The molecular and biochemical basis for varietal variation in sesquiterpene content in melon (*Cucumis melo* L.) rinds

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Abstract A combined chemical, biochemical and molecular study was conducted to understand the differential accumulation of volatile sesquiterpenes in melon fruits. Sesquiterpenes were present mainly in the rinds of climacteric varieties, and a great diversity in their composition was found among varieties. Sesquiterpenes were generally absent in non-climacteric varieties. Two climacteric melon varieties, the green-fleshed 'Noy Yizre'el', and the orange-fleshed 'Dulce' were further examined. In 'Noy Yizre'el' the main sesquiterpenes accumulated are δ-cadinene, γ-cadinene and α-copaene, while α-farnesene is the main sesquiterpene in 'Dulce'. Sesquiterpene synthase activities, mainly restricted to rinds of mature fruits, were shown to generate different sesquiterpenes in each variety according to the compositions found in rinds. EST melon database mining yielded two novel cDNAs coding for members of the *Tps* gene family termed *CmTpsNY* and *CmTpsDul* respectively, that are 43.2% similar. Heterologous expression in *E. coli* of *CmTpsNY* produced mainly δ-copaene, α-copaene, β-caryophyllene, germacrene D, α-muurolene, γ-cadinene, δ-cadinene, and α-cadinene, while *CmTpsDul* produced α-farnesene only. *CmTpsNY* was mostly expressed in 'Noy Yizre'el' rind while *CmTpsDul* expression was specific to 'Dulce' rind. None of these genes was expressed in rinds of the non-climacteric 'Tam Dew' cultivar. Our results indicate that different sesquiterpene synthases encoded by different members of the *Tps* gene family are active in melon varieties and this specificity modulates the accumulation of sesquiterpenes. The genes are differentially transcriptionally regulated during fruit development and according to variety and are likely to be associated with chemical differences responsible for the unique aromas of melon varieties.

Keywords *Cucumis melo* L. · Sesquiterpene synthases · α-Farnesene · δ-Cadinene · *CmTpsNY* · *CmTpsDul*

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Introduction

Melons (*Cucumis melo* L., Cucurbitaceae) comprise a broad array of wild and cultivated genotypes differing in many traits such as climactericity during fruit ripening, the patterns of sugar and acid accumulation, as well as in the

content and composition of secondary metabolites associated with color, taste and aroma (Seymour and McGlasson 1993; Perin et al. 2002; Morales et al. 2004; Burger et al. 2006). The aroma of melons, as is the case with most fruits, consists of complex mixtures of volatile compounds (Beaulieu and Grimm 2001; Jordan et al. 2001; Shalit et al. 2001; Yahyaoui et al. 2002; Aubert and Bourger 2004). Different cultivars display different aromas, and this is reflected in their volatile compositions (Shalit et al. 2001; Aubert and Bourger 2004; Aubert and Pitrat 2006; Beaulieu 2006). Volatile esters, mainly acetate derivatives, are prominent in climacteric melon varieties, together with lower amounts of norisoprenes, short-chain alcohols, aldehydes and sesquiterpenes (Shalit et al. 2000; Beaulieu and Grimm 2001; Shalit et al. 2001; Yahyaoui et al. 2002). Non-climacteric varieties often have lower levels of total volatiles, and especially lack the volatile esters. In contrast, volatile aldehydes and alcohols, albeit in low levels, are most abundant in non-climacteric melons (Shalit et al. 2001; Burger et al. 2006). This metabolic pattern is also prominent in ethylene-repressed transgenic melons (Flores et al. 2002). The variation in sesquiterpenes among cultivars has not been well characterized to date.

The biochemical and molecular events that control the generation of aroma compounds have recently been the focus of increased attention (Goff and Klee 2006; Pichersky et al. 2006). As a result, some genes involved in fruit aroma formation have been described (Aharoni et al. 2000; Sharon-Asa et al. 2003; Beekwilder et al. 2004; Pechous and Whitaker 2004; Simkin et al. 2004; Goff and Klee 2006). In melons, genes that encode acyl-CoA alcohol acetyltransferases (El-Sharkawy et al. 2005) and alcohol dehydrogenases (Manriquez et al. 2006), involved in the formation of volatile alcohols and esters that determine the unique aroma of fruit flesh, have been characterized. Orange-fleshed melons rich in β-carotene contain β-ionone, a key β-carotene-derived norisoprenoid with a profound typical aroma that is either absent or only present in minute quantities, in white and green-fleshed melon varieties. A gene encoding a carotenoid cleavage dioxygenase able to cleave carotenoids to release norisoprenoid compounds has recently been described (Ibdah et al. 2006). However, little is known about genes that control the unique aromas of rind tissues and the sesquiterpene content of these tissues.

Sesquiterpenes are fifteen-carbon compounds found in the majority of plant species and serve diverse roles in communication and defense (McGarvey and Croteau 1995; Trapp and Croteau 2001; Degenhardt et al. 2003; Ro et al. 2006). Sesquiterpenes are derived from farnesyl diphosphate by a group of enzymes termed sesquiterpene synthases, and encoded by members of the *Tps* gene family (Aubourg et al. 2002). Many sesquiterpene synthase genes

have been described in plants. For example, two sesquiterpene synthase genes are induced in cucumber (*Cucumis sativus* L.) leaves upon spider-mite attacks. Their respective gene products are able to catalyze the conversion of farnesyl diphosphate to (E,E)-α-farnesene and (E)-β-caryophyllene, respectively, when expressed in *E. coli*, (Mercke et al. 2004). Although sesquiterpenes have been found in the flesh of melon fruits (Horvat and Senter 1987; Homatidou et al. 1992; Beaulieu and Grimm 2001; Shalit et al. 2001; Aubert and Bourger 2004; Beaulieu 2007) little is known about the composition in rinds and the variation within cultivars as well as the factors and biological processes that control their occurrence and accumulation. Here we describe the identification, characterization and molecular regulation of two novel members of the *Tps* family that are primarily expressed in ripe melon rind tissues: *CmTpsNY* and *CmTpsDul*. Following heterologous expression in *E. coil* we functionally identified them as cadinene synthase and α-farnesene synthase, respectively. This work is another step towards the understanding of the factors that contribute to the formation of aroma compounds in melon fruits as part of a larger program aimed at the molecular and biochemical characterization of traits determining fruit quality and diversity.

Materials and methods

Plant material

Sixteen commercial melon varieties and breeding lines that differ in color, flavor, and aroma, as well as in rind color and texture, were examined (Table 1). Four varieties are related to the 'Galia' market type. 'Galia' is an Israeli-bred melon F1 hybrid (Karchi 2000). Galia-type varieties are characterized by juicy green flesh and a finely netted rind. 'Noy Yizre'el' is a powdery mildew resistant cultivar derived from 'Ha'Ogen' which serves as the female parent of 'Galia', while 'Krymka', a muskmelon, is the male parent. A cross between 'Krymka' (female) and Eshkolit Ha'Amaqim' (male) gave rise to another 'Galia' type variety named 'Arava' (Karchi 2000; Nuñez-Palenius et al. 2006). The remaining genotypes in this study are open pollinated accessions that represent the major marketing types of melons. Melons were grown in open field conditions at the Newe-Ya'ar Research Center under standard irrigation and fertilization during the summer seasons of 2004–2006. Female flowers were tagged, and fruits were harvested at the following stages of development: (1) young, green fruits (12 days after anthesis—DAA); (2) green fruits (25 DAA); (3) mature fruits (after change of color and the development of abscission zone); and (4) post-harvest fruits (mature fruits that were picked and kept

