Available online at http://www.ijims.com
ISSN: 2348 – 0343

Identification of fragrance-related transcripts from selected orchids using cDNA Representational Difference Analysis (cDNA-RDA) approach

Barudin Mohd-Aiman[1], Ab. Rahim Mohd-Hairul[1*], Tan Suat Hian[1]
[1]Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia.
*Corresponding Author: Ab. Rahim Mohd-Hairul

Abstract

Volatile components have been identified in hundreds of orchids including species and hybrids, but the biochemical and molecular biological knowledge on the biosynthesis of scent compounds in orchids is still limited. To date, a strategy combining chemical analysis, genomics and bioinformatics is adopted to unravel the scent biosynthetic pathways that are involved in floral scent biosynthesis in orchids. In this study, cDNA-RDA was used to identify fragrance-related transcripts in three selected fragrant-orchids including*Vanda* Mimi Palmer, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siamin comparison to *Vanda* Tan Chay Yan, a non-fragrant orchid. This cDNA-RDA approach allows us toisolate cDNA fragments that are presentin selected fragrant orchids but absent in non-fragrant orchid. Double-stranded cDNA of each fragrant orchid was hybridized separately with double-stranded cDNA of the selected non-fragrant orchid and followed by amplification of subtracted fragments. Three rounds of cDNA-RDAwere carried out to enrich difference cDNA fragments.A number of 21 clones of difference products were sequenced and sequence analysis has shown the presence of three different putative fragrance-related transcripts including methionine synthase, *S*-adenosyl-*L*-methionine synthase, and phenylalanine ammonia lyase. All of the identified transcripts might be involved in fragrance biosynthesis of those fragrant orchids.

Keywords:fragrant-orchids, non-fragrant orchid, cDNA-RDA, fragrance-related transcripts.

Introduction

Orchidaceae flowers display various shapes, sizes, colours, and also emission of different fragrances [1]. Limited information is available for the corresponding fragrance-related genes that are involved in fragrance production in orchids. Studies on fragrance-related genes have been reported in other scented plants previously including *Petunia hybrida, Antirrhinum majus,Nicotianatabacum* and *Rosa hybrida*[2]. In a well-known scented orchid, *Vanda* Mimi Palmer, a few fragrant-related transcripts have been isolated through suppression subtractive hybridization (SSH) approach including alcohol acyltransferase (VMPAAT) and sesquiterpene synthase(VMPSTS)[3]. Besides that, some other fragrance-related transcripts in monoterpene biosynthetic pathway have been identified in another scented orchid, *Phalaenopsisbellina* through EST library including

geranyl diphosphate synthase (GDPS), farnesyl diphosphate synthase (FDPS) and geranylgeranyl diphosphate synthase (GGDPS)[4,5].

Representational difference analysis (RDA), a PCR based subtractive cloning method is a sensitive and powerful PCR mediated subtraction approach for identification of DNA fragments that are the most different between two samples. Although the method was first developed by Lisitsyn*et al.* (1993)[6] for identifying the differences between two complex genomes, Hubank*et al.*(1994)[7] later adapted the method for use with cDNA to identify differentially expressed genes.cDNA-RDA is a notable method to detect differentially expressed genes in plants [8,9]. For example, a common bean, *Phaseolus vulgaris* was studied to identify drought-stress induced genes using cDNA-RDA prior to transcriptome analysis of contrasting genotypes [10].Besides that, there are numerous positive results reported on cDNA-RDA method that are applied in different plants including *Oryzasativa*[11], *Hordeumvulgare*[12] and *Solanumlycopersicum*[13].

In this study, we report the isolation of putative fragrance-related transcripts through cDNA-RDA. This method was applied to cDNAs from floral tissues of three selected fragrant-orchids including*Vanda* Mimi Palmer, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siam in comparison to *Vanda* Tan Chay Yan, a non-fragrant orchid. In this cDNA-RDA analysis, transcripts that are either differentially expressed or divergent in their sequences are enriched by PCR after successive rounds of subtractive hybridization, whereas non-differentially expressed or non-divergent sequences (which are of no interest) are suppressed and effectively removed. For vandaceous orchid, cDNA-RDA is the first attempt to be used for identifying fragrance-related transcripts that would provide better understanding of the molecular biology of fragrance in vandaceous orchids including*Vanda* Mimi Palmer, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siam.

