CHAPTER 7
ISOLATION AND CHARACTERIZATION OF ETHYLENE RELATED GENES IN POLLINATED DENDROBIUM POMPADOUR

7.1 INTRODUCTION

In many flowers pollination causes an initial, dramatic increase in ethylene production in the stigma and style and a subsequent increase in ethylene production by other floral organs (Larsen et al., 1995; O'Neill et al., 1993; Zhang and O'Neill, 1993). Ethylene is synthesized by plants through the conversion of S-adenosyl-L-Met to ACC, which is then oxidized to ethylene (Adams and Yang, 1979). The former is catalyzed by the enzyme ACCS, while the latter is catalysed by the enzyme ACCO. The importance of ACCO in ethylene regulation was demonstrated in transgenic tomatoes with antisense copies of ACCO where ethylene production was greatly reduced (Hamilton et al., 1990). Studies on Phalaenopsis showed that the production of ACCO increased following pollination alongside the increase in ethylene (Nadeau et al., 1993). Thousands of genes have been cloned from different plants and plant tissues (for review, see Zarembinski and Theologis, 1994; Klee and Clark, 2010). The role of ACCS during pollination in orchids has also been investigated. Bui and O’Neill (1998) reported increase in ACCS production especially in the ovary. They hypothesized that a portion of the ACC synthesized in the ovary is available for translocation to other floral organs, especially the perianth. The control of these enzymes may also be key in controlling ethylene synthesis in climacteric plants. Thus, many biochemical studies have been carried out to characterize this protein.
In concert with these biochemical studies, this chapter looks into the molecular characterization of this important enzyme. To complete the process of ethylene regulation, ethylene is then perceived by a group of ethylene receptors. They function as negative regulators as the binding of ethylene results in the inactivation of receptor function. The first and most widely studied ethylene receptor is the Ethylene Receptor 1, (ETR1), reported in Arabidopsis (Bleeker et al., 1988 and Chang et al., 1993), tobacco (Knoester et al., 1998), rice (Yau and Yip, 1997) and Phalaenopsis (Do et al., 1999). Subsequently, ETR2, ERS1, ERS2 and EIN4 were also identified as the ethylene receptors involved in ethylene perception (Hua et al., 1995, 1998; Sakai et al., 1998; Harada et al., 2011). Ethylene receptor genes with homology to receptors found in Arabidopsis have also been identified in flowers such as carnation, rose, delphinium and geranium. All ethylene receptors across species are predicted to contain three N-terminal transmembrane domains, a GAF domain and a kinase domain.

The aim of this chapter is to characterize these important genes using bioinformatics tools. With the progress of bioinformatics, many programmes and tools have been developed to allow swift data generation, processing, analysis and storage. Various bioinformatics centers and programmes offer free of charge tools for application in research and academics. In this chapter, the main source for gene retrieval used is the National Center for Biotechnology (NCBI) while analysis of the genes are carried out using tools available at the ExPASy Proteomics Server maintained by the Swiss Institute of Bioinformatics.
7.2 MATERIALS AND METHOD

7.2.1 Plant Material

*D* Pompadour flowers were obtained from the glasshouse of University of Malaya. Flowers were hand-pollinated stigmas explained in Chapter 3. Individual flowers were cut at the proximal end of the peduncles in water and placed in 20 ml water vials containing distilled water.

7.2.2 Extraction of RNA

Total RNA was extracted using GeneTACG RNA extraction kit. Approximately 0.1 g of flower material was ground using a mortar and pestle under liquid nitrogen and 500µl of PRX extraction buffer was added. The lysate was mixed and vortexed thoroughly and transferred to a shearing tube and centrifuged at 13,000 rpm for two minutes. The flow through was collected and the volume measured. Approximately half volume of absolute ethanol was added and the solution was mixed by gentle pipetting. The mixture was then transferred to a mini column and centrifuged at 10,000 rpm for two minutes. The flow through was discarded and 500µl of WF wash buffer and tubes were centrifuged at 13,000 for 1 minute. Flow through was discarded and 700 µl of WS wash buffer was added and tubes were centrifuged at 13,000 rpm for one minute. Washing step with WS buffer was repeated. Tubes were then centrifuged at 13,000 rpm for three minutes to discard any left-over wash buffer. The mini column was then transferred to an elution tube and 30µl of nuclease free water was added to elute the RNA at full speed for two minutes. RNA was stored at -20 until further usage.

7.2.3 Primer Design

Primers were designed for the amplification of *ACCO, ACCS, ETR1, ERS1* and *ERS2*. When primers were designed from conserved regions of existing genes, multiple gene
sequences from a number of species were retrieved from the Genbank and subsequently aligned using the ClustalW tool available on the Expert Protein Analysis System (ExPASy) website: [www.expasy.org](http://www.expasy.org). Primers were then designed based on the conserved nucleotides of the multiple aligned sequences. The following primers were used for gene amplification using RT-PCR.

i) **ACCO primers**

**Forward primers**  
5’ CTGAATAAATATATGGCAGTACTG 3’

**Reverse primers**  
5’ CTTATAGCCTCCAGGGGTAAT 3’

ii) **ACCS primers**

**Forward primers**  
5’ TGCTCTCGAGATAGCTACTAACA 3’

**Reverse primers**  
5’ GATTGTCAAGAGCCAGGATCT 3’

iii) **ETRI primers**

**Forward primers**  
5’ GCGTGTTTGCCAGCAGCGGCGG 3’

**Reverse primers**  
5’ TGACCTGAATTGTGAGCCCTGA 3’

iv) **ERS1 primers**

**Forward primers**  
5’ TTAAGGGGGGCTTGTGTTT 3’

**Reverse primers**  
5’ TTAAGGGGGGCTTGTGTTT 3’

v) **ERS2 primers**

**Forward primers**  
5’ ATTTTAGCATTCGCTGAACTGA 3’

**Reverse primers**  
5’ TGCTGATTTTCGAGCGATA 3’
7.2.4 Preparation of RT-PCR Reaction Mixture

RT-PCR (PromegaAccessQuick™) was carried out using a reaction mixture summarized below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccessQuick™ Master Mix 2X</td>
<td>25 μl</td>
</tr>
<tr>
<td>Forward primer (5μg μl⁻¹)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse primer (5μg μl⁻¹)</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNA template (1μg μl⁻¹)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The reaction mixture above was thoroughly mixed and 1μl of AMV Reverse Transcriptase was added and mixed by gentle vortexing.

7.2.5 RT-PCR Amplification of Genes

RT-PCR was carried out using a GeneAmp 9600 Thermocycler (Perkin Elmer). Amplification cycles were carried out as described below:

Reverse transcription (1 cycle) 48°C for 45 minutes

PCR amplification:

Denaturation (1 cycle) 95°C for two minutes

Annealing (40 cycles)

95°C for 30 seconds
55°C for one minute
72°C for one minute

Extension (1 cycle) 72°C for seven minutes

Soak cycle (hold) 4°C overnight

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cDNA was then analysed on a 1% electrophoresis and sized using a GeneMass DNA ladder (Fermentas). Purification of cDNA was carried out using the Wizard SV Gel and PCR Cleanup System (Promega).

### 7.2.6 Agarose Gel Preparation

Electrophoresis of all the RT-PCR products obtained was carried out on a 1% (w/v) agarose gel in 1x TAE buffer. A solution of 250 ml of 1 % (w/v) agarose was made by adding 2.5 g of agarose to 5 ml of 50x TAE (242.1 g of Tris, 100 ml of 0.5 M EDTA, pH 8.0 adjusted with glacial acetic acid) boiled and made up with sterile deionized water to its final volume. The gel solution was then cooled down to 50°C and mixed with 2.5 µl of 10 µg µl⁻¹ ethidium bromide before pouring into a gel casting tray to solidify. The gel was electrophoresed in 2 L of 1x TAE running buffer containing 80-100µl ethidium bromide (10µg ul⁻¹).

### 7.2.7 Purification of RT-PCR product

RT-PCR product was gel purified using the Wizard® SV gel and PCR purification Clean-up system from Promega. The RT-PCR product was added to an equal volume of Membrane Binding Solution and the mixture was transferred to SV mini-column and centrifuged for five min at 12000 rpm. The column was then removed and transferred to a fresh Eppendorf tube and the purified product was eluted using the PCR Clean-Up system.

### 7.2.8 Sequencing and Characterization of Isolated cDNA

Purified cDNA obtained from RT-PCR was commercially sequenced by First Base Laboratories Sdn Bhd, Selangor, Malaysia.
7.2.9 Bioinformatic Analysis Tools

DNA sequences were analysed using bioinformatic tools available online.

7.2.9.1 Translate

Translated amino acid sequence was obtained using the Translate tool available at the ExPASy site. The output format included amino acid and nucleotide sequences.

7.2.9.2 Prosite

The finding of biologically relevant sites and signatures in a sequence was done using Prosite programme which allows scanning of protein sequences against PROSITE databases (Castro et al., 2006). Detection of these similar sites gives clues to the function of a sequence or part of a sequence, as highly conserved regions are likely to be active sites.

7.2.9.3 Pfam

This software allows for the detection of homologous regions between knowns sequences stored in the database and the query sequence. Pfam gives information regarding important domains of a sequence and subsequently the family it belongs to.

7.2.9.4 BLAST NCBI

BLAST NCBI was accessed via the NCBI (National Center for Bioinformatics USA) website: www.ncbi.com. The programme is widely used for searching DNA and protein databases for sequence similarities. Protein blast or blastp is used in this study to search a number of non-redundant protein databases (Swiss Prot, PDB, PIR, PRF) for similarities with the query sequence.
7.2.9.5 ProtParam

ProtParam is a software used in this study to analyze the physico-chemical properties of the sequence of interest (Gasteiger et al., 2005). In this chapter, the parameter analysed were molecular weight, theoretical PI, three major amino acids and protein stability.

7.2.9.6 ClustalW

ClustalW was used to perform multiple sequence alignment, which allows identification of conserved amino acid regions, and detects relationships between the proteins of interest (Chenna et al., 2003). ClustalW by default uses a number of symbols to denote the degree of conversion; “*” (all residues are identical), “:” (conserved substitutions observed) and “.” (semi-conserved substitutions observed). A distance matrix phylogenetic tree was generated from the resultant sequence alignment using the neighbour joining method.

7.2.9.7 Swiss Model

This is a server for automated comparative modelling of three dimensional (3D) protein structures (Schwede et al., 2003). This involved generating a set of structures representative of most of the possible folds for specific protein domains and then solving the structures for new proteins based on known fold-structure relationships.