Table 1 Melon varieties and breeding lines used in this study

Genotype	Marketing type	Group[a]	Fruit ripening	Flesh color	Rind color	
					Young fruit	Mature fruit
Eshkolit Ha'Amaqim	Muskmelon-cantaloupe	Reticulatus	Climacteric	Green–white	Bright	Yellow-orange
Arava (F1 hybrid)	Galia	Reticulatus	Climacteric	Green	Dark green	Yellow-orange
Dulce	American shipper	Reticulatus	Climacteric	Orange	Dark green	Yellow-orange
Noy Yizre'el	Ha'Ogen	Cantalupensis	Climacteric	Green	Dark green	Yellow-orange
Krymka	Muskmelon	Reticulatus	Climacteric	Green-white	Dark green	Yellow-orange
Dudaim	Pocket melon	Dudaim	Climacteric	White	Dark green	Yellow-orange
Doya	Pickling	Flexuosus	Climacteric	Green	Bright	Cream-white
Doublon	Charentais	Cantalupensis	Climacteric	Orange	Bright	Cream-white
Faqqous	Snake melon	Flexuosus	Climacteric	Green	Bright	Cream-white
Noy Amid	Yellow Canary	Inodorus	Non-climacteric	Green–white	Bright	Yellow-orange
Rochet	Casaba	Inodorus	Non-climacteric	Green–white	Dark green	Dark Green
Piel De Sapo	Casaba	Inodorus	Non-climacteric	Green–white	Dark green	Dark green
Védrantais	Charentais	Cantalupensis	Climacteric	Orange	Bright	Cream-white
Tendral Verde Tardio	Casaba	Inodorus	Non-climacteric	Green–white	Dark green	Dark green
Tam Dew	Honey Dew	Inodorus	Non-climacteric	Green	Bright	White-Yellow
PI4142723	Cooking melon	Momordica	Climacteric	Pale orange	Bright	Yellow-orange

[a] According to Pitrat et al. 2000

for 7 days at 20°C). Fruit tissues were frozen in liquid nitrogen following the removal of seeds and the separation of the rind from the flesh by a manual vegetable peeler. Tissues from three separate fruits were collected for each developmental stage.

Analyses of volatiles

Solid Phase Microextraction (SPME)

Fresh melon tissues (1.5 g) were ground into a uniform powder under liquid nitrogen with a mortar and pestle. The powder was placed in a 20 ml SPME vial containing 3 ml of 20% (w/v) NaCl and 0.3 μg internal standard (2-heptanone), sealed and placed in 4°C until used. Headspace sampling was conducted immediately utilizing a 65 μm fused silica fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) (Supelco). After 40 min the SPME syringe was introduced into the injector port of the GC-MS apparatus for further analysis (see below).

GC-MS analysis

Volatile compounds were analyzed on a GC-MS apparatus (Agilent Technologies CA, USA) equipped with an Rtx-5SIL MS (30 m*0.25 mm*0.25 μm) fused-silica capillary column. Helium (1 ml/min) was used as a carrier gas. The injector temperature was 250°C, set for splitless injection. The oven was set to 50°C for 1 min, and then the temperature was increased to 220°C at a rate of 5°C/min. Thermal desorption was allowed for 10 min. The detector temperature was 280°C. The mass range was recorded from m/z 41 to 350, with electron energy of 70 eV. Identification of the main components was done by comparison of mass spectra and retention time data with those of authentic samples and supplemented with a Wiley7 N and HP CH1607 GC-MS libraries.

Sesquiterpene synthase enzymatic activity

Cell-free extracts derived from melon

Fruits were cut into small pieces (1–2 g) and frozen at −40°C for up to 1 month until use. The frozen pieces were placed in a chilled mortar and ground with a pestle in the presence of sand and 1 g polyvinylpolypyrrolidone (PVPP) until a uniform powder was obtained. Ice-cold extraction buffer [50 mM bis Tris, pH 6.9, 10% (v/v) glycerol, 10 mM dithiothretiol (DTT), 5 mM $Na_2S_2O_5$] was added (4:1 v/w) and the suspension was further extracted for an additional 30 s. The slurry was centrifuged at 20,000g for 10 min at 4°C. The supernatant (crude extract) was either used fresh or kept for up to 2 weeks at −40°C until its enzymatic activity determination (Shalit et al. 2000; Shalit et al. 2001).

Preparation of bacterial lysates

Individual bacterial pellets were suspended in 3 ml of lysis buffer containing 50 mM Bis tris pH 6.9, 10% v/v glycerol, 10 mM DTT, 5 mM $Na_2S_2O_5$ and 30 μg lysozyme (Sigma grade VI from chicken egg, 60,000 units mg^{-1} protein). The samples were vigorously mixed and incubated in ice water (4°C) for 15 min. After the cells lysed, the suspensions were centrifuged (20,000g for 10 min at 4°C). The supernatants were used for characterization of the enzymatic activity of the gene products.

Enzymatic GC-MS assay

Enzymatic assays were performed by mixing 100 μl of crude extract, 10 mM $MgCl_2$, 10 μM $MnCl_2$, 10 μM FDP and reaction buffer [50 mM bis Tris, pH 6.9, 10% (v/v) glycerol, 10 mM dithiothretiol (DTT), 5 mM $Na_2S_2O_5$] to a total volume of 300 μl. The reactions were incubated for 3 h at 30°C. After incubation the samples were analyzed by SPME GC-MS for the identification of volatile sesquiterpenes generated during the 30°C incubation.

Isolation and characterization of sesquiterpene synthase genes

Isolation of RNA and construction of melon fruit cDNA libraries

Total RNA was isolated using a modification to the method of LaClaire and Herrin (1997) from: (1) slices of the rind of young and mature fruits of cv. 'Noy Yizre'el' or (2) slices of mature fruit containing both rind and flesh tissues of 'Dulce' melons. Poly (A)⁺ mRNA was purified from 1 mg of total RNA by use of the Oligotex® mRNA purification kit according to manufacturer's recommendations (Qiagen, Hilden, Germany).

Two types of cDNA libraries were created: (1) A SSH library enriched for the mature rind (against the young rind) of 'Noy Yizre'el' was constructed using the PCR-Select™ kit (Clontech Laboratories, USA). The PCR products generated by the SSH were cloned into pGEM®-T Easy vector with the pGEM®-T Easy system kit (Promega, WI, USA). (2) An EST library of mature fruit of 'Dulce' was constructed using the ZAP cDNA Synthesis Kit and ZAP Express® cDNA Gigapack® III Gold Cloning Kit according to manufacturer's recommendations (Stratagene, USA). Phage clones were converted to pBK-CMV phagemid expression-vector following the manufacturer's instructions (Stratagene, USA).

Isolation of full length clones of CmTpsNY and CmTpsDul

Full length sequence of the *CmTpsNY* gene (1,716 bp) was obtained by 5′/3′ RACE (Roche, Mannheim, Germany) of the clone SSH9B15 (http://melon.bti.cornell.edu/). The 3′-Race was performed using the primer 5′-GACATCGC TTCCCACAAG-3′ while for the 5′-Race the primer 5′-CGT TTGTTACAATGTGATCACC-3′ was used. Full-length sequence of the second gene, FR15G19 (http://melon.bti. cornell.edu/), *CmTpsDul*, (1,683 bp) was obtained using a forward primer of (5′-ATGAGTTCAAACATATCAGCAA TTCC-3′) and a reverse primer from the homologous gene *Cucumis sativus E,E-α*-farnesene synthase (5′ TTAGAGTG GTACAGAATCAACAAGC-3′). DNA sequencing was performed by Macrogen Inc. (Seoul, Korea).

