Rodríguez-Baixauli, AM.; Peris-Rodrigo, JE.; Redondo, A.; Shimada, T.; Costell, E.; Carbonell, I.; Rojas, C.... (2017). Impact of D-limonene synthase up- or down-regulation on sweet orange
fruit and juice odor perception. Food Chemistry. 217:139-150.
doi:10.1016/j.foodchem.2016.08.076

Title: Impact of D-limonene synthase up- or down-regulation on sweet orange fruit and juice odor perception.

Authors: Ana Rodríguez[a,b], Josep E. Peris[a,b], Ana Redondo[c], Takehiko Shimada,[d] Elvira Costell[e], Inmaculada Carbonell[e], Cristina Rojas[f] and Leandro Peña[a,b,]*.

Affiliations:

[a] Departamento de Biotecnología y Mejora Vegetal de Especies Cultivadas. Instituto de Biología Molecular y Celular de Plantas - Consejo Superior de Investigaciones Científicas (IBMCP-CSIC). Av. Ingeniero Fausto Elio s/n. 46022 Valencia, Spain.

[b] Fundo de Defesa da Citricultura, 14807–040 Vila Melhado, Araraquara, São Paulo, Brazil.

[c] Centro de Protección Vegetal y Biotecnología. Instituto Valenciano de Investigaciones Agrarias (IVIA), carretera Moncada-Náquera Km. 4.5. 46113 Moncada, Valencia, Spain.

[d] National Institute of Fruit Tree Science (NIFTS), National Agriculture and Bio-oriented Research Organization (NARO), Sizuoka, Shizuoka 424-0292, Japan.

[e] Departamento de análisis sensorial. Instituto de Agroquímica y Tecnología de Alimentos - Consejo Superior de Investigaciones Científicas (IATA-CSIC). C/ Catedrático Agustín Escardino Benlloch, 7. 46980 Paterna, Valencia, Spain.

[f] Centro de Tecnología Poscosecha, Instituto Valenciano de Investigaciones Agrarias (IVIA).

*Other e-mail: lpenya@fundecitrus.com.br

Abstract

Citrus fruits are characterized by a complex mixture of volatiles making up their characteristic aromas, being the D-limonene the most abundant one. However, its role on citrus fruit and juice odor is controversial. Transgenic oranges engineered for alterations in the presence or concentration of few related chemical groups enable asking precise questions about their contribution to overall odor, either positive or negative, as perceived by the human nose. Here, either down- or up- regulation of a D-limonene synthase allowed us to infer that a decrease of as much as 51 times in D-limonene and an increase of as much as 3.2 times in linalool in juice were neutral for odor perception while an increase of only 3 times in ethyl esters stimulated the preference of 66% of the judges. The ability to address these questions presents exciting opportunities to understand the basic principles of selection of food.

Keywords

D-limonene, genetically-modified fruits, sensory panel, alcohols, ethyl esters, orange odor perception, OAV, *Citrus sinensis*

1. Introduction

Citrus types are the most economically relevant and extensively grown fruit tree crops in the world and their fruits are an important source of secondary metabolites for nutrition, health, and industrial applications. Moreover, they are one of the most aromatic edible fruits available (Sharon-Asa et al., 2003). Citrus fruit odor results from a complex combination of soluble and volatile compounds, the latter consisting mostly of mono- and sesquiterpenes, which are accumulated in specialized oil glands in the peel (flavedo) and oil bodies in the juice sacs. Among citrus, sweet orange fruits are the most popular ones (Dugo & Di Giacomo, 2002), as they are consumed both fresh and processed into juice. Additionally, orange peels containing abundant fragrant substances are widely used for extracting essential oils which are commercialized for flavoring foods, beverages, perfumes, cosmetics, etc. (Qiao et al., 2008).

The fruit quality attributes are classified into two groups: 1) internal quality attributes, including texture/mouthfeel, seed presence and number, juice percentage, juice color, flavor (governed by the balance between sugar:acid content plus the concentration of volatile compounds); and 2) external quality attributes, related to the appearance and especially important for fruit intended for fresh consumption, such as size, shape, peel color, presence of alterations and defects on the surface (blemishes, puffing,…), etc.; this also includes attributes related to post-harvest shelf life of the fruit, such as antifungal wax treatments, cold storage time and conditions, etc. Quality attributes have strong economical relevance because they are related to consumer perception and ultimately determine marketability, price and use of fruits. They may eventually constrain the success of a citrus industry (Moufida & Marzouk, 2003). Nowadays, many quality attributes are evaluated by subjective methods, but it would be desirable to develop objective standards of human liking.

Although different fruits often share many volatile compounds, each fruit has a distinctive odor that is a function of the proportion of key volatiles and the presence or absence of unique components (Baxter, Easton, Schneebeli, & Whitfield, 2005). It is known that in many cases only a limited number of flavor components contribute to the character of an odor (Heath & Reineccius, 1986). The olfactory sensory system and the food volatiles with which they interact provide the basis for the diversity of odors and flavors selected by men and found in the human diet (Goff & Klee, 2006).

Citrus fruits can be distinguished from other kinds of fruits by a characteristic “citrus-like” odor, but each citrus fruit type differs in cultivars, hybrids and genotypes according to its specific odor attributes. While esters are the most important aroma compounds responsible of the odor in several fruits (Jordán, Goodner, & Shaw, 2002; Jordán, Tandon, Shaw, & Goodner, 2001), the oxygenated terpenes and medium length aldehydes are generally considered the primary volatile compounds contributing to odor in citrus fruits and juices (Ahmed, Dennison, Dougherty, & Shaw, 1978). In general, in citrus, oxygenated compounds comprising alcohols and aldehydes, but also ketones, acids, and esters occur in relatively small amounts, though they are widely responsible for the odor and flavor profiles of fruits. D-limonene is the most abundant volatile component of all commercially grown citrus fruits and together with other monoterpene hydrocarbons makes up about 96% of total volatile compounds (Dugo & Di Giacomo, 2002). However, its role on citrus fruit and juice odor is controversial. There are reports indicating that it is a relatively important contributor (Buettner & Schieberle, 2001; Lin & Rouseff, 2001) but others report a minimal active effect on odor and flavor (Baxter et al., 2005; Plotto, Margaría, Goodner, & Baldwin, 2008). Högnadóttir & Rouseff (2003) suggested that D-limonene might play an odor activity by co-eluting other minor hydrophobic volatiles because it has a low odor threshold (Plotto, Margaría, Goodner, Goodrich, & Baldwin, 2004).