7.2.9.8 Rasmol

This programme interactively displays the molecule on the screen in a variety of colour schemes and molecular representations. Molecules can be shown as wire frame bonds, cylinder Dreiding stick bonds, alpha-carbon trace, space filling, spheres and macromolecular ribbons.
7.3 Results

7.3.1 ACCO gene of D. Pompadour

7.3.1.1 RT-PCR results of ACCO gene of D. Pompadour

RT-PCR using specific primers for the ACCO gene resulted in a 928bp product. A band indicating the presence of the product was visible on a 1% agarose gel (Fig 7.1).

Fig 7.1 RT-PCR results with ACCO primers. In Lane 1 is the GeneRuler DNA ladder Mix (Fermentas). Lane 2 is the PCR product of 928bp ACCO gene analysed through 1% agarose gel. 10 μl of ACCO was loaded into the well while 5 μl of ladder was used.
7.3.1.2 Nucleotide and amino acid sequence of ACCO gene of D. Pompadour

Fig 7.2 shows the nucleotide sequence of ACCO and the corresponding amino acid. The forward and reverse primers are italicized in red.

```plaintext
1 CTGAATAACTATATGGCAGTACTGAGGGACGCCTGTGAGAACTGGGGCTT
51 TTTTTGCGACTTTTCAGGGCGACACTCTCTCCAGAGTTGATGGACAAAGTGG
101 AGCATATTAGTGAAGGTATATGCAAAATTACCCAGAGGACAATGAAAGCTTTATCAGGCGACACTCTCTCACGAGTTGGACAAAGTGACACTTTACTCTCT
151 GATATATGCTAGCCATAACTCAGGAGTTTTTCTCGTAACACAACAGGTGATGGA
201 TATCGGGGACATCCACGTCTTAAGTGACAGCTTATAGACTTCATTCTTAC
251 TCGAAGAGCCCCAGAGTCCTGACGTCAATACAGAAAACTCGGGAAC
301 ATTCGCGGCACAAAATGAAAGCTTTATTACCTACCGCAAACTTCAACATC
351 GCGAAGAAGCTTGGACCAATCTAGAGGTCTCTGCGGCATACGGAAGC
401 GCTAAATGGAAGATCCGCAAACCTCTCTGAGACAAAGGTTGATCTAAATTACCT
451 ATGCCCGAAGCTTGGACCAATCTAGAGGTCTCTGCGGCATACGGAAGC
501 GTGAAATTCTATCTATTCTCTCAGGACGATACGATCTCCCGGGTTAACAATTG
551 TTGAGGAGATAAGAGTGGATTTGTAGATGTTCGCCAACCTGCTCACAGCGTTG
601 TCAGAATTCGCGGCACCAAACTAGAGCTTATACCATGGAAGTGGATGACA
651 GTGTCATGCACTGCTAATGCTCAGACAGATCGTAACCCGATCGTCAATA
701 GCCCTCTCTCTTAAACCCTGGTGTAGCTGATTTATATATCCGCTCCAC
751 ATTAGTGGAGatatattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
7.3.1.3 Homology of ACCO protein of D. Pompadour

BLAST homology search at www.ncbi.nlm.nih.gov/blast revealed the following match in the database:

1. 567/799 positive identities (71%) (4e-103) with ACCO of Dianthus caryophyllus (a)
2. 482/681 positive identities (71%) (3e-85) with ACCO of Nicotiana tabacum (b)
3. 486/691 positive identities (70%) (2e-80) with ACCO of Hevea brasiliensis (c)
4. 489/698 positive identities (70%) (7e-81) with ACCO of Prunus persica (d)
5. 492/698 positive identities (70%) (2e-80) with ACCO of Fragaria ananassa (e)
6. 478/681 positive identities (70%) (8e-80) with ACCO of Nicotiana suaveolens (f)
7. 477/675 positive identities (71%) (3e-78) with ACCO of Lactuca sativa (g)
8. 487/698 positive identities (70%) (3e-78) with ACCO of Prunus domestica (h)
9. 448/629 positive identities (71%) (4e-77) with ACCO of Antirrhinum majus (i)
10. 470/686 positive identities (69%) (3e-73) with ACCO of Solanum lycopersicum (j)
(b) Accession HQ418208      Moniuszko et al., 2010.
(c) Accession AY207387      Kongsawadworakul and Chrestin, 2002.
(d) Accession AF319166      Callahan et al., 1993.
(e) Accession AY706156      Kiss et al., 2006.
(f) Accession DQ984136      Roeder et al., 2009.
(g) Accession AB158345      Takahashi et al., 2010.
(h) Accession AJ890086      Fernandez et al., 2002.
(j) Accession AK324411      Aoki et al., 2010.
7.3.1.4 Features of ACCO protein of *D. Pompadour*

Analysis with Prosite resulted in the detection of four different sites; N-glycosylation, protein kinase C phosphorylation, Casein kinase II phosphorylation and Myristoylation. These four sites are found in almost all ACCO protein sequences found in the data bank, the difference being only in the number of each pattern site. Table 7.1 summarizes the sites, position of residues corresponding to the sites, the consensus pattern and the number of sites detected in ACCO.


<table>
<thead>
<tr>
<th>Site</th>
<th>Residue</th>
<th>Consensus Pattern</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glycosylation</td>
<td>86 - 89 NISK</td>
<td>of N-{P}-[ST]-{P}</td>
<td>One</td>
</tr>
<tr>
<td>Protein kinase C phosphorylation</td>
<td>40 - 42 SeR</td>
<td>[ST]-x-[RK]</td>
<td>One</td>
</tr>
<tr>
<td>Casein kinase II phosphorylation</td>
<td>90 - 93 SpeD 254 - 257 TlvE</td>
<td>[ST]-x(2)-[DE]</td>
<td>Two</td>
</tr>
<tr>
<td>Myristoylation</td>
<td>61 - 66 GNfeNT 125 - 130 GLekAY 137 - 142 GAngCN 140 - 145 GCnpTF 146 - 151 GTkvSN 232 - 237 GNrmSI 315 - 320 GgypGD</td>
<td>G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}</td>
<td>Seven</td>
</tr>
</tbody>
</table>
7.3.1.5 Pfam analysis of ACCO protein of D. Pompadour

Pfam analysis of ACCO protein showed the presence of Fe(2+) 2-oxoglutarate dioxygenase domain profile from positions 143 – 243 amino acids. Enzymes with the Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase domain typically catalyse the oxidation of an organic substrate using a dioxygen molecule, mostly by using ferrous iron as the active site cofactor and 2OG as a cosubstrate which is decarboxylated to succinate and CO₂. Fig 7.4 shows the amino acid sequence of the Fe(2+) 2-oxoglutarate dioxygenase domain in ACCO protein sequence. Residues conserved in the domain were detected which are Y, H, D, H and R.
Fig 7.3 Results from Pfam analysis show the information on the Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase domain. The #HMM line shows the consensus of the model, with capital letters representing the most conserved (high information content) positions, and dots (.) indicating insertions in the query sequence. Identical residues are coloured cyan, and similar residues are coloured dark blue. The #MATCH line indicates matches between the model and the query sequence, where a + indicates a “conservative substitution”; the #PP line represents the expected accuracy (posterior probability) of each aligned residue, where a 0 means 0–5%, 1 means 5–15%, and so on to 9 meaning 85–95% and a * meaning 95–100% posterior probability (pp); the #SEQ line is the query sequence, coloured according to the pp for each residue match on a scale from bright green for * through paler green and pale red down to bright red for 0. The Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase in the deduced ACCO protein is highlighted in yellow.
7.3.1.6 ProtParam analysis of ACCO protein of D. Pompadour

Table 7.2 summarises the physical and chemical properties of ACCO as analysed using the ProtParam programme accessed via the ExPASy website. The results show that ACCO protein of D. Pompadour share similar properties with other species. These include molecular weight within the range of 30-37 kDa and a theoretical PI that is acidic. The major amino acids in the ACCO protein of D. Pompadour, leucine and lysine are also major amino acids in Dtps sp, P. sp, D. caryophyllus and Rosa cultivar.

Table 7.2 Summary of protein parameters of ACCO protein analysed using ProtParam

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>PI</th>
<th>Major amino acid composition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrobium</em> Pompadour</td>
<td>321</td>
<td>36696.8</td>
<td>5.7</td>
<td>Leu (9%)</td>
<td>Stable</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lys (7.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Asp (6.2%)</td>
<td></td>
</tr>
<tr>
<td><em>Doritaenopsis</em> sp</td>
<td>317</td>
<td>36171.3</td>
<td>5.8</td>
<td>Leu (11%)</td>
<td>Unstable</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu (10.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lys (7.6%)</td>
<td></td>
</tr>
<tr>
<td><em>Phalaenopsis</em> sp</td>
<td>325</td>
<td>37131.4</td>
<td>5.2</td>
<td>Leu (10.5%)</td>
<td>Unstable</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu (9.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lys (6.8%)</td>
<td></td>
</tr>
<tr>
<td><em>Dianthus</em> caryophyllus</td>
<td>321</td>
<td>36819.2</td>
<td>6.03</td>
<td>Leu (9.3%)</td>
<td>Stable</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lys (8.7%)</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu (7.5%)</td>
<td></td>
</tr>
<tr>
<td><em>Rosa</em> cultivar</td>
<td>277</td>
<td>31663.1</td>
<td>5.12</td>
<td>Leu (10.5%)</td>
<td>Stable</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu (9.0%)</td>
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<td></td>
<td></td>
<td></td>
<td>Lys (8.7%)</td>
<td></td>
</tr>
</tbody>
</table>
7.3.1.7 Multiple sequence analysis of ACCO protein of D. Pompadour

Multiple sequence alignment of D. Pompadour ACCO protein with ACCO protein from Rosa hybrid, D. caryophyllus, Dtps. sp, and P. sp. and D. crumenatum using the ClustalW program (Fig 7.4) revealed the presence of four conserved regions, each region containing 10 or more consecutive conserved residue. Within these conserve regions, a Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase domain was present. Five conserved residues were also present in this domain.

A phylogenetic analysis of the relationship of D. Pompadour ACCO protein with ACCO proteins from Rosa hybrid, D. caryophyllus, Dtps sp, and P. True Lady and D. crumenatum was also carried out using the ClustalW program and is presented in Fig 7.5. The phylogenetic tree derived showed that D. Pompadour ACCO is clustered with Dianthus caryophyllus and Rosa hybrid. However, the ACCO from D. Pompadour is more closely related to that of D. caryophyllus. ACCO proteins of Dtps. sp and P. True Lady share a very close relationship while D. Anna and D. crumenatum also share the same cluster. The ACCO protein from D. Pompadour seems to diverge in evolution as it belongs to a different cluster than the orchids.
Fig 7.4 Comparison of the deduced amino acid sequence of D. Pompadour ACCO and sequences encoding ACCO from P. True Lady (AF004662) Dtps. Sp (L37103), D. crumenatum (Q9ZQZ1), D. Anna (GQ332400) Rosa (AF441282), and D. caryophyllus (M62380) using ClustalW. Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase domain are highlighted while conserved regions are boxed. Residues conserved in the Fe(2+) and 2-oxoglutarate (2OG)-dependent dioxygenase domain are highlighted in yellow.
Fig 7.5 Phylogenetic tree generated from the CLUSTALW multiple alignment programme. Figure shows relationship between *D. Pompadour* ACCO protein and ACCO proteins from *D. Anna*, *D. crumenatum*, *Rosa* hybrid, *Dtps.* sp, *P. Lady* and *D. caryophyllus*. The numbers indicate the distance matrix.
7.3.1.8 Three dimensional (3D) structure of ACCO protein of *D. Pompadour*

Analysis using Swiss Model and Rasmol indicated that the *ACCO* protein sequence had enough functional domains to generate a 3D protein structure. The structure consisted of 286 groups, 2322 atoms and 2377 bonds. Furthermore, the programme also identified 205 H bonds, 11 helices, 15 strands and 23 turns.

![3D Structure of the ACCO protein as generated by Swiss Model and Rasmol programmes](image)

**Fig 7.6 3D Structure of the ACCO protein as generated by Swiss Model and Rasmol programmes.** The structure is viewed as ribbons where secondary structures are identified. The alpha helix is represented as a coiled structure and the beta strand is represented as a pleated structure. The colour is a smooth spectrum from blue through green, yellow and orange to red. The N termini of proteins are coloured blue and the C termini, red. This shows folding from one end of the ribbon to the other.
7.3.2 ACCS gene of *D. Pompadour*

7.3.2.1 RT-PCR results of ACCS gene of *D. Pompadour*

RT-PCR using specific primers for the ACCS gene resulted in a 1.32Kb product. A clear band indicating the presence of the product was visible on a 1% agarose gel (Fig 7.7).

![RT-PCR results with ACCS primers](image)

**Fig 7.7 RT-PCR results with ACCS primers.** In Lane 1 is the GeneRuler DNA ladder Mix (Fermentas). Lane 2 is the PCR product of 1.32Kb ACCS gene analysed through 1% agarose gel. 10 µl of ACCS was loaded into the well while 5µl of ladder was used.
7.3.2.2 Nucleotide and amino acid sequence of ACCS gene of *D. Pompadour*

Fig 7.8 shows the nucleotide sequence of ACCS and the corresponding amino acid. The forward and reverse primers are italicized in red.

