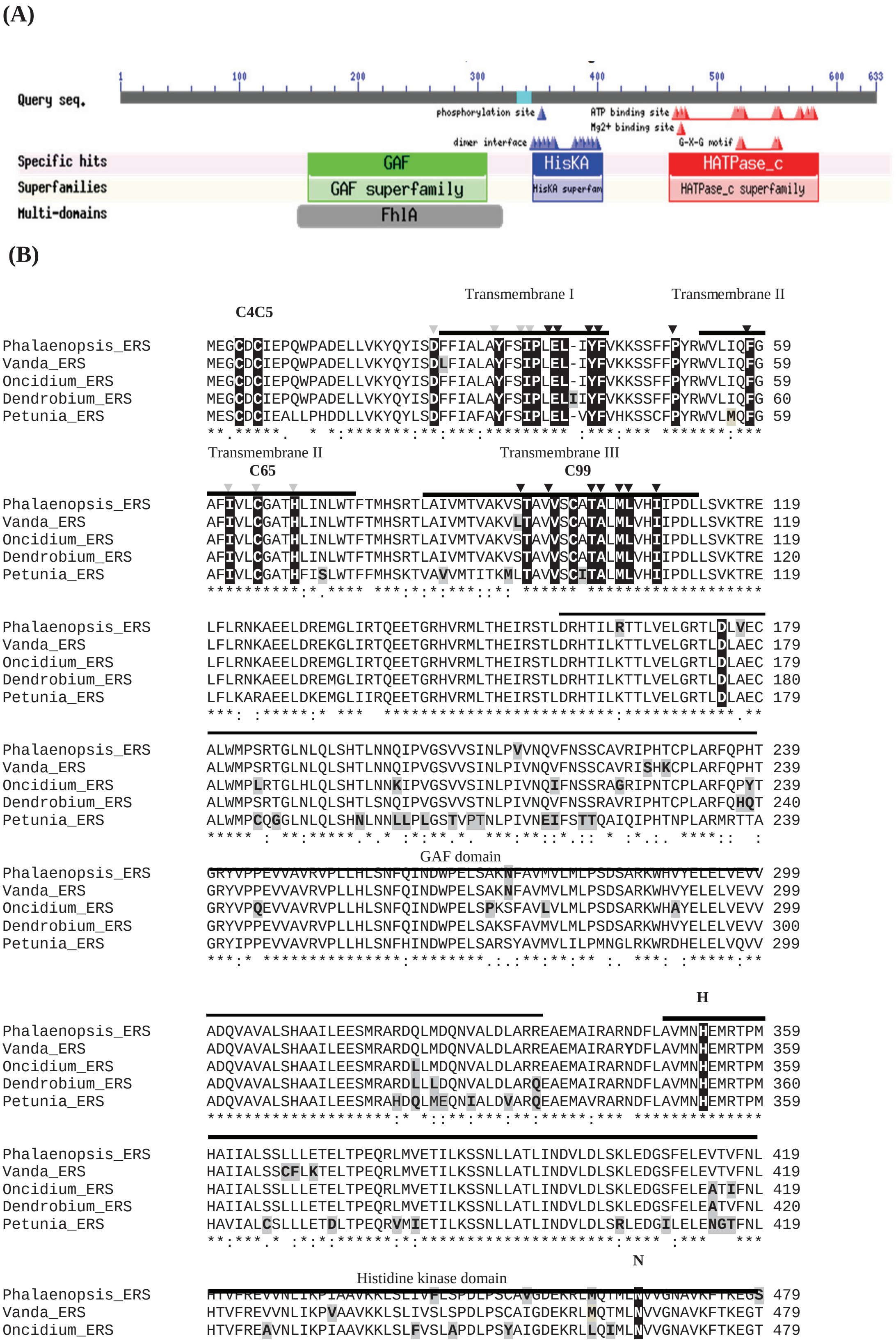
Amino acid sequence analysis of orchid ethylene response sensor polypeptides

The multiple alignment between the ERS amino acid sequences of *Vanda* and the other three orchid species revealed that the *Vanda* ERS was 95, 91, 90 and 70% identical to the ERS of *Phalaenopsis equestris, Dendrobium* Khao Sanan, *Oncidium* Gower Ramsey and the out group sample of the *Petunia* hybrid cultivar, respectively. The most conserved region was that of the three transmembrane domains (I–III) located at the N-terminus of the polypeptide, while the most variable region was found near the C-terminus downstream of the HATPase-like domain (Figure 1).