Odors and flavors are major determinants of fruit quality, but these traits are often genetically complex and difficult to score (Galili, Galili, Lewinsohn, & Tadmor, 2002), making them difficult targets for breeding. Natural variation and genetic engineering in flavor-associated odor volatiles have been used to evaluate the chemistry of tomato fruits, creating a predictive model of liking (Tieman et al., 2012). We have modified the volatile profile of sweet orange fruits by either down-regulating or over-expressing a citrus D-limonene synthase gene under the control of the CaMV 35S promoter (Rodríguez et al., 2011a; Rodríguez et al., 2011b). Antisense (AS) down-regulation of D-limonene synthase expression led to reduction in the accumulation of different monoterpene hydrocarbons (up to 100 times less D-limonene in the peel of downregulated fruits) and (likely due to a partial redirection of the pathway) to the accumulation of monoterpenes alcohols, further transformed into aldehydes and ethyl esters, which were only present in low concentrations in empty vector (EV) control fruits (Rodríguez et al., 2011a). AS fruits were found to be more resistant to important diseases caused by bacteria and fungi, such as *Xanthomonas citri* subsp *citri* and *Penicillium digitatum*, respectively, and less attractant to an important citrus pest, the Mediterranean fruit fly *Ceratitis capitata* (Rodríguez et al., 2011a). In D-limonene sense (S) over-expressing fruits, only a slight increase in the amount of D-limonene was found (Rodríguez et al., 2011b). These fruits are a promising tool for generating broad spectrum resistance against the most important pests and pathogens in citrus worldwide, allowing to reduce the use of highly toxic pesticides.

The availability of these transgenic fruits with the same genetic background in two different orange varieties, Navelina and Pineapple, were used here to assess whether the quantitative or qualitative alteration of several terpenoid volatile organic compounds (VOCs) in their fruits contributed positively, negatively or were neutral for fruit and juice odor perception.

2. Material and methods

2.1 Plant materials

Sweet orange transformants used in this work were generated previously in our laboratory (Rodríguez et al., 2011a; Rodríguez et al., 2011b). Briefly, *A. tumefaciens* EHA 105 containing the binary plasmid pBI121FLM with the D-limonene synthase gene from satsuma mandarin (*Citrus unshiu* Mark) in either sense (S) or antisense (AS) orientation under the control of the *Cauliflower mosaic virus* 35S promoter and the nopaline synthase gene (NOS) terminator was used in the different experiments as a vector for the transformation of two sweet orange types: Navelina and Pineapple sweet orange (*C. sinensis* L. Osb.). AS3, AS5 and EV Navelina and AS11, S13 and EV Pineapple transgenic lines were chosen for our experiments based on their efficient and stable either down-regulation (AS) or over-expression (S) of the limonene synthase gene and low transgene loci number. In the case of Navelina we selected two AS lines because we were unable to produce any S line showing phenotype. Ten plants per transgenic line were transferred to orchard conditions in 2008, together with their respective controls (EV; plants transformed with the pBI121FLM plasmid alone). The experimental orchard was located at Villarreal, Spain (latitude 39°56’40.4’’N, longitude 0°08’11.0’’W and elevation of 67 m; typical Mediterranean climate), and was approved by the biosafety regulatory authorities (permit B/ES/08/02). All scions were grafted onto Carrizo citrange rootstock and grown in a loamy clay soil using drip irrigation. The orchard was managed as for normal citrus cultivation in the Mediterranean region.

Navelina orange fruits are seedless and they reach optimum maturity in the second half of December, when the ratio of sugars/acids of the fruits reach more than eight, although they can be harvested from mid-October until the end of January depending on the year. Pineapple orange fruits are seeded and they reach optimum maturity in Spain in the second half of January, when the ratio of sugars/acids of the fruits reach nine, although they can be harvested from second half of December until the end of March depending on the year. For the first season, fruits were harvested on 24th November of 2011 for Navelina sweet orange and on 10th January 2012 for Pineapple sweet orange. For the second season analyzed, fruits were harvested on 17th January of 2013 for Navelina sweet orange and on 28th March 2013 for Pineapple sweet orange.

2.2 Phenology

The phenological cycle of every tree in the orchard was evaluated through weekly observations. The predominant phenological stage of development according to BBCH codifications was recorded and grouped into phases stressing flowering and fruit development stages as described in (Pons, Peris, & Peña, 2012). A visual representation of the phenological cycle of each line was produced by generating phenological calendars (Supplementary Figure S1).

2.3 Analysis of fruit quality

The assessment of fruit quality for the sweet orange lines was performed for the same 2 seasons in which the sensory analyses were performed. 30 fully mature fruits per tree (grouping in bags of 5 fruits each) were harvested and immediately processed. The following fruit quality parameters were measured and averaged for each sample: total soluble solids (TSS), titratable acidity (TA) and maturity index (MI). The juice with pulp was extracted from the fruit using a rotary citrus squeezer (the same used for sensorial evaluation; Lomi model 4) and, immediately, the TSS was determined in terms of Brix degrees using a refractometer (Atago PR-101 model 0-45 %, Tokyo, Japan). TA of the juice was determined by titration with 0.1 mol L^{-1} NaOH and expressed as the percentage of anhydrous citric acid by weight, using phenolphthalein as a visual endpoint indicator, according to AOAC methods (AOAC. 1980. Official Methods of Analysis, 13th ed. N°46024 and N° 22061. Association of Official Analytical Chemists, Washington. DC). MI was estimated as the TSS/TA ratio.

2.4 Extraction of Volatiles and Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Flavedo and juice with pulp tissue was obtained from orange fruits, immediately frozen in liquid nitrogen, and stored at -80 ºC until extraction.

The extraction of flavedo volatiles was performed as reported before (Rodríguez et al., 2011a). A Thermo Trace GC Ultra coupled to a Thermo DSQ mass spectrometer with electron ionization mode at 70 eV was used. Frozen ground material (200 mg) was weighed in screw-cap Pyrex tubes and then immediately 3 mL of cold pentane and 25 μg of 2-octanol (Fluka; internal standard) were added. Samples were homogenized on ice for 30 s with a Yellowline homogenizer (model DI 25). The suspension was vortexed for 15 s, and 3 mL of MilliQ water were added. The sample was further vortexed for 30 s and centrifuged at 1,800g for 10 min at 4 ºC. The organic phase was recovered with a Pasteur pipette, and the aqueous phase re-extracted two more times with 3mL of pentane. A 2-μL aliquot of the pooled organic phases was directly injected into the gas chromatograph-mass spectrometer (GC-MS) for volatile analysis; at least two extractions for each sample were performed.