```
1  GCTCTCGAAGATAGCTACTAACAATTCAACAGCTTTGCGCATTCTGCTTCAAA
TATATATCATACTTGAAGAATCTCAGAAATTGCGCCATATTCGAATTT
101 CGAAATAGCTGCTCTATTGACTCAAGGTAGGAGCGGTCTCTCATACTTCTG
151 GCCTGGAAGACCTATGAGGGTTAGCTTATGCTCTTGTGACTGCTTAC
201 TGAGAGTATTCTACGAGATATCTCCCTTCCCTCCCTGATATCGAAGCT
251 ACCCAGGAAGAGATGATGGTGCACGTGCTTGCGCATATCTCTGAT
301 AGAGGAGAAGACCTAAGGAGCTCAGAGGGTTAATTGCGGCTATACA
351 GTGTCGCGYCGAGGTCGAGATACGCTCTYCTGCTAGGTGGCTGTCA
401 ATCTCAAAAATACGGGCTCGGAGAATCTGCAGCTGCTTATATGAGAT
451 GAGTCCTAAATAACGTCAAGGGTAACGCTAAGGGGACCGCTGCTTAC
501 ACTCGGCTACATAGGTGATAAGGAAGACACTACGTACTGCGACTTT
551 TACCGAGGAAGAGTATCTATGCTACCTGAGGTAGGCTGCTATAT
601 GCCTTGCCGGAGAGAAGGAGGAGTACTTCTGCTGCAGACTCTTAT
651 GACATGATGAGGTGCTACTACTCTGCTGCTGCTTATATGAGAT
701 GTGTTCGCGCAGGAGAGATAGGGTACTTCTGCTGCTATATGAGAT
751 GGACATTCCAGAGTCAACATTAGTATTGACTATCTGCTATATGAGAT
801 CTTAAGGATAGATACTGTTGACTGCTATATGAGAT
851 GCTGTCAGATGAGGATTTACAATGAGATAGAGAGATAGAGAT
901 CTGAGAGAGAGATATGAATTGTTGAGTGCCAATGCTAGGTCTAATT
951 CTTGACCTTTCTCTTAAAACGCTGCGATGATATCTGCTATATGAGAT
1001 CTAGAGAGAGACTTATGGAATACCTTCTGCTGCAGACTCTTAT
1051 ACCGCTGTTTTATTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
1101 AACCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
1126 GCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
1127 AAACGTCTAAGAGCTCGTCTGCTGCTGCTGCTGCTGCTGCTG
1151 TAAAATCAATGATCTGCCTGCTGCTGCTGCTGCTGCTGCTGCTG
1201 GCTGTCAGATGAGGATTTACAATGAGATAGAGAGATAGAGAT
1251 CTGACTTCTCTATGATGAGATCTGCTGCTGCTGCTGCTGCTG
1301 GCCAGGATCCTATGATGAGATCTGCTGCTGCTGCTGCTGCTG

