FUNCTIONAL EXPRESSION OF *VANDA* MIMI PALMER MONOTERPENE SYNTHASES IN PICHIA PASTORIS, A YEAST SYSTEM

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PART I

SYNOPSIS

There are more than 100 orchid species and hybrids that have been reported to emit fragrance compounds including monoterpenoids, sesquiterpenoids, benzenoids and phenylpropanoids, which have potential to be commercialized in the fragrance, flavour and food industries. Unfortunately, the price of scented orchids is too expensive and the supply of scented orchids is insufficient for essential oil extraction due to the infrequent flowering throughout the year. This study aims to isolate monoterpene synthase transcripts from selected orchids for overexpression in selected microbial systems via *in vivo* biosynthesis to fulfill the demand of monoterpene compounds by the fragrance, flavor and food industries. Transcriptomic sequencing on four selected scented orchids including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren and *Vandachostylist* Sri-Siam in comparison to a non-scented orchid *Vanda* Tan Chay Yan was chosen to identify monoterpene synthase transcripts responsible for the biosynthesis of monoterpene compounds. Open reading frames of four monoterpene synthases including linalool and ocimene synthases have been isolated and successfully transformed in selected microbial systems including *Escherichia coli*, *Lactococcus lactis*, *Pichia pastoris* and *Kluvyromyces lactis.* Functional expression of all linalool and ocimene synthases isolated from the orchids were carried out by recombinant protein analysis using SDS-PAGE and Western blot while fragrance compounds produced by the recombinant systems were analysed using Gas Chromatography-Mass Spectrometry. The targeted monoterpene products from the recombinant systems will be beneficial for large scale supplies of monoterpenoids to the fragrance, flavour and food industries.

SINOPSIS

Terdapat lebih daripada 100 spesies orkid dan kacukan telah dilaporkan mengeluarkan sebatian wangian termasuk monoterpenoids, sesquiterpenoids, benzenoids dan phenyl-propanoids, yang mempunyai potensi untuk dikomersialkan dalam industri wangian, dan perisa makanan. Malangnya, harga orkid wangi adalah terlalu mahal dan bekalan orkid wangi tidak mencukupi untuk pengekstrakan minyak pati kerana berbunga kerap sepanjang tahun. Kajian ini bertujuan untuk mengasingkan transkrip monoterpene synthase dari orkid dipilih untuk diexpres dalam sistem mikroorganisma melalui biosintesis *in vivo* untuk memenuhi permintaan terhadap 'monoterpene' daripada industri wangian, dan perisa makanan. Jujukan Transcriptomic kepada empat orkid wangi termasuk *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren dan *Vandachostylist* Sri-Siam berbanding dengan orkid bukan wangi *Vanda* Tan Chay Yan telah dipilih untuk mengenalpasti transkrip monoterpene synthase bertanggungjawab biosintesis monoterpene sebatian. Sebanyak empat 'monoterpene synthases' termasuk 'linalool' dan 'ocimene synthases' telah diasingkan dan berjaya mengubah dalam sistem mikrob terpilih termasuk *Escherichia coli*, *Lactococcus lactis*, *Pichia pastoris* dan *Kluvyromyces lactis*. Analisis kefungsian kesemua 'linalool' dan 'ocimene synthases' tersebut telah dijalankan oleh analisis protein rekombinan menggunakan 'SDS-PAGE' dan 'Western blot' manakala sebatian wangian yang dihasilkan oleh sistem rekombinan telah dianalisis menggunakan 'Gas Chromatography-Mass spectrometry'. Produk 'monoterpene' yang dihasilkan daripada sistem rekombinan akan memberi manfaat untuk bekalan skala besar 'monoterpenoids' kepada industri wangian, dan perisa makanan.

PART II

INTRODUCTION

2.1 Introduction

Fragrant-orchids are commonly sold at higher price compared to orchids without any fragrance. In nature, volatiles from orchids and other plants are mainly used by the plants to attract specific pollinators to help in their pollination whereby different types of pollinators are attracted to different types of fragrance (Topik *et al.*, 2005). Unfortunately, extensive breeding work to produce attractive colours and shape of orchid flowers cause modern orchid hybrid to lose their fragrance characteristics because in previous traditional breeding, fragrance was not included as the main objective of the new hybrids (Liu *et al.*, 2008). To date, some orchid hybrids with fragrant characteristic are extensively cultivated in Southeast-Asian countries including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren and *Vandachostylis* Sri-Siam (Chew, 2008). The orchid hybrids are propagated through tissue culture approach, mainly in Thailand to be commercialised not only in Thailand and Malaysia but also all around the world.

Research on fragrant-orchids has been started since early 1990s on determination of their volatile components via analysis using Gas Chromatography-Mass Spectrometry (GC-MS). Meanwhile molecular work on fragrance-related transcripts isolation and characterization was only reported in 2006 by identification of geranyl diphosphate synthase (GDPS) and some other putative fragrance-related transcripts from *Phalaenopsis bellina* through the establishment of Expressed Sequence Tags (ESTs) library (Hsiao *et al.*, 2006; 2008). Subsequently, some other fragrance-related transcripts have been isolated by a research group from Malaysia on another fragrant-orchid, *V.* Mimi Palmer that has won several international awards for its sweet-strong fragrance (Chan *et al.*, 2011; Mohd-Hairul, 2011). Lately, the sesquiterpene synthase (VMPSTS) of *V.* Mimi Palmer has been reported to be functionally expressed in *Lactococcus lactis*, a gram positive bacterium. *In vitro* analysis has shown that VMPSTS is involved in catalysing the biosynthesis of multiple sesquiterpene compounds including germacrene D, copaene and nerolidol (Song *et al.*, 2012).

In this study, four selected monoterpene synthases including two linalool synthases and two ocimene synthases that have been isolated from selected fragrant-orchids including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren and *Vandachostylist* Sri-Siam were chosen for functional characterisation in selected microbial systems including *Escherichia coli*, *Lactococcus lactis*, *Pichia pastoris* and *Kluvyromyces lactis*. Functional expression of all linalool and ocimene synthases isolated from the orchids were carried out by recombinant protein analysis using SDS-PAGE and Western blot while fragrance compounds produced by the recombinant systems were analysed using Gas Chromatography-Mass Spectrometry. The targeted monoterpene products from the recombinant systems will be beneficial for large scale supplies of monoterpenoids to the fragrance, flavour and food industries.

2.2 Objectives of the study

The objectives of this research project were:

1. To **clone** and **express** orchid monoterpene synthases in *Pichia pastoris*
2. To **functionally characterize** the heterologous expressed orchid monoterpene synthases
3. To mass produce monoterpene compunds by regulating terpenoid biosynthetic pathway.

2.3 Significance of the study

Over-expression of precursors of monoterpene compounds by over-expressing the key regulator enzyme that control terpenoid pathway is possible to be done as terpenoid pathway in plants has been well studied in some plant models including *Arabidopsis thaliana*, *Clarkia breweri* and *Anthirrhinum majus*. Unfortunately, policy, rules and regulations on transgenic plants production pose a big obstacle to commercialization. Thus, an alternative approach is to clone and express monoterpene synthase transcripts of *Vanda* Mimi Palmer and other selected fragrant-orchids in several microbial systems including in *Escherichia coli*, *Lactococcus lactis*, *Pichia pastoris* and *Kluvyromyces lactis* as hosts to

express recombinant protein, which will open up more opportunities for exploitation of the monoterpene compounds because mass production of the compounds whenever required is possible as one does not have to face the poor yield constraint posed by its natural source (plant).

PART III

METHODOLOGY

3.1 Plant Materials

All orchid plants used for this study including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren and *Vandachostylist* Sri-Siam were bought from the United Malaysian Orchid, a nursery located at Rawang, Selangor. All the orchids were maintained at Taman Mahkota Aman, 26070 Kuantan, Pahang and exposed to 70-80% sunlight with the temperature range between 28-31°C. The whole pots of the plants were brought to Molecular Biology Laboratory, FIST, UMP for samples processing purpose.

3.2 Isolation of fragrance-related monoterpene synthases

Flowers of fragrant orchids were detached from their mother plants, cut into small pieces, wrapped in aluminium foil and frozen in liquid nitrogen before stored at -80°C for further use. Total RNA extraction was carried out using modified CTAB extraction method as decribed by Chan et al. (1999). The extracted RNA samples were then subjected to 5'- and 3'-RACE cDNA preparation using SMARTer RACE cDNA synthesis kit (Clonetech, USA) by following the manufacturer's instructions. Partial sequence of monoterpene synthase was isolated using 5'-RACE cDNA as template, together with set of primers that are designed at conserved region of monoterpene synthase mRNA sequences that are available at the NCBI GenBank database. Specific primers (were designed for isolation of 5'- and 3'-regions of the monoterpene synthase mRNA using optimised PCR protocol: pre-denaturation at 94ºC for 3 minutes; 35 cycles of 45 seconds denaturation at 94ºC, 45 seconds annealing at 65ºC and 3 minutes extension at 72ºC; final extension at 72ºC for 5 minutes. Open Reading Frame (ORF) of respective monoterpene synthase was isolated using specifically the same PCR protocol with specifically designed primers. Full ORF of all the monoterpene synthases were sequenced by Bioneer Corporation (Republic of Korea).

3.3 Sequence analysis of monoterpene synthases

Sequencing result of all the monoterpene synthases were subjected to BLASTX analysis to identify its putative function and further sequence analysis using various bioinformatics tools including ChloroP and MEGA softwares for phylogenetic tree construction. The deduced amino acid sequence of all the monoterpene synthases were further analysed using various protein analysis tools including BLAST-PDB, PSI-BLAST, SUPERFAMILY HMM, HHpred, pGenThreader and LOMETS.

3.4 Cloning of monoterpene synthases into PMAL-c5X plasmid vector

cDNA sequence of all the monoterpene synthases were amplified using another specifically forward and reverse primers containing additional *Not*I and *EcoR*I RE sites, respectively. In addition, was incorporated together in the reverse primer. The PCR amplification was carried out using the following protocol: pre-denaturation at 94ºC for 3 minutes; 35 cycles of 45 seconds denaturation at 94ºC, 45 seconds annealing at 65ºC and 3 minutes extension at 72ºC; final extension at 72ºC for 5 minutes. The PCR-amplified product of all the monoterpene synthases were further purified using ExpinTM Combo PCR purification kit (GeneAll, Korea) by following the manufacturer's instructions. The purified PCR product of all the monoterpene synthases were digested with *Not*I and *EcoR*I (NEB, USA) and followed by purification step using the same purification kit. Another similar RE digestion step was conducted on pMAL-c5X plasmid vector that is provided in the pMAL™ Protein Fusion and Purification System (NEB, USA) and followed by similar purification step. The digested product of GpMTS and pMAL-c5X plasmid were ligated together in molecular ratio of 3:1 using T4 DNA ligase (NEB, USA) by incubating at 16ºC for 16 hours. The ligation product was then subjected to heat-shock transformation into calcium chloride-treated *Escherichia coli* ER2353 (NEB, USA) competent cells. The heat-shock transformed cells were plated on LB agar containing 100µg/mL ampicillin and incubated at 37ºC for 16 hours for screening of successful transformants.