The sequences in the putative transmembrane region at the N-termini of the ERSs of the four orchids were the most highly conserved at the level of 99.6% identity. In considering the amino acid sequences in this transmembrane region of *Vanda* ERS, three hydrophobic conserved domains were found and defined as transmembrane I (^{26}LFIALAYFSIPLELIYFV^{43}), transmembrane II (^{53}WVLIQFGAFIVLCGATHLINLWT^{75}) and transmembrane III (^{82}LAIVMTVAKVLTAVVSCATALML^{104}) based on *Arabidopsis* ERS (Hua *et al.*, 1998). There were only three residue variations—leucine (L) L26 and L93 of *Vanda* ERS and an extra isoleucine (I) I40 of *Dendrobium* ERS (Table 1)—that caused the differences in the composition of the ERS in the orchid. Seven crucial residues responding for ethylene binding (D25, Y32, I35, P36, I62, C65 and H69), together with 13 conserved residues for receptor isoforms (E38, L39, Y41, F42, P50, F58, T94, V97, T101, A102, M104, L105 and I108) that have been reported in *Arabidopsis* ERS (Wang *et al.*, 2006), were consistent in the orchid ERSs (Figure 1B). In addition, four cysteine (C) residues—C4, C6, C65 and C99—that were potentially involved in the formation of disulfide bridges were also discovered in this region.

The second most conserved region with 97.5% amino acid identity of orchid ERSs was predicted to be the GAF domain involved in a dimer formation located at residues 158–335. Surprisingly, in all orchid ERSs, there was no signature GAF-conserved motif of the amino acids Asn (N), Lys (K), Phe (F), Asp (D), and Glu (E)—namely, the NKFDE motif—which is commonly found in all GAF-domain-containing enzymes that have cyclic capacity as reported by Turko *et al*. (1996). However, compared with the other orchid ERSs, the greatest amount of amino acid diversity in this putative GAF domain was found in *Oncidium* ERS (Figure 1B and Table 1).

Further analysis was undertaken on HisKA, the phosphoacceptor, located at residues 343–408 and the HATPase-like domain, the ATP binding site, located at residues 455–586. The less conserved sequences that shared 94.9% amino acid identity within the orchid ERSs are known as histidine kinase domains located from the middle to the C-terminus of the orchid ERS polypeptides. This longest region composed of the HisKA domain and the HATPase-binding domain contains five identified conserved sequences in *Vanda* ERS—namely, one conserved consensus sequence of ^{349}AVMN$\underline{\text{H}}$FMRTPM^{359} located in the histidine kinaseA domain and another four conserved consensus sequences presented in the HATPase-binding domain having sequences of ^{459}RLMQTML$\underline{\text{N}}$VVGN^{460}, ^{514}QIKDT$\underline{\text{G}}$CGIS^{523}, ^{529}HV$\underline{\text{F}}$TKFA^{535}and ^{547}GS$\underline{\text{G}}$LGI^{552} based on the bacterial histidine kinases domain identified by Mukjang and Burns (2006). These conserved sequences are designated the H, N, G1, F, and G2 motifs, which represent the amino acid residues of His353, Asn466,Gly519, Phe531 and Gly549, respectively, located in the middle of each sequence. Further analysis of the homologous amino acid diversity in this histidine kinase region of each orchid ERS (Table 1 and Figure 1B) revealed that *Dendrobium* ERS and *Oncidium* ERS contained substantially more divergent amino acids than *Vanda* ERS and *Phalaenopsis* ERS in the HATPase-binding domain of the histidine kinase activity.



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Phalaenopsis_ERS     MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFG 59
Vanda_ERS            MEGCDCIEPQWPADELLVKYQYISDLFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFG 59
Oncidium_ERS         MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLEL-IYFVKKSSFFPYRWVLIQFG 59
Dendrobium_ERS       MEGCDCIEPQWPADELLVKYQYISDFFIALAYFSIPLELIIYFVKKSSFFPYRWVLIQFG 60
Petunia_ERS          MESCDCIEALLPHDDLLVKYQYLSDFFIAFAYFSIPLEL-VYFVHKSSCFPYRWVLMQFG 59
                     **.*****.  *  *:*******:**:***:********* :***:*** *******:***

