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Isolation of Transcripts Related to Floral Scent Biosynthesis from Cempaka Putih (*Michelia alba*) Flower Using Subtractive Hybridization Approach

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ABSTRACT

Floral scent plays an important role in the reproductive processes of many plants and contributes a considerable economic value in guaranteeing yield and quality of many ornamental plants and cut flowers by enhancing their aesthetic properties. It is determined by a specific complex mixture of volatile low-molecular-mass molecules which fall into the terpenoid or phenylpropanoid/benzenoid classes of compounds. Although volatile compounds have been identified in several flower species, little is known about the enzymes and genes controlling the biochemical synthesis of floral scent production and the molecular mechanisms involved, which may differ from species to species. In this preliminary study, we have identified four genes associated directly with the monoterpene scent biosynthesis pathway in the local flower, Cempaka putih (*Michelia alba*) including geranyl diphosphate synthase, deoxyxylulose-5-phosphate synthase, cytochrome P450 and (+)-pulegone reductase, and two more, including benzoyl coenyme A: benzyl alcohol benzoyl transferase and salicyclic acid methyltransferase (SAMT) that might be involved with the phenylpropanoid/benzenoid scent pathway using subtractive hybridization. We have also identified four other genes that might be indirectly related to scent metabolism in this flower including lipoxygenase, peroxidase, heat shock protein and myb transcription factor.

Keywords: *Michelia alba*, monoterpene scent biosynthesis pathway, linalool biosynthesis, menthol biosynthesis, geranyl diphosphate synthase, deoxyxylulose-5-phosphate synthase, cytochrome P450, (+)-pulegone reductase, benzoyl coenyme A: benzyl alcohol benzoyl transferase, salicyclic acid methyltransferase (SAMT), subtractive hybridization, bioinformatics

INTRODUCTION

Many plants emit floral scents which attract a variety of animal pollinators, mostly insects. They may also play a critical role in plant defenses against herbivores and pathogens (Wink, 1999). Floral fragrances vary widely among species in terms of number, identity and relative amounts of constituent volatile compounds. Humans find an aesthetic value in certain types of floral scents, and while there is certainly a wide variation in human taste, most people prefer the scents described as 'sweet smelling' (Knudsen and Tollsten, 1993). Unfortunately, very few plants are currently cultivated primarily for their scent. Moreover, a large number of commercial flower varieties have lost their scent due to focus on maximizing post-harvest shelf-life, shipping, characteristics and visual aesthetic values (color, shape) and a lack of selection for the scent trait.

The chemical composition of floral scents has been extensively investigated for hundreds of years because of the commercial value of floral volatiles in perfumery. However, research on plant scents has been hampered mainly by the invisibility of this character, its dynamic nature, and the complex mixtures of components that are present in very small quantities. Gas chromatography and mass spectrometry (GC-MS) findings have shown that the floral scents are almost always a complex mixture of small

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(approximately 100-200D) volatile molecules and are dominated by terpenoids, phenylpropanoids/ benzenoids compounds (Pichersky and Dudareva, 2007). These metabolites may be produced at specific stages of flower development and the volatile compounds escape directly into the atmosphere.

Terpenes are synthesized from isopentenyl diphosphate by different mono and sesquiterpene synthases (Vainstein *et al.*, 2001) via two alternative pathways: the mevalonate pathway from acetyl-CoA, and the methylerythritol phosphate pathway from pyruvate and glyceraldehyde-3-phosphate (G3P) (Rodriguez-Concepcion and Boronat, 2002). One of the best studied examples are the monoterpenes, the C_{10} members of the terpenoid (isoprenoid) family of natural products. Monoterpenes such as linalool, limonene, myrcene, and trans-β-ocimene, and also some sesquiterpenes such as farnesene, nerolidol, and caryophyllene are common constituents of floral scent. They are colorless, lipophilic, volatile substances responsible for many of the characteristic odors of plants (Hay and Waterman, 1993) and are also frequent constituents of oils and resins (Fahn, 1979). The phenylpropanoids constitute another large class of secondary metabolites in plants, several of which have been found to be volatile. Work on *Clarkia breweri* flowers (Pichersky and Dudareva, 2000) have resulted in the identification and characterization of three volatile enzymes that catalyze the formation of the floral volatiles, (iso)methyleugenol, benzylacetate and methylsalicyclate, from this group.