3.5 Protein expression of all the monoterpene synthases

Overnight culture of recombinant *E.coli* ER2353 containing PMAL-c5X-MTS plasmid was used as starter culture and inoculated into fresh LB Broth containing 100µg/mL ampicillin at 10% (v/v) and incubated at 37ºC for ~1.5-2.0 hours until OD_{600} reached ~0.6. The culture was divided equally into two sub-samples consisting Non-induced culture and culture induced with 0.3 mM IPTG, respectively. Both IPTG-induced and Non-induced cultures were further incubated at 37ºC for 2 hours with shaking at 200 rpm. Next, 500µL of IPTG-induced' and Non-induced cultures were centrifuged at 13,200x*g* for 1 minute, separately. Pellets of IPTG-induced and Non-induced cultures were resuspended in 200µL of sample buffer containing 1X Bolt™ LDS Sample Buffer (Thermo Fisher, USA) and further heated at 95ºC for 5 minutes. Both samples were placed on iced and subjected to SDS-PAGE analysis on precast Bolt® 10% Bis-Tris Plus Gels (Thermo Scientific, USA) by following the manufacturer's instructions.

Purification of recombinant *Gp*MTS

Recombinant *E.coli* ER2353 containing PMAL-c5X-MTS plasmid was grown overnight at 37ºC with shaking at 200rpm as starter culture. The starter culture was transferred into 10ml fresh LB broth containing 100µg/mL ampicillin at 10% (v/v) and continue incubated in similar condition for 2-3 hours until reached OD_{600} ~ 0.5 and followed by induction with 0.3mM IPTG. After that, the IPTG-induced *E. coli* ER2353 transformant was further incubated in similar condition for 4 hours. Next, the recombinant bacteria was aliquoted into several 2.0ml microcentrifuge tubes and centrifuged at 13,200x*g* for 1 minute. Supernatant was discarded and recovered bacterial pellet was resuspended in 1mL of binding buffer containing 20mM sodium phosphate, 500mM NaCl, and 20mM imidazole. Next, freeze-thaw was carried out for cell lysis by five times of freezing at -80ºC freezer and thawing at 37ºC water bath alternately in 30-minute intervals. Cell debris was removed by another centrifugation step and supernatant containing crude protein extract was then transferred into fresh 2.0ml microcentrifuge tube. Recombinant monoterpene synthases protein was purified from the crude protein extract using His-tag purification using His Buffer Kit (GE Healthcare, UK) by following the manufacturer's instructions. Purified monoterpene synthases protein was further desalted by following the manufacturer's

instructions. Eluent containing recombinant monoterpene synthases protein recovered in different fractions were further verified by measuring OD_{280} using NanoVue Spectrophotometer (GE Healthcare, USA). Fractions with high protein concentration were subjected to SDS-PAGE, Western blot and monoterpene assay.

3.6 Monoterpene synthases enzymatic assay

Enzymatic assay on the recombinant monoterpane synthase was performed as described by Bouwmeester et al. (1998). Briefly, the assay was carried out in tightly close 20ml headspace vial containing monoterpene assay buffer with purified monoterpane synthase protein, 2mM DTT and 20µg/ml of GPP substrate. The mixture was incubated at 30ºC for three hours. Volatiles available in the headspace vial were captured by exposing polydimethylsiloxane (PDMS) fibre of Solid Phase Microextraction (SPME) device for 20 minutes. The volatile compounds trapped in the PDMS fibre were release from the fibre by injecting the needle of SPME device into the injected port at Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent, USA) that has been pre-set at 250ºC. The volatile compounds were further separated on HP5 column using the following protocol: Initial GC temperature 50ºC; increment 5ºC per minute until 100ºC; hold at 100ºC for 3 minutes; increment of 25ºC/min to 150ºC; Post run at 250ºC for 5 minutes. After the separation process, the spectrum of volatile compounds was compared to the NIST 2011 Library database that is available on the GC-MS system.

3.7 *In vivo* analysis on monoterpene compounds produced by the recombinant *E. coli*

A volume of 1,000µl of 16 hours recombinant bacterial culture was used to be inoculated into 10ml of fresh LB broth containing 100µg/ml ampicillin. The fresh culture was incubated for two hours at 37ºC with shaking at 250rpm. Subsequently, a volume of 4,000µl of the culture was transferred into a sterile 20ml headspace vial. Then, 0.167 M glucose and 1.0 mM IPTG was added into the bacterial culture and followed by incubation at 37ºC for 16 hours. After the incubation period, the volatile compounds were captured by exposing to PDMS fibre of Solid Phase Microextraction (SPME) device for 20 minutes.

The volatile compounds trapped in the PDMS fibre were release from the fibre by injecting the needle of SPME device into the injected port at Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent, USA) that has been pre-set at 250ºC. The volatile compounds were further separated on HP5 column using the following protocol: Initial GC temperature 50ºC; increment 5ºC per minute until 100ºC; hold at 100ºC for 3 minutes; increment of 25ºC/min to 150ºC; Post run at 250ºC for 5 minutes. After the separation process, the spectrum of volatile compounds was compared NIST 2011 Library database that is available on the GC-MS system.

PART IV

BACKGROUNDS AND SITES OF RESEARCH

4.1 Orchids: Introduction

In the Plantae kingdom, seed plants can generally be divided into two groups which are flowering plants (angiosperms) and non-flowering plants (gymnosperms) (Audesirk *et al.*, 2002). Orchidaceae which has been reported as the largest family of flowering plants comprises 17,000 to 35,000 species that belong to 880 genera. Orchids are estimated to cover almost 30% of monocotyledonous plants as well as 10% of total flowering plants from all around the world (Dressler, 1993; Hossain, 2011). Besides that, Orchidaceae family has received a great interest among evolutionary biologist, botanist and orchid enthusiasts from all around the world after the establishment of a book on orchids 'Fertilization of Orchids' by Darwin in 1862. Orchids in Orchidaceae family are divided into five recognised subfamilies namely Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae and Vanilloideae (Chase *et al.*, 2003). Among all the orchids in Orchidaceae family, more than 230 genera of orchids covering 898 to 4,000 species in 143 genera can be found in Peninsular Malaysia (Go *et al.*, 2010).

Orchid plants are naturally grown in a wide range of ecological habitats (Ramirez *et al.*, 2007) that are most suitable for their growth requirement based on their specialized morphological, structural and physiological characteristics (Dressler, 1990). Among all orchid species from the entire world, an estimated 70% of orchids are classified as epiphytic plants that grow on the trunks or branches of trees without consuming any nutrient from the host plants. In addition, epiphytic orchids have been reported to be nearly two-thirds of total epiphytic flowering plants from all around the world (Gravendeel *et al.*, 2004; Hsu *et al.*, 2011). Besides epiphytic orchids, there are an estimated 25% of orchids from all around the world which have been reported to be terrestrial orchids whereby the orchids grow on soil and absorb nutrients directly from soil.

Meanwhile the remaining 5% of orchids can be found to be grown naturally in various other supports (Atwood, 1986). It has been reported that almost all temperate orchids that grown in temperate regions are terrestrial orchids while tropical orchids grown in tropical regions are mostly epiphytic as well as lithophytic orchids (Dressler, 1990). Litophytic orchids are another type of orchids that grow naturally on exposed rocks whereby their roots are directly attached to the extremely rocky terrain with slight humus materials (McDonald, 1999).

Among all types of orchids, lithophytic orchids are rarely selected to be cultivated by botanists and orchid enthusiasts due to their natural requirement either for high level of humidity or warm temperature throughout the year that can be quite difficult to be maintained in housing areas (Alikas, 2009; McDonald, 1999). Meanwhile for terrestrial orchids, they are mostly cultivated for cut flower industry due to their suitability to be grown on soil as well as their easier maintenance and requirement compared to epiphytic and lithophytic orchids (Ramirez *et al.*, 2007). Besides that, semi-terrestrial orchids that can be grown on the ground are also chosen as orchids to be cultivated whereby the root of the orchids do not directly penetrate the soil hence the factors including pH, salt levels and other materials in the soil will not directly inhibit the growth of the orchids. Instead of directly attached to soil, the root of this type of orchids prefer to find supports as well as nutrients from humus and leaves that are available on the ground surface (McDonald, 1999). In addition, this type of semi-terrestrial orchids require open space areas including coastal shrubs, wetlands as well as open shrubby forests where they can obtain adequate level of sunlight for their photosynthesis process without depending on any other plants for support (Alikas, 2009). Examples of terrestrial orchids that are favoured by botanists as well as orchid enthusiasts to be cultivated are *Cymbidium* orchids as well as slipper orchids (Dharmani, 2013).

In orchid industry, there are more than 100,000 orchid hybrids that have been produced by orchid breeders and the number keeps increasing every year in order to produce orchids with better characteristics including the brightness and exotic shape of inflorescent as well as their scent to increase their commercial values (Hands, 2006). Orchid hybrids are produced either by crossing species in the same genera (inter-specific hybrid) or by crossing orchids from different genera (inter-generic hybrid) (Hands, 2006). Examples of orchid hybrids used in this study are *V.*

Mimi Palmer (*Vanda tessellata* x *Vanda* Tan Chay Yan), *V.* Tan Chay Yan (*Vanda dearea* x *Vanda* Josophine van Brero), *V.* Small Boy Leong (*Vanda tricolor* and *Vanda limbata*) and *Vandachostylis* Sri-Siam (*Vanda tessellata* x *Rhynchostylis gigantae*). From the list, *V.* Mimi Palmer, *V.* Tan Chay Yan and *V.* Small Boy Leong are inter-specific hybrids whereby all those orchids have been crossed between orchids in *Vanda* genera. Meanwhile, *Vandachostylis* Sri-Siam is an example of inter-generic hybrid whereby the orchid is produced by hybridisation between two orchid species from two different genera.

In floricultural industry, a large number of orchids have significant economic value where the products including cut-flowers and potted orchids from Malaysia, Singapore and Thailand have contributed an export value of RM200 millions annually (Raubeson *et al.*, 2005; Ooi, 2005). In Japan floral market, 23.4% of orchids have been imported from Malaysia (7,648 million yen), followed by Columbia (19.2%) and China (10.4%) as reported by the Japan Florists' Telegraph Delivery Association, 2010. In Malaysia and Singapore orchid industry, the development of the industry is enhanced by the cultivation of various orchid hybrids due to the easy cultivation of orchid hybrid compared to orchid species that are more difficult to be maintained, besides their free blooming habit as well as their various patterns of shapes, colours and a new array of flowers (Kishor *et al.*, 2006). The demand for orchid species and hybrids is still very high in the floricultural industry that is not limited to local market, but also from international market due to their aesthetic values (exotic and limited sources), including colour, scent and morphology (Hamdan, 2008).