Materials and Methods

Plant materials

Orchid plants (*Vanda*Mimi Palmer,*Vanda* Small Boy Leong, *Vandachostylis* Sri-Siam and*Vanda*Tan Chay Yan) used in this study were purchased from the United Malaysian Orchids Sdn. Bhd., a nursery located in Rawang, Selangor. Fully open flowers were detached from their mother plants, frozen in liquid nitrogen and stored at -80°C prior to total RNA extraction.

Double-stranded cDNA preparation

Total RNA was extracted from fully open flower of *Vanda* Mimi Palmer, *Vanda* Tan Chay Yan, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siam separately, following the method described by Chan et al. (2009)[14].PolyA$^+$ mRNA was then isolated from each sample using the PolyATract® mRNA Isolation Systems III (Promega, USA). PolyA$^+$ mRNA of each sample was subjected to double-stranded cDNA synthesis using the Universal Riboclone® cDNA synthesis system (Promega, USA), following the manufacturer's instructions.

Representational difference analysis (RDA)

In this study, cDNA Representational Difference Analysis (cDNA-RDA) was carried out in forward way. The cDNA of*Vanda* Mimi Palmer,*Vandachostylis* Sri-Siamand *Vanda* Small Boy Leong (fragrant-orchids) were designated as testers (the source of the sequences of interest to be isolated) while *Vanda* Tan Chay Yan (non-fragrant orchid) was chosen as a driver DNA (the source of the sequences to be eliminated) in this cDNA-RDA experiment (Table 1).

An amount of 2µg of each tester and a driver was digested at 37°C for 3 hours using three restriction enzymes (*Bgl*II,*Bam*HI and *Hin*dIII), separately.The digested testers and driver were then purified with phenol: chloroform: isoamyl alcohol (25:24:1) extraction before precipitating with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5volume of absolute ethanol at -20°C for 30 minutes. The precipitated testers and driver were then centrifuged at 4°C at 16,099*x*g for 20 minutes. After that, supernatants were discarded and a washing step was carried out by applying 200µl of 70%(w/v) ethanol on each cDNA pellet. All of the cDNA pellets were then air dried before dissolved in 30µl of TE buffer.

cDNA-RDA was carried out as described by Hubank and Schatz (1994)[7] with minor modifications. The major steps were: (1) generation of representations; (2) hybridization and selective PCR amplification; (3) cloning and sequencing of difference products. The modifications were made mainly in the tester:driver ratios that wereused in three rounds of cDNA-RDA to increase the detection of rare transcripts. An amount of 50ng of each digested testers and driver was ligated with 250µM of R adaptor pairs (Table 2) at 16°C for 20 hours in the presence of 400U of T4 DNA ligase (New England Biolabs, UK). The ligated products were bulked up by PCR amplification using the same 24-mer adaptors (R Bgl24, R Hind24 and R Bam24) as specific primers. The amplified PCR products were purified byphenol:chloroform:isoamylalcohol (25:24:1) extraction and precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol. The purified PCR products were then digested with the respective enzyme to remove the respective R adaptor.

In the first round of cDNA-RDA, the digested PCR products were ligated with the respective J adaptors. An amount of 30ng of each tester was mixed with 3,000ng of each driver, resulting for a ratio of 1:100 (tester:driver). The mixtures were denatured at 95°C for 5 minutes and hybridized at 67°C for 20 hours in a thermocycler (Biorad, USA). The hybridized products were adjusted to a final volume of 200µl by adding TE buffer. Then, 10µl of the diluted hybridized products were selectively PCR amplified in a 100µl reaction containing 1X PCR buffer, 0.2mM dNTPs, 2.5U of TaqDNA polymerase (GeneAll, Korea) and 24-mer J primers (J BgI24, J Hind24 and JBam24). The PCR mixtures were then incubated at 72°C for 5 minutes to fill in the ends of the re-annealed fragments. After that, a PCR cycle was performed using the following parameter: 94ºC for 3 minutes; 35 cycles at (94ºC for 1 minute; 72ºC for 2 minutes) and 72ºC for 5 minutes. The PCR products were then purified with phenol: chloroform: isoamyl alcohol (25:24:1) extraction and precipitated with 0.1 volume of 3M sodium acetate (pH5.2) and 2.5 volume of absolute ethanol. The precipitated pellets were dissolved in 40µl of TE buffer.