Although the chemical structures of many floral scent compounds have been determined (Guterman *et al.,* 2002), there have been few studies concerning the biochemical synthesis of floral scent compounds and the genes that control these processes. In the last few years, genes encoding the enzymes responsible for the synthesis of many monoterpenes and sesquiterpenes have been identified and characterized (Bohlman *et al.*, 1998). All of the enzymes responsible for the first dedicated steps of monoterpene (isoprenoid) biosynthesis from the deoxyxylulose-5-phosphate (DXP) pathway (Lange *et al.*, 1998, 1999) in peppermint, *Mentha × Piperita,* have been well established by *in-vitro* and cell-free studies (Colby *et al.*, 1993) including linalool synthase (LIS), (4S)-limonene synthase (LMS), myrcene synthase (MYS), 1,8-cineole synthase (CIS) and (-pinene synthase (PIS).

Earlier investigations on the floral scent production in *C. breweri* (an annual plant native to California) and *Antirrhinum majus* (cultivated snapdragron) have reported the isolation of several genes involved in the de novo synthesis of scent compounds in these flowers (Pichersky and Dudareva, 2007). Some of the genes encoding enzymes such as LIS, benzylalcohol acetyltransferase and 2-methyltransferases, involved in the biosynthesis of C. breweri scent volatiles (Dudareva and Pichersky, 2000) and methyltransferase that catalyzes methyl benzoate formation in the petals of the snapdragon, *A. majus* (Dudareva *et al.*, 2000) have been isolated and characterized. Other findings reported include enzymes involved in phenylpropene metabolism in sweet basil, *Ocimum basilicum* (Gang *et al.*, 2001), diterpene synthesis in Stevia rivaudiana of Asteraceae (Brandle *et al.*, 2002), terpene synthase in *Arabidopsis* (Aubourg *et al.*, 2002; Chen *et al.*, 2003) and sesquiterpene synthase from rose petals (Brandle *et al.*, 2002; Guterman *et al.*, 2002).

In general, expression of the genes involved in the synthesis of scent compounds have been found to be highest in petals and is restricted to the epidermal cell layers of floral tissues (Dudareva *et al.*, 1996; 1999; Dudareva and Pichersky, 2000). In *C. breweri* flowers, the expression of these genes were also found to be temporally and spatially regulated during flower development. The emission of the bulk of the volatiles was shown to occur from the petals of both the *C. breweri* and snapdragon flowers within few days of anthesis and thereafter declining gradually. LIS enzyme was found to increase in maturing buds and young flowers, then declined in old (5d) flowers, but activity remained relatively high even though emissions of linalool ceases. Accordingly the mRNA levels, encoding LIS enzyme, first detected in petal cells just before flower opening, increases until they peak at or around anthesis, then begin to decline (Dudareva *et al.*, 1996, 1998a; Wang *et al.*, 1997). Peak levels of mRNAs for this gene occur 1-2 days ahead of enzyme activity peaks and emission of corresponding compounds. Overall, a good positive correlation has been found between amount of mRNA, amount of protein and enzyme activity for each of these enzymes, and emission of corresponding components up to

2nd or 3rd day post anthesis. After that scent enzymes remain relatively high despite declining levels of corresponding mRNAs and also without concomittant emission of volatiles (Dudareva *et al.*, 1996, 1999). Thus, the level of enzyme activity involved in scent production (indirectly scent emission) is regulated mainly at mRNA levels at the site of emission in this flower.

