Genetic engineering of plant volatiles in fleshy fruits:

pest repellency and disease resistance through D-limonene downregulation in transgenic orange plants

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Dissertation submitted in partial fulfillment of the requirements for obtaining the degree of Doctor (PhD) in Biotechnology

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El Doctor Leandro Peña García, Investigador científico del Instituto Valenciano de Investigaciones Agrarias,

CERTIFICA:

Que la presente memoria titulada “Genetic engineering of plant volatiles in fleshy fruits: pest repellency and disease resistance through D-limonene downregulation in transgenic orange plants”, ha sido realizada por Ana María Rodríguez Baixauli, Ingeniero Agrónomo por la Universitat Politècnica de València, bajo su dirección y constituye su Memoria de Tesis para optar al grado de Doctor Internacional en Biotecnología.

Fdo: Dr. Leandro Peña García

Valencia, 06 de mayo de 2013

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El Doctor D. Alejandro Atarés Huerta, Profesor Titular de la Universidad Politécnica de Valencia,

CERTIFICA:

Que Dña. Ana María Rodríguez Baixauli, Ingeniero Agrónomo por la Universitat Politècnica de València, ha realizado bajo su tutela el trabajo que, con el título "Genetic engineering of plant volatiles in fleshy fruits: pest repellency and disease resistance through D-limonene downregulation in transgenic orange plants", presenta para optar al grado de Doctor Internacional en Biotecnología por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firma el presente certificado

Dr. Alejandro Atarés Huerta

Valencia, 06 de mayo de 2013

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Abstract

Terpenes, the largest group of secondary metabolites, are well known as constituents of essential oils, floral scents and defensive resins of aromatic plants, to which they impart their characteristic aromas and flavors. Terpene volatiles defend many species of plants, animals and microorganisms against predators, pathogens and competitors. Moreover, those compounds seem to serve as advertisements to attract pollinators and seed-dispersal agents as well as pest predators. The study of VOCs emitted during fruit development and after challenge with different biotic agents may help to determine the interactions of fleshy fruits not only with legitimate vertebrate dispersers and predators, but also with insects and microorganisms.

Fleshy fruits are particularly rich in volatiles. In citrus fruits, monoterpenes are the main components of the essential oil glands of the peel, being D-limonene the most abundant one (up to 95% in orange fruits). This characteristic makes citrus a good model system for studying the function of terpenes in plants. Modern molecular biology now enable experiments to test terpenoid function by the use of genetically transformed organisms in which terpene levels have been manipulated. In this work, a plasmid harboring the complete cDNA of a citrus limonene synthase gene (*CiTMTSE1*) in antisense (AS) or sense (S) orientation was used to modify the expression and accumulation of D-limonene of sweet orange (*Citrus sinensis* L. Osb) plants. D-limonene accumulation in AS fruits was dramatically reduced but the accumulation of other terpenoids was also modified, such as monoterpene alcohols, whose concentration increased in the peel of fruits. Genetically transformed plants were morphologically indistinguishable from wild-type (WT) and empty vector (EV) control plants.

Transgenic fruits were challenged against a pest and different pathogens to test whether volatile profile alteration results in an improvement in the response of the fruit flavedo against them. Males of the Mediterranean fruit fly (*Ceratitis capitata*) exposed to AS fruits versus EV in wind tunnel assays were significantly more attracted to the odor of EV control fruits. In separate experiments with the green mould rot of citrus fruits and citrus canker caused by *Penicillium digitatum* and *Xanthomonas axonopodis* subsp. *citri*, respectively, transgenic fruits with a reduced content in D-limonene showed resistance to both pathogens. High D-limonene content in mature orange peels may be a signal for attractiveness of pests and microorganisms which might be likely involved in facilitating the access to the pulp of seed dispersal frugivores.

A global gene expression analysis of the flavedo of AS transgenic fruits linked the decrease of D-limonene and monoterpene metabolism to the up-regulation of genes involved in the innate immunity response, including transcription factors together with Ca^{2+} entry into the cell and activation of MAPK cascades, contributing to activation of jasmonic acid (JA) signaling, which triggered the up-regulation of JA metabolism and drastically increased the accumulation

of JA in orange peels upon fungal challenge, explaining the resistance to necrotrophic fungi observed in AS fruits.

These results indicate that limonene accumulation in the peel of citrus fruit appears to be involved in the successful trophic interaction between fruits, insects, and microorganisms and provide a much more comprehensive view of roles of terpenes in nature. It also represents a very promising alternative for increasing resistance or tolerance of plants to pathogens.

Resumen

Los terpenos constituyen el mayor grupo de metabolitos secundarios, siendo componentes de las glándulas de aceites esenciales, de las flores y de las resinas defensivas de plantas aromáticas, a los que proporcionan sus aromas y sabores característicos. Los terpenos volátiles se asocian a la defensa de muchas especies de plantas, animales y microorganismos contra depredadores, patógenos y competidores. Por otra parte, estos compuestos parecen servir como señales para atraer a los polinizadores y agentes dispersores de semillas, así como a depredadores de plagas. El estudio de compuestos orgánicos volátiles emitidos durante el desarrollo del fruto y después del desafío con diferentes agentes bióticos puede ayudar a conocer las interacciones de los frutos carnosos no sólo con vertebrados dispersores y depredadores, sino también con insectos y microorganismos.

Los frutos carnosos son particularmente ricos en volátiles. En los frutos cítricos, los monoterpenos son los principales componentes de las glándulas del aceite esencial de la cáscara (flavedo), siendo el D-limoneno el más abundante (hasta 95% en la naranja). Esta característica hace que los cítricos sean un buen sistema modelo para el estudio de la función de los terpenos en los frutos. La biología molecular moderna permite la realización de experimentos para comprobar la función de terpenos por medio del uso de organismos transformados genéticamente en los que se han manipulado los niveles de acumulación de dichos compuestos. En este trabajo, se ha utilizado un plásmido que alberga el cDNA completo del gen de una limoneno sintasa de cítricos (*CiTMTSE1*) en orientación antisentido (AS) o sentido (S) para modificar la expresión y la acumulación de D-limoneno en plantas de naranjo dulce (*Citrus sinensis* L. Osb.). La acumulación de D-limoneno en las frutas AS se redujo drásticamente pero la acumulación de otros terpenos también se modificó, afectando a compuestos tales como alcoholes monoterpenos, cuya concentración se incrementó en la cáscara de las frutas. Las plantas transformadas fueron morfológicamente indistinguibles de las plantas control (WT) y de las plantas transformadas con el vector vacío (EV).

Los frutos transgénicos fueron desafiados con un insecto plaga y con diferentes patógenos para probar si la alteración de los niveles de acumulación de estos volátiles daba como resultado una mejora en la respuesta del flavedo frente a plagas y patógenos. Los machos de la mosca mediterránea de la fruta (*Ceratitis capitata*) expuestos a las frutas AS y EV en ensayos en túnel de viento fueron significativamente más atraídos por el aroma de los frutos control EV. En otros experimentos de desafío con el hongo de la podredumbre verde *Penicillium digitatum* y la bacteria causante de la cancrosis de los cítricos *Xanthomonas axonopodis* subsp. *citri*, las frutas transgénicas con un contenido reducido de D-limoneno mostraron elevada resistencia a estos patógenos. El alto contenido en D-limoneno en la cáscara de naranjas maduras puede ser una señal para la atracción de plagas y microorganismos que podrían estar involucrados en la facilitación del acceso a la pulpa de los frugívoros dispersores de semillas.

El análisis de la expresión génica global en el flavedo de las frutas transgénicas vinculó la disminución de D-limoneno y la reducción de la expresión de genes del metabolismo de monoterpenos con la activación de la expresión de genes implicados en inmunidad innata, incluyendo factores de transcripción, genes de quinasas implicadas en la entrada de Ca^{2+} en la célula y genes implicados en la activación de las cascadas de MAPKs, con la consiguiente activación de la ruta de señalización de ácido jasmónico (JA), lo que provocó la activación del metabolismo de JA y un aumentó drástico de la acumulación de JA en la cáscara de la naranja tras el desafío con *P. digitatum*, lo que explicaría la resistencia al menos a hongos necrotrofos observada en las frutas.

Estos resultados indican que la acumulación de D-limoneno en la cáscara de la naranja estaría implicada en la interacción trófica entre las frutas, insectos y microorganismos, lo cual proporciona una visión mucho más amplia de las funciones de los terpenos en la naturaleza. También representa una alternativa muy prometedora para incrementar la resistencia o tolerancia de las plantas frente a patógenos y plagas.

Resum

Els terpens constitueixen el major grup de metabòlits secundaris, i són components de les glàndules d'olis essencials, de les flors i de les resines defensives de plantes aromàtiques, als que proporcionen les seues aromes i sabors característics. Els terpens volàtils s'associen a la defensa de moltes espècies de plantes, animals i microorganismes contra depredadors, patògens i competidors. D'altra banda, aquests compostos pareixen servir com a senyals per atraure els pol·linitzadors i agents dispersors de llavors, així com a depredadors de plagues. L'estudi de compostos orgànics volàtils emesos durant el desenvolupament del fruit i després del desafiament amb diferents agents biòtics pot ajudar a conèixer les interaccions dels fruits carnosos no només amb vertebrats dispersors i depredadors, sinó també amb insectes i microorganismes.

Els fruits carnosos són particularment rics en volàtils. En els fruits cítrics, els monoterpens són els principals components de les glàndules de l'oli essencial de la corfa (flavedo), sent el D-limonè el més abundant (fins a 95% a la taronja). Aquesta característica fa que els cítrics siguen un bon sistema model per a l'estudi de la funció dels terpens en els fruits. La biologia molecular moderna permet la realització d'experiments per comprovar la funció de terpens per mitjà de l'ús d'organismes transformats genèticament en els que s'han manipulat els nivells d'acumulació d'aquests compostos. En aquest treball, s'ha utilitzat un plasmidi que alberga el cDNA complet del gen d'una limonè sintasa de cítrics (*CiTMTSE1*) en orientació antisentit (AS) o sentit (S) per modificar l'expressió i l'acumulació de D-limonè en plantes de taronger dolç (*Citrus sinensis* L. Osb.). L'acumulació de D-limonè en les fruites AS es va reduir dràsticament però l'acumulació d'altres terpens també es va modificar, afectant compostos tals com alcohols monoterpens, la concentració dels quals va augmentar a la corfa de les fruites. Les plantes transformades van ser morfològicament indistingibles de les plantes control (WT) i de les plantes transformades amb el vector buit (EV).

Els fruits transgènics van ser desafiats amb un insecte plaga i amb diferents patògens per a provar si l'alteració dels nivells d'acumulació d'aquests volàtils donava com a resultat una millora en la resposta del flavedo contra plagues i patògens. Els mascles de la mosca mediterrània de la fruita (*Ceratitis capitata*) exposats a les fruites AS i EV en assajos en túnel de vent van ser significativament més atrets per l'aroma dels fruits control EV. En altres experiments de desafiament amb el fong de la podridura verda, *Penicillium digitatum,* i el bacteri causant de la cancrosi dels cítrics, *Xanthomonas axonopodis* subsp. *citri*,, les fruites transgèniques amb un contingut reduït de D-limonè van mostrar elevada resistència a aquests patògens. L'alt contingut en D-limonè en les corfes de taronges madures pot ser un senyal per a l'atracció de plagues i microorganismes que podrien estar involucrats en la facilitació de l'accés a la polpa dels frugívors dispersors de llavors.

L'anàlisi de l'expressió gènica global en el flavedo de les fruites transgèniques va vincular la disminució de D-limonè i la reducció de l'expressió de gens del metabolisme de monoterpens amb l'activació de l'expressió de gens implicats en immunitat innata, incloent factors de transcripció, gens de quinases implicades en l'entrada de Ca^{2+} a la cèl·lula i gens implicats en l'activació de les cascades de MAPKs, amb la conseqüent activació de la ruta de senyalització d'àcid jasmònic (JA), el que va provocar l'activació del metabolisme de JA i un augment dràstic de l'acumulació de JA a la corfa de la taronja després del desafiament amb *P. digitatum*, el que explicaria la resistència almenys a fongs necròtrofs observada en les fruites.

Aquests resultats indiquen que l'acumulació de D-limonè en la corfa de la taronja estaria implicada en la interacció tròfica entre les fruites, insectes i microorganismes, la qual cosa proporciona una visió molt més àmplia de les funcions dels terpens en la naturalesa. També representa una alternativa molt prometedora per incrementar la resistència o tolerància de les plantes enfront de patògens i plagues.

1. INTRODUCTION

Fruit aromas in mature fleshy fruits as signals of readiness for predation and seed dispersal

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"… that a ripe strawberry or cherry is as pleasing to the eye as to the palate (…) will be admitted by every one. But this beauty serves merely as a guide to birds and beasts, in order that the fruit may be devoured and the matured seeds disseminated."
(Darwin, 1872).

Abstract

The dispersal of seeds away from parent plants seems to be the underlying selective force in the evolution of fleshy fruits attractive to animals. Secondary metabolites, which are not essential compounds for plant survival, are involved in the interaction of fleshy fruits with seed dispersers and antagonists. Plant volatile organic compounds (VOCs) are secondary metabolites that play important roles in biotic interactions and in abiotic stress responses. They are usually accumulated at high levels in specific plant tissues and organs, such as fleshy fruits. The study of VOCs emitted during fruit development and after different biotic challenges may help to determine the interactions of fleshy fruits not only with legitimate vertebrate dispersers but also with insects and microorganisms. The knowledge on fruit VOCs could be used in agriculture to generate attraction or repellency to pests and resistance to pathogens in fruits. This review provides a view of specific fruit VOC blends as signals for either seed dispersal or predation through simple or complex trophic chains, which may have consequences for understanding the importance of biodiversity in wild areas.

1. Introduction.

Besides phylogenetic and physiological constraints influencing fruit traits (Eriksson & Ehrlén, 1998, Whitehead & Poveda, 2011), the dispersal of seeds away from parent plants seems to be an important selective force in the evolution of fleshy fruits attractive to vertebrates (Van der Pijl, 1969, Snow, 1971). There is still little empirical evidence about the primary function of secondary metabolites in fleshy fruits, but it is widely assumed that they are involved in mediating two main goals, attracting seed dispersal organisms and avoiding consumption by seed predators. It is thought that the primary function of these specialized metabolites in immature fruit is to defend them against all types of potential consumers (Cipollini & Levey, 1997, Mack, 2000). Other hypotheses such as direct nutritional benefits, defense tradeoff, attraction/association, seed germination inhibition, and influence on protein assimilation and gut retention time have been proposed additionally (Cipollini, 2000). Changes in secondary metabolites occur during ripening in combination with changes in size, texture, taste, aroma and color, however, their biological role and whether they have evolved under the selective pressures of frugivores is largely unknown.

Fruit traits are perceived by animal frugivores in a hierarchical manner. The aroma and color are probably the first cues for the frugivore attraction at distance, once a frugivore contacts the fruit it perceives morphological traits, and finally fruit chemistry determines taste and digestibility. Visual signals have been extensively investigated lately and special attention has been paid to the function of anthocyanins in attracting mutualists and/or deterring antagonists (Schaefer, 2011, Valido *et al.*, 2011). However, the role of ripe fruit volatiles as olfactory signals directed to legitimate dispersers and predators has been scarcely investigated. Only recently it has been shown that aroma and color in wild fig fruits (actually, flower-bearing receptacles called syconia) in Papua New Guinea have evolved in concert and as predicted by differences in the behavior, physiology and morphology of their bird and bat dispersers, indicating that differences among vertebrate frugivores have shaped the evolution of fruit traits. This evidence experimentally supports for the first time the existence of the seed dispersal syndromes, at least for fruit aroma and color (Lomáscolo *et al.*, 2010).

Plant volatile organic compounds (VOCs) comprise a wide diversity of low molecular weight secondary metabolites, with an appreciable vapor pressure under ambient conditions. Whereas some VOCs are probably common to almost all plants, others are specific to only one or a few related taxa. To the first type belong the so-called `green leaf´ volatiles (GLVs) because of their `fresh green´ odor. This group comprises short chain (C6) acyclic aldehydes, alcohols and their esters produced by plants from most taxa as a wound response via the enzymatic metabolism of polyunsaturated fatty acids. On the other hand, species- or genus-specific VOCs have been described in some species, such as the sulfur-contaning VOCs of Alliaceae and Brassicaceae (Qualley & Dudareva, 2001). To understand the functional significance of VOCs in ripe fruits, it is necessary to know their biosynthesis and developmental regulation, their quantitative and qualitative accumulation and the responses triggered by VOCs

on organisms interacting with the fruit, including vertebrates, insects and microorganisms. In this review, we attempt to update and integrate all relevant references pertaining to this issue to obtain a clearer picture on the VOC biosynthetic patterns in fleshy fruits and on the putative roles of VOCs in the attraction or deterrence of seed dispersers and/or predators.

1.1. VOCs in plants

It is assumed that VOCs were originally antimicrobial compounds that later also served to combat pests, thus providing plants with a kind of immune system (Turlings & Tumlinson, 1992). In vegetative tissues, VOC patterns have coevolved with phytophagous insects, and their chemical diversity has escalated, likely to gain improved defenses (Becerra & Venable, 1999). It has been proposed that the different VOCs could act synergistically, as in conifer resins, for simultaneous protection against pests and pathogens (Phillips & Croteau, 1999). Recent data have demonstrated that VOCs serve as signals for communication between plants and between distal parts within the same plant (Qualley & Dudareva, 2001). They are also involved in protecting the plant against abiotic stress, defending the plant against pests and pathogens, and attracting herbivore predators and pollinators (Gershenzon & Dudareva, 2007, Kessler *et al.*, 2008). It is well documented through genetic engineering experiments that specific terpenoid compounds emitted by leaves can intoxicate, repel or deter herbivores (Aharoni *et al.*, 2003), or they may attract natural predators and parasitoids of damaging herbivores thus protecting plants from further damage (Kappers *et al.*, 2005). It has also been demonstrated that specific volatile compounds emitted by flowers greatly contribute to the plant's reproductive success and survival in natural ecosystems (Kessler *et al.*, 2008).

Our knowledge regarding VOCs synthesis and accumulation in fruits is much less extensive than that related to flowers and leaves. There are few references that have considered specific VOCs or VOC blends in mature fleshy fruits for attraction of legitimate disperser organisms (Lomáscolo *et al.*, 2010) and no references considering VOCs in interactions with putative predators, probably due to the difficulties and complexities involved in measuring and analyzing VOC contents and emission from fruits under different developmental and environmental conditions in ecological contexts. In contrast, the importance of the interaction of fruit VOCs with specific insects or microorganisms in agricultural contexts has been a subject of extensive research due to its economic impact (Bruce *et al.*, 2005), though there are few works on the interactions of fruit VOCs with vertebrates in crops (Borges *et al.*, 2011).

1.1.1. VOCs in fleshy fruits

In general, flowers and fruits release the widest variety of VOCs, with emission rates peaking before pollination and at ripening, respectively (Dudareva *et al.*, 2004). Additionally, flowers, leaves and fruits often show different VOC profiles, suggesting that their functions in

different tissues or organs may be also different (Fig. 1, our unpublished results). For example, mono- and sesquiterpenes are major compounds of mango leaves and fruits, although specific VOCs can be ascribed to each tissue, such as esters that are not detected in leaves (Lalel *et al.*, 2003, Silva *et al.*, 2012). Similarly, important scent VOCs in ripe peach (linalool and C10 lactones) are absent from leaves (Horvat & Chapman, 1990). Specific fruit and leaf VOCs have also been reported in citrus (Dugo & Di Giacomo, 2002) and in the wild *Schinus molle* (Maffei & Chialva, 1990). Based on principal component analysis, Oliveira *et al.* (2010) showed that the peel, pulp and leaves from different fig cultivars can be distinguished by their distinct abundance of monoterpenes, sesquiterpenes and aldehydes.

VOCs in fruits are diverse, consisting of different chemical products comprising only 10^{-7}-10^{-4} of the fresh fruit weight (Jiang & Song, 2010). Hundreds of VOCs are identified in most fruits, and this diversity is partially responsible for the unique scent found in different fruit species and cultivars. The aroma properties of fruits depend upon the combination of VOCs produced and on the concentration and odor threshold of each in the blend. Most of them can be divided into four major classes according to their metabolic origin (Negre-Zakharov *et al.*, 2009): terpenoids (e.g. mono- and sesquiterpenes and apocarotenoids), phenylpropanoids/benzenoids (e.g. eugenol, benzaldehide), fatty acid derivatives (e.g. hexenal, hexenol) and amino acid derivatives (e.g. thiazole, 2- and 3-methylbutanal). Among them,

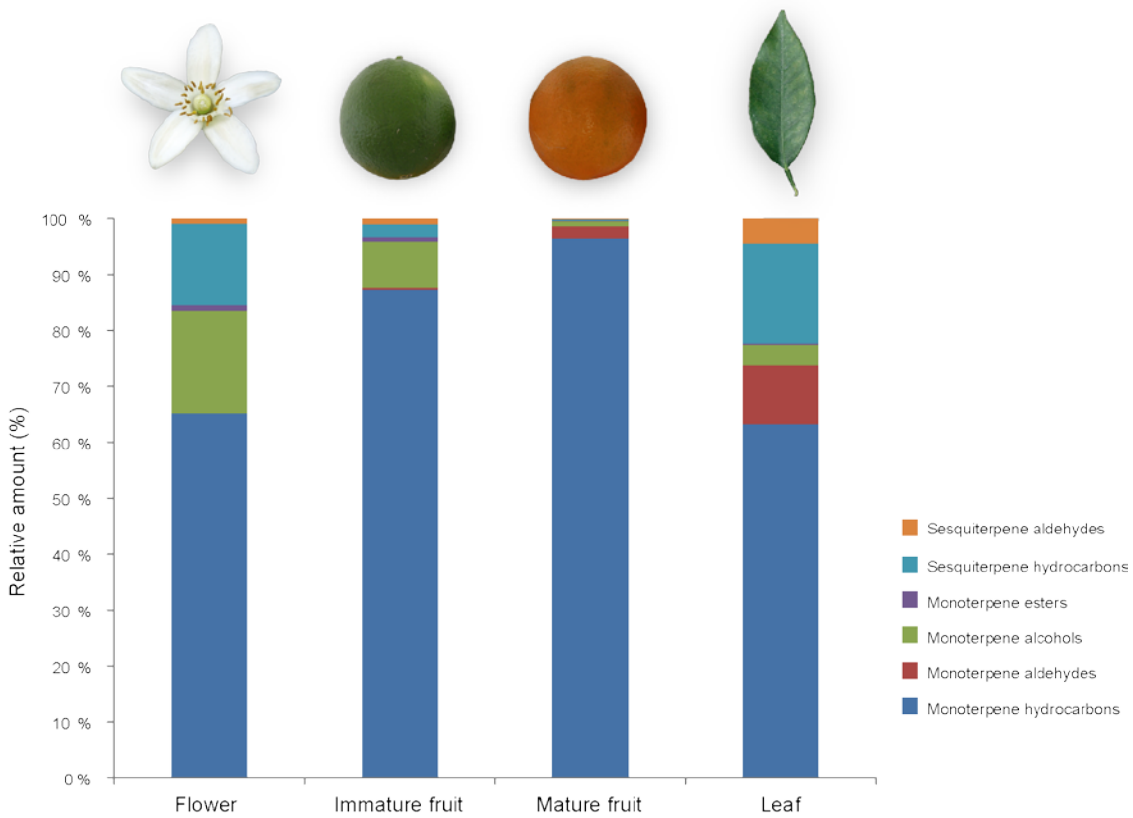

Figure 1. Terpene volatile profile of different citrus tissues and organs: flower, peel from immature fruit, peel from mature fruit and leaf. Relative amount of sesquiterpene and monoterpene derived volatiles is presented as a percentage of each class with respect to the total.

terpenoids and lipid derivatives are likely the most abundant and expensive to produce in terms of energy and nutrients (Gershenzon & Dudareva, 2007). From a chemical view, these VOCs can be classified as esters, alcohols, aldehydes, ketones, lactones and terpenoids.

To attract seed disseminators and thus to ensure reproductive and evolutionary success, many plants release diverse blends of VOCs from their fruits. With their huge number of compounds and high structural diversity, terpenoids are one of the largest classes of VOCs in fruits, especially monoterpenes, sesquiterpenes and irregular terpenes of low molecular weight. In lulo, myrtle, coriander, mango and citrus mature fruits, monoterpenes are the most representative type of volatiles, its content varying between 50 and almost 100% of the total VOCs (Table 1). Esters are also key contributors to the fruity aroma. For example, the ester fraction has been described as the determinant for the characteristic varietal aroma in apple cultivars and Lambrusco and hybrid grapes (Jiang & Song, 2010, Yang *et al.*, 2011). In *Ficus racemosa*, esters dominate volatile profiles (86-94% of total) as it also occurs in *F. benghalensis* fruits during the night (Borges *et al.*, 2011). Some other groups of minor volatiles in terms of concentration are also important for fruit scent. For example, apocarotenoids, also called norisoprenoids, derived from carotenoids by oxidative cleavage. Studies in tomato, melon, peach and watermelon indicate that the carotenoid profile has a clear impact on aroma via determining the suite of synthesized apocarotenoids (Lewinsohn *et al.*, 2005, Rodrigo *et al.*, 2012). Other compounds such as sulfur volatiles, mainly arising as degradation products of cysteine, cystine, methionine, glutathione and some vitamins, are also characterized by their extremely low aroma thresholds (Du *et al.*, 2011). VOCs derived from aminoacids are important flavor constituents of many ripe fruits, such as strawberries, tomato, melon and apples (Goff & Klee, 2006, Gonda *et al.*, 2010).

1.1.2. VOC changes during ripening

The following examples, without being an extensive review of the published literature, illustrate how VOC profiles change during fruit ripening. VOCs production increases between 1 and more than 1000 times during the maturation of most fruits (Table 1). Concomitantly, qualitative changes of VOC profile take place along ripening (Table 1). For example, short-chained aldehydes, which provide the `green fresh´ odor, are abundant in numerous unripe green fruits, and their concentration decrease with ripening in fruits such as nectarines, guavas, apples, coriander, strawberries and kiwis. In other fruits, such as neutral grapes, few volatiles other than C6 compounds accumulate (Yang *et al.*, 2011). In this case, VOCs with a green flavor increase until the period of ripening and then decrease. Monoterpene profile also changes during ripening of lulo, myrtle, coriander and citrus fruits (Table 1). For example, in oranges, there is a reduction in the linalool content as maturation progresses, whereas the limonene content increases from 30- to 100-fold between the green and the color break stages (Dugo & Di Giacomo, 2002, Rodríguez *et al.*, 2011). In white guava, mono- and sesquiterpenes, which are absent in unripe fruit tissues, accumulate and increase during ripening (Soares *et al.*, 2007).

The concentration of esters and lactones, responsible for the spicy floral and fruity scent of many fruits, increases extraordinarily along ripening. In apple, mango, strawberry, kiwi, papaya, guava and lulo fruits, ester production increases by a factor ranging from 1.3 to more than 2300 during maturation and, at the ripe stage, esters can account for up to 97% of total VOCs (Table 1). It is interesting to remark that, in the case of strawberries, apples and lulos, a burst in ester production has been associated with the onset of ripening (Suárez & Duque, 1992, Menager *et al.*, 2004, Villatoro *et al.*, 2008, Table 1). In nectarines, lactones are characteristic of ripe fruits, their concentration increasing during maturation to reach up to 45.7% of the total VOCs (Engel *et al.*, 1988).

Accumulation of low-strength ripeness-specific fruity aromas, such as apocarotenoids, sulfur volatiles and furan-related compounds, also increase along fruit ripening. In most carotenogenic fruits, the pigment profile changes during maturation, thus changing the apocarotenoid profile as well. In tomato, apocarotenoid VOCs are not emitted until relatively late in fruit ripening, and, during this process, the amount of apocarotenoids increases by a factor of 40 (Mathieu *et al.*, 2009). In peaches, the apocarotenoid level also increases during fruit ripening, representing approximately 40-60% of the total VOCs at the ripe stage (Aubert *et al.*, 2003, Brandi *et al.*, 2011). In strawberries, most sulfur volatiles increase by as much as 100% with maturity (Du *et al.*, 2011). Methyl sulfanyl compounds increase considerably in kiwi with ripening, being dimethyl sulfide one of the key components that differentiate the aroma of yellow and green cultivars (Garcia *et al.*, 2012). Finally, furanone-derived compounds also increase in concentration with the maturation of some fruits. In peaches, furan-related VOC accumulation starts just before color change, and its concentration reaches its maximum at the ripe stage (Brandi *et al.*, 2011). In strawberries, furan-derived compounds are not detected until the fruit reaches red color and their content increase by around 100-fold along maturation (Menager *et al.*, 2004).

In summary, the influence of the ripening stage on fruit scent is clearly evident, and it is well documented that VOC composition changes both quantitatively and qualitatively during maturation. Indeed, analysis of principal components has been successfully applied to discriminate between ripening stages depending on the presence/absence of some VOCs in many fruits such as apples (Villatoro *et al.*, 2008), grapes (Yang *et al.*, 2011), mangos (Lebrun *et al.*, 2008), strawberries (Azodanlou *et al.*, 2004), figs (Hodgkison *et al.*, 2007) peaches and nectarines (Lavilla *et al.*, 2002). Some of these VOC modulations have been related to aroma chemical changes associated with ripening. For example, sugars, the concentration of which increases with ripening, are precursors of furanones, and in tomatoes a direct relationship has been established between sucrose and VOC production (Zanor *et al.*, 2009). Fatty acids are quantitatively the major precursors responsible for the synthesis of esters, aldehydes, alcohols and acids found in fleshy fruits. Because lipid biosynthesis and membrane fluidity increase during ripening, a wider assortment of lipid-derived precursors of aroma contributing VOCs is found in the tissues of fully ripe fruits (Sanz *et al.*, 1997).

Table 1. Changes in the total amount and in selected groups of volatile organic compounds during the development and ripening of different fleshy fruits.

	Increase along ripening	Aldehydes		Esters		Monoterpenes		Sesquiterpenes		References
		Change	Total	Change	Total	Change	Total	Change	Total	
Solanum vestissimum	> x 30	*	1.4	> x 6.0	75.0	> x 0.5	1.2	nd	nd	(Suárez & Duque, 1992)
Schinus molle	·/· 1.5	nd	nd	nd	nd	x 1.0	85.9	x 1.0	10.8	(Hosni *et al*., 2011)
Myrtus communis	x 3.0	nd	nd	·/· 3.3	0.1	x 0.9	71.6	·/· 4.5	3.06	(Aidi Wannes *et al*., 2009)
Coriandrum sativum	x 30.7	·/· 2.1	1.2	·/· 14.7	2.4	x 1.6	90.4	·/· 1.9	2.0	(Msaada *et al*., 2009)
Psidium guajava	x 2.9	·/· 12.5	2.3	x 8.8	54.0	∞	10.0	∞	33.5	(Soares *et al*., 2007)
Psidium salutare	·/· 2.2	nd	nd	·/· 1.2	2.5	·/· 2.2	58.5	·/· 2.6	20.0	(Pino & Queris, 2008)
Mangifera indica	nd	nd	nd	x 44.6-∞	7.7-38.4	·/· 1.2-1.6	53.6-78.3	·/· 1.9	7.9-10.8	(Lalel *et al*., 2003)
Fragaria ananassa	x 4.0-19.0	·/· 60.3	0.5-4.2	x 1.3	78.0-91.0	x 3.3	nd	nd	nd	(Menager *et al*., 2004, Azodanlou *et al*., 2004)
Actinidia deliciosa	x 5.6	·/· 1.7-2.0	9.3-15.8	x 330-409	71.6-82.8	x 1.0-1.1	0.0-0.1	nd	nd	(Garcia *et al*., 2012, Wang *et al*., 2011)
Actinidia chinensis	x 60-117	·/· 1.1-x 1.6	0.3-0.7	x 1116-2381	76.0-83.0	x 1.0-4.4	0.0-0.9	nd	nd	(Wang *et al*., 2011)
Malus x domestica	x 1-30	x 1.7-18.3	3.3-54.3	x 9-515	11.5-97.0	nd	nd	nd	nd	(Villatoro *et al*., 2008, Ortiz *et al*., 2011)
Prunus persica	·/· 1.0-4.6	·/· 3.1-x 1.2	0.3	nd	nd	·/· 1.7- ∞	0.3-40	nd	nd	(Engel *et al*., 1988, Aubert *et al*., 2003)
Carica papaya	x 3.3	nd	nd	x 1.6	50.8-95.1	∞	1.6	nd	nd	(Almora *et al*., 2004, Fuggate *et al*., 2010)
Capsicum annuum	·/· 1.2-1.3	x 1.3	13.8	·/· 1.7	52.8	x 1.9	18.1	·/· 1.4	10.4	(Forero *et al*., 2009)
Solanum lycopersicum	x 1.9-1077.8	·/· 2.7-x 5.5	13-82.0	nd	nd	x 1.3-1.6	<0.1	nd	nd	(Birtic *et al*., 2009, Ortiz-Serrano & Gil, 2010)
Ficus scortechinii	x 18.0	nd	nd	x 3.1	61.1	nd	nd	·/· 17.3	0.1	(Hodgkison *et al*., 2007)
Ficus hispida	x 30	nd	nd	x 2.6	46.4	nd	nd	·/· 6.9	0.1	(Hodgkison *et al*., 2007)
Ficus benghalensis										
Diurnal	nd	nd		nd	13.34	nd	12.16	nd	35.86	(Borges *et al*., 2011)
Nocturnal	nd	nd	4.6	nd	46.9	nd	10.61	nd	6.67	
Ficus racemosa	nd	nd	0.5-0.6	nd	85.6-93.2	nd	3.1-3.6	nd	nd	(Borges *et al*., 2011)
Citrus sinensis	x 4.2	·/· 48	0.48	∞	0.01	x 1.2	96	·/· 8	0.34	(Rodríguez *et al*., 2011)

∞ , specific of ripening and or mature fruits
* , -slight increase from the onset of ripening
·/·, Reduction in accumulation.
X, Increase in accumulation.
nd, non-detailed.

Additionally, recent molecular findings support the idea that *de novo* synthesis of VOCs is induced at ripening. Transcriptional regulation has been described for terpene, carotenoid, fatty acid and phenylpropanoid derived VOCs, and in most cases gene expression is induced upon ripening concomitantly with the production of important flavor compounds (Rodrigo *et al.*, 2012). Additionally, some genes have been shown to display a fruit-specific expression, as those involved in different steps of alcohol and ester biosynthesis in melon (Yahyaoui *et al.*, 2002, Manríquez *et al.*, 2006). Expression of genes involved in the biosynthesis of aminoacid-derived VOCs is also much higher in ripe fruits than in vegetative and unripe fruits (Gonda *et al.*, 2010). Similarly, sesquiterpene synthase activity is evident in the rind from ripe melon, while it is null in the flesh and unripe rind, where no sesquiterpenes accumulate (Portnoy *et al.*, 2008). Moreover, sesquiterpene synthase genes are found to be transcriptionally regulated during fruit development and are likely to be associated with VOC differences responsible for the unique aroma of different melon varieties (Portnoy *et al.*, 2008). It is common in VOCs that a single enzyme catalyzes the synthesis of multiple products from different substrates (Pichersky & Gang, 2000). Therefore, it has been proposed that this broad substrate specificity is the result of convergent evolution in which new enzymes with the same function have evolved independently in separate plant lineages from a shared pool of related enzymes with similar but not identical functions, providing an extraordinary versatility to VOC blend production patterns in specific plant tissues (Pichersky & Gang, 2000).