A volume of 20µl of the purified amplified hybridization products were then subjected to mung bean nuclease digestion. The digestion mixture was carried out in a total volume of 40µl containing 1X mung bean nuclease buffer and 2U of mung bean nuclease enzyme (New England Biolabs, UK). The digestion reaction was carried out at 30°C for 30 minutes and followed by addition of 160µl of 50mM Tris-Cl (pH 8.9). The mung bean

nuclease activity of the mixture was then terminated at 99°C for 5 minutes. The first round RDA products were used as drivers for second round of RDA. Second round of RDA was carried out by digesting the RDA products with the respective restriction enzymes (*Bgl*II, *Bam*HI and *Hin*dIII). Then, the digested products were ligated with N adaptors at the ratio of tester:driver (1:1000). The next steps were carried out in the same manner until mung bean nuclease digestion step. Subsequently, third round of RDA was carried out using J adaptors with the ratio of tester:driver (1:100,000). Products of each round of RDA were electrophoresed on 1.2%(w/v) agarose gel containing 1X TAE buffer and 0.2X gel-red solution (Biotium, USA) and viewed under a UV gel documentation system (Figure 1).

Cloning, sequencing and sequence analysis of cDNA-RDA products

Each difference productin third round of cDNA-RDA was excised from 1.2%(w/v) agarose gel and purified using GeneAllExpin Combo GP kit (GeneAll, Korea) following the manufacturer's instructions. The purified RDA products were cloned into pGEM-T easy vector (Promega, USA) and transformed into DH5α*Escherichia coli* competent cells using heat-shock method as described bySambrook and Russell (2001) [15]. Positive clone of each RDA products were cultured overnight in 5ml LB broth containing 20mg/ml ampicillin. The bacterial culture of each clone was subjected to plasmid mini preparation using DNA spin plasmid DNA purification kit (GeneAll, Korea) following the manufacturer's instructions. The purified plasmid of each clone was sent for sequencing using universal T7 and SP6 primers (Macrogen, Korea).Sequencing result of each clone was subjected to vector sequence removalusing Vecscreen tool in NCBIGenBank database [16]. Then, all the nucleotide sequences were compared to thecloselyrelatedsequencesavailableat NCBI GenBank database using the BLASTX tool[17].

Results and discussion

cDNA Representational Difference Analysis (cDNA-RDA) of selected fragrant-orchids including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siam in comparison to *Vanda* Tan Chay Yan, a non-fragrant orchid has shown the presence of a total number of 21 cDNA bands (Figure 1c) with the size range of 250 to 600bp.BLASTX analysis of all the 21 cloneshas shown the presence of three fragrance-related transcripts including phenylalanine ammonia-lyase (VMP/*Bam*HI_1), *s*-adenosyl methionine synthase (VSBL/*Bgl*II_1) and methionine synthase (VSBL/*Bgl*II_3 and VSSS/*Bgl*II_3) (Table 3).

Sequence analysis on VMP/*Bam*HI_1 clone, using BLASTX tool in the NCBI GenBank database shows that it might resemble nucleotide sequence of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). Phenylalanine ammonia lyase (PAL) has been reported to be involved in benzenoid and phenylpropanoid pathway in plants including *Cistanchedeserticola*[18], *Musa acuminata*[19], *Rhuschinensis*[20] and*Dendrobiumcandidum*[21].PAL is the first and the key enzyme in the regulationof overall carbon flux in thebenzenoid andphenylpropanoid pathway [22,23]. This enzyme catalyzes the deamination of L-phenylalanine to produce cinnamic acid (trans-cinnamate) and ammonia [23]. It is very important as flower pigments, antibiotics, protection against ultraviolet radiation, insect repellent,molecular signals in the microbe–plant interaction, and precursors of suberin, lignin and other

components of cell wall [24,25]. Besides that, phenylpropanoid resulted from the catalysis of PAL can be indirectly or directly produce many secondary metabolic products including plant hormones[26,27].

BLASTX analysis on VSBL/*Bgl*II_1 clone has shown the transcript encodes for *s*-adenosyl-*L*-methionine synthase (SAMS; EC 2.5.1.6) with the highest score of 97.1 and E-value of $2e^{-22}$(Table 3). *S*-adenosyl-*L*-methionine synthase (SAMS) enzyme has been reported to be involved as the key enzyme in *S*-adenosyl-*L*-methionine (SAM) biosynthesis. SAMS is known to be a crucial key enzyme in SAM cycle,catalyzing SAM biosynthesis withthe adenylation of methionine (Met) and ATP [28,29]. The gene encoding SAMS has been studied from several plants including*Oryzasativa* (rice) and*Brassica rapa* (chinese cabbage) [30,31,32].

