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Effects of Nucleo-cytoplasmic Interactions on Leaf Volatile Compounds from Citrus Somatic Diploid Hybrids

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Three diploid citrus somatic hybrids (cybrids) were produced by fusions combining nucellar callus-derived protoplasts of Willow Leaf mandarin (*Citrus deliciosa* Ten.) and Commune clementine (*Citrus clementina* Hort. ex Tan.) with, respectively, leaf protoplasts of Eureka lemon [*Citrus limon* (L.) Burm.] and Marumi kumquat [*Fortunella japonica* (Thunb.) Swing.] and leaf protoplasts of Marumi kumquat. Ploidy and origins of the nuclear, chloroplastic, and mitochondrial genomes were investigated by flow cytometry and nuclear and cytoplasmic simple sequence repeat analyses. Volatile compounds were extracted from the leaves of the three cybrids by a pentane/ether (1:1) mixture, analyzed by GC-MS, and compared to those of their parents. The cybrids were found to be very close to their nucleus-giving parent, suggesting that the main information for volatile compounds biosynthesis is contained in the nucleus. However, nucleo-cytoplasmic interactions occurred: the (mandarin + lemon) cybrid, possessing nucleus and chloroplasts of lemon and mitochondria from mandarin, synthesizes more monoterpene alcohols and esters than its nucleus-giving parent; the (clementine + kumquat) cybrid, possessing nucleus from kumquat and organelles from mandarin, synthesizes more monoterpene and sesquiterpene hydrocarbons and sesquiterpene alcohols than its nucleus-giving parent.

KEYWORDS: Citrus; Rutaceae; diploid somatic hybrids (cybrids); leaf volatile compounds; nucleo-cytoplasmic interactions; flow cytometry; SSR markers; nuclear DNA; mitochondrial DNA; chloroplast DNA

INTRODUCTION

Somatic hybridization by fusion of parental protoplasts is a technique allowing a tremendous increase of the genetic diversity by combining sexually compatible or incompatible parents or distantly related genera and species. It produces allo- and autotetraploid, triploid, and also diploid hybrids (also called *cybrids*) as byproducts (*1, 2*). Tetraploid hybrids inherit nuclear genomes of their parents (addition of chromosomes) and various parental combinations of cytoplasmic organelles (chloroplasts and mitochondria), whereas diploid ones inherit the nuclear genome of one of the parents and various parental combinations of chloroplasts and mitochondria. Numerous protoplast fusions have been performed in the *Citrus* and related genera and, to date, about 200 kinds of somatic hybrids have been generated in the world (*1*), of which only 30 are diploids.

Among the aims of somatic hybridization (e.g., production of rootstocks with improved cold hardiness, nematode and *Phytophtora* resistances, ...) (*2*−*5*), obtaining plants bearing fruits of good sensory characteristics is a major goal. Aroma being a major factor of fresh citrus fruit quality and also of their derived products (juices, essential oils, ...), improvement of citrus cultivars through this technique must include the analysis of volatile compounds of the produced hybrids. It has been shown that citrus allotetraploids, although possessing all chromosomes of their parents, do not keep all their parental traits with regard to the biosynthesis of volatile compounds (*6*−*10*). On the other hand, cybrids are of considerable interest because, possessing the nuclear genome of only one parent and various combinations of the parental cytoplasmic genomes (chloroplastic and mitochondrial), they are good models for studying the influence of nucleo-cytoplasmic interactions on volatile compound synthesis. To our knowledge, until now, only one paper has been released giving the composition of leaf volatile compounds of a cybrid obtained by fusion of sweet orange and lemon (*11*).

Diploid somatic hybrids have been obtained by CIRAD (*2*) and are cultivated at the Station de Recherches Agronomiques INRA-CIRAD (San Giuliano, Corsica, France). They were characterized by flow cytometry and single sequence repeat

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(SSR) analyses. With the aim of studying the genetic control of volatile compound biosynthesis (i.e., the nucleo-cytoplasmic interactions), we analyzed leaf volatile compounds from three somatic diploid hybrids obtained by fusion of various combinations of mandarin (*Citrus deliciosa* Ten.), lemon [*Citrus limon* (L.) Burm.], clementine (*Citrus clementina* Hort. ex Tan.), and kumquat [*Fortunella japonica* (Thunb.) Swing.]. Leaves of the parents were also analyzed, and the results are presented hereafter.

MATERIALS AND METHODS

Plant Materials. The 1-year-old parents, all grafted onto volkameriana rootstock (*Citrus limonia* Osb.) and growing in the same field of the Station de Recherches Agronomiques (INRA-CIRAD) of San Giuliano, were of the following species: mandarin (cv. Willow Leaf; hereafter designated WLM in tables and figures), lemon (cv. Eureka, EUR), clementine (cv. Clémentine Commune, CLM), and kumquat (cv. Marumi, MK). We also analyzed three 1-year-old somatic diploid hybrids (cybrids), obtained by the fusion of protoplasts from (i) nucellar callus line of mandarin and, respectively, leaf-derived protoplasts of lemon [(mandarin + lemon) hybrid = (WLM + EUR)] and kumquat [(mandarin + kumquat) hybrid = (WLM + MK)] and from (ii) nucellar callus line of clementine and leaf-derived protoplasts of kumquat [(clementine + kumquat) hybrid = (CLM + MK)]. Callus-derived protoplasts of clementine were haploid. These hybrids were all grafted onto volkameriana rootstock and planted as their parents the same week in the same field at the Station de Recherches Agronomiques INRA-CIRAD of San Giuliano. For each parent and hybrid, three individual plants were cultivated under totally randomized design.