MCLPGRNIGAVLSDKATNQLAHHSPISPIYIHHLKLIEITIRRCRCHRKLWCLMLMESALD
TSLPGKRMKRLVRDLMIHELQFSDIANFQDYHGLFKFRQAIAHFMKLRAGGRVTF
DPESVMSAGATGNETVMFICADPGDVFLIPSPYYAARFDRLRRCRAIVFVRCS
SDNLKIAVDAAEACYAENKSNKVRNLILTNPSNPLGMLDKDTLTNLVRVTGRNIH
LVVDIEIAYATFVAGDFVSVNNVVDSEXNVLIVHSVYLSKDMGLPFRGVIVY
SFNDSVYSTTLELKKREECVQVSFLFLLKLRCSCCCLLCQMSRSLQDRIRERDMY
MGRKQSNAGLFWMDLRHLLDRNVSVEISELAIIIIDRVKINVSPGSSFRCTEPWVF
RICFANMMSLRCVIINDMRLNVSPGSSFDQEPGSEN
```

Fig 7.8 Nucleotide sequence of *ACCS* from *D. Pompadour* and the corresponding amino acid as translated using the Translate tool accessed via [www.expasy.org](http://www.expasy.org). Forward and reverse primers are printed in red.
7.3.2.3 Homology of ACCS protein of *D. Pompadour*

BLAST homology search at [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast) revealed the following match in the database:

1. 918/1041 positive identities (88%) (0.0) with *ACCS* of *Arabidopsis thaliana* (a)
2. 797/1042 positive identities (87%) (0.0) with *ACCS* of *Brassica oleracea* var. italic (b)
3. 231/269 positive identities (97%) (2e-57) with *ACCS* of *Doritaenopsis* sp (c)
4. 230/269 positive identities (96%) (9e-56) with *ACCS* of *Phalaenopsis* True Lady (d)
5. 232/295 positive identities (88%) (2e-39) with *ACCS* of *Dendrobium* Karen (e)
6. 356/500 positive identities (71%) (9e-56) with *ACCS* of *Pyrus pyrifolia* (f)
7. 215/271 positive identities (79%) (4e-41) with *ACCS* of *Dendrobium Anna* (g)
8. 214/270 positive identities (79%) (9e-37) with *ACCS* of *Dendrobium crumenatum* (h)
9. 339/478 positive identities (71%) (3e-29) with *ACCS* of *Pyrus communis* (i)
10. 428/637 positive identities (67%) (1e-48) with *ACCS* of *Rosa Kardinal* (j)
(a) Accession NM_179241 Liang et al., 1993.
(b) Accession AF338652 Gonzalez and Botella, 2001.
(c) Accession L07882 Bui and O’Neill, 1998.
(d) Accession AF004663 Huang et al., 1997.
(e) Accession EF488014 Warin et al., 2010.
(f) Accession EF566865 Qiao et al., 2007.
(g) Accession GU138671 Nagtong et al., 2009.
(h) Accession DCU64031 Yang et al., 1996.
(i) Accession AF386518l Sharkawi et al., 2004.
(j) Accession AY061946 Lei et al., 2002.
7.3.2.4 Features of ACCS of D. Pompadour

Analysis with Prosite resulted in the detection of seven different sites: Aminotransferases class-I pyridoxal-phosphate attachment, N-glycosylation, cAMP- and cGMP-dependent protein kinase phosphorylation, Protein kinase C phosphorylation, Amidation, Casein kinase II phosphorylation site and N-myristoylation. These four sites are found in almost all ACCS gene sequences found in the data bank, the difference being only in the number of each pattern site. Table 7.3 summarizes the sites, position of residues corresponding to the sites, the consensus pattern and the number of sites detected in ACCS.

Table 7.3 Protein sites detected in ACCS using the Prosite tool accessed via [www.expasy.org](http://www.expasy.org).

<table>
<thead>
<tr>
<th>Site</th>
<th>Residue</th>
<th>Consensus Pattern</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glycosylation</td>
<td>131 to 134 NETV 293 to 296 NDSV</td>
<td>N - {P} - [ST] - {P}</td>
<td>Two</td>
</tr>
<tr>
<td>cAMP- and cGMP-dependent protein kinase phosphorylation</td>
<td>351 to 354 RKQS</td>
<td><a href="2">RK</a> - x - [ST]</td>
<td>One</td>
</tr>
<tr>
<td>Protein kinase C phosphorylation</td>
<td>38 to 40 TIR 194 to 196 SNK 227 to 229 TRK 397 to 399 SFR</td>
<td>[ST] - x - [RK]</td>
<td>Four</td>
</tr>
<tr>
<td>Amidation</td>
<td>62 to 65 PGKR 349 to 352 MGRK</td>
<td>x - G - [RK] - [RK]</td>
<td>Two</td>
</tr>
<tr>
<td>Casein kinase II phosphorylation site</td>
<td>55 to 58 SALD 163 to 166 TGAE 173 to 176 SCSD</td>
<td>[ST] - x(2) - [DE]</td>
<td>Ten</td>
</tr>
</tbody>
</table>
7.3.2.5 Pfam analysis of ACCS protein of *D. Pompadour*

Pfam analysis of ACCS protein showed the presence of two Aminotransferases class-I and II pyridoxal-phosphate attachment site at positions 30-246 and 301-351 amino acids. Aminotransferases share certain mechanistic features with other pyridoxal-phosphate dependent enzymes, such as the covalent binding of the pyridoxal-phosphate group to a lysine residue. All residues conserved in the domains were detected which are G, N, P, G, D, Y, K, G, G, R and G.
Fig 7.9 Results from Pfam analysis show the information on the two Aminotransferases class-I and II pyridoxal-phosphate domains. The #HMM line shows the consensus of the model, with capital letters representing the most conserved (high information content) positions, and dots (.) indicating insertions in the query sequence. Identical residues are coloured cyan, and similar residues are coloured dark blue. The #MATCH line indicates matches between the model and the query sequence, where a + indicates a “conservative substitution”; the #PP line represents the expected accuracy (posterior probability) of each aligned residue, where a 0 means 0–5%, 1 means 5–15%, and so on to 9 meaning 85–95% and a * meaning 95–100% posterior probability (pp); the #SEQ line is the query sequence, coloured according to the pp for each residue match on a scale from bright green for * through paler green and pale red down to bright red for 0. The aminotransferases class-I and II pyridoxal-phosphate domains in the deduced ACCS protein is highlighted in yellow.
7.3.2.6 ProtParam analysis of ACCS protein of *D. Pompadour*

The table below summarizes the physical and chemical properties of ACCS. The results show that ACCS protein of *D. Pompadour* share similar properties with other species. These include molecular weight within the range of 44-51 kDa. The theoretical PI of ACCS that is basic is similar to ACCS from *A. thaliana*. The major amino acids in the ACCS protein of *D. Pompadour*, Leu and Ser also major amino acids in *A. thaliana*, *D. crumenatum*, *D. Karen*, *D. Anna* and *P. True Lady*.

Table 7.4 Summary of protein parameters of *D. Pompadour ACCS* protein analysed using ProtParam.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>PI</th>
<th>Major amino acid composition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrobium</em></td>
<td>443</td>
<td>49995.1</td>
<td>7.82</td>
<td>Leu (10.6%) Val (8.1%) Ser&amp;Arg (7.7%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>Pompadour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em></td>
<td>496</td>
<td>55531.5</td>
<td>7.20</td>
<td>Leu (9.1%) Ser (8.3%) Asp &amp; Val (7.3%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>thaliana</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dendrobium</em></td>
<td>435</td>
<td>48699.6</td>
<td>5.47</td>
<td>Leu (11.5%) Ser (9.2%) Glu (7.6%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>crumenatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dendrobium</em></td>
<td>435</td>
<td>48793.8</td>
<td>5.98</td>
<td>Leu (11.0%) Ser (9.2%) Ala (8.0%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>Karen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dendrobium</em></td>
<td>435</td>
<td>48528.7</td>
<td>5.90</td>
<td>Leu (11.5%) Ser (9.0%) Ala (8.0%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Anna</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phalaenopsis</em></td>
<td>445</td>
<td>49666.7</td>
<td>6.53</td>
<td>Leu (9.9%) Ser (9.7%) Ala (7.0%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>True Lady</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.3.2.7 Multiple sequence analysis of ACCS protein of *D. Pompadour*

Multiple sequence alignment of *D. Pompadour* ACCS protein with ACCS protein from *D. crumenatum, D. Karen, D. Anna, Dtps. Sp, P. true Lady, D. caryophyllus* and *A. thaliana* using the ClustalW program (Fig 7.10) revealed the presence of Aminotransferases class-I pyridoxal-phosphate attachment site.

A phylogenetic analysis of the relationship of *D. Pompadour* ACCS protein with ACCS proteins *D. crumenatum, D. Karen, D. Anna, Dtps. Sp, P. true Lady, D. caryophyllus* and *A. thaliana* was also carried out using the ClustalW program and is presented in Fig 7.11. The phylogenetic tree derived showed that the ACCS proteins were divided into three clusters. *D. Pompadour* ACCS was clustered with *D. crumenatum, Dtps sp, P. True Lady, D. caryophyllus and A. thaliana*. However, ACCS of *D. Pompadour* had the closest relationship with *A. thaliana*. 
Fig 7.10 Comparison of the deduced amino acid sequence of *D. Pompadour* ACCS and sequences encoding ACCS from *D. Karen* (EF488014), *D. Anna* (GU138671) *D. crumenatum* (DCU64031), *Dtps. sp* (L07882), *P. True Lady* (Z77854), *D. caryophyllus* (Q43753) and *A. thaliana* (Q06402). The aminotransferases class-I pyridoxal-phosphate attachment site is underlined. Residues conserved in aminotransferases are highlighted in yellow.
**Fig 7.11** Phylogenetic tree generated from the CLUSTALW multiple alignment programme. Figure shows the relationship between *D. Pompadour* ACCS protein and ACCS proteins from *D. Anna*, *D. Karen*, *D. crumenatum*, *Dtps. sp.*, *P. True Lady*, *D. caryophyllus* and *A. thaliana*. The numbers indicate the distance matrix.
7.3.2.8 Three dimensional (3D) structure of ACCS protein of D. Pompadour

Analysis using Swiss Model and Rasmol indicated that the ACCS protein sequence had enough functional domains to generate a 3D protein structure. The structure consisted of 420 groups, 3330 atoms and 3386 bonds. Furthermore, the programme also identified 270 H bonds, 18 helices, 14 strands and 43 turns.

Fig 7.12 3D Structure of the ACCS protein as generated by Swiss Model and Rasmol programmes. The structure is viewed as ribbons where secondary structures are identified. The alpha helix is represented as a coiled structure and the beta strand is represented as a pleated structure. The colour is a smooth spectrum from blue through green, yellow and orange to red. The N termini of proteins are coloured blue and the C termini, red. This shows folding from one end of the ribbon to the other.
7.3.3 ETR1 gene of D. Pompadour

7.3.3.1 RT-PCR results of ETR1 gene of D. Pompadour

RT-PCR using specific primers for the ETR1 gene resulted in a 692bp product. A clear band indicating the presence of the product was visible on a 1% agarose gel (Fig 7.13).

Fig 7.13 RT-PCR results with ETR1 primers. In Lane 1 is the MassRuler DNA ladder Mix (Fermentas). Lane 2 is the PCR product of 692bp gene analysed through 1% agarose gel. 10µl of ETR1 was loaded into the well while 5 µl of ladder was used.
7.3.3.2 Nucleotide and amino acid sequence of ETR1 gene of D. Pompadour

Fig 7.14 shows the nucleotide sequence of ETR1 and the corresponding amino acid sequence. The forward and reverse primers are italicized in red.

1  GCGTGTTTTGGCAGCAGCGCGCGGTTGCGCATTTCCCCCGCATCACGCTGGCG
51  CGCAGCCAGCCGACATACCCGCCGCTGCAGATCGCTGACGATTTACCCCGCATCAG
101  ATTTACCGCAGTTAACCAGCTGAGCAACTTTTCAAGATTAACAACAGCAACT