4.2 Vandaceous Orchids

Vandaceous orchids (subfamily Vandoideae) that consist of several genera including *Aranda*, *Arachnis*, *Ascocentrum*, *Mokara*, *Phalaenopsis* and *Vanda* are among the most popular orchids that have been cultivated in orchid industry where these types of orchids receive a high demand as cut-flowers as well as potted flowering plants for both local and international markets in this two decades (Lekawatana, 2010; Fadelah *et al.*, 2001). Among the Vandaceous orchids, orchids from *Vanda* genera are the second most popular orchids after *Phalaenopsis* (Cheam *et al.*, 2009). *Vanda* orchids are favoured by botanists and orchid enthusiasts due to their fast

growth and frequent blooming compared to other orchids (Allikas, 2009). *Vanda* orchids are categorised as monopodial orchids that bloom frequently within three to five times in a year and the flowers in full-bloom stage have longer life-time (two to three weeks) compared to other orchids with shorter life-time (Dharmani, 2013). In addition, orchids from this genera have magnificent flowers with broad colour range including maroon, green, orange, red and white (Cheam *et al.*, 2009). Besides that, popularity of *Vanda* orchid keeps increasing due to a lot of successful orchid hybrids which have been produced by crossing between different species in this genera as well as different genera (Lekawatana, 2010). Exportation activity on Vandaceous orchid in cut flower industry has been initiated in Singapore in mid-1950 (Ng, 1984). To date, many different types of orchid cut-flowers have been exported to over 30 countries including Japan, Australia, USA and Western Europe (Goh and Kavaljian, 1989; Koay, 1993).

Vanda orchids have originated from tropical countries including Malaysia, Philippines and Thailand as their natural habitats (Chew, 2007). There are about 50 species from *Vanda* genera that have been identified to be native to tropical Asian countries and distribution of *Vanda* species has been reported from Sri Lanka and southern India to Papua New Guinea and, northern Australia and Solomon Islands as well as to the north region of China, Taiwan and the Philippines (Chew, 2008). *Vanda* orchids are also known as sympodial epiphytic orchids that can grow easily in full sun exposure, bright light, warm temperatures and in high humidity areas. Other than that, *Vanda* orchids have long and trailing roots to absorb nutrients and moisture from the atmosphere (Kishor *et al*., 2006). Most orchids in *Vanda* genera are epiphytic but some of them have been reported to be either lithophytic or terrestrial that can survive very well in warm and full-sun habitats (Dharmani, 2013). However, there are some orchid species in *Vanda* genera that have been identified to grow naturally in cool and low temperature highland regions including the north of India, Himalaya, Southeast Asia, Papua New Guinea, south China as well as northern Australia (Cheam *et al.*, 2009).

All *Vanda* orchids possess thick stems and roots and the orchids normally require at least for three and a half to 10 years to reach their maturity stage as flowering plants (Kishor *et al*., 2008). Leaves of *Vanda* orchids are flat, strap-shaped and close together but might be different in size and morphology which depend on their habitats (Cheam *et al.*, 2009). Basically, *Vanda*

orchids can be categorised into three main groups according to their leaf' morphological structures which are strap-leaves, semi-terete and terete *Vanda*. Strap-leaf *Vanda* is characterised by the presence of flat leaves while terete *Vanda* orchids is described by their round and pencil-shape leaves. Meanwhile, semi-terete *Vanda* is characterised by the hybrid of both leaf characters, with intermediate size of leaves (American Orchid Society, 2013).

Vanda Mimi Palmer is an orchid hybrid that emits strong sweet fragrance from its floral tissues (Figure 1(a)). This orchid is produced from the crossing between a well-known fragrant-orchid *Vanda tessellata* with an orchid hybrid *Vanda* Tan Chay Yan. *Vanda* Mimi Palmer received its sweet fragrance as well as tri-colour (purplish-green-brown) flower from *Vanda tessellata* while the shape of petal, sepal and leaves might be inherited from *Vanda* Tan Chay Yan (Mohd-Hairul, 2011). The fragrance emitted by floral parts of this orchid has been reported to be developmentally regulated, whereby none of fragrance emission has been detected in bud stage and starts to be detected in half-open flower stage and reaches the highest peak during fully-open flower stage (Mohd-Hairul *et al.*, 2010a). Interestingly, this fragrant-orchid has not only received high demand among orchid enthusiasts but also received several international awards for its strong sweet-smelling fragrance including The Champion Award of Fragrant-Orchids by the Royal Horticultural Society of Thailand in 1993 and the Best Fragrant Orchid in the 17th World Orchid Conference in 2002 (Nair and Arditti, 2002). Besides that, another reason why this orchid hybrid receives high demand among orchid lovers is due to its frequent blooming within a year with maximum emission of floral scent during fully-open flower stage (Chan *et al.*, 2011). Scientific studies on this fragrant-orchid by researchers from Universiti Putra Malaysia have reported the identification, isolation and characterisation of fragrance-related transcripts that are involved in fragrance biosynthesis in this fragrant-orchid via molecular and genetic engineering works (Mohd-Hairul *et al.*, 2010b; Teh *et al.*, 2011; Chan *et al.*, 2011; Song *et al.*, 2012). Interestingly, a sesquiterpene synthase transcript from this orchid has been successfully expressed in *L. lactis*, a gram positive bacterial system whereby the recombinant enzyme has been reported to be involved in catalysing the biosynthesis of multiple sesquiterpene compounds including germacrene D, copaene and nerolidol (Song *et al.*, 2012).

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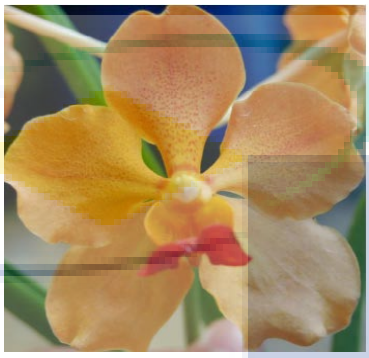
(a)

Vanda Mimi Palmer

(b)

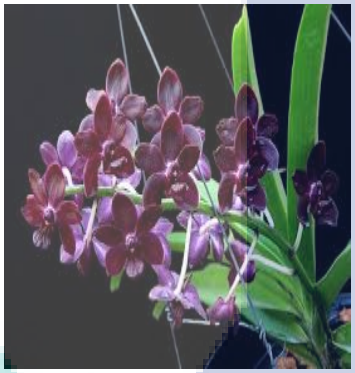


Vanda Tan Chay Yan

(c)

Vanda Small Boy Leong

(d)



Vandachostylis Sri-Siam

Figure 1: Orchids used in this study; (a) *Vanda* Mimi Palmer, (b) *Vanda* Tan Chay Yan, (c) *Vanda* Small Boy Leong and (d) *Vandachostylis* Sri-Siam.

Vanda Tan Chay Yan (Figure 1(b)) that was used in this study is an orchid hybrid that had been produced by the crossing between *Vanda* Josephine and *Vanda dearei* (Yeoh, 1978) with golden colour of round and flat petals and sepals (Mohd-Hairul *et al*., 2010a). This orchid hybrid has been reported to be popular in 1960s and had won several international awards including the First Class certificate in 1954 by the Royal Horticultural Society and the Trophy for The Best *Vanda* at the Second World Conference in Hawaii. This orchid hybrid was first

produced by Robert Tan Hoon Siang, son of a Malaysian rubber plantation merchant, Tan Chay Yan (http://eresources.nlb.gov.sg/). However, this golden colour beautiful orchid hybrid has been reported to lose its popularity as cut flower due to its infrequent blooming of twice a year (Yeoh, 1978). Studies on this orchid by Mohd-Hairul *et al.* (2010a) has shown that this orchid hybrid emits β-ocimene compound that is normally produced by plants for pollination and plant defense. In molecular aspects, none of the β-ocimene synthase gene, transcript and enzyme that are responsible for the biosynthesis of β-ocimene has been reported so far from any orchid.

Vanda Small Boy Leong is a delightful primary orchid hybrid with brown yellowish sepals and petals containing white spots (Figure 1(c)). This orchid hybrid was produced by the crossing of two orchid species, *Vanda tricolor* (seed parent) and *Vanda limbata* (pollen parent). This orchid hybrid was registered by International Orchid Registrar under the Royal Horticultural Society, England on 1st January 1955 (https://www.rhs.org.uk/). This orchid is classified as fragrant-orchid that emits fragrance by its fully-open flower and might receive its strong fragrance characteristics from its pollen parent *Vanda tricolor*. Unfortunately, no study on the biochemical analysis of its fragrance as well as fragrance-related genes, transcripts and enzymes have been reported so far.

Vandachostylis Sri-Siam (Figure 1 (d)) is an orchid hybrid of *Vanda tessellata* and *Rhynchostylis gigantea*. Seed parent for this orchid hybrid is *Vanda tessellata* while the pollen parent is *Rhynchostylis gigantea*. The purplish-grape colour of *Vandachostylis* Sri-Siam as well as the shape and size of this orchid hybrid might be inherited from *Rhynchostylis gigantea* while its fragrant characteristic might be inherited from the other parent, *Vanda tessellata*. This orchid hybrid was registered with the Royal Horticultural Society in England on 1st January 1978 (https://www.rhs.org.uk/). Unfortunately, until this moment, no scientific finding has been reported on any biochemical and molecular biology aspects of this fragrant orchid.

4.3 Biology of Floral Scent

Floral scent or floral fragrance is due to the production of a complex mixture of low molecular mass molecules including monoterpenes, sesquiterpenes, benzenoids,

phenylpropanoids and fatty acid derived compounds that play a very important role in pollination in floral tissues and plant defense in vegetative tissues (Raguso, 2008; Knudsen *et al.*, 1993). In general, emission of floral scent by flowering plants appears during anthesis stage when the petals are already opened and ready to be pollinated by specific or non-specific pollinators (Schade *et al.*, 2001). Specific odours are commonly produced by flowering plants in order to attract specific pollinators such as bees, moths and butterflies to help in their pollination (Dobson, 1994). Besides that, specific emission time of floral scent by floral organ of flowering plants have been reported to be different due to the different active time of specific pollinators as well as the control by circadian clock and photoperiod (Verdonk *et al.*, 2003). This plant-insect interaction is very important to determine the accomplishment of pollination that leads to fruit development in many crop species (Shuttleworth and Johnson, 2009). In addition, biochemical compounds that are present in floral scent play a very important role for plant protection against their natural enemies including herbivores, ants and other pathogens (Schiestl, 2010).

Floral scents of different plant species have been identified to be different due to the combination and compositional level of each compound (Dudareva *et al.*, 2000; Knudsen *et al.*, 1993). Among flowering plants that have been reported to synthesise and emit floral scent, petals of the flowers play an important role for scent emission rather than the entire floral organ (Pichersky *et al.*, 1994) whereby synthesised essential oils are temporarily stored in special oil glands that exist in petals of the flowers before being released as volatiles to the environment (Effmert *et al.*, 2006). However, this concept is different in orchids whereby sepals and lips of orchid flowers also contribute to the fragrance biosynthesis and emission. This is due to the fact that both petals and sepals share almost the same morphological structure in matured flowers where petal becomes the first whorl while sepals become the second whorl of the flowers (Schiestl, 2010). Meanwhile, a special structure of lip or labellum which is a modified petal in orchid flowers is totally different compared to petals and sepals where expression of fragrance-related genes have been detected in the organ but in much lower level compared to petals and sepals (Raguso, 2008). The component of floral scent or floral volatiles that are emitted by flowers can be detected using Gas Chromatography-Mass Spectrometry (GC-MS) which has specific database containing mass spectra of isolated and well-studied compounds (Knudsen and Gershenzon, 2006).