Batches of leaves were randomly hand-picked, revolving around the shrubs on the same day (February 2003), and immediately air-freighted to our laboratory. Three individual shrubs were sampled for each parent and hybrid, and each batch of leaves was analyzed separately as follows. Leaves (50 g) were cut in half with scissors after removal of the central rib and then ball-milled in liquid N_2 with a Dangoumilll 300 grinder for 2 min. Finely pulverized leaf powder was then stored under argon at −80 °C before extraction and analysis of volatile compounds the day after.

DNA Extraction. Total genomic DNA was extracted according to the method of Doyle and Doyle (*12*) from leaves of parents (mandarin, lemon, and kumquat) and of the three cybrids and then kept at −80 °C before analysis.

Cytometry and SSR Analysis. The ploidy of parents and hybrids was determined by flow cytometry (*1*). For SSR analysis, 22 primers for nuclear microsatellite amplifications were used: Ci01B10, Ci01C07, Ci01D06a, Ci01E02, Ci01H05, Ci02A04, Ci02A09, Ci02B07, Ci06A08, Ci06A12, Ci06B05, Ci06B07, Ci07B05, Ci07B09, Ci07C09, Ci07D06, Ci07D07, Ci07E05, Ci07E06, Ci07F11, Ci08B08, and Ci08C05 (*13*). Three chloroplast primers were used: ntcp9 (*14*), ccmp5 and ccmp6 (*15*). PCR amplifications of the samples were performed using a PTC-200 thermocycler (MJ Research Inc.) in a 15 μL final volume containing 0.8 unit of *Taq* DNA polymerase (Eurogentec), 10 ng of citrus DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 μM reverse primer, and 0.2 μM forward primer. The following PCR program was applied: denaturation at 94 °C for 5 min and subjected to 35 repeats of the following cycle: 30 s at 94 °C, 1 min at 50 or 55 °C, 45 s at 72 °C; and a final elongation step of 4 min at 72 °C. Samples were then kept at 4 °C prior to analysis. After the addition of 15 μL of loading buffer [98% formamide, 10 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol], the mixture was denatured at 92 °C for 3 min and kept at 70 °C during gel loading. Six microliters of each sample was loaded in 5% polyacrylamide sequencing gel with 7.5 M urea in 0.5% TBE buffer prior to electrophoresis at 60 W for 1.5−2.5 h. Gels were silver stained using an improved method adapted from that of Beidler et al. (*16*).

Extraction of Volatile Compounds. The solvents (*n*-pentane and ether) were of analytical grade. Reference compounds, when available, and *n*-alkane (C_5−C_{22}) standards were from Aldrich Chimie (Saint Quentin Fallavier, France). The internal standard (30 μg of *n*-hexanol) was added to leaf powder (500 mg), which was then homogenized using a Potter Elvejhem homogenizer with 20 mL of pentane/ether (1:1) for 5 min. The slurry was then filtered on a glass crucible (porosity = 4) filled with anhydrous sodium sulfate. The extract was finally concentrated at 42 °C to a volume of 2 mL with a 25 cm Vigreux distillation column.

GC and GC-MS Analysis. Solvent extracts were analyzed by GC-FID using two fused silica capillary columns of DB-Wax (column A, J&W Scientific, Folsom, CA) (60 m × 0.32 mm i.d. × 0.25 μm film) and DB-1 (column B, J&W Scientific) (30 m × 0.32 mm i.d. × 0.25 μm film). Oven temperature was increased from 40 °C at a rate of 1.5 °C min^{-1} (DB-Wax) or 3 °C (DB-1) to 245 °C, at which it was held for 20 min. On-column injector was heated from 20 to 245 °C at 180 °C min^{-1}. Detector temperature was 245 °C. Hydrogen was the carrier gas at 2 mL min^{-1}. Injected volumes were 2 μL of concentrated extract.

Solvent extracts were also analyzed by GC-MS using a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass spectrometer with electron ionization mode (EI) generated at 70 eV. The ion source and quadrupole temperatures were 230 and 150 °C, respectively, and the filament emission current was 1 mA. Volatile compounds were separated on a DB-Wax column (column A, J&W Scientific) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film) and on a DB-1 column (column B, J&W Scientific) (30 m × 0.25 mm i.d. × 0.25 μm film). Oven temperature was increased from 40 °C at a rate of 3 °C min^{-1} to 250 °C, at which it was held for 20 min. On-column injector was heated from 20 to 245 °C at 180 °C min^{-1}. Detector temperature was 245 °C. Helium was the carrier gas at 1.1 mL min^{-1}. Electron impact mass spectra were recorded in the 40−600 amu range at 1 s intervals. Injected volumes were 1 μL of concentrated extract. Compounds were identified on the basis of linear retention indices on both columns (DB-Wax and DB-1) and EI mass spectra (Wiley 275.L library) from the literature or from authentic standard compounds.