1.2. Fruit VOCs and interactions with vertebrates

Vertebrate seed dispersers of fleshy fruits are primarily birds and mammals, though fishes and some reptiles have also been described as minor seed dispersal agents (Fleming & Kress, 2011). It is generally assumed that 140 million years ago, when angiosperms probably originated, seeds were small and had very few dispersal attributes, indicating that dispersal was probably unassisted (Tiffney, 2004). Around the Tertiary (65 million years ago), plant and fruit sizes became larger, strongly affecting evolution of biotic dispersal via the production of fleshy fruits. At this stage probably began the radiation of mammals and birds in the Early Tertiary that mediated more efficient dispersal of larger seeds (Fleming & Kress, 2011).

It is widely assumed that birds use primarily visual stimuli for detecting fleshy fruits because the smell sense is less developed in avian dispersers (Schaefer, 2011). Obviously, this is not the case of nocturnal birds, which have well-developed olfactory bulbs (Corlett, 2011). Moreover, recent works show that at least some birds are able to detect VOCs (Mardon *et al.*, 2010), and they use VOCs as cues to detect insect-infested trees (Mäntylä *et al.*, 2008) or to recognize the fleshy fruits of figs (Borges *et al.*, 2008, Borges *et al.*, 2011). The VOC profiles in ripe fruits of different fig species are quite variable, and different VOC profiles have been observed in bat-dispersed versus bird-dispersed figs (Borges *et al.*, 2008). Interestingly, in the case of *Ficus benghalensis* fruits, night VOCs, when seeds are dispersed by bats, are dominated by esters whereas diurnal VOCs, when figs are consumed by birds, have a greater

representation of terpenes (Borges *et al.*, 2011). Without underestimating color cues, these evidences suggest that VOCs emitted by fig fruits are olfactory cues for either birds or bats.

Bat attraction by fruit VOCs has been investigated in further detail. Different bat species are able to distinguish by smell and clearly prefer ripe over unripe fruits of different plant genera (Luft *et al.*, 2003, Schlumpberger *et al.*, 2006, Hodgkison *et al.*, 2007). Other bat species are attracted by artificial fruits impregnated with essential oils of *Piper gaudichaudianum* and *Ficus insipida* (Bianconi *et al.*, 2007). Among the diverse aromatic profiles of mature fruits from *Ficus* species (Table 1), bats use smell, as well as color, as one of the components of the bat syndrome dispersion (Lomáscolo *et al.*, 2010). This evidence has been used to estimate the occurrence of bat vs. bird dispersal syndromes in 42 co-occuring *Ficus* species in Papua New Guinea. The results indicated that odor (and color) in figs have evolved as predicted by the selective pressures of their frugivores (Lomáscolo *et al.*, 2010). In this study, VOC production was considered quantitatively and not qualitatively and, on average, the number and total peak area of VOCs was lower in bird-dispersed than in bat-dispersed figs, although the differences between these values were not statistically significant. It would be worth testing now whether specific VOCs of VOC mixtures are actually preferred by either bird or bat dispersers.

Most frugivorous mammals rely on olfactory stimuli to detect ripe fruits. Dominy *et al.* (2001) proposed that early primates that were insectivorous and nocturnal were also frugivores, eating dull-colored and smelly fruits. Although most primates later acquired trichromatic vision, which permitted them to become more efficient in selecting ripe fruits, some primates still show extremely acute sensitivity to odors associated with fleshy fruits (Laska *et al.*, 2006). Dichromatic white-faced capuchins rely on olfaction more than trichromatic individuals to detect fig fruits (Melin *et al.*, 2009). Night monkeys (*Aotus*) could detect banana fruits by smell alone in laboratory trials, but diurnal monkeys could not (Bicca-Marques & Garber, 2004). Therefore, it is possible that odor cues remain important in primates (especially in nocturnal primates) to detect fleshy fruits, but it is reasonable to think that other senses such as sight or touch are used almost simultaneously to decide whether to eat a fruit (Dominy *et al.*, 2001). Mammals other than primates with an extraordinary sense of smell, including rodents, also use VOCs to recognize fleshy fruits (Corlett, 2011).

1.3. Fruit VOCs and interactions with insects

Seed consumption by herbivorous invertebrates, mainly insects, dates back to the Devonian (approximately 416 million years ago). However, those insects were probably granivorous and contributed little to the evolution of fleshy fruits (Mack, 2000, Fleming & Kress, 2011). Frugivore insects comprise mainly taxa from the orders Lepidoptera, Hemiptera, Coleoptera, Hymenoptera and Diptera (Sallabanks & Courtney, 1992). Fruit location is a key issue for feeding, mating and reproduction of specialist insects, and it involves the perception of a sequence of olfactory and visual cues (Schoonhoven *et al.*, 2005). Generally, specialized insects are able to distinguish the VOCs emitted by vegetative tissues and unripe and ripe fruits; they are mainly attracted by particular VOC blends of ripe fleshy fruits and in some cases they

are repelled by green tissues (Vallat & Dorn, 2005, Piñero & Dorn, 2009). For example, the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) is attracted by mature apple fruits, but repelled by green fruits, likely through the emission of benzaldehyde and butyl acetate (Vallat & Dorn, 2005). The preference for mature fruits has also been shown for females of the oriental fruit moth (*Cydia molesta*; Lepidoptera: Tortricidae) in apple and peach fruits, while VOCs released by vegetative tissue are behaviorally ineffective (Piñero & Dorn, 2009). For *Ceratitis capitata* (Diptera: Tephritidae) females, the odor of ripe or almost ripe coffee drupes is more attractive than that of unripe drupes, leaves or stems (Prokopy & Vargas, 1996).

Host-fruit recognition usually depends on specific blends of VOCs and not just on the detection of a single compound; however, some blend components are biologically more important than others for the interaction (Light *et al.*, 2001, Reddy & Guerrero, 2004). Moreover, the recognition of a host plant by insects could occur by using either specific ratios of ubiquitous compounds or species-specific compounds (Bruce *et al.*, 2005). For example, polyphagous insects like *Anastrepha obliqua* and *C. capitata* fruit flies (Diptera: Tephritidae) are attracted by different blends of monoterpene compounds emitted by mango and citrus fruits (Papadopoulos *et al.*, 2006, Malo *et al.*, 2012). In contrast, monophagous insects like the olive fly *Bactrocera oleae* (Diptera: Tephritidae) are attracted by a specific VOC blend present in ripening fruits and in leaves. Therefore, those specific VOC cues may have evolutionary significance for monophagous insects. In this context, the case of the monophagous apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae) is particularly interesting, because this insect shifted from its ancestral host hawthorn (*Crataegus spp.*) to cultivated apple approximately 150 years ago, and it has been shown that apple and hawthorn native flies use fruit VOCs to distinguish between both hosts (Linn *et al.*, 2003). Genetic analysis of F2 and backcross hybrid insects indicate that differences in host choices based on VOC discrimination pertain to a few loci, imply cytonuclear gene interactions, and have resulted in reproductive isolation, which has facilitated sympatric insect speciation in the absence of geographic isolation (Dambroski *et al.*, 2005).

Additionally, insects are sensitive to volatiles for social communication and some of them acquire host plant compounds to use them as sex pheromones or sex pheromone precursors (Bruce *et al.*, 2005). Insects such as Tephritidae and Drosophilae Diptera release sex pheromones in response to host fruit chemical emissions that additionally enhance the response of insects to sex pheromones. For example, the combination of male pheromone and host fruit odor is more attractive to female papaya fruit flies, *Toxotrypana curvicauda* (Diptera: Tephritidae), than is either male pheromone or host fruit aroma alone (Landolt *et al.*, 1992). Oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) males are attracted to and feed on methyl eugenol, a VOC emitted by *Terminalia catappa* ripe fruits (Siderhurst & Jang, 2006). Males that have eaten methyl eugenol are more successful in courting and mating with females than males that have not (Shelly & Dewire, 1994).

1.4. Fruit VOCs and interactions with microbes

Microbes are the most abundant frugivores of fleshy fruits, though they have been scarcely studied as such in ecological contexts (Levey, 2004). Fungi that naturally infect a wide range of wild fruits included *Colletotrichum*, *Phomopsis*, *Cladosporium*, *Penicillium* and *Fusarium* species predominantly (Tang *et al.*, 2003, Tang *et al.*, 2005). Coincidently these species cause the most conspicuous opportunistic diseases in commercial fleshy fruits. Fruit softening during ripening facilitates the establishment of opportunistic microbial infections. Ripening is a developmental process usually associated with increased susceptibility to microbial infections in crops (Prusky, 1996) and in wild plants (Tang *et al.*, 2003, Tang *et al.*, 2005).

The effect of VOCs emitted by different plant organs and tissues on microorganisms, either as volatiles or through direct contact, has been widely investigated in crops and forest trees. Many studies indicate that VOCs are toxic to diverse fungi, yeasts and bacteria; however the studies were performed with individual compounds *in vitro* and sometimes these assays used levels far in excess of what is actually present in a fruit (Dorman & Deans, 2000, Daferera *et al.*, 2003). For fleshy fruits of agricultural importance, it has been proposed that VOCs could be used as inhibitors of postharvest fruit spoilage (Archbold *et al.*, 1997). However, when compounds able to control fungal or bacterial growth *in vitro* were tested in fruits, they were inefficient or even stimulated microbial growth. For example, (*E*)-2-hexenal vapor at different doses inhibited *Botrytis cinerea* spore germination and mycelial growth in Petri dishes, but the same doses of this compound applied on strawberry fruits enhanced fungus incidence during storage (Fallik *et al.*, 1998) indicating that VOC toxicity experiments should be performed *in planta*. In orange fruits, D-limonene content is usually low in the exocarp during the 2 to 3 months postanthesis, it dramatically increases when the green fruit develops seeds and remains at high level until the fruit becomes fully mature (Dugo & Di Giacomo, 2002). When oranges were engineered to accumulate very reduced levels of this monoterpene, they became resistant to the bacterium *Xanthomonas citri* subsp. *citri*, to *Penicillium digitatum* and other specialized fungi (Rodríguez *et al.*, 2011). Therefore, D-limonene is required for pathogens to establish infections in mature oranges.

1.5. VOCs as mediators of indirect interactions

Recent works show that considering the third (and the fourth) partners within the community context converts a previously considered parasitism into a multispecies mutualism (Dunn *et al.*, 2008, Palmer *et al.*, 2010). In the fleshy fruit-frugivore context, the ecological importance of such interactions is difficult to predict because, in many cases it is not unequivocally known whether consumers are actually seed dispersal or predator agents under different environmental circumstances. Pulp feeder “antagonists” may have a positive effect on seed and seedling fate (Fedriani *et al.*, 2012), seed predators may facilitate seed dispersal (Norconk *et al.*, 1998), legitimate seed dispersal animals may have negative effects on plant

population dynamics (Loayza & Knight, 2010), and the interference between mammal seed dispersers and insect seed predators may ultimately benefit seed dispersal (Visser *et al.*, 2011). Considering legitimate vertebrate frugivores and insects/microbes as competitors for the fleshy fruit, vertebrates should take the fruit earlier than microbes and/or pests for successful seed dispersal.

1.5.1. Fruit VOCs and interactions among insects

In spite of the high cost for the plants to produce VOCs in ripe fruits and the importance of ripe fruit aromas for the life cycle of specialized insect frugivores, as mentioned previously, insects are considered harmful to plant fitness and are much more involved in fruit and seed predation than in seed dispersal (Janzen, 1977). Fruit VOCs might be involved in indirect defense against insect consumers by attracting pest parasites. For example *Leptopilina boulardi* (Hymenoptera: Figitidae), a parasite of *Drosophila melanogaster*, is attracted to VOCs emitted by fly-infested banana or pear fruits but not to non-infested ones (Couty *et al.*, 1999). VOCs emitted by coconut fruits infested by *Aceria* guerreronis (Acari: Eriophyidae) are more attractive for two mite predators (*Neoseiulus baraki* and *Proctolaelaps bickleyi*) than uninfected ones (Melo *et al.*, 2011). While indirect defenses are widely documented in plant vegetative tissues (Heil, 2008), there are no reported cases of indirect defenses against pathogens in ripe fruits.

1.5.2. Fruit VOCs and interactions with insects and vertebrates

VOCs may be involved in vertebrates distinguishing between infested and uninfested fruits. In general, avian consumers prefer intact mature fruits and reject fleshy fruits infested by insects (Traveset *et al.*, 1995, García *et al.*, 1999). Deterrent effects of infested fruits on avian frugivores are considered an evolutionary necessity for insect frugivores to escape predation (Sallabanks & Courtney, 1992). In another scenario, legitimate seed dispersers may be attracted by VOCs from infested fruits and consume them without major problems (Drew, 1987, Valburg, 1992). In a recent work the attraction of birds to heavily insect-infested trees is directly correlated with the emission of several specific terpene VOCs by the trees (Mäntylä *et al.*, 2008). In these cases, insects would directly benefit birds by enhancing the nutrient content of the fruit and indirectly benefit host plants by facilitating vertebrate seed dispersal. Additionally, a recent review shows several examples of insects inhabiting seeds from wild ripe fruits that can survive passage through the entire digestive tract of seed dispersing vertebrates, including many bird species and also primates, which suggests that this process may also favor insect dispersal (Hernández, 2011). As described above, most mammals may primarily use the sense of smell instead of sight to locate fruits. There are references of ungulates, primates and rodents being attracted by fruits infested by insects (Redford *et al.*, 1984, Rader & Krockenberger, 2007, Bravo, 2008), which suggests that they may be able to also distinguish VOCs of infested fruits. However, the results for any of the intervening elements of these

tritrophic interactions are often unpredictable and could conversely lead to killed larvae, destroyed seeds or toxicity for the vertebrate (Or & Ward, 2003).

1.5.3. Fruit VOCs and interactions with microorganisms and insects

Ripe fruit VOCs are also important in trophic interactions involving microbes and insects. Insects that feed on overripe, wounded or decomposing fruits commonly exploit VOCs induced by microbial action on damaged tissues for host finding (Hammons *et al.*, 2009). The microbial detoxification of pulp secondary metabolites and the breakdown of carbohydrates refractory to insect digestive enzymes on one hand, and microbial dissemination on the other, may explain such mutualisms (Berenbaum, 1988). The Japanese beetle *Popillia japonica* (Scarabaecidae: Rutelinae) facilitates feeding of the green June beetle *Cotinis nitida* (Scarabaecidae: Cetoniinae) on grapes by biting through the skin and introducing yeasts in such wounds. Yeasts eliciting fermentation VOCs are exploited by both sexes of *C. nitida* for host finding (Hammons *et al.*, 2009). Nitidulid sap beetles (*Carpophylus humeralis*; Coleoptera: Nitidulidae) are attracted to VOCs from fermenting fruits and vegetables (Nout & Bartelt, 1998). There are other insects that prefer damaged fruit, such as the Asian lady beetle *Harmonia axyridis* (Coleoptera: Coccinellidae) (Koch *et al.*, 2004) or the medfly *Ceratitis capitata* (Papadopoulos *et al.*, 2006). Fruit flies, such as *Bactrocera tryoni* and *B. oleae*, have symbiotic bacterial associations, which can improve the nutritive quality of their fruit diet and may play a role in detoxifying plant secondary chemicals (Fletcher, 1987). Recently, it has been demonstrated that specific odors from rotten fruits sexually attracted male fruit flies (*Drosophila melanogaster*; Diptera: Drosophiladae) (Grosjean *et al.*, 2011). *D. melanogaster* larvae consume yeasts growing on rotting fruit and have evolved resistance to fermentation products. Ethanol is produced in overripe and rotten fruits through sugar fermentation by infecting microorganisms. Interestingly, it has been shown that alcohol protects *D. melanogaster* from endoparasitoid wasps, thus flies consuming alcohol do not need to activate the stereotypical antiwasp immune response. Therefore, fly larvae seek for ethanol containing food and likely use it as an antiwasp medicine (Milan *et al.*, 2012).

Many butterflies in tropical forests feed on fruits that have fallen to the ground. This substrate differs in many ways from floral nectar, and it has been established that fruit-feeding butterflies use specific VOC cues from the fruits and fermentation products to locate their food (Molleman *et al.*, 2005, Sourakov *et al.*, 2012). From the plant's perspective, the presence of microbes and insects in damaged fruits for predation may favor the possibility of undamaged fruit to attract legitimate seed dispersers. Alternatively, VOC compounds emitted by wounded fruits may play an indirect role in plant defense by facilitating attraction of natural enemies of the damaging fungus and/or insect.

1.5.4. Fruit VOCs and interactions with microorganisms and vertebrates

Little is known about whether VOCs emitted by ripe fleshy fruit infected by microbes are distinguished by vertebrates. Primates, rodents and bats have demonstrated sensitivity to ripe fruit-associated odors, such as those of esters, aldehydes and alcohols (Laska *et al.*, 2006, Sánchez *et al.*, 2008). Thus, it is possible that these frugivores are able to recognize rotten fruits through the VOCs emitted by the fruit, the microbe or both. The only volatiles from rotten fruits that have been studied with some detail for their interaction with vertebrates are alcohols, specifically ethanol. Dudley (2000) proposed that ethanol could represent an important sensory cue to primates because of its association with caloric and physiological rewards. Moreover, Dominy (2004) have suggested that the ethanol content (together with soft texture) could have been cues with strong adaptive advantages for primates, and the selection of fruits on this basis may be a long-standing trend in primate evolution. However, Levey (2004) concluded that frugivores usually prefer ripe, non-rotting fruits over damaged or rotting fruits (in which the concentration of ethanol is supposed to be higher). In Egyptian fruit bats, ethanol neither stimulated visits to nor ingestion of ripe fruit (Sánchez *et al.*, 2008). Studies performed with wild individuals of several frugivorous and nectarivorous bat species have shown that these animals tolerate relatively high levels of ethanol without negative effects on their flight and echolocation performance (Orbach *et al.*, 2010). These authors believe that frugivorous bats may be used to eating fruits rich in ethanol when other healthy fruits are unavailable.

Most birds and small mammals prefer ripe, uninfected fruits to rotten fruits (Borowicz, 1988, Cipollini & Stiles, 1993) except for some specialized rodents (Borowicz, 1988). The omnivorous diet of such rodents may be an adaptation for enhanced tolerance to microbes in rotten fruits and for efficient competition with most vertebrates for these resources. Nevertheless, rotten fruits are generally non toxic to vertebrates. When just rotten fruits are offered or ripe fruits are scarce, those are readily consumed (Borowicz, 1988, Cipollini & Stiles, 1993, Levey, 2004, Sánchez *et al.*, 2008). Therefore, microbes and vertebrates may not be strong competitors, especially when ripe fruit resources are limited. Microbes may benefit by being ingested by frugivores and dispersed in their feces (Abranches *et al.*, 1998). From the plant's perspective, there could be two different scenarios. Deterrence to microbes may be important for the fruit if those infected seeds may compromise their viability (Janzen, 1977), particularly in the case of small fruits from shrubs and small trees or in the case of seeds without coats. Neutral or attraction responses may be favored by ripe fruits when seed dispersal is not compromised, mainly in the cases of large fruits with large pericarps and/or coated seeds. Microbial infection and/or insect infestation would favor fruit crushing and/or abscission and then access of terrestrial animals to the fruit.

1.6. VOCs in fruit crops and agriculture

Most VOC research has been conducted in agricultural species; it has contributed greatly to our understanding of the role of VOCs in plant-insect and plant-microbe interactions and it is providing many applied tools in agriculture. For example, the pear ester ethyl (2*E*, 4*Z*)-2,4-decadienoate is highly attractive and used to monitor both males and females of the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) (Light *et al.*, 2001). Field-trapping tests show that *Argyresthia conjugella* (Lepidoptera: Yponomeutidae) females are attracted to VOCs identified from rowanberries, and that a blend of 2-phenyl ethanol and anethole is sufficient to show a strong attraction (Bengtsson *et al.*, 2006). In addition, identification of VOCs specifically emitted from infested fruits and attractive to natural enemies would allow the development of lures to be used in integrated pest control programs. Alternatively, based on the synergism between insect pheromones and VOCs, it has been suggested that mating disruption dispensers could be developed for certain pests using small amounts of expensive pheromonal ingredients and small amounts of inexpensive plant VOCs (Reddy & Guerrero, 2004). In addition, pheromone based mass annihilation strategies are nowadays successfully employed to control Diptera and Coleoptera insects in agriculture (Witzgall *et al.*, 2010). These strategies, unlike detection and monitoring (where only a small proportion of a population needs to be sampled) requires the use of the most attractive lure and may become far more efficacious if lures include fruit VOCs involved in ovipositional and/or feeding cues. On the other hand, blends of VOCs emitted by non-hosts are usually neutral but they could be also repellent, though this aspect has been largely overlooked (Reddy & Guerrero, 2004). For example, the psyllid *Diaphorina citri* (Hemiptera: Psyllidae), transmission vector of the bacterium that causes the Huanglongbing (HLB) disease of citrus, is attracted to VOCs emitted by citrus host plants (Patt & Sétamou, 2010), while VOCs from the non-host guava have been shown to be repellent and also to inhibit the psyllid response to the normally attractive citrus odor (Rouseff *et al.*, 2008, Onagbola *et al.*, 2011). Identification of repellent VOC blends from guava leaves and fruits would allow developing strategies to control the psyllid population and thus HLB spreading. Finally, the growing number of reports on the involvement of specific VOCs in plant defense together with the current progress on the knowledge of their biosynthesis and regulation is allowing the use of plant genetic engineering for improving plant resistance to pests and diseases. For example, D-limonene production, which represents up to 97% of total VOCs in orange fruit peel, has been downregulated by overexpressing an antisense construct of a D-limonene synthase gene (Rodríguez *et al.*, 2011). Transgenic orange fruit peels with up to 85 times reduced D-limonene accumulation were less attractant to males of the citrus pest medfly (*Ceratitis capitata*, Diptera*:* Tephritidae) and strongly resistant to fungal and bacterial pathogens (Rodríguez *et al.*, 2011). This work illustrates how fruit VOC emissions can be manipulated providing novel strategies for pest and disease management without altering important agronomic traits. Our most recent results indicate that D-limonene upregulation is highly associated with a general depletion of defenses in mature fruit peels (our unpublished results),

suggesting that there is a tradeoff between costly production of monoterpenes for attraction of frugivores and decreased general defense.

The foundations for the aromas associated with most fruits existed long before crop domestication (Goff & Klee, 2006). However, in recent times, breeding with the aim of improving yield, size and postharvest fruit shelf life has affected some original sensory qualities, including aromas. For example, in a commercial tomato cultivar, the modification in some VOCs concentration has been detected when comparing with a wild relative (Goff & Klee, 2006). In strawberry, marked differences in the production of specific sesqui- and monoterpenes between cultivated and wild strawberries species have been related with the activity of just one enzyme (Aharoni *et al.*, 2004). The sesquiterpene profile also varies greatly in the rind of melons resulting from breeding programs (Portnoy *et al.*, 2008).

Some fruit tree crops, such as *Malus* (apples), *Pyrus* (pears) and *Prunus* (peachs, nectarines, plums, etc.) species, have been subjected to extensive breeding programs, but in general fruit trees have very long juvenile periods that have delayed the possibilities of producing new varieties through breeding, at least when comparing with annual crops. Consequently, varieties from *Citrus* (including oranges, lemons, mandarins, limes, grapefruits, etc.), and most (when not all) varieties from other tropical and subtropical fruit trees including some with highly odorous fruits (mango, guava, avocado, durian, passionfruit, breadfruit, pitanga, mangosteen, loquat, quince, etc.) are species, natural hybrids or budsport mutants selected in nature by men in more or less recent times. Within a given genus, VOC profiles could be similar, at least qualitatively, in ancestral types (maintained in germplasm banks) and in relatively recent cultivated hybrids (e.g., citrus types; Table 2) or could be variable among close species with drastic changes in some specific major compounds (e.g., *Psidium* species; Table1).

1.7. Concluding remarks and future prospects

As illustrated in this review, fruit VOC profiles are diverse, change during ripening, and have important effects on both mutualists and antagonists. Most research in this topic has been conducted so far in agricultural species and information regarding wild fruits is scarce, totally absent when pertaining to the role of VOCs in interactions with vertebrates, insects or microbes in nature. Therefore, it would be indispensable additional information on wild fruit VOCs and on their interaction with frugivores in order to assess their ecological relevance and to make any strong evolutionary inference about how aromas may have evolved due to selective pressure from surrounding living organisms. Moreover, there is still little information on how VOCs changes might affect species interaction. Wild and domesticated species and cultivars with different VOC profiles are excellent tools to investigate the importance of VOCs for fruit interactions with their frugivores. Moreover the possibility of generating mutants and transgenic plants affected in VOC biosynthetic or signal transduction pathways could allow determining key compounds involved in fruit-frugivore interactions.

Table 2. Changes(%) in the total amount of the volatile organic compounds in different ancestral, wild and natural hybrid *Citrus* types.

	Monoterpenes		Sesquiterpenes
	Total	**limonene**	**Total**
Ancestral species			
Citrus grandis	50.3-100.0	48.9-95.6	Tr-4.5
Citrus medica	53.4-100.0	51.2-93.6	Tr-8.3
Citrus reticulata	90.6-100.0	87.4-91.7	Tr
Wild species from subgenus *Papeda*			
Citrus hystrix	55.3-100.0	2.8-14.2	Tr-3.1
Hybrids			
Citrus aurantium	82.9-100.0	80.1-95.8	Tr
Citrus paradisi	86.31-100	83.4-93.8	Tr-5.1
Citrus aurantifolia	69.7-94.9	38.4-50.0	5.36-12.87
Citrus clementina	85.1-100.0	83.0-95.1	Tr-2.2
Citrus bergamia	31.5-100	24.1-54.9	Tr-2.2
Citrus limon	71.6-100	59.6-76.2	Tr-3.0
Citrus junos	70.7-100.0	60.4-82.4	Tr-4.7
Citrus unshiu	42.8-100.0	41.2-90.7	Tr-2.7
Citrus sinensis	93.4-100.0	91.0-97.0	Tr-1.1

Obtained from Dugo & Di Giacomo, 2002.

Tr, traces.

Information about fruit VOCs evolution and the influence of frugivores in this process is also scarce. Selective pressures on fruit VOC production and emission may be exerted not only by legitimate seed dispersal animals but also modulated and/or abolished by less apparent but often more common frugivore agents. Whether attraction of seed dispersal and fruit predator agents through fleshy ripe fruit-emitted VOCs is positive or detrimental for plant fitness, and therefore the net effect of these trophic interactions with multiple partners should be carefully considered and investigated. Seed predation could be a selective force on fruit VOC emission in some cases, as has been suggested for fruit color polymorphisms in *Acacia ligulata* (Whitney & Stanton, 2004). Therefore, to understand whether and to what extent diversification of VOCs in fleshy fruits has been shaped by frugivores will require overcoming the traditionally considered dichotomy of seed dispersers vs. seed predators and investigating the interactions among the multiple partners of the network as a whole. Additionally, a broad view of VOCs is necessary, together with other traits in each specific fruit species as integrated cues for frugivory, because it is unlikely that such different cues have evolved independently. Furthermore, it is necessary to

deep our understanding on how VOCs are perceived by frugivores animals and to what extent the response to odors are learned or innate, which may have important consequences when considering the presumed co-evolution of fleshy fruits and frugivores. As envisaged from this review the role of VOCs in species interaction may be quite complex due to multi-trophic, direct and indirect interactions, synergistic effects of compounds, etc, thus integrative studies are necessary that take into account the full fitness costs and benefits of particular traits.

Unraveling how differences and singularities between fruit VOCs of different species, cultivars and mutants or transgenic plants are explained from a molecular and biochemical perspective and how they are linked to different direct and indirect trophic interactions will require multidisciplinary collaborative work from chemists, geneticists, ecologists and biologists in coming years. Comparative transcriptomic, proteomic and metabolomic datasets in both fruits and vertebrates/insects/microbes would provide new valuable data for clarifying these highly complex and interactive processes. Since not all studies accomplished to date were reproducible out of laboratory settings, it will be of major importance the ecological and/or agricultural realism of new experiments. In agricultural contexts, studies on fruit VOCs may help to develop potential alternatives to toxic synthetic agrochemicals for the control of devastating pests and diseases. In conclusion, future work can improve our basic understanding of plant ecology and evolution and may have important applications in agriculture.

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2. OBJECTIVES

2. Objectives

1. To modify the D-limonene content in the peel of sweet orange fruits cv. Navelina and cv. Pineapple by using two genetic transformation strategies:

a. Down-regulation of D-limonene and related terpenes by using a D-limonene synthase gene in antisense under the control of the constitutive promoter El2p35S

b. Up-regulation of D-limonene and related terpenes by overexpression of a D-limonene synthase gene under the control of the constitutive promoter El2p35S

2. To perform the genotypic, phenotypic and biochemical analyses of the fruits from the genetically transformed plants.

3. To determine the possible effects of down-regulation of D-limonene content in orange peels over an important citrus pest.

4. To determine the possible effects of down-regulation of D-limonene content in orange peels over different specialized citrus pathogens.

5. To study the transcriptome of antisense vs. empty vector control fruit peels by using cDNA microarrays to identify changes associated to the D-limonene down-regulation in orange fruits, putatively involved in defense responses against specialized pathogens. To analyze phytohormone accumulation and signaling before and after challenge inoculation to further elucidate the resistance mechanism.

3. RESULTS: CHAPTER 1.

Terpene downregulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens

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Abstract

Plants use volatile terpene compounds as odor cues for communicating with the environment. Fleshy fruits are particularly rich in volatiles that deter herbivores and attract seed dispersal agents. We have investigated how terpenes in citrus fruit peels affect the interaction between the plant, insects and microorganisms. Because limonene represents up to 97% of the total volatiles in orange fruit peel, we chose to downregulate the expression of a limonene synthase gene in orange plants by introducing an antisense construct of this gene. Transgenic fruits showed reduced accumulation of limonene in the peel. When these fruits were challenged with either the fungus *Penicillium digitatum* or with the bacterium *Xanthomonas citri* subsp*. citri*, they showed marked resistance against these pathogens that were unable to infect the peel tissues. Moreover, males of the citrus pest *Ceratitis capitata* were less attracted to low limonene-expressing fruits than to control fruits. These results indicate that limonene accumulation in the peel of citrus fruit appears to be involved in the successful trophic interaction between fruits, insects and microorganisms. Terpene downregulation might be a strategy to generate broad-spectrum resistance against pests and pathogens in fleshy fruits from economically important crops. In addition, terpene engineering may be important for studying the basic ecological interactions between fruits, herbivores and pathogens.

Introduction

Plants produce a wide variety of secondary metabolites, many of which are volatile compounds that are released by leaves, flowers, fruits and roots. These compounds serve as signals between plants and within distal parts of the same plant (Baldwin, et al., 2006). They are also involved in protecting the plant against abiotic stress (Gershenzon and Dudareva, 2007), defending the plant against pests and pathogens (Vickers, et al., 2009; Bednarek and Osbourn, 2009), and attracting herbivore predators (Kessler and Baldwin, 2001; Degenhardt, et al., 2009) and pollinators (Kessler, et al., 2008). Volatile compounds that are emitted by flowers greatly contribute to the plant's reproductive success and survival in natural ecosystems (Kessler, et al., 2008). In addition, fruits are generally rich in terpene compounds that determine their specific bouquet and may attract mutualists and repel antagonists, as in animal-pollinated flowers (Junker and Bluthgen, 2010). Flavor volatiles in plants (particularly in fruits) are linked to human selection of genotypes and their use for nutritional, health or industrial purposes (Goff and Klee, 2006).

It is widely accepted that the primary function of terpene compounds in immature fruit is to defend against all types of potential consumers. Changes in these substances occur during maturation, in combination with changes in texture, taste and color. These changes are necessary to attract frugivorous animals for fruit predation and seed dispersal (Janzen, 1977; Herrera, 1982; Sallabanks and Courtney, 1992). Fruit traits are thought to evolve in response to the sum of selective pressures exerted by mutualists and antagonists (Whitney and Stanton, 2004; Cazetta, et al., 2008). Nonetheless, proof that a specific fruit terpene acts as an attractant or repellent for specific pests or pathogens has not been obtained (Dudareva and Pichersky, 2008).

In the last decade, a series of important studies have been published on plant volatiles as repellents of pests and as attractants of herbivore predators (Aharoni, et al., 2003; Arimura, et al., 2000; De Moraes, et al., 2001). The results from these studies seem to suggest that it may be possible to modulate plant volatile emission through metabolic engineering to improve the plant's defense against pests. The overexpression of the precursor for a linalool/nerolidol synthase from strawberry in transgenic *Arabidopsis* led to accumulation of high levels of linalool and consequently to the induction of resistance against aphids (Aharoni, et al., 2003). The overexpression of this transgene in mitochondria of *Arabidopsis thaliana* leads to the accumulation of nerolidol and a derived homoterpene, (*E*)-DMNT, which attract insect carnivore predators that are natural enemies of pest mites (Kappers, et al., 2005). In addition, the overexpression of the gene encoding a sesquiterpene synthase, *TPS10*, in transgenic *Arabidopsis* plants attracts parasitic wasps due to the emission of high levels of sesquiterpenes, which are normally released when the larvae of these wasps chew the leaves (Schnee, et al., 2006). More recently, the overexpression of the gene of a trans-caryophyllene synthase from oregano in transgenic corn makes the roots attract nematodes that protect the plant from beetles (Degenhardt, et al., 2009). The transgenic overexpression of a precursor gene of a

pachulol synthase in tobacco together with the farnesyl diphosphate synthase, a precursor of sesquiterpenes, leads to high accumulations of pachulol and 13 other sesquiterpenes, which make the plants highly resistant to larvae of insect pests (Wu, et al., 2006).

The role of different terpenoid compounds in pathogen resistance is well documented, particularly in forest trees, but the overexpression of precursors of these genes as a biotechnology strategy for plant protection has not yet been reported (Trapp and Croteau, 2001).

In summary, the use of metabolic engineering to induce resistance against biotic agents represents an alternative technology to the use of expensive and highly toxic fungicides, bactericides and pesticides. The use of this technology could also result in increased product quality.