151  GGGCAGCGCGCGAAAAACTTTTGCAGGTTGATGGGATGCTGATGCTGCTCCCGGAC
201  GCAGCCGACGTTGGATGCTGATGCTGCTCCCGGACGTTGATGGGATGCTGATGCTG
251  GCAACAGCGCGCGGTTGCACTCTGAGCGATGCTGATGCTGCTCCCGGACGTTGATGG
301  GCGCGCAGCCGCGCAGCCGCGCAGCCGCGCAGCCGCGCAGCCGCGCAGCCG
351  CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
401  GTGATGATTACAGATGACAGATGACAGATGACAGATGACAGATGACAGATGACAGA
451  CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
501  AAAACCTGGAGACGACCGACGGACCGGACCGGACCGGACCGGACCGGACCGGACCGG
551  CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
601  CGGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
651  CGGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG

GAGCGCTGATTGTGAGCGT

ILGSVQSINLPVNRVFNSSRAVRIPTPHQLARSQPHGQDMPIIFRKFTQFNQLSNF
QINNSNWPSAKNFAVMMLPSHRDKWHYELVELELVADQVAVALSAAILEEESMR
ERDQLEMQVNAVLARREAMAIRARNDFAVMHEMTRTPMHIAILSSLLEETLTP
EQRLMVEILKSSNLLATLINDVLDLSKLEDGSFELEVTVFNLHTVFLMVNLKPIA
AVDPLSLIVSLSPD

Fig 7.14 Nucleotide sequence of ETR1 from D. Pompadour and the corresponding amino acid as translated using the Translate tool accessed via www.expasy.org. Forward and reverse primers are printed in red.
7.3.3.3 Homology of ETR1 protein of D. Pompadour

BLAST homology search at www.ncbi.nlm.nih.gov/blast revealed the following match in the database:

1. 369/533 positive identities (69%) (1e-50) with ETR1 of Phalaenopsis sp. 'True Lady' (a)
2. 274/380 positive identities (72%) (2e-49) with ETR1 of Brassica rapa subsp. Chinensis (b)
3. 275/382 positive identities (72%) (5e-49) with ETR1 of Pyrus pyrifolia (c)
4. 367/533 positive identities (69%) (7e-48) with ETR1 of Phalaenopsis equestris (d)
5. 274/382 positive identities (72%) (7e-48) with ETR1 of Malus x domestica (e)
6. 274/383 positive identities (72%) (8e-47) with ETR1 of Arabidopsis thaliana (f)
7. 306/434 positive identities (71%) (3e-46) with ETR1 of Prunus persica (g)
8. 300/425 positive identities (71%) (1e-45) with ETR1 of Petunia x hybrid (h)
9. 349/510 positive identities (68%) (2e-42) with ETR1 of Oncidium Gower Ramsey (i)
10. 354/517 positive identities (68%) (6e-42) ETR1 of Dendrobium Khao Sanan (j)
(a) Accession AF055894   Do et al., 1999.
(b) Accession GU296679   Liu and Zhu, 2010.
(c) Accession AB042108   Itai et al., 2004.
(f) Accession NM_105305   Swarbreck et al., 2011.
(g) Accession AF124527   Basset et al., 2002.
(i) Accession AF276237   Liu et al., 2000.
(j) Accession FJ628419   Thongkum et al., 2009.
7.3.3.4 Features of ETR1 protein of D. Pompadour

Analysis with Prosite resulted in the detection of four different sites; N-glycosylation, protein kinase C Phosphorylation, Casein Kinase C Phosphorylation and N-Myristoylation. Table 7.5 summarizes the sites, position of residues corresponding to the sites, the consensus pattern and the number of sites detected in ETR1.

### Table 7.5: Protein sites detected in ETR1 using the Prosite tool accessed via www.expasy.org.

<table>
<thead>
<tr>
<th>Site</th>
<th>Residue</th>
<th>Consensus Pattern</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glycosylation</td>
<td>211 - 214 NSSR</td>
<td>N-{P}-[ST]-{P}</td>
<td>One</td>
</tr>
<tr>
<td>Protein kinase C phosphorylation</td>
<td>115 - 117 SvK 143 - 145 TgR 212 - 214 SsR</td>
<td>[ST]-x-[RK]</td>
<td>Three</td>
</tr>
<tr>
<td>Casein kinase II phosphorylation</td>
<td>78 - 81 TahE 139 - 142 TqeE</td>
<td>[ST]-x(2)-[DE]</td>
<td>Two</td>
</tr>
<tr>
<td>N-myristoylation</td>
<td>135 - 140 GLirTQ 196 - 201 GSvqSI</td>
<td>G-{EDRKHPFYW}-x(2)- [STAGCN]-{P}</td>
<td>Two</td>
</tr>
</tbody>
</table>

7.3.3.5 Pfam analysis of ETR1 protein of D. Pompadour

Pfam analysis of ETR1 protein showed the presence of histidine kinase domain at positions 148-208 amino acids. Histidine kinase domains are signature domains present in all ethylene receptor genes and have similar characteristics to a two-component bacteria system. Residues conserved in this domain were detected which are H, E, P and L. A conservative substitution of L/M was also occurred.
Fig 7.15 Results from Pfam analysis show the information on the histidine kinase domain. The #HMM line shows the consensus of the model, with capital letters representing the most conserved (high information content) positions, and dots (.) indicating insertions in the query sequence. Identical residues are coloured cyan, and similar residues are coloured dark blue. The #MATCH line indicates matches between the model and the query sequence, where a + indicates a “conservative substitution”; the #PP line represents the expected accuracy (posterior probability) of each aligned residue, where a 0 means 0–5%, 1 means 5–15%, and so on to 9 meaning 85–95% and a * meaning 95–100% posterior probability (pp); the #SEQ line is the query sequence, coloured according to the pp for each residue match on a scale from bright green for * through paler green and pale red down to bright red for 0. Histidine kinase domain in the ETR1 protein is highlighted in yellow.
7.3.3.6 ProtParam analysis of ETR1 protein of D. Pompadour

The table below summarizes the physical and chemical properties of ETR1. The results show that ETR1 protein of D. Pompadour share similar properties with other species. The size of ETR1 fragment that is around 28 kDa is much smaller compare to ETR1 protein from the other species which range from 180 kDa to 22 kDa. The theoretical PI of D. Pompadour ETR1 that is acidic is similar to ETR1 from P. hybrid, D. Sonia, A. thaliana and O. Gower Ramsey. The major amino acids in the ETR1 protein of D. Pompadour, Thr is also the major amino acid in all the species analysed.

Table 7.6: Summary of protein parameters of D. Pompadour ETR1 protein analysed using ProtParam

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>PI</th>
<th>Major amino acid composition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrobium Pompadour</td>
<td>248</td>
<td>28374.9</td>
<td>6.44</td>
<td>Leu (10.5%) Val (8.9%) Thr (7.7%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>2574</td>
<td>213796.8</td>
<td>4.86</td>
<td>Thr (30.3%) Ala (28.3%) Gly (22.6%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>Dendrobium Sonia</td>
<td>2234</td>
<td>184879.2</td>
<td>4.88</td>
<td>Thr (28.8%) Ala (27.8%) Gly (23.6%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Phalaenopsis hybrid</td>
<td>2564</td>
<td>212449.3</td>
<td>4.91</td>
<td>Thr (29.8%) Ala (27.3%) Gly (23.7%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Oncidium Gower Ramsey</td>
<td>2409</td>
<td>199702.5</td>
<td>4.92</td>
<td>Thr (29.5%) Ala (28.2%) Gly (23.0%)</td>
<td>Stable</td>
</tr>
</tbody>
</table>
7.3.3.7 Multiple sequence analysis of *ETRI* protein of *D. Pompadour*

Multiple sequence alignment of *D. Pompadour* *ETRI* protein with *ETRI* from *P. hybrid*, *D. Sonia*, *O. Gower Ramsey* and *A. thaliana* using the ClustalW program (Fig 7.17) revealed the presence of a histidine kinase domain and three conserved regions. Residues conserved in a histidine kinase domain were also present.

A phylogenetic analysis of the relationship of *D. Pompadour* *ETRI* protein with *ETRI* proteins of *P. hybrid*, *D. Sonia*, *O. Gower Ramsey* and *A. thaliana* was also carried out using the ClustalW program and is presented in Fig 7.17. The phylogenetic tree derived showed that the proteins were divided into three clusters. *D. Pompadour* *ETRI* appeared to diverge in evolution since it shares the same cluster with and had the closest relationship to *A. thaliana*. 
Fig 7.16 Comparison of the deduced amino acid sequence of *D. Pompadour* ETR1 and sequences encoding ETR1 from *O. Gower Ramsey* (AF276233), *D. Sonia* (ABJ91124.1), *P. True Lady* (AF055894) and *A. thaliana* (AY174554). Histidine kinase domain is underlined while conserved regions are boxed. Conserved residues of histidine kinase domain is highlighted in yellow.
Fig 7.17 Phylogenetic tree generated from the CLUSTALW multiple alignment programme. Figure shows the relationship between D. Pompadour ETR1 protein and ETR proteins from P. hybrid, D. Sonia, O. Gower Ramsey and A. thaliana. The numbers indicate the distance matrix.
7.3.3.8 Three dimensional (3D) structure of ETRI protein of D. Pompadour

Analysis using Swiss Model and Rasmol indicated that the ETRI protein sequence had enough functional domains to generate a 3D protein structure. The structure consisted of 110 groups, 854 atoms and 864 bonds. Furthermore, the programme also identified 84 H bonds, 4 helices, and 4 turns.

Fig 7.18 3D Structure of the ETRI protein as generated by Swiss Model and Rasmol programmes. The structure is viewed as ribbons where secondary structures are identified. The alpha helix is represented as a coiled structure and the beta strand is represented as a pleated structure. The colour is a smooth spectrum from blue through green, yellow and orange to red. The N termini of proteins are coloured blue and the C termini, red. This shows folding from one end of the ribbon to the other.
7.3.4 *ERS1* gene of *D. Pompadour*

### 7.3.4.1 RT-PCR results of *ERS1* gene of *D. Pompadour*

RT-PCR using specific primers for the *ERS1* gene resulted in a 955bp product. A clear band indicating the presence of the product was visible on a 1% agarose gel (Fig 7.19).

**Fig 7.19 RT-PCR results with *ERS1* primers.** In Lane 1 is the GeneRuler DNA ladder Mix (Fermentas). Lane 2 is the PCR product of 955bp gene analysed through 1% agarose gel. 10µl of *ERS1* was loaded into the well while 5µl of ladder was used.
7.3.4.2 Nucleotide and amino acid sequence of \textit{ERS1} gene of \textit{D. Pompadour}

Fig 7.20 shows the nucleotide sequence of \textit{ERS1} and the corresponding amino acid. Both the forward and reverse primers are italicized in red.

\begin{verbatim}
1       TTAAGGGGGGGGGGGGCTTTGTTTTTCCATGCTGCCATCTTGAGGAATCC    51
        ATTCGGGCAAGGAGATCTCTCTCTTGTGATCGAATGTTGCTTTAGATTAG
101     CACGAGACGAGCTGGCCATTCTCGAGAGCAATGATTTTATAGCT
151     GTCATGAACATGAAATGCGGACTCCCCATGCAATCATTTGCCTTTTC
201     CTCCCTGCTCTTTGGAAACTGACTCCAGACGCAAGCGAGCTGGT
251     AAACCATCTTAAGATAGTAAGCTTGCGACCTAACTCAATATGAGG
301     TTAGACCTTCATAAAGCAGGATGGCGACGTTTCTGAGATTAGG
351     TTTCATTCTCTACTGCTATTTGAGTGGGACTTTTTTTTTTTT
401     CATACGCTTTAGGCGACCTAGTATTTATTTATTTATTTATTT
451     GCAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
501     ATATATATAGTCTGTAAGAAAATGGTACAGCAGCTTTACAGGG
551     GTGACTTTCCTCTCTTTGAACTGACTCCAGACGCAAGCGAGCT
601     AAGTTTTTAAAGAGGGTATGATATATCATATTACTCGACTATTT
651     CCATTACATATCCATTTGAAAGGATGAGCTTTGAGTTAGAGGCC
701     TTTCGTACGAGCAGAATCCGACGTTTATTTATTTATTTTTT
751     ATGGGCATTTCTATTTGAGTACAGTGACTACTGTAATGATTG
801     TTTCATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
851     GCAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
901     ATGGCTCTTTTACAAAAATTTGTGAGATAAAACCGAGACTACTCT
951     GTCCC
\end{verbatim}