Besides that, two partial sequences of methionine synthase transcripts have been identified in clones VSSS/*Bgl*II_3 and VSBL/*Bgl*II_3. Methionine synthase (MS; EC 2.1.1.14) functions in metabolic regeneration of SAM through the SAM cycle by performing the methylation of homocysteine to methionine and tetrahydrofolate[33]. The resulted methionine derived from the previous catalysis of methionine synthase after converting homocysteine in metabolic process of SAM cycle [34]. Besides that, methionine synthase has been studied in several plants including *Orobanche ramose* and*Nicotianasuaveolens*[33,35].

Both *S*-adenosyl-*L*-methionine synthase (SAMS) and methionine synthase (MS) are similarly involved in the same SAM cycle with different function of catalysis. The SAM cycle is a fundamental metabolic pathway providing cells and tissues with SAM, methionine and related metabolites [36]. To date, a few studies have been done for identifying the SAM cycle genesthat are expressed in leaves, while there is no information available on floral tissues [37]. In addition, SAMS, which produces *S*-adenosyl-*L*- methionine, the methyl donor of enzymes involved in the production ofvolatile methylated benzenoids including methylbenzoate[38]. The SAM cycle contributes to benzenoidsbiosynthesis by providing methyl groups from the methyl donor SAM, which is regenerated in several steps.Regulation of volatile benzenoid production is at the level of precursor production from the shikimate pathway via chorismate and phenylalanine [39]. In addition, *S*-adenosyl-*L*-methionine (SAM) is an important biological methyl donor that is involved in the methylation of many cellular compounds, volatiles and a precursor for the synthesis of metabolites, such as ethylene, polyamine, nicotianamine and biotin [30].

However, ten sequences ($^{\neq}$) belong to RNA-dependent RNA polymerase from *Cymbidium* mosaic virus (*Cym*MV) have been identified from the three sets of cDNA-RDA (Table 3). *Cym*MV infection is dominant and extremely stable in Orchidaceae, and it was found to be prevalent in *Vanda* Mimi Palmer. Previous screening on *Vanda* Mimi Palmer floral cDNA library revealed a 1.6% contamination rate with *Cym*MV genes [40]. More than 50 orchids infecting viruses have been detected worldwide, with *cymbidium* mosaic virus (*Cym*MV) and *odontoglossum* ringspot tobamovirus (*O*RSV) infection being the most prevalent [41].

Benzenoids production in fragrant orchids might be regulated by the transcriptional control of shikimate pathway with the production of chorismate which is then converted to phenylalanine (Phe) and further metabolized to benzenoids and related products. The role of PAL becomes important when it also converts Phe

to trans- cinnamic acid which is a precursor for downstream production of benzoic acid. The indirect relationship between PAL and *S*-adenosyl-*L*-methionine (SAM) cycle genes (SAMS and MS) might happen when SAM cycle contributes to volatile benzenoid synthesis by providing methyl groups from the methyl donor SAM to resulted benzoic acid. After the methylation of benzoic acid with the catalytic activity of benzoic acid salicylic acid methyltransferase (BSMT) enzyme, methylbenzoate (MeBA) is produced. MeBA is also benzenoid products besides phenylethyl alcohol and phenylacetaldehyde that are also synthesized from phenylalanine. Thus, in fragrant-orchids, it is suggested that the theory onbenzenoids production is based on the previous study on *Petunia hybrida* 'Mitchell'[42]. From this study, we prefer to suggest further studies need to be done in order to enable further elucidation on the regulation of benzenoid biosynthesis such as full-length isolation and molecular characterization of SAM cycle-regulated biosynthetic genesas well as PAL genein *vandaceous* orchids.

