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COMPARISON OF VOLATILES AND WAXES IN LEAVES OF GENETICALLY ENGINEERED TOMATOES

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Key Word Index—*Lycopersicon esculentum*; *L. hirsutum*; Solanaceae; tomato; essential oils; leaf waxes; alkanes; somatic hybrids; transgenic plants; *Agrobacterium rhizogenes*.

Abstract—The essential oils and leaf waxes of several putative somatic hybrid plants of *Lycopersicon esculentum* [+] *L. hirsutum* were compared with those of transgenic plants of *L. hirsutum* and non-transgenic plants of *L. esculentum*, and also of wild-type *L. hirsutum*. The latter contained β-myrcene, 3,7-dimethylocta-1,3,6-triene and a high proportion of undecan-2-one whereas the transgenic *L. hirsutum* and the somatic hybrids all contained similar proportions of 2-carene and β-phellandrene, but only traces of alkan-2-ones. *Lycopersicon esculentum* contained a very low proportion of essential oils and no alkan-2-ones. Hentriacontane (n-C_{31}) and 3-methylhentriacontane (*anteiso*-C_{32}) were the predominant components in all the leaf waxes, and a comparison of the ratio between these compounds suggested that the somatic hybrids contained at least some contributions from both the parent species.
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INTRODUCTION

Tomato is a vegetable crop which has been the focus of intensive breeding programmes in order to introduce useful traits, including resistance to pathogens and improved fruit quality [1–3]. Several agronomically useful traits are found in the wild species, *Lycopersicon hirsutum* [2, 4–8]. However, in the genus *Lycopersicon*, the use of such wild germplasms is often limited by sexual incompatibility. Consequently, sexual hybridization of *L. hirsutum* with its cultivated relative, *L. esculentum*, has met with only limited success [9, 10]. One approach to the improvement of tomato has been through the generation of novel somatic hybrids [11–15]. In a previous study at Nottingham, protoplasts of *L. esculentum* have been fused with those of transgenic *L. hirsutum* to generate a spectrum of somatic hybrid plants [16]. It was of interest to determine if, irrespective of chromosomal status and extent of genomic asymmetry, the relationship between the somatic hybrids and their parents could be monitored by comparison of their secondary metabolites.

In early studies, Soost *et al.* [17] reported marked differences between the composition and yield of the essential oils and, in particular, of the alkan-2-ones, from different *Lycopersicon* species. The oil from *L. hirsutum* contained 70% dodecan-2-one, but that from *L. hirsutum* f. *glabratum* contained 68% tridecan-2-one and 26% undecan-2-one, with less than 4% dodecan-2-one. The leaves of *L. esculentum* yielded considerably less oil, which contained 30% tridecan-2-one as the only major ketone and 15.8% limonene. Three other species, *L. peruvianum*, *L. pimpinellifolium* and *L. esculentum* var. *minor*, yielded insufficient oil for analysis. It has been suggested that these differences in secondary metabolites could be related to pest and insect resistances and, as such, could be relevant in breeding programmes. Williams *et al.* [5] reported the presence of a high content of tridecan-2-one in the insect-resistance wild tomato *L. hirsutum* f. *glabratum*, but a lower amount in insect-susceptible plants of *L. esculentum*. They suggested that hybridization of these two species might form the basis of commercially viable tomato accessions with enhanced insect resistance. Subsequently, this proposal was assessed by Fery and Kennedy [18], who found a correlation between tridecan-2-one content, leaf trichome characteristics and insect resistance. In further studies, Kashyap *et al.* [19] examined the tridecan-2-one and undecan-2-one glandular trichome-based resistance to insects of *L. hirsutum* f. *glabratum*.