The external colored peel of citrus fruits, known as the flavedo, is embedded with thousands of oil glands containing terpene volatile compounds. (+)-Limonene is the most abundant of these compounds (97% of total terpene in orange fruits) (Dugo and Di Giacomo, 2002). The extraordinarily high amount of limonene that accumulates in orange oil glands suggests an important biological role for this terpene compound in fruit aroma and in the plant's interactions with the environment. Recently, cDNAs for monoterpene synthases have been isolated from citrus, including several (+)-limonene synthases (Lucker, et al., 2002; Shimada, et al., 2004). The genetic modification of tobacco plants with three of these monoterpene synthases and their subsequent combination in one plant by crossing, showed that it was possible to increase the amount and alter the composition of monoterpenoids produced in those plants (Lucker, et al., 2004).

To determine whether the accumulation of limonene in fruits has a defensive function *in planta*, we manipulated the terpene content in oil glands with an antisense downregulation of the (+)-limonene synthase gene in mature sweet orange plants (*Citrus sinensis* L. Osb. cv. Navelina). Unexpectedly, transgenic fruits were resistant to economically important fungal and bacterial citrus pathogens and showed the repulsion of a major citrus insect pest.

Results and Discussion

Molecular characterization and volatile composition of transgenic plants

Transgenic plants expressing a citrus limonene synthase gene (*CitMTSE1*) in the antisense orientation were generated, and integration of the transgene was confirmed by both PCR and Southern blot analyses of the genomic DNA (Supplemental Fig. S1). Antisense (AS) transformants and their fruits were visually indistinguishable from those transformed with an empty vector (EV) and wild-type plants. Moreover, fruit quality traits (weight and volume, color index, acidity, maturity index, juice volume and vitamin C content) were not affected by this genetic modification. The transgenic lines AS1, AS3, AS6 and AS7 were further investigated.

Total terpene profiles in fruit peels from the AS lines showed a range of phenotypes, with slight (AS6) to strong (AS1, AS3 and AS7) decreases in limonene accumulation compared to fruits from the EV control (Fig. 1). The accumulation of other monoterpenes, sesquiterpenes and monoterpene aldehydes also decreased, whereas the level of monoterpene alcohols increased. Thus, lines AS1, AS3 and AS7 produced at least 85 and 50 times less (+)-limonene and β-myrcene, respectively, than the EV control but increased the production of monoterpene alcohols (more than 10 times for β-citronellol and nerol) and some esters (more than three times for geranyl acetate)(Supplemental Table S1), likely due to a partial redirection of the pathway. Downregulation of monoterpenes other than limonene might be also explained by the formation of multiple products from a single monoterpene synthase (Lucker, et al., 2002; Shimada, et al., 2004).

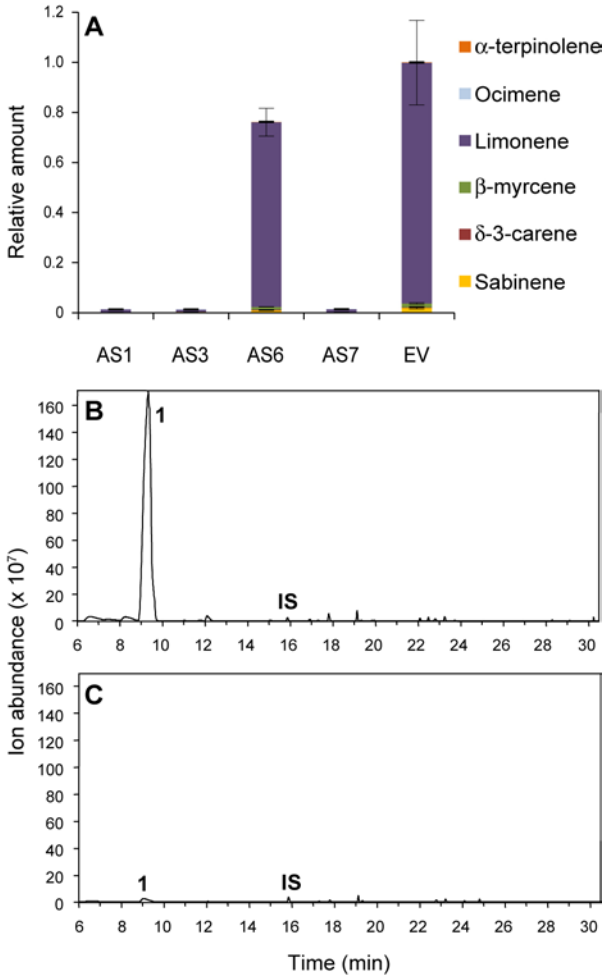

Figure 1. Phenotypes of orange flavedo in antisense (AS) and control-treated (EV) Navelina sweet orange plants. (A) Relative amount of individual terpenes is presented as a percentage (given as a fraction of unity) area of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant. (B,C) Representative total ion chromatograms (GC-MS) of the volatile profile for orange fruit flavedo from EV (B) and AS7 transgenic plants (C). Peaks number one and IS correspond to limonene and the internal standard (2-octanol), respectively.

Response of citrus pathogens to limonene downregulation in transgenic fruits

To test whether terpene downregulation confers resistance or susceptibility to different citrus pathogens, transgenic AS fruits were challenged with *Penicillium digitatum* (Pers.) Sacc, a fungus that causes the green mould rot in citrus fruits, and *Xanthomonas citri* subsp.*citri* (ex Hasse), the bacterium that causes citrus canker disease. Terpene downregulation in AS flavedo was confirmed in samples taken five days after inoculation (Supplemental Figs. S2 and S3).

P. digitatum causes the most damaging post-harvest disease of citrus fruits worldwide. It does not cause the decay of other non-citrus fruits or vegetables. The etiology of the disease is well understood. Dormant *Penicillium* spores present on the fruit's surface become active if the peel is injured. The spores germinate rapidly and colonize the injured tissue. Citrus fruit volatiles play an important role in host recognition by *P. digitatum*. Flavedo oil from several citrus species and volatiles emitted from injured oranges were reported to stimulate *in vitro* germination of *P. digitatum* conidia (Droby, et al., 2008). In the case of citrus fruit, volatiles are released from ruptured oil glands following mechanical wounding, facilitating the infection process (Droby, et al., 2008). Inhibitory effects have also been attributed to citrus monoterpenes, however, including limonene and derivatives (Ben-Yehoshua, et al., 2008).

When mature AS and EV fruits were inoculated with *P. digitatum*, the percentage of infected wounds and wounds with spores in EV fruits eight days post-inoculation were 60.8% and 54.9%, respectively, but only 18.5% and 7.4%, in AS7 fruits. Results with AS1 and AS3 lines were similar, with no significant difference found in the area under the disease progress curve (AUDPC) for infected wounds ($P < 0.05$, Fig. 2). To assess whether the reduced content of limonene and other terpenes causes an increased susceptibility to other non-pathogenic fungi, we inoculated AS and EV fruits with *P. minioluteum*. No infection occurred in either AS or EV fruits, indicating that general terpene downregulation does not alter the interaction with other non-specialized microorganisms.

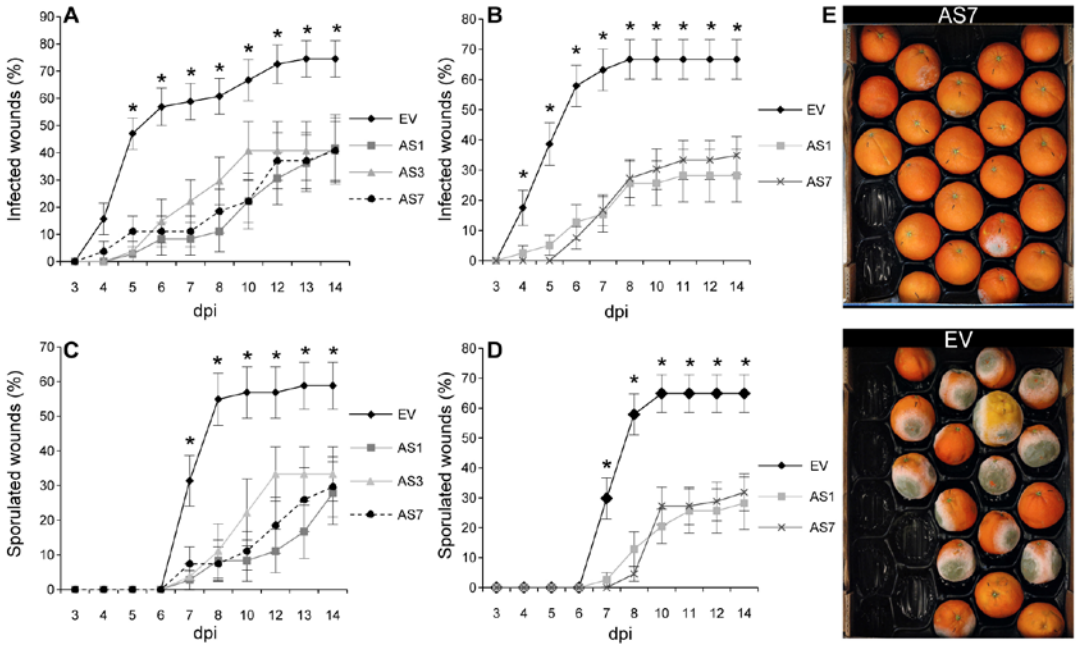

Figure 2. Transgenic expression of *CitMTSE1* in the antisense orientation in orange plants confers fungal resistance. (A, B, C, D, E). Evolution of the disease caused by the fungus *Penicillium digitatum* in mature orange fruits inoculated with 1×10^4 spores mL^{-1}: percentage of infected (A, B) and sporulated (C, D) wounds in orange fruits of EV and AS lines in two consecutive fruiting seasons—season 1 (A, C) and season 2 (B, D). Results are the average ± s.e.m. ($n \geq 10$). dpi: days post-inoculation. *, $P < 0.05$ using Fisher's Protected LSD test. We repeated all experiments at least twice and obtained similar results. (E) AS and EV fruits eight days after inoculation.

To study whether the resistance phenotype was related to limonene downregulation or was indirectly induced as a consequence of increased monoterpene alcohols, *P. digitatum* challenge assays were performed *in vitro* with pure (+)-limonene and nerol. Results showed that both compounds had a pronounced stimulatory effect on germination of *P. digitatum* spores directly related to their concentration (Fig. 3). Germ tube elongation response was much higher with limonene at low concentrations, while nerol at high levels had just a slight inhibitory effect (Fig. 3).

To provide further evidence that downregulation of limonene was directly responsible for the resistance response, AS and EV orange fruits were supplemented with limonene and then inoculated with *P. digitatum*. The percentage of infected wounds in EV and AS3 fruits four days post-inoculation were 77.3% and 80.0%, respectively. Results with AS7 were similar, with no significant difference found for infected wounds ($P < 0.05$, Fig. 4). This confirmed the critical importance of limonene accumulation levels on fruit susceptibility to *P. digitatum*.

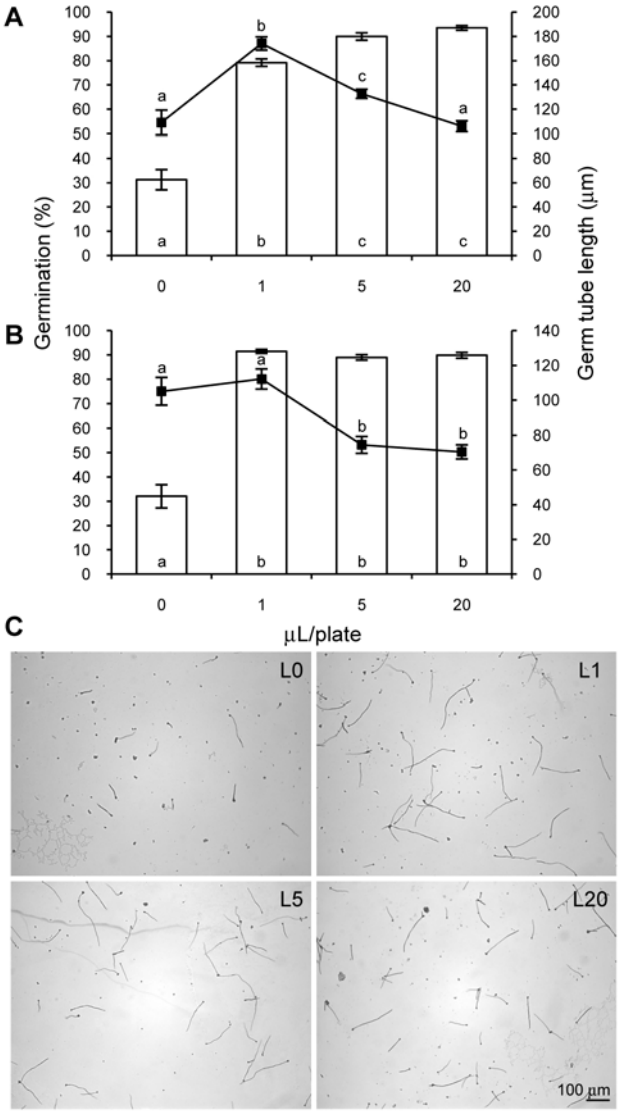

Figure 3. Effect of the monoterpenes limonene (A) and nerol (B) in Petri dish assay on percent germination (□) and growth (■) of *Penicillium digitatum*. Results are average of three microscopic fields of different colonies containing at least thirty spores each ± s.e.m. (n = 15). Treatments with different letters are significantly different at $P < 0.05$ using Fisher's Protected LSD test. (C) Images shown are light micrographs at 10x magnification of germinating spores in different concentrations of limonene. Scale bar indicate 100 µm.

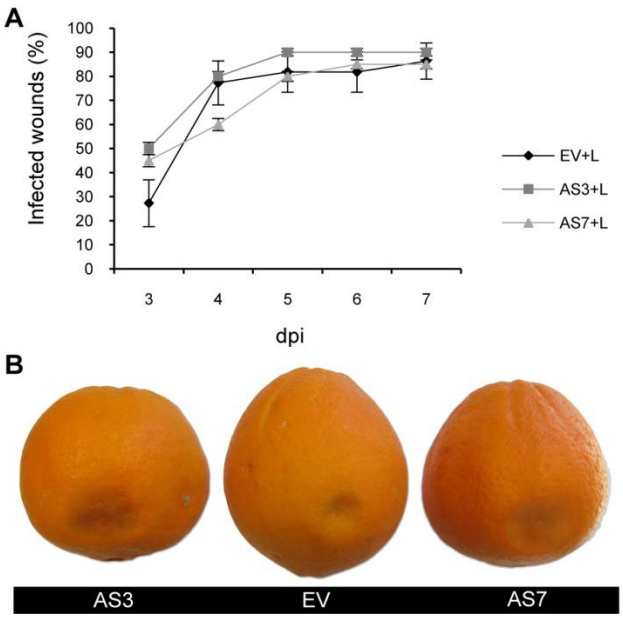

Figure 4. Supplementation of pure limonene to the peel of EV and AS fruits confers early infection by the fungus *Penicillium digitatum*. (A, B) Evolution of the disease caused by the fungus *P. digitatum* in mature orange fruits inoculated with 1×10^4 spores mL^{-1}. (A) percentage of infected wounds in orange fruits of EV and AS lines with 5 µL of limonene applied to the wound. Results are the average ± s.e.m. (n = 10). dpi: days post-inoculation. No significant differences were found at $P < 0.05$ using Fisher's Protected LSD test. (B) AS and EV fruits four days after inoculation.

We also challenged fruits with *X. citri* subsp*. citri*, an economically important citrus pathogen that reduces fruit yield and quality and causes quarantine restrictions for the movement of fresh fruit from affected areas (Graham, et al., 2004). This bacterium enters the host plant tissues through stomates and wounds and multiplies in the lesions in leaves, stems and mainly in the fruits. All above-ground tissues of the citrus plant are maximally susceptible to infection by *X. citri* subsp*. citri* during the last half of the expansion phase of growth (Graham, et al., 2004). The percentage of infected wounds in green fruits inoculated with the bacterium at four weeks post-inoculation was 65.7% in EV fruit, whereas few infections were observed in inoculated AS fruits ($P < 0.05$, Fig. 5). Peel pieces from lesions of inoculated AS and EV fruits yielded *X. citri* subsp. *citri* colonies when cultivated in an appropriate medium. This result suggests that the presence of a threshold amount of limonene may be necessary for the bacterium to establish infection in citrus fruits. However, we cannot rule out in this case that other up- or down-regulated compound/s in AS fruits may contribute to the resistance phenotype observed.

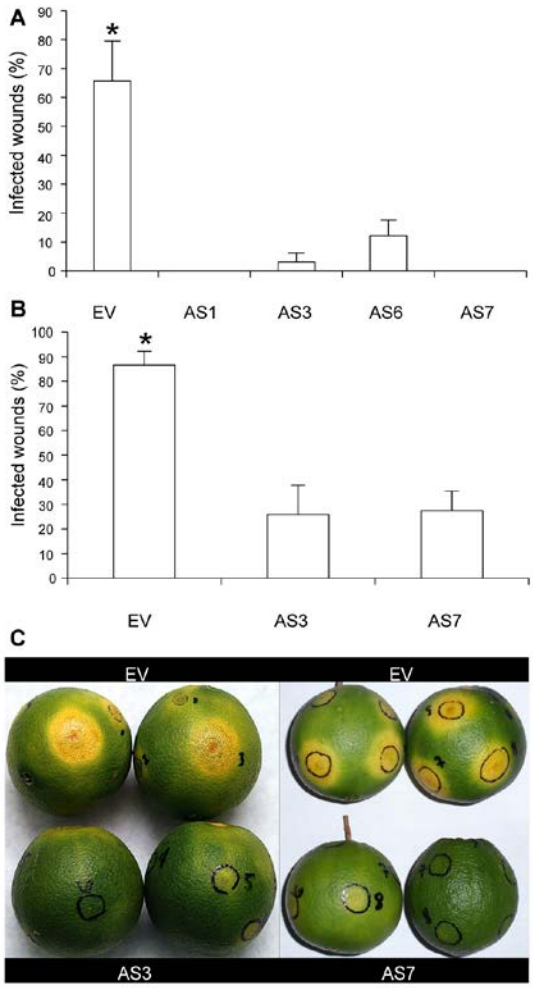

Figure 5. Transgenic expression of *CitMTSE1* in the antisense orientation in orange plants confers bacterial resistance. (A, B) Number of wounds with symptoms after inoculation of green mature orange EV and AS fruits with 10^6 CFU mL^{-1} of the bacterium *Xanthomonas citri* subsp. *citri* in two consecutive seasons. Results are the average ± s.e.m. ($n \geq 10$). *, $P < 0.05$ using Fisher's Protected LSD test. We repeated all experiments at least twice and obtained similar results. (C) AS and EV fruits at four weeks post-inoculation.

To assess whether or not this response is genotype-dependent, both pathogens were inoculated onto Pineapple sweet orange fruits with or without terpene downregulation. The results paralleled those of the Navelina sweet orange (Fig. 6 and Supplemental Table S2). This finding suggests that disease resistance is directly correlated with limonene downregulation. Thus, this control strategy could be extended to other citrus species and varieties that accumulate high levels of limonene in the flavedo, such as most sweet oranges, mandarins, grapefruits, and their hybrids. It would be worth testing whether this resistance phenotype could be extended to other important bacterial and fungal citrus pathogens. The relationship between terpenoid production and the activation of defense mechanisms is not fully understood and further research is required. Recent work has shown that glucosinolates, a group of secondary metabolites that are important for preventing damage caused by herbivores in brassicas, are required for the plant's defense against certain pathogens (Clay, et al., 2009; Bednarek, et al., 2009).

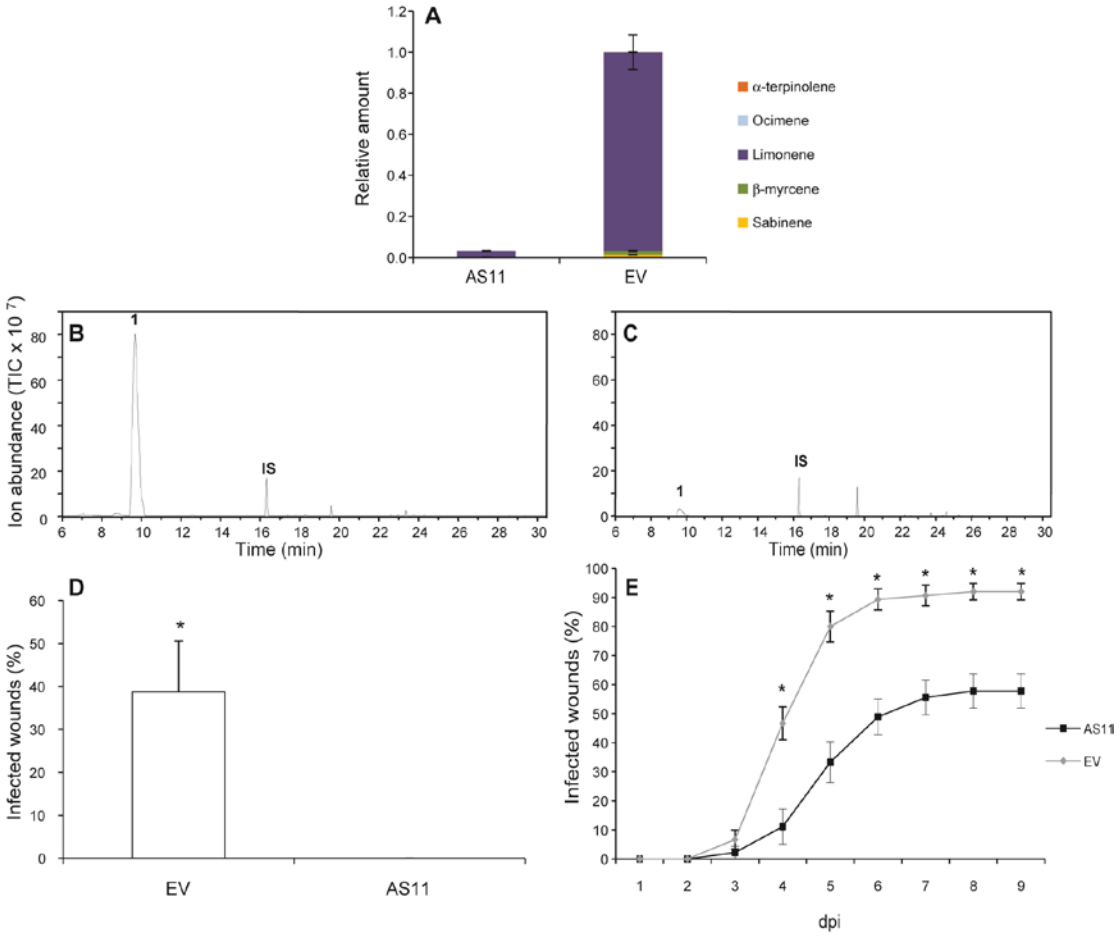

Figure 6. Phenotypes and behavioral responses of orange flavedo to pathogens in antisense and control-treated (EV) Pineapple sweet orange plants. (A) Relative amounts of individual terpenes are presented as a percentage area relative to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant. (B, C) Representative total ion chromatograms (GC-MS) of the volatile profile of orange fruit flavedo from EV (B) and AS11 transgenic plants (C). Peaks number one and IS correspond to limonene and the internal standard (2-octanol), respectively. (D) Number of infected wounds at four weeks after inoculation of EV and AS fruits with the bacterium *Xanthomonas citri*. (E) Evolution of the disease caused by the fungus *Penicillium digitatum* and the percentage of infected wounds in the orange fruits of the EV and AS lines. Results are average ± s.e.m. ($n \geq 20$).*, $P < 0.05$ using Student's *t*-test.

Response of a citrus insect pest to limonene downregulation in transgenic fruits

There is evidence to suggest that limonene and other terpene compounds of the citrus peel confer partial resistance in fruits to the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (medfly), a major pest of citrus species worldwide (Papachristos and Papadopoulos, 2009), and to other tephritid pests (Back and Pemberton, 1915). We assessed the behavioral response of *C. capitata* to terpene downregulation in sweet orange fruits by no-choice and two-choice flight tunnel assays.

No-choice assays showed that the oviposition response of medfly females after three days of exposure to AS or EV fruits was similar (Supplemental Fig. S4), supporting the notion that medfly females are capable of counteracting the hypothetical deterrent effect induced by the high levels of essential oils that are present in orange flavedo.

It has been suggested that the acquisition of a certain aroma in the flavedo is responsible for increased mating success of medfly males, as demonstrated by exposing entire rooms of mass-reared medfly males to the aroma of orange oil (Shelly, et al., 2008). Flight tunnel assays with medfly males exposed to different pure synthetic compounds ((+)-limonene, nerol and citronellol) in disk assays revealed a preference for these monoterpenes over control water, being limonene the most attractant one (Supplemental Fig. S5). Accordingly, we examined how the behavioral response of medfly males could be affected by the modification of the terpene profile in AS fruits. Flight tunnel assays with medfly males exposed to AS and EV fruits showed that males were more attracted to EV than to AS fruits in green (19% vs. 5%, $P < 0.05$, Supplemental Fig. S6) and mature fruits (32% vs. 2%, $P < 0.05$, Fig. 7), suggesting that limonene emission attracts the male flies. Moreover, when medfly males in the field were exposed to AS vs. EV fruits together (Supplemental Movie S1, online), they were strongly attracted to and landed preferentially on EV fruits. The addition of pure limonene to the peel of mature AS fruits confirmed that this compound was responsible for this behavior in cage assays, because AS fruits were so attractive as EV fruits to medfly males under these conditions (Fig. 8). This olfactory-mediated flight behavior might decrease the mating success of those medfly males exposed to AS fruit in the field.

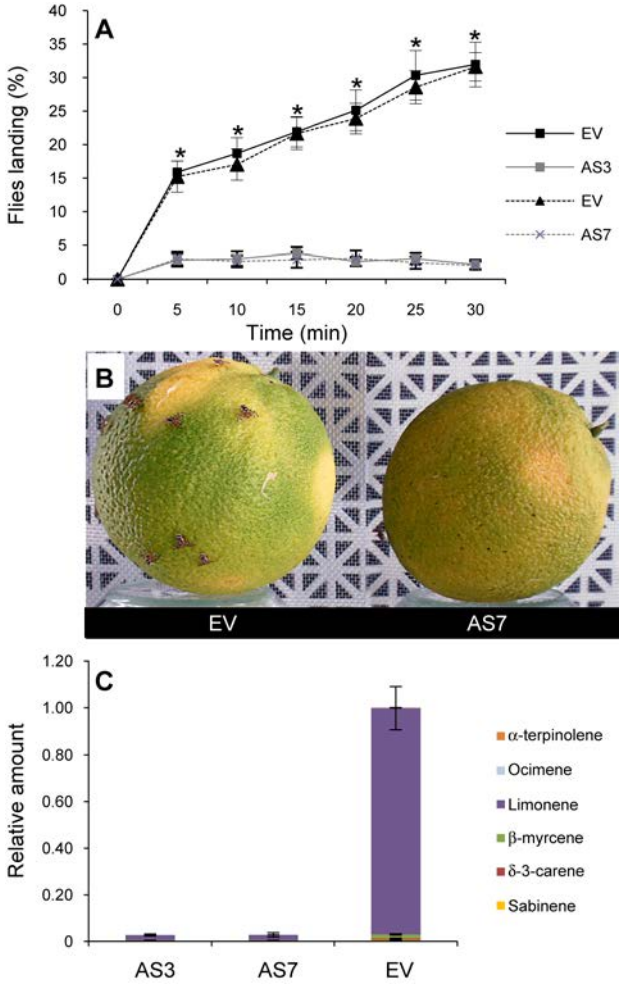

Figure 7. Terpene-mediated response of *Ceratitis capitata* males exposed to AS and EV fruits in flight tunnel assays. (A) Mean percentage of flies landing on AS and EV control fruits. Results are average ± s.e.m. (n = 10). *, $P < 0.05$ using Student's *t*-test. We repeated all experiments with two different AS lines and obtained similar results. (B) Terpene-mediated landing of flies on EV (left) and AS7 (right) fruits. (C) Relative amounts of individual terpenes are presented as a percentage area of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant.

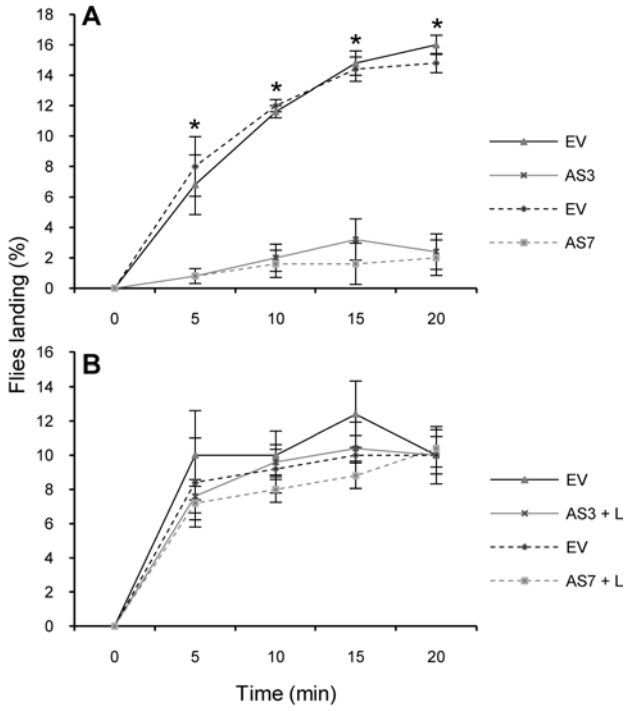

Figure 8. Terpene-mediated response of *Ceratitis capitata* males exposed to AS and EV fruits in cage assays. (A) Mean percentage of flies landing on AS and EV control fruits. Results are average ± s.e.m. (n = 10). *, $P < 0.05$ using Student's t-test. (B) Mean percentage of flies landing on AS supplemented with 100 µL of pure limonene (L) and EV control fruits. Results are average ± s.e.m. (n = 10). No significant differences were found at $P < 0.05$ using Student's t-test. We repeated all experiments with two different AS lines and obtained similar results.

The effects of limonene downregulation in fruit flavedo on medfly attraction and fungal and bacterial infections strongly indicate that the high accumulation of this monoterpene in the peel of citrus fruits is required for the success of the fruit. In nature, limonene content is usually low in orange fruits during the two-three months post-anthesis; it then drastically increases when the fruit is still green but contains seeds and remains at high level until the fruit becomes fully mature (Fig. 9)(Dugo and Di Giacomo, 2002; Kekelidze, et al., 1989). In contrast to the view that animal-dispersers of fleshy fruit seeds compete with microbes for food resources (Janzen, 1977; Herrera, 1982; Cipollini and Levey, 1997), our data indicate that once a fruit has completely developed seeds, it advertises its condition to potential legitimate dispersers by inducing changes in terpene volatile signals, which also serve to attract specialized insects and microorganisms. In this way they could indirectly increase seed dispersal by providing a nutritional benefit to vertebrates that eat insect-infested or pathogen-infected fruits (Sallabanks and Courtney, 1992; Cazetta, et al., 2008). Dispersal could occur when the terpene-rich peel barrier is broken, making the seeds more accessible to terrestrial mammals, or by releasing volatiles that attract specialized vertebrates. This peel would otherwise be toxic or a deterrent for seed dispersing animals. It has been recently reported that the attraction of birds to heavily insect-infested trees is directly correlated with the emission of several specific terpene compounds (Mäntylä, et al., 2008)

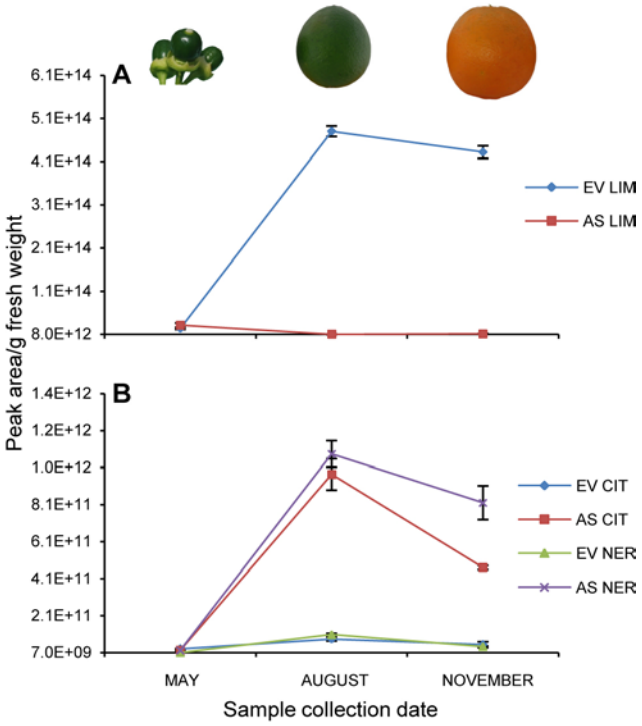

Figure 9. Evolution of citrus peel volatiles during EV and AS fruit development. (A) Limonene (LIM) variation. (B) Citronellol (CIT) and nerol (NER) variation.

Our results provide a more comprehensive view of the potential co-evolution of fruit terpene volatiles that act as signals in multiple trophic chains for insect herbivores and pathogens and seed-dispersing vertebrates. Moreover, these results demonstrate for the first time that genetic engineering of volatile terpenoids represents a promising method for developing broad-spectrum resistance or tolerance to pests and pathogens in fleshy fruits and potentially in other economically important crops.

Materials and Methods

Citrus transformation

Mature transformants from *Citrus sinensis* L. Osbeck plants (cv. 'Navelina' and 'Pineapple') were generated as previously described (Rodríguez, et al., 2008). A binary vector (pBI121FLM) was constructed containing the limonene synthase gene from satsuma mandarin (*Citrus unshiu* Mark.) (*CitMTSE1,* accession AB110636) in an antisense orientation (AS) under the control of the *Cauliflower mosaic virus* (CaMV) 35*S* promoter and the nopaline synthase gene (*NOS*) terminator using standard restriction and ligation DNA techniques. The T-DNA of this binary vector also included the neomycin phosphotransferase II gene (*nptII*) driven by the *NOS* promoter and terminator sequences (Supplemental Fig. 1A). The binary plasmid pBI121FLM was used as the vector system for transforming empty vector (EV) control plants (Supplemental Fig. 1B).