In addition to the chemical composition, the physiological factors that regulate the production of these natural products have to be examined as well. In most cases that have been analyzed, the scent of flowers has been shown to be markedly reduced soon after pollination. The cessation of scent emission is often due to the senescense and wilting of petals (which usually constitute the bulk of the flower and the main source of scent emission), stigma and style. The effect of temperature on fragrance emission has also been shown to have a strong effect on the quantity of fragrance emitted. However, it is not clear if the increase in emission is due solely to the greater volatility of these compounds at the higher temperature, or if it is also due to biological processes, including increased synthesis.

In this report, we describe the combined use of subtractive hybridization and bioinformatics to partially deduce the scent biosynthesis pathway/s in the local flower Cempaka putih *(Michelia alba)* by subtracting RsaI-digested cDNAs of stage 1 flower buds from the full bloom flower at stage 10. This is a first attempt to identify the scent-related genes in this flower species and we hope that the results will provide useful biochemical insights into the scent biosynthesis pathway/s that exists in this flower.

MATERIALS AND METHODS

All plant materials were collected from the outskirts of Serdang town, Selangor. The development of *M. alba* flowers was divided into 12 stages (*Fig. 1*) according to size and morphology. At stage 1, the flower bud is very small and closed. The petals are green and no fragrance emitted. At stage 10, *M. alba* flowers are in full bloom and have a strong fragrance. Stage 1 was chosen as the driver so as to remove as many ribosomal proteins and housekeeping genes that might mask the low copy number of fragrance genes. Stage 10 was chosen as the tester as previous studies on GC-MS (*Fig. 2*) (Suri Roowi, personal communication) had shown that the major fragrance compound, linalool, increased sharply from stage 8 and peaked at stage 10 before declining towards stage 11 when the flower begins to senesce.

Fig. 1: Michelia alba *at different stages of flower development*

Subtraction Hybridization

Total RNA was extracted from flower stages 1 and 10 using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Chang *et al.*, 1993). Poly(A) mRNA was isolated using the μMax Poly(A) RNA Isolation kit (Miltenyi Biotech, Germany). The subtraction hybridization was carried out using the BD PCR-Select cDNA Substraction Kit (BD BioSciences Clontech, USA). RsaI-digested cDNAs of stage 1 flower buds were subtracted from those at stage 10 and the cDNA clones synthesized were non-directionally mass cloned into the pGEM-T vector system (Promega, USA).

DNA Sequencing and Analysis

Plasmid DNAs were purified from overnight cultures using the Qiagen miniprep kit (Germany). The sequencing of the plasmid clones was outsourced (First Base Sdn. Bhd.) and sequencing was performed from the 5' end of the sense strand using the T7 universal primer. Raw sequence data was analyzed using our in-house iDNAs customized sequencing bioinformatics software (KooPrime Pte Ltd., Singapore). The PHRED and LUCY programs (Ewing *et al.*, 1998; Chou and Holmes, 2001)