Urbasch [20] has compared extracts of eight cultivars of *L. esculentum*, which yielded only traces of tridecan-2-one (<0.1% of the extract), with four wild tomato species, *L. peruvianum* and *L. glandulosum*, both of which also yielded only traces of tridecan-2-one (0.2–0.7%), *L. pimpinellifolium*, which had a low proportion (2–3%) and *L. hirsutum*, which yielded a high propor-

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tion of tridecan-2-one (60–70%). In contrast, Lundgren *et al.* [21] found that the major components in an extract of *L. hirsutum* f. *glabratum* from Ecuador were undecan-2-one (32%) and zingiberene (26.8%) (this compound has been subsequently characterized as 7-epizingiberene [22]), but only a trace of tridecan-2-one. Tridecan-2-one (2.7%), but not undecan-2-one, was found in a plant of *L. hirsutum* and Lundgren *et al.* commented that with the wide geographical range of *L. hirsutum*, chemotype differentiation is not unexpected. No ketones were found in *L. esculentum* and an F1 hybrid of *L. esculentum* × *L. hirsutum* f. *glabratum* contained an intermediate amount of tridecan-2-one.

The terpenoids in *Lycopersicon* species have also been compared as a possible aid to plant breeders. Andersson *et al.* [23] collected the vapours around tomato plants on adsorbent tubes and examined the eluted extracts by gas chromatography. They found a common pattern of volatile constituents in 'woundograms' for seven cultivars of *L. esculentum*, the major constituents being β-phellandrene (>45%), α-terpinolene and limonene. In contrast, *L. peruvianum* yielded caryophyllene (>50%), humulene, α-pinene and two unidentified sesquiterpenes. Vapours from a sexual hybrid of *L. peruvianum* and *L. esculentum* closely resembled the *L. esculentum* pattern, although some β-caryophyllene, characteristic of the *L. peruvianum* parent, was present. *Lycopersicon hirsutum* contained a major unidentified sesquiterpene (83%) (which corresponds to 7-epizingiberene [22]), but almost no β-caryophyllene or β-phellandrene. Later, Urbasch [24] examined leaves, petioles and stems of *L. esculentum* and reported the presence of β-phellandrene, hex-2-enal and caryophyllene as the major constituents. Buttery *et al.* [25] identified 33 compounds from *L. esculentum*. The major components (>1 ppm) in the vapours above the plant were β-phellandrene (25 ppm), 2-carene (7 ppm), limonene (4 ppm), caryophyllene (3 ppm), α-phellandrene (1.3 ppm) and eugenol (1 ppm). In a further study, Buttery and Ling [26] reported a series of lipid-derived C_5 to C_8 alkanols, alkanals and alkan-2-ones from the leaves.

From previous studies on the composition of the secondary metabolites of *Lycopersicon* species and their F1 hybrids, it is clearly difficult to determine whether any components are characteristic of individual species, because of the diversity of natural chemotypes within many of the species and the frequent lack of a direct genetic relationship between different plant samples. The present investigation was therefore undertaken in order to examine changes in the volatile secondary metabolites following genetic manipulation of *Lycopersicon* species, where the relationships between the parents and hybrids were clearly delineated. Any changes in the composition profile could then be used to provide evidence of hybridity and/or intragenic complementation. The essential oil constituents of wild-type *L. hirsutum* following its genetic transformation using *Agrobacterium rhizogenes* and of normal *L. esculentum* have been compared with those of a range of somatic hybrids and products of protoplast fusion. In addition, the leaf waxes of these species have also been compared as possible markers for genetically derived traits evident in the somatic hybrid plants.

RESULTS AND DISCUSSION

Generation of somatic hybrid plants

Inoculation of *L. hirsutum* seedlings with the 'super-virulent' *A. rhizogenes* strain R1601 [27] induced 'hairy' roots at the sites of inoculation. The Ri plasmid of this strain carried the neomycin phosphotransferase II gene, conferring resistance to the antibiotic, kanamycin sulphate, on plant cells, in addition to the oncogenic *rol A*, *B*, *C*, and *D* genes. 'Hairy' roots were confirmed as being transgenic by their ability to grow in the presence of kanamycin sulphate at 50 mg l^{-1} and by their biosynthesis of agropine. *Lycopersicon hirsutum* plants regenerated from these transgenic roots also grew in the presence of kanamycin sulphate. Leaf protoplasts from kanamycin-resistant transgenic plants of *L. hirsutum* were fused electrically with leaf protoplasts of *L. esculentum*. Plants regenerated from selected somatic hybrid tissues were designated HSH1–HSH8 following kanamycin selection. A second series of plants, HSH9–HSH18, was recovered following iodoacetamide treatment of *L. hirsutum* protoplasts prior to fusion, combined with kanamycin selection. Regenerated putative somatic hybrids were transferred to the glasshouse and their hybridity was confirmed by morphological, cytogenetic, isoenzyme and molecular (restriction fragment length polymorphism) analyses [16].