In 1990s, research on floral scent was focused on isolation, structural elucidation of fragrance compounds as well as their chemical synthesis mechanisms in order to fulfil high demand from perfumery and food industries (Knudsen, 1993). Significant research on natural fragrance compounds are very important where the information on their precursors and compound derivatives is very important in production of synthetic perfume that mimic natural floral scent such as rose, jasmine, lavender and petunia for commercialization in perfumery industry (Verdonk *et al.*, 2003; Guterman *et al.*, 2002; Zuker *et al.*, 1998; Kaiser, 1993). Unfortunately, commercialisation of plants in different regions of the world has faced problem on the subsequent progeny production due to the lack of natural local pollinators while traditional breeding of flowering plants to produce better hybrid features has also faced problems to attract their natural pollinators due to the drastic changes of their morphology and biochemistry (Pichersky and Dudareva, 2007). In addition, effort to bring their natural pollinators into the new areas or territory was also unsuccessful the failure of their natural pollinators to adapt to the new environment (Buchmann and Nabhan, 1996).

Thus, introduction of scent engineering approach by transforming fragrance-related genes into plant of interest has opened a new opportunity for pollination where local pollinators that are attracted to the scent emitted by the plants, may visit the plants for honey hunting and indirectly become their pollinating agents (Pichersky and Dudareva, 2007). Besides that, transformation of fragrance-related genes into hybrid plants might restore the original scent produced by their mother plants where original scent from their mother plants had been suppressed due to previous selective breeding that focused on traits other than their fragrant characteristic (Spitcer *et al.*, 2007). Moreover, introduction of fragrance biosynthesis and emission in scentless flowering plants by genetic engineering has been reported to increase their commercial values in cut flower industry (Verdonk *et al.*, 2003).

4.4 Fragrance Biosynthesis Mechanisms

Studies on fragrance biosynthetic pathways via biochemical and molecular biology approaches have revealed that the complex mixture of floral volatile from various flowering

plants consist of compounds derived from terpenoid, benzenoid and phenylpropanoid pathways (Pichersky and Dudareva, 2007). Terpenoid pathway that has been reported to occur in both plastid and cytosol of plants is responsible for the presence of a wide number of terpenoid compounds including monoterpenoids (C10 compounds), sesquiterpenoids (C15 compounds) and diterpenoids (C20 compounds) (Knudsen and Gershenzon, 2006). Examples of monoterpenoid compounds that have been identified as the constituents of floral scent are linalool, ocimene, β-pinene, citral and geraniol while germacrene D, nerolidol, farnesene, copaene and caryophyllene are some of the natural sesquiterpenoids that have been identified to be the constituents of various scents produced by different species of flowering plants (Knudsen and Gershenzon, 2006). Besides terpenoid pathway, benzenoid and phenylpropanoid biosynthetic pathways comprise more than 300 compounds including methylbenzoate, methylsalicylate, phenylacetaldehyde, phenylethyl acetate, benzyl acetate, phenylethanol, eugenol and isoeugenol which are considered as the second largest contributor for floral scent (Pott *et al*., 2002; Raguso *et al*., 2003; Verdonk *et. al*, 2003). In addition, there is another important class of floral scent compounds known as fatty acid derived compounds such as hexanol, hexanal, nonanal, pentadecane, decanal and dodecanal that are synthesised via lipoxygenase pathway and by fatty acid degradation that have been reported to contribute extensively for the constituent of floral scent (Knudsen and Gershenzon, 2006). Volatile of fatty acid derivatives are mostly detected in vegetative tissues in high levels, specifically playing a very important role in plant defense (Schaller, 2001). However this class of compound has also been reported as minor constituent of floral scent that might contribute to their odour that specifically participate in attracting pollinators for pollination purpose (D'Auria *et al*., 2007).

Volatile compound identification that has been focused by researchers in early 1990s has led to fragrance-related enzyme isolation and characterization starting from the middle of 1990s whereby *in vitro* characterisation has been carried out by purification of specific enzymes (Guterman *et al*., 2002; Vainstein *et al*., 2001). Many fragrance-related enzymes have been identified and characterised including linalool synthase, eugenol synthase, salicylic acid carboxyl methyltransferase and geranyl diphosphate synthase from *C. breweri*, phenylacetaldehyde synthase and eugenol synthase from *P. hybrida* as well as ocimene synthase and mycrene synthase from *A. majus* (Chen *et al*., 2003; Dudareva *et al*., 2003; Nagegowda *et al*., 2008;

Pichersky *et al*., 1995). After that, with the development of molecular biology approach, a lot of work has been carried out employing Expressed Sequence Tags (ESTs) library construction for identification of fragrance-related transcripts from scented flowers including *R. hybrida*, *P. hybrida* and fragrant orchids such as *Phalaenopsis bellina* and *V.* Mimi Palmer (Hsiao *et al*., 2006; 2008; Teh *et al*., 2011). In recent years, development of Next Generation Sequencing (NGS) approach that can directly sequence total cDNA that has been transcribed from polyA$^+$ mRNA has enhanced the identification of fragrance-related transcripts involved in fragrance biosynthetic pathway in scented flower with much cheaper cost, faster speed and larger output of data compared to conventional EST libraries that are extensively studied in the previous decade (Graciet *et al*., 2014).

4.5 Benzenoid and phenylpropanoid biosynthetic pathway

Benzenoid and phenylpropanoid biosynthetic pathway that has shown a high contribution in fragrance compound biosynthesis produces the second largest class of volatile organic compounds including benzenoids, phenylpropanoids and their derivatives including alcohols and esters (Gang *et al*., 2001). This pathway involves the main important precursor which is phenylalanine, an aromatic amino acid that is synthesised via shikimate pathway, one of the most important pathways for plant secondary metabolite biosynthesis (Boatright *et al*., 2004). Biochemical compounds synthesised via this pathway are categorised into two major groups based on their carbon skeleton structure whereby benzenoids are referred to C6-C1 backbone while C6-C2 backbone is categorised as phenylpropanoid related compounds (Knudsen and Gershenzen, 2006). Among available benzenoid and phenylpropanoid compounds, about 24% of the compounds have been found to be derived from phenylalanine compound (Pichersky and Dudareva, 2007). Meanwhile the remaining compounds (76%) are derived from non-phenylalanine compounds including benzylbenzoate, benzylacetate, methylbenzoate and phenylethyl acetate (Boatright *et al*., 2004; Pichersky and Dudareva, 2007).

In benzenoid and phenylpropanoid pathway, biosynthesis of fragrance compounds is initiated by a well characterised enzyme which is *L*-phenylalanine ammonia lyase that is very important in catalysing the deamination of *L*-phenylalanine to trans-cinnamic acid. The step is

followed by benzaldehyde formation via C2 shortening of trans-cinnamic acid. Formation of benzenoids (C6-C1) from cinnamic acid involves shortening of the propyl side chain by two carbons and was shown to proceed either via β-oxidative pathway, non-β-oxidative pathway or a combination of both routes (Boatright *et al.* 2004; Orlova *et al.* 2006). The β-oxidative pathway has only recently been fully elucidated in the flower of *P. hybrida* and appears to resemble to fatty acid catabolism and certain branched chain amino acids that are localized in peroxisomes. The pathway begins with an activation of cinnamic acid to cinnamoyl-CoA (CoA thioester), which undergoes hydration, oxidation and cleavage of the β-keto thioester, resulting in subsequent formation of benzoyl-CoA (Van Moerkercke *et al.*, 2009; Klempien *et al.*, 2012; Qualley *et al.*, 2012). Benzoyl-CoA which is localised in peroxisomes for β-oxidative pathway might be transported to cytosol using a special mechanism for benzyl benzoate and phenylethyl benzoate biosynthesis (Kaminaga *et al*., 2006).

In contrast to benzenoids and phenylpropanoids, biosynthesis of volatile phenylpropanoid related compounds (C6-C2), such as phenylacetaldehyde and 2-phenylethanol has been reported to be synthesised via another route whereby phenylalanine is utilized as the main substrate to phenylacetaldehyde synthase as a competitor to cinnamic acid route (Kaminaga *et al.*, 2006; Tieman *et al.*, 2007). Interestingly, the genes involved in both phenylacetaldehyde and 2-phenylethanol biosynthesis have been isolated and characterised (Hirata *et al.*, 2012). In addition, phenylacetaldehyde biosynthesis has been reported to be different in both petunia (*P. hybrida*) and rose (*R. hybrida*) petals in the presence of two main routes which are decarboxylation-amine oxidation reaction and decarboxylation of formed phenylpyruvate intermediate (Kaminaga *et al.*, 2006; Sakai *et al.*, 2007). Specifically, conversion of phenylacetaldehyde to 2-phenylethanol is catalysed by a phenylacetaldehyde reductase that has been reported to be present in rose flowers while decarboxylation reaction catalysed by phenylacetaldehyde synthase (PAAS) has been detected to occur in petals of both *P. hybrida* and *R. hybrida* (Kaminaga *et al.*, 2006; Farhi *et al*., 2010). Besides that, another alternative route has been reported whereby deamination process of phenylalanine by aromatic amino acid aminotransferase first occurs to form phenylpyruvate as intermediate compound and is subsequently followed by decarboxylation of phenylpyruvate to phenylacetaldehyde, representing second alternative route that only occurs in *R. hybrida* (Farhi *et al.*, 2010). Other than that, the deamination of phenylalanine in tomato also occurs via two

separate steps, where it is first converted to phenylethylamine by an aromatic amino acid decarboxylase and then, the action of a hypothesised amine oxidase, dehydrogenase, or transaminase for the formation of phenylacetaldehyde (Tieman *et al.*, 2006). Meanwhile, Gonda *et al.* (2010) discovered that melon fruit (*Cucumis melo* L.) has a third enzymatic route whereby transaminated phenylalanine is converted to its corresponding α-keto acid, phenylpyruvate and subsequently followed by decarboxylation to phenylacetaldehyde.

Meanwhile, formation of floral volatile phenylpropanoids (C6-C3) such as eugenol, isoeugenol, methyleugenol, methyl-isoeugenol, chavicol and methylchavicol has been identified to share the same initial biosynthetic steps with lignin biosynthetic pathway up to the step of coniferyl alcohol biosynthesis (Koeduka *et al*., 2006). Subsequent esterification step of coniferyl alcohol to coniferyl acetate that has been identified to occur in petals of *P. hybrida* is catalysed by a coniferyl alcohol acetyltransferase (Dexter *et al.*, 2007). Coniferyl acetate is subsequently converted to phenylpropanoid compounds which are eugenol and isoeugenol by eugenol synthase (EGS) and isoeugenol synthase (IGS), respectively. EGS and IGS have been reported to belong to pinoresinol-lariciresinol reductase, isoflavone reductase and phenylcoumaran benzylic ether reductase (PIP) family of NADPH dependent reductases (Koeduka *et al*., 2006; 2008).