Real-time PCR analysis

Total RNA was isolated separately from the rind and flesh tissues of 'Dulce' and 'Noy Yizre'el' fruits at various developmental stages as described above. This RNA was used as template for cDNA synthesis. RNA samples (50 μg) were treated with RNAse-free DNAse I (20 units) (EPI-CENTRE®, USA) for 15 min at 37°C. First-strand cDNA was synthesized from 0.8 μg of total RNA by Reverse-iT™ MAX 1st Strand Synthesis kit (ABgene®'s Inc., Epsom, UK), using a blend of random hexamers and anchored oligo-dT primers (3:1). The cDNA was then diluted in a total volume of 100 μl of 3 mM Tris–HCl, pH 7.2, 0.2 mM EDTA solution. A 2-μl aliquot of cDNA was used for each real-time PCR performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster, CA). Amplifications were conducted using the ABsolute™ QPCR SYBR® Green Mixes (ABgene®'s Inc., Epsom, UK). The following primer sequences (0.2 μm final concentration) were used: (1) *cyclophiline* (a house-keeping gene, accessions no. DV632830) forward primer 5′-GATGGAGCTCT ACGCCGATGTC-3′ and reverse 5′-CCTCCCTGGCACAT GAAATTAG-3′; (2) *CmTpsNY*: forward primer 5′-CGAGG TTCTTCAATGGCTTTCAA-3′ and reverse primer 5′-GCG ATGTCGTCCATGAGTCTACA-3′; (3) *CmTpsDul*: forward primer 5′-AATGAGCCAAAAGGAAACAACA CC-3′ and reverse primer 5′-CCTTACACCATTTGGCCTC TACC-3′.

Thermal cycling was initiated by 15 min at 95°C, followed by 40 cycles of 90°C, 15 s; 60°C, for 1 min. A melting curve analysis was performed for each reaction to confirm the specificity of the amplification. Real-time PCR was performed in duplicate for each primer combination. Three biological samples of each developmental stage were tested. C_t values were determined by the ABI Prism 7000 SDS software and exported into an MS Excel workbook

(Microsoft Inc., Redmond, WA) for statistical analysis. Real-time efficiencies (E) were calculated from the slopes of standard curves for each gene ($E = 10^{[-1/\text{slope}]}$). The relative expression ratio (R) was calculated according to Pfaffl (2001) while the control was the sample of rind of the respective young fruits (12 DPA).

Northern blot analysis

Twenty-five micrograms of total RNA pooled from three fruits for each developmental stage or tissue, were separated on 1% formaldehyde-agarose gel and transferred to Hybond N$^+$ nylon (Amersham Biosciences) (Sambrook and Russell 2001). DNA fragments of *CmTpsNY* and *CmTpsDul* were labeled using the Rediprime TM II Prime Labeling System kit (Amersham Biosciences). Hybridization and washing were carried out as described by Church and Gilbert (1984). The radioactively labeled membranes were exposed to Kodak Biomax MS film and kept at $-70°C$ for several days, according to the intensity of the radioactive signal.

Cloning of sesquiterpene synthase genes

CmTpsNY and *CmTpsDul* were subcloned into expression vector T7/NT-TOPO (Invitrogen) by PCR with the appropriate primers according to the manufacturer's instructions.

Expression of *CmTpsNY* and *CmTpsDul* in *Escherichia coli*

Recombinant *E. coli* BL21 (DE3) Gold (Stratagene, La Jolla, CA) bacteria were plated in Luria-Bertani broth (LB)-agar containing 50 μg ml^{-1} ampicillin. Individual colonies were grown in 2 ml of LB liquid medium containing 50 μg ml^{-1} ampicillin overnight to be used as starter cultures. Five hundred microliters of bacterial cell suspensions were transferred into 50 ml of LB liquid medium containing ampicillin and grown at 37°C with shaking (200 rpm) until the OD600 reached 0.6. Isopropylthio-β-galactoside was then added to a final concentration of 0.3 mM, and the cultures were grown for another 4–5 h at room temperature and aliquoted into 1.5-ml aliquots. The cells were harvested by centrifugation at 20,000g for 10 min at 4°C and frozen at $-20°C$ until use (Lavid et al. 2002).

Results and discussion

Sesquiterpene content in melon varieties

Sesquiterpene content and composition greatly differed according to the variety examined (Table 2). The highest levels of sesquiterpenes were found in 'Eshkolit Ha'Amaqim' rinds. 'Eshkolit Ha'Amaqim' is a climacteric smooth yellow elite breeding line. Indeed, 'Eshkolit' is one of the parental lines of 'Arava' an important economic line that has a very similar rind sesquiterpene composition as compared to 'Eshkolit' (Table 2).

The main sesquiterpene compounds in these varieties are γ-cadinene, δ-cadinene, α-copaene, E-caryophyllene and α-muurolene. Other lines analyzed, including 'Noy Yizre'el' and 'Dudaim', displayed a great variability in sesquiterpene compositions while 'Krymka' rinds contained mainly α-copaene. Interestingly, none of the above varieties contained α-farnesene, the only sesquiterpene component of 'Dulce' rinds. All of the non-climacteric varieties examined lacked significant levels of sesquiterpenes, but some of the climacteric varieties such 'Vedrantais' and 'PI414723' also lacked sesquiterpenes in their rind (Table 2). This indicates that sesquiterpene patterns in melon rinds are highly polymorphic and their levels might be influenced by climacteric traits.

To further investigate the developmental and tissue compartmentalization of sesquiterpene accumulation, two varieties, 'Noy Yizre'el' and 'Dulce', that differ in sesquiterpene composition were further analyzed. The main sesquiterpenes found in 'Noy Yizre'el' rind are α-copaene, β-caryophyllene, germacrene D, α-muurolene, γ-cadinene, δ-cadinene, and α-cadinene. No α-farnesene was detected in 'Noy Yizre'el' rinds, but conversely, the only sesquiterpene found in 'Dulce' rind was E,E α-farnesene (Tables 2 and 3). In both varieties, the flesh (both ripe and unripe) and the unripe rind tissues lacked noticeable levels of sesquiterpenes.

Low levels of sesquiterpenes have been previously found in the flesh of melon cultivars (Horvat and Senter 1987; Homatidou et al. 1992; Shalit et al. 2001). Sesquiterpenes have also been found in fruit rinds of apple and orange (Sharon-Asa et al. 2003; Pechous and Whitaker 2004). In apple rind, α-farnesene levels increased when the plant was attacked by pests (Pechous and Whitaker 2004). Citrus fruits contain sesquiterpenes (such as the sesquiterpene aldehydes α- and β-sinensal) that might serve defensive roles or greatly contribute to its aroma. Valencene, an important sesquiterpene hydrocarbon present in oranges and mandarins, is a precursor of the oxygenated derivative nootkatone, a compound with a typical grapefruit aroma present in pummelos and grapefruits (Sharon-Asa et al. 2003). Valencene is accumulated in oranges only upon maturation, or after exogenous ethylene applications (Sharon-Asa et al. 2003). Orange rinds also contain the sesquiterpene β-farnesene, which might play a role in plant defence against aphids (Maruyama et al. 2001). The role of the sesquiterpenes in melons, apart from imparting unique notes to melon rind aroma, is presently unknown.
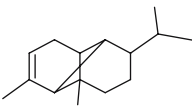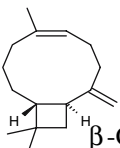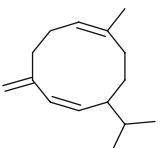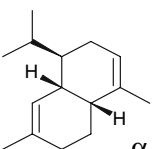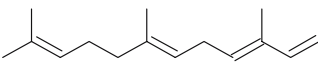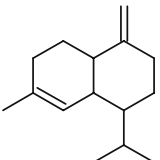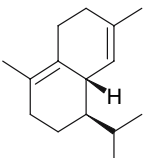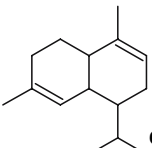
Table 2 Sequiterpenes identified in mature melon rinds