Conclusion

In this study, cDNA Representational Difference Analysis (cDNA-RDA) is one of the molecular approaches that usecDNA in identifying putative fragrance-related transcripts after performing three rounds of hybridization between cDNA of fragrant and non-fragrant orchids. Sequence analysis of all the difference products has shown the presence of three putative fragrance-related transcripts that are predicted to be involved in benzenoid and phenylpropanoid pathway in fragrant orchids including putative methionine synthase (MS), *S*-adenosyl-*L*-methionine synthase (SAMS) and phenylalanine ammonia-lyase (PAL). In future, full-length sequences of all the three fragrance-related transcripts need to be isolated for further molecular characterization of the transcripts that might contribute for fragrance biosynthesis in *vandaceous* orchids.

Acknowledgements

We thank Ministry of Sciences, Technology and Innovation for Sciencefund Research Grant (02-01-16-SF0049) (RDU 120501) and Universiti Malaysia Pahang for the Short Term Research Grant (RDU 110353)to support this project.

References

1) Chai D and Yu H. Recent advances in transgenic orchid production. Orchid Science and Biotechnology. 2007; 1: 34-39.
2) Dudareva N, Pichersky E and Gershenzon J. Biochemistry of plant volatiles. Plant Physiology. 2004; 135: 1839-1902.
3) Chan WS, Abdullah JO and Namasivayam P. Isolation, cloning and characterization of fragrance-related transcripts from *Vanda* Mimi Palmer. ScientiaHorticulturae. 2011; 127: 388-397.
4) Hsiao YY, Tsai W, Kuoh C, et al. Comparison of transcripts in *Phalaenopsisbellina* and *Phalaenopsisequestris* (*Orchidaceae*) flowers to deduce monoterpene biosynthesis pathway. BMC Plant Biology. 2006; 6: 14-27.
5) Hsiao YY, Jeng MF, Tsai WC, et al. A novel homodimericgeranyl diphosphate synthase from the orchid *Phalaenopsisbellina* lacking a DD(X)2–4D motif. Plant J. 2008; 55: 719–733.
6) Lisitsyn N, Lisitsyn NM and Wigler M. Cloning the differences between two complex genomes. Science. 1993; 259: 946–951.
7) Hubank M and Schatz DG. Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. 1994; 22: 5640–5648.
8) Bowler LD. Representational difference analysis of cDNA. Methods Mol Med. 2004; 94(2): 49-66.
9) Mishra RN, Ramesha A, Kaul T, et al. A modified cDNA subtraction to identify differentially expressed genes from plants with universal application to other eukaryotes. Anal. Biochem. 2005; 345: 149–157.