Quantitative data were obtained from the GC-FID analyses. Integration was performed on compounds eluted from the DB-Wax column between 3 and 110 min. Response factors of 10 reference compounds from different classes (monoterpenes, sesquiterpenes, monoterpene alcohols and aldehydes, esters) were determined and found to range from 0.85 to 1.2 versus *n*-hexanol, averaging 1.0. Response factors were therefore taken as 1.0 for all compounds with reference to the internal standard. It was also confirmed that the internal standard was fully recovered after extraction and concentration from a leaf powder, by the separate injection of 2 μL of a standard solution of *n*-hexanol (15 μg mL^{-1}) in pentane/ether (1:1). Amounts were expressed as micrograms of *n*-hexanol equivalent per gram of dry weight. Linear retention indices were calculated with reference to *n*-alkanes (C_5−C_{22}). Concentrations (see **Table 2**) are given as the average of data from three individual shrubs. The total content in volatile compounds of leaves from hybrids and their parents was calculated by summing concentrations of all volatile compounds eluted from the DB-Wax column between 3 and 110 min and expressed as percent of dry weight.

Statistical Analyses. For each combination, Euclidian distances were calculated (@DARwin 4.0 software, CIRAD, Montpellier, France) between parents and hybrids (**Figure 2**). Calculations were based on the average concentrations of each volatile compound from leaves of three individual shrubs (**Table 2**). The statistical comparison of data was performed by ANOVA using the GLM procedure of SAS (SAS Institute Inc., 1989) to reveal significant differences among cybrids and their parents. Least significant differences between means were assessed using Sheffe's test (at $P < 0.01$ and $P < 0.05$).

RESULTS AND DISCUSSION

A prerequisite to our investigation on nucleo-cytoplasmic interactions influencing volatile compound biosynthesis was to determine the origins in the cybrids of nuclei and organelles (mitochondria and chloroplasts).

Molecular Analysis. Analyses by flow cytometry showed that all hybrids were diploids ($2n = 18$). The three citrus somatic

Table 1. Characteristics of the Parents and Their Diploid Hybrids (= Cybrids)

parent	ploidy
mandarin (cv. Willow Leaf, WLM)	$2n = 18$
lemon (cv. Eureka, EUR)	$2n = 18$
kumquat (cv. Marumi, MK)	$2n = 18$
clementine (cv. Commune, CLM; haploid)	$n = 9$

cybrid	parent 1	protoplasts	parent 2	protoplasts	nucleus	chloroplasts	mitochondria	ploidy
WLM + EUR	WLM	callus	EUR	leaf	EUR	EUR	WLM	$2n = 18$
WLM + MK	WLM	callus	MK	leaf	MK	MK	WLM	$2n = 18$
CLM + MK	CLM	callus	MK	leaf	MK	WLM[a]	WLM[a]	$2n = 18$

[a] Clementine has chloroplasts and mitochondria from mandarin.

hybrids were produced by fusion of callus-derived protoplasts of mandarin or clementine (embryogenic parents) with leaf-derived protoplasts of lemon or kumquat (leaf mesophyll parents). In citrus somatic hybrids obtained by fusion of leaf protoplasts and embryogenic callus protoplasts, the mitochondrial genome is inherited from the embryogenic parent (*2*, *17*): that was the case of our three cybrids, which inherited mitochondria from their mandarin or clementine parent. As the clementine parent inherited mitochondria from its Willow Leaf mandarin parent (*18*), we can consider that our three cybrids possess the same WLM mitochondrial genome (**Table 1**). Determination of nuclear and chloroplast DNA origins was performed by SSR analysis. All nuclear and chloroplast primers succeeded in amplifying the three hybrids and their parents. Nuclear and chloroplast primers have been chosen to display polymorphism between parents and therefore allowed us to identify the genome origins of our cybrids (**Figure 1**). The (mandarin + kumquat) hybrid, (WLM + MK), combined the nucleus and chloroplasts from kumquat (**Table 1**). The (clementine + kumquat) hybrid, (CLM + MK), combined the nucleus from kumquat with chloroplasts from mandarin. Concerning the (mandarin + lemon) hybrid, (WLM + EUR), the nucleus and chloroplasts came from lemon. Moreover, analysis of nuclear DNA by 22 SSR markers revealed that the three hybrids possessed bands from one parent only: nuclear genome was apparently transmitted without punctual recombination and/or deletion.

Volatile Compounds. The total contents in volatile compounds of leaves (percent dry weight) from the parents were as follows: kumquat, 1.64; mandarin, 1.20; and Eureka lemon, 1.29. The leaf volatile contents of hybrids were as follows: (clementine + kumquat), 1.98; (mandarin + kumquat), 1.70; and (mandarin + lemon), 1.41. It is worth noting that the contents measured in the hybrid leaves were higher than those of their parent leaves (by 4−21%). The compositions of leaf extracts from the hybrids and their respective parents are given in **Table 2**. Each component is given as micrograms of *n*-hexanol equivalent per gram of leaf (dry weight), response factors being taken as 1.0 for all compounds with reference to the internal standard. For each combination, the volatile component profile of the hybrid was qualitatively similar to that of the mesophyll leaf parent. Euclidean distances between cybrids and their parents were calculated on the basis of the average concentration of each volatile compound from leaves of three individual shrubs (**Figure 2**): they shows that each cybrid was closer to its leaf mesophyll parent (lemon or kumquat) than to its embryogenic one (mandarin). In the same way, results obtained with methyl *N*-methylanthranilate (compound **74**), a mandarin-specific compound, lead to the same conclusion. This compound was observed in high amount in the leaves of the mandarin parent (embryogenic parent) (8711 μg g^{-1}; 72.9% of the volatile compounds). It was absent in leaves of the other parents and in those of the cybrids.