Compound	Retention time	Genotype																Method of compound's identification	
		Eshkolit Ha'Amakim	Arava	Dulce	Noy Yizre'el	Krymka	Dudaim	Doya	Doublon	Faqqous	Noy Amid	Rochet	Piel De Sapo	Vedrantais	Tendral Verde Tardio	Tam Dew	PI 414723	RI	MS
		Compound content (ng/gFW)																	
α-Copaene	20.2	33.5 (25.7)	56.9 (21.1)	nd	24.5 (12.3)	62.7 (40.8)	0.5 (0.4)	0.8 (0.3)	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
β-Elemene	20.6	4.8 (4.8)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
α-Gurjunene	21.1	1.7 (1.7)	0.4 (0.4)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
β-Caryophyllene	21.5	34.8 (30.1)	42.1 (17.8)	nd	3.6 (1.9)	nd	33.3 (25.5)	1.1 (0.3)	4.0 (0.3)	0.6 (0.4)	nd	nd	nd	nd	nd	nd	nd	+	+
β-Farnesene	22.2	6.5 (4.4)	10.5 (6.2)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
α-Humulene	22.4	5.9 (5.9)	6.4 (3.4)	nd	1.1 (0.6)	nd	4.8 (3.9)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
allo aromadendrene	22.6	0.6 (0.6)	1.0 (1.0)	nd	0.5 (0.2)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	*	+
γ-Muurolene	22.9	32.4 (29.7)	17.7 (9.1)	nd	3.2 (0.6)	nd	0.3 (0.2)	0.4 (0.3)	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
Germacrene D	23.1	23.0 (21.2)	13.2 (9.7)	nd	nd	nd	0.8 (0.6)	nd	nd	0.5 (0.2)	nd	nd	nd	nd	nd	nd	nd	+	+
Unidentified sesquiterpene	23.3	37.2 (18.9)	11.6 (6.1)	nd	10.0 (2.2)	0.5 (0.5)	nd	0.8 (0.6)	nd	nd	nd	nd	nd	nd	nd	nd	nd		
α-Muurolene	23.5	23.2 (11.0)	30.3 (7.5)	nd	6.0 (1.4)	0.5 (0.3)	0.4 (0.3)	0.5 (0.4)	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
E,E-α-Farnesene	23.6	nd	nd	154.4 (70.3)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
γ-Cadinene	23.9	151.7 (65.7)	174.2 (43.7)	nd	33.1 (6.1)	nd	1.0 (0.8)	1.4 (0.4)	0.1 (0.1)	0.6 (0.2)	nd	nd	nd	nd	nd	nd	nd	+	+
δ-Cadinene	24.1	145.7 (69.4)	128.6 (51.1)	nd	7.9 (3.9)	nd	2.0 (1.6)	1.9 (1.6)	0.4 (0.4)	2.6 (1.1)	nd	nd	nd	nd	nd	nd	nd	+	+
α-Cadinene	24.5	13.4 (5.9)	17.9 (3.3)	nd	2.2 (0.5)	nd	nd	0.1 (0.1)	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+
Total sesquiterpenes	514.3	510.6	154.4	92.1	63.7	43.1	6.9	4.5	4.3										

Volatile analysis was done using SPME coupled to GC–MS (see materials and methods). The sequiterpenes were identified by comparison to published retention times (RI) and mass spectra (MS) as indicated. The results shown are an average of three biological replicates

nd—Non detected

Table 3 Sesquiterpene volatiles in the rind of melons during various stages of development

Sesquiterpenes (ng/gFW)	Variety and days after anthesis (DAA)							
	'Noy Yizre'el'				'Dulce'			
	12 DAA	25 DAA	Mature	Post-harvest	12 DAA	25 DAA	Mature	Post-harvest
α-Copaene	0.0	0.0	6.3	80.5	0.0	0.0	0.0	0.0
β-Caryophyllene	0.2	0.9	14.4	23.5	0.0	0.0	0.0	0.0
Germacrene D	0.0	0.0	7.0	10.5	0.0	0.0	0.0	0.0
α-Muurolene	0.00	0.0	9.37	32.07	0.0	0.0	0.0	0.0
α-Farnesene	0.0	0.0	0.0	0.0	0.0	0.0	95.71	54.16
γ-Cadinene	0.0	0.	30.25	66.43	0.0	0.0	0.0	0.0
δ-Cadinene	0.0	0.0	64.46	103.5	0.0	0.0	0.0	0.0
α–Cadinene	0.0	0.0	6.9	10.3	0.0		0.0	0.0

Volatile analysis was done by SPME coupled to GC-MS (see materials and methods). The volatiles were identified by comparison to published retention time and mass spectra. The results shown are an average of three replicates (SE)

Sesquiterpene synthase activity in flesh and rind of ripe 'Noy Yizre'el' and 'Dulce' melons

The bulk of sesquiterpenes in melon fruit are present in the rind (Tables 2 and 3). It was therefore of interest to determine if sesquiterpenes are biosynthesized in rind tissues or imported from the adjacent flesh tissues. To address this question, we compared the levels of sesquiterpene synthase activity in cell-free extracts derived from either rind or flesh tissues of ripe 'Noy Yizre'el' and 'Dulce' melons. Our results clearly indicate that enzymatic activity is measurable only in the rind of each cultivar, while flesh tissues displayed almost no sesquiterpene synthase activity (Fig. 1 and S1). There was a marked difference between the products generated *in vitro* from farnesyl diphosphate by the enzymatic extracts derived from each variety. The products generated by 'Noy Yizre'el' cell-free extracts were the same as those present in 'Noy Yizre'el' mature rinds and included α-copaene, caryophyllene, germacrene D, γ-, α- and δ-cadinene. In contrast, α-farnesene (the only sesquiterpene that accumulates in 'Dulce' rind) was the major product generated in cell-free extracts derived from the rind tissues of 'Dulce' (Fig. 1 and S1).

Optimization of the levels of sesquiterpene synthase activity was performed using cell-free extracts derived from the rinds of ripe 'Noy Yizre'el' melons. The pH optimum was pH 7 (within the pH range of 6.5–9) and the enzyme activity had an absolute requirement for the presence of divalent metal ions for efficient catalysis. Optimal

levels of activity were obtained in the presence of either 10 mM $MgCl_2$ or 10 μM $MnCl_2$. These conditions are in accordance with results obtained for other enzymes of the terpene synthase family that also require the presence of divalent metal ions for catalysis (Alonso and Croteau 1993; Croteau and Cane 1985), and thus these conditions were adopted for the rest of the experiments.

Sesquiterpene synthase activity during fruit development

Since both the aroma properties of melons and the levels of sesquiterpenes are enhanced during fruit ripening (Table 3) it was of interest to assess whether sesquiterpene synthase activity limits the accumulation of sesquiterpenes during development. Sesquiterpene synthase activity was measured in protein extracts derived from rind tissues of 'Noy Yizre'el' and 'Dulce' melons at four different developmental stages. Extracts derived from young green fruits (12 and 25 days after anthesis) showed no detectable sesquiterpene synthase activity. Only the extracts derived from the rind of mature and post-harvest (see material and methods section) fruits produced sesquiterpenes following incubation with farnesyl diphosphate. As before, the sesquiterpene products obtained with the 'Noy Yizre'el' extracts were α-copaene, caryophyllene, germacrene D, γ-, α- and δ-cadinene (Fig. 2). Extracts from mature and post-harvest 'Dulce' rind tissues yielded only E-E-α-farnesene