Head-space analyses

On abrasion, leaves of transgenic *L. hirsutum* and somatic hybrid plants exhibited odours which were characteristically different from those of leaves from wild-type plants of *L. hirsutum*. Samples of the leaves from non-transgenic plants of *L. esculentum* and *L. hirsutum* and two independently transgenic plants of *L. hirsutum* (LhT1 and LhT2) were compared with the hybrids from the two selection strategies by head-space followed by GC–mass spectrometry. Where possible, mass spectral identifications were confirmed by the injection of authentic samples. For two hybrids, HSH3 and HSH9, samples collected at the same time from different positions or from both new and older leaves on the same plant yielded comparable compositions.

Two distinctive chromatographic patterns were obtained (Table 1) when leaf samples were heated to 60° two days after collection and the head-space gases examined by GC–mass spectrometry. The first pattern, which was only observed from wild-type (non-transgenic) *L. hirsutum*, contained primarily β-myrcene, a dimethyloctatriene (tentatively identified from the mass spectrum as 3,7-dimethylocta-1,3,6-triene) and

Table 1. Relative proportion of volatile components from head-space analysis of leaves of *Lycopersicon* species

Compound	Retention time (min)	Plant samples* (proportion of peak area, %)										
		L.e.	*L.h.*	LhT1	LhT2	HSH1	HSH2	HSH3	HSH4	HSH9	HSH10	HSH16
Unidentified	7.70			6	4							
Unidentified	7.90		4									
Unidentified	8.50			4	2							
β-Myrcene	9.08		26									
2-Carene	9.20	39		34	33	33	37	47	39	37	42	29
β-Phellandrene	9.83	61	2	56	54	68	62	53	61	63	58	71
Unidentified	10.05		2									
3,7-Dimethyl-1,3,6-octatriene	10.25		52		3							
Undecan-2-one	14.30		11									
Tridecan-2-one	16.07		2									

*Plant material: *L.e.*, *L. esculentum*; *L.h.*, wild-type *L. hirsutum*, LhT1 and LhT2 transgenic *L. hirustum*; HSH1-16, somatic hybrids between LhT1 and *L. esculentum*.
GC conditions; see Experimental.
Blank spaces indicate peak not detected or less than 1% of peak area.

Table 2. Relative peak areas of alkanes in leaf waxes of *Lycopersicon* species

Compound	Identification* (*I*)	Retention time (min)	Plant samples* (peak area, %)								
			L.e.	*L.h.*	LhT1	LhT2	HSH1	HSH4	HSH9	HSH10	HSH16
		24.45	1		9	2					
n-C_{27}	2700, MS	26.95		12	12	5	8	1	2	1	
n-C_{29}	2900, MS	28.43		8	10	5	7	2	2	2	4
anteiso-C_{30}	2975	28.95		3	4	4	4				
iso-C_{31}	3065, MS	29.58		10	7	6	9	2	2	2	5
n-C_{31}	3100, MS	29.83	73	34	30	40	32	61	61	64	51
(peak height)			(3.3)	(0.2)	(0.1)	(0.6)	(0.1)	(6.3)	(3.3)	(6.6)	(0.5)
anteiso-C_{32}	3175, MS	30.33	5	25	17	22	33	13	11	13	16
n-C_{32}	3200	30.51	1			2		2	2	2	7
n-C_{33}	3300	31.31	6	5	6	4	5	9	8	9	12

**n*-Alkanes identified by direct comparison; MS, identified by mass spectra; *I*, retention indices measured isothermally; remaining peak could not be positively identified.
†Plant materials as Table 1.
GC conditions: see Experimental.
Blank spaces indicate peak not detected or less than 1% of peak area.