10) Müller BSF, Sakamoto T, Dias Silveira RD, et al. Differentially Expressed Genes during Flowering and Grain Filling in Common Bean (*Phaseolus vulgaris*) Grown under Drought Stress Conditions. Plant MolBiol Rep. 2013.
11) Jun HC, Gi HY, Nam SC, et al. Cloning of albino-associated genes from rice (Dongjinbyeo) by cDNA-representational difference analysis. 2005.
12) Kwasniewski M and Szarejko I. Molecular Cloning and Characterization of β-Expansin Gene Related to Root Hair Formation in Barley. Plant Physiol. 2006; 141(3): 1149–1158.
13) Kok EJ, Nicole LW, Franssen-van Hal, et al. Assessment of representational difference analysis (RDA) to construct informative cDNA microarrays for gene expression analysis of species with limited transcriptome information, using red and green tomatoes as a model. Journal of Plant Physiology. 2007; 164: 337-349.
14) Chan WS, Abdullah JO, Namasivayam P, et al. Molecular characterization of a new 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) transcript from *Vanda* Mimi Palmer. ScientiaHorticulturae. 2009; 121: 378-382.
15) Sambrook J and Russell D. Molecular Cloning: a Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.
16) NCBI GenBank database [Online] Available from: http://www.ncbi.nlm.nih.gov/ [Accessed February 2015].
17) BLASTX program [Online] Available from: http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastx [Accessed February 2015].
18) Gao SH, Jing MJ, Yeon JH, et al. Molecular characterization of phenylalanine ammonia lyase gene from *Cistanchedeserticola*. Mol. Biol. Reps. 2011; 38(6): 3741-3750.
19) Alvarez JC, Rodriguez HA, Rodriguez-Arango E, et al. Characterization of a Differentially Expressed Phenylalanine Ammonia-Lyase Gene From Banana Induced During*Mycosphaerellafijiensis* Infection. Journal of Plant Studies. 2013; 2(2).
20) Ma W, Wu M, Wu Y, et al. Cloning and characterisation of a phenylalanine ammonia-lyase gene from *Rhuschinensis*. Plant Cell Rep. 2013; 32: 1179-1190.
21) Jin Q, Yao Y, Cai Y, et al. Molecular Cloning and Sequence Analysis of a Phenylalanine Ammonia-Lyase Gene from *Dendrobium*. PLoS ONE. 2013; 8(4): 62352.
22) Camm EL. Towers GHN. Review article: phenylalanine ammonia lyase. Phytochemistry. 1973; 12: 961-73.
23) Xiang L and Moore BS. Inactivation, complementation, and heterologous expression of encP, a novel bacterial phenylalanine ammonia-lyase gene. J Biol Chem. 2002; 277: 32505-9.
24) Liang X, Dron M, Cramer CL, et al. Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. J Biol Chem. 1989; 264: 14486–14492.
25) Calabrese JC, Jordan DB, Boodhoo A, et al. Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. Biochemistry. 2004; 43: 11403-16.
26) Hahlbrock K and Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol. 1989; 40:347–36.
27) Kim SH, Virmani D, Wake K, et al. Cloning and disruption of a phenylalanine ammonialyase gene from *Ustilagomaydis*. Curr Genet. 2001; 40:40-8.
28) Barbara AM and Elizabeth AW. Sustaining S-adenosyl-L -methionine-dependent methyltransferase activity in plant cells. Physiol. Plant. 2001; 113:435-442.
29) Lim CC, Liu JZ and Pua EC. Characterization of S -adenosylmethioninesynthetase genes and its expression is associated with ethylene synthesis in mustard (*Brassica juncea*). Physiol. Plant. 2002; 116: 522-530.
30) Li W, Han Y, Tao F, et al. Knockdown of SAMS genes encoding S-adenosyl-L-metininesynthetases causes methylation alterations of DNAs and histones and leads to late flowering in rice. J. Plant Physiol. 2011; 168: 1837-1843.
31) Jae-Gyeong Y, Gi-Ho L and Young-Doo P. Physiological Role of Endogenous S-adenosyl-L-methionine Synthetase in Chinese Cabbage. Hort. Environ. Biotechnol. 2012; 53(3): 247-255.
32) Saet-Byul K, Jae-Gyeong Y, Gi-Ho L, et al. Characterization of *Brassica rapa* S-adenosyl-L-methionine Synthetase Gene Including Its Roles in Biosynthesis Pathway. Hort. Environ. Biotechnol. 2012; 53(1):57-65.
33) Roeder S, Dreschler K, Wirtz M, et al. SAM levels, gene expression of SAM synthetase, methionine synthase and ACC oxidase, and ethylene emission from *N. suaveolens* flowers. Plant Mol. Biol. 2009; 70: 535-546.
34) Gómez-Gómez L and Carrasco P. Differential expression of the S-adenosyl-L methionine synthase genes during pea development. Plant Physiol. 1998; 117:397-405.
35) Gonza´lez-Verdejo CI, Die JV, Nadal S, et al. Isolation and expression analysis of a cobalamin-independent methionine synthase gene from the parasitic plant *Orobancheramosa*. ScientiaHorticulturae. 2008; 116:337–341.
36) Ravanel S, Block MA, Rippert P, et al. Methionine metabolism in plant: Chloroplasts are autonomous for de novo methionine synthesis and can import S-adenosyl-methionine from the cytosol. J Biol Chem. 2004; 279: 22548–22557.
37) Kim SB, Yu JG, Lee GH, et al. Characterization of *Brassica rapa* S-adenosyl-L-methionine synthetase gene including its roles in biosynthesis pathway. Hort. Environ. Biotechnol. 2012; 53: 57-65.
38) Boerjan W, Bauw G, Van Montagu M, et al. Distinct phenotypes generate by over-expression and suppression of S-adenosyl- L-methionine synthetase reveal developmental patterns of gene silencing in tobacco. The Plant Cell. 1994; 6: 1401–1414.
39) Roje S. S-Adenosyl-L-methionine: Beyond the universal methyl group donor. Phytochemistry. 2006; 67: 1686-1698.
40) Teh SL, Chan WS, Abdullah JO, et al. Development of expressed sequence tag resources for *Vanda* Mimi Palmer and data mining for EST-SSR. Molecular Biology Reports. 2011; 38: 3903-3909.