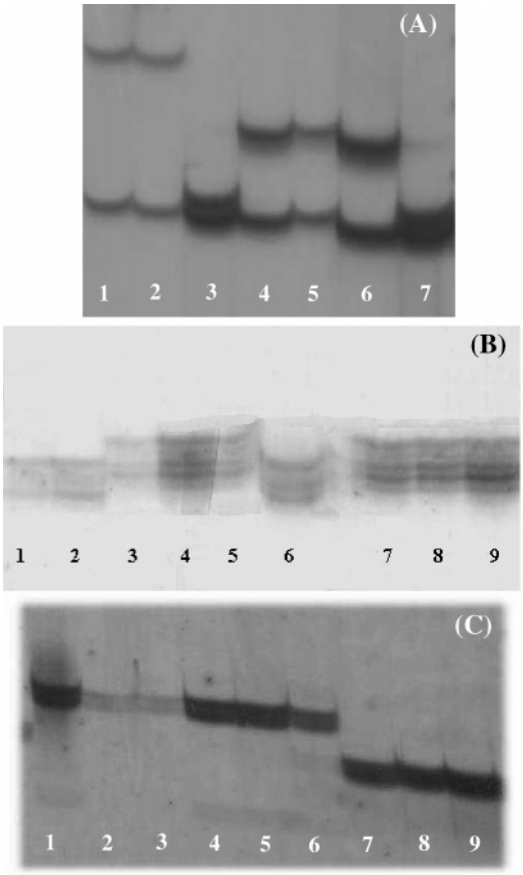

Figure 1. (**A**) Nuclear SSR analyses with Ci01C07 primer. Leaf DNA samples were as follows: lane 1, lemon = EUR; lane 2, (mandarin + lemon) cybrid = (WLM + EUR); lane 3, mandarin = WLM; lane 4, (mandarin + kumquat) cybrid = (WLM + MK); lane 5, kumquat = MK; lane 6, (clementine + kumquat) cybrid = (CLM + MK); lane 7, mandarin. (**B**) Chloroplast SSR analyses with ccmp6 primer. Leaf DNA samples were as follows: lanes 1 and 2, (mandarin + kumquat) cybrid; lanes 3–5, (clementine + kumquat) cybrid; lane 6, kumquat; lanes 7 and 8, (clementine + kumquat) cybrid; lane 9, mandarin. (**C**) Chloroplast SSR analyses with ntcp9 primer. Leaf DNA samples were as follows: lanes 1–3 (mandarin + lemon) cybrid; lanes 4–6, lemon; lanes 7–9, mandarin.

Monoterpene Hydrocarbons. These compounds were weakly represented in kumquat leaves (56 μg g^{-1}; 0.3% of total volatiles) (**Table 2**); mandarin and lemon leaves were richer in this class of volatiles with, respectively, 2881 μg g^{-1} (24.1%) and 5340 μg g^{-1} (42.0%).

The (mandarin + lemon) hybrid, (WLM + EUR), which inherited nucleus and chloroplasts from lemon and mitochondria

Table 2. Volatile Compounds of Leaves (Micrograms per Gram of Dry Weight and Class Relative Percent) from Parents and Their Diploid Hybrids