Fig. 1 Sesquiterpene synthase activity in the flesh and rind of ripe 'Noy Yizre'el' and 'Dulce' melon fruits. Cell-free extracts prepared from the flesh (**A** and **C**) and from the rind (**B** and **D**) of 'Noy Yizre'el' and 'Dulce' respectively, were incubated with FDP under the assay conditions. The products formed in rind of 'Noy Yizre'el' (left panels) were: 1—a-Copaene, 2—Caryophyllene-E, 3—Germacrene D, 4—α-Muurolene, 5—γ-Cadinene, 6—δ-Cadinene, 7—α Cadinene. The product formed by 'Dulce' extracts (right panels) was α-farnesene. See Table 3 for structures and the chemical identification and supplemental material for the mass spectra

(Fig. 2). Our results further indicate that sesquiterpenes in melon rinds are biosynthesized in situ, and this process is developmentally regulated by the levels of sesquiterpene synthase activity. In citrus fruit, the levels of the sesquiterpene valencene are enhanced upon ripening or upon exogenous ethylene treatments (Sharon-Asa et al. 2003). Although ethylene has been shown to influence the biosynthesis of many volatiles in melons, it is presently unknown if it has any effect on sesquiterpene levels (Flores et al. 2002; Yahyaoui et al. 2002; Manriquez et al. 2006). Non-climacteric melon varieties do not display sesquiterpenes (Table 2 and Shalit et al. 2001), but not all the climacteric varieties display sesquiterpenes in their mature rinds (Table 2).

Isolation and characterization of *CmTpsNY* and *CmTpsDul*

The melon EST Database (http://melon.bti.cornell.edu/) contains a comprehensive annotation of clones from a collection of EST libraries, most of them from fruit, has proven to be a good source for identifying genes affecting quality characteristics in melon, including aroma traits (Lewinsohn et al. 2004; Yariv et al. 2004; Burger et al. 2006; Ibdah et al. 2006). Data mining of this database yielded two different ESTs with significant similarity to members of the *Tps* gene family. The first clone, SSH9B15, a 306 bp sequence mined from an SSH library enriched for rind of ripe 'Noy Yizre'el' fruit versus the rind of young 'Noy Yizre'el' fruit, showed a relatively high similarity

(59% at the amino acid level) to a sequence coding for a germacrene D synthase from poplar (Arimura et al. 2004). The full-length clone was obtained using rapid amplification of cDNA ends (RACE). The resulted full length gene, termed *CmTpsNY*, encoded for 571 aa and displayed a high similarity (72% at the amino acid level) to the β-caryophyllene synthase from *Cucumis sativus* leaves (Mercke et al. 2004) (Fig. 3). The second clone, FR15G19, consisting 432 bp, was isolated from an EST library of mature 'Dulce' fruit and displayed a high similarity (86% at the amino acid level) to the α-farnesene synthase from *Cucumis sativus* leaves (Mercke et al. 2004). A full-length cDNA clone of this gene was obtained by PCR using a forward primer based on the melon sequence and a reverse primer based on the cucumber sequence. The full-length clone was designated *CmTpsDul*. The largest open-reading frame of *CmTpsDul* encoded a predicted protein of 560 aa (Fig. 3).

A comparison of the predicted amino acid sequences of *CmTpsNY* and *CmTpsDul* indicates that the level of similarity between them is relatively low (43.2%). The melon genes share three common conserved motifs known to be found in members of the *Tps* gene family, including (a) RR(x)$_8$W motif previously described to be essential for the initial diphosphate migration of the isomerisation-cyclization reaction of limonene synthase in *Mentha*. (b) RxR and DDxD motifs suggested to be involved in catalysis (Croteau and Cane 1985; Alonso and Croteau 1993; Back and Chappell 1995; Steele et al. 1998; Williams et al. 1998; Davis and Croteau 2000; Aubourg et al. 2002). The