PCR and Southern blot analysis

Standard PCR techniques were used to detect the limonene synthase gene construct sequence. The primers used were 5'-ATCTCCACTGACGTAAGGGATGACG -3' (p35S) and 5'-ATGTCTTCTTGCATTAATCCCT-3' (*CitMTSE1*). Reactions were performed in 25 μL containing 1 μL of DNA (50 ngμL^{-1}), 200 μM dNTPs, 3 mM MgCl2, 50 mM KCl, 20 mM Tris-HCl pH 8.4, 0.25 μM of each primer and 0.5 U of Taq DNA polymerase (Roche). Reactions were subjected to 35 cycles of 0.5 min at 95 °C, 0.5 min at 58 °C and 2 min at 72 °C for the *CitMTSE1* gene. Amplified DNA was detected with ultraviolet (UV) light after electrophoresis on 1% agarose-ethidium bromide gels. Genomic DNA was isolated from leaves as previously described (Dellaporta, et al., 1983). To detect *CitMTSE1*, Southern blot experiments were performed on samples digested with 20 μg of *Hin*dIII, separated on 1% agarose gels and blotted onto nylon membranes (Hybond-N+, Amersham Pharmacia). Filters were probed with digoxigenin-labeled (DIG-11-dUTP) (Roche Diagnostics) fragments of the 35*S* promoter prepared by PCR, fixed by UV irradiation and detected by chemiluminescence with the CSPD substrate (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) (Roche Diagnostics).

Chemicals

Synthetic compounds used in the assays and as references for identification of the citrus volatiles were: (R)-(+)-limonene (99%), nerol (97%), citronellol (95%) and 2-octanol all supplied by Sigma–Aldrich.

Extraction of volatiles and GC-MS analysis

Flavedo tissue was obtained from orange fruits, immediately frozen in liquid nitrogen and stored at -80 ºC until extraction. A Thermo Trace GC Ultra coupled to a Thermo DSQ mass spectrometer with electron ionization mode (EI) 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 were added as an internal standard. 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 1800 g 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 3 mL of pentane. A 2-μL aliquot of the pooled organic phases was directly injected into the GC-MS for volatile analysis; at least two extractions for each sample were performed**.**

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 i.d. x 0.25 μm film). 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 30-400 amu range with a scanning speed of 0.5 scans^{-1}. Compounds were identified by matching the acquired mass spectra with those stored in the reference libraries (Wiley6 and NIST) or from authentic standard compounds when available.

Data were quantified by integrating the peak areas of total ion chromatograms (TIC) and normalizing to the recovery rate of the internal standard (2-octanol). The data in Figs. 1, 6, 7 and Supplemental Figs. S2 and S3 represent relative amounts of individual terpenes and are presented as a percentage area of each terpene (given as a fraction of unity) with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one. The data in Supplemental Tables S1 and S2 represent fold-changes for each volatile in the AS lines relative to the EV line. Negative values indicate a decrease and positive values an increase of the specific volatile with respect to the reference EV line. Values represent at least two independent experiments and are shown as means ± standard error (s.e.m).

Inoculation of fruit with *Penicillium digitatum and P. minioluteum*

AS and EV mature oranges (*Citrus sinensis* L. Osbeck) cvs. 'Navelina' and 'Pineapple' were used throughout this study. *P. digitatum* isolate NAV-7 and *P. minioluteum* isolate GAA-2 were obtained from the culture collection of the Laboratory of Pathology, Postharvest Technology Center, IVIA. Fruits were used immediately after harvest and were surface disinfected (1-min immersion in a sodium hypochlorite solution (4 gL^{-1})), rinsed with fresh water, and left to air dry at room temperature before inoculation.

The concentration of the spore suspension was measured with a hemocytometer and adjusted to 1×10^4 spores mL^{-1} by dilution with sterile water. Fruit inoculation with *P. digitatum* or *P. minioluteum* was conducted as described previously (Palou, et al., 2001). Spore suspensions were prepared from 7 to 10-day old cultures on potato dextrose agar (PDA, Difco, Detroit, USA) incubated in the dark at 25 ºC. Spores were removed from sporulating colonies with a sterile loop and suspended in Tween 80 (0.05% w/v) in sterile distilled water. After vigorous agitation for 3 min in a vortex mixer, the remaining mycelial fragments were removed by filtration through two layers of cheesecloth.

A Petri dish assay system was developed to assess the effect of different concentrations of synthetic compounds limonene and nerol on spore germination and germ tube development as previously described (Droby, et al., 2008).

Oranges were inoculated by immersing a stainless steel rod with a probe tip 1 mm in width and 2 mm in length into the spore suspension and wounding the rind once in the equator. The wound penetrated the albedo tissue but not the juice sacs, simulating natural infection. Three different rind sites around the equator of each fruit were inoculated. Different fruits were

used for each fungus. For the assays of limonene supplementation, 5 μL of the pure compound were allowed to penetrate in the wound and the same procedure for inoculation was performed. For each season and treatment, replicates of 10 to 25 fruit per transgenic line were used. Inoculated fruit were placed on plastic cavity trays on open cardboard trays that prevent fruit contact and incubated at 20ºC and 80% RH for two weeks. Disease incidence and sporulation, which are considered to be the number of infected wounds and sporulated infections, respectively, and disease severity, which is measured by the lesion diameter, were checked daily. Severity values were used to calculate the area under the disease progress curve (AUDPC (de Capdeville, et al., 2002)). Data on disease severity (AUDPC) and arcsine-transformed data on the percentage of infected wounds and sporulated lesions were subjected to the analysis of variance using Statgraphics v.5.1 software (Manugistics Inc., Rockville, USA). When appropriate, Fisher's Protected Least Significant Difference (LSD) test ($P < 0.05$) was used to separate the means.

Inoculation of fruit with *Xanthomonas citri* subsp. *citri*

AS and EV green oranges, the most susceptible developmental stage, (*Citrus sinensis* L. Osbeck) cvs. 'Navelina' and 'Pineapple' were used throughout this study. Fruits were used immediately after harvest and were ethanol-surface-disinfected, rinsed with fresh water, and left to air dry at room temperature before inoculation. The inoculum was prepared with strain 306 of *X. citri* subsp. *citri* at 10^6 CFU mL^{-1} isolated in Brazil and obtained from the culture collection of the Laboratory of Bacteriology, IVIA. Inoculations were performed as previously reported (Viloria, et al., 2004). Fruits were inoculated using a 1-mL syringe without a needle with phosphate buffered bacterial suspensions at 10^6 CFU mL^{-1} obtained from an overnight culture in Nutrient Broth (NB) (Difco) or with phosphate buffer alone as control. Using a stainless steel rod equipped with a top, all lesions were ensured to be 1-mm depth; all wounds penetrated the flavedo tissue. Fruits were punctured at 5-8 inoculation points. Lesions were evaluated at 7, 15 and 30 days after inoculation. Between fifteen and twenty fruits per repetition of each transgenic line were used.

Inoculated fruits were placed in plastic trays covered with transparent film in a temperature and humidity controlled incubator (28ºC/80% RH, respectively) for four weeks until symptoms of an infection halo and canker lesions were visible. Disease incidence was estimated by measuring the number of developed lesions, the number of fruits with a developed halo and the diameter of this halo. Fisher's Protected Least Significant Difference (LSD) test ($P < 0.05$) was used to evaluate the data on the percentage of infected wounds.

Insect assays

Larvae and adults of *Ceratitis capitata* were obtained from a laboratory population maintained at IVIA since 2002. Larvae were reared on an artificial diet as previously described

(San Andrés, et al., 2007). After emergence, adults were kept in ventilated Perspex cages (20x20x20cm) and fed with a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1, w:w) and water until they were five days old. Larvae and adult flies were maintained in an environmental chamber at 25ºC ± 2°C, 60% ± 10% RH and 16:8 h (L: D) photoperiod.

No-choice assays were performed in an insecticide-free greenhouse at 26 ± 2 ºC day temperatures, with a relative humidity between 60%-80%. The experimental arena consisted of aluminum framed cages of 150x150x90 cm covered with gauze. Groups of 100 five-day-old adults (50 males and 50 females) were released inside each cage containing a single plant with one fruit. Flies were fed as described before and allowed to lay eggs for three days.

For two-choice flight assays, a laboratory Plexiglass tunnel model OLFM-WT (Analytical Research Systems, Gainesville, Florida, USA) measuring 180x60x60 cm producing a laminar flow of air was used to compare AS against EV fruit. Air was pulled through the chamber at 0.2 $msec^{-1}$ connected to the downwind end. Air exiting the chamber was directly removed. In addition, this tunnel contained inlet and outlet vents to bring new air into the room from the outside and remove air from the room to the outside. Fruits were placed in the upwind end of the tunnel, and flies were released at the downwind opening. For two-choice cage assays, cages measuring 50x30x30 cm were used to compare AS against EV fruit. For paper assays, 30 μL of pure compounds were applied to 8-cm-diameter filter paper disks. For each assay, 50 five-day-old medfly males were released from the downwind end of the flight tunnel or inside the cages and allowed to respond freely between 20 and 30 min. Both EV and AS fruits were placed in the peel eight times with a 2-mm long steel rod. All assays were performed at 25 ± 2°C, under fluorescent lights (2,000 lux). In cage assays, 100 μL of synthetic limonene was used to cover the AS fruit. Ten to twenty replications were conducted in all experiments.

The oviposition response, number of punctures per fruit, number of pupae per fruit and percentage of emergence data from no-choice assays were compared using Mann-Whitney *U* test ($P < 0.05$), and a *t*-test of arcsine-transformed data was performed to examine the mean percentage of male medflies landing on the transgenic (AS) and control (EV) fruits in the two-choice assays.

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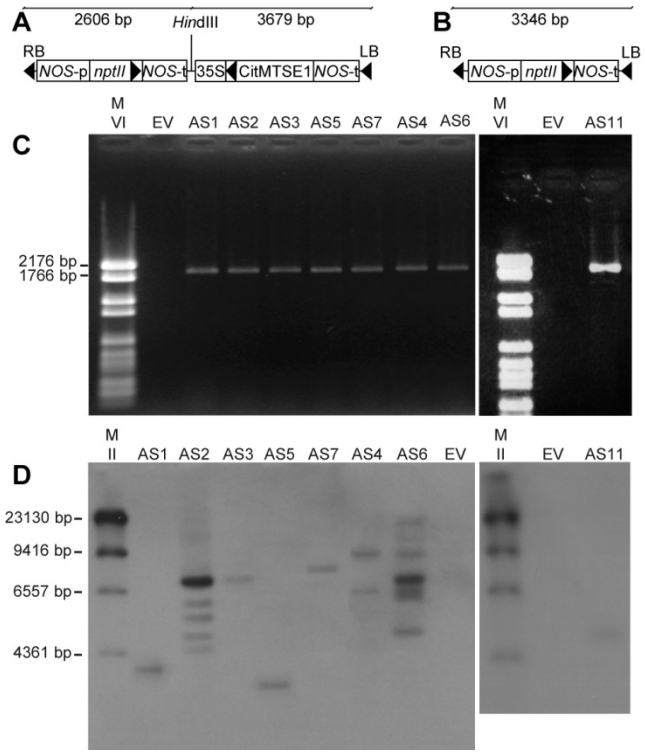
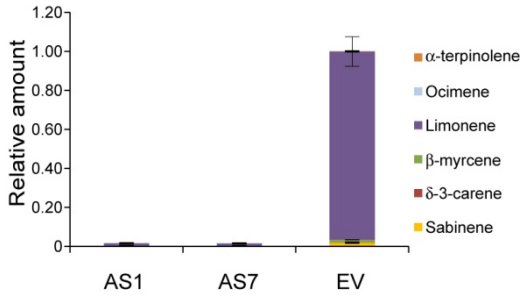
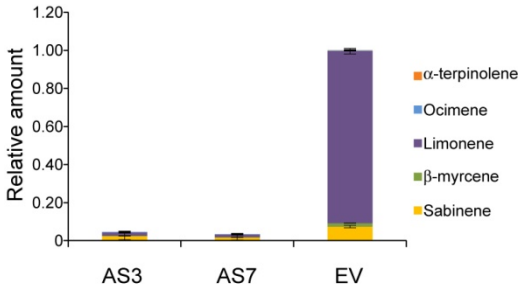
Supplemental Files Chapter 1



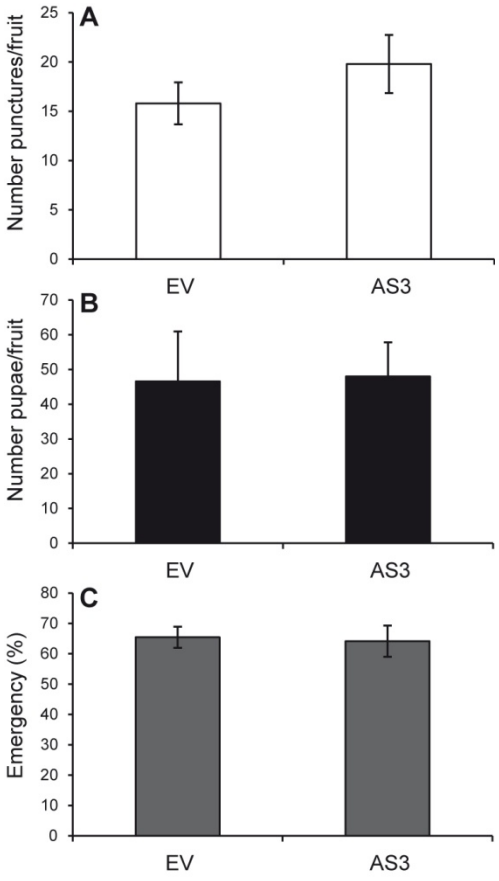
Supplemental Figure S1. Molecular analyses of Navelina (AS1-AS7) and Pineapple (AS11) citrus plants transformed with the limonene synthase gene in an antisense orientation. (A, B) Map of the T-DNA region of the binary vector used to transform AS (A) and EV plants (B). LB, left T-DNA border region; RB, right T-DNA border region; *nptII*, gene conferring kanamycin resistance under the control of the *NOS* promoter and terminator regions; *CitMTSE1*, limonene synthase gene in antisense orientation under control of the CaMV35S promoter and *NOS* terminator. (C) PCR analysis. (D) Southern blot analysis indicating the loci number of the transgene. The 35*S* promoter was used as probe. M: DNA molecular weight markers II and VI from Roche Applied Science.



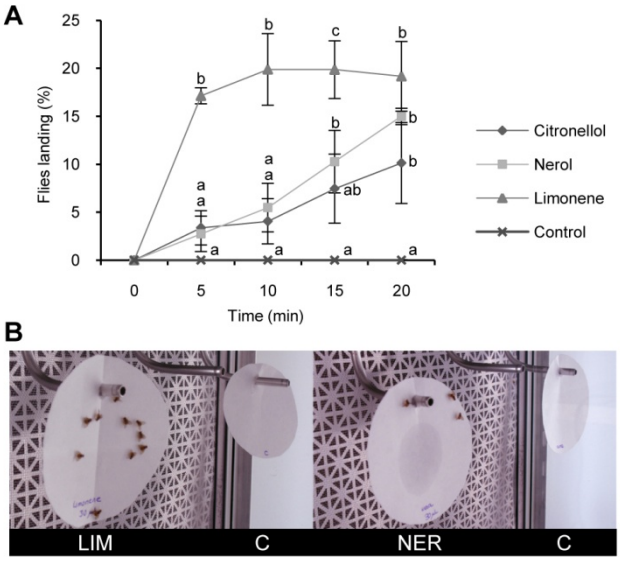
Supplemental Figure S2. Relative amounts of individual terpenes in fruits used for the assays, presented as a percentage area (fraction of unity) of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in *Penicillium digitatum* assays. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant.



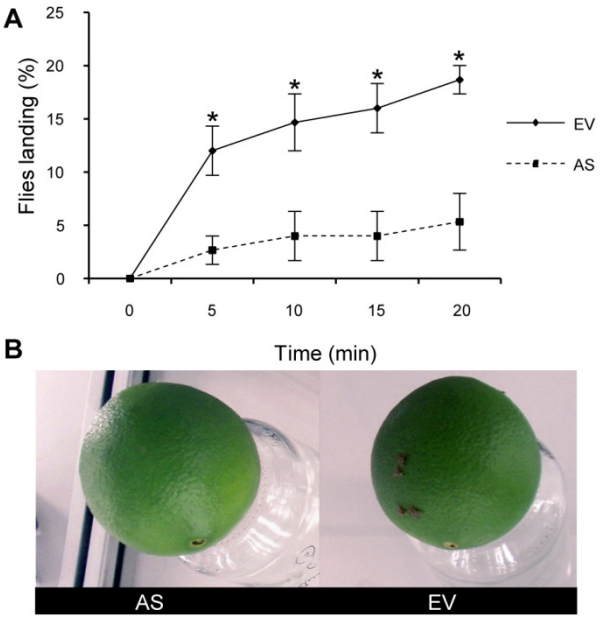
Supplemental Figure S3. Relative amounts of individual terpenes in fruits used for the assays, presented as a percentage area (fraction of unity) of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in *Xanthomonas citri* subsp. *citri* assays. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant.



Supplemental Figure S4. Oviposition response of *Ceratitis capitata* females in no-choice assays after three days of exposure to the orange odor of AS and EV plants. (A) Number of punctures per fruit. (B) Number of pupae per fruit. (C) Percentage of emergence. Results are average ± s.e.m. (n = 5). No significant differences were found at $P < 0.05$, using a Mann-Whitney U test. We repeated all experiments at least twice and obtained similar results.



Supplemental Figure S5. Terpene-mediated response of *Ceratitis capitata* males exposed to pure synthetic monoterpene compounds in flight tunnel assays. (A) Mean percentage of flies landing on paper disks supplemented with 30 µL of water or pure synthetic compounds. Results are average ± s.e.m. (n = 10). Treatments with different letters are significantly different at $P < 0.05$ using Fisher's Protected LSD test. (B) Terpene-mediated landing of flies on limonene (LIM), nerol (NER) or water control (C) disks.



Supplemental Figure S6. Terpene-mediated response of *Ceratitis capitata* males exposed to AS and EV green fruits in flight tunnel assays. (A) Mean percentage of flies landing on AS and EV control fruits. Results are average ± s.e.m. (n = 5). *, $P < 0.05$ using a Mann-Whitney U test. (B) Terpene-mediated landing of flies on AS (left) and EV (right) fruits.

Supplemental Table S1. Fold-change of volatiles in AS transgenics compared to EV control Navelina sweet orange mature flavedo. The level of accumulation of individual volatiles in EV flavedo was arbitrarily set to 1.0. Negative values indicate decreases and positive values reflect increases of the specific volatile with respect to the reference EV line. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant.

Compounds	**Transgenic line**								
	AS1		**AS3**		**AS6**		**AS7**		**EV**
	MEAN	**S.E.M.**	**MEAN**	**S.E.M.**	**MEAN**	**S.E.M.**	**MEAN**	**S.E.M.**	
Monoterpenes									
Hydrocarbons									
Sabinene	- 8.72	0.01	- 5.71	0.00	- 2.07	0.01	-6.82	0.02	
δ-3-carene	nd		nd		- 1.08	0.08	nd		
β-myrcene	- 51.02	0.00	- 57.38	0.00	- 1.38	0.01	- 82.69	0.01	
D-Limonene	- 90.88	0.00	- 107.39	0.00	- 1.33	0.07	- 85.95	0.00	
Ocimene	- 3.28	0.07	- 1.84	0.32	1.63	0.42	- 3.07	0.10	
α-terpinolene	nd		nd		- 1.06	0.33	nd		
Relative (%)	43.5		45.3		95.8		49.7		96.0
Aldehydes									
***Z*-citral**	- 3.02	0.05	- 5.65	0.07	- 1.28	0.16	- 5.33	0.05	
E*-citral	nd		nd		- 1.19	0.17	nd		
Citronellal	-1.31	0.21	- 1.16	0.26	-1.15	0.12	-1.48	0.21	
Perilla aldehyde	nd		nd		- 1.23	0.10	nd		
Relative (%)	3.6		3.0		0.5		2.7		0.5
Alcohols									
***E*-sabinene hydrate**	- 10.07	0.00	- 6.20	0.04	- 1.61	0.07	- 7.71	0.03	
***Z*-sabinene hydrate**	- 26.65	0.04	- 11.64	0.09	- 1.40	0.13	- 3.98	0.02	
Linalool	1.07	0.27	1.05	0.02	1.40	0.36	- 2.02	0.03	
1-octanol	- 1.91	0.29	-1.20	0.35	1.22	0.37	1.10	0.32	
4-terpineol	- 4.66	0.04	- 1.87	0.03	- 1.42	0.02	- 3.92	0.00	
***E*-p-mentha-2,8-dienol**	nd		nd		1.64	0.03	- 12.22	0.08	
α-terpineol	- 21.23	0.01	- 11.78	0.03	-1.41	0.02	-15.77	0.02	
β-citronellol	18.65	3.88	10.45	1.26	3	0.45	11.43	0.28	
Nerol	14.71	2.09	9.98	2.64	4.45	2.84	16.57	4.70	
***E*-carveol**	nd		nd		- 1.06	0.31	nd	0.00	
Geraniol	3.95	0.24	4.61	0.90	1.61	0.82	4.45	2.88	
***Z*-carveol**	nd		nd		1.68	0.51	nd		
Relative (%)	34.5		31.7		1.6		21.7		0.9
Esters									
Neryl acetate*	p		p		nd		p		
Geranyl acetate	3.25	1.47	3.85	0.87	1.49	0.60	3.65	0.92	
Perilla acetate	nd		nd		nd		nd		
Limonen-10-yl acetate	nd		nd		nd		nd		
Relative (%)	7.9		7.2		0.01		8.3		0.01
Sesquiterpenes									
Hydrocarbons									
α-copaene	nd		nd		- 4.57	0.22	nd		
β-cubebene	nd		nd		- 2.36	0.11	- 44.50	0.02	

Germacrene D	nd		nd		- 1.81	0.32	nd		
β-elemene	- 3.51	0.00	- 1.81	0.30	1.11	0.04	- 1.30	0.19	
α-caryophyllene	- 7.46	0.02	- 11.58	0.03	- 3.09	0.03	- 5.33	0.04	
β-farnesene	nd		- 6.96	0.14	- 1.86	0.54	nd		
Valencene	- 1.89	0.16	- 1.39	0.35	1.08	0.24	- 1.34	0.01	
δ-Cadinene	- 6.08	0.00	- 5.45	0.01	-1.29	0.00	- 5.71	0.00	
α-muurolene	nd		nd		nd		nd		
β-sesquiphellandrene	- 3.47	0.07	- 1.95	0.24	1.28	1.01	1.02	0.04	
Relative (%)	4		5.4		0.4		6.7		0.4
Aldehydes									
β-sinensal	- 6.27	0.09	- 12.30	0.02	- 2.64	0.07	- 6.75	0.05	
α-sinensal	- 4.93	0.03	- 3.41	0.08	1.23	0.07	- 4.46	0.02	
Relative (%)	0.6		0.8		0.1		0.7		0.1
Alcohols									
d-nerolidol	nd		nd		-4.32	0.23	nd		
Elemol	- 44.76	0.02	nd		- 2.23	0.21	nd		
Relative (%)	0.5		0		0.01		0		0.02
Aliphatic aldehydes									
Octanal	- 35.58	0.00	- 38.16	0.00	- 1.90	0.03	- 20.34	0.01	
Nonanal	- 15.12	0.00	- 22.68	0.00	-1.90	0.00	-9.94	0.00	
Decanal	- 10.57	0.02	- 11.58	0.01	- 1.44	0.07	- 6.39	0.06	
Undecanal	- 5.95	0.05	- 9.62	0.10	- 1.75	0.06	- 5.28	0.05	
2-decenal	nd		nd		- 1.15	0.53	nd		
Relative (%)	3.3		3		1.3		5.5		1.8
Others/Irregular									
(+)-Isopiperitenone	nd		nd		-1.58	0.63	nd		
***Z*-limonene oxide**	nd		nd		1.29	0.29	nd		
***E*-limonene oxide**	- 6.07	0.07	nd		- 1.07	0.26	- 7.47	0.13	
Caryophyllene oxide	1.11	0.12	- 1.07	0.29	1.94	0.28	2.36	0.63	
β-cyclocitral	1.37	0.65	- 1.23	0.26	1.02	0.51	1.17	0.83	
Relative (%)	1.0		1.2		0.2		1.9		0.2

nd: non-detectable

p: present (impossible to quantify fold-change because it is not present in EV plants)

* Compound differing from EV (E-citral) and AS plants (Neryl acetate)

Supplemental Table S2. Fold-change of volatiles in AS transgenics compared to EV control Pineapple sweet orange mature flavedo. The level of accumulation of individual volatiles in EV flavedo was arbitrarily set to 1.0. Negative values indicate decreases and positive values reflect increases in the specific volatile with respect to the reference EV line. Data represent the mean values ± s.e.m. and are derived from at least five fruits per plant.

	Transgenic line		
	AS11		**EV**
Compounds	**MEAN**	**S.E.M.**	
Monoterpenes			
Hydrocarbons			
Sabinene	- 16.13	0.00	
β-myrcene	- 33.46	0.00	
D-Limonene	- 32.48	0.00	
Ocimene	- 1.83	0.03	
α-terpinolene	nd		
Relative (%)	51.6		97.2
Aldehydes			
***Z*-citral**	- 1.84		
E*-citral	nd		
Citronellal	- 1.03	0.04	
Perilla aldehyde	nd		
Relative (%)	1.4		0.1
Alcohols			
***E*-sabinene hydrate**	- 6.76	0.03	
***Z*-sabinene hydrate**	nd		
Linalool	2.23	0.02	
1-octanol	nd		
4-terpineol	nd		
a-terpineol	- 15.74	0.00	
β-citronellol	13.73	0.57	
Nerol	5.49	0.31	
***E*-carveol**	nd		
Geraniol	2.47	0.15	
***E*-p-mentha-2,8-dienol**	nd		
p-mentha-1(7),8(10)-dien-9-ol	nd		
Relative (%)	37.6		1.0
Esters			
Citronellyl acetate	p		
Neryl acetate*	p		
Geranyl acetate	p		
Limonen-10-yl acetate	nd		
Relative (%)	6.3		0.01
Sesquiterpenes			
Hydrocarbons			
α-copaene	- 5.24	0.02	
β-cubebene	nd		
Germacrene D	nd		

α-gurjunene	nd		
β-elemene	nd		
β-selinene	nd		
α-caryophyllene	nd		
β-caryophyllene	- 1.82	0.01	
Z-β-farnesene	nd		
Valencene	- 30.48	0.00	
α-selinene	nd		
Germacrene B	nd		
δ-cadinene	- 10.34	0.00	
Relative (%)	0.7		0.7
Aldehydes			
β-sinensal	nd		
α-sinensal	- 2.43	0.06	
Relative (%)	0.2		0.2
Alcohols			
d-nerolidol	nd		
Elemol	- 6.87	0.00	
Relative (%)	0.03		0.04
Aliphatic aldehydes			
Octanal	- 3.63	0.01	
Nonanal	nd		
Decanal	- 18.66	0.00	
2-decenal	nd		
Decadienal	nd		
Relative (%)	1.4		0.4
Others			
(+)-isopiperitenone	- 4.09	0.00	
***E*-limonene oxide**	- 3.70	0.01	
Caryophyllene oxide	nd		
β-cyclocitral	nd		
Cyclohexane, 2-ethenyl-1,1-dimethyl-3-methylene-	nd		
***Z*-3-hexen-1-ol**	-1.55	0.00	
Relative (%)	0.2		0.2

nd: non-detectable

p: present (impossible to quantify fold-change because it is not present in EV plants)

* Compound differing from EV (E-citral) and AS plants (Neryl acetate)

4. RESULTS: CHAPTER 2

The monoterpene limonene in orange peels attracts pests and microorganisms

Plant signaling & behavior (2011) **6**, 1820-1823 (doi: 10.4161/psb.6.11.16980)
Rodríguez A, San Andrés V, Cervera M, Redondo A, Alquézar B, Shimada T, Gadea J, Rodrigo MJ, Zacarías L, Palou L, López MM, Castañera P, Peña L

Addendum to: Rodríguez A, San Andrés V, Cervera M, Redondo A, Alquézar B, Shimada T, et al. Terpene down-regulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens. Plant Physiol 2011; 156:793-802; PMID:21525333; DOI: dx.doi.org/10.1104/pp.111.176545.

Abstract

Plant volatiles include terpenoids, which are generally involved in plant defense, repelling pests and pathogens and attracting insects for herbivore control, pollination and seed dispersal. Orange fruits accumulate the monoterpene limonene at high levels in the oil glands of their fruit peels. When limonene production was downregulated in orange fruits by the transgenic expression of a limonene synthase (*CitMTSE1*) in the antisense configuration, these fruits were resistant to the fungus *Penicillium digitatum* (Pers.) Sacc. and the bacterium *Xanthomonas citri* subsp. *citri* and were less attractive to the medfly pest *Ceratitis capitata*. These responses were reversed when the antisense transgenic orange fruits were treated with limonene. To gain more insight into the role of the limonene concentration in fruit responses to pests and pathogens, we attempted to overexpress *CitMTSE1* in the sense configuration in transgenic orange fruits. Only slight increases in the amount of limonene were found in sense transgenic fruits, maybe due to the detrimental effect that excessive limonene accumulation would have on plant development. Collectively, these results suggest that when limonene reaches peak levels as the fruit develops, it becomes a signal for pest and pathogen attraction, which facilitate access to the fruit for pulp consumers and seed dispersers.

Introduction

In recent years, large efforts have been made to understand the biosynthetic pathways regulating the production of terpene volatiles in plants as well as the metabolism and physiological effects of these compounds.[1] Evaluating volatile emission as a language for communication between plants and the environment is gaining increasing interest.

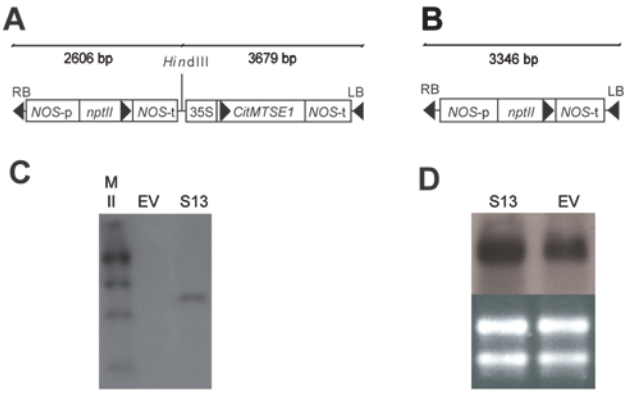
Upon maturation, fleshy fruits modify their color, taste, texture and aroma to become more attractive to frugivore animals for seed dispersal. The volatile content and emission profiles undergo drastic changes during fruit development and maturation, but little is known about the roles of specific volatiles in interactions with herbivores and other seed-dispersing agents. Regarding the impact of specialized insect pests and pathogens on fleshy fruits, most studies have focused largely on pests and pathogens as competitors of seed dispersers.[2-4] A broader exploration of the pathogen–plant–vertebrate relationship from the perspective of evolutionary ecology is enticing.

The monoterpene limonene represents up to 97% of the total volatiles in orange fruit peel. Previously, we have shown that overexpression of an antisense (AS) construct of a limonene synthase gene from mandarin (*CitMTSE1*) in transgenic oranges resulted in a downregulation of their synthesis and reduced accumulation of limonene as well as other related monoterpenes, and increased amounts of monoterpene alcohols such as nerol, geraniol and citronellol.[5] AS fruits showed a marked resistance against the fungus *Penicillium digitatum* (Pers.) Sacc. and the bacterium *Xanthomonas citri* subsp. *citri*, two important citrus fruit pathogens, which were unable to infect transgenic peel tissues. In addition, males of one of the most polyphagous pests of citrus fruits, *Ceratitis capitata*, were much less attracted to the AS fruits than empty vector (EV) control fruits.[5] Interestingly, the resistant/less attractant phenotype was fully reversed when AS fruits were treated with exogenous limonene (and not with nerol or citronellol), indicating that limonene accumulation in the orange peel modulates fruit interactions with insects and microorganisms.

Results and discussion

To determine whether the constitutive increase in the production of limonene could lead to a higher level of attraction of *C. capitata* and greater susceptibility to the tested pathogens, transgenic orange plants overexpressing the same limonene synthase gene from mandarin (*CitMTSE1*)[6] in the sense orientation were generated (Fig. 1A-D). Limonene synthase transcript accumulation was higher in sense (S) than in EV lines (Fig. 1D). However, total terpenes profiling in fruit peels from the sense lines showed very slight increases in limonene accumulation compared to fruits from the EV control line (Fig. 1E). Although we found increases of more than four-fold in the levels of some monoterpenes, such as α-terpinolene and δ-3-carene, in leaf tissues in S lines compared to EV controls, none of the S lines showed an

increase in limonene in fruit peels of more than 1.4 times, and the levels of related mono- and sesquiterpenoid compounds remained basically unaltered (Supplementary Table 1).



E

	S13	EV
α-terpinolene	0.00074 ± 0.00005	0.00034 ± 0.00013
Ocimene	0.00052 ± 0.00010	0.00029 ± 0.00003
Limonene	1.33466 ± 0.13350	0.96935 ± 0.06178
β-myrcene	0.02490 ± 0.00186	0.01764 ± 0.00148
Sabinene	0.00933 ± 0.00062	0.01235 ± 0.00009

Figure 1. Molecular analyses and phenotypes of orange flavedo in sense (S) and control-treated (EV) Pineapple sweet orange plants. (A, B) Map of the T-DNA region of the binary vector used to transform S (A) and EV plants (B). LB, left T-DNA border region; RB, right T-DNA border region; *nptII*, gene conferring kanamycin resistance under the control of the *NOS* promoter and terminator regions; *CitMTSE1*, limonene synthase gene in sense orientation under control of the CaMV35S promoter and the *NOS* terminator. (C) Southern blot analysis indicating the loci number of the transgene. The 35S promoter was used as a probe. M: DNA molecular weight marker II from Roche Applied Science. (D) Northern blot analysis of total RNA extracted from flavedo of transgenic plants. RNA was separated by electrophoresis on a formaldehyde-containing agarose gel, transferred to a nylon membrane, and hybridized with a whole limonene synthase gene-specific RNA probe under stringent conditions (upper panel). Ethidium bromide staining of the same gel showing that equivalent amounts of RNA were loaded in the different lanes (lower panel). (E) The relative amounts of individual terpenes are presented as the percent (given as a fraction of unity) areas of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one. The data represent the mean values ± SEM and were derived from at least five fruits per plant.

Further, fruits of the S lines were challenged with *P. digitatum* and *X. citri subsp. citri*. When mature S and EV orange fruits cv. Pineapple were inoculated with *P. digitatum*, the percentage of infected wounds and wounds with spores in EV fruits 10 d postinoculation were 90.66% and 86.66%, respectively, as compared with 82.05% and 71.79%, respectively, in S fruits (Fig. 2A-D). S fruits also showed a response similar to that of EV fruits when challenged with *X. citri subsp. citri*; the percentage of infected wounds at 4 weeks postinoculation in green fruits inoculated with the bacterium was 80.83% in EV fruits and 88.09% in S fruits (Fig. 2E, F) and no significant differences were found, with $P<0.05$. Similar results were obtained for the S and EV lines from another transformation experiment with the Navelina orange cultivar (data not shown).