The volatile compounds of juice with pulp were extracted by headspace solid-phase microextraction (HS-SPME) and analyzed by GC-MS. A 100 μm fiber coated with polydimethylsiloxane (PDMS, Supelco, USA) was used. The fiber was conditioned in the GC injector as indicated by the manufacturer prior to use. 1.5 g of the ground juice with pulp sample was placed in a 7 mL headspace vial containing a stirring bar and sodium chloride (0.45 g) and capped with a 13 mm diameter PFTE/silicone septum. 10 μg of 2-octanol was added as internal standard. The sample was then equilibrated at 37 °C for 10 min under stirring (500 rpm). Afterwards, the vial was incubated with the fiber at 40 ºC for 30 min without stirring. After sampling the headspace volatiles, the fiber was retracted into its sheath and then immediately transferred to the injector port of the GC–MS at 220 ºC and 4 min. Each analytical sample was measured in triplicate. The ion source and the transfer line were set to 200 ºC and 260 ºC, respectively. Volatile compounds were separated on a HP-INNOWax (Agilent J&C Columns) column (30 m x 0,25 mm x 0,25 μm) coupled to a Termo DSQ mass spectrometer. The column temperatures were programmed as follows: 40 ºC for 5 min, raised to 150 ºC at 5 ºCmin-1, then raised to 250 ºC at 20 ºCmin-1 and held for 2 min at 250 ºC. The injector temperature was 220 ºC. Helium was the carrier gas at 1.5 mLmin-1 in the splitless mode. Electron impact mass spectra were recorded in the 30 to 400 amu range with a scanning speed of 0.5 scans-1. Compounds in both pentane or HS-SPME extractions were identified by matching the acquired mass spectra with those stored in the reference libraries (Wiley6, MAINLIB, REPLIB and National Institute of Standards and Technology) and/or by comparison with authentic standard compounds when available. Data were analyzed by integrating the peak areas of total ion chromatograms using Xcalibur 1.4.z software and quantified by using calibrating curves previously obtained in the laboratory of authentic chemical compounds. The recovery rate of each extraction was calculated with the internal standard (2-octanol) to assure the uniformity of the procedure. The amount of every compound in each sample was calculated as its corrected peak area (by weight and volume) divided by its response factor and recovery rate of the internal standard. The results are reported as the mean values of peak area percent ± SE or in ng/g ± SE from the total identified volatiles in each case.

Published odor thresholds in an orange juice matrix (Plotto et al., 2004, 2008) were used to determine the contribution of the identified compounds to the orange juice aroma by calculating their odour activity values (OAVs). Thus, the interaction between the orange juice matrix and the volatile compound is considered. The OAV is the ratio between a compound concentration and its odor threshold. An OAV higher than 1 is assumed to contribute to that juice aroma.

2.5 Preparation of samples for sensory evaluation

Navelina and Pineapple sweet oranges were harvested in the morning of the day of the odor testing and immediately selected for uniformity in size and absence of defects. Navelina is consumed as fresh fruit while Pineapple is used for juice processing.

Fresh fruits. Right after harvesting, Navelina oranges were cut transversely and each half was immediately placed/faced down in a white dish that was completely tasteless and odorless and presented to the panelists at a uniform room temperature.

Fresh juice with pulp. In each analysis, at least 200 fruits were harvested in the morning of the day of the odor testing and groups of 20 oranges each were taken for every juice evaluation session. The juice from each group was extracted using a rotary citrus squeezer with a strainer (Lomi model 4) and immediately pour (including the pulp that passed through filters) into 15 mL-aliquots in a 40 mL-flask with cup and served at a uniform room temperature.

Each sample was identified by a random 3-digit number, different for every assay and the order in which the sample appeared for each level was also random and balanced among subjects.

2.6 Sensorial evaluation

Each panel consisted of volunteers (n=54–70, males and females, age range 20-65 years old) from two Research Institutes: Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Spain) and Instituto de Agroquímica y Tecnología de Alimentos (IATA, Paterna, Spain) being all of them frequent citrus fruit and juice consumers. Most panelists participated in all tests, and have performed the same task for the two seasons analyzed. Panels took place in individual booths under white light at room temperature (ISO 8595:2007), usually from 10:00 a.m. to 14:00 p.m. Samples were prepared within 1 h prior to evaluation. Panelists were able to make comments after the evaluation session.

For cut fruit (flavedo and pulp with juice) odor evaluation, a paired comparison was performed (ISO 5495:2005). Panelists were presented with two halves of unpeeled fresh Navelina oranges, one of them being the EV control line (AS3 or AS5 vs. EV halves). They were asked to choose which of the samples they preferred or whether they were able to differentiate between them. In another test, they were asked to choose which sample between both was more intense. Panelists were first instructed to peel a piece of flavedo of each sample, smell both of them and answer the question. After that, they were instructed to smell the juice with pulp and answer the question. If they could not perceive a difference, they were instructed to guess (forced choice).

For juice with pulp odor evaluation, a ranking test was performed (ISO 4121:2003). Panelists were presented with 3 flasks, corresponding to juice from the three transgenic lines tested of each variety (AS3, AS5 and EV for Navelina or AS11, S13 and EV for Pineapple juice comparison). Panelists were first instructed to uncap the flaks in the appropriate order near their nose and smell. Orange juice odor was scored on a 9-point hedonic category scale varying from 1 (extremely dislike) to 9 (extremely like). For the Friedman tests, the acceptability scores (1 to 9) given by each consumer were converted into rank order numbers (1,2,3 = low quality; 4,5,6 = acceptable quality and 7,8,9 = high quality).

2.7 Statistical analysis

For the analysis of the parameters of fruit quality, the variables were checked for normality, and those that deviated were transformed appropriately. Means were compared by the least significance difference (LSD) test. The statistical analyses were all performed using the software package Statgraphics v.5.1 software (Manugistics Inc.) and a significance level (α) of 0.01 was taken into consideration to protect against Type I errors.

For the analysis of data obtained in the paired comparison test of sensory panels, tables based on binomial distribution were used, in which the minimum number of correct judgments to establish significance at various probability levels are given (Roessler, Pangborn, Sidel, & Stone, 1978). Discrimination tests (paired comparisons) and hedonic ranking score were analyzed using Fizz Calculations software (Biosystemes, France). A Friedman test was also applied to data obtained from ranking tests (sensory evaluation of juice). In this case the acceptability scores (1 to 9) given by each panelist to the evaluated samples were converted into rank order numbers.

Juice with pulp volatile emission data were compared among lines and together with sensorial evaluations served to establish correlations between chemistry and liking. Flavedo volatile content was tested just for Navelina fruits, as the panelists were taught to cut transversally the flavedo of oranges from this variety, disrupting oil glands and thus releasing the oils directly to the nose.

3. Results

3.1 Phenological calendars and fruit quality attributes were comparable in transformants showing either suppressed or enhanced accumulation of D-limonene and empty vector controls

Making use of comparative analyses of phenology conducted over two years, we evaluated the equivalence of field-grown D-limonene synthase up- or down-regulated transgenic sweet orange trees relative to their EV controls in terms of plant growth and fruit development. The comparison between AS3, AS5 and EV Navelina and AS11, S13 and EV Pineapple transgenic lines showed that the expression of D-limonene transgenes did not cause any alteration of the main phenotypic and agronomic plant and fruit characteristics (Supplementary Figure S1). Therefore, the modification of D-limonene accumulation in fruit tissues *per se* did not affect the morphological appearance or phenological cycle of the trees.