Phalaenopsis_ERS     AFIVLCGATHLINLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRE 119
Vanda_ERS            AFIVLCGATHLINLWTFTMHSRTLAIVMTVAKVLTAVVSCATALMLVHIIPDLLSVKTRE 119
Oncidium_ERS         AFIVLCGATHLINLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRE 119
Dendrobium_ERS       AFIVLCGATHLINLWTFTMHSRTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRE 120
Petunia_ERS          AFIVLCGATHFISLWTFFMHSKTVAVVMTITKMLTAVVSCITALMLVHIIPDLLSVKTRE 119
                     **********:*.**** ***:*:*:*:***::*: ****** ******************

Phalaenopsis_ERS     LFLRNKAEELDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILRTTLVELGRTLDLVEC 179
Vanda_ERS            LFLRNKAEELDREKGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAEC 179
Oncidium_ERS         LFLRNKAEELDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAEC 179
Dendrobium_ERS       LFLRNKAEELDREMGLIRTQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAEC 180
Petunia_ERS          LFLKARAEELDKEMGLIIRQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAEC 179
                     ***: :*****:* *** ************************:************ .**

Phalaenopsis_ERS     ALWMPSRTGLNLQLSHTLNNQIPVGSVVSINLPVVNQVFNSSCAVRIPHTCPLARFQPHT 239
Vanda_ERS            ALWMPSRTGLNLQLSHTLNNQIPVGSVVSINLPIVNQVFNSSCAVRISHKCPLARFQPHT 239
Oncidium_ERS         ALWMPLRTGLHLQLSHTLNNKIPVGSVVSINLPIVNQIFNSSRAGRIPNTCPLARFQPYT 239
Dendrobium_ERS       ALWMPSRTGLNLQLSHTLSNQIPVGSVVSTNLPIVNQVFNSSRAVRIPHTCPLARFQHQT 240
Petunia_ERS          ALWMPCQGGLNLQLSHNLNNLLPLGSTVPTNLPIVNEIFSTTQAIQIPHTNPLARMRTTA 239
                     ***** : **:*****.*.* :*:**:*:.***:**::*.:: * :*.:. ****:: :

Phalaenopsis_ERS     ~~GRYVPPEVVAVRVPLLHLSNFQINDWPELSAKNFAVMVLMLPSDSARKWHVYELELVEVV~~ 299
Vanda_ERS            GRYVPPEVVAVRVPLLHLSNFQINDWPELSAKNFAVMVLMLPSDSARKWHVYELELVEVV 299
Oncidium_ERS         GRYVPQEVVAVRVPLLHLSNFQINDWPELSPKSFAVLVLMLPSDSARKWHAYELELVEVV 299
Dendrobium_ERS       GRYVPPEVVAVRVPLLHLSNFQINDWPELSAKSFAVMVLMLPSDSARKWHVYELELVEVV 300
Petunia_ERS          GRYIPPEVVAVRVPLLHLSNFHINDWPELSARSYAVMVLILPMNGLRKWRDHELELVQVV 299
                     ***:* ***************:********.:.:**:**:**.:. ***: :.*****:**

Phalaenopsis_ERS     ADQVAVALSHAAILEESMRARDQLMDQNVALDLARREAEMAIRARNDFLAVMNHEMRTPM 359
Vanda_ERS            ADQVAVALSHAAILEESMRARDQLMDQNVALDLARREAEMAIRARYDFLAVMNHEMRTPM 359
Oncidium_ERS         ADQVAVALSHAAILEESMRARDLLMDQNVALDLARREAEMAIRARNDFLAVMNHEMRTPM 359
Dendrobium_ERS       ADQVAVALSHAAILEESMRARDLLLDQNVALDLARQEAEMAIRARNDFLAVMNHEMRTPM 360
Petunia_ERS          ADQVAVALSHAAILEESMRAHDQLMEQNIALDVARQEAEMAVRARNDFLAVMNHEMRTPM 359
                     ********************:* *::**:***:**:*****:*** **************

Phalaenopsis_ERS     HAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEVTVFNL 419
Vanda_ERS            HAIIALSSCFLKTELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEVTVFNL 419
Oncidium_ERS         HAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEATIFNL 419
Dendrobium_ERS       HAIIALSSLLLETELTPEQRLMVETILKSSNLLATLINDVLDLSKLEDGSFELEATVFNL 420
Petunia_ERS          HAVIALCSLLLETDLTPEQRVMIETILKSSNLLATLINDVLDLSRLEDGILELENGTFNL 419
                     **:***.* :*:*:*:*****:*:*********************:**** :***     ***