Fig 7.20 Nucleotide sequence of \textit{ERS1} from \textit{D. Pompadour} and the corresponding amino acid as translated using the Translate tool accessed via \texttt{www.expasy.org}. Forward and reverse primers are printed in red.
7.3.4.3 Homology of ERS1 protein of D. Pompadour

BLAST homology search at [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast) revealed the following match in the database:

1. 900/917 positive identities (98%) (0.0) with ERS1 of *Dendrobium* hybrid KhaoSanan (a)
2. 360/365 positive identities (99%) (2e-176) with ERS1 of *Dendrobium* cv. 'Sonia' (b)
3. 337/364 positive identities (93%) (5e-146) with ERS1 of *Oncidium* cv. 'Gower Ramsey' (c)
4. 331/359 positive identities (92%) (3e-142) with ERS1 of *Phalaenopsis* equestris (d)
5. 332/359 positive identities (92%) (3e-142) with ERS1 of *Phalaenopsis* sp. 'KCbutterfly' (e)
6. 299/355 positive identities (84%) (6e-101) with ERS1 of *Gladiolus* hybrid cultivar (f)
7. 290/359 positive identities (81%) (3e-86) with ERS1 of *Musa acuminata* AAA (g)
8. 282/354 positive identities (80%) (7e-81) with ERS1 of *Lilium formosanum* x *Lilium* longiflorum (h)
9. 282/354 positive identities (70%) (5e-76) with ETR1 of *Prunus persica* (i)
10. 274/348 positive identities (79%) (8e-74) with ERS1 of *Actinidia* deliciosa (j)
(a) Accession FJ644936    Thongkum et al., 2009.
(b) Accession AY746972    Suwanagul et al., 2004.
(c) Accession AF276234    Liu et al., 2000.
(d) Accession AJ563284    Chang et al., 2005.
(e) Accession AF113541    Chai et al., 1998.
(f) Accession AB180247    Arora et al., 2006.
(g) Accession AB266315    Yamane et al., 2008.
(i) Accession AF396830    Bonghi et al., 2001.
(j) Accession EU170627    Yin et al., 2008.
### 7.3.3.4 Features of ERS1 protein of D. Pompadour

Analysis with Prosite resulted in the detection of four different sites; N-Myristolation, protein kinase C Phosphorylation, N-glycosylation and Casein Kinase II Phosphorylation. Table 7.7 summarizes the sites, position of residues corresponding to the sites, the consensus pattern and the number of sites detected in ERS1.

#### Table 7.7: Protein sites detected in ERS1 using the Prosite tool accessed via [www.expasy.org](http://www.expasy.org).

<table>
<thead>
<tr>
<th>Site</th>
<th>Residue</th>
<th>Consensus Pattern</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-myristoylation</td>
<td>2 - 7: GlrmCl</td>
<td>G - {EDRKHPFYW} - x(2) - [STAGCN]</td>
<td>Two</td>
</tr>
<tr>
<td></td>
<td>69-74: GCspSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase C phosphorylation</td>
<td>28 - 30:ScR</td>
<td>[ST] - x - [RK].</td>
<td>Seven</td>
</tr>
<tr>
<td></td>
<td>53 - 55:ScK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>59 - 61:SdR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>65 - 67:TtR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90 - 92:SaK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>103 - 105:SdR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>162 - 164:SdK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-glycosylation</td>
<td>47 - 50:NSTY</td>
<td>N - {P} - {ST} - {P}</td>
<td>Two</td>
</tr>
<tr>
<td></td>
<td>160 - 163:NRSR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein kinase II phosphorylation</td>
<td>57 - 60:StsD</td>
<td>[ST] - x(2) - [DE]</td>
<td>Three</td>
</tr>
<tr>
<td></td>
<td>132 - 135:SleE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>162 - 165:SrkD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 7.3.4.5 Pfam analysis of ERS1 protein of D. Pompadour

Pfam analysis of ERS1 protein showed the presence of histidine kinase domain at positions 32-101 amino acids. Residues conserved in this domain were also present which are H, P, E and L. A conservative substitution of L/M was detected.
**Table:**

<table>
<thead>
<tr>
<th>Family</th>
<th>Description</th>
<th>Entry type</th>
<th>Clan</th>
<th>Envelope Start</th>
<th>Alignment Start</th>
<th>Alignment End</th>
<th>HMM From</th>
<th>HMM To</th>
<th>Bit score</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HisKA</td>
<td>His Kinase A (phospho-acceptor) domain</td>
<td>Domain CL0025</td>
<td>45</td>
<td>110</td>
<td>46</td>
<td>110</td>
<td>2</td>
<td>68</td>
<td>64.9</td>
<td>4e-18</td>
</tr>
</tbody>
</table>

**Fig 7.21:** Results from Pfam analysis show the information on the histidine kinase domain. The #HMM line shows the consensus of the model, with capital letters representing the most conserved (high information content) positions, and dots (.) indicating insertions in the query sequence. Identical residues are coloured cyan, and similar residues are coloured dark blue. The #MATCH line indicates matches between the model and the query sequence, where a + indicates a “conservative substitution”; the #PP line represents the expected accuracy (posterior probability) of each aligned residue, where a 0 means 0–5%, 1 means 5–15%, and so on to 9 meaning 85–95% and a * meaning 95–100% posterior probability (pp); the #SEQ line is the query sequence, coloured according to the pp for each residue match on a scale from bright green for * through paler green and pale red down to bright red for 0. The histidine kinase domain in the deduced ERS1 protein is highlighted in yellow.
7.3.4.6 ProtParam analysis of ERS1 protein of D. Pompadour

The table below summarizes the physical and chemical properties of ERS1. The results show that ERS1 protein of D. Pompadour share similar properties with other species. The size of ERS1 fragment that is around 22 kDa is much smaller compared to ERS1 protein from the other species which range from 69 kDa to 71 kDa. The theoretical PI of D. Pompadour ERS1 that is basic is similar to ERS1 from D. Khao Sanan and Sonia. The major amino acid in the ERS1 protein of D. Pompadour, Leu is also the major amino acid in D. Khao Sanan, Sonia, P. Equestris and KcButterfly.

Table 7.8: Summary of protein parameters of D. Pompadour ERS1 protein analysed using ProtParam

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>PI</th>
<th>Major amino acid composition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrobium Pompadour</td>
<td>199</td>
<td>22389.8</td>
<td>7.06</td>
<td>Ser (12.6%) Leu (9.0%) Ala (6.5%)</td>
<td>Unstable</td>
</tr>
<tr>
<td>Dendrobium Khao Sanan</td>
<td>622</td>
<td>69876.5</td>
<td>7.29</td>
<td>Leu (13.5%) Ser (7.6%) Ala (6.9%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Phalaenopsis Equestris</td>
<td>633</td>
<td>70997.7</td>
<td>6.61</td>
<td>Leu (12.6%) Val (8.2%) Ala (7.3%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Dendrobium Sonia</td>
<td>621</td>
<td>69763.4</td>
<td>7.29</td>
<td>Leu (13.5%) Val (8.1%) Ser (7.6%)</td>
<td>Stable</td>
</tr>
<tr>
<td>Phalaenopsis KcButterfly</td>
<td>633</td>
<td>71086.8</td>
<td>6.88</td>
<td>Leu (13.0%) Val (8.1%) Ala (7.4%)</td>
<td>Stable</td>
</tr>
</tbody>
</table>
7.3.4.7 Multiple sequence analysis of ERS1 protein of D. Pompadour

Multiple sequence alignment of D. Pompadour ERS1 protein with ERS1 protein from D. Khao Sanan, P. Equestris, D. Sonia and P. KCButterfly using the ClustalW program (Fig 7.22) revealed the presence of a histidine kinase domain and three highly conserved regions where each region has ten or more consecutive conserved residue. Residues conserved in a histidine kinase domain were also present.

A phylogenetic analysis of the relationship of D. Pompadour ERS1 protein with ERS1 proteins D. Khao Sanan, P. Equestris and P. KC butterfly and D. Sonia was also carried out using the ClustalW program and is presented in Fig 7.23. The phylogenetic tree derived showed that these proteins were divided into three clusters. D. Pompadour ERS1 did not share the same cluster with any of the other orchids.
Fig 7.22 Comparison of the deduced amino acid sequence of *D. Pompadour* ERS1 and sequences encoding *ERS1* from *P. KCButterfly* (AF113541.1), *P. Equestris* (AJ563284.1), *D. Sonia* (AY746972.2) and *D. Khaos Sanan* (FJ628419.1). The histidine kinase domain is underlined while conserved regions are boxed. Residues conserved in the histidine kinase domain are highlighted in yellow.
Fig 7.23 Phylogenetic tree generated from the CLUSTALW multiple alignment programme. Figure shows the relationship between D. Pompadour ERSI protein and proteins from P. Equestris and KCbutterfly, D.Sonia and D. Khao Sanan. The numbers indicate the distance matrix.
7.3.4.8 Three dimensional (3D) structure of *ERSI* protein of *D. Pompadour*

Analysis using Swiss Model and Rasmol indicated that the *ERSI* protein had enough functional domains to generate a 3D protein structure. The structure consisted of 109 groups, 1131 atoms and 1157 bonds. Furthermore, the programme also identified 109 H bonds, 6 helices, 2 strands and 4 turns.

![3D Structure of the *ERSI* genes as generated by Swiss Model and Rasmol programmes.](image)

**Fig 7.24 3D Structure of the *ERSI* genes as generated by Swiss Model and Rasmol programmes.** The structure is viewed as ribbons where secondary structures are identified. The alpha helix is represented as coiled structure and the beta strand is represented as a pleated structure. The colour is a smooth spectrum from blue through green, yellow and orange to red. The N termini of proteins are coloured blue and the C termini, red. This shows folding from one end of the ribbon to the other.
7.3.5 *ERS2* gene of *D. Pompadour*

7.3.5.1 RT-PCR results of *ERS2* gene of *D. Pompadour*

RT-PCR using specific primers for the *ERS2* gene resulted in a 756bp product. A clear band indicating the presence of the product was visible on a 1% agarose gel (Fig 7.25).

![Fig 7.25 RT-PCR results with *ERS2* primers.](image) In Lane 1 is the MassRuler DNA ladder Mix (Fermentas). Lane 2 is the PCR product of 756 bp gene analysed through 1% agarose gel. 10µl of *ERS2* was loaded into the well while 5µl of ladder was used.
7.3.5.2 Nucleotide and amino acid sequence of ERS2 gene of D. Pompadour

Fig 7.26 shows the nucleotide sequence for ERS2 and the corresponding amino acids. The forward and reverse primers are italicized in red.

The forward and reverse primers are italicized in red.

Fig 7.26 Nucleotide sequence of ERS2 from D. Pompadour and the corresponding amino acid as translated using the Translate tool accessed via www.expasy.org. Forward and reverse primers are printed in red.
7.3.4.3 Homology of **ERS2** protein of *D. Pompadour*

BLAST homology search at [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast) revealed the following match in the database:

1. 579/781 positive identities (74%) (6e-137) with **ERS** of *Lilium formosanum* x longiflorum (a)
2. 553/760 positive identities (73%) (4e-114) with **ERS** of *Dendrobium cv Sonia* (b)
3. 552/763 positive identities (72%) (9e-110) with **ERS** of *Dendrobium Khao Sanan* (c)
4. 535/762 positive identities (70%) (8e-85) with **ERS** of *Delphinium Magic fountain* (d)