undecan-2-one. In freshly collected leaves, the proportion of the alkan-2-one could be as high as 83%, but decreased fairly rapidly after collection. The presence of a high proportion of an alkan-2-one in wild-type *L. hirsutum* agreed with previous work [18,19]. The second pattern was found from the transgenic *L. hirsutum* plants and the HSH hybrid series, and consisted predominately of 2-carene, β-phellandrene and sometimes traces of 3,7-dimethylocta-1,3,6-triene, but no peaks for the less volatile alkan-2-ones. Clearly the transformation of *L. hirsutum* by *A. rhizogenes* had resulted in a marked change in the metabolic pathways leading to the essential oils. The pattern of the peaks from *L. esculentum* was very similar to this second pattern, but the peak intensities were much lower. The pattern was similar to that reported for *L. esculentum* by Buttery and co-workers [25, 26]. The relatively high yields of the volatile constituents in the somatic hybrids suggested that the genetic traits determining the formation of these compounds was derived from the transgenic *L. hirsutum* parent rather then the *L. esculentum* parent.

Solvent extracts of leaves

Samples of leaves were extracted with hexane in a semi-quantitative study. In this case, the chromatographic examination was continued up to 300°, as the presence of high M_r alkanes had been observed in trial separations. The extract of wild-type *L. hirsutum* plants contained the monoterpenes detected in the head-space analysis, but the predominant component was undecan-2-one (76%) with traces of dodecan-2-one and tridecan-2-one. Unlike the earlier study by Lundgren *et al.* [21], no sesquiterpenes, such as β-caryophyllene or zingiberene (epizingiberene [22]), were detected. The chromatograms of the extracts of the transgenic *L. hirsutum* plants yielded small amounts of undecan-2-one, as well as 2-carene and β-phellandrene, and traces of tridecan-2-one. Transformation of *L. hirsutum* has therefore also suppressed alkan-2-one formation and from the earlier studies it would be anticipated to result in an increased susceptibility to insect attack. The somatic hybrids all contained effectively the same principal constituents as the transgenic *L. hirsutum* plants and, apart from traces of undecan-2-one, closely matched their head-space analysis. In contrast to these samples, the extract of *L. esculentum* yielded virtually no readily volatile constituents, mirroring the low yield from the head-space assay.

In addition, distinctive groups of peaks were detected in the extract of each accession at high oven temperatures, which were attributed to leaf wax alkanes. Earlier studies have suggested that such compounds could be used as chemotaxonomic markers [28, 29]. Consequently, they were considered in detail in the present study. Unlike the essential oil components, the waxes of *L. hirsutum* appeared to be unaffected by the transformation and both the wild-type and transgenic plants showed very similar patterns (Table 2), which differed from those of *L. esculentum* and many of the hybrids. The major constituent in each case was *n*-hentriacontane (*n*-C_{31}), comprising 30–65% of the alkane mixture. The recorded intensity of this peak (Table 2) differed between solutions containing the same ratio of wet weight of the leaves of hexane. This suggested that there was a much lower yield of these leaf waxes from the *L. hirsutum* leaves compared to those from *L. esculentum* leaves. In the latter case, the waxes were the major components in the chromatogram, in agreement with the weak response for the terpenes. There were also significant differences between the yields of waxes from the hybrids.

In each case, there was a significant peak (*ca* 4–11% of the alkanes) for tritriacontane (*n*-C_{33}), smaller C_{29} and C_{27} *n*-alkane peaks and sometimes a C_{32} *n*-alkane peak. Each of the extracts also yielded a peak with a relative area of 12–33% of the total alkanes, which was identified as *anteiso*-dotriacontane (*anteiso*-C_{32}) (3-methyl branched alkane). This compound was identified from its characteristic mass spectrum with a $[M-29]^+$ peak at *m/z* 421 [30]. It was usually accompanied by a small peak attributed to *anteiso*-triacontane (*anteiso*-C_{30}). In an isothermal separation, these peaks had retention indices, respectively, of 3175 and 2975, characteristic of 3-methylalkanes [31]. It was noticeable that the ratio of the *n*-C_{31} peak to the *anteiso*-C_{32} peak was highest in samples with the stronger peak intensities, suggesting that the two alkanes were derived from different characteristic of the species.