Phalaenopsis_ERS     ~~HTVFREVVNLIKPIAAVKKLSLIVFLSPDLPSCAVGDEKRLMQTMLNVVGNAVKFTKEGS~~ 479
Vanda_ERS            HTVFREVVNLIKPVAAVKKLSLIVSLSPDLPSCAIGDEKRLMQTMLNVVGNAVKFTKEGT 479
Oncidium_ERS         HTVFREAVNLIKPIAAVKKLSLFVSLAPDLPSYAIGDEKRLLQIMLNVVGNAVKFTKEGT 479
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Dendrobium_ERS        HTVFREVVNLIKPIAAVKKLSVFVSLSPDLPSLAIGDEKRLIQTMLNVVGNAVKFTKEGS 480
Petunia_ERS           HGILREAVNLIKPIASLKKLSITLALALDLPVLAVGDAKRLIQTLLNVAGNAVKFAKEGH 479
                      * ::**.******:*::****: : *: ***  *:** ***:* :***.******:***

                                                             G1          F
Phalaenopsis_ERS      ISITASIAKPDSLRDPRDPEFYPIPSDGHFYLRVQIKDTGCGISPQELPHLFTKFAHAQN 539
Vanda_ERS             ISITASIAKPDSLRDPRDPEFYPSPTDGHFYLRVQIKDTGCGISPQELPHVFTKFAHAQN 539
Oncidium_ERS          VFITVNIAKPDSLRDP---DFYPAPTDGHFYLRVQVKDTGCGISPQELPHLFTKFAHTQN 536
Dendrobium_ERS        ISITATIAKSDSLRDSRDPEFHPIPSDGYFYLRVQVKDTGCGISPLELPRLFTKFAHTQN 540
Petunia_ERS           ISIEASVAKPEYARDSHPPEVYPMPSDGQFYLRVQVRDSGCGISPQDIPLVFTKFAEAQS 539
                      : *  ..:**.:  **.   :.:* *:** ******::*:****** ::* :*****.:*.

                               G2
Phalaenopsis_ERS      GSDKGYNGSGLGLAICKRFVNIMKGHIWPESEGIGKGCTTIFIVKLGISEDPAHRFQHKL 599
Vanda_ERS             GSDKGYNGSGLGLAICKRFVNLMKGHIWLESEGIGKGCTTIFIVKLGISEDSAFRYQHKL 599
Oncidium_ERS          GSDKGYSGSGLGLAICKRFVNLMKGHIWLESEGIGKGCTTIFIVKLGMSEDPTLRYQQKL 596
Dendrobium_ERS        GSYKGYTGSGLGLAICKRFVNLMKGRIWLESEGIGKGCTTIFIVKLGISEDPTLRYQQKL 600
Petunia_ERS           TSNRSTGGAGLGLAICRRFIQLMKGNIWIESEGLGKGTTVTFVVKLGVCNHPNAMPLLPT 599
                       * :.  *:*******:**:::***.** ****:*** *. *:****:.:..

Phalaenopsis_ERS      LPPIRAGQSEADAFGSKP-TPTDLIPLK--NRYQRSL 633
Vanda_ERS             LPPIRAAQSEADPFGSKP-MPKDLIPLK--NRYQRSL 633
Oncidium_ERS          LPPIRAGQAEADPFGLKPAAPKDELMPK--IRYQRSL 631
Dendrobium_ERS        LPPIPKDE-------------KNSIPSK--IRHQRSL 622
Petunia_ERS           VPRSRLNQGSDDLFRYRQFHGDDSAMSVTVQRYQRSL 636
                      :*     :              :        *:****
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Figure 1 (A) Diagram of four predicted conserved domains of ethylene response sensor (ERS) protein in *Vanda* Miss Joaquim. The black bar represents a polypeptide chain with one white area for a spacer. The boxes indicate four conserved domains of the transmembrane, GAF (so-called after its components which may be cGMP-specific phosphodiesterases, adenylyl cyclases or a formate hydrogen lyase transcriptional activator, FhlA) superfamily, histidine kinaseA (HisKA) domain and histidine ATPase (HATPase_C)-binding domain that are placed along the polypeptide.