5. 530/760 positive identities (70%) (8e-85) with **ERS** of *Phalaenopsis sp. 'KCbutterfly'* (e)
6. 533/766 positive identities (70%) (4e-83) with **ER2** of *Oncidium Gower Ramsey* (f)
7. 529/760 positive identities (70%) (1e-83) with **ERS** of *Phalaenopsis equestris* (g)
8. 529/760 positive identities (70%) (1e-83) with **ERS** of *Phalaenopsis‘True lady’* (h)
9. 530/762 positive identities (70%) with (1e-76) with **ERS** of *Delphinium Belladonna* (i)
10. 525/760 positive identities (69%) (2e-79) with **ERS** of *Gladiolus hybrid cultivar* (j)
11.

(a) Accession DQ408428  Pan and Chen, 2006.
(b) Accession AY746972  Suwanagul et al., 2004.
(c) Accession FJ628419  Thongkum et al., 2009.
(d) Accession AB055430  Kuroda et al., 2003.
(e) Accession AF113541  Chai et al., 1998.
(f) Accession AF276234  Liu et al., 2000.
(g) Accession AJ563284  Chang, 2005.
(h) Accession AF055894  Do et al., 1999.
(i) Accession AB201245  Tanase and Ichimura 2006.
(j) Accession AB180248  Arora et al., 2006.
7.3.5.4 Features of ERS2 protein of D. Pompadour

Analysis with Prosite showed that ERS2 isolated from D. Pompadour contained five different protein site, cAMP- and cGMP-dependent protein kinase phosphorylation, Protein kinase C phosphorylation, N-myristoylation and Casein kinase II phosphorylation N-glycosylation. These sites are all common sites found in ethylene receptors.

Table 7.9: Protein sites detected in ERS2 using the Prosite tool accessed via www.expasy.org.

<table>
<thead>
<tr>
<th>Site</th>
<th>Residue</th>
<th>Consensus Pattern</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP- and cGMP-dependent protein kinase phosphorylation</td>
<td>18-21 KKsS</td>
<td><a href="2">RK</a>-x-[ST]</td>
<td>One</td>
</tr>
<tr>
<td>Protein kinase C phosphorylation</td>
<td>88-90 SvK</td>
<td>[ST]-x-[RK]</td>
<td>Five</td>
</tr>
<tr>
<td></td>
<td>116 – 118 TgR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>194 – 196 SsR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>213 – 215 TgR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>243 – 245 SaK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-myristoylation</td>
<td>108 - 113:GlirTQ</td>
<td>G-{EDRKPFWY}-x(2)-[STAGCN]-[P]</td>
<td>Two</td>
</tr>
<tr>
<td></td>
<td>178 - 183:GSvvST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein kinase II phosphorylation</td>
<td>112 – 115 TqEe</td>
<td>[ST]-x(2)-[DE]</td>
<td>Four</td>
</tr>
<tr>
<td></td>
<td>129 – 132 StlD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>140 – 143 TlvE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>261 – 264 SphE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-glycosylation</td>
<td>193 – 196 NSSR</td>
<td>N-{P}-[ST]-{P}</td>
<td>One</td>
</tr>
</tbody>
</table>

7.3.5.5 Pfam analysis of ERS2 protein of D. Pompadour

Pfam analysis of ERS2 protein showed the presence of GAF domain at positions 132 - 220 amino acids. The GAF domain has been reported to be present in ethylene receptors, though its function in ethylene receptors is unknown.
Fig 7.27 Results from Pfam analysis show the information on GAF domain. The #HMM line shows the consensus of the model, with capital letters representing the most conserved (high information content) positions, and dots (.) indicating insertions in the query sequence. Identical residues are coloured cyan, and similar residues are coloured dark blue. The #MATCH line indicates matches between the model and the query sequence, where a + indicates a “conservative substitution”; the #PP line represents the expected accuracy (posterior probability) of each aligned residue, where a 0 means 0–5%, 1 means 5–15%, and so on to 9 meaning 85–95% and a * meaning 95–100% posterior probability (pp); the #SEQ line is the query sequence, coloured according to the pp for each residue match on a scale from bright green for * through paler green and pale red down to bright red for 0. The GAF domain in the deduced ERS2 protein is highlighted in yellow.
7.3.5.6 ProtParam analysis of ERS2 protein of *D. Pompadour*

The table below summarizes the physical and chemical properties of ERS2. The results show that ERS2 protein of *D. Pompadour* share similar properties with other species. The size of ERS2 fragment that is around 29 kDa is much smaller compared to ERS2 protein from the other species which range from 69 kDa to 71 kDa. The theoretical PI of *D. Pompadour* ERS2 that is basic is similar to ERS2 from *D. Khao Sanan, D. Sonia, P. True Lady, P. KcButterfly* and *O. Gower Ramsey*. The major amino acids in the ERS2 protein of *D. Pompadour*, Leu and Val are also the major amino acids in the other species.

Table 7.10 Summary of protein parameters of *D. Pompadour* ERS2 protein analysed using ProtParam

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>PI</th>
<th>Major amino acid composition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrobium Pompadour</em></td>
<td>265</td>
<td>29955.1</td>
<td>9.21</td>
<td>Leu (13.1%) Val (9.4%) Thr (8.3%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>Dendrobium Khao Sanan</em></td>
<td>622</td>
<td>69876.5</td>
<td>7.29</td>
<td>Leu (13.7%) Val (8.9%) Ser (7.6%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>Dendrobium Sonia</em></td>
<td>621</td>
<td>69763.4</td>
<td>7.29</td>
<td>Leu (13.4%) Val (8.1%) Ser (7.6%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>Phalaenopsis True Lady</em></td>
<td>633</td>
<td>71084.7</td>
<td>6.88</td>
<td>Leu (13.0%) Val (8.4%) Ala (7.4%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>Phalaenopsis KcButterfly</em></td>
<td>633</td>
<td>71086.8</td>
<td>6.88</td>
<td>Leu (13.0%) Val (8.1%) Ala (7.4%)</td>
<td>Stable</td>
</tr>
<tr>
<td><em>Oncidium Gower Ramsey</em></td>
<td>631</td>
<td>70721.6</td>
<td>6.82</td>
<td>Leu (13.6%) Ala (7.9%) Val (7.1%)</td>
<td>Stable</td>
</tr>
</tbody>
</table>
7.3.5.7 Multiple sequence alignment of *ERS2* protein of *D. Pompadour*

Multiple sequence alignment of *D. Pompadour* *ERS2* protein with *ERS* protein from *D. Khao Sanan*, *P. True Lady*, *D. Sonia* and *P. KCButterfly* using the ClustalW program (Fig 7.28) revealed the presence of a GAF domain and eight highly conserved regions where each region has ten or more consecutive conserved residue.

A phylogenetic analysis of the relationship of *D. Pompadour* *ERS2* protein with *ERS* proteins from *D. Khao Sanan*, *D. Sonia*, *P. True Lady* and *P. KCButterfly* was also carried out using the ClustalW program and is presented in Fig 7.29. The phylogenetic tree derived showed *D. Pompadour* *ERS2* is more closely related to *D. Khao Sanan* and *Sonia* compared to the other orchid hybrids.
### Fig 7.28 Comparison of the deduced amino acid sequence of *D. Pompadour* ERS2 and sequences encoding ERS2 from *D. Khao Sanan* ethylene (FJ628419), *D. Sonia* (AY746972), *P. True Lady* (AF055894), *P. KCButterfly* (AF113541) and *O. Gower Ramsey* (AF276233).

The GAF domain is underlined while conserved regions are boxed.
Fig 7.29 Phylogenetic tree generated from the CLUSTALW multiple alignment programme. Figure shows the relationship between *D. Pompadour* ERS2 protein and ERS proteins from *D. Khao Sanan*, *P. True Lady*, *D. Sonia*, *P. KCButterfly* and *O. Gower Ramsey*. The numbers indicate the distance matrix.
7.3.5.8 Three dimensional (3D) structure of ERS2 protein of D. Pompadour

Analysis using Swiss Model and Rasmol indicated that the ERS2 protein had enough functional domains to generate a 3D protein structure. The protein structure consisted of 117 groups, 1219 atoms and 1107 bonds. Furthermore, the programme also identified 86 H bonds, 5 helices, 6 strands and 11 turns.

Fig 7.30 3D Structure of the ERS2 genes as generated by Swiss Model and Rasmol programmes. The structure is viewed as ribbons where secondary structures are identified. The alpha helix is represented as a coiled structure and the beta strand is represented as a pleated structure. The colour is a smooth spectrum from blue through green, yellow and orange to red. The N termini of proteins are coloured blue and the C termini, red. This shows folding from one end of the ribbon to the other.
7.4 DISCUSSION

The regulation of pollination-induced senescence in flowers by ethylene has long been an accepted dogma (Arditti, 1979). In the earlier section (3.2), results confirm the climacteric nature of pollinated *D. Pompadour* flowers, producing a burst of ethylene and eventually senescing within 48 after pollination. In Arditti’s review on orchids, this climacteric nature was found to be present in all the orchids studied in literature. However, this is not unique to orchids, as other flowers such as carnations, rose, petunia, hibiscus and *Arabidopsis* have also been established as climacteric flowers.

**ACCO** requires Fe(II) and ascorbate as cofactors for enzymatic activity (McGarvey and Christoffersen 1992), and 12 amino acid residues of **ACCO** participate in the interaction with these cofactors (Zarembinski and Theologis 1994). The 321 amino acid protein encoded by the gene isolated from *D. Pompadour* in this study contains a highly conserved Fe(2+) 2-oxoglutarate dioxygenase domain. This domain is also present in **ACCO** isolated in all the other orchids reported in the NCBI GeneBank which are *C. hybrid*, *Dtps. sp*, *P. True Lady*, *O. Gower Ramsey*, *D. Crumenatum*, *D. Sonia*, *D. Anna* and *D. Khao Sanan* as well as other flowers such as carnations, *Arabidopsis*, *Pelargonium* and *Rosa* hybrid. This further confirms the Fe(2+) 2-oxoglutarate dioxygenase domain as the signature domain which is not only unique to orchid **ACCO** but also other climacteric flowers as well. In plants, Fe(II) 2OG dioxygenase domain enzymes catalyse the formation of plant hormones, such as ethylene, gibberellins, anthocyanidins and pigments such as flavones. The protein encoded also contained five conserved residues with seven conservative substitutions. These substitutions may not affect the function of the **ACCO** protein as has been demonstrated in petunia where
substitution of alanine with glycine still resulted in a functional ACCO, suggesting that amino acids such as alanine may play the same role (Do et al., 2005).

Analysis with ScanProsite showed the presence of two histidine and an aspartate residues which are conserved in all the Fe(II) requiring families of enzymes (Tang et al., 1993). It also contains the sequence motifs of 2OG-dependent oxygenases, [RK], which was found to bind to the terminal carboxylate of 2OG. The histidine and aspartate residues catalytically bind a metal ion, generally iron, and are directly involved in catalysis. Mutagenesis and spectroscopic investigations found that [HX--H] motif of the conserved domain is employed in the binding of iron in ACCO (Schofield and Zhang, 1999). Moreover, the substitution of histidine or aspartate in tomato ACCO with glutamate resulted in a lower rate of ACC conversion to ethylene (Nakatsuka et al., 1998).