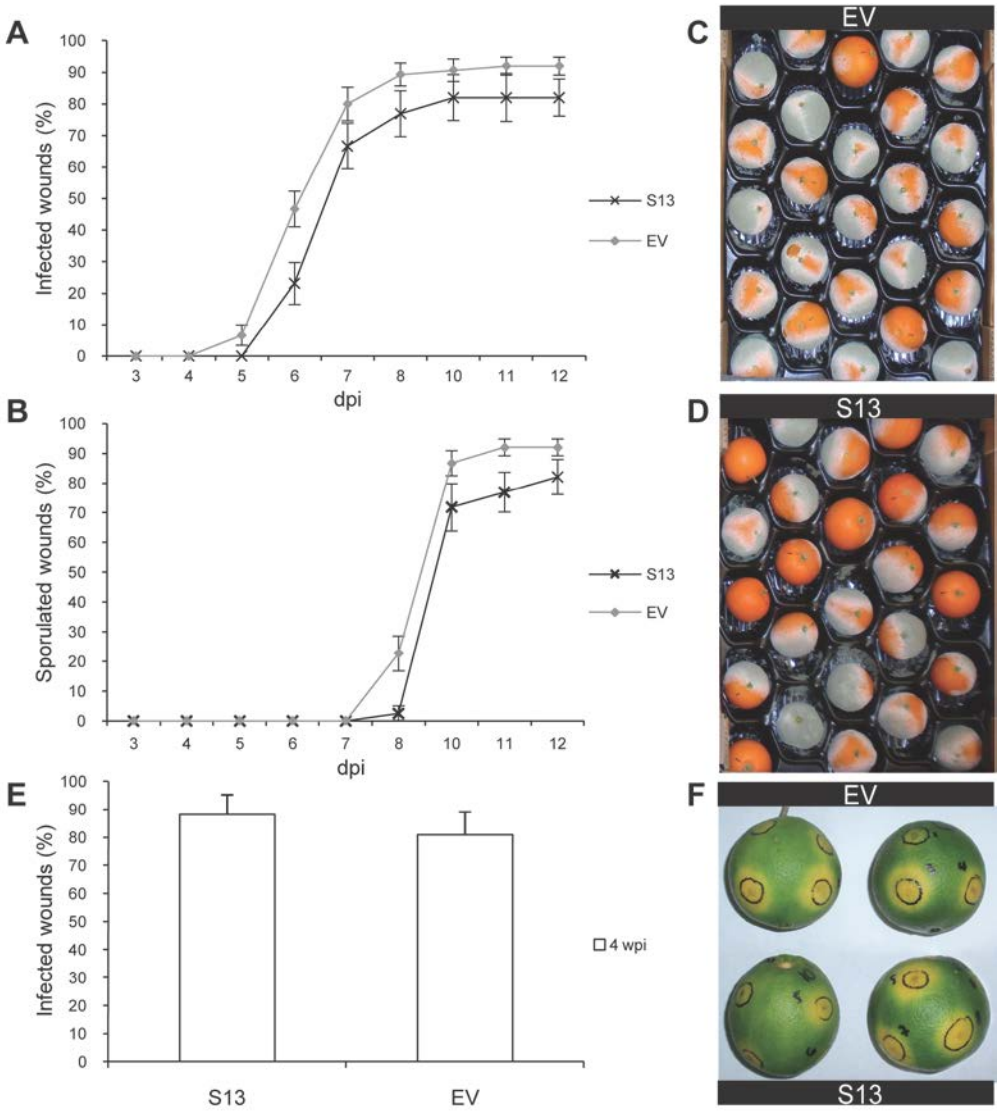


Figure 2. Transgenic expression of *CitMTSE1* in the sense orientation in orange plants did not modify susceptibility to fungal and bacterial infections. A to D, Evolution of the disease caused by the fungus *Penicillium digitatum* in mature orange fruits inoculated with $1x10^4$ spores mL^{-1}: percentage of infected (A) and sporulated (B) wounds in orange fruits of the EV and S lines. The results are the average ± SEM ($n \geq 20$). dpi, days postinoculation. (C, D) S and EV fruits 10 d after inoculation. E to F, Evolution of the disease caused by the bacterium *Xanthomonas citri* subsp. *citri* in green mature orange fruits using 10^6 CFU mL^{-1}. (E) Number of wounds with symptoms in EV and S fruits. The results are the average ± SEM ($n \geq 10$). (F) S and EV fruits at 4 weeks postinoculation. We repeated all experiments several times during two consecutive seasons and obtained similar results. No significant differences were found at $P < 0.05$ using Student's *t*-test.

Attempts to overexpress terpenoids in transgenic plants have resulted in enhanced accumulation of the expected compound in several cases,[7-9] but the growth and/or development of these plants were usually affected, with the strength of the phenotype being correlated with the accumulation level of the transgenically produced terpenoid.[10-12] S orange lines did not show noticeable changes in plant and fruit morphology compared to controls. It may be possible that S lines did not overproduce limonene because such high levels of production could be

detrimental to orange development. Following this rationale, S lines would accumulate limonene to those maximum levels that would not compromise cell and plant viability. It may be also hypothesized that under normal growing conditions, oil glands synthesize and accumulate near-saturating concentrations of limonene (and/or its geranyl diphosphate precursor) and overexpression of limonene synthase is not able to increase further the large amount of limonene occurring in the oil glands.

Our results together with the fact that in nature limonene reaches peak levels in fruit peels at the end of the growth phase, when fruits are still green but the seeds are fully developed, suggest that such high limonene doses exert an important signal effect to attract insect pests and microbial pathogens that break the peel barrier to facilitate eating of the fruit pulp by vertebrate consumers and seed dispersers.

Volatiles are important determinants of the overall aroma properties and taste of fruits.[13] The compounds that are produced during the first period of fruit growth make eating by vertebrates an unpleasant experience. It is generally accepted that a primary function of secondary metabolites in immature fruits is defense from pathogens and pre-dispersal seed predators.[2,4] The consumption of immature fruits would always be detrimental or repulsive because the seeds are not yet viable.[4] By the end of the first period of growth, when seeds are developed, the goal is to make the fruit as appealing as possible so that seed dispersal can occur. In that phase, volatiles become an important part of the attractiveness to animals and a signal of readiness for seed dispersal.[14] The identification of the limonene as a key compound in citrus fruits involved in pathogen interactions as well as insect attraction and its likely effect on seed dispersal could greatly increase our knowledge about fresh fruit trophic interactions. Just as in the pollination of flowers by insects, these interactions are complex and fine-tuned among the different organisms involved. We showed here that terpene engineering may be important for studying the basic ecological interactions between fruits, herbivores and pathogens. Determining how vertebrate dispersers fit into this tritrophic framework will provide new perspectives on these interactions.

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Supplemental Files Chapter 2

Supplementary Table 1. Fold-change of volatiles in S transgenics compared to EV control Pineapple sweet orange mature flavedo. The level of accumulation of individual volatiles in EV flavedo was arbitrarily set to 1.0. Negative values indicate decreases and positive values reflect increases in the specific volatile with respect to the reference EV line. Data represent the mean values ± s.e.m. and are derived from at least five fruits per plant.

	Transgenic line		
	S13		EV
Compounds	**MEAN**	**S.E.M.**	
Monoterpenes			
Hydrocarbons			
Sabinene	- 1.32	0.05	
β-myrcene	1.41	0.11	
D-Limonene	1.38	0.14	
Ocimene	1.77	0.33	
α-terpinolene	2.18	0.13	
Relative (%)	97.51		97.40
Aldehydes			
Z-citral	1.31	0.10	
E-citral	1.35	0.09	
Citronellal	1.18	0.09	
Perilla aldehyde	1.00	0.14	
Relative (%)	0.19		0.21
Alcohols			
E-sabinene hydrate	- 1.26	0.10	
Z-sabinene hydrate	- 1.08	0.10	
Linalool	1.33	0.11	
1-octanol	1.32	0.05	
4-terpineol	1.10	0.27	
α-terpineol	1.37	0.13	
β-citronellol	- 1.34	0.05	
Nerol	-1.74	0.04	
E-carveol	1.35	0.04	
Geraniol	1.16	0.05	
E-p-mentha-2,8-dienol	-1.16	0.12	
p-mentha-1(7),8(10)-dien-9-ol	1.05	0.09	
Relative (%)	0.80		0.87
Esters			
Citronellyl acetate	nd		
Neryl acetate	nd		
Geranyl acetate	nd		
Limonen-10-yl acetate	1.08	0.14	
Relative (%)	0.01		0.01

Sesquiterpenes			
Hydrocarbons			
α-copaene	1.34	0.13	
β-cubebene	1.54	0.08	
Germacrene D	1.75	0.02	
α-gurjunene	1.63	0.09	
β-elemene	1.94	0.29	
β-selinene	1.93	0.42	
α-caryophyllene	1.35	0.06	
β-caryophyllene	1.18	0.02	
Z-β-farnesene	- 1.17	0.09	
Valencene	1.61	0.11	
α-selinene	1.83	0.07	
Germacrene B	1.07	0.07	
δ-cadinene	1.35	0.09	
Relative (%)	0.85		0.75
Aldehydes			
β-sinensal	- 1,13	0.02	
α-sinensal	- 1.28	0.07	
Relative (%)	0.02		0.04
Alcohols			
d-nerolidol	- 1.33	0.18	
Elemol	1.37	0.07	
Relative (%)	0.04		0.04
Aliphatic aldehydes			
Octanal	1.26	0.09	
Nonanal	1.18	0.07	
Decanal	1.20	0.01	
2-decenal	1.52	0.13	
Decadienal	1.20	0.06	
Relative (%)	0.37		0.41
Others			
(+)-isopiperitenone	1.39	0.09	
E-limonene oxide	1.10	0.09	
Caryophyllene oxide	1.61	0.08	
β-cyclocitral	nd		
Cyclohexane, 2-ethenyl-1,1-dimethyl-3-methylene-	1.11	0.13	
Z-3-hexen-1-ol	-1.04	0.04	
Relative (%)	0.08		0.12

nd: non-detectable

5. RESULTS: CHAPTER 3

Terpene downregulation triggers innate immunity constitutively and jasmonate-mediated defense in transgenic oranges leading to resistance against fungal pathogens

Manuscript submitted

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Abstract

Terpenoid volatiles are isoprene compounds that are emitted by plants to communicate with the environment. In addition to their function in repelling herbivores and attracting carnivorous predators in green tissues, the presumed primary function of terpenoid volatiles released from fruits is the attraction of seed-dispersing animals. Orange fruit primarily accumulates terpenes in mature peel oil glands, with D-limonene accounting for approximately 97% of the terpene content. In a previous report, we showed that the antisense downregulation of a D-limonene synthase gene alters monoterpene levels in orange fruit peels leading to resistance against *Penicillium digitatum* infection. A global gene expression analysis of transgenic fruits expressing antisense vs. empty vector constructs revealed that the downregulation of D-limonene upregulated genes involved in the innate immune response, including transcription factors likely involved in jasmonic acid (JA) signaling and secondary metabolite biosynthesis, which triggered the upregulation of JA metabolism and drastically increased the accumulation of JA in orange peels upon fungal challenge, explaining the resistance to necrotroph fungi observed in antisense fruits. In nature, D-limonene levels increase in orange fruit once the seeds are fully viable. The inverse correlation between the increase in D-limonene content and the decrease in the jasmonate-mediated defense response suggests that D-limonene promotes the infection by microorganisms that are likely involved in facilitating access to the pulp for seed-dispersing frugivores.

Introduction

Plants are sessile organisms that produce and emit a vast array of volatile organic compounds (VOCs) to communicate between parts of the same plant and with other plants. It is generally accepted that the original role of these compounds in nature is related to defense functions (Degenhardt et al., 2003). Most VOCs are terpenoids, fatty acid degradation compounds, phenylpropanoids and amino acid-derived products. Among these, terpenoids are likely to be the most abundant and expensive to produce (Gershenzon, 1994). Terpenoids are also isoprenoid-derived compounds as they are synthesized through the condensation of C5 isoprene units, a process that is catalyzed by a wide diversity of terpene synthases (TPS) using geranyl diphosphate (GPP) and farnesyl diphosphate (FDP) as substrates. These reactions give rise to the C5 hemiterpenes, the C10 monoterpenes and the C15 sesquiterpenes (Dudareva et al., 2006).

In green tissues, terpenoid synthesis is either induced upon wounding or occurs constitutively; terpenes can be then stored in specific organs or tissues where they would be most effective in defense responses, such as leaf trichomes, resin ducts and lacticifers, pockets near the epidermis or in secretory cavities in citrus species (Langenheim, 1994; Turner et al., 2000; Trapp and Croteau, 2001; Voo et al., 2012). Genetic engineering experiments have demonstrated that specific terpenoid compounds emitted by leaves can intoxicate, repel or deter herbivores (Aharoni et al., 2003; Wu et al., 2006), or they may attract the natural predators and parasitoids of damaging herbivores to protect plants from further damage (Kappers et al., 2005; Schnee et al., 2006). These terpenoids are naturally found in complex mixtures, and it has been proposed that they can act synergistically, as in conifer resin, for simultaneous protection against pests and pathogens (Phillips and Croteau, 1999). Although fatty acid degradation products (such as jasmonates) and phenylpropanoids (such as salicylates) as well as their volatile and non-volatile precursors are clearly involved in many induced defense responses against pests and pathogens (Glazebrook, 2005), much less is known regarding the participation of terpenoid volatiles in the defense against microorganisms in plants and regarding the possible interactions of these terpenoids with phytohormones.

In contrast to their function in leaves, when released from flowers and fruits, the main function of terpenoid volatiles is in the attraction of pollinators (Pichersky and Gershenzon, 2002; Kessler et al., 2008; Junker and Blüthgen, 2010; Schiestl, 2010) and seed-dispersing animals (Lomáscolo et al., 2010; Rodríguez et al., 2011b), respectively. Fruit maturation and ripening are usually associated with large increases in the synthesis and accumulation of specific flavored volatiles, which are proposed to function as signals for seed dispersal (Auldridge et al., 2006; Goff and Klee, 2006; Rodríguez et al., 2013).

Upon wounding, plant responses to biotic stresses are orchestrated locally and systemically by signaling molecules. Among these molecules, the jasmonates regulate defenses against arthropod herbivores and necrotroph fungal pathogens as well as biotrophic pathogens, such as some mildews (Ellis and Turner, 2001; Stintzi et al., 2001; Kessler et al.,

2004; Li et al., 2005; Wasternack, 2007; Browse and Howe, 2008). In addition to jasmonates, molecules such as salicylic acid (SA) and ethylene (ET) are major synergistic or antagonistic regulators of plant innate immunity. Plants produce a specific blend of these alarm signals after pathogen or pest attacks and the production of these alarm signals vary greatly in quantity, composition and timing. These signals activate differential sets of defense-related genes that eventually determine the nature of the defense response against the attacker (Reymond and Farmer, 1998; Rojo et al., 2003; De Vos et al., 2005). All genes that encode enzymes involved in the biosynthesis of jasmonates are jasmonic acid (JA)-inducible (Wasternack, 2006), indicating that JA biosynthesis is regulated by positive feedback. The precursor for the biosynthesis of JA is α-linolenic acid. The activity of the 13-lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) enzymes converts α-linolenic acid to *cis*-(+)-12-oxophytodienoic acid (OPDA). OPDA reductase 3 (OPR3) catalyzes the reduction of OPDA (and dinor-OPDA) to oxo-pentenyl-cycloheptane-octanoic acid (OPC-8), which, in turn, undergoes three rounds of β-oxidation leading to jasmonyl-CoA (JA-CoA) formation. JA-CoA is then cleaved by a putative thioesterase yielding (+)-7-iso-JA, which equilibrates to the more stable (-)-JA (Wasternack and Kombrink, 2010).

The exogenous application of jasmonates on plants and the existence of mutant and/or transgenic plants that are altered in JA biosynthesis or signaling have led to altered susceptibility or resistance to pathogens. For example, *Arabidopsis* mutants defective in JA perception (e.g. *coi1*) or biosynthesis (e.g. *aos* and *dad1*) are susceptible to pathogen infections (Feys et al., 1994; Xie et al., 1998; Park et al., 2002; Turner et al., 2002). In contrast, mutants (e.g., *cev1, ap2c1*) with constitutive or wound-induced activation of the JA pathway exhibit enhanced resistance to fungal pathogens and pests and phenotypes characteristic of JA-treated plants (Ellis and Turner, 2001; Ellis et al., 2002; Schweighofer et al., 2007). Impaired JA biosynthesis or signaling is generally associated with decreased levels of defensive compounds, including VOCs, and reduced plant biomass and/or fitness under insect attack (Howe et al., 1996; Halitschke and Baldwin, 2004).

Sweet orange (*Citrus sinensis* (L) Osb.) is a perennial tree species that is exposed to recurrent biotic and abiotic challenges during its decades of growth in orchards. Orange fruits undergo a non-climacteric maturation process in which the biochemistry, physiology and structure of the organ are altered to complete the release of mature seeds. These changes typically include fruit growth and texture modification; color change through the degradation of chlorophylls and a parallel induction of carotenogenesis in the peel (flavedo) and pulp; flavonoid accumulation in the pulp; increases and decreases in the sugar and acid contents, respectively; and global accumulation and selective emission of volatile terpenoids (Spiegel-Roy and Goldschmidt, 1996). In nature, D-limonene accumulates gradually in the oil glands of the peel during fruit development and reaches its maximum level shortly before the breaker stage; followed by a steady decline during maturation (Attaway et al., 1967; Kekelidze et al., 1989; Rodríguez et al., 2011b). The high amount of D-limonene that accumulates in orange peels has

a tremendous metabolic cost, suggesting an important biological role for this terpene and other related compounds in the interactions between fruits and the biotic environment.

Previously, we examined the biological role of D-limonene, by manipulating oil gland chemistry via the antisense overexpression of a D-limonene synthase gene from Satsuma mandarin in orange fruits. Compared to empty vector (EV) controls, fruit peels from antisense transformants (AS) showed a dramatic reduction in D-limonene accumulation; decreased levels of other monoterpenes, sesquiterpenes and monoterpene aldehydes; and increased levels of monoterpene alcohols. When challenged with the necrotroph fungus *Penicillium digitatum* (Pers.:Fr.), the causal agent of green mold rot, AS-transformed fruits were highly resistant to fungal infection. Because full susceptibility to *P. digitatum* infection was restored when AS fruits were supplemented with D-limonene but not other monoterpene alcohols, indicating that D-limonene accumulation in the orange peel was required for the successful progress of this plant-pathogen interaction (Rodríguez et al., 2011a; Rodríguez et al., 2011b). Green mold rot is the most important postharvest disease of citrus fruit worldwide, accounting for up to 60-80% of total losses during postharvest life of the fruit. *P. digitatum* is considered to be a specialist pathogen of citrus fruits that efficiently infects the peel through injuries in which ubiquitous fungal spores germinate and rapidly colonize the surrounding areas (Droby et al., 2008). The control of this pathogen relies heavily on the use of synthetic chemicals, but concerns regarding their potential negative effects on human health and also the generation of fungicide-resistant strains has encouraged finding alternatives, such as the generation of citrus trees with fruits that are genetically resistant to the pathogen.

In this work, to better understand the mechanism underlying the constitutive resistance to *P. digitatum* conferred by the reduction of limonene in AS orange fruits, we analyzed the pattern of fruit growth and the morphological and biochemical developmental characteristics, and performed a global analysis of gene expression using a 20K citrus microarray. The study is supplemented by examining the possible involvement of key hormone signals such as those eliciting JA accumulation in the fruit peel. We report here the reduced level of D-limonene in AS fruits is tightly associated with the constitutive activation of defense response signaling cascades and the accumulation of JA rapidly after inoculation. Together, our results establish for the first time a correlation between increased volatile terpene content and the decline of JA-mediated defense responses in a fleshy fruit during maturation, which would facilitate necrotroph fungal infections in citrus fruits.

Results

Downregulation of a D-limonene synthase gene leads to fungal resistance in the flavedo of transgenic orange fruits

It is generally accepted that flavedo of Citrus fruit is the entrance for fungal colonization and offer higher resistance to *P. digitatum* infection than the albedo (inner white area without oil

glands) (Kavanagh and Wood, 1967; Ballester et al., 2006). To examine the contribution of flavedo terpenes and the albedo to the susceptibility of orange fruit to infection, the flavedo of orange fruits was partially peeled off and the remaining fruit was left on the bench at room temperature to facilitate the germination of ubiquitous fungal spores. Whereas EV control fruits were infected by several fungi by the third day after peeling, samples from AS3 and AS7 antisense lines became infected to a much lower extent by the seventh day. This experiment was repeated monthly from August to December over two consecutive fruiting seasons with identical results that were independent of the developmental stage of the fruit (Supplemental Fig. S1) and the orange cultivar tested (Navelina, Fig. 1; Pineapple, results not shown). During the first week after peeling, fungal infection was exclusively restricted to wounded flavedo areas (Fig. 1A and B), and resistance was linked to very low D-limonene levels in the oil glands of AS transformants (Fig. 1C and D, Supplemental Table SI). The infecting fungi were morphologically identified as *P. digitatum*, *P. italicum* and *Aspergillus sp.* (Fig. 1E to H). Therefore, D-limonene and related terpenes produced in the flavedo of EV control fruits appeared to act as the primary inducers of fungal germination and growth (see also Rodríguez et al., 2011b).

Morphological and biochemical characteristics of the orange fruit flavedo were not altered in transformants showing constitutive downregulation of the D-limonene synthase gene

D-limonene accounts for approximately 97% of the total terpenes in oil glands from the flavedo of orange fruit (Dugo and Di Giacomo, 2002). To assess whether changes in D-limonene and other mono- and sesquiterpenoid accumulation in AS vs. EV transgenic fruits (Rodríguez et al., 2011b) could have affected peel morphology, the number and size of oil glands in green and mature flavedo from transgenic Navelina and Pineapple oranges were determined. As shown in Fig. 2A and B, as well as Supplemental Fig. S2A and B, oil glands increased in size as fruit grows, but they were comparable in number and diameter in AS and EV fruits. Moreover, peel thickness was also similar between AS and EV samples at the different developmental stages that were analyzed (Fig. 2C and Supplemental Fig. S2C).

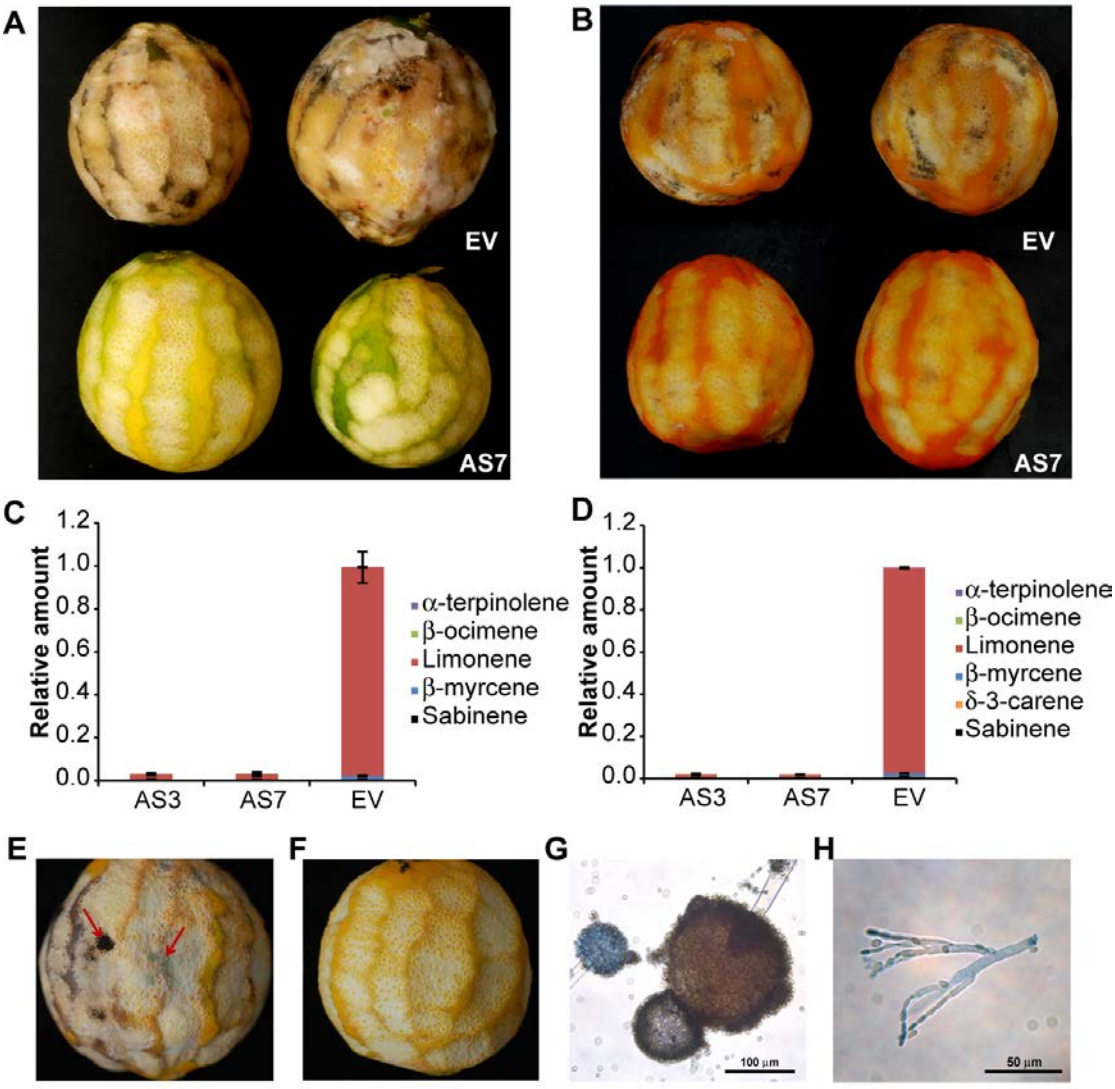

Figure 1. Fungal infection in partially peeled D-limonene transgenic (AS) and EV control fruits. (A, B) Disease incidence at five days after peeling in EV lines compared to AS lines in green (A) and mature (B) fruits. (C, D) The relative amount of individual terpenes is presented as a percentage area (given as a fraction of unity) of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in green (C) and mature (D) flavedo. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant. (E, F) Magnification of fruits in EV (E) and AS7 lines (F). The red arrows indicate flavedo-infected zones of EV fruit. (G, H) Microscopic identification of fungi-infected fruits. Images shown are light micrographs of *Aspergillus* sp. (G) at 20x magnification and *Penicillium* sp. (H) at 40x magnification. Scale bars indicate 100 mm and 50 mm, respectively.

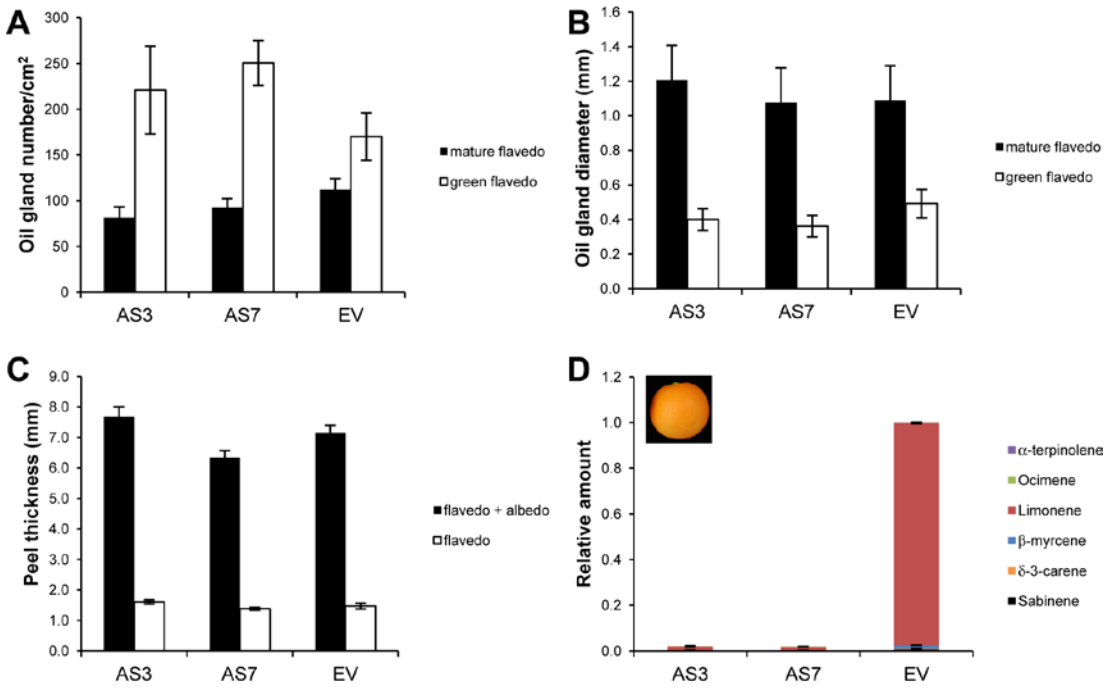

Figure 2. Characteristics of green (70 mm diameter) and mature (90 mm diameter) flavedo in antisense (AS) and control (EV) Navelina sweet orange plants. (A, B) Secretory oil gland number and size in green and mature flavedo. (C) Peel thickness in mature fruits. No significant differences were found at $P < 0.05$ using Fisher's Protected LSD test at each stage. (D) The relative amount of individual terpenes is presented as a percentage area (given as a fraction of unity) of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in the mature flavedo. Data represent mean values ± s.e.m. and are derived from at least five fruits per plant.

We then tested whether the transgenic manipulation of monoterpene biosynthesis in fruits may have induced a metabolic diversion and affected the levels of other related isoprenoids that share common precursors, particularly those important during the development of orange fruit, such as chlorophylls or carotenoids (Supplemental Fig. S3). Chlorophyll and total carotenoid contents in EV control green and mature flavedo from Navelina oranges were similar to those found in AS7 and AS3 flavedo (Fig. 3). The degreening of the fruits followed the same pattern in AS and EV control lines (Supplemental Fig. S1). Chlorophyll and carotenoid values were also similar in EV and AS fruits from the Pineapple orange (Supplemental Fig. S4). In addition, the percentage of individual xanthophylls and carotenes remained at nearly the same level in both EV and AS lines (Supplemental Fig. S5). Taken together, these results confirmed that fruit growth and development were not substantially altered by the drastic changes in monoterpene accumulation; thus, other factors must be responsible for the increased disease resistance found in the peel of D-limonene antisense plants.

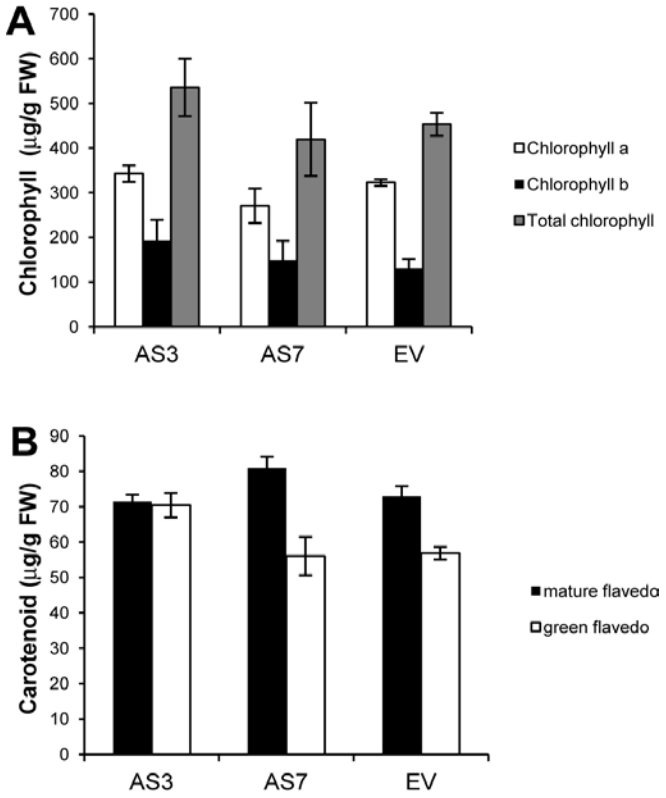

Figure 3. Total chlorophyll and carotenoid content in green and mature flavedo of Navelina sweet orange plants. No chlorophyll was detected in the flavedo of mature fruits. Data represent mean values ± s.e.m. and are derived from at least ten fruits per plant. No significant differences were found at $P < 0.05$ using Fisher's Protected LSD test in each stage.

D-limonene downregulation induced the expression of genes involved in the innate immune response against pathogens

To understand the mechanisms underlying the induced resistance of AS orange fruits to *P. digitatum* and other fungi, large-scale gene expression analysis was carried out using a 20k citrus cDNA microarray (Martinez-Godoy et al., 2008). Using intact mature flavedo tissue, gene expression in the AS3 and AS7 lines was compared to that of two independent EV control lines. The ectopic upregulation or downregulation of genes involved in eliciting defense responses against herbivores and plant pests usually results in phenotypic aberrations because such genes are also important for growth and development (Bedon et al., 2010; Kallenbach et al., 2010; Yang et al., 2012). As AS orange plants and fruits were visually indistinguishable from EV controls, we hypothesized that the impact of D-limonene synthase downregulation on the general transcript profile would not be very high quantitatively. Then, common genes from both AS lines showing at least a 1.6-fold expression change vs. EV lines were identified as

differentially expressed (Table I). We found differential gene expression in the AS3 line, with 82.9% of genes upregulated (Supplemental Table SIIA). Among the genes deregulated in the AS7 line, 93% were upregulated (Supplemental Table SIIB). To elucidate key processes that were altered in AS fruits, functional enrichment categories were searched for the full robust set of differentially expressed genes (Fig. 4, schematic representation of the full figure). Based on gene ontology (GO) terms, the genes downregulated in the flavedo of AS fruits were primarily involved in biological processes associated with secondary metabolism (Fig. 4). Antisense downregulation of the D-limonene synthase gene was found to reduce the transcription of nine genes, of which four encode enzymes that would be required for volatile terpenoid biosynthesis, such as a monoterpene (R)-limonene synthase gene and a putative germacrene-D synthase gene whose expression was reduced four-fold in the AS vs. EV samples in the microarray analysis (Fig. 5A) and ten- and five-fold in qRT-PCR analyses, respectively, in the AS3 line (Supplemental Fig. S6).

Table I. Common genes differentially expressed genes in the intact mature flavedo of two independent transgenic AS Navelina sweet orange plants (vs. EV plants).