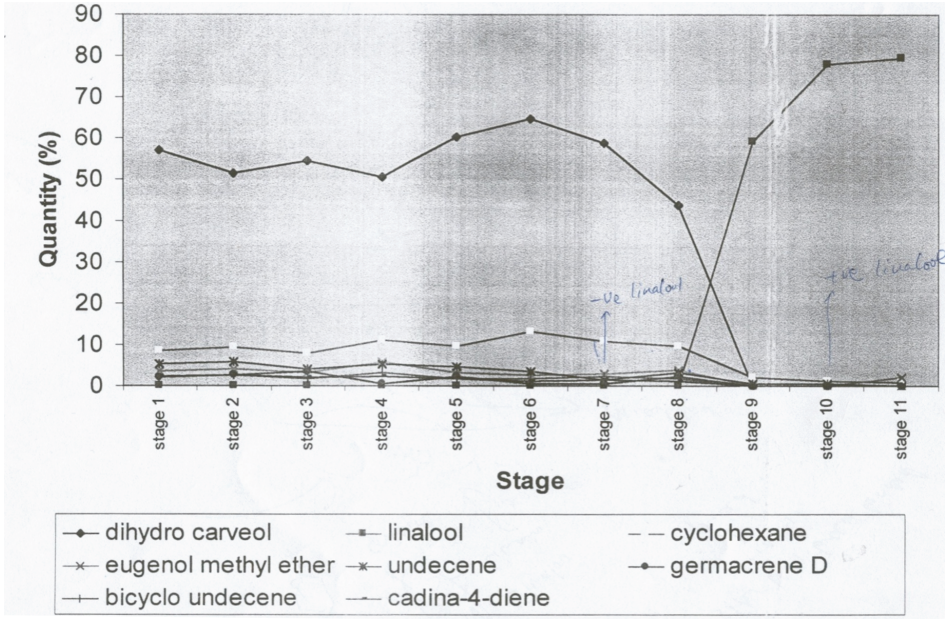

Fig. 2: Percentage of scent-related compounds obtained at different stages of M. alba *flower development using GC-MS*

were employed for base calling and sequence quality assessment (remove vector sequences, poly(A), adaptors and ambiguous sequences), respectively. CAP3 fragment assembly program was used to organize the redundant complementary DNA (cDNA) sequences into unigenes of overlapping contigs (Huang and Madan, 1999). The individually trimmed sequences were then submitted to BLASTX (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) analysis against our in-house nucleotide non-redundant protein database updated on 23rd May 2007 from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to search for similarities. The sequences were functionally characterized using Gene Ontology (GO) (http://www.geneontology.org) (Ashburner *et al.*, 2000). The putative floral biosynthesis enzymes deduced for *Phalaenopsis bellina* (Hsiao *et al.*, 2006) were used as a guide for the identification of the scent-related genes.

RESULTS AND DISCUSSION

A total of 420 cDNA clones were retained after subtraction. Vector trimming and homology search revealed 413 sequences varying in length between 50 and 773 bp. Fragment assembly of the clones generated 154 unigenes: 81 contigs and 73 singletons. 80.9% (334) of the clones showed similarities to known sequences in the non-redundant GenBank database, (E-value ≤ 1.0×10^{-4}), while the remaining 19.1% (79) had very low or no significant match (E-value > 1.0×10^{-4}) based on the highest scoring results.

Putative functions were assigned to the above sequences based on the classification proposed by GO. Details of the gene species included in each group are given in Table 1. GO allowed 36.1% of the total sequences to be placed in the molecular function category, 22.8% in the biological process category and 0.5% in the cellular component category. The remaining 46.7% either showed insufficient similarities to any proteins (no hits, 19.1%) or hit proteins without a GO identifier (unclassified, 27.6%). Among the molecular functions, the categories most highly represented were the other enzyme activity excluding transferases, synthases, hydroxylases, oxidases and oxygenases (15.0 %), transferases (8.0%) and synthases (4.6%). Among the biological processes, the largest proportion (13.3%) of functionally assigned sequences fell into the other metabolic processes (excluding protein metabolism, DNA metabolism, electron transport, energy pathways and transcription);

TABLE 1
Functional classification of *M. alba* flower genes from stage 10 using GO Classification and based on first hit blast results (E-value ≤1 x 10^{-4})

Categories	Putative functions	Frequency	%
Molecular process	Protein binding	3	0.7
	DNA binding	4	1.0
	Other binding	2	0.5
	Transporter activity	16	3.6
	Synthase activity	19	4.6
	Transferase activity	33	8.0
	Other enzyme activity	62	15.0
	Structural molecule activity	10	2.4
Biological process	Protein metabolism	16	3.6
	DNA metabolism	2	0.5
	Electron transport	3	0.7
	Energy pathway	5	1.2
	Transcription	6	1.5
	Other metabolic processes	55	13.3
	Other physiological processes	5	1.2
	Other cellular processes	2	0.5
Cellular process	Other cellular components	2	0.5
Unclassified	Unknown, unnamed, hypothetical and others	114	27.6
No hits		79	19.1
TOTAL		413	

other cellular processes excluding signal transduction, cell organization and biogenesis and transport accounted for only 0.5% while the physiological processes accounted for 1.2%. Together, these two categories of molecular and biological processes accounted for 58.9% of the assigned sequences (Table 1).