During ripening there is a decline in titratable acidity of fruits (TA) mostly due to catabolism of citric acid in citrus juice and an increase in sugars, usually expressed as total soluble solids (TSS). The typical taste and aroma of citrus fruits is determined, besides the accumulation of volatile compounds, by the maturity index (MI) that is the TSS/TA ratio. To assess whether the modification of D-limonene accumulation affected the quality of the transgenic fruits, TSS, TA and MI were evaluated in fruit samples from the orchard-grown transgenic trees of the two varieties in two different harvest seasons. We found no significant differences for any of the parameters analyzed with $P<0.01$ in Navelina fruits (Table 1A). For Pineapple, we only found a significant difference in TSS between AS11 and EV, but not influencing the final MI (Table 1B). Small differences in TSS and MI values between the first and second season for both cultivars are explained by the fact that fruits were harvested at the beginning and the end of the season, respectively, for both varieties. In this way, we could infer that specific differences in VOC profiles for a given season were mostly attributable to the influence of environmental conditions on fruit development and maturation (within a range of standard commercial MIs for fruit harvesting) and that common differences in both seasons were attributable to the genetic modification performed. We had previously shown that morphological and biochemical characteristics of the orange fruit flavedo were not altered in transformants showing constitutive either up- or down-regulation of the D-limonene synthase gene (Rodríguez et al., 2014, 2015).

Chlorophyll and total carotenoid contents in EV control green and mature flavedo from Navelina and Pineapple oranges were similar to those found in AS lines (Rodríguez et al., 2014).

3.2 Different and distinctive VOC profiles were found in fruits from D-limonene synthase antisense and sense vs. empty vector control transformants

As a whole in Navelina, EV fruits contained and emitted much more total VOCs than AS fruits (Supplementary Figure S2). For Pineapple juice with pulp, there were quantitative differences between the first and second years for VOC emission in the three transgenic lines, but S13 and EV emitted comparable amounts of total VOCs while AS11 always emitted much less VOCs than S13 and EV for a same year (Supplementary Figure S3).

For both sweet orange juice with pulp types, the most conspicuous difference between AS and EV samples was the 2.6 to over 51-fold decrease in emission of D-limonene and the very much reduction in the emission of related monoterpene hydrocarbons including α- and β-myrcene and α-pinene to levels which made some of them undetectable for specific transgenic lines/seasons (Tables 2 and 3). D-limonene synthase down-regulation led to partially blocked accumulation of D-limonene, which caused a diversion of the pathway leading to the about two- to more than three-fold enhanced emission of linalool and additionally, in some samples, related monoterpene alcohols such as β-citronellol and nerol (Tables 2 and 3; Supplementary Tables S1 and S2). As a consequence of this, monoterpene and aliphatic aldehyde emission levels were also generally altered, particularly for both (*Z*)- and (*E*)-citral forms together with hexanal, octanal, nonanal and decanal, especially in the second season evaluated for both sweet orange varieties. Derived from aldehydes, esters and their levels were also modified slightly in some samples. Somehow unrelated sesquiterpene hydrocarbons as valencene, and other terpenes as β-ciclocitral and nootkatone showed significantly lower concentrations in AS than EV samples (Tables 2 and 3, see Additional Data in brief).

D-limonene synthase over-expression in Pineapple S13 juice caused the opposite phenotype at least for major terpene compounds. However, differences were not significant or were only significant for linalool (almost three-fold decreased) and some aldehydes (generally decreased) during the second season when compared with EV juices. Importantly, S13 juice emitted 2 times more ethyl hexanoate than EV juice in the second season (ethyl hexanoate was not found in EV juice in the first season), 3 times more ethyl octanoate in both seasons, and 9 and 4.4 times more ethyl 3-hydroxyhexanoate in the first and second seasons, respectively, than EV juice (Table 3; Supplementary Tables S2). Therefore, AS juice was characterized by the higher influence of the oxygen fraction and S juice emitted less linalool but much more esters than AS and EV juices (Tables 2 and 3; see Additional Data in brief).

Regarding Navelina sweet orange peel, AS samples generally showed a strong decrease in the accumulation of D-limonene and β-myrcene, enhanced levels of linalool and other alcohols (nerol, geraniol and β-citronellol) but reduced concentrations of α-terpineol, and reduced levels of aldehydes, both monoterpene (citral) and aliphatic (octanal, nonanal and decanal) ones when compared with EV controls, resembling major differences found in AS vs. EV juices with pulp. However, valencene and β-ciclocitral were only detected in both AS peels and not in EV samples the second season evaluated (Table 4; Supplementary Table S3; see Additional Data in brief).

To assess whether these distinctive VOC profiles could lead to different odor activity values (OAV) for the citrus juices and peel, we evaluated which of these compounds were present in concentrations higher than their threshold value (Tables 2, 3 and 4). In Navelina sweet orange juice, the monoterpene hydrocarbons D-limonene and β-myrcene contributed to odor perception only in the case of EV control fruits, while reaching values much lower than 1 in AS juices. The alcohol linalool was the only compound important in juice odor for all the three AS3, AS5 and EV juices for both seasons analyzed, showing higher OAV usually in AS juices. Additionally, ethyl hexanoate contributed to odor of only AS5 juice the first season and the aliphatic aldehydes octanal, nonanal and decanal had an impact on odor of EV juices just the second season (Table 2).

In Pineapple sweet orange juices, D-limonene contributed to the odor perception of all the three juices types, but OAVs were much lower in AS11 and slightly higher in S13, compared to EV (Table 3). The other major monoterpene hydrocarbon β-myrcene (plus α-pinene the second season) as well as the ethyl esters ethyl butyrate and ethyl hexanoate (just the second season) were affecting odor perception of S13 and EV, but not AS11 juices. Moreover, OAVs of ethyl esters were much higher in S13 than in EV juices and ethyl hexanoate contributed to the odor of only S13 the first season. As in Navelina juices, linalool was the most influential alcohol for AS odor juice perception, especially the first season in which it was contributing to global OAV of only AS11 juice. Moreover, the second season, one of the aliphatic aldehydes, either nonanal or decanal, had an impact on the OAV of AS11 and S13 juices, while both compounds enriched the OAV of EV controls. Additionally, valencene had a positive OAV in S13 and EV but not AS11 juices the second season (Table 3).

In the case of Navelina sweet orange flavedo, almost all the compounds mentioned before and represented in Table 4 had a positive influence on global OAV, but values were generally much reduced in AS compared to EV fruits, in such a way for minor compounds that α-terpineol (both seasons) and (*E*)-citral (the second season) enriched the global OAV of only EV samples. However, the second season, valencene and β-ciclocitral contributed to global OAV of AS but not EV fruits (Table 4).

The odor thresholds in an orange juice matrix are higher than those obtained in water, but some VOCs showing highly divergent concentrations in AS vs. EV transgenic juices did not show positive OAVs (Tables 2, 3, 4; Supplementary Tables S1, S2 and S3; Data in brief). The possible contribution of VOCs such as the alcohols nerol, β-citronellol or geraniol to odor and flavor perception in AS fruits and juices remains to be further investigated.

3.3 Sensory panelists made fruit and juice with pulp choices correlated with the lack or presence and abundance of certain specific volatile compounds

We next attempted to correlate the different VOC and OAV profiles with sensory responses of citrus cut fruit and juice with pulp of the panelists to generate an estimate of the overall impact of specific VOCs or VOC groups on odor perception. Half-cut fruits or orange juices with pulp were offered to panels from two different research centers consisting of 54-70 volunteers, who were used to consume and evaluate citrus fruits and juices.