(B) Amino acid sequence alignment of four orchid ERS polypeptides of *Dendrobium* Khao Sanan, *Oncidium* Gower Ramsey, *Phalaenopsis equestris*, *Vanda* Miss Joaquim compared with *Petunia* hybrid, as an out group sample. All the conserved residues are highlighted with white letters on a black background and the divergent residues are in light gray. The conserved domains of the transmembrane region together with crucial residues responding for ethylene binding (D25, Y32, I35, P36, I62, C65 and H69) (▼) and for receptor isoforms (E38, L39, Y41, F42, P50, F58, T94, V97, T101, A102, M104, L105 and I108) (▼); GAF domain for components of cGMP-specific phosphodiesterases, adenylyl cyclases or a formate hydrogen lyase transcriptional activator; and histidine kinase domain with sequence motifs (H, N, G1, F, and G2). The conserved motifs are denoted by the closed triangle symbols, in particular four residues for potential disulfide bridge formations (C4, C6, C65 and C99). Identical residues are represented by *, strongly homologous residues by :, weakly homologous residues by . and no consensus residue by -.

Table 1 List of divergent amino acid residues in three putative functional domains presented in ethylene response sensor proteins of four orchids.

	Functional domain		
	Transmembrane (78 amino acids)	GAF (178 amino acids)	Histidine kinase (241 amino acids)
Dendrobium	I40	H238, Q239, L323, L325 Q336,	A415, V442, F443, L453 T462, S480, T486, S490, T504, S506, Y509, L526 T538, Y543, T547
Oncidium		L185, K200, I217, G224 Y238, Q245, P270, L276, A290, L322	A414, I416, A426, F442 A446, Y452, L461, V480 F481, V484, N485, D496 A500, T534, S543, M584
Phalaenopsis	-	R164, V177, V213, N272	F444, V454, M461, I503, S505, I515, P568
Vanda	L26, L93	S227, K229, N272	C368, V433, M461, S503, I515, V530
Petunia	F30, V41, M56, F70, S72, V85, I89, M92, L93, I100	C185, G187, N196, L200, L201, L203, T206, P208 T209, E216, I217, T220 T221	N414, L452

GAF = so-called after its components which may be cGMP-specific phosphodiesterases, adenylyl cyclases or a formate hydrogen lyase transcriptional activator, FhlA.

Phylogenetic tree

The genetic relationships between the available orchid *ERS* genes were analyzed by aligning their protein sequences, either for the full proteins or the putative functional domains, as shown in Figure 2. The topology of all three phylogenetic relationships was well supported with the ERSs from the two ethylene-sensitive species, (*Vanda* Miss Joaquim and *Phalaenopsis equestris*) consistently grouped together. The phylogenetic tree generated from the amino acid sequences in the histidine kinase regions substantially exhibited a distinctive subgroup between the close-related ERS proteins from the ethylene-sensitive species (*Vanda* Miss Joaquim and *Phalaenopsis equestris*) and the ethylene-insensitive species (*Dendrobium* Khao Sanan and *Oncidium* Gower Ramsey). The petunia that is more distantly related is superfluous, since that is the meaning of its designation as an out-group.

Expression pattern of ethylene response sensor gene in *Vanda* Miss Joaquim

To evaluate the *ERS* mRNA transcript levels in the various *Vanda* orchid tissues, qPCR was carried out using the *ERS* gene cloned from *Vanda* flowers in pERSJ27 as a gene copy number standard and 5.8 *rRNA* used as an internal control. Higher expression of *ERS* transcripts was detected in the flower tissues (column, lip and perianth) than in the vegetative tissues (root and leaf) of *Vanda* orchids. In particular, the abundance of