Furthermore, the diversification of phenylpropanoid and benzenoid compounds is further increased by modification process on the compounds by methylation, hydroxylation and acetylation reaction that enhance their volatility or olfactory properties of scent compounds (Muhlemann *et al*., 2014). In addition, further methylation reactions on the modified benzenoids and phenylpropanoids are catalysed by either O-methyltransferases (OMTs) or carboxyl methyltransferases to eugenol, isoeugenol and chavicol for the downstream production of methyleugenol, isomethyleugenol and methylchavicol (Gang *et al.*, 2002). Meanwhile, O-methyltransferases (OMTs) have been reported to be responsible for the biosynthesis of a diverse array of benzenoids and phenylpropanoids including veratrole in white campion flower (*Silene latifolia*) (Gupta *et al.*, 2012; Akhtar and Pichersky, 2013), 3,5- dimethoxytoluene and 1,3,5-trimethoxybenzene in roses (Lavid *et al.*, 2002; Scalliet *et al.* 2002) as well as methyleugenol and isomethyleugenol in *C. breweri* (Wang and Pichersky, 1998).

Besides that, in benzenoid and phenylpropanoid pathway, *S*-adenosyl-*L*-methionine: salicylic acid carboxyl methyltransferase (SAM:SAMT), *S*-adenosyl-*L*-methionine: benzoic acid carboxyl methyltransferase (SAM:BAMT) and theobromine synthase (SABATH) are well-known as carboxyl methyltransferases (D'Auria *et al.*, 2003) responsible for volatile ester biosynthesis such as methylbenzoate in *A. majus* and *P. hybrida* flowers (Murfitt *et al.*, 2000; Negre *et al.*, 2003), as well as methylsalicylate in *C. breweri* and *P. hybrida* (Ross *et al.*, 1999; Negre *et al.*, 2003). In addition, enzymes from the benzyl alcoholacetyl-,anthocyanin-O-hydroxy-cinnamoyl-,anthranilate-N-hydroxy-cinnamoyl/benzoyl deacetyl vindoline acetyl trans ferase (BAHD) superfamily of acyltransferases including acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase, benzylalcohol O-acetyltransferase, benzoyl-CoA benzyl alcohol phenylethanol benzoyltransferase, and benzoyl-CoAbenzylalcohol O-benzoyltransferase (D'Auria, 2006) has been shown to be responsible for acetylation of scent compounds such as benzyl acetate in *C. breweri* (Dudareva *et al.*, 1998), benzoyl benzoate in *C. breweri* and *P. hybrida* (D'Auria *et al.*, 2002; Boatright *et al.*, 2004; Orlova *et al.*, 2006), and phenylethyl benzoate in *P. hybrida* (Boatright *et al.*, 2004; Orlova *et al.*, 2006).

4.6 Scientific studies on fragrant-orchids

Identification of biochemical compounds in complex mixture of fragrant-orchid volatiles has been initiated in late 1980s using Gas Chromatography-Mass Spectrometry (GC-MS) (Kaiser, 1993). A scientific book on orchid scent by Kaiser (1993) has shown GC-MS analysis data of more than 180 orchid species and hybrids including *Cattleya araguaiensis*, *Cymbidium formosanum*, *Dendrobium carniferum*, *Dendrobium superbum*, *Oncidium curcutum*, *Phalaenopsis violacea* and *Vanda tessellata*. While in 2006, scent constituent of *Phalaenopsis bellina* was reported by Hsiao *et al.* (2006) and followed by *V.* Mimi Palmer (Mohd-Hairul *et al.*, 2010a) whereby most of their scents are dominated by terpenoids as well as benzenoids and phenylpropanoids. Monoterpenoids and sesquiterpenoids are the highly distributed compounds among orchid species based on the GC-MS analysis on scented orchids including linalool, mycrene, ocimene, germacrene D and nerolidol. Besides terpenoids, benzenoids and phenylpropanoid compounds are also found to be described among in scented orchids including methylbenzoate, benzyl benzoate, benzyl acetate, phenylethyl acetate, eugenol and isoeugenol.

In addition, there were traces of other compounds detected in scented orchids such as fatty acid derivatives, indole and formanilide (Kaiser, 1993; Hsiao *et al.*, 2006). More recently, Gallego *et al.* (2012) reported that scent constituents of three orchid species namely *Himantoglossum robertianum*, *Ophrys apifera* and *Gymnadenia conopsea* containing 106 different volatile compounds have been identified in their scent. Among the volatile compounds, 54% of the compounds have been confirmed including α-pinene, β-pinene and limonene in *Himantoglossum robertianum*, 1-butanol, butyl ether and caryophyllene in *Ophrys apifera* and phenethyl acetate, eugenol and benzaldehyde in *Gymnadenia conopsea*.

Meanwhile, knowledge on molecular biology aspects of fragrance biosynthesis in orchid plants was firstly reported on *Phalaenopsis bellina* by Taiwanese researchers by identifying fragrance-related transcripts from constructed cDNA library (Hsiao *et al.*, 2006). Subsequently, characterisation of a monoterpene synthase geranyldiphosphate synthase (PbGDPS) that lack DXXD motif has been reported for the same orchid (Hsiao *et al.*, 2008). In addition, expression analysis using real-time PCR and Northern-blot has shown that PbGDPS gene expression is developmentally regulated whereby the transcript level has been detected to increase gradually once the bud opened and reached the highest peak on the fifth-day after bud-opening, and followed by a gradual decrease of its expression towards the end of the flower life-cycle. Subsequently, several fragrance-related transcripts have been identified from *V.* Mimi Palmer, a well-known fragrant-orchid that received several international awards for its strong sweet smelling fragrant (Chan *et al.*, 2009; 2011; Mohd-Hairul *et al.*, 2010b; Mohd-Hairul, 2011; Teh *et al.*, 2011; Song *et al.*, 2012). More recently, another work has been reported on isolation and characterisation of a eugenol synthase transcript from an orchid species *Gymnadenia odoratissima* where functional expression of the transcript in *Escherichia coli* system has shown that the recombinant enzyme was capable of catalysing the biosynthesis of eugenol from its substrate coniferyl acetate (Gupta *et al.*, 2014).

4.7 Molecular and biochemical studies on the scent of *Vanda* Mimi Palmer

Biochemical analysis on the scent of *V.* Mimi Palmer has been reported by Mohd-Hairul *et al.* (2010a) whereby the volatile comprises high level of benzenoids as well as phenylpropanoid compounds including methylbenzoate, benzylacetate, phenylethyl acetate and phenylethyl alcohol besides terpenoid compounds including linalool, ocimene and nerolidol. Besides that, emission of volatile from the orchid hybrid has been reported to be developmentally regulated, in the same pattern of *Phalaenopis bellina* that has been reported by Hsiao *et al.* (2006). In addition, emission pattern of volatiles has been reported to be time regulated whereby the volatile emission has been detected at very low level at 6.00am, and increased gradually until it reached the highest peak in the afternoon and decreased gradually in the evening. The volatile emission could not be detected at night (Mohd-Hairul *et al.*, 2010a).

In molecular biology aspects, a floral cDNA library has been constructed by Chan *et al.* (2009) and several fragrance-related transcipts have been identified from the library covering both terpenoid as well as benzenoid and phenylpropanoid biosynthetic pathway (Chan *et al.*, 2009; Mohd-Hairul, 2011; Teh *et al.*, 2011). From their findings, the expression of most fragrance-related transcripts showed the same expression pattern whereby the transcripts level in the floral tissues are developmentally regulated. The expression of fragrance-related transcripts including *V.* Mimi Palmer 1-deoxyd-xylulose 5-phosphate reductoisomerase (VMPDXR), *V.* Mimi Palmer phenylacetaldehyde synthase (VMPPAAS), *V.* Mimi Palmer alcohol acyltransferase (VMPAAT), *V.* Mimi Palmer sesquiterpene synthase (VMPSTS), *V.* Mimi Palmer 4-(cytidine 5 -diphospho)-2-C-methyl-d-erythritol kinase (VMPCMEK), *V.* Mimi Palmer cytochrome P450 (VMPCyP450) and *V.* Mimi Palmer Acetyl-CoA-C-acetyltransferase (VMPACA) started to be detected during the opening of the bud, increased gradually until reached the highest peak during fully-open flower stage and decreased gradually until their senescence. Interestingly, expression analysis on the fragrant-related transcripts in different tissues has shown that the transcripts have been detected at very high level in petal and sepal compared to bud and vegetative tissues. The results suggested that sepals of the orchid are playing the same role as petals for fragrance biosynthesis in which they share the same morphological structures and functions. Recently, genetic engineering work on *V.* Mimi Palmer

Sesquiterpene synthase (VMPSTS) was successfully carried out by expressing the transcript in *L. lactis*, a gram positive bacterium (Song *et al.*, 2012) where the sesquiterpene synthase has been found to be responsible in catalysing the biosynthesis of Germacrene D as the major compound besides some other sesquiterpene compounds as its by-products including nerolidol and copaene.

4.8 Economic Importance of fragrant-orchids

For orchid, trait such as floral scent is a primary novel marker because it is a key determinant of consumer choice (Hsiao *et al*., 2006). However, traditional breeding programmes have caused the lost of the scent of orchid varieties. Many orchids have been focused on other non-fragrance purpose like improving the visual aesthetic and vase life, and for producing either cut-flower or ornamental-type orchids (Hsu *et al*., 2011). Orchid industry thrives on novelty for producing high quality varieties which also focused on colour and shape of orchid flower as well as their floral scent (Hsiao *et al*., 2011). Studies on orchid scent biology is difficult due to different odours, genome size, period of life cycle, regeneration time and inefficient transformation systems (Chang *et al*., 2011). For production of orchid hybrids, several scented and scentless orchids which are cross-incompatible probably lead to restriction of scented progeny, either with diluted scent or complete lost of the scent characteristics (Hsiao *et al*., 2008). Other than that, fragrance biosynthetic pathway of orchids remains yet to be understood because limited studies have been carried out so far, especially in discovery of scent-related enzymes and genes in monocotyledonous plants such as orchids generally, and vandaceous orchids specifically (Teh *et al*., 2011). In addition, new scented orchid varieties may have been developed successfully for cut-flower, but, the rational design of suitable choice of species is also important for genetic manipulation in order to understand the composition of scent components, their specific fragrance biosynthetic pathways, key scented enzymes and other few related features (Hsiao *et al*., 2011).