Fig. 3 Alignment of the deduced amino acid sequences of CmTpsNY, CmTpsDul and selected sesquiterpene synthases: *Cucumis sativus beta*-caryophyllene synthase (accession no. AAU05952), *Populus balsamifera* (−)-germacrene-D-synthase (accession no. AAR99061), *Gossypium arboreum* (+)-delta-cadinene synthase (accession no. CAA65289), *Cucumis sativus* E,E-*alpha*-farnesene synthase (accession no.AAU05951). The amino acids marked are the conserved motifs: RRx₈W; RxR and DDXXD

```
CmTpsNY                  MSSQVSNFPASIMKTNDIPDVKRSLANFHPNIWKEHFLSFTFDDALKIDEGMKERTEKLK 60
beta-caryophyllene s.    MS---SHFPASIMKTNDIYDTKRSLANFHPTIWKEHFLSFTFDDALKVDGGMKERIEKLK 57
(+)-delta-cadinene s.    MASQVSQMPSSSPLSSNK-DEIRPKADFQPSIWGDFFLNCP---DKNIDAGTEKRHQQLK 56
(-)-germacrene s.        MSVEGSAIFST---ATVEPNVSRRSAGYSPSIWGDHFLSYA---TDSMETSDKAEHKKLK 54
E,E-alpha-farnesene s.   MSSNVSAIPNS-------FELIRRSAQFQASVWGDYFLSYHSLPPEKGNKVMEKQTEELK 53
CmTps_Dul                MSSNISAIPNS-------LEVIRRSAQFQASVWGDYFLSYHSLSPEKGNKVMEKQTEELK 53
                         *: . * : :   :.   :   * . *: . .* :.**.        . :   : . ::**

CmTpsNY                  EEIRMMMIAYVENQ-LIKLNLVDSIQRLGVSYHFEDEVDEFLEHIYVSYNNSLLLSNKNS 119
beta-caryophyllene s.    EEIRMMVIASVQNP-LVKLNLVDSIQRLGVSYHFEDEIDQFLEHMYVSYNNSLLFSSNDS 116
(+)-delta-cadinene s.    EEVRKMIVAPMANS-TQKLAFIDSVQRLGVSYHFTKEIEDELENIYH---------NNND 106
(-)-germacrene s.        EEVKRELMANINKP-SQTLDFIDAIQRLGISYHFEIEIDEILREMYKSHCDFDNGDDDDH 113
E,E-alpha-farnesene s.   EEIKMELVSTTKDE-PEKLRLIDLIQRLGVCYHFENEINNILQQLHH-----ITITSEKN 107
CmTps_Dul                EEIKRELNSTTKDEEPEKLRLIDSIQRLGVCYHFEYEINKILEQLHH-----ITITSKNN 108
                         **::   : :    .    .* ::* :****:.*** **:. *..:: :  .    ...

CmTpsNY                  NGEDLHITALLFRLLRQQGYRISCDIFLKFMDDNGKFKESLVEDERGLLSLYEASHMMGH 179
beta-caryophyllene s.    QDDDLHISALLFRLLRQHGYRISCDIFLKFMDNNGKFKESLVEDERGILSLYEASHMRGH 176
(+)-delta-cadinene s.    AENDLYTTSLRFRLLREHGYNVSCDVFNKFKDEQGNFKSSVTSDVQGLLELYQASYLRVH 166
(-)-germacrene s.        HHNDLYAISLKFRLLRQQGYKISCDVFGKFKNSQGTFNDSLANDTRGILSLYEATHLRVH 173
E,E-alpha-farnesene s.   GDDNPYNMTLCFRLLRQQGYNVSSEPFDRFR---GKWESSYDNNVEELLSLYEASQLRMQ 164
CmTpsDul                 GDDHPYNMTLRFRLLRQQGYNISSKSFERFR---GKWESSYDKNVEELLSLYEASQLRMR 165
                         :. :  :* *****::**.:*.. * :* ...*.::.*  .:  . :*.**:*: :  :

CmTps NY                 GEALLEEALEFTTTHLQTYIHRYSNINPSFASEVSNALKLPIRKSVPRIKAREYLEIYQQ 239
beta-caryophyllene s.    GEALLEEALEFTTTHLKAYIHLYSNINPNFASEVSNALKLPIRKCVPRVKAREYFEIYQQ 236
(+)-delta-cadinene s.    GEDILDEAISFTTNHLS---LAVSSLDHPLSEEVSHALKQSIRRGLPRVEARHYLSVYQD 223
_-_-germacrene s.        GDEVLEEALVFTTSHLE---FLATHSSSPLRAKINHALKQPVRKNIPRLEARHYFSIYQE 230
E,E-alpha-farnesene s.   GEEALDEAFCFATAQLEA-IVQDPTTDPMVAAEIRQALKWPMYKNLPRLKARHHIGLYSE 223
CmTpsDul                 GEEALDEAFRFATAQLEA-IVQDPTTDPTVVGEVCQALKWPMYKNLPRLQASHYIGLYSE 224
                         *:  *:**: *:* :*.:      . . .  :: :*** .: : :**::* .:: :*.:

CmTpsNY                  HPSHNETLLEFSKLDFNILQKLHQKELSEICRWWKDLDVPTKFPFARDRIVECYFWTLGA 299
beta-caryophyllene s.    QPSHNETLLTFSKLDFNILQKLHQKEIAEICRWWKDLNVSTNFPFARDRIVECYFWILSI 296
(+)-delta-cadinene s.    IESHNKALLEFAKIDFNMLQFLHRKELSEICRWWKDLDFQRKLPYARDRVVEGYFWISGV 283
(-)-germacrene s.        DPSCSEVLLNFAKLDFNILQKQHQKELSEIANWWKELDFAKKLPFARDRVIECYFWILGV 290
E,E-alpha-farnesene s.   KPWRNESLLNFAKMDFNKLQNLHQTEIAYISKWWDDYGFAEKLSFARNRIVEGYFFALGI 283
CmTpsDul                 KPWRNESLPNFAKMDFSKLQKLHQKEIAYISKWWDDYGFAEKLSFARNRIVEGYFFALGI 284
                         .: *  *:*:**. ** *:.*:: *..**.: ..  ::.:*::*:* **:   .

CmTps_NY                 YFEPQYSVGRKMLTKVIAIASILDDIYDAYGTFEELQVLTPAIQRWDRSMVHTLPLYMKP 359
beta-caryophyllene s.    YFEPYFKFGRKILTKVIAMTSIMDDIYDAYGTFEELQLFTLAIKRWDMSMVNLLPQYMKV 356
(+)-delta-cadinene s.    YFEPQYSLGRKMLTKVIAMASIVDDTYDSYATYEELIPYTNAIERWDIKCIDELPEYMKP 343
(-)-germacrene s.        YLEPEYFLARRILTKVIAMTSVIDDIYDVYGTPEELELFTDAIERWEITAVDQLPEYMKV 350
E,E-alpha-farnesene s.   FFEPQLLTARLIMTKVIAIGSMLDDIYDVYGTFEELKLLTLALERWDKSETKQLPNYMKM 343
CmTpsDul                 FFEPQLSTARLIMTKIIAIGSVLDDIYDVYGTFEELKLLTLALERWDKSETKKLPKYMKM 344
                         ::** .* ::**:**: *::** ** *.* ***   * *::**:.  . ** :***

CmTpsNY                  FYVAMLELYEEIGKEIDKD---QNSLHLQVAIGGIKRLSESYFEEAKWLNKEYKPSFKEY 416
beta-caryophyllene s.    HYTTLLDLFEEMDKGIVND---GISYRSCFGKEAMKRQAESYFKEAEWLNKNYKPKYGEY 413
(+)-delta-cadinene s.    SYKALLDVYKEMEQLVAEH---GRQYRVEYAKNAMIRLAQSYLVEARWTLQNYKPSFEEF 400
(-)-germacrene s.        TYKALLDVYTEIEENMVTE---ERSYRVYYAKEAMKNLVRAYYLESKWFHQKHTPTMEEY 407
E,E-alpha-farnesene s.   YYEALLDVFEEIEQEMSQKETETTPYCIHHMKEATKELGRVFLVEATWCKEGYTPKVEEY 403
CmTps_Dul                YYEALLDVFEEIEQEMSQK--ETTPYCIHQMKEATKELGRVFLVEAKWCKEGYTPTVEEY 402
                         * ::*::: *: : :  .          .  . : *: *  : :.*. *:

CmTpsNY                  MELALKTTGYTMLISISFLGLGDHIVTNEVLQWLSNGPQIIKASTIICRLMDDIASHKFE 476
beta-caryophyllene s.    MEVALASSGYELLSTISFVCMGD-IATKEVFEWLFDCPQILKASTTISRLMDDVVSYKFE 472
(+)-delta-cadinene s.    KANALPTCGYAMLAITSFVGMGD-IVTPETFKWAAANDPKIIQASTIICRFMDDVAEHKFK 459
(-)-germacrene s.        MAVALVTSGYAMLAATSFVGMGH-VVTKDSFDWLFRGPKILKASEIICRLMDDIVSHKFE 466
E,E-alpha-farnesene s.   LDIALISFGHKLLMVTALLGMGSHMATQQIVQWITSMPNILKASAVICRLMNDIVSHKFE 463
CmTpsDul                 LDIALISFGHKLLMVTALLGMGSTIATQQIVQWITSMPNILKASAIICRLMNDIVSHKFE 462
                         ** : *: :*   ::: :*  :.* : .:.* : ..*   *:*::** *.*:*:*:..:**:

CmTpsNY                  QEREHVASAVECYMKQYDCSEEEACIELHKEVVDAWKDTNE---AFYRPFNVPVPVLMRV 533