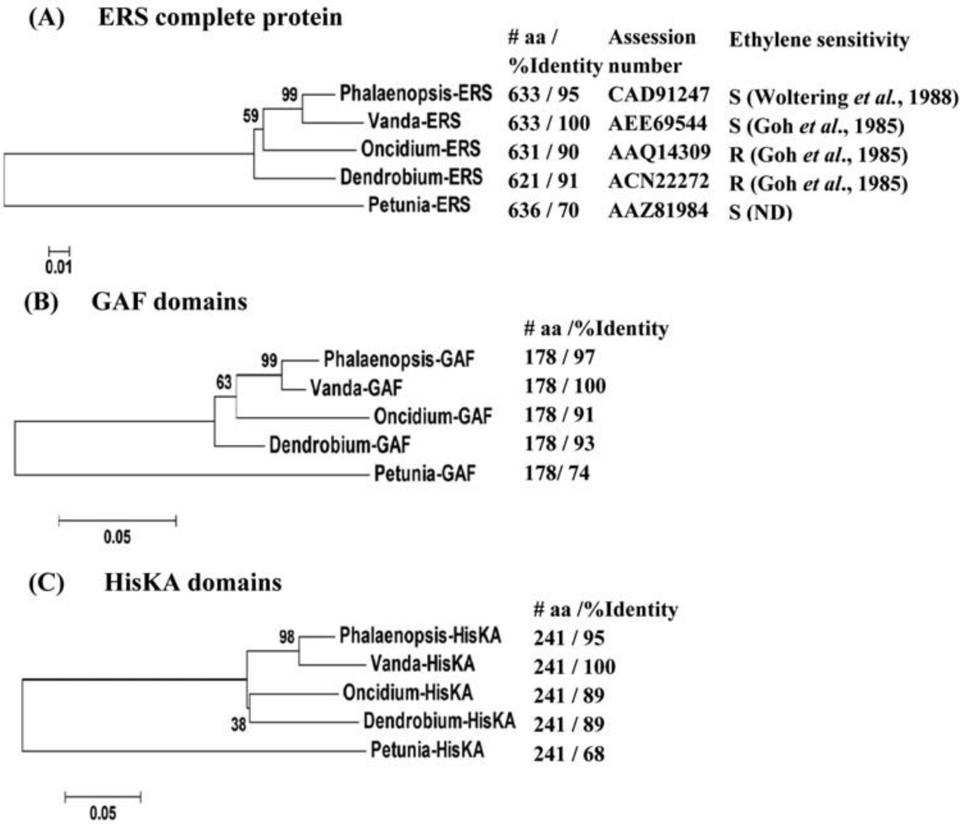

Figure 2 Neighbor-joining phylogenetic relationship of *Vanda* ethylene response sensor (ERS) to other orchid ERSs. The ERS polypeptides used in the construction were retrieved from the database as: (A) complete proteins; (B) GAF domain (so-called after its components which may be cGMP-specific phosphodiesterases, adenylyl cyclases or a formate hydrogen lyase transcriptional activator, FhlA); and (C) histidine kinaseA (HisKA) domain. The clustering was performed with 1,000 replicates for bootstrapping analysis using the MEGA package version 5.1 (Tamura *et al.*, 2011). The percentage of amino acid identity of ERSs and the level of sensitivity to ethylene of orchid flowers and the petunia flower are also presented. S and R denote sensitivity and insensitivity, respectively, to ethylene reaction of orchid flowers. The term # aa defines the number of amino acid residues.

ERS transcripts found in the fully opened flower (199.0 × 10^3 copies per microgram of total RNA) was significantly 2.7 and 2.2 fold greater than *ERS* transcripts in the leaf tissue (72.1 × 10^3 copies per microgram of total RNA) and root tissue (89.6 × 10^3 copies per microgram of total RNA), respectively (Figure 3). However comparable expression levels of the *ERS* transcripts were found in the column (168.9 × 10^3 copies per microgram of total RNA), lip (157.7 × 10^3 copies per microgram of total RNA) and perianth tissues (132.3 × 10^3 copies per microgram of total RNA). Similar results were obtained from the triplicate measurements. The mean and standard deviation of the copy number of each gene were calculated throughout and the differences were considered significant at the $P < 0.05$ level.