Identification of Scent Biosynthesis Pathway/s in M. alba *Using the iDNAs Customized Bioinformatics Package*

Monoterpene synthase genes have been identified in both floral and vegetative organs of several angiosperms and gymnosperms (Aubourg *et al.*, 2002; Iijima *et al.*, 2004). The terpene synthases are of special interest, which are a large class of enzymes that appear to be responsible for most of the structural variation among terpenes (Wise and Croteau, 1999). In comparison to the floral scent biosynthesis pathway deduced in *P. bellina,* (Hsiao *et al.*, 2006), we identified four transcripts that were directly involved in monoterpene scent biosynthesis including deoxylylulose-5-phosphate synthase (DXPS), geranyl diphosphate synthase (GDPS), cytochrome P450 and (+)-pulegone reductase (PR), and two others including benzoyl coenyme A: benzyl alcohol benzoyl transferase and salicyclic acid methyltransferase (SAMT) that might be involved with the phenylpropanoid/ benzenoid scent pathway. Four other genes including lipoxygenase (LOX), peroxidase, heat shock protein and myb transcription factor were also identified that might be indirectly involved in scent biosynthesis (Table 2). GDPS was significantly expressed (4.36%) followed by DXPS (3.39%), cytochrome P450 (2.18%), LOX (1.21%), peroxidase (0.48%), heat shock protein 90 (0.24%), (+)-PR (0.24%), benzoyl coenzyme A: benzyl alcohol benzoyl transferase (0.24%) and SAMT (0.24%) (Table 2).

Analysis of volatiles by GC-MS data obtained earlier (Suri Roowi, personal communication), have shown that linalool (>80.0%) is a major fragrance compound of *M. alba* flower scent at stage 10 flower development as shown in *Fig. 2.* The high expression level of GDPS in *M .alba* flowers also suggests that scent biosynthesis in this species is predominantly due to production of linalool from GDP. GDPS has been shown to