UP-REGULATED GENES

Description	Citrus unigene	AS3 fold-change	AS7 fold-change	Most similar Ath gene
Defense response				
Cyclic nucleotide regulated ion channel (CNGC2)	aC32102F03EF_c	2.03	1.88	AT5G15410
Cyclic nucleotide regulated ion channel (CNGC2)	aCL5832Contig1	3.60	2.90	AT5G15410
Disease resistance protein (NBS-LRR class)	aCL5233Contig1	1.75	2.12	AT3G14460
Harpin-induced family protein (YLS9)/HIN1 family protein	aCL2389Contig2	2.06	1.63	AT2G35980
Nonspecific lipid transfer protein 1 (LTP1)	aCL4Contig13	3.31	3.46	AT2G38540
Phenylalanine ammonia-lyase 1 (PAL1)	aCL1166Contig2	3.47	1.96	AT2G37040
Protein phosphatase 2C, putative (PP2C)	aCL683Contig1	2.33	3.41	AT2G30020
Similar to zinc finger (CCCH-type) family protein (CZF1)	aC31603G11EF_c	2.38	3.53	AT2G40140
Chalcone synthase/naringenin-chalcone synthase	aCL27Contig2	1.85	1.94	AT5G13930
Encodes a member of the ERF subfamily B-3 of ERF/AP2 (ATERF-6)	aCL337Contig1	1.63	3.43	AT4G17490
R2R3-MYB family transcription factor (MYB73)	aCL693Contig1	3.18	2.34	AT4G37260
No apical meristem (NAM) family protein (NAC72)	aCL35Contig5	2.02	1.99	AT4G27410
Sodium-inducible calcium-binding protein (ACP1)	aCL1345Contig2	1.75	3.97	AT5G49480
Vacuolar processing enzyme gamma	aCL554Contig1	1.62	2.11	AT4G32940
Peptidase U7 family protein (SPPA)	aCL27Contig1	1.84	1.66	AT1G73990
Putative serine/threonine kinase SRK2F	aC04002A03SK_c	1.61	1.76	AT4G40010
Ser-thr protein kinase	aCL5546Contig1	1.62	1.75	AT2G40270
cellular component organization and biogenesis				
Eukaryotic translation initiation factor SUI1	aCL1184Contig4	1.99	1.78	AT5G54940
FAD-binding domain containing protein	aCL246Contig1	4.10	2.43	AT2G34790
Heavy-metal-associated domain-containing protein	aCL2730Contig1	2.21	1.67	AT4G08570
Histone H1-3 (HIS1-3)	aCL517Contig2	2.62	1.69	AT2G18050
Peptidase S41 family protein similar to	aCL7817Contig1	1.87	2.02	AT3G57680

PSII D1 protein processing enzyme				
Cellulose synthase family protein	aCL1355Contig1	1.73	1.82	AT2G32540
Other				
CCR4-not transcription complex protein	aCL206Contig1	3.26	3.61	AT5G22250
Strictosidine synthase family protein	aC31201B02EF_c	2.09	1.90	AT3G59530
WD-40 repeat family protein	aCL6446Contig1	2.19	1.90	AT1G53090
DC1 domain-containing protein	aCL2160Contig1	1.71	1.78	AT1G60420
Copper chaperone (CCH)-related	aCL4708Contig1	1.68	1.90	AT5G63530
Unknown				
3-oxo-5-alpha-steroid 4-dehydrogenase family protein	aC01011F03SK_c	2.14	2.26	AT5G16010
Calcium-binding protein (CML4)	aCL7914Contig1	2.09	1.71	AT1G21550
Calmodulin	aCL535Contig3	2.85	2.93	AT3G10190
Calcium-binding EF hand family protein	aCL8972Contig1	1.67	1.81	AT1G05150
Chac-like family protein	aCL283Contig1	1.71	1.91	AT4G31290
Chac-like family protein	aC05802B02SK_c	1.87	2.79	AT4G31290
Esterase/lipase/thioesterase family protein	aCL5939Contig1	2.07	1.75	AT1G54570
Expressed protein	aC08031A08SK_c	2.03	2.08	AT5G41110
Expressed protein	aCL8468Contig1	1.87	2.13	AT1G69760
Expressed protein	aCL6840Contig1	2.68	2.26	AT3G52740
UDP-glucoronosyl/UDP glucosyl transferase family protein	aC02002E10SK_c	1.87	2.04	AT3G02100
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aCL5570Contig1	2.85	1.95	AT2G36970
Remorin-like protein	aCL1490Contig1	1.82	1.69	AT2G41870
ATP-sulfurylase 1 (APS1)	aCL438Contig2	1.73	1.70	AT3G22890
No similar protein found	aCL8681Contig1	5.08	2.57	
No similar protein found	aC08007E01SK_c	4.64	9.66	
No similar protein found	aCL50Contig2	3.38	2.56	
No similar protein found	aCL1714Contig1	2.44	3.83	
No similar protein found	aC03007D01SK_c	2.39	1.87	
No similar protein found	aCL101Contig2	2.28	1.65	
No similar protein found	aC08007C02SK_c	2.24	1.99	
No similar protein found	aC31006C04EF_c	2.13	1.84	
No similar protein found	aC31206E07EF_c	2.08	1.86	
No similar protein found	aCL4787Contig1	2.06	1.83	
No similar protein found	aC31807H02EF_c	2.03	2.09	
No similar protein found	aC31305G08EF_c	1.97	1.97	
No similar protein found	aC19003E02T7_c	1.90	1.82	
No similar protein found	aCL2819Contig1	1.85	2.80	
No similar protein found	aC18001E11Rv_c	1.66	1.94	
No similar protein found	aC19006C07T7_c	1.61	2.62	

DOWN-REGULATED GENES

Description	Citrus unigene	AS3 fold-change	AS7 fold-change	Most similar Ath gene
Secondary metabolic process				
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aC02013A08SK_c	-4.65	-4.68	AT3G25820
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aCL2450Contig1	-3.48	-3.12	AT3G25820
Encodes a sesquiterpene synthase (germacrene-D synthase)	aCL4874Contig1	-4.03	-5.48	AT5G23960
Flavonol 3-O-methyltransferase 1 / Caffeic acid	aCL38Contig8	-3.01	-2.41	AT5G54160
cellular component organization and biogenesis				
TIP1;3 | major intrinsic family protein / MIP family protein. Aquaporin	aCL824Contig2	-1.94	-2.40	AT4G01470

Unknown				
Alcohol dehydrogenase	aC34109F01EF_c	-2.27	-2.20	AT5G42250
O-methyltransferase family 2 protein, similar to caffeic acid O-methyltransferase	aCL3052Contig1	-1.82	-2.27	AT4G35160
O-methyltransferase family 2 protein, similar to caffeic acid O-methyltransferase	aCL4905Contig1	-1.72	-2.32	AT4G35160
F4H5.19 protein	aC20001E01SK_c	-3.55	-3.13	AT1G06720

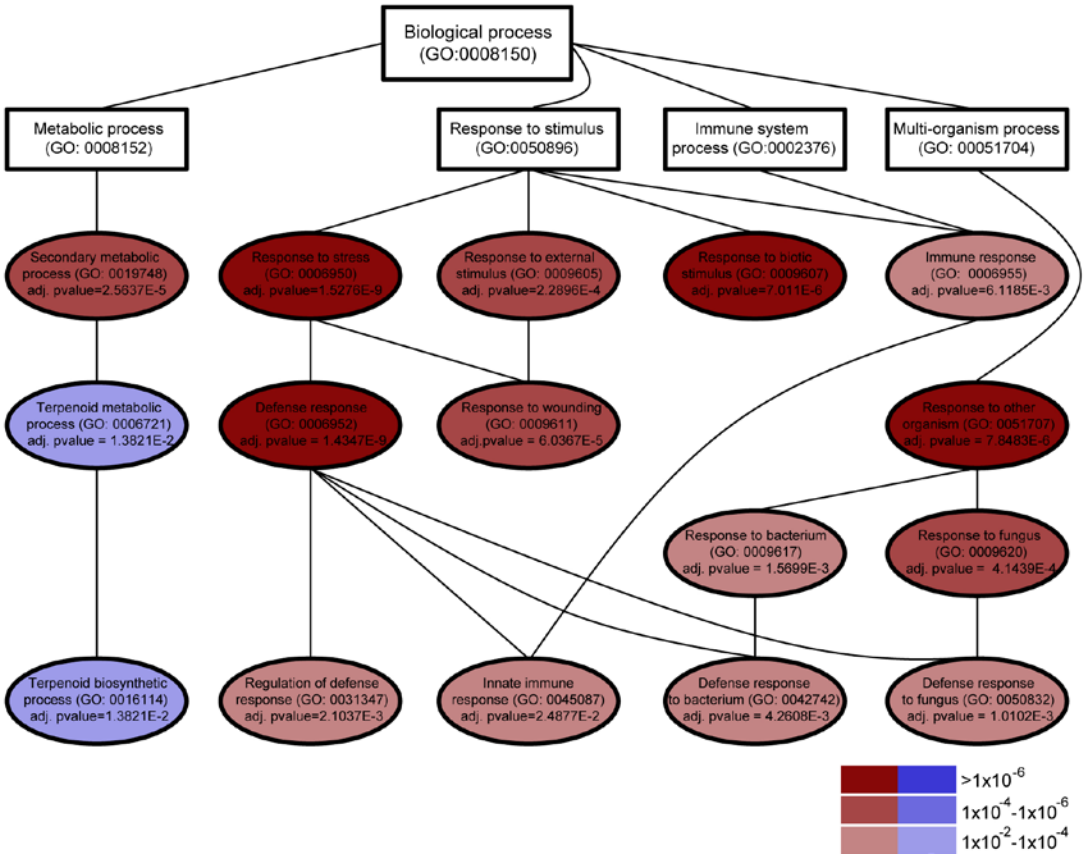

Figure 4. Antisense downregulation of the D-limonene synthase gene causes the downregulation of genes required for monoterpenoid biosynthesis and the upregulation of genes related to different defense responses in plants. A hierarchical view of gene ontology (GO) biological categories significantly deregulated in the intact flavedo of AS7 plants compared with EV control plants. Significant categories (adjusted $P < 0.05$) are shown using color scaling according to their significance level and to up- (red) or down-regulation (blue). Other categories required to complete the hierarchy are shown in white. A similar graph was obtained for AS3 plants.

Conversely, the biological processes that were over-represented in the AS flavedo compared with the EV controls were primarily associated with defense responses against biotic stresses, including defense responses to fungus and bacteria and the response to wounding (Fig. 4, schematic representation of the full figure). Fatiscan analysis allowed us to perceive a clear connection between the downregulation of secondary metabolism and upregulation of the response to biotic stresses at a global level. At least half of the 58 upregulated genes in both AS

lines were related to defense. Most of the other upregulated genes had unknown functions or did not match any known protein-coding gene in the databases. Increases in cytoplasmic calcium mediated by calcium influx (Nicaise et al., 2009) are critical for triggering defense pathways in plant cells. The expression of two cyclic nucleotide-regulated ion channel genes that are likely to be involved in cellular calcium entry was two to three times higher in the AS3 and AS7 lines than in the EV lines (Fig. 5; Table I). Several genes coding for calcium-binding proteins, including at least one calmodulin, were also upregulated in the AS lines (Table I). This calmodulin-like protein gene was confirmed to be upregulated by approximately two-fold in further qRT-PCR analysis (Supplemental Fig. S6). Calcium signals are sensed by calcium-dependent protein kinases (CDPK). Together with mitogen-activating protein kinases (MAPK), CDPK are essential elements for reprogramming transcriptional cascades that underlie the immune response in plants and animals (Akira et al., 2006; Boudsocq et al., 2010). Although putative CDPK genes, such as homologs of CDPK19 or CPK7, and MAPK genes, such as a homolog of MPK3 or MKK9, were found to be slightly upregulated (more than 1.5-fold) in one of the AS lines (Fig. 5; Supplemental Tables SIIA and SIIB). The citrus homolog of the early response *YLS9* gene (also known as *NHL10*) (Zipfel et al., 2004) was found to be upregulated approximately two-fold in AS3 and AS7 fruits (Table I). It has also been shown that several CPKs strongly induce *YLS9* (Boudsocq et al., 2010). Our results indicate that defense cascades were activated in terpene-downregulated orange fruits. Moreover, a putative protein phosphatase 2C gene that directly regulates several MAPKs (Schweighofer et al., 2007) was found to be strongly induced (by three-fold) in both AS lines (Fig. 5B; Table I).

The target genes of these signaling cascades include transcription factors (TFs) belonging to Zn finger (CCCH-type), MYC, ERF/AP2, MYB, WRKY and NAC family transcription factors, which have been associated with a suite of diverse mechanism leading to defense responses (Fujita et al., 2006) and were found to be upregulated in both AS lines (Fig. 5B; Table I; Supplemental Tables SIIA and SIIB). Citrus homologs of R2R3-MYB73 and ATERF6 were constitutively upregulated in the flavedo of AS lines and confirmed to be upregulated by three- and seven-fold, respectively, by qRT-PCR analyses (Fig. 5B; Supplemental Fig. S6). Genes encoding putative WRKY6 and WRKY33 transcription factors were induced more than four- and two-fold, respectively, but this was only observed in line AS7 (Fig. 5B; Supplemental Table SIIB). Moreover, several “no apical meristem (NAC domain)” genes were upregulated by approximately two-fold in both lines (Fig. 5B; Table I; Supplemental Tables SIIA and SIIB). Most of these TFs have been related to the JA-mediated elicitation of secondary metabolism and defense (Bedon et al., 2010; De Geyter et al., 2012).

Additionally, a large proportion of the remaining misregulated genes in either AS line or that were common to both of them could be linked to the phenylpropanoid biosynthetic pathway. Congruently, several FAD-binding domain-containing proteins were upregulated by more than two-fold in AS lines (Fig. 5C, Supplemental Table SII). Although homologs of *CHS* (Chalcone synthase) and *PAL1* (Phenylalanine ammonia-lyase 1) genes were upregulated by approximately two-fold in both AS lines, several *OMT1* (flavonol O-methyltransferases)

homologs were clearly downregulated by up to three-fold (Fig. 5C; Table I; Supplemental Table SII). *PAL1* was confirmed to be upregulated by four- to seven-fold in qRT-PCR analyses (Supplemental Fig. S6). This finding is consistent with the well-known role of some of the upregulated TFs as positive and negative regulators of enzymes required for the biosynthesis of phenylpropanoids (Grotewold, 2005).

Other defense related genes, such as *LTP1* (nonspecific lipid transfer protein 1) and *NBS-LRR* (nucleotide-binding site–leucine-rich repeat), were highly induced in the AS lines (Table I; Supplemental Table SII). *LTP1* was confirmed to be upregulated by four-fold in subsequent qRT-PCR analyses (Supplemental Fig. S6), suggesting its possible involvement in the induction of disease resistance responses in AS citrus fruits (Table I; Fig. 5B). Regarding cell wall organization and biogenesis, several homologs of cellulose synthase and other xyloglucan endotransglycosylase genes were found to be upregulated in both AS3 and AS7 fruits (Supplemental Tables SIIA and SIIB). Genes for other enzymes putatively involved in starch biosynthesis or electron transport were also upregulated (Supplemental Tables SIIA and SIIB; Table I). Overall, these results indicate that terpene downregulation activates constitutive defense responses in the fruit flavedo.

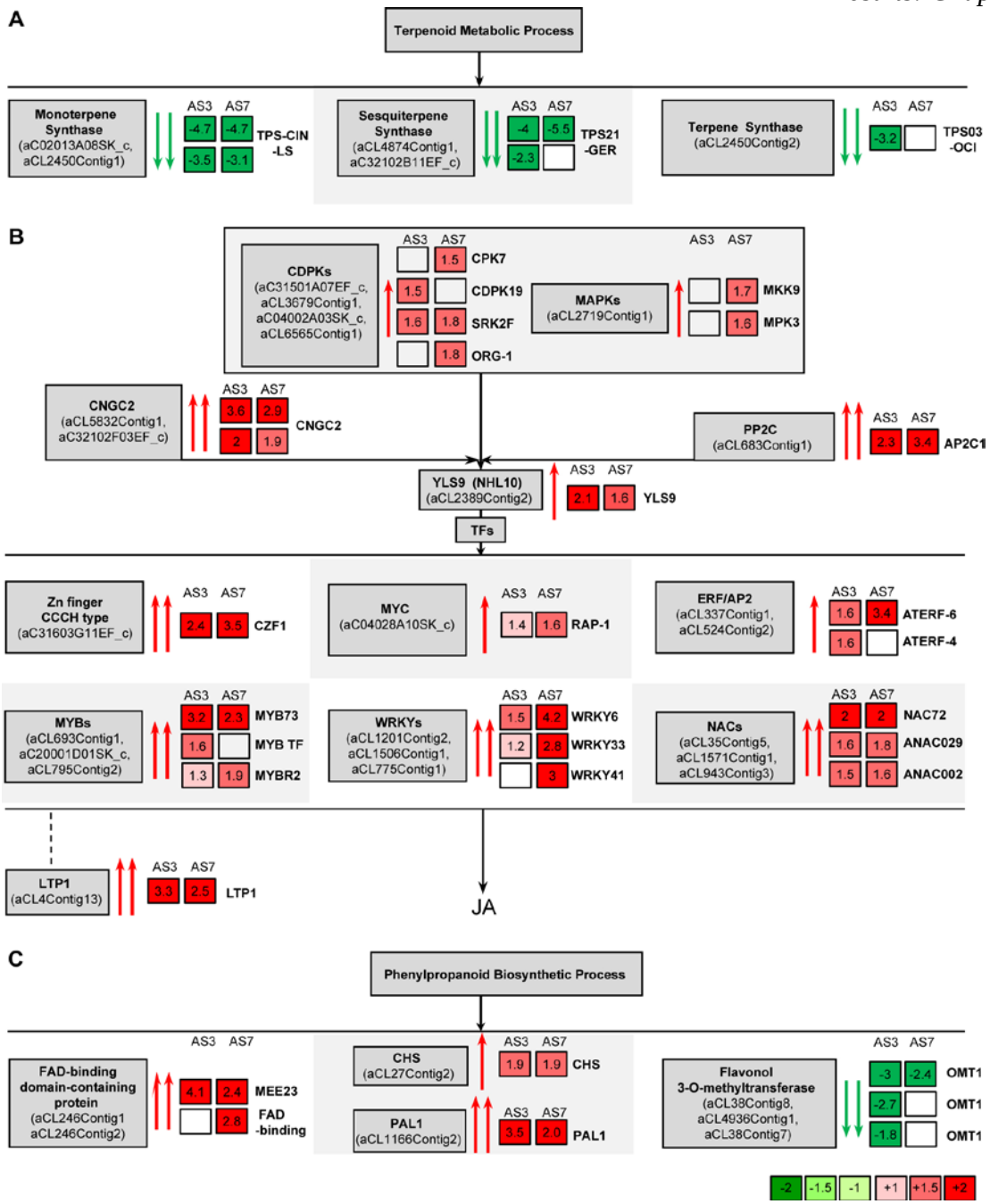

Figure 5. Graphical representation of the genes that were differentially expressed in the flavedo of intact oranges. (A) Genes involved in the terpenoid metabolic process that were downregulated in transgenic orange plants. (B) Defense-related genes that were upregulated in transgenic orange plants. (C) Genes involved in the phenylpropanoid biosynthetic process that were misregulated in transgenic orange plants. Numbers in squares indicate the ratio of expression in AS fruits compared to EV fruits. Blank square: gene not detected. The most similar *Arabidopsis thaliana* gene functions are listed at the right side of the squares. TPS-CIN-LS: Monoterpene synthase similar to Limonene synthase; TPS21-GER: Sesquiterpene synthase similar to Germacrene-D synthase; TPS03-OCI: Terpene synthase similar to β-ocimene/α-pharnesene synthase; CDPKs: Calcium-dependent protein kinases (CPK7, CDPK19, SRK2F, ORG-1); MAPKs: Mitogen-activated protein kinases (MKK9, MPK3); CNGC2: Cyclic nucleotide-regulated ion channel; PP2C: Protein phosphatase 2C (AP2C1); YLS9: Harpin-induced family protein (YLS9); Zn finger CCCH type family protein (CZF1); MYC: Basic helix-loop-helix (bHLH) protein (RAP-1); ERF/AP2: Ethylene response factor/AP2 domain (ATERF-6, ATERF-4); MYB: Members of the MYB family transcription factor (MYB73, MYB TF, MYBR2); WRKY: Members of the WRKY family transcription factor (WRKY6, WRKY33, WRKY41); NAC: No apical meristem (NAM) family protein (NAC72, ANAC029, ANAC002); LTP1: Nonspecific lipid transfer protein 1; FAD: Flavin adenine dinucleotide-binding proteins (MEE23, FAD-binding); CHS: Chalcone synthase; PAL1: Phenylalanine ammonia-lyase 1; Flavonol 3-O-methyltransferase (OMT1).

Downregulation of D-limonene and related terpenes triggered the accumulation of JA in orange peels upon fungal inoculation

Because the upregulation of specific TFs such as those described above has been linked to wound-, pathogen- and herbivore-induced JA accumulation, JA, together with SA and ABA levels, were quantified in the flavedo of AS and EV lines before and after inoculation with *P. digitatum* to assess whether these defense signaling molecules were activated or repressed by D-limonene downregulation. Whereas low levels of JA were observed in AS fruits before fungal inoculation (compared to EV controls), an approximately 7- to 20-fold increase in JA content was observed in AS flavedo 2 h after wounding (from 45 to 327 ng/g FW in the AS3 line and from 15 to 323 ng/g FW in the AS7 line) (Fig. 6A), reaching levels higher than those of flavedo of EV controls. A small decrease in JA levels was observed in the EV control samples after wounding (from 252 to 170 ng/g FW), indicating that fungal infection have a minimal effect on JA levels in these fruits. Moreover, the EV controls accumulated less JA than the AS samples after fungal inoculation (Fig. 6A), indicating that *Penicillium* infection enhanced JA content in AS fruits. These results were consistent in different independent transgenic lines over several months of two consecutive fruiting seasons (Supplemental Fig. S7). Our results suggested that the downregulation of D-limonene and related terpenes in AS fruits induced the accumulation of JA upon inoculation, which was the most likely in vivo regulator of resistance against necrotroph fungi in AS orange fruits. Conversely, high D-limonene contents in EV fruits might be related to the lack or depletion of jasmonate-mediated defense responses.

The SA content was constitutively low in all samples but increased in the flavedo of both AS and EV lines 2 h after inoculation; however, SA reached much higher levels in EV samples (nine-fold) compared to AS samples (six-fold in AS3) (Fig. 6B). The attenuated increases of SA in AS samples observed upon inoculation may be related to the inhibitory effect of JA, as antagonistic interactions between these two compounds are common and well documented in plants (Glazebrook, 2005). ABA levels were slightly reduced in AS samples when compared with EV controls, although they were strongly decreased in all samples after inoculation (Fig. 6C). These results might be better explained by the crosstalk of ABA with JA and/or SA signaling pathways and may not be directly related to constitutive monoterpene downregulation (Anderson et al., 2004; Flors et al., 2008).

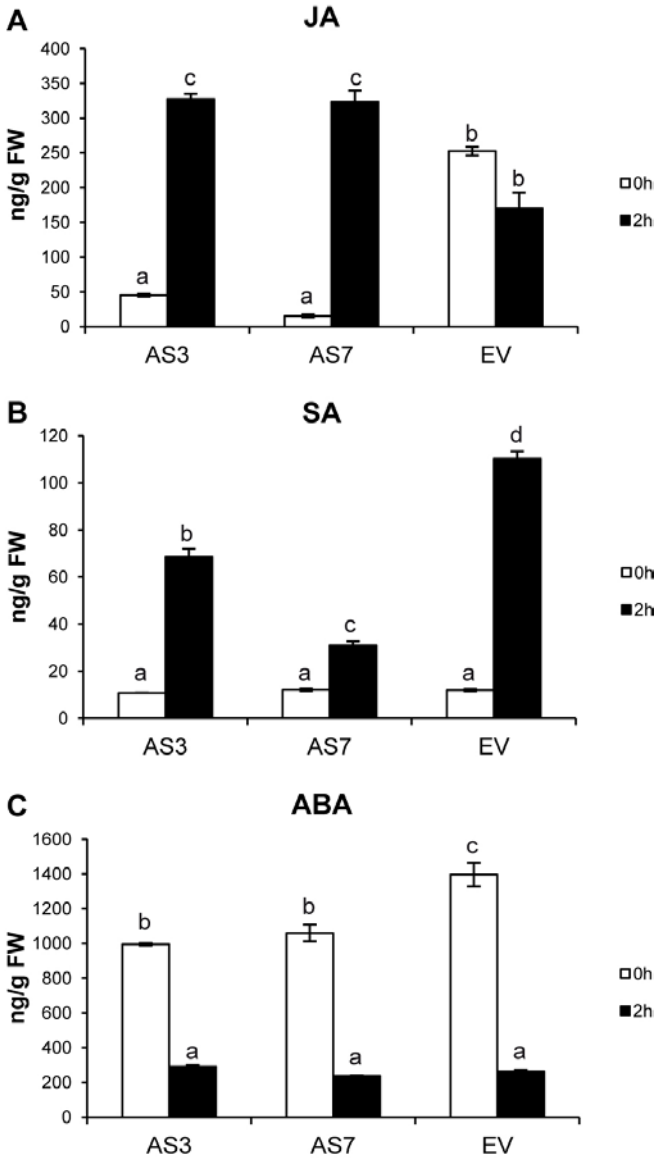

Figure 6. Phytohormone measurement in the flavedo of AS transgenic and EV control plants. The content of jasmonic acid (A, JA), salicylic acid (B, SA) and abscisic acid (C, ABA) were measured before (0 h) and after (2 h) fungal inoculation. Data represent mean values ± s.e.m. and are derived from at least six fruits per plant. Different letters indicate significant differences at $P < 0.05$ using Fisher's Protected LSD test.

The AS fruit response to fungal challenge inoculation mimics the exogenous application of JA to regular fruit resulting in protection against *P. digitatum* and the upregulation of genes of the JA biosynthesis and signaling pathways

Pretreatment of plants with exogenous jasmonates such as methyl jasmonate or JA has been shown to induce protection against different necrotroph pathogens in many plants, including *Arabidopsis* and grapefruit (*Citrus paradisi* Macf.) (Thomma et al., 1998; Droby et al., 1999). To examine the effect of the exogenous application of JA on induced resistance in citrus

fruits, ordinary untransformed Navelina oranges and clementine mandarins were wounded, and either water (W) or JA were immediately applied to the wounds, and then fruits were inoculated with *P. digitatum*. Pretreatment with JA conferred significant levels of induced resistance, as shown by the reduced percentage of infection in pretreated fruits three and four days after challenge inoculation (Fig. 7; Supplemental Fig. S8).

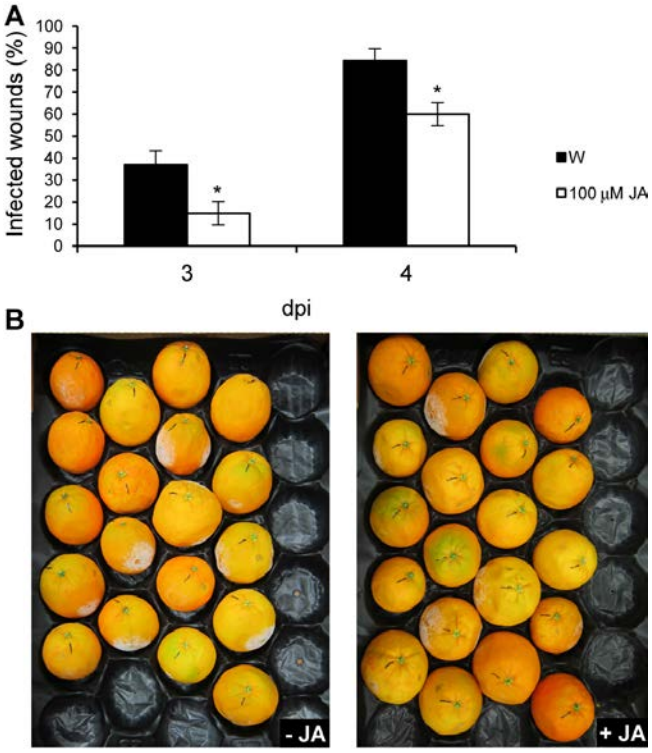

Figure 7. Exogenous application of jasmonic acid (JA) in the control orange plants confers fungal resistance. (A, B) Evolution of the disease caused by the fungus *Penicillium digitatum* in mature orange fruits inoculated with 1×10^5 spores mL^{-1} and treated with water (W) or jasmonic acid (JA). (A) Percentage of infected wounds in inoculated points three and four days after inoculation. The results are the average ± s.e.m. ($n \geq 19$). dpi: days post-inoculation. *, $P < 0.05$ using Student's *t*-test. We repeated all experiments at least twice and obtained similar results. (B) Control fruits four days after inoculation.

To verify that the observed resistance was directly related to the application of JA, the upregulation of several genes of the JA biosynthesis (such as sweet orange homologs of *LOX*, *AOS* and *AOC*) and signaling (such as sweet orange homologs of *COI1*, *MYC2* and *PDF1.2*) pathways was analyzed by qRT-PCR in AS and EV fruits before and after fungal inoculation. Transcript levels of *AOS*, coding for the bottleneck enzyme of the pathway (Schaller, 2001), were rapidly induced in both "inoculated + W" and "inoculated + JA" samples 2 h after treatment; but levels of AOS transcripts were much higher in JA- than in W-treated plants (6.8- vs. 3.1-fold changes, respectively) (Fig. 8A), indicating that exogenous application of JA positively boosted JA biosynthesis. Among TFs acting downstream of JA in the stress responses, the bHLHzip-type transcription factor AtMYC2, a master switch in JA signaling, is essential regulating networks that modulate phytohormone and secondary metabolism in plants (De Geyter et al., 2012; Wasternack and Hause, 2013). Additionally, AtMYC2 negatively

regulates the defensin gene *PDF1.2* (Lorenzo et al., 2004). The sweet orange homolog of *MYC2* was upregulated in JA-treated samples whereas *PDF1.2* was downregulated in both W- and JA- treated fruits. No differences were found in the relative expression of the other genes analyzed (Fig. 8A).

Next, we investigated whether such genes were misregulated in the transgenic AS vs. EV control fruits. Before inoculation with *P. digitatum*, most genes of the JA pathway showed only slight, non-significant differences in expression, which was consistent with the microarray data. Significant downregulation was found only in the case of the *PDF1.2* gene in AS7 (Fig. 8B). One explanation for the discrepancy in basal JA accumulation (but not JA pathway gene expression) between AS and EV fruits is likely related to the post-transcriptional or post-translational modification of certain enzymes in the JA biosynthesis pathway, affecting enzyme abundance and/or activity (Yang et al., 2012).

After inoculation with *P. digitatum*, AS samples showed a marked *AOS* activation (6.1- and 5.9-fold changes in AS3 and AS7, respectively), an increase in *MYC2* expression (1.8- and 2.3-fold change) and a slight decrease in *PDF1.2* expression, similar values to those found in JA-treated untransformed fruits (Fig. 8C). Collectively, the upregulation of JA biosynthetic and signaling genes and the drastically increased JA levels upon inoculation mostly explain the resistant phenotype shown by AS fruits against fungal infection.

To assess whether the upregulation of JA metabolism upon challenge inoculation altered isoprenoid pathways in both JA-treated and AS fruits, the expression of sweet orange homologs of 1-deoxyxylulose 5-phosphate synthase (*DXS*), geranylgeranyl diphosphate synthase (*GGDP*), geranyl diphosphate synthase (*GDP*) and farnesyl diphosphate synthase (*FDP*) was analyzed by qRT-PCR in “inoculated + W” and “inoculated + JA” EV fruits and in EV vs. AS fruits before and after fungal challenge. Inoculation upregulated *GGDP* in control fruits, but JA application enhanced this effect (Fig. 9A). This gene was constitutively upregulated in AS7 but not in AS3 compared to EV control samples (Fig. 9B), but it was 2 h after inoculation when its expression dramatically increased, by more than ten-fold, in both AS lines (Fig. 9C). No significant differences were found in the expression of the other prenyl transferase genes in JA-treated and in transgenic AS fruits either exposed or not exposed to fungal challenge when compared to corresponding controls (Fig. 9A to C). Therefore, enhanced JA perception and signaling in AS fruits upon inoculation additionally triggered alterations in the expression of *GGDP* and likely in isoprenoid metabolism.

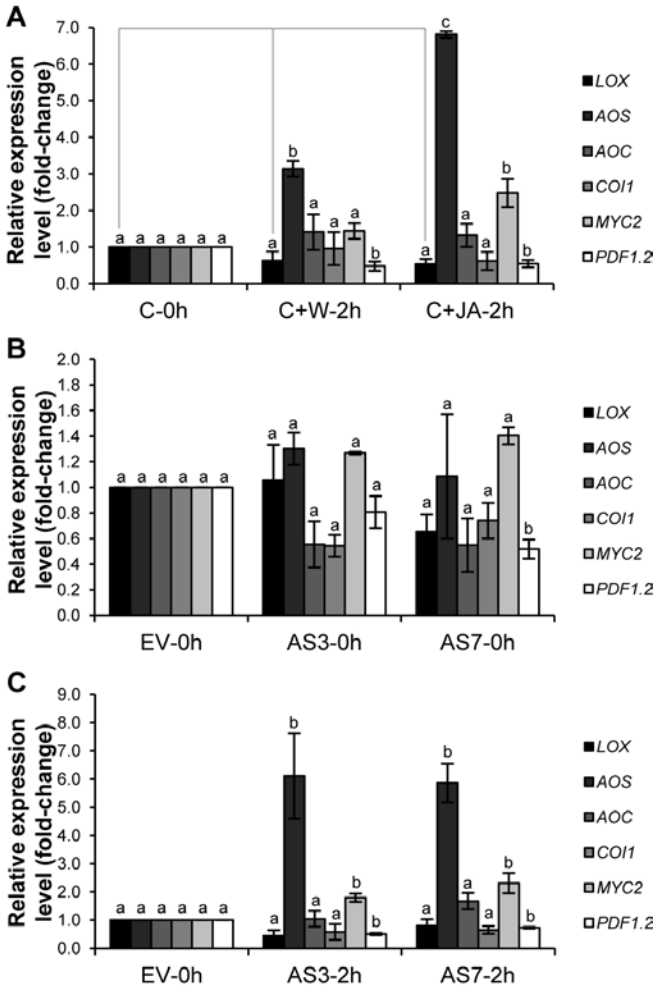

Figure 8. Quantitative real-time PCR analyses of genes involved in jasmonic acid (JA) biosynthesis (sweet orange homologs of *LOX*, *AOS* and *AOC*) and signaling (sweet orange homologs of *COI1*, *MYC2* and *PDF1.2*) before and after fungal challenge inoculation. Differential expression of these genes in regular untransformed fruits without (C+W-2h) or with application of JA (C+JA-2h) (A) and in different independent transgenic lines without application of JA (B, C) was measured before (0 h) and after (2 h) *Penicillium digitatum* inoculation. Expression of each gene was analyzed in at least 12 independent technical replicates using four different 96-well plates. Fold-change was calculated in relation to two independent regular and EV control lines before inoculation, to which an arbitrary value of one was assigned. Different letters indicate significant differences in the expression of each gene at $P < 0.05$ using Fisher's Protected LSD test. *LOX*, Lipoxygenase; *AOS*, Allene oxide synthase; *AOC*, Allene oxide cyclase; *COI1*, Coronatine-insensitive 1; *MYC2*, bHLHzip-type transcription factor; *PDF1.2*, Defensin-like gene.