no.	compound	RI DB-Wax	RI DB-1	WLM[a]	EUR[b]	MK[c]	WLM + EUR[d]	WLM + MK[e]	CLM + MK[f]	reliability of identification[g]
monoterpene hydrocarbons										
1	α-pinene	1015	925	120	**136	–[h]	**79	–	2	1
2	α-thujene	1017	920	91	6	–	6	–	–	2
3	camphene	1046	935	–	10	–	5	–	4	1
4	β-pinene	1092	962	131	**1735	–	**818	–	–	1
5	sabinene	1110	961	17	*365	–	*184	–	–	1
6	δ-3-carene	1135	994	–	*56	4	*208	1	5	1
7	β-myrcene	1152	983	44	133	11	164	13	20	1
8	α-phellandrene	1153	989	–	10	–	12	–	4	1
9	α-terpinene	1165	1000	11	–	–	–	–	–	1
10	limonene	1190	1018	689	2546	8	3139	15	21	1
11	β-phellandrene	1194	1010	–	56	–	51	–	–	1
12	(*Z*)-β-ocimene	1225	1028	14	48	2	42	1	8	1
13	γ-terpinene	1232	1043	1497	2	–	9	–	–	1
14	(*E*)-β-ocimene	1240	1039	40	222	*31	192	55	*106	1
15	*p*-cymene	1252	1002	181	–	–	–	–	–	1
16	α-terpinolene	1269	1072	46	*15	–	*45	–	–	1
	total			2881	5340	*56	4954	85	*170	
	rel %			24.1	42.0	0.3	35.9	0.5	0.9	
monoterpene aldehydes										
17	citronellal	1460	1133	–	114	–	146	–	–	1
18	neral	1660	1213	–	2100	–	2481	–	–	1
19	geranial	1715	1245	–	3325	–	3955	–	–	1
	total			0	5539	0	6582	0	0	
	rel %			0.0	43.6	0.0	47.7	0.0	0.0	
monoterpene alcohols										
20	linalool	1537	1086	29	133	18	162	21	16	1
21	terpinen-4-ol	1578	1145	–	–	–	5	–	–	1
22	1,8-menthadien-4-ol	1644	–	17	–	–	–	–	–	2
23	α-terpineol	1680	1172	32	47	4	44	4	7	1
24	citronellol	1755	1213	-	15	–	15	–	–	1
25	nerol	1784	1213	5	**83	–	**200	1	3	1
26	geraniol	1893	1246	8	108	–	134	–	–	1
	total			91	*386	22	*560	26	26	
	rel %			0.8	3.0	0.1	4.1	0.2	0.1	
monoterpene esters										
27	linalyl acetate	1525	1211	2	–	–	4	1	4	2
28	citronellyl acetate	1655	1336	–	–	–	9	–	–	1
29	neryl acetate	1719	1344	–	*135	–	*237	–	–	1
30	geranyl acetate	1746	1363	–	192	–	209	–	–	1
	total			2	*327	0	*459	1	4	
	rel %			0.0	2.6	0.0	3.3	0.0	0.0	
sesquiterpene hydrocarbons										
31	α-cubebene	1449	1332	–	–	17	–	11	23	1
32	δ-elemene	1460	1320	–	–	**46	–	52	**72	2
33	α-ylangene	1472	1346	–	–	*37	–	53	*70	2
34	α-copaene	1481	1349	–	–	*19	–	32	*54	1
35	β-bourbonene	1504	1362	–	–	182	–	165	280	2
36	β-cubebene	1522	1374	–	–	36	–	68	49	2
37	β-elemene	1573	1370	–	–	39	–	42	53	2
38	*trans*-α-bergamotene	1573	1420	–	129	-	106	–	–	2
39	sesquiterpene[i]	1580	–	–	–	92	–	100	123	
40	(*E*)-β-caryophyllene	1581	1396	209	553	91	670	90	93	1
41	3,7-guaiadiene	1594	1414	–	–	239	–	265	295	2
42	sesquiterpene	1605	–	–	–	159	–	187	190	
43	β-guaiene	1622	1483	–	–	33	–	30	46	2
44	α-humulene	1649	1429	16	43	210	49	218	235	1
45	(*E*)-β-farnesene	1661	1443	–	–	212	–	233	255	1
46	germacrene D	1693	1471	–	–	7813	–	7030	8154	2
47	β-selinene	1702	1458	–	–	*16	–	*56	*43	2
48	bicyclogermacrene	1718	1472	12	165	**26	145	23	**41	2
49	sesquiterpene	1724	–	–	–	–	–	–	10	
50	(*E*,*E*)-α-farnesene	1736	1490	–	–	27	–	17	36	2
51	germacrene A	1745	1480	–	–	739	–	778	928	2
52	germacrene C	1754	1493	–	–	377	–	387	450	2
53	δ-selinene	1756	1509	–	–	1131	–	1161	1351	2
54	germacrene B	1806	1528	–	–	2472	–	2480	2856	2
	total			237	890	14013	970	13478	15707	
	rel %			2.0	7.0	80.8	7.0	79.5	79.8	

Table 2 (Continued)

no.	compound	RI DB-Wax	RI DB-1	WLM[a]	EUR[b]	MK[c]	WLM + EUR[d]	WLM + MK[e]	CLM + MK[f]	reliability of identification[g]
sesquiterpene alcohols										
55	δ-cadinol	1955	1555	–	–	490	–	496	574	2
56	(*E*)-nerolidol	2028	1548	–	41	**79	43	105	**145	1
57	sesquiterpenol[j]	2050	1560	–	–	147	–	144	179	
58	elemol	2058	1520	–	–	**64	–	72	**105	2
59	sesquiterpenol	2068	–	–	–	56	–	55	65	
60	sesquiterpenol	2074	–	–	–	84	–	84	98	
61	sesquiterpenol	2125	–	–	–	52	–	49	63	
62	sesquiterpenol	2150	–	–	–	149	–	183	183	
63	sesquiterpenol	2155	–	–	–	744	–	802	780	
64	sesquiterpenol	2159	–	–	–	183	–	141	208	
65	sesquiterpenol	2161	–	–	–	90	–	70	107	
66	sesquiterpenol	2169	–	–	–	32	–	30	32	
67	sesquiterpenol	2176	–	–	–	172	–	178	187	
68	α-eudesmol	2193	1620	–	–	316	–	333	355	2
69	β-eudesmol	2199	1615	–	–	472	–	496	521	2
70	spathulenol	2220	1561	–	–	**86	–	93	**129	2
71	sesquiterpenol	2250	–	–	–	30	–	28	34	
	total			0	41	3246	43	3359	3765	
	rel %			0.0	0.3	18.7	0.3	19.8	19.1	
aliphatic aldehydes										
72	nonanal	1380	1083	–	22	–	20	–	–	1
73	decanal	1485	1184	–	6	–	6	–	–	1
	total			0	28	0	26	0	0	
	rel %			0.0	0.2	0.0	0.2	0.0	0.0	
mandarin esters										
74	methyl *N*-methylanthranilate	2033	1376	8711	–	–	–	–	–	2
75	methyl anthranilate	2188	1332	5	–	–	–	–	–	1
	total			8716	0	0	0	0	0	
	rel %			72.9	0.0	0.0	0.0	0.0	0.0	
others										
76	1,8-cineole	1198	1021	–	40	–	71	–	–	1
77	*trans*-limonene oxide	1439	1121	3	–	–	–	–	–	1
78	*trans*-sabinene hydrate	1456	1050	8	15	–	16	–	–	2
79	thymyl methyl ether	1577	1217	3	–	–	–	–	–	2
80	*trans*-caryophyllene oxide	1962	1580	–	39	–	49	–	–	1
81	thymol	2153	1283	10	–	–	–	–	–	1
82	geranic acid	2287	–	–	55	-	80	–	–	2
	total			24	**149	0	**216	0	0	
	rel %			0.2	1.2	0.0	1.6	0.0	0.0	
total hydrocarbons				3118	6230	14069	5924	13563	15877	
	rel %			26.1	49.1	81.2	42.9	80.0	80.7	
total oxygenated compounds				8833	*6470	3268	*7886	3386	3795	
	rel %			73.9	50.9	18.8	57.1	20.0	19.3	