More recently, orchids have become the centre of attention to new areas of plant research, including genetic engineering, functional genomics, proteomics and metabolomics (Hsu *et al*., 2011; Chang *et al*., 2011), which require advanced transgenic strategies (da Silva *et al*., 2011) to achieve their different goals. The successful application of these new approaches will help to

further improve orchids and orchid products. However, different situation happened in cut-flower industry when breeding of cultivated flowers has been extensively studied in order to improve their vase life, shipping characteristics and visual aesthetic values such as shape and colour, leading to the lost of floral scent originality eventually (Vainstein *et al.*, 2001). Moreover, due to the rising demand and popularity of orchids, it is nowadays a multimillion dollar business, primarily as pot plants and cut flower stalks (Winkelmann *et al.*, 2006). To meet the demand on orchids in the future, as well as to conserve their biodiversity, the development and deployment of new technologies are very important for improving flower quality, resistance to biotic and abiotic stresses, and to rapidly produce mass propagation members of this phylum (Raubeson *et al.*, 2005).

4.9 Genetic engineering of terpene synthases

Terpene synthases are typically present in plants at low levels and hence not easily to be purified (Bohlmann *et al.*, 1998). Therefore, heterologous expression of these enzymes is crucial in obtaining high yield of pure proteins for analysis purpose. Terpene synthases are often expressed successfully in *E. coli* using standard plasmid expression vectors. Although the good success rate, there are some possible difficulty for the expression of plant terpene synthases in *E. coli*. The synthases sequences may contain N-terminal transit peptides (Targeting sequences that target the nuclear-encoded preproteins to the plastids for proteolytic processing to the mature forms) and in some cases these sequences promote the formation of inclusion bodies when expressed in *E. coli* (Bohlmann *et al.*, 1998). Removing the targeting sequences from the cDNA of the synthases will likely solve the problem. Another common problem is the frequency of arginine residues that use rare tRNAs in the prokaryotic host. Co-expression of the terpene synthase cDNA with the required tRNA can provide solution to this translational difficulty (Steele *et al.*, 1998).

Due to the obstacles faced by *E. coli* expression system, other methodology had been explored as to provide an alternative protein expression for terpene synthases. As an example, an optimized Agrobacterium-mediated transient expression assay had been tested in *Nicotiana benthamiana* for plant diterpene synthase expression (Brückner and Tissier, 2013). This method

has successfully produced sufficient quantities of the enzyme required for analysis of unknown diterpenes. Such method may one day provide alternative expression vehicle for various terpenes synthases that faced difficulty being synthesized by *E. coli*. Interestingly, Lücker *et al.* (2004) had conducted the first study on the introduction and simultaneous expression of multiple terpene synthases in plants by metabolic engineering. The authors had engineered a transgenic tobacco plant line with three foreign cDNA from lemon that encodes different monoterpene synthases. The resulting tobacco plant had elevated expression of β-pinene, limonene, and γ-terpinene and a few additional products of the monoterpene synthases.

4.10 *Escherichia coli* expression system

E. coli is gram-negative microorganism that can be found living in the lower intestines of humans and other warm-blooded animals. It has rod-shape morphology which is about 2.5µm long and 0.8µm in diameter and characterized with hemispherical end caps (Berg, 2008). *E. coli* is a widely used expression system for recombinant proteins. As the biochemistry of *E. coli* had been unravelled extensively and the research community is very familiar with it, that figures why *E. coli* is so widely used in each laboratory. Not just in laboratories, *E. coli* is also utilized for industrial and pharmaceutical large-scale protein production (Terpe, 2006). However, Terpe (2006) had pointed out that the use of *E. coli* as cell factory has a major disadvantage where there will be undesired lipopolysaccharide (endotoxins), which is pyrogenic to humans, mixing in the biosynthesized recombinant proteins. This provides a challenge for downstream processing of pharmaceutical proteins.

The recombinant proteins can be expressed either in *E. coli* cytoplasm (cytoplasmic expression) or secreted into the periplasm (periplasmic expression). In most cases, researchers prefer to conduct cytoplasmic expression of heterologous proteins as this approach allows a significantly higher yield of the proteins (Terpe, 2006). Each mode of expression has its own advantages and disadvantages (Baneyx, 1999). First, cytoplasmic expression offers high yield expression but the overproduction of proteins in cytoplasm may lead to the formation of aggregates known as inclusion bodies. Second, eukaryotic proteins expressed in cytoplasm may misfold due to the lack of disulphide bond formation mechanism. Third, heterologous proteins in

cytoplasm must be obtained by cell lysis, which will break out all the native cellular proteins that are mixed with proteins of interest. In certain circumstances, periplasmic expression is more viable. Periplasm is an oxidizing environment and it harbours enzymes which catalyse the formation and rearrangement of disulphide bonds which is ideal for disulphide bond formation in eukaryotic proteins (Missiakas and Raina, 1997).

In the past, (-)-limonene synthase, myrcene synthase, and (-)-pinene synthase from Grand Fir had been expressed successfully in *E. coli* expression system (Bohlmann *et al.*, 1997). Three monoterpene synthases from common sage (*Salvia officinalis*): (+)-Sabinene synthase, bornyl diphosphate synthase and 1,8-cineole synthase had also been successfully expressed in *E. coli* expression system (Wise *et al.*, 1998). A α/β-pinene synthase gene from Cotton (*Gossypium hirsutum L.*) had also been heterologously expressed in *E. coli* and the expressed enzyme converted the substrate GPP to α-pinene and β-pinene with 9:1 ratio (Huang *et al.*, 2013). Other than that, first α-pinene fed-batch fermentation using *E. coli* had been reported (Yang *et al.*, 2013). Yang and colleagues had assembled a biosynthetic pathway of α-pinene in an *E. coli* strain using the heterologous MVA pathway and the geranyl diphosphate synthase (GPPS2) as well as α-pinene synthase gene (Pt30) from *Pinus taeda*. Other than that, toxicity of β-pinene to *E. coli* culture will not be a limiting factor as it was reported that β-pinene is significantly less toxic than α-pinene to cell cultures (Sarria *et al.*, 2014).

CHAPTER V

RESULTS

5.1 Transformation into *E. coli* competent cells and clones verification

pMAL:MTS had been primarily transformed into DH5α *E. coli* strain. After that, the extracted plasmid from the DH5α *E. coli* clone was transformed into BL21 and Rosetta *E. coli* strains. On the other hand, pMAL:*Ec*DXR:MTS, pMAL:*Ec*DXS:MTS and pMAL:*Ec*GPP:MTS plasmids were initially transformed into BL21 *E. coli* strain. Later, the plasmids were extracted from the BL21 *E. coli* clones and the plasmids were transformed into Rosetta *E. coli* strain for protein expression.

To verify the successful transformants, colony PCR was carried out and the PCR products were analyzed using agarose gel electrophoresis. Verification of pMAL:*MTS* clones was successful where the PCR yielded single bands (Figure 4.6). However, the verification of pMAL:*Ec*DXR:MTS, pMAL:*Ec*DXS:MTS and pMAL:*Ec*GPP:MTS clones were proven to be challenging as *Ec*DXR, *Ec*DXS and *Ec*GPP genes are naturally present in the *E. coli* genomic DNA. Hence, another clone verification approach had been utilized. The pMAL:*Ec*DXR:MTS, pMAL:*Ec*DXS:MTS and pMAL:*Ec*GPP:MTS plasmids were extracted and were used as template for multiplex PCR. Multiplex PCR works different only by the application of both *MTS* set 1 and respective *Ec*DXR, *Ec*DXS or *Ec*GPP set 2 primers in each reaction. The PCR products were analyzed using agarose gel electrophoresis (Figure 2). Despite that, the results of multiplex PCR was unsatisfactory. Thereby, a few clones had been selected directly for protein expression instead.

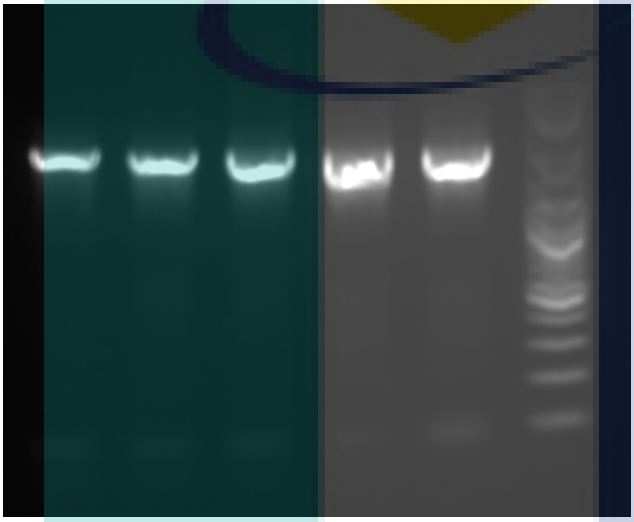


Figure 2: Colony PCR amplification of pMAL:MTS clones. Lane M: 100 bp Ladder Mix (Thermo Fisher, USA); Lane 1-5: PCR product of five different colonies of pMAL:MTS clones. The size of the amplified gene was ~12.5 kb. The estimation of the size of the DNA was not accurate due to the distortion of the DNA ladder.

5.2 Expression of recombinant clones

Heterologous expression of the MBP-MTS recombinant protein or MBP-MTS protein fused with endogenous *Ec*DXR, *Ec*DXS or *Ec*GPP proteins was analyzed by SDS-PAGE and Western blotting. There were a total of three different strains of *E. coli* (DH5α, BL21 and Rosetta) that harbored pMAL:MTS plasmid. On the other hand, pMAL:*Ec*DXR:MTS, pMAL:*Ec*DXS:MTS and pMAL:*Ec*GPP:MTS plasmids had been harbored by BL21 and Rosetta *E. coli* strains.

Recombinant MBP-MTS protein expression of DH5α harboring pMAL:MTS plasmid was analyzed using SDS-PAGE (Figure 3). However, it was observed that the induction of the clones with IPTG decreased expression of MBP-MTS recombinant protein and the results were analyzed using SDS-PAGE. BL21 strain had failed to express the MBP-MTS recombinant protein (Figure 4). Then, Rosetta strain, which was designed to compensate *E. coli* rare codons, had successfully produced the MBP-MTS recombinant protein (Figure 5).