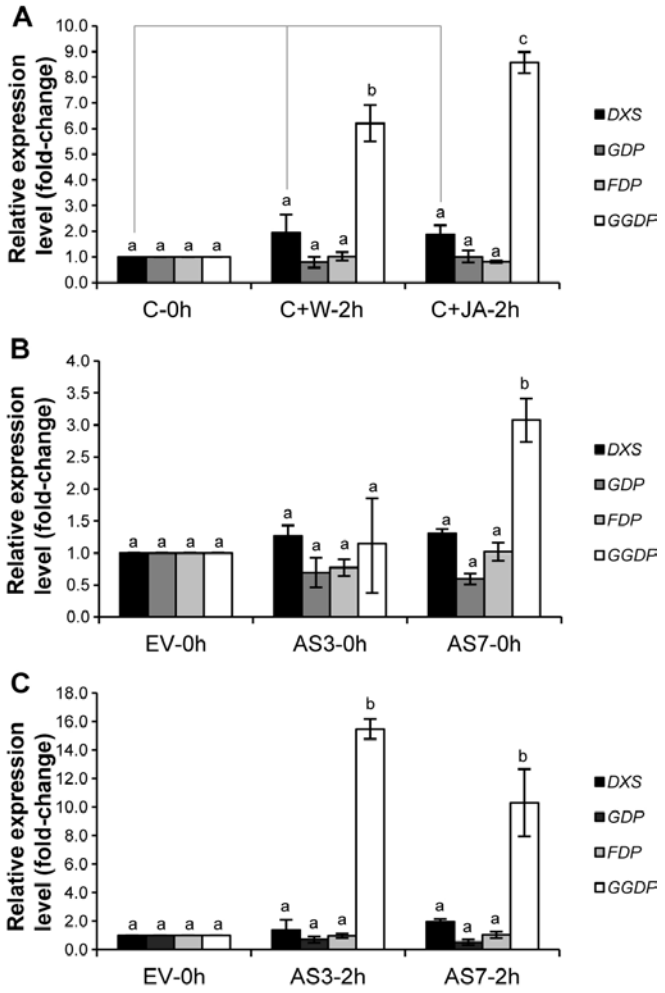

Figure 9. Quantitative real-time PCR analyses of genes involved in terpenoid biosynthesis (sweet orange homologs of *DXS*, *GGDP*, *GDP* and *FDP*) before and after fungal challenge inoculation. Differential expression of these genes in regular untransformed fruits without (C+W-2h) or with application of jasmonic acid (JA) (C+JA-2h) (A) and in different independent transgenic lines without application of JA (B, C) was measured before (0 h) and after (2 h) *Penicillium digitatum* inoculation. Expression of each gene was analyzed in at least 12 independent technical replicates using four different 96-well plates. Fold-change was calculated in relation to two independent regular and EV control lines before inoculation, to which an arbitrary value of one was assigned. Different letters indicate significant differences in the expression of each gene at $P < 0.05$ using Fisher's Protected LSD test. *DXS*, 1-deoxyxylulose 5-phosphate synthase; *GDP*, Geranyl diphosphate synthase; *FDP*, Farnesyl diphosphate synthase; *GGDP*, Geranylgeranyl diphosphate synthase.

Discussion

Terpenoids are ecologically important molecules (Degenhardt et al., 2003) due to various properties such as their volatility, flavor/aroma and toxicity, which give them important roles in plant defense, plant-to-plant communication and pollinator attraction (Pichersky and Gershenzon, 2002). Transgenic plants with modified terpenoid production can contribute to fundamental studies aimed to understand their function in plant/environment relationships

(Aharoni et al., 2005). In a previous study, we showed that the transgenic downregulation of a D-limonene synthase gene led to a dramatic reduction in the levels of D-limonene and other monoterpene and sesquiterpene hydrocarbons whereas monoterpene alcohols, such as nerol or citronellol, were substantially increased. Consequently, fruits were more resistant to *P. digitatum* and bacterial pathogens (Rodríguez et al., 2011a; Rodríguez et al., 2011b). Resistance to *P. digitatum* was related to D-limonene down-regulation and not to the increased accumulation of monoterpene alcohols, as in vitro assays with pure (+)-limonene, nerol or citronellol showed that these compounds were not toxic to the fungus but, instead, had a pronounced stimulatory effect on the germination of *P. digitatum* spores that was directly related to the concentration of the pure compound used.

Microarray-mediated transcriptional profiling has been successful in identifying constitutively activated defense signaling pathways in the flavedo of AS citrus fruit potentially related to the increased fungal resistance. Characteristic CDPK and MAPK cascades were upregulated in AS samples in addition to early response and protein phosphatase kinase targets (Asai et al., 2002; Boudsocq et al., 2010) that phosphorylate TFs belonging to the R2R3-MYB, MYC, WRKY, ERF/AP2 and NAC domains, and the Zn finger (CCCH-type) families, which likely participate in defense (Wang et al., 2008; Guo et al., 2009; Birkenbihl and Somssich, 2011; De Geyter et al., 2012). Recently, many of these TFs, often JA-activated themselves, have been related to the JA-modulated regulation of defense and elicitation of secondary metabolism. For example, the homolog *MYB14* (as well as other members of the R2R3-MYB TF family with a conserved EAR motif) is activated in the conifer trees *Picea glauca* (white spruce) and *Pinus taeda* (loblolly pine) after JA application (or wounding when overexpressed transgenically) and contributes to the accumulation of terpenoids and broad defense responses (Bedon et al., 2010). WRKY33, a JA- or pathogen-inducible TF, is required for the MPK3/MPK6-induction of biosynthesis of the major defense compound camalexin in *Arabidopsis thaliana* (Mao et al., 2011), and the silencing of WRKY3 or WRKY6 generates plants highly vulnerable to herbivores by impairing JA accumulation and the synthesis of sesquiterpene volatiles in *Nicotiana attenuata* (Skibbe et al., 2008).

Microarray results suggested that the coordinated upregulation of these TFs together with Ca^{2+} entry into the cell and activation of MAPK cascades could contribute to defense of orange fruits against fungal infection through the activation of the JA metabolism (Wasternack and Hause, 2013). Consistent with these results, the accumulation of JA and transcripts encoding the sweet orange homologs of *AOS* and *MYC2* dramatically increased in AS fruits after inoculation with *P. digitatum*, mimicking the response of untransformed orange fruits to JA treatment. Although almost all enzymes involved in the biosynthesis pathway of JA have been identified and characterized, the signaling pathways regulating the production of JA remain largely unknown (Yang et al., 2012). For example, in *Arabidopsis* the expression of *AOS* is activated by a variety of signals, including wounding, JA, OPDA and SA, and the regulation of the expression of the AOS protein exerts a major control on JA signaling (Laudert and Weiler, 1998). However, the overexpression of *Arabidopsis AOS* in transgenic *Arabidopsis* and tobacco

plants did not alter the basal level of JA. These transgenic plants produced higher levels of JA only after wounding when compared to wounded, untransformed plants, suggesting that the accumulation of AOS might be a way of controlling defense responses when actually required, at least in these plants (Laudert et al., 2000). It might also be possible that plants counter-regulates the production of higher amounts of these signaling molecules through yet unknown mechanisms. Regarding *MYC2*, *AtMYC2*, together with *AtMYC3* and *AtMYC4*, activates JA responses in *Arabidopsis* (Fernández-Calvo et al., 2011). Nevertheless, the overexpression of *AtMYC2* does not promote a constitutive response to JA in the absence of the hormonal signal, suggesting that additional JA-regulated factors cooperate with *AtMYC2* in the activation of the responses to this hormone (Lorenzo et al., 2004). Additionally, *AtMYC2* plays a central role in priming against pathogens during induced systemic resistance (Stein et al., 2008). In orange fruits, D-limonene downregulation was not accompanied by massive constitutive changes in the transcriptomic profile. Instead, it seems that mild induction of defense-related genes was able to prime fruits for JA-mediated defense upon challenge inoculation. Defense responses are vital but costly for the plant. Thus, plants may activate different inducible mechanisms depending on the attacker rather than maintaining them continuously. These mechanisms are subjected to tight regulation because their rapid activation may be vital for a successful defense (Pozo et al., 2004; De Geyter et al., 2012). One of these inducible mechanisms involves the accumulation of JAs that play a major role in basal and induced resistance against necrotroph pathogens in addition to their role in plant-herbivore interactions. The JA signal needs to persist as long as the plant is under attack. However, multiple regulatory mechanisms have been also developed to keep such energy-consuming responses silent under normal conditions (Glazebrook, 2005; Wasternack, 2007). Such a precise modulation of JA-mediated defense responses seems to be modulated by the D-limonene accumulated in oil glands of orange fruits.

A second mechanism by which D-limonene downregulation may activate defenses against pathogens is through the upregulation of disease resistance/LRR protein genes family (Chen et al., 2002) or other genes that code for proteins that are either antimicrobial themselves or that catalyze the production of antimicrobial compounds such as LTP1, which was upregulated in AS lines and is predicted to be a member of the PR-14 pathogenesis-related protein family. Various LTPs have been shown to have in vitro antimicrobial activity against fungi and bacteria (Sels et al., 2008). LTP1 is localized in the cell wall and binds calmodulin in a Ca^{2+}-independent manner (Thoma et al., 1994; Wang et al., 2004). The endogenous overexpression of three *LTP*-like genes in *A. thaliana* resulted in enhanced tolerance to *Botrytis cinerea* (Chassot et al., 2007). Moreover, transgenic *A. thaliana* plants overexpressing a barley *LTP1* gene exhibited enhanced resistance against *Pseudomonas syringae* pv. *tomato* and *B. cinerea* (Jung et al., 2005).

To understand the basis of the induction of resistance against *P. digitatum* in citrus fruits, Ballester et al. (2011) using a 12K citrus cDNA microarray studied transcriptional changes in elicited fruits. Elicitation consisted of inoculation with the fungus followed by a curing treatment one day later (37 ºC for three days with high RH) that strongly reduced the incidence

of green mold in oranges. Several days after infection, the most highly induced genes belonged to the phenylpropanoid and ET pathways. Although wounding, infection and successive curing treatments would likely cause the upregulation of many stress-responsive genes, including those from both pathways, the expression of *PAL1*, the first gene in the phenylpropanoid pathway, was consistently increased in elicited fruits along with other downstream genes. We show here that some genes of the phenylpropanoid pathway, such as *PAL1*, together with *CHS*, were constitutively activated in the flavedo of D-limonene AS fruits before inoculation with *P. digitatum*. Other putative genes of the pathway, such as *C4H*, *CAD* and *REF8*, were also slightly (approximately 1.5-fold) upregulated in our transcriptional profiling analyses, although several O-methyltransferases were downregulated in the case of AS fruits. Elicited and AS fruits showed altered phenylpropanoid biosynthetic pathways. However, elicitation and curing had no effects on JA metabolism, suggesting that enhanced JA levels and signaling in AS fruits upon inoculation were a direct consequence of drastic changes in monoterpene levels and the constitutive induction of defense responses that primed the fruits for resistance.

Transgenic plants with constitutive changes in terpenoid or JA production often present altered phenotypes compared to their corresponding wild-type counterparts (Estévez et al., 2001; Aharoni et al., 2003; Glazebrook, 2005; Wu et al., 2006). One possible explanation for the growth retardation phenotype in these transgenic lines is the depletion of the precursors, which may lead to reductions in the levels of essential compounds. These compounds include growth regulators and other vital components such as carotenoids, chlorophyll and quinones. However, transgenic orange fruits with strong D-limonene synthase downregulation did not exhibit growth retardation or changes in chlorophyll/carotenoid levels when compared to control fruits. Oil gland and peel morphology were also not affected by the drastic reduction of D-limonene levels in AS peels. Congruently, prenyl transferase genes were not consistently misregulated in AS fruits before fungal challenge inoculation. These results indicate that in orange fruits, neither a reduction in D-limonene nor its metabolic consequences caused morphological alterations or other pleiotropic effects. In the same sense, the constitutive upregulation of TFs, such as MYBs or WRKYs, was sufficiently moderated in AS oranges to avoid the diversion of resources away from fruit growth and development. Such phenotypical alteration conversely occurred in transgenic plants either upregulating or downregulating genes encoding similar TFs (Bedon et al., 2010; Kallenbach et al., 2010; Yang et al., 2012).

The application of JA or related members of the octadecanoid pathway to plant leaves mediates the induction of VOCs, increases the level of certain toxins and upregulates defense gene expression (Baldwin, 1998; Dicke et al., 1999; Thaler, 1999). Many of the VOCs induced in response to mechanical damage or herbivory are fatty acid degradation products that share precursors with jasmonates. In addition, a tight correlation between MYC2 and terpenoids has recently been demonstated, as MYC2 positively regulates sesquiterpene (and likely monoterpene) biosynthesis in *Arabidopsis* (Hong et al., 2012). Whether D-limonene downregulation and subsequent JA metabolism activation upon inoculation lead to changes in the isoprenoid and defense-related pathways in orange fruits remains to be investigated.

However, this is plausible as we showed here that *GGPD* was dramatically upregulated in AS fruits after fungal inoculation as well as in JA-treated untransformed fruits.

The downregulation of a D-limonene synthase gene in orange flavedo triggers innate immunity defense cascades linked to the activation of the jasmonate signaling pathway and a consequent strong resistance to necrotroph pathogens. The molecular basis for this crosstalk between D-limonene downregulation and the signaling pathways that mediate pathogen resistance is unknown. Recent findings have shown that glucosinolates, which are another group of secondary metabolites previously identified as important VOCs involved in the avoidance of herbivory damage in Brassicaceae, are required for the plant defense response against fungal and bacterial pathogens in leaves; specifically, glucosinolates are essential for the MAMP-triggered callose innate immune response to both adapted and non-adapted pathogens (Bednarek et al., 2009; Clay et al., 2009). In the present study, the drop in D-limonene accumulation in AS plants produced an increase in the constitutive defense-related genes leading to the accumulation of JA upon inoculation. Because AS fruits exhibited a broad resistance to different pathogens (Rodríguez et al., 2011b; results not shown), it would be interesting to test whether JA metabolism could also be activated by such different pathogens and/or under distinct plant-pathogen-pest interaction scenarios.

In nature, the D-limonene concentration is lower in the first stages of orange development. However, once the fruit has almost attained its final size and the seeds are fully viable, D-limonene levels drastically increase, and it becomes the predominant constituent of flavedo oil glands until fruit maturation (Dugo and Di Giacomo, 2002; Flamini and Cioni, 2010; Rodríguez et al., 2011b). We have shown here that high D-limonene and related terpene levels are tightly associated with a general depletion of defense-related genes. Because an increase in D-limonene occurs once the seed is formed and this coincides with a general enhanced susceptibility to opportunistic pathogens (Rodríguez et al., 2011b), our results indicate that the high accumulation of D-limonene and related terpenes might be a signal that attracts frugivores, including microorganisms. As the accumulation of D-limonene at high levels in flavedo of mature fruits is common to all *Citrus* species, including relatives and ancestral types (Dugo and Di Giacomo, 2002), our results may also indicate that *P. digitatum* and other microorganisms have acted to shape the evolution of D-limonene content in citrus fruit peel. Whether this additionally serves to attract legitimate vertebrate dispersers or facilitates their access to the fruit pulp and seeds requires further investigation.

Materials and methods

Plant material

Fruits of independent lines of Navelina and Pineapple sweet orange plants (*Citrus sinensis* L. Osbeck) at different developmental stages (August and December; see

Supplemental Fig. 1) were harvested over three consecutive years. To determine the oil gland size and number in orange peels, a defined area of 200 mm^2 along the equator of the fruit was measured using 10 fruits for each developmental stage. Gland density was measured using fruits of 70 and 90 mm in diameter (green and mature Navelina flavedo, respectively) and 60 and 80 mm in diameter (green and mature pineapple flavedo, respectively). Images were taken with a Leica DFC490 digital camera mounted on a magnifying glass, and secretory glands visible on the surface were counted and measured using the UTHSCSA ImageTool software (version 3.0, Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas). For all fruit, gland density was expressed as number of glands per cm^2. Peel thickness was measured with a caliper (as both flavedo and albedo or flavedo only) in four different sections around the equator of 10 mature fruits.

For the analysis of chlorophyll and total carotenoid content, the flavedo tissue (outer colored part of the fruit peel) was separated from the fruits. The flavedo was frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C until analysis. The data for oil gland diameter, oil gland number, chlorophyll and carotenoid content are presented as the means ± s.e.m. of 10 replicate samples.

For phytohormone quantification in fruit flavedo, mature fruits that were 90 mm in diameter were used. Flavedo samples were excised with a razor blade before (0 h) and after (2 h) inoculating the fruit in the equatorial region with a stainless steel rod as described in (Rodríguez et al., 2011b). Data were obtained from the analysis of at least six fruits per line, and this analysis was repeated several times during the fruiting season and over two consecutive years.

Fungal assays

For the experiments of natural infection by fungi, fruits were harvested monthly during a five-month period for two consecutive years. Ten fruits per independent transgenic line were used for each experiment. Fruits were partially peeled and put into plastic trays for germination of ubiquitous spores. Observations were made daily for the appearance and progress of symptoms. Samples placed on slides for microscopic identification were obtained from fungi-infected fruits.

For the assays of jasmonic acid (JA) supplementation to the regular Navelina sweet orange control and Clementine mandarins (*Citrus clementina* Hort. ex Tan.) fruits, 25 μL of a 100 μM jasmonic acid (JA, Sigma-Aldrich) aqueous solution containing 0.1% Tween-20 (Sigma-Aldrich) as surfactant were allowed to penetrate in the wound and the same procedure for inoculation and incubation described before (Rodríguez et al., 2011b) was followed. Inoculation was performed with 1×10^5 spores mL^{-1}. For control treatment, a solution of water supplemented with Tween-20 (0.1% v/v) was used. For each treatment, replicates of at least 18 and 30 fruits per line were used with orange and clementines, respectively. Disease incidence was estimated as the number of infected wounds per total number of inoculated points.

Chlorophyll and total carotenoid extraction and quantification

Fruit pigments were extracted as described previously (Rodrigo et al., 2003). The chlorophyll (*a* + *b*) content was determined by measuring the absorbance at 644 and 662 nm and calculated according to the Smith and Benitez equations (Smith and Benitez, 1955). After chlorophyll measurements, the pigment ethereal solution was dried and saponified using a 10% methanolic KOH solution. The carotenoids were subsequently re-extracted with diethyl ether until the hypophase was colorless. An aliquot of the ethereal extract was used for quantification of total carotenoid content. The total carotenoid content was calculated by measuring the absorbance of the saponified extracts at 450 nm using the extinction coefficient of β-carotene, $E^{1\%}$ = 2500 (Davies, 1976). The samples were dried under N_2 and kept at -20 °C until high-performance liquid chromatography (HPLC) analysis. All operations were carried out on ice under dim light to prevent photodegradation, isomerizations, and structural changes in the carotenoids.

HPLC of Carotenoids

For HPLC analysis of carotenoids, the peels of fruits at two maturation stages were selected as follows: fruits harvested in August (green) and fruits harvested in December (mature). The samples were prepared for HPLC by dissolving the dried residues in MeOH:acetone (2:1, v/v). Chromatography was carried out using a Waters liquid chromatography system equipped with a 600E pump, a model 996 photodiode array detector and Millennium Chromatography Manager software (version 2.0) (Waters, Barcelona, Spain) as described previously (Rodrigo et al., 2004). A C_{30} carotenoid column (250 mm x 4.6 mm, 5 μm) coupled to a C_{30} guard column (20 mm x 4.0 mm, 5 μm) (YMC Europe GMBH, Schermbeck, Germany) were used with MeOH, water, and methyl *tert*butyl ether. Carotenoid pigments were analyzed by HPLC using a ternary gradient elution that was reported previously (Rouseff et al., 1996). The photodiode array detector was set to scan from 250 to 540 nm throughout the entire elution profile. The area of each peak was obtained, and the percentage of each individual carotenoid was calculated over the total area of carotenoid peaks, as integrated by the Maxplot chromatogram. Each sample was extracted twice, and two replicate injections from each extraction were performed. The β-carotene and α-carotene standards were obtained from Sigma-Aldrich (Madrid, Spain). The β-cryptoxanthin, lutein, and zeaxanthin standards were obtained from Extrasynthese (Lyon, France).

Extraction of Volatiles and Gas Chromatography-Mass Spectrometry Analysis

Flavedo tissue was obtained from orange fruits, immediately frozen in liquid nitrogen, and stored at -80 ºC until extraction. A Thermo Trace GC Ultra coupled to a Thermo DSQ mass spectrometer with the electron ionization mode set at 70 eV was used. Extraction and analysis was carried out as described before (Rodríguez et al., 2011b). Frozen ground material (200 mg) was weighed in screw-cap Pyrex tubes and then 3 mL of cold pentane and 25 μg of 2-octanol (Fluka) were immediately added as an internal standard. 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 was added. The sample was further vortexed for 30 s and centrifuged at 1,800 g for 10 min at 4 ºC. The organic phase was recovered with a Pasteur pipette, and the aqueous phase was re-extracted two more times with 3 mL of pentane. A 2-μL aliquot of the pooled organic phases was directly injected into the GC-MS for volatile analysis; at least two extractions for each sample were performed.

The ion source and the transfer line were set to 200 ºC and 260 ºC, respectively. Volatile compounds were separated on an HP-INNOWax (Agilent J&C Columns) column (30 m x 0.25 mm i.d. x 0.25 μm film). The column temperatures were programmed as follows: 40 ºC for 5 min, raised to 150 ºC at 5 ºC min^{-1}, then raised to 250 ºC at 20 ºC min^{-1} and held for 2 min at 250 ºC. The injector temperature was 220 ºC. Helium was the carrier gas at 1.5 mL min^{-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 were identified by matching the acquired mass spectra with those stored in the reference libraries (Wiley6 and the National Institute of Standards and Technology) or from authentic standard compounds when available. Data were quantified by integrating the peak areas of total ion chromatograms and normalizing to the recovery rate of the internal standard (2-octanol). The data in Fig. 1, Fig. 2, Supplemental Table SI and Supplemental Fig. S2 represent the relative amounts of individual terpenes and are presented as the percentage area of each terpene (given as a fraction of unity) with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one.

RNA extraction

Total RNA was isolated from flavedo as previously described (Rodrigo et al., 2004). For quantitative real time RT-PCR analyses, RNA was cleaned up with the RNeasy mini kit (QIAGEN) and treated with DNase I (Rnase-Free DNase Set; QIAGEN) following the manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer.

Microarray experimental design, hybridization, data acquisition and data analysis

Microarray experiments were performed with the mature orange flavedo (90 mm in diameter) of two independent AS and two EV transgenic lines, comparing transgenic vs. control samples on the same slide. Three plants per line were used in every experiment. The total RNA from each line was duplicated for dye swap labeling.

Gene expression analysis was conducted using a citrus cDNA microarray containing 21,081 putative unigenes (Martinez-Godoy et al., 2008). Microarray labeling, hybridization and scanning were performed as described previously (Forment et al., 2005). Microarray slides were scanned with a GenePix 4000B scanner (Molecular Devices, USA) using GenePix 6.0 image acquisition software. Spots with a net intensity in both channels that was lower than the median spot signal background plus two standard deviations were not used for further analysis. Data were normalized using an intensity-based Lowess function, a normalization procedure based on robust local regression, to accommodate different types of dye biases and the use of control sequences spotted on the array (Yang et al., 2002) and analyzed only for features with at least three values. Differentially expressed genes were identified using the one-class SAM test (Tusher et al., 2001). A common set of genes was identified based on the overlap between the lists from each transgenic line. A gene was considered to be differentially expressed if the false discovery rate (FDR) was < 5%, and it had at least a 1.6-fold average change in expression between AS and EV plants.

Functional categorization of differentially expressed genes

Genes that were differentially expressed were grouped into gene ontology (GO) categories according to their biological function. Because very limited functional information is available for the sequences represented on the citrus genome array, the transcripts were annotated by finding orthologs in *Arabidopsis thaliana* using The Arabidopsis Information Resource (TAIR).

Results from Fatiscan analyses (Al-Shahrour et al., 2007) were used to represent statistically significant GO biological processes from levels 3 to 9. GO categories were grouped into five main groups (Table I), including “Defense response”, which covers GO categories such as defense response, response to biotic stimulus, immune response, plant-type hypersensitive response and death, response to abiotic stimulus, response to stress, response to chemical stimulus, response to endogenous stimulus and response to external stimulus; “Cellular component organization and biogenesis”, which covers GO categories such as establishment of localization, cellular component organization and biogenesis, plant type cell wall organization and cell communication; “Other”, which covers GO categories such as cellular metabolic process, primary metabolic process, regulation of biological process, regulation of transcription,

macromolecule metabolic process, regulation of biological quality and nitrogen compound metabolic process; "Secondary metabolic process", which covers GO categories such as monoterpenoid biosynthetic process, sesquiterpenoid biosynthetic process and phenylpropanoid biosynthetic process; and "Unknown", which covers genes without a match in the databases.

Quantitative Real-Time RT-PCR

Expression of selected genes chosen from microarray analyses was estimated by quantitative real-time RT-PCR using the SYBR Green assay and the LightCycler480 System (Roche) equipped with LightCycler 480 v.1.5 Software. The genes selected were *LS*, Limonene synthase; *GER*, Germacrene-D synthase; *CALMOD*, Calmodulin; *MYB*, MYB73 transcription factor; *PAL1*, Phenylalanine ammonia-lyase 1; *LTP1*, Nonspecific lipid transfer protein 1; and *ERF*, Ethylene response factor (ATERF-6). For jasmonic acid and terpenoid biosynthesis, the homolog genes from citrus, *LOX*, Lipoxygenase; *AOS*, Allene oxide synthase; *AOC*, Allene oxide ciclase; *COI1*, Coronatine-insensitive 1; *MYC2*, bHLHzip-type MYC transcription factor, *PDF1.2*, Defensin-like gene; *DXS*, 1-deoxyxylulose 5-phosphate synthase; *GGDP*, Geranylgeranyl diphosphate synthase*; GDP*, Geranyl diphosphate synthase; and *FDP*, Farnesyl diphosphate synthase were selected. The primers were designed based on the corresponding sequences available in the database of the CFGP (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB) (Supplemental Table SIII).

For the microarray genes selected, one-step RT-PCR was carried out with 25 ng of DNase-treated RNA by adding 1.6 units of Superscript II Reverse Transcriptase (Invitrogen), 0.8 units of Protector Rnase Inhibitor (Roche), 6.25 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), and optimized amounts of gene-specific primers (Supplemental Table SII) in a total volume of 12.5 μL. Incubations were carried out as follows: 45 ºC for 30 min, 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 40 s, and 70 ºC for 15 s. Fluorescence intensities were acquired during the 70 ºC step.

For the JA and terpenoid biosynthesis genes selected, two-step RT-PCR was carried out. First-strand cDNA was synthesized from 1 μg total RNA using Superscript II Reverse Transcriptase following the manufacturer's protocol. cDNA samples were diluted 1:5 with nuclease-free water before analysis and 2 μL of this dilution was used for the subsequent steps. Reactions were carried out with 10 μL of LightCycler® 480 DNA SYBR Green I Master (Roche) and 2 μL of gene-specific primers (Supplemental Table SIII) in a total volume of 20 μL. Incubations were carried out as follows: 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 10 s, 60 ºC for 10 s, and 72 ºC for 20 s. Fluorescence intensities were acquired during the 72 ºC step.

The specificity of the amplification reactions was assessed by post-amplification dissociation curves. To transform the fluorescence intensity measurements into relative mRNA

levels, a standard curve was generated with a 10-fold dilution series of an RNA sample. Relative mRNA levels were normalized to the citrus actin gene (GenBank Acc: CX289161) following the efficiency method (Pfaffl, 2001).

Induction values of one-fold were arbitrarily assigned to the control sample before inoculation. The quantification of each transcript in each cDNA source was accomplished using at least nine independent technical replicates (using at least three different 96-well plates) with two AS and two EV control independent lines. Means ± s.e.m. were calculated.

Phytohormone quantification

Hormone extraction and analysis were carried out as described in Durgbanshi et al. (2005) with slight modifications. Briefly, 0.5 g of frozen plant material was extracted in 5 mL of distilled water after spiking with a mixture of d_6-ABA, d_6-SA and dihydrojasmonic acid as internal standards. After centrifugation at 4000 g at 4 ºC, supernatants were recovered and the pH was adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice with 3 mL of di-ethyl ether. The organic upper layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The dry residue was then resuspended in a 10% MeOH solution by gentle sonication. The resulting solution was filtered through regenerated cellulose 0.22 μm membrane syringe filters (Albet S.A., Barcelona, Spain) and directly injected into the HPLC system (Waters Alliance 2695, Waters Corp., Milford, MA, USA). Separations were carried out on a C18 column (Kromasil 100, 5 μm particle size, 100×2.1 mm, Scharlab, Barcelona, Spain) using a gradient of MeOH:H_2O supplemented with 0.01% acetic acid at a flow rate of 300 μL min^{-1}. Hormones were quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source (Arbona et al., 2010).

Statistical analysis

Data on characterization of orange peels, quantitative RT-PCR, phytohormone levels or arcsine-transformed data on the percentage of infected wounds in JA assays were subjected to the analysis of variance using Statgraphics v.5.1 software (Manugistics Inc., Rockville, USA). A *t*-test or Fisher's Protected Least Significant Difference (LSD) test ($P < 0.05$) were used to separate the means, when appropriate.