[a] Mandarin. [b] Lemon. [c] Kumquat. [d] (Mandarin + lemon). [e] (Mandarin + kumquat). [f] (Clementine + kumquat). [g] Key for reliability of identification: 1, identified by linear retention index and mass spectrum of reference compounds; 2, tentatively identified by linear retention index and mass spectrum similar to mass libraries. [h] Not detected. [i] MW = 204. [j] MW = 222. Hybrids and their nucleus-giving parents are significantly different for compounds (>10 μg g^{-1}) and classes of compounds (>10 μg g^{-1}) bearing one ($P < 0.01$) or two asterisks ($P < 0.05$).

from mandarin, had a total monoterpene hydrocarbon content not significantly different [4954 μg g^{-1} (35.9%)] from that of its lemon parent. However, when monoterpene hydrocarbons were considered individually, various situations were encountered:

(1) The cybrid leaf contents in α-pinene, β-pinene, and sabinene were significantly lower (−50% on average) than those measured in its lemon parent. Furthermore, the relative proportions of these three compounds were similar in the cybrid and its lemon parent (α-pinene/β-pinene/sabinene, 0.09/1.00/0.22); these proportions compare favorably with those observed in the major reaction products of (−)-β-pinene synthase from *Citrus limon* flavedo (α-pinene/β-pinene/sabinene, 0.05/1.00/0.14) (*19*); this enzyme is nuclear encoded as a preprotein bearing a transit peptide signal and is imported into the chloroplasts, where it is proteolytically processed into its mature functional form. Thus, it appears that the expression of this nuclear encoded chloroplastic enzyme is strongly influenced by the mitochondrial genome of the mandarin embryogenic parent: although not understood, and admittedly the nuclear genome was transmitted without any punctual recombination and/or deletion, this could be a form of nucleo-cytoplasmic interaction. However, one must bear in mind that chloroplastic-mitochondrial interactions may also exist (*20*).

(2) Conversely, the cybrid leaf contents in δ-3-carene and α-terpinolene were significantly higher (×3.7 and 3, respectively) than those measured in its lemon parent. Although no δ-3-carene synthase has been purified yet from *Citrus limon*, it was shown that a δ-3-carene synthase from *Picea abies* (*21*) most closely resembles in its deduced primary structure a terpinolene synthase from *Abies grandis* (*22*). These enzymes employ a very similar cyclization mechanism: δ-3-carene and terpinolene are formed from geranyl diphosphate by, respectively, 1,3-elimination or 1,2-elimination of a proton from the

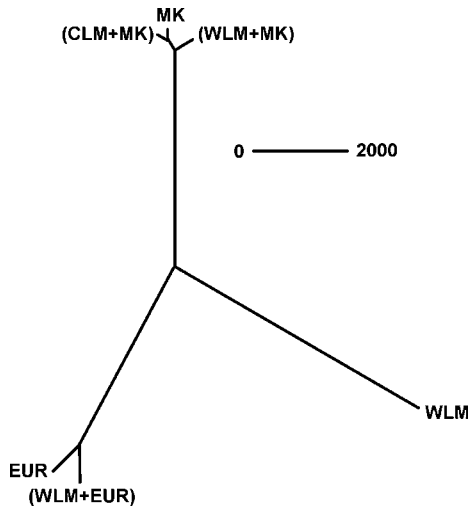

Figure 2. Euclidian distances between hybrids and their parents. Mandarin = WLM; lemon = EUR; kumquat = MK; (mandarin + lemon) cybrid = (WLM + EUR); (mandarin + kumquat) cybrid = (WLM + MK); (clementine + kumquat) cybrid = (CLM + MK).

(4*S*)-α-terpinyl carbocation (*23*). Thus, it appears that the biosynthesis of these two monoterpenes is strongly influenced by the mitochondrial genome of the mandarin embryogenic parent: again, although not understood, this could be a form of nucleo-cytoplasmic interaction.

(3) Other cybrid monoterpenes were not significantly different from those of its lemon parent.