Next, BL21 clones harboring pMAL:*Ec*GPP:MTS plasmids had successfully expressed the recombinant MBP-*Ec*GPP-*MTS* protein (Figure 6). Subsequently, the expression of recombinant protein by Rosetta clones harboring pMAL:*Ec*DXR:MTS, pMAL:*Ec*DXS:MTS or pMAL:*Ec*GPP:*MTS* plasmids were studied using SDS-PAGE. The expression of MBP-*Ec*DXR-*MTS* and MBP-*Ec*DXS-*MTS* proteins were not successful (Figure 7 and Figure 8). The clones harboring pMAL:*Ec*DXR:*MTS* produced protein bands with size of ~45 kDa which may be MBP

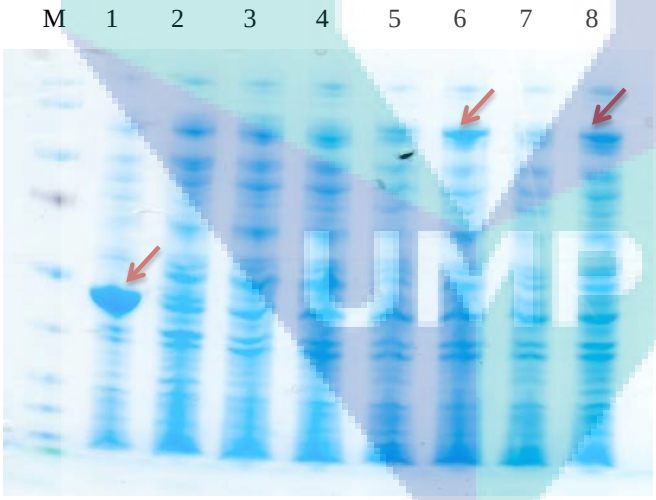


Figure 3: Protein expression of DH5α clones harboring the pMAL:*MTS* or empty pMAL (with no gene inserted) plasmid induced at 0.3 mM IPTG for four hours. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1-2: Clone harboring empty pMAL

plasmid, induced and non-induced respectively. Induced clone produced a MBP protein band with size of ~45 kDa; Lane 3-4: Clone 1 harboring pMAL:*MTS* plasmid, induced and non-induced respectively. No protein expression was observed; Lane 5-6: Clone 2 harboring pMAL:*MTS* plasmid, induced and non-induced respectively. The non-induced clone expressed an intense protein band with size ~115 kDa.; Lane 7-8: Clone 3 harboring pMAL:*MTS* plasmid, induced and non-induced respectively. The non-induced clone expressed an intense protein band with size ~115 kDa.

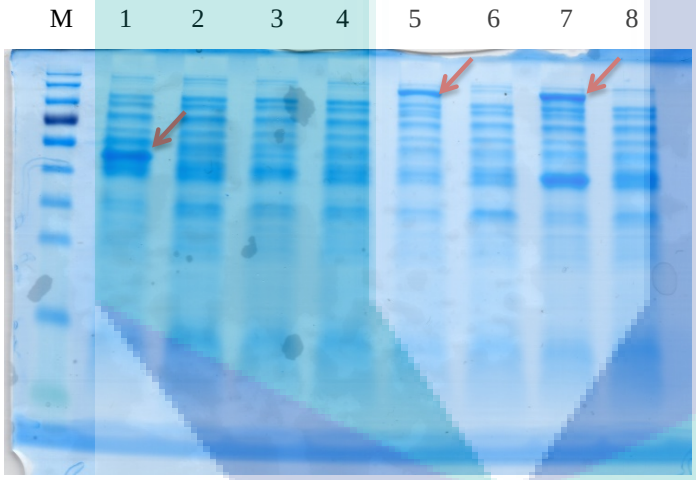


Figure 4: Protein expression of BL21 clones harboring the pMAL:*MTS* and pMAL:*Ec*GPP:*MTS* plasmids induced at 0.3 mM IPTG for two hours. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1-2: Putative clone harboring empty pMAL plasmid, induced and non-induced respectively. Induced clone produced a protein band with size of ~45 kDa; Lane 3-4: Clone harboring pMAL:*MTS* plasmid, induced and non-induced respectively. No protein expression was observed; Lane 5-8: Clones one and two harboring pMAL:*Ec*GPP:*MTS* plasmids, with lanes alternating between induced and non-induced clones. Both induced clones expressed intense protein bands with size ~130 kDa.

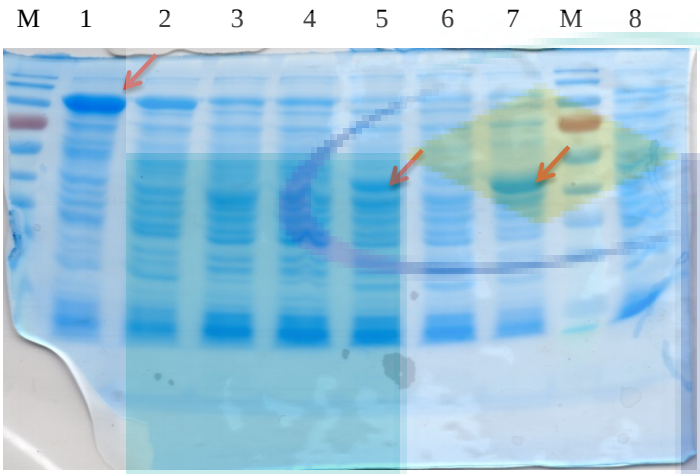


Figure 5: Protein expression of Rosetta clones harboring the pMAL:*MTS* and pMAL:*Ec*DXR:*MTS* plasmids induced at 1.0 mM IPTG for two hours. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1-4: Clones one and two harboring pMAL:*MTS* plasmid. The lanes alternate between induced and non-induced. Induced clone one produced an intense protein band with size of ~110 kDa. Clone two had no recombinant protein expression; Lane 5-8: Clones one and two harboring pMAL:*Ec*DXR:*MTS* plasmid. The lanes alternate between induced and non-induced. However, the induced clones produced protein bands with size of ~45 kDa which may be MBP proteins. The MBP-*Ec*DXR-*MTS* recombinant protein was not successfully expressed.

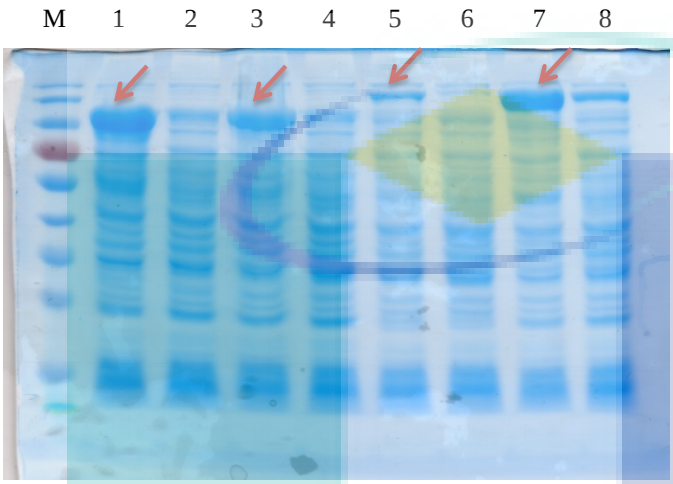


Figure 6: Protein expression of Rosetta clones harboring the pMAL:*Ec*DXS:*MTS* and pMAL:*Ec*GPP:*MTS* plasmids induced at 1.0 mM IPTG for two hours. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1-4: Clones one and two harboring pMAL:*Ec*DXS:*MTS* plasmid. The lanes alternate between induced and non-induced. Induced clones produced intense protein bands with size of ~110 kDa. However, this size observation do not agrees with the estimated MBP-*Ec*DXS-*MTS* recombinant protein size; Lane 5-8: Clones one and two harboring pMAL:*Ec*GPP:*MTS* plasmid. The lanes alternate between induced and non-induced. The induced clones produced protein bands with size of ~130 kDa. This size observation agrees with the estimated MBP-*Ec*GPP:*MTS* recombinant protein size.

proteins. The clones harboring pMAL:*Ec*DXS:*MTS* produced intense protein bands with size of ~110 kDa. However, this size observation do not agrees with the estimated MBP-*Ec*DXS-*MTS* recombinant protein size which is 173.8 kDa. Despite clones harboring pMAL:*Ec*DXR:*MTS* or pMAL:*Ec*DXS:*MTS* did not produce expected results, the clones harboring pMAL:*Ec*GPP:*MTS* had successfully expressed the MBP-*Ec*GPP-*MTS* recombinant protein with size of ~130 kDa (Figure 4.12). This size observation agrees with the estimated MBP-*Ec*GPP-*MTS* recombinant protein size.

Subsequently, in order to perform enzymatic assays, the MBP-*MTS* recombinant protein was purified using affinity chromatography. Then, SDS-PAGE analysis was conducted using purified MBP-*MTS* protein as to verify the success of the purification process (Figure 7). It is concluded that the protein had been successfully purified using the affinity chromatography method.

For clones harboring the pMAL:*MTS* or pMAL:*Ec*GPP:*MTS*, Western blot showed bands demonstrating the presence of recombinant protein with his-tag (Figure 8). However, during the process of transferring the protein from acrylamide gels to nitrocellulose membranes, it was discovered that the gels are much bigger than the membrane. Thereby, in the process of accommodating the transfer of sample proteins, the PageRuler Protein Ladder was not fully enclosed with the membrane, and hence not transferred to the membrane. By this, the Western blot analysis did not have the protein ladder and the protein size of the sample bands cannot be determined. Furthermore, Western blot analysis for clones harboring the pMAL:*Ec*DXR:*MTS* or pMAL:*Ec*DXS:*MTS* did not yield any bands formation (Figure 8).

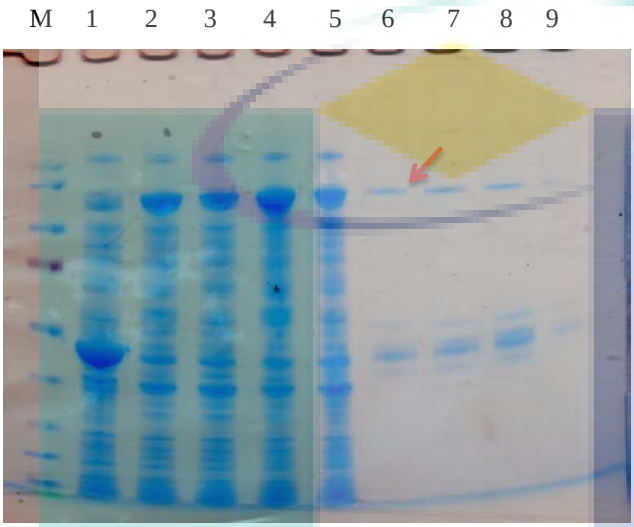


Figure 7: SDS-PAGE analysis of purified MBP-*MTS* recombinant protein using affinity chromatography. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1: Clone harboring empty pMAL plasmid induced with IPTG. MBP band was observed at ~45 kDa; Lane 2-5: Clones one and two harboring pMAL:*MTS* plasmid. The lanes alternate between induced and non-induced. The induced clones produced bigger bands at ~120 kDa which represents MBP-*MTS* recombinant protein; Lane 6-9: Replicates of the purified MBP-*MTS* recombinant protein using affinity chromatography. The recombinant protein was observed at ~120 kDa. Other than that, there were bands at ~45 kDa which were MBP.