In spite of the great differences found in the accumulation of total VOCs and OAVs (mainly D-limonene) in Navelina AS compared to EV fruits (Tables 2 and 4, and Supplementary Figure S2 and Data in brief), the members of both panels did not perceive any significant difference in the odor intensity of flavedo or juice with pulp between AS3 and EV fruits in any of the two seasons analyzed at $P<0.01$ (Figure 1). They significantly distinguished the odor of the EV cut fruits from that of AS5 ones in the first season but odor choices were comparable between these two lines for the second season (Figure 1). As there were not differences in the total OAVs of AS3 and AS5 vs. EV samples, and the only conspicuous difference in the VOC profile of AS5 peel between the first and second years was a higher accumulation of β-citronellol, nerol and geraniol the first year and this difference was additionally observed when compared to AS3 peels, these compounds may explain panelists' perceptions. Alternatively, much higher OAV for linalool in AS5 vs. EV together with the contribution of ethyl hexanoate to the global OAV of AS5 (and not AS3 and EV) juice with pulp may have also influenced panelists' discriminations. Panelists also found a higher intensity of the juice with pulp odor of AS5 vs. EV fruits in the second season and were able to differentiate between them (Figure 1G and 1H). That season, AS5 juice with pulp emission was characterized by a higher contribution of linalool to total OAV when compared to AS3 one. Additionally, D-limonene and β-myrcene were lacking in the global OAV of AS5 when compared to that EV juices and the opposite occurred for aliphatic aldehydes (Table 2), which as a whole may explain consumers' discrimination of both juices.

However, all AS3, AS5 and EV fruits were considered to have an "acceptable quality" in a 9-point hedonic evaluation of the juice with pulp odor (results not shown). Some panel members noticed a similarity between AS fruits peel odor and lemon-like or sour orange-like odor, likely related to the increased accumulation of linalool in peel and juice with pulp of AS fruits. Most panelists described the odors associated with AS fruits as with rose or geranium-like notes, in accordance with their VOC composition (Supplementary Tables S1 and S3). Overall, the sweet aroma derived from linalool (and perhaps other alcohols as nerol, β-citronellol or geraniol) would not contribute in AS fruits to any "off-odor" when accumulated and emitted at levels similar to those found in the AS lines.

For Pineapple orange juices with pulp, panelists distinguished S13 smell from that of EV for the first season and found S13 more intense than EV odor for the second season (Figure 2A-D). In addition, using hedonic ratings, sensory panels judged S13 juice to have the highest hedonic score of the three transgenic juices evaluated, with significant differences over AS11 and EV control juices in both seasons (Figure 2E-H). Some panelists reported a "special" smell in S13 fruits compared to EV and AS ones. In spite of showing much lower peak areas in the chromatograms than other VOCs, the relative increase of key ethyl hexanoate and ethyl butyrate esters and their qualitative (1^{st} season) and qualitative (2^{nd} season) contribution to total

OAVs in S13 compared to EV juice probably impacted on the organoleptic attributes of this juice, explaining its hedonic evaluation, mostly in the first season when ethyl hexanoate enriched global OAV of only S13 juice.

On the other hand, panelists did not find statistically significant differences at $P<0.01$ between AS11 and EV control juices and their hedonic ratings were also comparable (Figure 2), even when AS11 juice showed a much reduced OAV for D-limonene and lacked β-myrcene (and α-pinene the second year) when compared with OAVs of S13 and EV juices. As in the case of Navelina sweet orange AS juices, AS11 emitted much more linalool than EV juice, making both qualitative (1st season) and quantitative (2nd season) contributions to its global OAV. The higher production of linalool (and other alcohols; see Supplementary Table S2 and Data in brief) did not affect negatively to panelist scores in this case.

4. Discussion

In the context of plant genetics, breeding for quality means improving traits such as flavor, nutrition, appearance and postharvest processing (Klee, 2010). In citrus fruits, genetic engineering have been already used to achieve resistance to an important postharvest disease as the green mold rot caused by *Penicillium digitatum*, fruit resistance to citrus canker caused by the bacterium *Xanthomonas citri subsp. citri* and less attraction to the Medfly pest *Ceratitis capitata* (Rodríguez et al., 2011a), and to increase β-carotene content of the juice, thus enhancing its antioxidant properties *in vivo* (Pons et al., 2014). The potential for plant metabolic engineering to increase the accumulation and emission of specific fruit odor compounds could allow transferring such desirable quality traits into mature tissues of elite genotypes. However, before that, it is essential uncovering chemical groups of compounds that may be discriminated by our olfactory sensory system from complex mixtures and either improve or decrease the quality of a blend. In tomato, fruit-specific geraniol synthase over-expression led to a highly increased accumulation of monoterpene alcohols, aldehydes, esters and oxides as well as hydrocarbons as expense of reduced lycopene, but these fruits were preferred over control counterparts by panelists (Davidovich-Rikanati et al., 2007). In another work, transgenic tomato plants were modified to no longer express a 13-lipoxygenase gene (*LoxC*) whose product catalyzes the first step in the metabolic pathway that converts 18:2 and 18:3 fatty acids to C6 volatiles such as cis-3-hexenal, hexanal, cis-3-hexen-1-ol, hexyl alcohol and hexyl acetate. Consumers were able to distinguish the transgenic (unable to produce C6 volatiles) from control fruits but it did not affect their preferences (Tieman et al., 2012).

D-limonene synthase up- or down-regulated orange fruits offer an unprecedented tool to study the influence of D-limonene and related terpene compounds (mainly qualitatively but also quantitatively altered) in whole cut fruit and juice quality as perceived by odor panelists. D-limonene is the most abundant terpene compound in sweet orange as well as in most citrus fruits (Dugo & Di Giacomo, 2002). In AS fruits, its concentration was reduced at least 90 times in the peel, reaching very low OAVs, and 6 times in the juices, thus lacking OAV, when compared to EV controls. However, panelists did not differentiate and neither find significant differences in intensity between both AS and EV transgenic types and in both orange cultivars, Navelina and Pineapple. In spite of its high accumulation, the role that D-limonene plays in orange fruit and juice odor is not clear. It was rated as a prominent contributor of citrus juice aromas (Selli & Kelebek, 2011), a barely aroma active compound (Perez-Cacho & Rouseff, 2008), a mid-potency VOC (Choi, 2005) and a negative contributor to citrus juice aromas (Tietel, Plotto, Fallik, Lewinsohn, & Porat, 2011). In flavor modeling studies, D-limonene was considered to be important to mimic orange juice odor (Ahmed et al., 1978; Buettner & Schieberle, 2001). Our results indicate that D-limonene contributed little to sweet orange odor but we cannot discard the idea that it is acting in the complex VOC mixture through additive or synergistic effect with other orange odor components, serving as a solvent for the other compounds (Perez-Cacho & Rouseff, 2008).