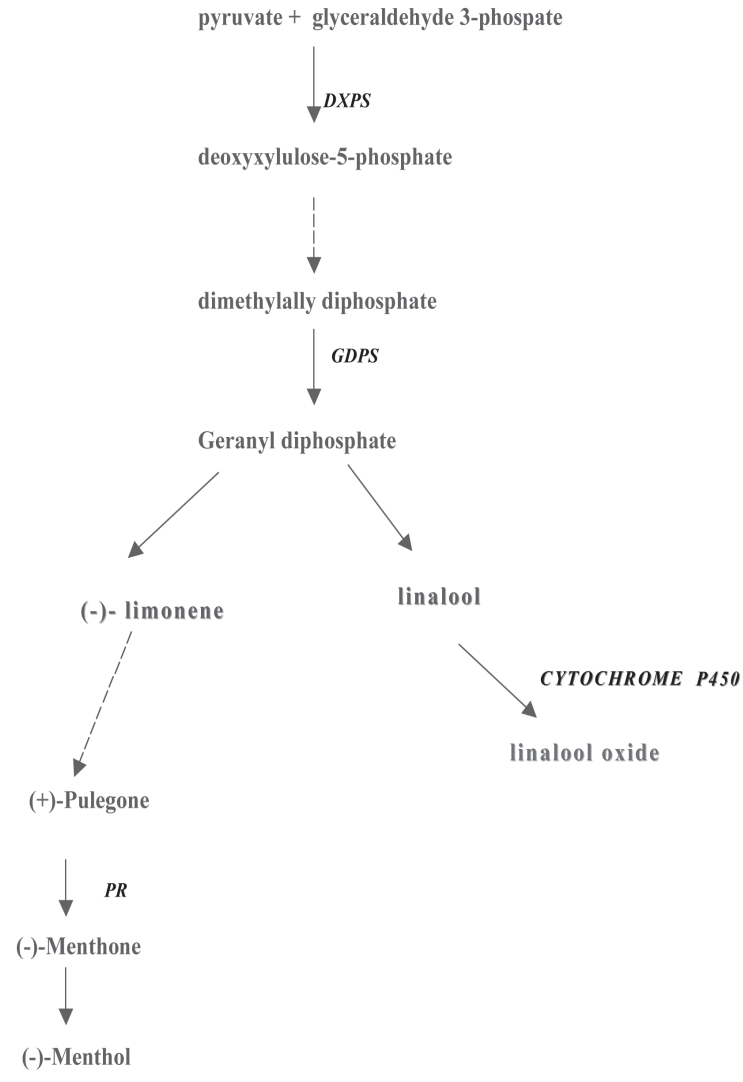

Fig. 3: Putative metabolic pathway from pyruvate and glyceraldehyde-3-phosphate to monoterpene scent biosynthesis, and its related enzymes, in M. alba. *DXPS: deoxylylulose-5-phosphate synthase; GDPS: geranyl diphosphate synthase; cytochrome P450; PR: Pulegone reductase*

participate in the biosynthesis of monoterpenes in plastids (Sommer *et al.*, 1995) primarily by supplying the essential precursor. Based on the above findings and the fact that all monoterpenes are formed from geranyl diphosphate (GDP), which is synthesized from dimethylallyl diphosphate and isopentenyl diphosphate (Tholl *et al.*, 2004), it is therefore reasonable to speculate that monoterpenoids are biosynthesized in these flowers.

However, we were not able to detect the transcripts for linalool synthase or limonene synthase, the enzymes responsible for the formation of linalool and limonene en route to pulegone, respectively. These enzymes were also not detected in *P. bellina* (Hsiao *et al.*, 2006). They have been reported to belong to families with high diversity in non-conserved regions (Lange *et al.*, 1998) and therefore, the low sequence-relatedness among them (Iijima *et al.*, 2004) might have added to the difficulty in identifying them. Alternatively, the pool of subtracted clones obtained for *M. alba* may be insufficient, so that not all genes in the scent

TABLE 2
Major classes of scent and scent-related transcripts in *M. alba* flowers during full bloom (stage 10) based on first hit blast results (E-value ≤ 1 x 10^{-4})

Scent and scent-related genes identified	Frequency	% (out of 413 sequences)
Deoxyxylulose-5-phosphate synthase (DXPS)	14	3.39
Geranyl diphosphate synthase (GDPS)	18	4.36
Cytochrome P450	9	2.18
(+)-pulegone reductase	1	0.24
Benzoyl Coenyme A: benzyl alcohol benzoyl transferase	1	0.24
Salicyclic acid methyltransferase (SAMT)	1	0.24
Lipoxygenase (LOX)	5	1.21
Heat shock protein 90	1	0.24
Myb transcription factor	1	0.24
Peroxidase	2	0.48

biosynthesis pathway were represented. It could also be due to regulation of the scent biosynthesis at the precursor level, and the enzymes responsible for synthesis are not transcriptionally regulated. Previous studies have shown that LIS levels and activities in *C. breweri* remain high while linalool emission decreases, suggesting that regulation of terpenoid precursors occurs in this species (Dudareva and Pichersky 2000; van Schie *et al.*, 2006).

Although we did not detect the transcripts for linalool synthase at this stage of flower development, the presence of cytochrome P450 indicated that linalool synthase could have been expressed much earlier. Our results showed that cytochrome P450 is involved with the formation of linalool oxide from linalool in *M. alba* as shown by Hsiao *et al.* (2006) (*Fig. 3*).This enzyme has also been reported to act as a hydroperoxide lyase and catalyze the cleavage of lipoxygenase products (fatty acid hydroperoxides), forming omega-oxoacids and volatile C6- and C9-aldehydes and alcohols (Noordermeer *et al.*, 2001).

