Food Chemistry 217 (2017) 139–150

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

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

[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), carretera Moncada-Náquera Km. 4.5, 46113 Moncada, Valencia, Spain

ARTICLE INFO

Article history:
Received 9 February 2016
Received in revised form 21 August 2016
Accepted 23 August 2016
Available online 24 August 2016

Keywords:
D-Limonene
Genetically-modified fruits
Sensory panel
Alcohols
Ethyl esters
Orange odor perception
OAV
Citrus sinensis

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.

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

* Corresponding author at: 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.
E-mail address: lpenya@fundecitrus.com.br (L. Peña).

http://dx.doi.org/10.1016/j.foodchem.2016.08.076
0308-8146/

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 and 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, 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 Fig. 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