Apart from drastically reduced D-limonene concentrations, AS juices showed higher accumulation of monoterpene alcohols, mainly linalool, which strongly contributed both quantitatively and qualitatively to their total OAVs. Other alcohols as nerol, β-citronellol and geraniol also showed increased concentrations in AS vs. EV juices thought none of them reached OAVs above 1. However, floral notes generally provided by them were perceived by most panelists. Although their accumulation levels varied between transgenic lines and seasons (but not much between varieties), some of these alcohols reached concentrations typically found in certain sour orange, lemon and lime genotypes and such distinctive blend was also noticed by panelists. It is possible that having a much reduced amount of D-limonene as a solvent in AS juices would increase the volatility of these compounds thus influencing their perception. Nevertheless, typical AS odor had not influence on panelist differentiations, odor intensities and hedonic scores, considering that they were chosen or classified at comparable rates to EV control fruits and juices for both Navelina and Pineapple varieties. However, in the specific case of Navelina AS5 samples panelists perceived them as different, less intense than EV ones in the first season for the cut fruit and in the second season for the juice. In the first case, it coincided with the important contribution of linalool together with ethyl hexanoate to the global OAV of AS5 (and not AS3) juice with pulp as well as with the lack of OAV for D-limonene and other monoterpene hydrocarbons. However, panelists did not find the odor of AS5 whole cut fruit or juice unpleasant, but different, being considered by some panelists as oranges smelling like lemons or limes. Considering that TSS and TA of AS5 fruit was characteristic of mature oranges and comparable to those of EV and AS3 fruits, it worth testing how panelists would feel the taste and aroma of AS5 fruit and its juice compared to EV counterparts.

It is widely considered that the alcohol linalool has a substantial contribution to orange fresh fruit and juice flavor (Ahmed et al., 1978; Bazemore, Rouseff, & Naim, 2003), being pondered as one of the three most prominent constituents of good quality peel oil and orange juice (Macleod, Macleod, & Subramanian, 1988). It also characterizes the floral odor of fresh and processed mandarins and the peel oil of clementines (Buettner, Mestres, Fischer, Guasch, & Schieberle, 2003; Schieberle, Mestres, & Buettner, 2003) and contributes to the refreshing floral aroma of orange peel and juice (Macleod et al., 1988; Qiao et al., 2008). Other terpene alcohols such as β-citronellol and geraniol have also been found to add fruity aromas to the essence oils of oranges (Högnadóttir & Rouseff, 2003). Therefore, it could be expectable that the relative increase in the concentration of these alcohols, especially linalool, in orange fruits may lead to generation of new varieties with more pleasant odor and aroma, similar to those of lemons, limes or bergamots. Our results seem to contradict in part these expectations, although in our transgenic fruits linalool increases were generally correlated to D-limonene strong decreases and vice versa. It is possible that a better compensated concentration of both compounds may generate more pleasant fruits.

S13 juice was characterized by the increased OAVs for ethyl hexanoate and ethyl octanoate esters together with slightly enhanced levels of D-limonene and other related monoterpene hydrocarbons. It was preferred by panelists and had significantly higher hedonic ratings than AS11 or EV ones. Ethyl esters, including branched chain esters, have been generally described as ‘sweet’ or ‘fruity’ at concentrations above their odor thresholds (Plotto et al., 2008). Ethyl hexanoate was perceived as ‘fruity’ at low concentrations (Plotto et al., 2008). Evaluations of odor active compounds in orange juices showed that the main odor contributors to the fresh, fruity note odor quality of freshly hand squeezed orange juices were mainly esters together with aldehydes (Buettner & Schieberle, 2001). It was also found that ethyl hexanoate as well as ethyl butyrate presence had a significant positive correlation with hedonic flavor scores (Miyazaki, Plotto, Goodner, & Gmitter Jr, 2011; Obenland et al., 2009) and both esters have been identified as contributors to fresh orange flavor (Ahmed et al., 1978; Buettner & Schieberle, 2001). The presence or light (but significant) increases in the OAVs of these esters in S13 juice were likely responsible of their preference and higher hedonic ratings compared to AS11 or EV samples. It is generally accepted that orange odor and aroma are the result of a collection of active VOCs present at low concentrations (Bazemore, Goodner, & Rouseff, 1999) and that their sensory relevance is due to considerably lower odor thresholds (Grosch, 2001). Our results generally agree with this view because esters in S13 samples were present and emitted at much lower concentrations than for example D-limonene and other terpene hydrocarbons, but certainly they were the most representative compounds in S samples most likely determining the fresh citrusy of these juices.

We have previously shown that antisense down-regulation of D-limonene synthase in the sweet orange peel induced a drastic decrease in the accumulation of D-limonene plus related monoterpene hydrocarbons while concentrations of other terpene compounds including monoterpene alcohols, aldehydes and esters were also altered (Rodríguez et al., 2011a). This led to constitutive activation of plant natural defenses and consequently to resistance to diverse fungal and bacterial pathogens as well as less attraction to an important citrus pest (Rodríguez et al., 2011a; 2014). Here, we have been interested in investigating whether differences in the accumulation and emission of terpene compounds by these genetically modified sweet orange fruits would affect negatively odor perception by potential consumers, thus precluding further development of this promising biotechnological product. Moreover, the availability of AS fruits and juices with null OAVs for D-limonene and related monoterpene hydrocarbons as well as much higher OAVs for linalool, S fruits and juices with much higher OAVs for esters, and their isogenic counterparts with regular concentrations and OAVs for these compounds, allowed us to study the role of specific VOCs or VOC groups in the odor of orange fruit and juice. We show here that the lack of D-limonene and monoterpene hydrocarbons in the global OAV of sweet orange juices was neutral for intensity and panelists did not perceive them as different to regular controls. Conversely, in spite of the important role widely attributed to linalool as well as other oxygenated terpenes as positive contributors to orange odor, in our case, the unbalance of not only linalool but also D-limonene and other minor compounds in the same fruit and juice backgrounds could be responsible of the consideration of increased linalool concentrations as neutral. More studies are needed to assess whether linalool and/or the other oxygenated terpenes may play a different role in flavor panels. Increased OAVs for ethyl esters in S juices made their odor more intense and attractive supporting the role of esters as markers of odor liking for orange juice. Our data provide clues for understanding which specific chemical groups influence odor juice and fruit perception. This is essential to better select targets for molecular engineering of aroma and flavor.

In conclusion, our results indicate that AS down-regulation of D-limonene synthase and the consequent modification of fruit odor by genetic engineering did not affect negatively sweet orange fruit and juice intensity and discrimination. Moreover, as AS fruits have antimicrobial and pesticide activities, such modifications may also improve shelf-life of stored fruits and/or reduce synthetic pesticide use, which could influence positively to the consumers perception.

Acknowledgements

We are grateful to Drs. Lorenzo Zacarías and M.Jesús Rodrigo (IATA-CSIC) for the GC-MS facilities and support. We would like to acknowledge also to Drs. Berta Alquézar and Elsa Pons for their critical review of the manuscript and to all the panelists that participated in the sensory panel. This research is being funded in part by Fundo de Defesa da Citricultura (Fundecitrus) and FAPESP project 2014/12616-9.