Interestingly, we also detected (+)-pulegone reductase, a central intermediate in the biosynthesis of (-)-menthol, the most significant component of peppermint essential oil (Soheil and Rodney, 2003). Depending on environmental conditions, the cyclization of GDP, the universal monoterpene precursor will lead to the production of (-)-limonene and after a sequence of several steps, to produce the branch point metabolite, pulegone, which may be reduced to (-)-menthone en route to menthol, by PR.

Two different kinds of LOX transcripts (LOX1 and LOXC) accounted for the relatively high percentage (1.21). LOX genes may be involved in converting storage lipids into substrates for further oxidation to provide energy for scent emission as shown by the presence of many lipid bodies found in the petal epidermis of *P. bellina* by transmission electron microscopy (Hsiao *et al.*, 2006). LOX genes have also been reported to be involved in plant growth and development; biosynthesis of regulatory molecules such as jasmonic acid and traumatin; and biosynthesis of volatile compounds such as hexanal, hexenal and hexenol, which are involved in flavor, insect attraction and defense (Chen *et al.*, 2004; Feussner and Wasternack, 2002). The role of the LOX pathway in plant-pathogen interactions and their product, jasmonate, in resistance against insects and pathogens have been analyzed in numerous pathosystems (Howe and Schilmiller, 2002). Although the biological function of the relatively high levels of LOX expression in *M. alba* flowers is not clear, their expression may indirectly control the synthesis of some signal for flower scent formation or emission.

Our results also showed that peroxidase and heat shock protein 90 might be related indirectly to scent metabolism although they were found in very low abundance (0.48 and 0.24% respectively). Interestingly, anthocyanin colour biosynthesis genes were also identified although the flower is white. Transcripts encoding signal transduction factors (Table 1, cellular processes) such as membrane proteins were also identified,

suggesting that scent emission may be related to stimuli that causes a series of signal transduction processes leading to gene expression and scent production. We also detected Myb transcription factor (0.24%) which could be related to the Myb family protein (Table 2) shown to regulate the biosynthesis of petunia flower fragrance (Verdonk *et al.*, 2005).

From the chemical profiling, data mining and bioinformatic analyses, we partially deduced a monoterpene biosynthesis pathway of 4 steps in the *M. alba* flower, leading from pyruvate and G3P to GDP, linalool, limonene and their derivatives (*Fig. 3*). We also managed to show a weak existence of the phenylpropanoid/ benzenoid scent pathway in this flower at this stage, although the 2 enzymes were present in relatively low abundance (0.24%). The sequences of these transcripts will be deposited in the public database after publications of these findings.

CONCLUSIONS

In this study, we have shown how a combination of genomics and EST database mining can be applied for the construction of a putative scent metabolism pathway in *M. alba* and the identification of the genes encoding the enzymes involved in this pathway. We used the customized in-house integrated DNA sequencing (iDNAs) bioinformatics package to identify the scent genes in *M. alba* including those for DXPS, GDPS, cytochrome P450 and (+)-pulegone reductase, all involved in the DXP-linalool-limonene pathway. For a non-model plant with no genomic information at all, EST analysis of its transcriptome profile becomes a very efficient and informative tool and may be applicable for comparative genomics.

It is clear from the above, that a major priority of scent research should be, to continue to understand the biochemical pathways leading to scent biosynthesis and the identification and characterization of genes controlling these pathways. In addition to this, the sub-cellular location of the synthesis of most of the scent compounds still needs to be determined, as well as the mechanisms controlling developmental changes of the pathways. It would also be useful to examine the molecular processes that bring about the variability in floral scent characteristics among different species, whether they are on the level of gene regulation, post transcriptional regulation, or protein evolution. Finally, the availability of scent genes should allow us to create transgenic lines with optimum fragrance production.

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