beta-caryophyllene s.    KEREHIVSAVECYMSNHGRSEDETCAELLKQVEDAWKTINE---CCLHPMNVPMPFLICL 529
(+)-delta-cadinene s.    HRREDDCSAIECYMEEYGVSAQEAYDVFNKHVESAWKDVNQ---EFQKPTEMPTEVLNRS 516
(-)-germacrene s.        QKRGHVASSIECYMKQHGTTEQETVHEFRKQVTDAWKDLNE---EFLHPTAVPMPLLTRM 523
E,E-alpha-farnesene s.   QERGHVASAIECYMEQNHLSEYEALIALRKQIDDLWKDMVENYCAVITEDEVPRGVLMRV 523
CmTpsDul                 QERGHVASAIECYMEQNYMSEHDVLIILGKQIDEFWKDMVENYCVVITEEEVPRGVLMRV 522
                         :.* . *::****.:  :  :.   : *.: . **   :     :* .*

CmTpsNY                  LNFSRVINLLYLDEDGYTNAKSGTKFLIKSLLVDPLPC 571
beta-caryophyllene s.    LNLTRVMALLYSHEDGYTNSKGRTKLLIQSLLIDPLHL 567
(+)-delta-cadinene s.    LNLARVMDVLYREGDGYTYVGKAAKGGITSLLIEPIAL 554
(-)-germacrene s.        LNLARVIDVVYKDEDGYTNAGTVLKDLVSALLIDPVPM 561
E,E-alpha-farnesene s.   LNLTRLFNVIYKDGDGYTQSHGSTKAHIKSLLVDSVPL 561
CmTpsDul                 LNLTRLFNVIYKDGDGYTQSHGSTKTHIKSLLVDSLPL 560
                         **::*:: ::* . ****    *  : :**::.:
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RR(x)₈W motif of *CmTpsDul* is conserved while that of *CmTpsNY* has a known variation in which a serine substitutes an arginine RS(x)₈W. Based on phylogenetic analysis of the two melon *Tps* genes with respect to other members of the *Tps* gene family we conclude that *CmTpsNY* and *CmTpsDul* belong to the *Tps*-a group (class III) (Bohlmann et al. 1998; Trapp and Croteau 2001; Aubourg et al. 2002) (Fig. 4).

Fig. 4 Phylogenetic tree of the deduced amino acid sequences of functionally characterized sesquiterpene synthases genes, including *CmTpsDul* and *CmTpsNY*. The deduced protein sequences were clustered using simple default parameters of ClustalW program (www.ebi.ac.uk/) and the radial tree representation was realized using the TREEVIEW application (Page 1996). Subdivision of the *TPS* gene family into six subfamilies, designated *TPSa* to *TPSf*, each distinguished by sharing a minimum of 40% identity among members (Bohlmann et al. 1998). Class-I, class-II and class-III designation followed the classification of Aubourg et al. (2002), except for the genes of *C. melo*, *C. sativus* and *Malus domestica*. This classification is based on gene structure (Trapp and Croteau 2001), that is not available for the latter five genes. The accession numbers for the newly described *TPS* genes are: *Cucumis melo*—*CmTpsNy* (EU158098) and *Cucumis melo*—*CmTpsDul* (EU158099). The accession numbers for the additional TPS genes represented in the tree are: *Abies grandis*—*4S*-limonene synthase (AF006193); *Abies grandis*—myrcene synthase (U87908); *Arabidopsis thaliana*—TPS10 (AF178535); *Arabidopsis thaliana*—TPSGA1 (U11034); *Arabidopsis thaliana*—TPSGA2 (AF034774); *Artemisia annua*—*epi*-cedrol synthase (AF157059); *Clarkia breweri*—*S*-linalool synthase (U58314); *Cucumus sativus*—*β*-caryophyllene synthase (AAU05952); *Cucumus sativus*—*E,E*-*α*-farnesene synthase (AAU05951); *Cucumis sativus*—*ent*-kaurene synthase (AB045310); *Cucurbita maxima*—copalyl diphosphate synthase (AF049905); *Cucurbita maxima*—*ent*-kaurene synthase (U43904); *Elaeis oleifera*—sesquiterpene synthase (AAC31570); *Malus × domestica*—*E,E*-*alpha*-farnesene synthase (AY787633); *Mentha longifolia*—*4S*-limonene synthase (AF175323); *Mentha × piperita*—*E-beta*-farnesene synthase (AF024615); *Nicotiana tabacum*—5-*epi*-aristolochene synthase (L04680); *Pisum sativum*—copalyl diphosphate synthase (U63652); *Populus balsamifera*—germacrene-D-synthase (AAR99061); *Salvia officinalis*—(+)-sabinene synthase (AF051901); *Vitis vinifera*—terpene synthase (AAS66357)

Functional expression of *CmTpsNY* and *CmTpsDul* in *E. coli*

In order to functionally determine the biochemical role of *CmTpsNY* and *CmTpsDul* the genes were heterologoulsy expressed in *E. coli* and the recombinant gene products were assayed in vitro for sesquiterpene synthase activity. Bacterial lysates derived from recombinant *E. coli* expressing *CmTpsNY* catalyzed the formation of three sesquiterpenes from farnesyl diphosphate: *δ*-cadinene, *γ*-cadinene and lower levels of *α*-copaene (Fig. 5a). In parallel, heterologous expression of *CmTpsDul* in *E. coli* yielded a protein extract possessing sesquiterpene synthase activity able to catalyze the conversion of farnesyl diphosphate into *α*-farnesene (Fig. 5b). These findings further indicate that the database mining yielded genes with biological relevance and probably account for the chemical variation observed within the melon varieties (Table 2, Fig. S1).

The cucumber *CsαFs* gene and the melon *CmTpsDul* share a relatively high degree of similarity (89% at the aa level and 93.2% at the nucleotide level). Both genes encode for proteins able to catalyze the conversion of farnesyl diphosphate to *α*-farnesene in vitro when functionally analyzed (Fig. 5b and Mercke et al. 2004), thus they seemingly have identical biochemical functions. Nevertheless they are expressed in different tissues (the cucumber gene is expressed in leaves after spider mite attack while the melon gene in ripe melon rind). These findings possibly indicate the recruitment of similar genes

Fig. 5 GC-MS of the products generated in vitro by recombinant *CmTpsNY* (**A**) and *CmTpsDul* (**B**) genes. Bacterial lysates derived from *E. coli* overexpressing recombinant CmTpsNY or CmTpsDul were incubated with FDP under the conditions described in Materials and Methods. Identification of the products was done by GC-MS according to the retention time and by comparing the compound mass spectrum to literature. Recombinant CmTpsNY produced three different sesquiterpenes (**A**) while recombinant CmTpsDul produced a single product (**B**). No product was detected in controls without substrate FDP, without cofactors, or construct devoid of the recombinant gene (data not shown)