References

Ahmed, E. M., Dennison, R. A., Dougherty, R. H., & Shaw, P. E. (1978). Flavor and odor thresholds in water of selected orange juice components. *Journal of Agricultural and Food Chemistry*, *26* (1), 187–191.

Baxter, I. A., Easton, K., Schneebeli, K., & Whitfield, F. B. (2005). High pressure processing of Australian navel orange juices: Sensory analysis and volatile flavor profiling. *Innovative Food Science & Emerging Technologies*, *6* (4), 372–387.

Bazemore, R., Goodner, K., & Rouseff, R. (1999). Volatiles from unpasteurized and excessively heated orange juice analyzed with solid phase microextraction and GC-olfactometry. *Journal of Food Science*, *64* (5), 800–803.

Bazemore, R., Rouseff, R., & Naim, M. (2003). Linalool in orange juice: origin and thermal stability. *Journal of Agricultural and Food Chemistry*, *51* (1), 196–199.

Buettner, A., Mestres, M., Fischer, A., Guasch, J., & Schieberle, P. (2003). Evaluation of the most odour-active compounds in the peel oil of clementines (*Citrus reticulata* Blanco cv. clementine). *European Food Research and Technology*, *216* (1), 11–14.

Buettner, A., & Schieberle, P. (2001). Evaluation of aroma differences between hand-squeezed juices from Valencia Late and navel oranges by quantitation of key odorants and flavor reconstitution experiments. *Journal of Agricultural and Food Chemistry*, *49* (5), 2387–2394.

Choi, H. (2005). Characteristic odor components of kumquat (*Fortunella japonica* Swingle) peel oil. *Journal of Agricultural and Food Chemistry*, *53* (5), 1642–1647.

Davidovich-Rikanati, R., Sitrit, Y., Tadmor, Y., Iijima, Y., Bilenko, N., Bar, E., Carmona, B., Fallik, E., Dudai, N., Simon, J.E., Pichersky, E. & Lewinsohn, E. (2007). Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway. Nature biotechnology, 25 (8), 899-901.

Dugo, G., & Di Giacomo, A. (2002). *Citrus: the genus Citrus.* (G. Dugo & A. Di Giacomo, Eds.) (Vol. 26). New York: Taylor & Francis Group, CRC Press.

Galili, G., Galili, S., Lewinsohn, E., & Tadmor, Y. (2002). Genetic, molecular, and genomic approaches to improve the value of plant foods and feeds. *Critical Reviews in Plant*

Sciences, *21* (3), 167–204.

Goff, S. A., & Klee, H. J. (2006). Plant volatile compounds: sensory cues for health and nutritional value? *Science*, *311* (5762), 815–819.

Grosch, W. (2001). Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chemical Senses*, *26* (5), 533–545.

Heath, H. B., & Reineccius, G. (1986). *Flavor chemistry and technology*. (Westport : Avi Publishing, Ed.). Macmillan Publishers Ltd.

Högnadóttir, A., & Rouseff, R. L. (2003). Identification of aroma active compounds in orange essence oil using gas chromatography-olfactometry and gas chromatography-mass spectrometry. *Journal of Chromatography A*, *998* (1-2), 201–211.

Jordán, M., Goodner, K. L., & Shaw, P. E. (2002). Characterization of the aromatic profile in aqueous essence and fruit juice of yellow passion fruit (*Passiflora edulis* Sims F . Flavicarpa degner) by GC−MS and GC/O. *Journal of Agricultural and Food Chemistry*, *50* (6), 1523–1528.

Jordán, M. J., Tandon, K., Shaw, P. E., & Goodner, K. L. (2001). Aromatic profile of aqueous banana essence and banana fruit by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O). *Journal of Agricultural and Food Chemistry*, *49* (10), 4813–4817.

Klee, H. J. (2010). Improving the flavor of fresh fruits: genomics, biochemistry, and biotechnology. *The New Phytologist*, *187* (1), 44–56.

Lin, J., & Rouseff, R. L. (2001). Characterization of aroma-impact compounds in cold-pressed grapefruit oil using time-intensity GC-olfactometry and GC-MS. *Flavour and Fragrance Journal*, *16* (6), 457–463.

Macleod, A. J., Macleod, G., & Subramanian, G. (1988). Volatile aroma constituents of orange. *Phytochemistry*, *27* (7), 2185–2188.

Miyazaki, T., Plotto, A., Goodner, K., & Gmitter Jr, F. G. (2011). Distribution of aroma volatile compounds in tangerine hybrids and proposed inheritance. *Journal of the Science of Food and Agriculture*, *91* (3), 449–460.

Moufida, S., & Marzouk, B. (2003). Biochemical characterization of blood orange, sweet orange, lemon, bergamot and bitter orange. *Phytochemistry*, *62* (8), 1283–1289.

Obenland, D., Collin, S., Mackey, B., Sievert, J., Fjeld, K., & Arpaia, M. L. (2009). Determinants of flavor acceptability during the maturation of navel oranges. *Postharvest Biology and Technology*, *52* (2), 156–163.

Perez-Cacho, P. R., & Rouseff, R. L. (2008). Fresh squeezed orange juice odor: a review. *Critical Reviews in Food Science and Nutrition*, *48* (7), 681–695.

Plotto, A., Margaría, C. A., Goodner, K. L., & Baldwin, E. A. (2008). Odour and flavour thresholds for key aroma components in an orange juice matrix: esters and miscellaneous compounds. *Flavour and Fragrance Journal*, *23* (6), 398–406.

Plotto, A., Margaría, C. A., Goodner, K. L., Goodrich, R., & Baldwin, E. A. (2004). Odour and flavour thresholds for key aroma components in an orange juice matrix: terpenes and aldehydes. *Flavour and Fragrance Journal*, *19* (6), 491–498.

Pons, E., Alquézar, B., Rodríguez, A., Martorell, P., Genovés, S., Ramón, D., Rodrigo, M. J., Zacarías, L., & Peña, L. (2014). Metabolic engineering of β-carotene in orange fruit increases its in vivo antioxidant properties. *Plant Biotechnology Journal*, *12* (1), 17–27.

Pons, E., Peris, J. E., & Peña, L. (2012). Field performance of transgenic citrus trees: assessment of the long-term expression of *uidA* and *nptII* transgenes and its impact on relevant agronomic and phenotypic characteristics. *BMC Biotechnology*, *12* (1), 41.

Qiao, Y., Xie, B. J., Zhang, Y., Zhang, Y., Fan, G., Yao, X. L., & Pan, S. Y. (2008). Characterization of aroma active compounds in fruit juice and peel oil of Jinchen sweet orange fruit (*Citrus sinensis* (L.) Osbeck) by GC-MS and GC-O. *Molecules*, *13* (6), 1333–1344.

Rodríguez, A., San Andrés, V., Cervera, M., Redondo, A., Alquézar, B., Shimada, T., Gadea, J., Rodrigo, M. J., Zacarías, L., Palou, L., López, M. M., Castañera, P., & Peña, L. (2011a).