In the (mandarin + kumquat) hybrid, (WLM + MK), that inherited nucleus and chloroplasts from kumquat and mitochondria from mandarin, concentration in total monoterpenes was similar to that of the kumquat parent [85 μg g^{-1} (0.5%)]. Conversely, in the (clementine + kumquat) hybrid, (CLM + MK), with nucleus from kumquat and organelles from mandarin, concentration of total monoterpene hydrocarbons was significantly higher [170 μg g^{-1} (0.9%)] than that of the kumquat parent. This increase was due to a significantly higher amount of (*E*)-β-ocimene: thus, at least for this compound, the presence of the mandarin chloroplastic DNA in this cybrid seems to stimulate monoterpene hydrocarbon synthesis. The fact that biosynthesis of monoterpenes occurs in chloroplasts (*24*) should be related with this observation.

Sesquiterpene Hydrocarbons. Concentration ranges were as follows (**Table 2**): 237 μg g^{-1} (2.0%) for the mandarin, 890 μg g^{-1} (7.0%) for the lemon, and 14013 μg g^{-1} (80.8%) for the kumquat parent. The three hybrids showed concentrations not significantly different from those found in their mesophyll parents. Similarly to monoterpenes, when sesquiterpene hydrocarbons were considered individually, various situations were encountered:

(1) The (clementine + kumquat) cybrid, (CLM + MK), showed leaf contents in δ-elemene, α-ylangene, and α-copaene significantly higher (by ×1.6−2.8) than those measured in its kumquat parent. This is also the case of bicyclogermacrene. It must be noted that α-ylangene and α-copaene are two tricylic diastereomers obtained by cyclization of the cadinyl carbocation; they were observed as minor products of grand fir δ-selinene synthase (*25*). The (mandarin + kumquat) cybrid, (WLM + MK), does not exhibit such differences: because these two above kumquat-derived cybrids differ only by their chloroplasts (**Table 1**), this emphasizes the possible role of mandarin chloroplasts in the stronger synthesis of these compounds.

(2) The two kumquat-derived cybrids were significantly richer in β-selinene than their kumquat parent.

Such an increase was observed for the sesquiterpene (*E*)-β-caryophyllene in the leaves of a (sweet orange + lemon) cybrid with regard to its nucleus-giving lemon parent (*11*). Thus, although it is not possible to link the above effects to an up-regulation of one or several citrus sesquiterpene synthases, we may have here forms of nucleo-cytoplasmic interactions.

Oxygenated Compounds. The amounts of total oxygenated compounds in the parent leaves varied from 3268 μg g^{-1} (18.8%) for the kumquat to 6470 μg g^{-1} (50.9%) for the lemon and to 8833 μg g^{-1} (73.9%) for the mandarin (**Table 2**). Levels in total oxygenated compounds in two of the cybrids, (mandarin + kumquat) [(WLM + MK); 3386 μg g^{-1} (20.0%)] and (clementine + kumquat) [(CLM + MK); 3795 μg g^{-1} (19.3%)], were not significantly different from those found in the kumquat parent. Conversely, oxygenated compounds were significantly overproduced in the (mandarin + lemon) hybrid [(WLM + EUR); 7886 μg g^{-1} (57.1%)] with regard to its lemon parent. This is due to nerol and neryl acetate, which were significantly overproduced by, respectively, ×2.4 and ×1.8 in the (mandarin + lemon) cybrid, (WLM + EUR), with regard to its lemon parent. Conversely, geraniol and geranyl acetate contents were not significantly different from those measured in the lemon.

Similarly to the cases of monoterpene and sesquiterpene hydrocarbons, the (clementine + kumquat) cybrid, (CLM + MK), exhibited increases in three sesquiterpene alcohols, (*E*)-nerolidol, elemol, and spathulenol, compared with its nucleus-giving kumquat parent: once again, it is distinguishable from the (mandarin + kumquat) cybrid, (WLM + MK), which suggests a nucleo-chloroplastic interaction affecting the biosynthesis of these compounds.

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LITERATURE CITED

(1) Grosser, J. W.; Ollitrault, P.; Olivares-Fuster, O. Somatic hybridization in *Citrus*: an effective tool to facilitate variety improvement. *In Vitro Cell. Dev. Biol.-Plant*. **2000**, *36*, 434−449.

(2) Ollitrault, P.; Dambier, D.; Froelicher, Y.; Carreel, F.; d'Hont, A.; Luro, F.; Bruyère, S.; Cabasson, C.; Lofty, S.; Jouma, A.; Vanel, F.; Maddi, F.; Treanton, K.; Grisoni, M. Apport de l'hybridation somatique pour l'exploitation des ressources génétiques des agrumes. *Cahiers Agric.* **2000**, *9*, 223−236.

(3) Grosser, J. W.; Gmitter, F. G., Jr.; Castle, W. S.; Chandler, J. L. Production and evaluation of citrus somatic hybrid rootstocks: progress report. *Proc. Fla. State Hortic. Soc.* **1995**, *108*, 140−143.

(4) Grosser, J. W.; Gmitter, F. G., Jr.; Castle, W. S.; Chandler, J. L. Somatic hybridisation: a new approach to citrus rootstock improvement. *Fruits* **1998**, *53*, 331−334.

(5) Guo, W. W.; Cheng, Y. J.; Deng, X. X. Regeneration and molecular characterization of intergeneric somatic hybrids between *Citrus reticulata* and *Poncirus trifoliata*. *Plant Cell. Rep.* **2002**, *20*, 829−834.