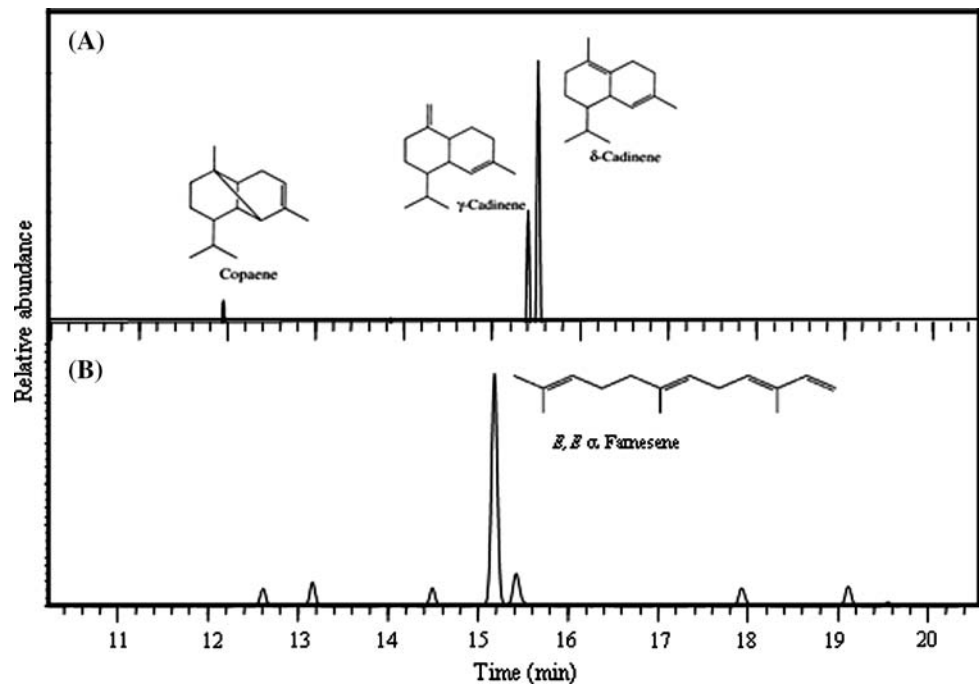
to different biological functions and emphasize the versatility of the terpene pathway in directing sesquiterpene biosynthesis in different physiological situations.

It is well known that some enzymes of the *Tps* family utilize a single substrate to generate a variety of products (Croteau and Cane 1985; Lewinsohn et al. 1992; Bohlmann et al. 1998; Mercke et al. 1999; Kollner et al. 2004; Deguerry et al. 2006), but others generate single products (Prosser et al. 2002; Sharon-Asa et al. 2003; Iijima et al. 2004; Deguerry et al. 2006; Pichersky et al. 2006). Interestingly, melon cultivars exhibit both of these types of enzymes, CmTpsNY produces several products, and CmTpsDul produces only one sesquiterpene product from farnesyl diphosphate.

Expression of *CmTpsNY* and *CmTpsDul* in melon rind and flesh during fruit development

In both varieties, sesquiterpene compounds (Table 3) and the sesquiterpene synthase activities (Fig. 1) are mainly restricted to the rind tissues of the ripe fruit. Northern blots and real-time PCR analyses were performed to explore for possible transcriptional tissue and varietal specific regulation of sesquiterpene accumulation. The results indicate that the levels of expression of *CmTpsNY* are negligible in 'Noy Yizre'el' during the early stages (days 12 and 25 after anthesis) of development but dramatically rise during ripening (Fig. 6a and b), in accordance with the levels of the extractable sesquiterpene synthase activity. Moreover, expression is found exclusively in rind tissues. Similarly,

the expression patterns of *CmTpsDul* in 'Dulce' melons follow a pattern parallel to sesquiterpene synthase activity in this variety. The highest levels of expression were found in mature rind tissues, though lower but significant levels of expression were also found in the rind of post-harvest fruits as well as in flesh of mature and post-harvest fruits (Fig. 6c and d).

Interestingly, *CmTpsDul* was not expressed in either the rind or flesh of 'Noy Yizre'el' melons at any developmental stage and, reciprocally, *CmTpsNY* was not expressed in 'Dulce' tissues.

Two other melon cultivars: 'Tam-Dew' (*C. melo* subspecies *melo* group *inodorus*) and 'PI 414723' (*C. melo* subspecies *agrestis* group *momordica*) that do not contain sesquiterpenes in their mature rinds (Table 2) were further examined for possible expression of *CmTpsNY* or *CmTpsDul*. In both of these varieties, gene expression was negligible in the mature flesh or rind tissues (not shown). This further indicates that sesquiterpene biosynthesis in melon rinds is regulated at the transcriptional level, in accordance to most of the cases tested, including in elicited tobacco cell cultures (Chappell 1995), potato and tomato leaves (Yoshioka et al. 1999; Kant et al. 2004), basil glandular trichomes (Iijima et al. 2004) cucumber leaves (Mercke et al. 2004), rose flowers (Guterman et al. 2002), orange fruit (Sharon-Asa et al. 2003) and Arabidopsis (Tholl et al. 2005).

Our results not only further corroborate that sesquiterpene formation is transcriptionally regulated during fruit development, but we have also demonstrated that each variety examined expresses unique sesquiterpene synthases

Fig. 6 Expression of *Tps* genes in melon tissues during development Real-time PCR analysis of *CmTpsNY* (**a**) and *CmTpsDul* (**c**). RNA was extracted separately from the rind and flesh at various stages of fruit development (12 DAA, 25 DAA, mature and post-harvest). Expression levels were normalized with respect to the internal control cyclophilin and are plotted relative to the expression of the 12 DAA samples. Values presented represent the mean ± SE derived from three biological replicates. (**b**) and (**d**) Northern blot analysis of sesquiterpene synthase genes in melon fruit. Each lane was loaded with 25 μg of total RNA extracted from the rind and flesh of 'Noy Yizre'el' and 'Dulce' at the stages of fruit development listed above. Blots were hybridized to *CmTpsNY* (**b**) or to *CmTpsDul* (**d**) probes. Ribosomal RNA was visualized by ethidium bromide staining and served as a loading reference
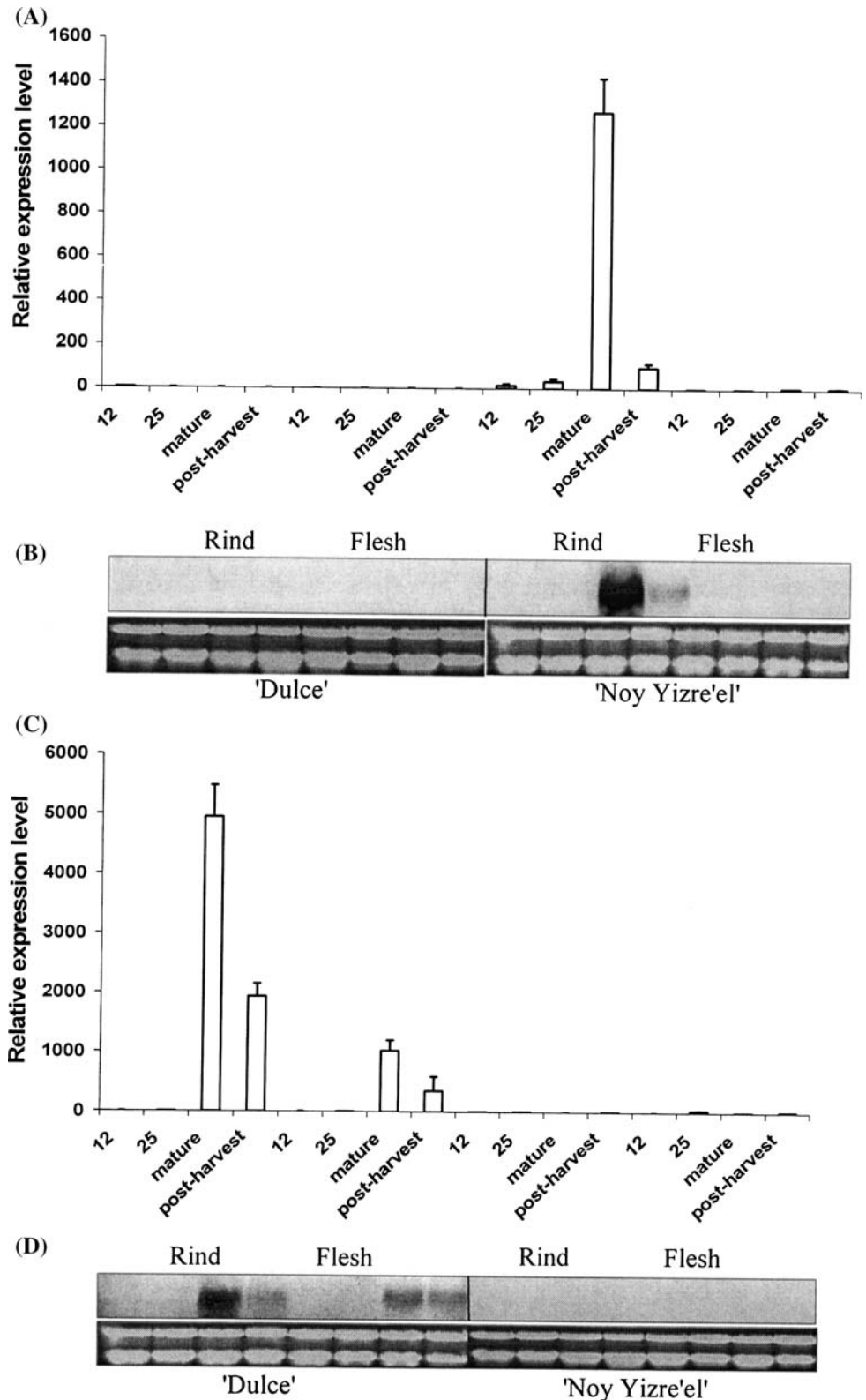
that are not expressed in other varieties examined. The unique expression patterns of each gene in a particular variety leads to the formation of unique sesquiterpenes that may contribute to the diversity in the characteristic aromas of melon cultivars. Rind aroma carries a great economical value affecting consumers' choice of melons. Therefore, our understanding of the underlying biosynthetic pathways to sesquiterpenes and their molecular regulation will enable

the development of tools for obtaining novel crop varieties with improved quality and aroma traits without compromising other agronomically important characteristics. The understanding of the diverse ecological roles played by the sesquiterpene family, as defence compounds, pollinator and seed-disperser attractants (McGarvey and Croteau 1995; Trapp and Croteau 2001; Degenhardt et al. 2003), will also benefit from our knowledge of the molecular control of their synthesis.

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