A further peak present in many of the extracts was identified as *iso*-hentriacontane (*iso*-C_{31}) (2-methyl-branched alkane) from its characteristic $[M-15]^+$ and $[M-43]^+$ fragments and retention index (3065) [31]. The presence of odd numbered *n*-alkanes (and *iso*-alkanes) in the waxes from these *Lycopersicon* species, dominated by *n*-hentriacontane and even-numbered 3-methyl-alkanes, is characteristic of many plant waxes [28, 29]. Although the waxes of HSH2 and HSH3 were not analysed in detail, the ratios of their peaks were very similar to those of HSH4.

The composition of the odd numbered *n*-alkanes in the leaf waxes was generally similar to those reported earlier by Hirooka *et al.* [32] for *L. esculentum*. They found that the major constituents (%) were C_{27} (2.1), C_{29} (4.8), C_{30} (2.5), C_{31} (62.2), C_{32} (9.8) and C_{33} (16.5). Zygadlo *et al.* [33] reported a broader *n*-alkane composition (%) for *L. esculentum*, including traces of C_{17}, C_{19} and C_{21} (7.9), C_{29} (4.3), C_{31} (26.1), C_{32} (5.8), C_{33} (14.0), C_{34} (3.7), C_{35} (6.0) and C_{36} (3.4). However, although both groups of workers described the presence of a significant proportion of the even-numbered alkanes, only traces of these alkanes were found in the present study. It is possible that, because of the limited resolution of the packed columns that were used in previous studies, the *anteiso*-C_{32}-alkane observed in the current investigation had been misidentified. Branched alkanes have not been reported previously from the leaves of *Lycopersicon* species. They have been detected in other Solanaceous species,

including *Solanum tuberosum* (potato) and *Hyoscyamus niger* (henbane) [34], but the *n*-alkane patterns were more complex in those cases. However, the epicuticular waxes of immature tomato fruit (<12 mm diameter) contained a high proportion (%) of *iso*-C_{31} (24) and C_{29} (11) alkanes with significant amounts of *anteiso*-C_{30} (5) and C_{32} (8) hydrocarbons, but these declined markedly and the proportion of *n*-C_{31} and *n*-C_{29} alkanes increased as the fruit ripened [35].

As it was thought that the ratio of the *anteiso*-C_{32} peak to the *m*-C_{31} peak in the *Lycopersicon* species might be diagnostically useful, a subsequent study was carried out later in the growing season in order to compare the diversity within two of the hybrids. Five samples collected on the same date from different positions on a single plant of the somatic hybrid HSH3 exhibited a variation from 46 to 59% for *n*-C_{31} and 16 to 28% for *anteiso*-C_{32}. The corresponding figures for four examples from HSH9 were from 41 to 53% and from 22 to 32%, respectively. In this case, the range of variation did not include the result on the sample of HSH9 collected earlier in the year (Table 2); however, similar changes in wax composition with the maturity of the plant have been reported previously [36].

The results given in Table 2 were also compared by principal components analysis. The first principal component showed large negative contributions from the C_{31}, C_{32} and C_{33} *n*-alkanes and positive contributions from the branched alkanes and the smaller *n*-alkanes (C_{27}–C_{29}). The main conclusion from this data analysis was that the range of results could be represented by *L. esculentum* at one end and by wild-type and transgenic *L. hirsutum* plants at the other. The HSH1 hybrid was similar to the transgenic *L. hirsutum* (LhT1) parent and both contained a characteristically low proportion of the *n*-alkanes compared with the *anteiso*-alkanes and a low overall yield of the alkanes (Table 2). As the ratio of the *n*- to *anteiso*-alkanes increased, higher yields were obtained and the patterns became more closely related to that of *L. esculentum*. This suggests that the hybrids exhibited inherited traits from both of their parents in different proportions.