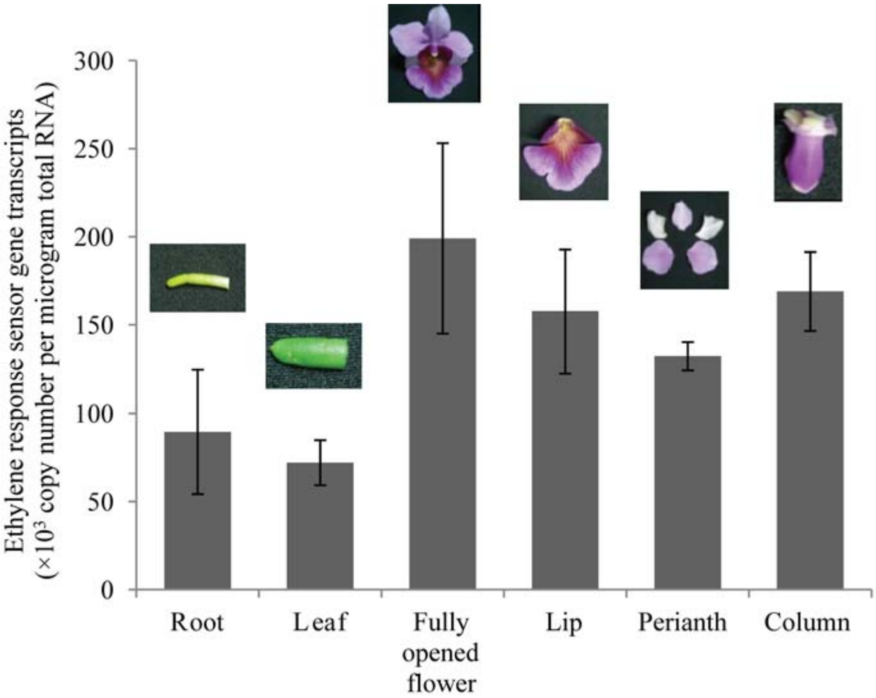

Figure 3 Expression analysis of ethylene response sensor gene transcripts in various tissues of *Vanda* Miss Joaquim evaluated by quantitative real time polymerase chain reaction with 5.8 *rRNA* used as an internal control. Error bars show the mean ± SD.

DISCUSSION

The orchid flowers showed a wide range of sensitivity to ethylene; *Vanda* Miss Joaquim was the most sensitive; *Cattleya, Cymbidium and Paphiopedilum* were less sensitive, while *Dendrobium and Oncidium* were insensitive, which agreed with the study of Goh *et al*. (1985). Therefore the ethylene receptor genes involved in the ethylene responses that were isolated in the current study were the same as those identified from *Dendrobium* (Suwanagul *et al*., 2008; Thongkum *et al*., 2009) and *Oncidium* species (Huang *et al*., 2007). In the current study, the full-length ethylene response sensor gene was successfully cloned from the mRNA extracted from the fully opened flowers of *Vanda* Miss Joaquim. The cloned *Vanda ERS* was classified as being from the ethylene receptor gene subfamily I based on its gene organization with three conserved transmembrane domains (Hua *et al*., 1998), the GAF domain (Martinez *et al*., 2005; Narumi *et al*., 2005) and histidine kinase (Marina *et al*., 2005) containing HisKA and HATPase-binding domains, and the lack of receiver domains at the C-terminus. The same protein organization is common in ERSs isolated from all orchid species, including *Phalaenopsis equestris* (Do *et al*., 1999), *Oncidium* Gower Ramsey (Huang *et al*., 2007) and *Dendrobium* orchids (Suwanagul *et al*., 2008; Thongkum *et al*., 2009) and some other monocotyledonous and also dicotyledonous plants, such as gladiolus (*Gladiolus* hybrid cultivar; Arora *et al*. (2006), rice (*Oryza sativa*; Mukjang and Burns (2006) and Arabidopsis (*Arabidopsis thaliana*; Wang *et al*. (2006), but different from petunia (*Petunia hybrid;* Wang and Kumar (2007).

The multiple alignment of four orchid ERSs from *Dendrobium* Kao Sanan (ACN22272), *Oncidium* Gower Ramsey (AAQ14309), *Phalaenopsis equestris* (CAD91247) and *Vanda* Miss Joaquim (AEE69544) illustrated that the sequences at the N-terminus up to the end of the transmembrane regions were in the most conserved region, while highly divergent residues were found in the middle region of both the GAF domain and the HATPase-binding domain of the histidine kinase regions. Three different homologous residues were found in the transmembrane regions—the I26 in transmemembrane I of *Vanda* ERS, the L93 in transmembrane III of *Vanda* ERS and an extra I40 in the transmemembrane I of *Dendrobium* ERS which had a similar position to I40 in a previous report (Thongkum *et al.*, 2009). However, it is unlikely that the I26, L93 and I40 divergent residues in this domain led to differences in function, since all the crucial residues involved in ethylene binding (Wang *et al.*, 2006), receptor isoforms (Wang *et al.*, 2006) and covalently linked disulfide bridges of homodimer (Schaller *et al.*, 1995; Huang *et al.*, 2007) were identical and extremely conserved in all orchid ERSs as shown in Figure 1A and 1B.