(6) Fatta Del Bosco, S.; Palazzolo, E.; Scarano, M. T.; Germanà, M. A.; Tusa, N. Comparison between essential oil yield and constituents of an allotetraploid somatic hybrid of *Citrus* and its parents. *Adv. Hortic. Sci.* **1998**, *12*, 72−77.

(7) Alonzo, G.; Fatta Del Bosco, S.; Palazzolo, E.; Saiano, F.; Tusa, N. Citrus somatic hybrid leaf essential oil. *Flavour Fragrance J.* **2000**, *15*, 258−262.

(8) Gancel, A.-L.; Ollé, D.; Ollitrault, P.; Luro, F.; Brillouet, J.-M. Leaf and peel volatile compounds of an interspecific Citrus somatic hybrid [*Citrus aurantifolia* (Christm.) Swing. + *Citrus paradisi* Macfayden]. *Flavour Fragrance J.* **2002**, *17*, 416−424.
(9) Alonzo, G.; Ramirez, S.; Saiano, F.; Abbate, L.; Ferrante, S.; Scarano, M. T.; Tusa, N. "Redblush" grapefruit + "Avana" mandarin somatic hybrids. Plantlet leaf essential oil study. *J. Essent. Oil-Bearing Plants* **2002**, *5*, 60−70.
(10) Gancel, A.-L.; Ollitrault, P.; Froelicher, Y.; Tomi, F.; Jacquemond, C.; Luro, F.; Brillouet, J.-M. Leaf volatile compounds of seven citrus somatic tetraploid hybrids sharing willow leaf mandarin (*Citrus deliciosa* Ten.) as their common parent. *J. Agric. Food Chem.* **2003**, *51*, 6006−6013.
(11) Alonzo, G.; Fatta Del Bosco, S.; Palazzolo, E.; Saiano, F.; Tusa, N. Citrus cybrid leaf essential oil. *Flavour Fragrance J*. **2000**, *15*, 91−95.
(12) Doyle, J. J.; Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11−15.
(13) Froelicher, Y.; Dambier, D.; Costantino, G.; Lotfy, S.; Didout, C.; Beaumont, V.; Scotto, C.; Brottier, P.; Risterucci, A.-M.; Luro, F.; Ollitrault, P. Isolation and characterisation of 24 microsatellite markers in mandarin, *Citrus reticulata* Blanco. *Mol. Ecol. Notes* **2005**, submitted for publication.
(14) Bryan, G. J.; McNicoll, J.; Ramsay, G.; Meyer, M. C.; De Jong, W. S. Polymorphic simple sequence repeat markers in chloroplast genomes of *Solanaceae* plants. *Theor. Appl. Genet*. **1999**, *99*, 859−867.
(15) Weising, K.; Gardner, R. C. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome* **1999**, *42*, 9−19.
(16) Beidler, J. L.; Hilliard, P. R.; Rill, R. L. Ultrasensitive staining of nucleic acids with silver. *Anal. Biochem.* **1982**, *126*, 374−380.
(17) Cabasson, C. M.; Luro, F.; Ollitrault, P.; Grosser, J. W. Non-random inheritance of mitochondrial genomes in *Citrus* hybrids produced by protoplast fusion. *Plant Cell Rep.* **2001**, *20*, 604−609.
(18) Nicolosi, E.; Deng, Z. N., Gentile, A.; La Malfa, S.; Continella, G.; Tribulato, E. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theor. Appl. Genet.* **2000**, *100*, 1155−1166.
(19) Lücker, J.; El Tamer, M. K.; Schwab, W.; Verstappen, F. W. A.; Van der Plas, L. H. W.; Bouwmeester, H. J.; Verhoeven, H. A. Monoterpene biosynthesis in lemon (*Citrus limon*). cDNA isolation and functional analysis of four monoterpene synthases. *Eur. J. Biochem.* **2002**, *269*, 3160−3171.
(20) Zubko, M. K.; Zubko, E. I.; Ruban, A. V.; Adler, K.; Mock, H.-P.; Misera, S.; Gleba, YuYu; Grimm, B. Extensive developmental and metabolic alterations in cybrids *Nicotiana tabacum* (+*Hyoscyamus niger*) are caused by complex nucleo-cytoplasmic incompatibility. *Plant J.* **2001**, *26*, 1−14.
(21) Fäldt, J.; Martin, D.; Miller, B.; Rawat, S.; Bohlmann, J. Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol. Biol.* **2002**, *51*, 119−133.
(22) Bohlmann, J.; Phillips, M.; Ramachandiran, V.; Katoh, S.; Croteau, R. cDNA cloning, characterization, and functional expression of four new monoterpene synthase members of the *Tpsd* gene family from grand fir (*Abies grandis*). *Arch. Biochem. Biophys.* **1999**, *368*, 232−243.
(23) Hoelscher, D. J.; Williams, D. C.; Wildung, M. R.; Croteau, R. A cDNA clone for 3-carene synthase from *Salvia stenophylla*. *Phytochemistry* **2003**, *62*, 1081−1086.
(24) Laule, O.; Fürholz, A.; Chang, H. S.; Zhu, T.; Wang, X.; Heifetz, P. B.; Gruissem, W.; Lange, B. M. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6866−6871.
(25) Steele, C. L.; Crock, J.; Bohlmann, J.; Croteau, R. Sesquiterpene synthases from grand fir (*Abies grandis*). *J. Biol. Chem.* **1998**, *273*, 2078−2089.

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