A comparison of previous results for leaf essential oils from tomato and allied *Lycopersicon* species with the present study demonstrates a diversity of composition of the essential oils of *L. hirsutum* reflecting different sources and shows the importance of choosing clearly defined parents in somatic hybridization studies. Our study demonstrates that, in species such as *L. hirsutum*, transformation by the oncogenic *rol A*, *B*, *C* and *D* genes of *A. rhizogenes* can have a significant effect on secondary metabolism. Although there was little difference between the somatic hybrids, even when compared by different selection strategies, they all contained components characteristic of the transgenic *L. hirsutum* parent. Comparison between the leaf waxes suggested that both parents may have contributed to the composition of the hybrids. These comparisons clearly provide an insight into the spectrum of changes associated with the genetic manipulation of tomato, whether this be by transformation and/or protoplast fusion. It is now important to couple such observations with detailed analyses of chromosomal events occurring in such plants, including ploidy changes, transgene copy number and the extent of genomic asymmetry and somatic recombination. Chromosome-painting techniques [37] will play a crucial part in achieving these objectives.

EXPERIMENTAL

Plant material and generation of somatic hybrids. Generation of somatic hybrid plants of *L. esculentum* [+] *L. hirsutum* was as described previously [16]. Seeds of *L. hirsutum* (Accession LA1353 obtained from Dr C. M. Rick, Tomato Genetic Stock Centre, University of California, Davis, U.S.A.) were germinated on agar-solidified Murashige and Skoog (MS) [38] based medium lacking growth regulators. Decapitated hypocotyls of 10–15 day-old seedlings were inoculated with 'supervirulent' strain R1601 of *A. rhizogenes* [26]. Transgenic ('hairy') roots which developed at the sites of inoculation were excised from parent plants and cultured on the same medium [39]. Roots were induced to regenerate shoots by transfer to MS-based agar medium containing 2 mg l^{-1} zeatin and 0.1 mg l^{-1} indoleacetic acid (designated MSZ1). Agropine-positive, kanamycin-resistant plants were used as source of leaf protoplasts. Protoplasts were isolated enzymically from leaves of transgenic plants of *L. hirsutum* and from leaves of wild-type plants of *L. esculentum*. In some experiments, protoplasts of *L. hirsutum* were treated with the metabolic inhibitor, iodoacetamide (0.4 mM), prior to electrofusion with leaf protoplasts of *L. esculentum* cv. Roma. Heterokaryon-derived somatic hybrid tissues were selected by their resistance to kanamycin sulphate derived from the *L. hirsutum* parent or by their antibiotic-resistance combined with metabolic complementation from *L. esculentum*. Putative somatic hybrid plants (designated HSH1-18) were regenerated from selected tissues by culture of the latter on MSZ1 medium. Confirmation of somatic hybridity was based on morphological, cytogenic, isoenzyme and molecular analyses [16]. The molecular analyses employed tomato DNA RFLP probes provided by Dr S. Tanksley, Department of Plant Biology and Biometry, Cornell University, Ithaca, NY, U.S.A. Plants HSH1–4 and HSH9, 10 and 16 were chosen for this study.

Gas chromatography. Volatile constituents in leaf samples were sepd using a J and W (Fulton CA) DB-5MS column (15 m × 0.25 mm i.d. and 0.25 μm film thickness). Sepns were carried out in splitless injection mode. Peaks were identified by comparison with the NIST Mass Spectral library and direct comparison with standard samples. Hexane was pesticide grade from Fisons Scientific Apparatus, Loughborough, U.K. Standards of 2-carene, 3-carene, undecan-2-one, tridecan-2-one and hydrocarbons were obtained from various sources.

Head-space analysis. Samples of leaves in sample vials were heated to 60° for 20 min and a 250 μl sample of the head-space vapour was injected using a heated syringe. The column was programmed at 30° for 5 min, followed by an increase at 10° min^{-1} to 150°.

Leaf extracts. Leaf samples were mixed with hexane (2 × fr. wt) and shaken for 20 min A 2 μl sample of the hexane extract was injected on to the GC column, which was held at 50° for 5 min. The column was then programmed at 10° min^{-1} from 50 to 300° and held at 300° for 5 min.

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