In considering the amino acid similarity in the unpredicted function of the putative GAF domain in orchid ERSs, there was no conserved signature NKX$_n$D motif for GAF-domain-containing enzymes that have the cyclic-nucleotide-binding capacity of the cGMP-binding site (Turko *et al.*, 1996) and cAMP-binging site (Martinez *et al.*, 2005). In addition, with regard to the high percentage of dissimilarity in the GAF regions among orchid ERSs (Table 1 and Figure 1B) and between the orchid ERSs and the GAF-conserved domains of smart0065 and pfam13492 (phytochrome and cGMP-specific phosphodiesterase) analyzed by Conserved Domain Database domain searching (Marchler-Bauer *et al.*, 2013; data not shown), the GAF domains of orchid ERSs are similar in domain architecture to phytochrome. This result agreed with a previous study on the *Arabidopsis* ETR1 (Aravind and Ponting, 1997) as highly conserved sequence similarity was found in the ERS1 and ETR1 (Hall *et al.*, 1999). However, the *Oncidium* ERS was predominantly divergent in the GAF domain, with twice as many divergent residues as the *Dendrobium* ERS and *Phalaenopsis* ERS and three times as many as *Vanda* ERS (Table 1). It could be suggested that the differences in the binding portion within the orchid ethylene receptors will be established if GAF plays an important role in being involved in binding capacity.

Further analysis is required of the histidine kinase domain containing HisKA (the phosphoacceptor which contains a conserved H residue and is activated via trans-autophosphorylation) and the HATPase-biding domain (ATP binding site). The orchid ERSs commonly shared five conserved motifs (H, N, G1, F, and G2 motifs) being the histidine kinase domain homologous to the bacterial histidine kinases domain (Mukjang and Burns, 2006). Four out of five consensus sequences located in the HATPase-binding domain (residues 455–586) of *Dendrobium* ERS and *Phalaenopsis* ERS had the most divergent amino acid residues (Table 1). This finding suggests that the amino acid composition in the HATPase-binding domain affects the genetic relationships between the four orchid ERSs. The clustering of the phylogenetic relationship constructed by multiple amino acid sequence alignment of the HATPase-binding domain was well established for the close relationship between *Vanda* ERS and *Phalaenopsis* ERS in terms of ethylene-sensitive types and the ERS ethylene-insensitive types of *Dendrobium* and *Oncidium* orchid species (Figure 2C)

The *Vanda ERS* gene isolated from *Vanda* Miss Joaquim flower was detected at different expression levels in various tissues of the *Vanda* Miss Joaquim orchid. High *ERS* expression was found mostly in *Vanda* flowers, particularly in the column tissues followed by the lip and perianth of fully opened flowers. The vegetative tissues of *Vanda*, both root and leaf, had much lower *ERS* expression. The similar patterns of *ERS* expressions were consistent with *ERS* expression in *Dendrobium* Pompadour, with the accumulation of *Dendrobium ERS1* transcripts being found in the lip, column and ovary both in the bud flower and opened flower (Thongkum *et al*., 2009). However, the *ERS* gene expression in the root that was substantially higher than in the leaf of *Vanda* suggested that the *Vanda ERS* transcripts may be involved in the establishment of aerial root cells of *Vanda* Miss Joaquim as well. However the expression pattern of *Oncidium ERS* encoding less amino acid identical to *Vanda* ERS was expressed abundantly in roots and flower buds, and to a lesser extent in pseudobulbs, leaves, and fully opened flowers in the *Oncidium* Gower Ramsey after pollinia dislodgment and the mRNA levels of *Oncidium ERS* initiated by exogenous ethylene treatments (Huang *et al*., 2007).

CONCLUSION

The full-length ethylene response sensor gene isolated from the ethylene-sensitive flowers of *Vanda* Miss Joaquim had a common gene organization with the conserved transmembrane region at the N-terminus, a GAF domain and the variable region of HATPase-binding domain of histidine kinase domains at the C-terminus. The most variable region of the HATPase-binding domain in the histidine kinase region was proposed as a representative sequence region for distinction among ERS relationships of closely related ERSs. In addition, the obtained *Vanda* ERS was highly specific and was expressed in flowers but not in root and leaf tissues.

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