41) Sherpa A, Hallan V, Pathak P, et al. Complete nucleotide sequence analysis of *cymbidium* mosaic virus Indian isolate: further evidence for natural recombination among potexviruses. Journal of Bioscience. 2007; 32: 663-669.
42) Schuurink RC, Haring MA and Clark DG. Regulation of volatile benzenoid biosynthesis in petunia flowers. Trends in Plant Science. 2006; 11:20–25.

Table 1: Three sets of RDA for identifying fragrance-related transcripts

RDA set	RE digestion	Tester	Driver
A	*Bgl*II *Bam*HI *Hin*dIII	cDNA of *Vanda* Mimi Palmer	cDNA of *Vanda* Tan Chay Yan
B	*Bgl*II *Bam*HI *Hind*III	cDNA of *Vanda* Small Boy Leong	cDNA of *Vanda* Tan Chay Yan
C	*Bgl*II *Bam*HI *Hin*dIII	cDNA of *Vandachostylis* Sri-Siam	cDNA of *Vanda* Tan Chay Yan

Table 2: Oligonucleotide adaptor sequences used in three rounds of cDNA-RDA

RE	**Adaptors**	**Name**	**Sequences 5'-3'**
*Bgl*II	R oligonucleotide pair	R Bgl24	AGCACTCTCCAGCCTCTCACCGAG
		R Bgl12	GATCCTCGGTGA
	J oligonucleotide pair	J Bgl24	ACCGACGTCGACTATCCATGAACG
		J Bgl12	GATCCTCCCTCG
	N oligonucleotide pair	N Bgl24	AGGCAACTGTGCTATCCGAGGGAG
		N Bgl12	GATCCTCCCTCG
*BamH*I	R oligonucleotide pair	R Bam24	ACCGACGTCGACTATCCATGAACG
		R Bam12	GATCCGTTCATG
	J oligonucleotide pair	J Bam24	AGGCAACTGTGCTATCCGAGGGAG
		J Bam12	GATCCTCCCTCG
	N oligonucleotide pair	NBam24	AGCACTCTCCAGCCTCTCACCGAG
		NBam12	GATCCTCGGTGA
*Hind*III	R oligonucleotide pair	RHind24	AGCACTCTCTCCAGCCTCTCACCGCA
		RHind12	AGCTTGCGGTGA
	J oligonucleotide	J Hind24	ACCGACGTCGACTATCCATGAACA

pair	JHind12	AGCTTGTTCATG
N oligonucleotide	NHind24	AGGCAGCTGTGGTATCGAGGGAGA
pair	NHind12	AGCTTCTCCCTC

Table 3: The BLASTX analysis of 6 difference products of three RDA sets.

Set A [*Vanda* Mimi Palmer (Tester) vs *Vanda* Tan Chay Yan (Driver)]

No	Clone name	Sequence homology	Putative identity	Score	E value	Genbankacession number
1	**VMP**/*Bgl*II_1	*Sorexaraneus*	Cobalamin biosynthesis protein	38.1	0.92	JZ480886
2	**VMP**/*Bam*HI_1	*Dendrobiumcandidum*	Phenylalanine ammonia- lyase*	167	$5e^{-46}$	JZ480887
3	**VMP**/*Bam*HI_2	*Glycine max*	Uncharacterized protein	90.5	$8e^{-19}$	JZ480888
4	**VMP**/*Hin*dIII_1	*Cymbidum*mosaic virus	RNA-dependent RNA polymerase#	100	$1e^{-22}$	-
5	**VMP**/*Hin*dIII_2	*Ophyrsqarqanica*	Stearoyl-acyl carrier protein desaturase	123	$4e^{-32}$	JZ480889
6	**VMP**/*Hin*dIII_3	*Cymbidum*mosaic virus	RNA-dependent RNA polymerase#	119	$3e^{-29}$	-

Set B [*Vanda* Small Boy Leong (Tester) vs *Vanda* Tan Chay Yan (Driver)]

No	Clone name	Sequence homology	Putative identity	Score	E value	Genbankacession number
1	**VSBL**/*Bgl*II_1	*Hostaventricosa*	s-adenosyl methionine synthase*	97.1	$2e^{-22}$	JZ480893
2	**VSBL**/*Bgl*II_2	*Pisumsativum*	Gag-pol polyprotein	71.6	$2e^{-12}$	JZ480894
3	**VSBL**/*Bgl*II_3	*Camellia sinensis*	Methionine synthase*	273	$3e^{-84}$	JZ480895
4	**VSBL**/*Bam*HI_1	*Oryzasativa*	Clathrin assembly protein	80.9	$2e^{-15}$	JZ480896
5	**VSBL**/*Hin*dIII_2	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	183	$7e^{-51}$	-
6	**VSBL**/*Hin*dIII_3	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	115	$9e^{-28}$	-