Terpene down-regulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens. *Plant Physiology*, *156* (2), 793–802.

Rodríguez, A., San Andrés, V., Cervera, M., Redondo, A., Alquézar, B., Shimada, T., Gadea, J., Rodrigo, M. J., Zacarías, L., Palou, L., López, M. M., Castañera, P., & Peña, L. (2011b). The monoterpene limonene in orange peels attracts pests and microorganisms. *Plant Signaling & Behavior*, *6* (11), 1820–1823.

Rodríguez, A., Shimada, T., Cervera, M., Alquézar, B., Gadea, J., Gómez-Cadenas, A., De Ollas, C. J., Rodrigo, M. J., Zacarías, L., & Peña, L. (2014). Terpene down-regulation triggers defense responses in transgenic orange leading to resistance against fungal pathogens. *Plant Physiology*, *164* (1), 321–339.

Rodríguez, A., Shimada, T., Cervera, M., Redondo, A., Alquézar, B., Rodrigo, M. J., Zacarías, L., Palou, L., López, M. M., & Peña, L. (2015). Resistance to pathogens in terpene down-regulated orange fruits inversely correlates with the accumulation of D-limonene in peel oil glands. *Plant Signaling & Behavior*, *10* (6), e1028704.

Roessler, E. B., Pangborn, R. M., Sidel, J. L., & Stone, H. (1978). Expanded statistical tables for estimating significance in paired-preference, paired-difference, duo-trio and triangle tests. *Journal of Food Science*, *43*(3), 940–943.

Schieberle, P., Mestres, M., & Buettner, A. (2003). Characterization of aroma compounds in fresh and processed mandarin oranges. In K. R. Cadwallader & H. Weenen (Eds.), *Freshness and Shelf Life of Foods* (Vol. 836, pp. 162–174). Washington, DC: American Chemical Society.

Selli, S., & Kelebek, H. (2011). Aromatic profile and odour-activity value of blood orange juices obtained from Moro and Sanguinello (*Citrus sinensis* L. Osbeck). *Industrial Crops and Products*, *33* (3), 727–733.

Sharon-Asa, L., Shalit, M., Frydman, A., Bar, E., Holland, D., Or, E., Lavi, U., Lewinsohn, E., & Eyal, Y. (2003). Citrus fruit flavor and aroma biosynthesis: isolation, functional characterization, and developmental regulation of *Cstps1*, a key gene in the production of the sesquiterpene aroma compound valencene. *The Plant Journal*, *36* (5), 664–674.

Tieman, D., Bliss, P., McIntyre, L. M., Blandon-Ubeda, A., Bies, D., Odabasi, A. Z., Rodríguez, G. R., van der Knaap, E., Taylor, M. G., Goulet, C., Mageroy, M. H., Snyder, D. J., Colquhoun, T., Moskowitz, H., Clark, D. G., Sims, C., Bartoshuk, L., & Klee, H. J. (2012). The chemical interactions underlying tomato flavor preferences. *Current Biology*, *22* (11), 1035–1039.

Tietel, Z., Plotto, A., Fallik, E., Lewinsohn, E., & Porat, R. (2011). Taste and aroma of fresh and stored mandarins. *Journal of the Science of Food and Agriculture*, *91* (1), 14–23.

Figure captions

Main text

Figure 1. Organoleptic evaluation of fresh-cut fruit and juice with pulp of transgenic Navelina sweet oranges. (A-H) Smell (orthonasal route) evaluations for the odor intensity and discrimination (perceived as different) in fresh-cut fruit and juice with pulp in the comparison of Navelina AS5 vs. EV and AS3 vs. EV samples performed by panelists for two different seasons (*n*=62 for the first season (A-D) and *n*=54 for the second season (E-H)). Differences found are statistically significant by two-tailed paired comparisons at $P\leq0.01$ (*) and $P\leq0.001$ (**). (I-L) Details of the sensory facility for the odor tests. (I) Individual booths with the two-paired samples presented to the panelists. (J) Situation of the panelist inside the booth. (K) A panelist cutting a Navelina orange fruit before smelling the peel. (L) A panelist before smelling the fresh juice with pulp of a Navelina orange.

Figure 2. Organoleptic evaluations of fresh-juice with pulp of transgenic Pineapple sweet oranges. (A-D) Smell (orthonasal route) evaluations for the juice-odor intensity and discrimination (perceived as different) in the comparison of Pineapple AS11 vs. EV and S13 vs. EV samples performed by panelists for two different seasons (*n*=65 for the first season (A, B) and *n*=70 for the second season (C, D)). Differences found are statistically significant by two-

tailed paired comparisons at $P \leq 0.01$ (*) and $P \leq 0.001$ (**). (E-H) Mean hedonic scores and ranking (Friedman tests) after the sensory evaluation of the fresh juice from different transgenic Pineapple oranges using an hedonic scale where 1=dislike extremely to 9=like extremely. Scaled values were grouped using ranks where Rank 1 included values 7 to 9, Rank 2 included values 4 to 6 and Rank 3 included values 1 to 3 in Friedman tests (F and H). Means followed by the same letter are not significantly different ($P \leq 0.01$). (I-J) Details of the sensory facility for the smelling tests. (I) Individual booths with the juice samples presented to the panelists for the juice-odor intensity and preference tests. (J) Juice samples presented to the panelists for the hedonic tests.

Supplementary Figure S1. Schematic representation of the phenological cycle of trees from the transgenic sweet orange lines Navelina AS3, AS5 and EV, and Pineapple AS11, S13 and EV. Phenological stages were recorded weekly according to the BBCH codification for citrus and grouped into 3 main phases including shoot formation and flowering (yellow), fruit development (green) and maturation (orange) stages.

Supplementary Figure S2. Total normalized volatiles peak areas of Navelina fruits for flavedo (A, C) and juice with pulp (B, D) in the first (A, B) and second (C, D) seasons analyzed.

Supplementary Figure S3. Total normalized volatiles peak areas of Pineapple fruits for juice with pulp in the first (A) and second (B) seasons analyzed.

Table 1. Average values for the fruit quality variables evaluated for oranges cv. Navelina (1A) and Pineapple (1B). TA = titratable acidity; SSC = soluble solids content; MI = maturity index. Means separation done by the least significance difference (LSD) test. Means in a column with different letters are statistically different ($P < 0.05$)

Table 2. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Navelina sweet orange juices in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

Table 3. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Pineapple sweet orange juices in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

Table 4. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Navelina sweet orange flavedo in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

Supplementary Table S1. Volatile components identified (%) in juice with pulp of cv. Navelina fruits analyzed by GC-MS in the first season (S1A) and second season (S1B).

Supplementary Table S2. Volatile components identified (%) in juice with pulp of cv. Pineapple fruits analyzed by GC-MS in the first season (S2A) and second season (S2B).

Supplementary Table S3. Volatile components identified (%) in flavedo of cv. Navelina fruits analyzed by GC-MS in the first season (S1A) and second season (S1B).