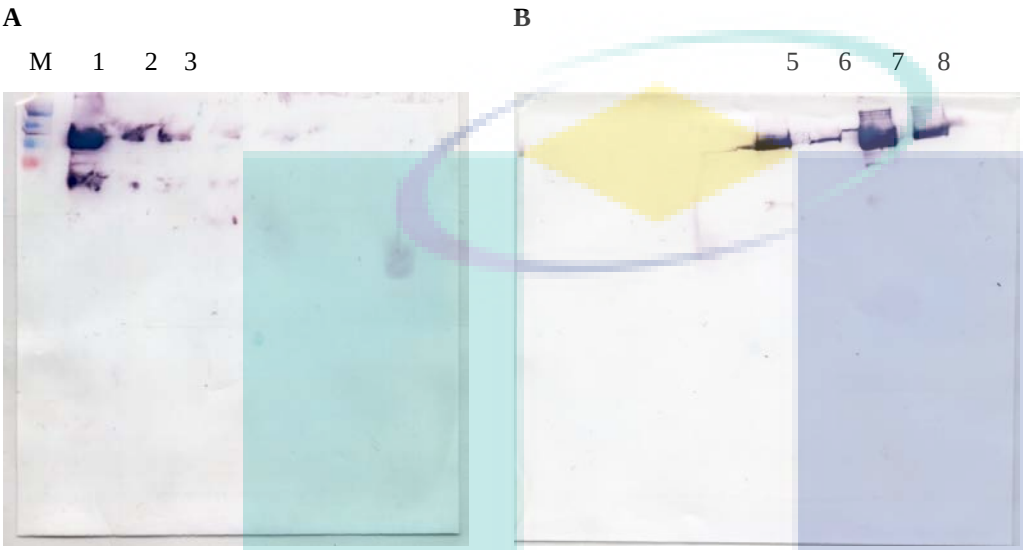
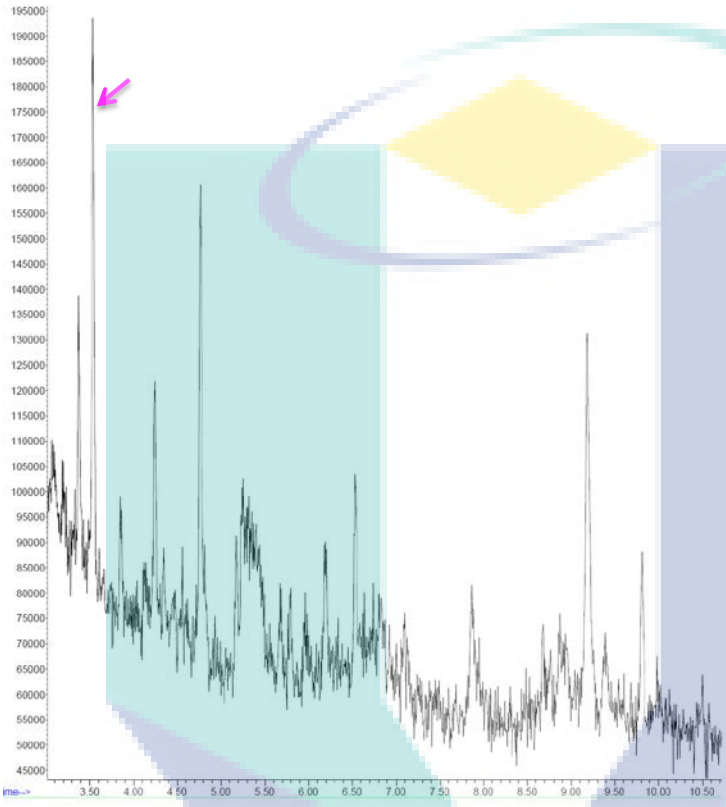


Figure 8: Western blot results for clones harboring the (A) pMAL:*MTS* and pMAL:*Ec*DXR:*MTS* as well as (B) pMAL:*Ec*DXS:*MTS* and pMAL:*Ec*GPP:*MTS* plasmids. The protein samples used for Western blot were the same used for SDS-PAGE in Figure 4.11 and Figure 4.12. Lane M: PageRuler Prestained Protein Ladder (Thermo Fisher, USA); Lane 1: Induced Rosetta clone one harboring pMAL:*MTS* plasmid; Lane 2-3: Non-induced Rosetta clone one harboring pMAL:*MTS* plasmid; Lane 5-8: Rosetta clones one and two harboring pMAL:*Ec*GPP:*MTS* plasmid. The lanes alternate between induced and non-induced. Empty spaces: Induced and non-induced Rosetta clones harboring pMAL:*Ec*DXR:*MTS* or pMAL:*Ec*DXS:*MTS* did not form any bands.

5.3 Determination of plasmid-expressed protein functionality

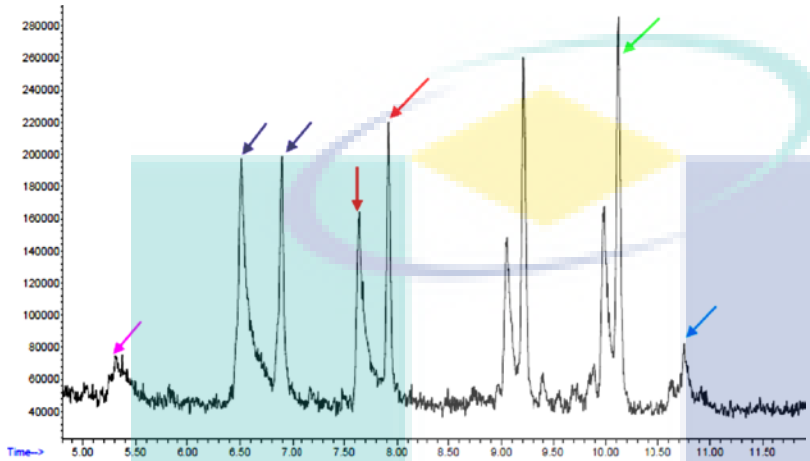
The functionality of heterologous expressed recombinant protein was analyzed by *in vivo* analysis and *in vitro* enzymatic assay. *In vivo* analysis on monoterpene compounds produced by recombinant Rosetta *E. coli* harboring pMAL:*MTS* plasmid had been carried out. Subsequently, PDMS fibre was used to trap the volatile compounds and later analysed using GC-MS equipment. The procedure had been conducted three times and the first two *in vivo* analysis yield no results. The third test resulted in an identification of a monoterpene compound. The result is a seen in Figure 9. From the result, only α-pinene compound had been detected at high level.

In vitro enzymatic assay using purified MBP-*MTS* recombinant protein had been carried out with GPP utilized as substrate. Subsequently, PDMS fibre was used to trap the volatile compounds and later analysed using GC-MS equipment. The result is a seen in Figure 10. From the result, the putative β-pinene synthase enzyme can produce multiple products as determined in this study. The products of the recombinant enzyme include β-pinene, linalool, methyl ester of linalool, (2E)-1-Methoxy-3,7-dimethylocta-2,6-diene and geraniol. Furthermore, another three replicates of *in vitro* enzymatic assay had been conducted and it was discovered that MBP-*MTS* may be able to produces β-ocimene and β-myrcene.



Label arrow colour	Time (min)	Compound
Pink	3.536	α-pinene

Figure 9: GC-MS result of *in vivo* monoterpene synthesis by Rosetta *E. coli* harboring pMAL:*MTS* plasmid.



Label arrow colour	Time (min)	Compound
Pink	5.321	β-pinene
Deep blue	6.513	Linalool
	6.899	
Red	7.642	Linalool,
	7.918	methyl ether
Green	10.121	(2E)-1-Methoxy-3,7-dimethylocta-2,6-diene
Baby blue	10.755	Geraniol

Figure 10: GC-MS result of *in vitro* enzymatic assay using purified *MTS* recombinant protein.

PART VI

INTERPRETATION

After confirming the recombinant proteins biosynthesis by *E. coli* harbouring the pMAL:*MTS* or pMAL:*Ec*GPP:*MTS* plasmid, the functional role of the novel recombinant monoterpene synthase in catalysing the biosynthesis monoterpene compounds was studied. *In vitro* enzymatic assay using purified MBP-*MTS* recombinant protein had been carried out with GPP utilized as substrate. Subsequently, PDMS fibre was used to trap the volatile compounds and later analysed using GC-MS equipment. The test showed that linalool, methyl ester of linalool and (2E)-1-Methoxy-3,7-dimethylocta-2,6-diene are the main products while β-pinene and geraniol are produced in minute amount. This finding agrees with the study hypothesis that *MTS* enzyme can catalyse GPP to form multiple monoterpene molecules. However, it is shown that β-pinene is not the main product of *MTS* enzyme. Furthermore, the amounts of each respective monoterpenes are relative and not quantitatively accurate. This is due to the qualitative nature of this study's methodology. Thereby, the ratio of products' quantities cannot be calculated.

In comparison, β-pinene synthase from *Artemisia annua* produces β-pinene and α-pinene in the ratio of 94:6, respectively (Lu *et al.*, 2002). Other than that, a monoterpene cyclase from *A. grandis* produces α-pinene and β-pinene at a ratio of 2:3 (Lewinsohn *et al.*, 1992). Furthermore, there are other monoterpene synthases that produce β-pinene as minor product. Limonene synthase of Spearmint (*Mentha spicata*) produces limonene (94%) as the major compound and traces of minor monoterpene by products including α-pinene (2%), β-pinene (2%), and myrcene (2%) (Colby *et al.*, 1993; McGarvey and Croteau, 1995). Next, 1,8-cineole synthase from common sage (*Salvia officinalis*) produces1,8-cineole as the major product and several monoterpene compounds as by products including α-pinene, β-pinene, myrcene and (+)-sabinene (Wise *et al.*, 1998). Interestingly, there are no monoterpene synthase found that produces linalool as main product and β-pinene as minor product. The *Ec*GPP:*MTS* fusion recombinant protein has not been tested for its functionality and whether it will increases the *in vivo* production of monoterpene compounds

PART VII

CONCLUSIONS AND SUGGESTIONS

The current study describes the development of *E. coli* as a heterologous host for the production of plant monoterpene compounds. In the past, various monoterpene synthases genes had been expressed into *E. coli*. However, it is note-worthy that monoterpene synthases from fragrant-orchids have not been characterized and expressed into *E. coli* expression system. In this study, monoterpene synthases from fragrant-orchids have been successfully cloned and expressed into *E. coli* expression host by utilizing PMAL-c5X as expression vector.

The recombinant protein is successfully expressed in the system and *in vitro* analysis on the purified enzyme has shown its involvement in biosynthesis of multiple monoterpene compounds including β-pinene, linalool, methyl ether of linalool, geraniol, β-ocimene and β-myrcene. Besides that, the recombinant system has been identified to produce α-pinene compound *in vivo*. These results indicate that this enzyme can be a viable candidate for fragrance and flavor industrial application. In future, additional metabolic engineering and growth medium optimization need to be carried out to mass produce monoterpene compounds in the recombinant system for industrial scale production.

In addition, metabolic engineering effort has been carried out in this study in an attempt to increases the biosynthesis of monoterpene compounds. A total of three native enzymes, *Ec*DXR, *Ec*DXS and *Ec*GPP belonging to MEP pathway in *E. coli* have been selected and their full ORF have been isolated from *E. coli*. In the end, only *Ec*GPP enzyme has been successfully co-expressed with β-pinene synthase. However, further *in vivo* analysis and *in vitro* enzymatic assay are required to study it further. Other than that, different strategy can be applied for further metabolic engineering such as integration of the gene into *E. coli* host genome and increasing the copies of monoterpene synthase genes in the host.

PART VIII

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