Set C [*Vandachostylis* Sri-Siam (Tester) vs *Vanda* Tan Chay Yan (Driver)]

No	Clone name	Sequence homology	Putative identity	Score	E value	Genbankacession number
1	**VSSS**/*Bgl*II_1	*Oryzasativa*	Auxin-repressed protein	51.2	$2e^{-06}$	JZ480890
2	**VSSS**/*Bgl*II_2	-	No significant similarity found	-	-	JZ480891
3	**VSSS**/*Bgl*II_3	*Orobancheramosa*	Methionine synthase*	271	$1e^{-83}$	JZ480892
4	**VSSS**/*Bam*HI_1	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	145	$7e^{-38}$	-
5	**VSSS**/*Bam*HI_2	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	106	$2e^{-24}$	-
6	**VSSS**/*Hin*dIII_1	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	119	$3e^{-29}$	-
7	**VSSS**/*Hin*dIII_2	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	184	$5e^{-50}$	-
8	**VSSS**/*Hin*dIII_3	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	183	$7e-^{51}$	-
9	**VSSS**/*Hin*dIII_4	*Cymbidium* mosaic virus	RNA-dependent RNA polymerase#	179	$8e^{-50}$	-

Note: Putative fragrance-related sequences were denoted in asterisk form (*). Putative sequence of RNA-dependent RNA polymerase of *Cymbidium* mosaic virus denoted as (*). The abbreviation for *Cymbidium* mosaic virus is *Cy*MV.

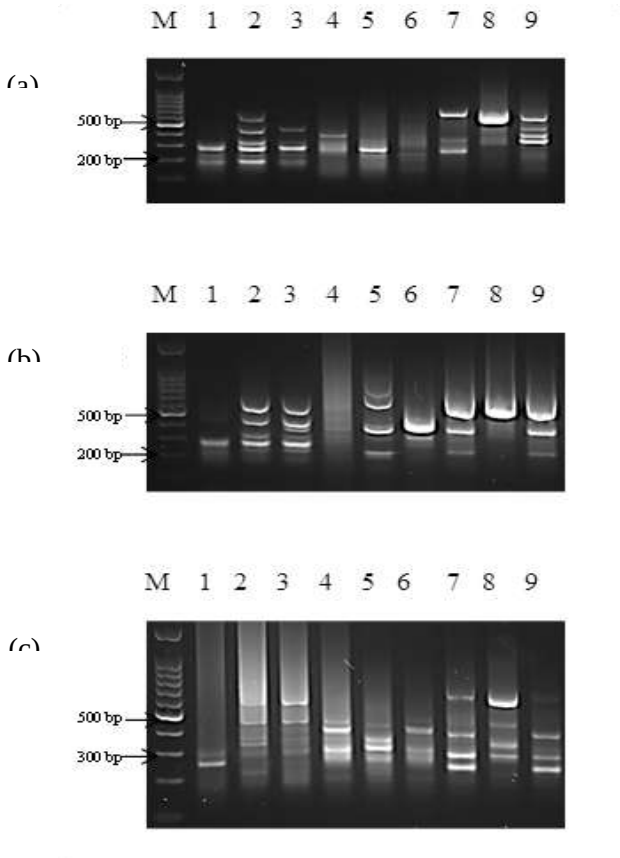

Figure 1 Difference products in three different rounds of cDNA-RDA: **(a)** first round cDNA-RDA; **(b)** second round cDNA-RDA; **(c)** third round cDNA-RDA. Each sample was electrophoresed on 1.2% (w/v) agarose gel. Lane M: 100 bp DNA marker (Vivantis, Malaysia); lane 1: Set A*Bgl*II; lane 2: Set A *Bam*HI; Lane 3: Set A *Hin*dIII; Lane 4: Set B *Bgl*II; Lane 5: Set B *BamH*I; Lane 6: Set B *Hin*dIII; Lane 7: Set C *Bgl*II; Lane 8: Set C *Bam*HI; Lane 9: Set C *Hin*dIII.