A conserved glycosylation site [NISK] was also detected in the deduced ACCO protein. This site allows for oligosaccharide attachment to allow for protein folding. Correctly folded proteins will eventually be exported from the endoplasmic reticulum. A study done by Meli et al., (2010) showed that when N-glycoproteins (precursors for glycosylation) were suppressed in transgenic Tomato (cv. Pusa ruby), expression of ACCO was down regulated and vase life of the fruits was extended. The results from that study further validated the importance of glycosylation sites in ensuring that ACCO is able to function. Analysis using ProtParam showed that the ACCO of D. Pompadour is soluble. This is similar to ACCO of Phalaenopsis that was readily extracted with water (Chung et al., 2002). The author suggested that the association of ACCO with the pellet fraction as was reported in apple and pear fruit might be an artifact.
Homology analysis showed that although *D. Pompadour* belonged to the same genus and family of *D. Crumenatum* and *Doritaenopsis sp.* respectively, the degree of homology was closer to that of the *ACCO* protein isolated from *Dianthus caryophyllus* and *Rosa* hybrid, both with more than 70% homology compared to 65-67% homology with the other orchid species. The close relationship with *Dianthus caryophyllus* is further confirmed by the phylogram where the *ACCO* from *D. Pompadour* and *Dianthus caryophyllus* share the same branch. Since *D. Pompadour* is a hybrid line, the different genotype may contribute to its variation from the other orchid species. Similar findings have been observed in *D. Anna*. The size of *D. Pompadour* *ACCO* was similar to *ACCO* proteins found in the GenBank.

*ACCS* belongs to the family of pyridoxal 5’-phosphate- dependant enzyme. This enzyme is part of a multigene family and transcription of different forms is induced by different physiological and environmental conditions (Stearns and Glick, 2003). RT-PCR amplification with specific primers resulted in a 1.32Kb nucleotide that translates into 440 amino acids. The protein contains 10 invariant residues in this subgroup and a conserved lysine residue which has been postulated as the essential residue for ensuring the function of *ACCS* protein. It was reported that this lysine residue is responsible for the binding of PLP and covalent linking of 2-aminobutyrate portion of AdoMet during the inactivation (Yip *et al.*, 1992). The deduced amino acid contained one Aminotransferases class-I pyridoxal-phosphate attachment site, the signature domain for this enzyme within which lies the lysine residue. Alignment of *D. Pompadour* *ACCS* with other species within this site detected one amino acid substitution (L/I). This substitution is unique to *D. Pompadour* as the *ACCS* from *Cymbidium* (BAF 36561), *D. crumenatum* (AAB67882), *D. Sonia* (ACY 72181), *Doritaenopsis sp* (AAB05849) and *Oncidium* Gower Ramsey (AAQ07441) maintain the L residue within this site. Amino
acid substitution however is not unique to *D. Pompadour* as in other flowers such as *Arabidopsis* and *Rosa* hybrid, (G/S) and (A/V) substitutions were found.

A study on mutant *ACCS* in tomatoes found that mutations which involved variant residues between *ACCS* and subgroup 1 aminotransferase did not result in a loss of function, concluding that some of the residues may be tolerant to substitution. These residues include Y92 and Y240. This, according to the author may mean that these residues are not essential for the functioning of *ACCS* (Tarun *et al.*, 1998). Studies on the effects of residue substitution on the function of *ACCS* are crucial as they could give insight into the accurate role of these residues.

The alignment of *D. Pompadour* *ACCS* with other species also showed a conserved Thr-Asn-Pro (TNP), a tripeptide implicated to be a stability determinant in *ACCS*. Poor expression of *ACCS* was observed when no TNP was present in an *Arabidopsis* *ACCS1* isoform (Liang *et al.*, 1995) while mutation of the tripeptide in tomato *ACCS* resulted in inactivity in mutants (Tarun *et al.*, 1998). *ACCS* isolated from *D. Pompadour* shared a high degree of homology with *ACCS* from other plants species, the highest being 81.9% with *ACCS* isolated from pelargonium. More than 70% homology was shared with *Petunia, Nicotiana, Dianthus caryophyllus* and *Arabidopsis thaliana*. Analysis with ProtParam further highlights the similarity between *ACCS* protein of the different species with Leu and Ser as the major amino acids. The *ACCS* protein of *Dendrobium Pompadour* was found to be unstable; a similar characteristic shared by *ACCS* isolated from *Petunia, Nicotiana* and *Dianthus caryophyllus*. Instability of *ACCS* coupled with its low concentrations in ethylene producing tissues has made purification of this enzyme a difficult task. When comparing *D. Pompadour* *ACCS* with proteins available in the GenBank, it was found that the size of the isolated *D. Pompadour* *ACCS* was
comparable as the average size of proteins submitted was approximately 450 aa. Multigene family for ACCS sequences has been reported for zucchini (Huang et al., 1991), tomato (Sato and Theologis 1989; van Der Straeten et al., 1990; Rottmann et al., 1991), Arabidopsis (Liang et al., 1992; van Der Straeten et al., 1992), mung beans (Botella et al., 1992), rice (Zarembinski and Theologis 1993), potato (Beltran Destefano et al., 1995) and carnation (ten Have and Woltering 1997).

Ethylene receptors have been widely studied at both biochemical and molecular level in many flowers including Arabidopsis thaliana, Dianthus caryophyllus and Petunia hybrida. In orchids, a number of ethylene receptors have been sequenced, mostly in Phalaenopsis sp. In this chapter, three partial cds of ETR1, ERS1 and ERS2 were isolated and further characterised using the bioinformatics tool. Using degenerate primers designed via alignment of ethylene receptors retrieved from the GenBank, four partial cds were isolated sized 692bp, 955bp and 756 bp for ETR1, ERS1 and ERS2 respectively. Analysis of the deduced amino acids detected a histidine kinase domain in ETR1 and ERS1 while a GAF domain was detected in ERS2. A typical His protein kinase has five conserved signature motifs, H, N, G1, F, and G2, with the conserved His as the central feature in the H motif. The other four motifs define the nucleotide-binding cleft (Grebe and Stock, 1999). The GAF domain binds cyclic nucleotides in a number of bacterial proteins, and the chromophore in the plant photoreceptor phytochrome (Aravind and Ponting, 1997). However, the function of this domain in the ethylene receptors is unknown.

These sequence data suggests that amino acids sequenced could be putative ethylene receptors with the ability to bind ethylene. The histidine kinase domain detected in ETR1 and ERS1 was highly conserved at the H residue, presumed as autophosphorylation site.
for the domain. The mechanism of histidine phosphorylation is initiated as a response to a stimulus, received by the response regulator and followed through by the effector domain. Following autophosphorylation, a phosphoryl group is transferred by the response regulator which in turn catalyses the transfer to an aspartate residue in the receiver domain (Stock et al., 2000).

Early investigations on ethylene receptors assumed that the presence of a histidine kinase is compulsory in order for signal transduction to occur. This hypothesis was established as ETR1 in both Arabidopsis and tomatoes were found to have conserved histidine kinase domains (Chang et al., 1993; Tieman and Klee, 1999). More recent published data however have come to a contradictory hypothesis where histidine kinase may not be the primary means for signal transduction (Wang et al., 2003). The isolation of ethylene receptors from subfamily 2 that contain a degenerate histidine kinase domain but still allow for signal transduction suggest the less significant role of histidine kinase. Furthermore, serine substitutions in the ethylene receptors, except for ETR still allowed for autophosphorylation to occur. In fact, it was postulated that the receptors may have evolved into serine-phosphorylation proteins (Moussatche and Klee 2004).

Phylogenetic analysis of ETR1 isolated from D. Pompadour resulted in a closer relationship with Arabidopsis thaliana, compared to three other orchid flowers, P. hybrid, D. Sonia and O. Gower Ramsey. ERS1 on the other hand showed high degree of similarity with four other orchid species and hybrids, D. Khao Sanan, Sonia, P. Equestris and KCbutterfly. ERS2 shared high degree of similarity with other orchids. The diversion from orchids, in the case of ETR1 may be contributed by changes in genotype caused by hybridization of the flower. Amongst the three receptors isolated,
ETR1 showed different major amino acid composition. Where ETR1 is concerned (in Dendrobium Pompadour and the other species, the major amino acids composed of Thr, whereas in ERS1 and ERS2, Leu seemed to be the major amino acid for all the species analysed. The difference in the composition may affect the folding of secondary and tertiary structure of the proteins which in turn results in the different molecular weights.

Though the molecular weight of the ethylene receptors isolated is smaller compared to the complete sequence of the ethylene receptors retrieved, the average size of ethylene receptor proteins retrieved is within the range of 19kDa to 71kDa. ETR1 proteins are within the range of 20kDa while the size of ERS1 and ERS2 reaches up to 71kDa. Analysis on solubilised ethylene receptors from Arabidopsis using gel filtration technique has shown that ethylene receptors exists as components of high mass protein complexes (Chen et al., 2010).The data from that study suggests the existence of heterogeneity among the ethylene receptors in the form of their protein complexes. They also reported that upon ligand binding, different response were exhibited by ETR1 and ERS1, which indicates a certain amount of independence between the protein complexes. Since the isolation of the five receptors from Arabidopsis, the number of ethylene receptors deposited in the GeneBank has increased tremendously. Members of the subfamily have also grown to include ETR3- ETR7.

In conclusion, this data obtained in this chapter has successfully characterise five genes that play are integral to the control of pollination induced senescence. The determination and analysis of these characteristics is important to ensure accurate identification of the role they play in both ethylene biosynthesis and perception. Moreover, this will also allow for effective downstream utilization which may include expression and manipulation of genes.