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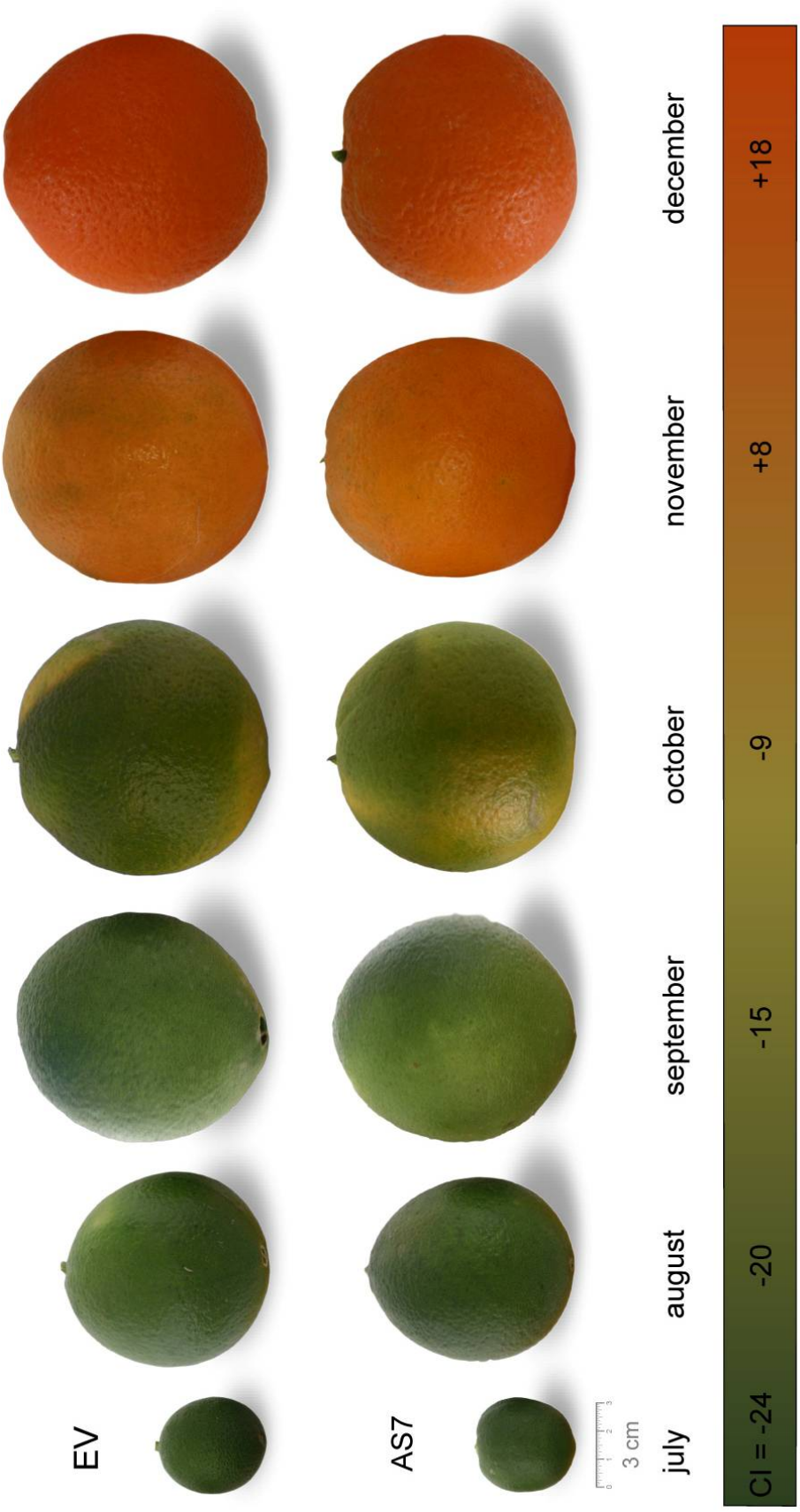
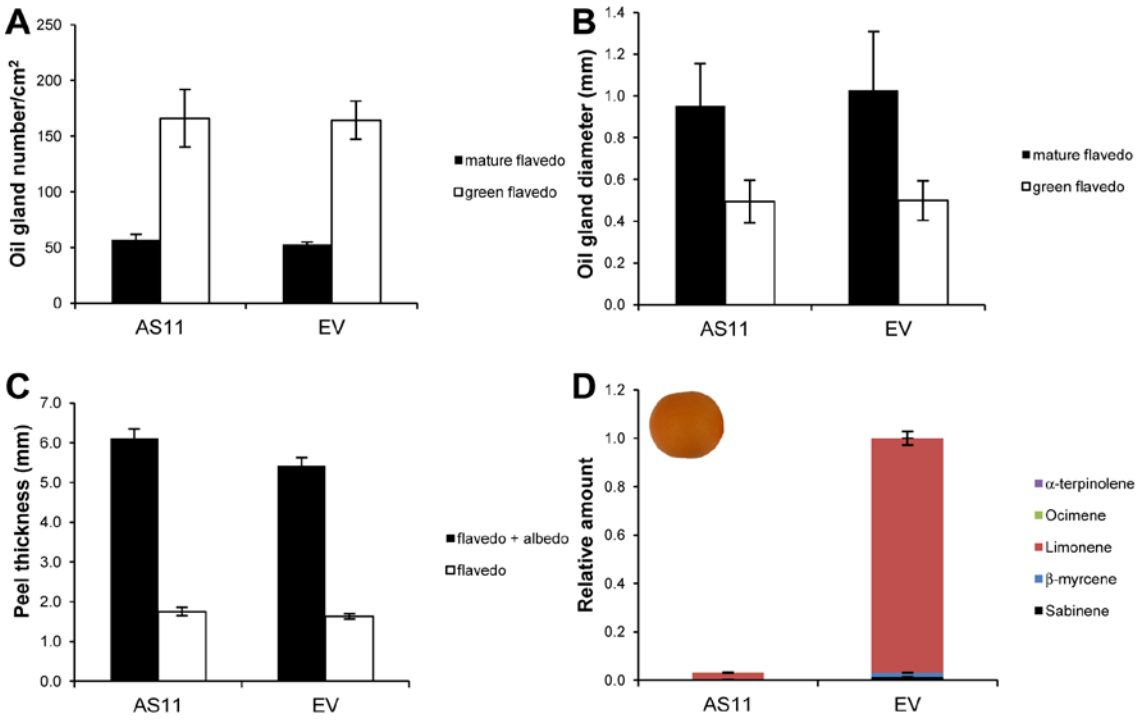
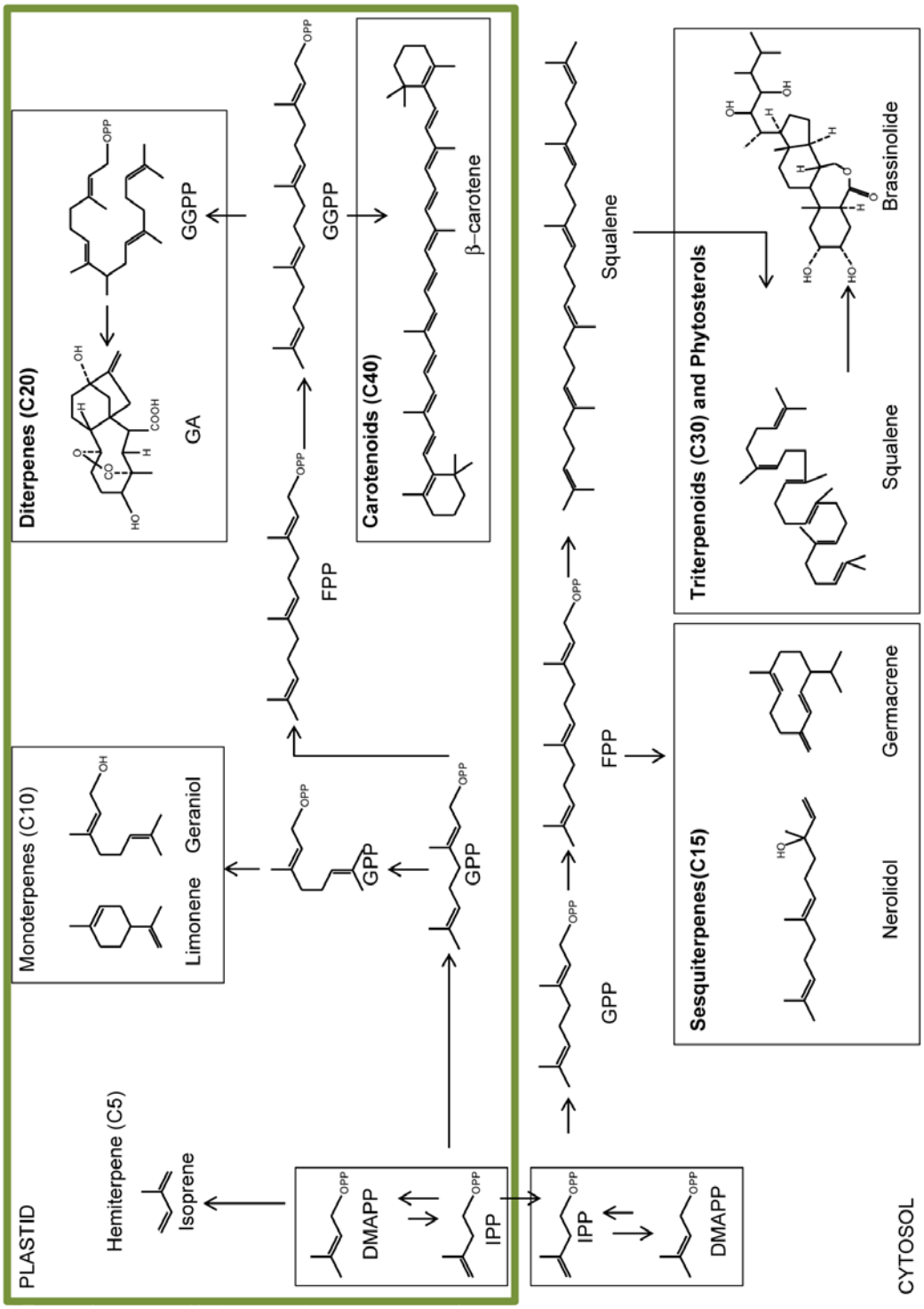
Supplemental Files Chapter 3



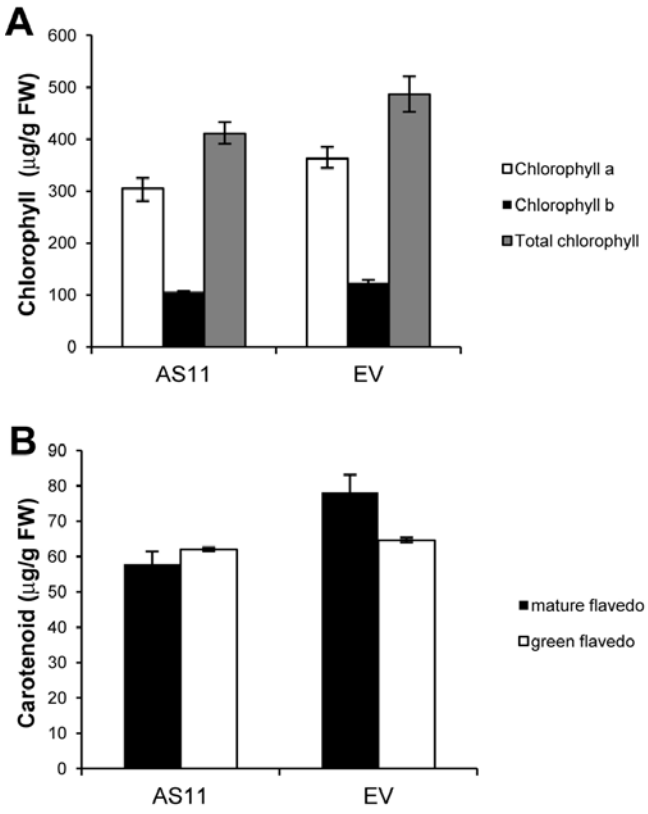
Supplemental Figure S1. Evolution of color during fruit development and ripening of Navelina oranges (*C. sinensis* L. Osbeck). CI: Color index based on Hunter parameters.



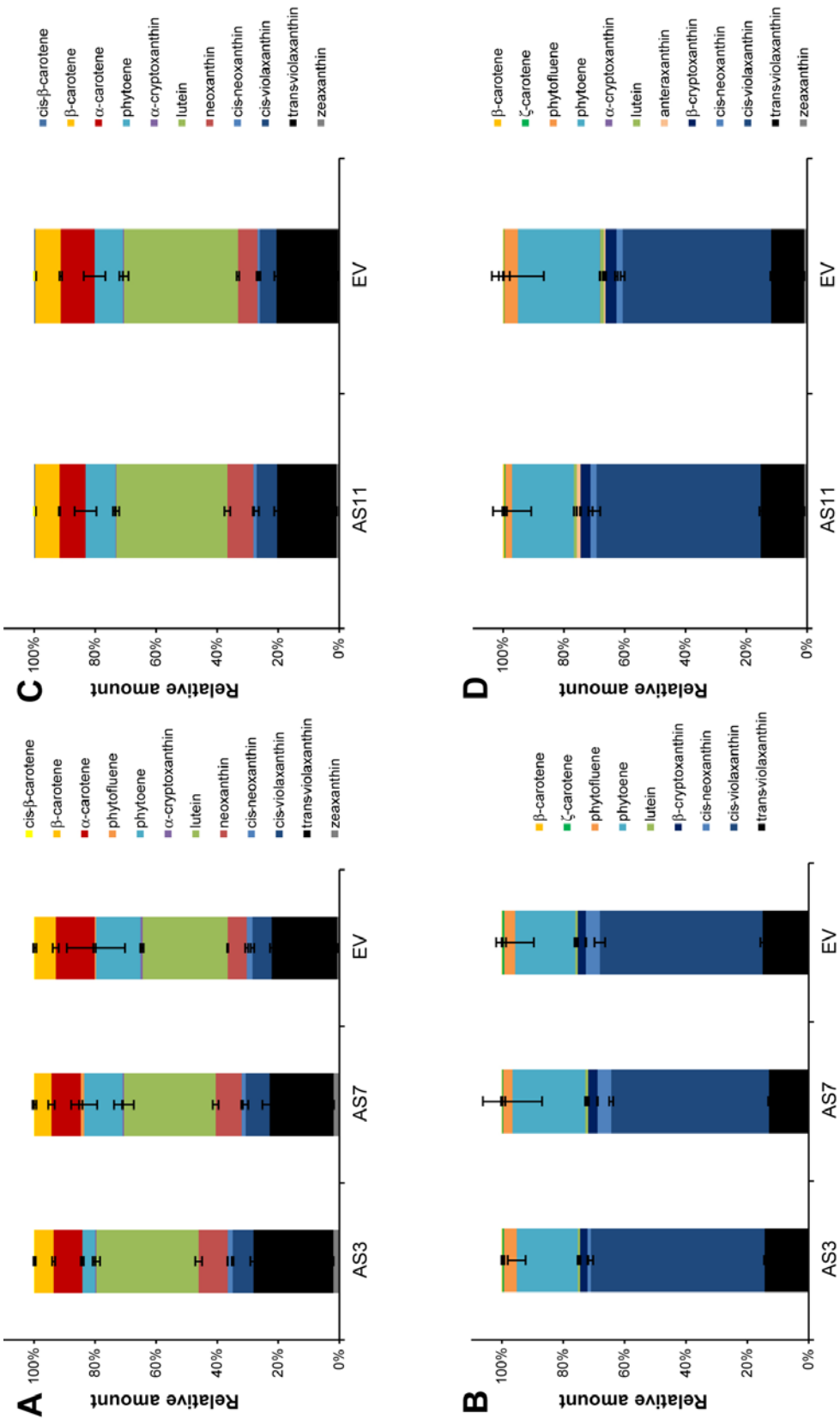
Supplemental Figure S2. Phenotypes of green (60 mm diameter) and mature (80 mm diameter) flavedo in antisense (AS) and control (EV) Pineapple sweet orange plants. (A, B) Secretory oil gland number and size in green and mature flavedo. (C) Peel thickness in mature fruits. No significant differences were found at $P < 0.05$ using Student's *t*-test in each stage. (D) The relative amount of individual terpenes is presented as a percentage area (given as a fraction of unity) of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in mature flavedo. Data represent mean values ± s.e.m and are derived from at least six fruits per plant.



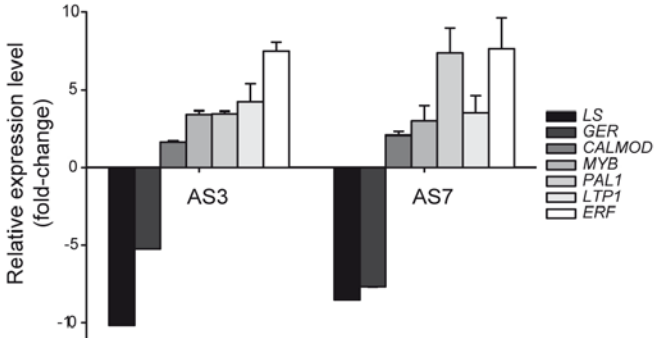
Supplemental Figure S3. Schematic representation of the metabolic diversion of the terpenoid pathway



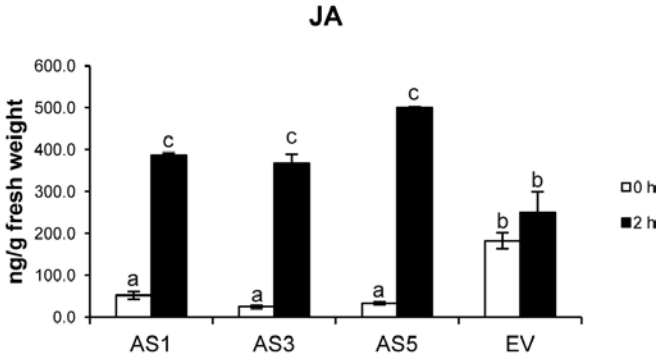
Supplemental Figure S4. Total chlorophyll and carotenoid content in Pineapple sweet orange plants in green and mature flavedo. No chlorophyll was detected in the flavedo of mature fruits. Data represent mean values ± s.e.m. and are derived from at least ten fruits per plant. No significant differences were found at $P < 0.05$ using Student's *t*-test in each stage.



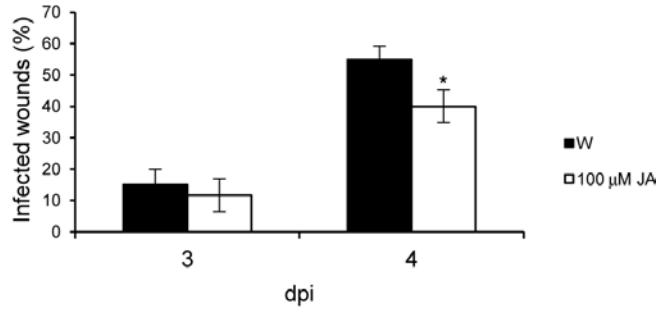
Supplemental Figure S5. Percentage of carotenoids in Navelina (A, B) and Pineapple (C, D) sweet orange transgenic and EV plants in green (A, C) and mature flavedo (B, D). The relative amount of individual carotenoids is presented as a percentage area of each carotenoid with respect to the total peak area for carotenoids in each line. Data represent mean values ± s.e.m. and are derived from at least ten fruits per plant. No significant differences were found at $P < 0.05$ using using Fisher's Protected LSD test or Student's *t*-test.



Supplemental Figure S6. Quantitative real-time PCR analyses of selected genes showing differential expression of several genes identified by microarray analyses with two independent transgenic lines. The expression of each gene was analyzed in at least nine independent technical replicates using three different 96-well plates. Fold-change was calculated in relation to two independent EV lines, to which an arbitrary value of 1 was assigned. *LS*, Limonene synthase; *GER*, Germacrene-D synthase; *CALMOD*, Calmodulin; *MYB*, MYB73 transcription factor; *PAL1*, Phenylalanine ammonia-lyase 1; *LTP1*, Nonspecific lipid transfer protein 1; *ERF*, Ethylene response factor (ATERF-6).



Supplemental Figure S7. Phytohormone measurement in the flavedo of AS transgenic and EV control plants. The content of jasmonic acid (JA) was measured before (0 h) and after (2 h) fungal inoculation. Data represent mean values ± s.e.m. and are derived from at least six fruits per plant. Different letters indicate significant differences at $P < 0.05$ using Fisher's Protected LSD test.



Supplemental Fig S8. Exogenous application of jasmonic acid (JA) in the control mandarin plants confers fungal resistance. Evolution of the disease caused by the fungus Penicillium digitatum in mature Satsuma mandarin fruits inoculated with 1×10^5 spores mL^{-1} and treated with water (W) or jasmonic acid (JA). Percentage of infected wounds in inoculated points three and four days after inoculation. The results are the average ± s.e.m. ($n \geq 30$). dpi: days post-inoculation. *, $P < 0.05$ using Student's *t*-test. We repeated all experiments at least twice and obtained similar results.

Supplemental Table SI. Monoterpene accumulation in AS and EV control fruits. The relative amounts of individual terpenes are presented as the percent (given as a fraction of unity) areas of each terpene with respect to the total terpene peak area for monoterpene hydrocarbons in the EV line, which was assigned an arbitrary value of one in green and mature flavedo. The data represent the mean values ± s.e.m. and were derived from at least five fruits per plant.

	GREEN FLAVEDO			MATURE FLAVEDO		
	AS3	AS7	EV	AS3	AS7	EV
α-terpinolene	0.00000 ± 0.00000	0.00000 ± 0.00000	0.00012 ± 0.00008	0.00000 ± 0.00000	0.00000 ± 0.00000	0.00091 ± 0.00028
β-ocimene	0.00140 ± 0.00079	0.00101 ± 0.00070	0.00441 ± 0.00010	0.00046 ± 0.00027	0.00023 ± 0.00001	0.00076 ± 0.00020
Limonene	0.01880 ± 0.00495	0.01537 ± 0.00097	0.90418 ± 0.01451	0.00881 ± 0.00032	0.01125 ± 0.00050	0.96091 ± 0.16870
β-myrcene	0.00170 ± 0.00104	0.00139 ± 0.00090	0.01799 ± 0.00041	0.00028 ± 0.00003	0.00019 ± 0.00010	0.01564 ± 0.00297
δ-3-carene	-	-	-	0.00000 ± 0.00000	0.00000 ± 0.00000	0.00189 ± 0.00107
Sabinene	0.02365 ± 0.01880	0.01621 ± 0.01341	0.07329 ± 0.00676	0.00362 ± 0.00000	0.00288 ± 0.00053	0.01988 ± 0.00472

Supplemental Table SII.

A) Differentially expressed genes in AS3 line

Description	Citrus unigene	fold-change	q-value(%)	Most similar Ath gene
no annotation available	aCL5425Contig1	7.17	0.00	
no annotation available	aC08009G11SK_c	6.37	0.00	
no annotation available	aCL8681Contig1	5.08	3.23	
no annotation available	aC08007E01SK_c	4.64	0.00	
FAD-binding domain-containing protein	aCL246Contig1	4.10	0.00	AT2G34790
calcium-binding EF hand family protein	aCL7645Contig1	3.93	0.00	AT2G44310
cyclic nucleotide-regulated ion channel (CNGC2)	aCL5832Contig1	3.60	0.00	AT5G15410
signal transducer of phototropic response (RPT2)	aCL1254Contig1	3.56	0.57	AT2G30520
Putative xyloglucan endotransglycosylase	aC05133B06SK_c	3.47	0.00	AT4G30270
phenylalanine ammonia-lyase 1 (PAL1)	aCL1166Contig2	3.47	0.00	AT2G37040
no annotation available	aCL50Contig2	3.38	0.00	
Putative trehalose-6-phosphate synthase	aCL5366Contig1	3.35	0.00	AT1G68020
nonspecific lipid transfer protein 1 (LTP1)	aCL4Contig13	3.31	0.00	AT2G38540
CCR4-NOT transcription complex protein	aCL206Contig1	3.26	0.00	AT5G22250
expressed protein	aC18025G03Rv_c	3.21	1.06	AT4G35560
R2R3-MYB family transcription factor (MYB73)	aCL693Contig1	3.18	0.00	AT4G37260
Putative membrane transporter	aCL854Contig2	3.17	0.00	AT2G43330
no annotation available	aC04005G08SK_c	3.07	0.92	
transducin family protein / WD-40 repeat family protein	aC18019A01Rv_c	2.97	0.00	AT4G34280
protein phosphatase 2C PP2C	aCL393Contig1	2.96	0.00	AT5G53140
no annotation available	aC18018D09Rv_c	2.93	3.23	
beta-amylase (Glycosyl hydrolase family 14)	aCL5Contig5	2.90	0.00	AT3G23920
no annotation available	aC05808F01SK_c	2.89	0.00	
calmodulin	aCL535Contig3	2.85	0.00	AT3G10190
Putative glucosyltransferase	aCL5570Contig1	2.85	0.00	AT2G36970
zinc finger (B-box type) family protein	aCL3547Contig1	2.75	0.00	AT3G21150
phosphomannose isomerase type I family protein	aCL1387Contig1	2.73	0.00	AT3G02570
no annotation available	aCL5263Contig1	2.71	0.00	
expressed protein	aCL6840Contig1	2.68	0.00	AT3G52740
Dehydration responsive element binding protein (AP2 domain)	aCL51Contig5	2.63	0.00	AT1G46768
Stress-inducible H1 histone-like protein (HIS1-3)	aCL517Contig2	2.62	0.00	AT2G18050
Putative trehalose-6-phosphate synthase	aCL9275Contig1	2.59	0.00	AT1G68020
no annotation available	aCL1714Contig1	2.44	0.00	
no annotation available	aC03007D01SK_c	2.39	0.00	
zinc finger (CCCH-type) family protein (CZF1)	aC31603G11EF_c	2.38	0.00	AT2G40140
Protein phosphatase 2C	aCL683Contig1	2.33	0.00	AT2G30020
no annotation available	aCL26Contig2	2.31	0.00	
no annotation available	aCL101Contig2	2.28	0.00	
no annotation available	aC06023B09SK_c	2.28	0.00	AT2G24540
no annotation available	aC08007C02SK_c	2.24	0.00	
heavy-metal-associated domain-containing protein	aCL2730Contig1	2.21	0.00	AT4G08570
no annotation available	aC08002C02SK_c	2.20	1.36	
WD-40 repeat family protein / phytochrome A-related	aCL6446Contig1	2.19	0.00	AT1G53090
no annotation available	aC01020E10SK_c	2.17	0.00	

autophagy 8h (APG8h)	aC18016G09Rv_c	2.15	3.23	AT3G06420
similar to glucose-1-phosphate adenylyltransferase large subunit 2 (APL2)	aCL9143Contig1	2.14	0.00	AT2G21590
3-oxo-5-alpha-steroid 4-dehydrogenase family protein	aC01011F03SK_c	2.14	0.00	AT5G16010
L-allo-threonine aldolase-related protein	aC08036F01SK_c	2.13	0.00	AT1G08630
no annotation available	aC31006C04EF_c	2.13	0.00	
fringe-related protein	aCL5584Contig1	2.11	0.46	AT1G05280
expressed protein	aCL3900Contig1	2.10	0.00	AT5G20790
expressed protein	aCL1991Contig1	2.10	0.00	AT2G15020
calcium-binding protein	aCL7914Contig1	2.09	0.00	AT1G21550
strictosidine synthase family protein	aC31201B02EF_c	2.09	0.00	AT3G59530
no annotation available	aCL123Contig3	2.09	0.00	
no annotation available	aC31206E07EF_c	2.08	0.00	
esterase/lipase/thioesterase family protein	aCL5939Contig1	2.07	0.00	AT1G54570
harpin-induced family protein (YLS9) / HIN1 family protein	aCL2389Contig2	2.06	0.00	AT2G35980
no annotation available	aCL4787Contig1	2.06	0.00	
Putative NAC domain protein NAC29	aCL1571Contig2	2.06	0.00	AT1G69490
no annotation available	aC08018E12SK_c	2.05	0.00	
no annotation available	aC31807H02EF_c	2.03	0.00	
expressed protein	aC08031A08SK_c	2.03	0.00	AT5G41110
No apical meristem (NAM) family protein (NAC72)	aC32102F03EF_c	2.03	0.00	AT5G15410
No apical meristem (NAM) family protein (NAC72)	aCL35Contig5	2.02	0.00	AT4G27410
no annotation available	aC01018F12SK_c	2.01	0.00	
Mitogen-activated protein kinase (WNK4)	aCL8779Contig1	2.01	1.36	AT5G58350
no annotation available	aCL5112Contig1	1.99	0.00	AT3G07565
BZip transcription factor	aCL9402Contig1	1.99	0.00	AT2G46270
Translation initiation factor-like protein	aCL1184Contig4	1.99	0.00	AT5G54940
no annotation available	aC31305G08EF_c	1.97	0.00	
no annotation available	aC16015A05SK_c	1.94	0.00	
zinc finger (C3HC4-type RING finger)	aCL9084Contig1	1.92	0.17	AT1G18660
homeobox-leucine zipper protein 7 (HB-7)	aCL5941Contig1	1.92	0.00	AT2G46680
Syringolide-induced protein 19-1-5	aCL107Contig2	1.91	0.00	AT4G25810
Putative calcium-transporting ATPase 13	aC18021G04Rv_c	1.91	2.81	AT3G22910
no annotation available	aC19003E02T7_c	1.90	0.00	
Beta-amylase (Glycosyl hydrolase family 14)	aCL5Contig21	1.89	0.00	AT3G23920
bZIP protein HY5 (HY5)	aC08022H08SK_c	1.88	0.00	AT5G11260
reverse transcriptase	aC02002E11SK_c	1.88	0.00	AT4G29090
UDP-glucosyltransferase HRA25	aC02002E10SK_c	1.87	0.74	AT3G02100
expressed protein	aCL8468Contig1	1.87	0.00	AT1G69760
Putative protease	aCL7817Contig1	1.87	0.00	AT3G57680
ChaC-like family protein-like	aC05802B02SK_c	1.87	0.00	AT4G31290
no annotation available	aC34009E05EF_c	1.85	0.00	
Chalcone synthase/naringenin-chalcone synthase	aCL27Contig2	1.85	0.00	AT5G13930
no annotation available	aCL2819Contig1	1.85	0.00	
Protein phosphatase 2C	aCL143Contig2	1.85	0.00	AT3G11410
peptidase U7 family protein	aCL27Contig1	1.84	0.00	AT1G73990
expressed protein	aCL2203Contig2	1.84	0.00	AT5G02020
no annotation available	aCL2606Contig1	1.84	0.00	
CBL-interacting protein kinase 14 (CIPK14)	aCL2213Contig1	1.83	0.00	AT5G01820
remorin family protein	aCL1490Contig1	1.82	0.00	AT2G41870

nodulin family protein	aC08023C08SK_c	1.82	0.00	AT1G74780
digalactosyldiacylglycerol synthase 1 (DGD1)	aCL2418Contig1	1.82	0.00	AT3G11670
similar to amino acid transporter family protein	aCL5219Contig1	1.82	0.57	AT3G30390
Granule-bound starch synthase 1	aCL60Contig1	1.81	0.00	AT1G32900
alcohol oxidase-related	aC08034C10SK_c	1.81	4.32	AT4G28570
expressed protein	aCL6738Contig1	1.81	0.57	AT5G24690
Serine/threonine protein kinase	aC02026D10SK_c	1.80	0.74	AT5G47750
expressed protein	aCL1020Contig1	1.80	0.00	AT4G29780
no annotation available	aCL2116Contig1	1.79	0.00	
Root iron transporter protein IRT1	aC34108F04EF_c	1.79	0.00	AT4G19690
bZIP transcription factor family protein	aCL3546Contig1	1.78	0.17	AT3G62420
no annotation available	aCL5461Contig1	1.78	0.00	
zinc finger (B-box type) family protein / salt-tolerance protein (STO))	aC31709D11EF_c	1.77	0.00	AT1G06040
ABA-responsive element-binding protein 2 (AREB2)	aCL474Contig1	1.76	0.00	AT3G19290
isoflavone reductase	aC34008H08EF_c	1.76	0.00	AT4G39230
Glycosyl transferase-like protein	aCL3226Contig1	1.75	0.00	AT3G28340
disease resistance protein (NBS-LRR class)	aCL5233Contig1	1.75	0.57	AT3G14460
sodium-inducible calcium-binding protein (ACP1)	aCL1345Contig2	1.75	0.00	AT5G49480
WRKY family transcription factor	aC04005E02SK_c	1.73	0.17	AT4G01720
early-responsive to dehydration stress protein (ERD4)	aCL5413Contig1	1.73	0.00	AT1G30360
sulfate adenylyltransferase 1 / ATP-sulfurylase 1 (APS1)	aCL438Contig2	1.73	0.00	AT3G22890
Beta-D-galactosidase	aC31805H10EF_c	1.73	0.00	AT4G36360
protein phosphatase 2C, PP2C	aCL5289Contig1	1.73	0.00	AT5G59220
no annotation available	aC03006F07SK_c	1.73	0.00	
cellulose synthase family protein	aCL1355Contig1	1.73	0.00	AT2G32540
30S ribosomal protein S1	aCL5730Contig1	1.72	4.32	AT5G30510
CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	aC08029D05SK_c	1.72	1.78	AT5G12840
disease resistance protein (TIR-NBS-LRR class)	aCL7525Contig1	1.72	0.74	AT5G36930
no apical meristem (NAM) family protein	aC31402A11EF_c	1.72	0.17	AT2G27300
ChaC-like family protein	aCL283Contig1	1.71	0.00	AT4G31290
protein kinase, putative, similar to protein kinase ATMRK1	aCL7535Contig1	1.71	0.00	AT3G22750
DC1 domain-containing protein	aCL2160Contig1	1.71	0.00	AT1G60420
no annotation available	aC01011A04SK_c	1.70	0.74	
cytochrome P450 98A3, putative (CYP98A3)	aCL627Contig1	1.70	0.00	AT2G40890
T-complex protein 11	aCL3898Contig1	1.69	0.00	AT1G22930
pectinesterase	aCL3119Contig1	1.69	3.23	AT3G10720
no annotation available	aC03010H04SK_c	1.69	0.00	
ChaC-like family protein	aC31807B06EF_c	1.68	0.00	AT4G31290
Sesquiterpene synthase (Valencene synthase)	aCL7292Contig1	1.68	0.00	AT5G23960
no annotation available	aC04027B03SK_c	1.68	0.92	
copper chaperone (CCH)-related	aCL4708Contig1	1.68	0.17	AT5G63530
calcium-binding EF hand family protein	aCL8972Contig1	1.67	0.00	AT1G05150
Xyloglucan galactosyltransferase KATAMARI 1	aC08005B05SK_c	1.67	0.00	AT2G20370
no annotation available	aCL986Contig2	1.67	0.00	
no annotation available	aC18001E11Rv_c	1.66	0.00	
Cystinosin homolog	aCL1587Contig1	1.66	0.00	AT5G40670
Putative BP-5 protein	aC02020G06SK_c	1.66	1.78	AT1G09530
no annotation available	aC02019F06SK_c	1.66	1.36	
no annotation available	aCL5559Contig1	1.65	2.81	
expressed protein	aC02027C10SK_c	1.65	0.74	AT3G14850

Homeodomain leucine zipper protein HDZ2	aC31108D05EF_c	1.63	0.00	AT3G01470
similar to MA3 domain-containing protein	aCL8107Contig1	1.63	0.00	AT4G24800
Encodes a member of the ERF subfamily B-3 of ERF/AP2 (ATERF-6)	aCL337Contig1	1.63	0.74	AT4G17490
Plasma membrane intrinsic protein 2-2	aCL102Contig2	1.63	0.00	AT3G54820
cation efflux family protein	aCL1011Contig2	1.62	0.46	AT2G39450
Ser-thr protein kinase	aCL5546Contig1	1.62	1.78	AT2G40270
Centromere protein-like	aC08027B06SK_c	1.62	1.78	AT3G55060
Encodes a member of the ERF subfamily B-1 (AP2 domain) (ATERF-4)	aCL524Contig2	1.62	0.00	AT3G15210
Vacuolar processing enzyme precursor	aCL554Contig1	1.62	0.00	AT4G32940
Chlorophyllide a oxygenase	aCL7904Contig1	1.62	0.57	AT1G44446
no annotation available	aCL1Contig26	1.62	0.00	
no annotation available	aC19006C07T7_c	1.61	0.17	
no annotation available	aC18004D10Rv_c	1.61	0.74	
Hydroquinone glucosyltransferase	aCL1737Contig1	1.61	0.00	AT4G01070
Putative serine/threonine kinase SRK2F	aC04002A03SK_c	1.61	0.00	AT4G40010
Cytochrome P450 90A1	aCL1811Contig1	1.60	0.17	AT5G05690
no annotation available	aCL7401Contig1	1.60	1.36	
no annotation available	aC08012C07SK_c	1.60	0.92	
zinc finger (C3HC4-type RING finger) family protein	aC08031G10SK_c	1.60	0.92	AT2G44950
Topoisomerase-like protein	aCL6553Contig1	1.60	0.00	AT5G63190
Zinc finger protein CONSTANS-LIKE 5	aC31501E05EF_c	1.59	0.17	AT5G57660
expressed protein	aC08034B11SK_c	1.59	0.00	AT1G73650
BPF-1 protein	aC08031A03SK_c	1.59	0.00	AT1G07540
ANAC029 | no apical meristem (NAM)	aCL1571Contig1	1.59	0.00	AT1G69490
early-responsive to dehydration stress protein (ERD4)	aCL3691Contig1	1.59	0.00	AT1G30360
MYB transcription factor	aC20001D01SK_c	1.58	0.57	AT2G38090
no annotation available	aC31801G05EF_c	1.58	0.00	
BTB/POZ domain-containing protein	aC05146H05SK_c	1.58	1.78	AT1G55760
Sesquiterpene synthase (Germacrene-D synthase)	aCL6701Contig1	1.57	0.00	AT5G23960
protein phosphatase 2C, PP2C	aCL1106Contig1	1.57	0.00	AT3G51370
senescence-associated family protein	aCL3777Contig1	1.57	1.06	AT5G66170
zinc finger (C2H2 type) family protein	aCL382Contig2	1.56	0.00	AT3G49930
ethylene receptor 1 (ETR1)	aCL3291Contig1	1.56	0.46	AT1G66340
Putative phosphatase	aCL4964Contig1	1.56	0.57	AT1G73010
Disease resistance protein, LRR	aCL9146Contig1	1.56	1.36	AT1G74180
SP2G	aCL6873Contig1	1.56	1.78	AT1G18100
Plasma membrane aquaporin	aCL3500Contig1	1.56	0.00	AT2G45960
Calcium homeostasis regulator CHoR1	aCL6545Contig1	1.55	2.81	AT3G55250
zinc finger (C3HC4-type RING finger) family protein	aC02003G02SK_c	1.55	3.23	AT5G08750
Putative ripening-related protein	aCL1973Contig2	1.55	0.00	AT5G02230
Biotin synthase	aC04033D07SK_c	1.55	0.57	AT2G43360
similar to DRE-binding protein (DREB2B)	aC34205B09EF_c	1.55	1.06	AT5G05410
basic helix-loop-helix (bHLH) family protein	aC04033B02SK_c	1.55	0.00	AT5G08130
Proline-rich protein	aCL2832Contig1	1.54	0.00	AT4G16380
Calcium-dependent protein kinase-like (CRK1).	aCL6018Contig1	1.54	0.74	AT2G41140
no annotation available	aC31702G12EF_c	1.54	0.00	
kinase interacting family protein	aCL8404Contig1	1.54	1.36	AT3G22790
expressed protein	aC08031B06SK_c	1.53	0.57	AT5G42760
pectin methylesterase	aCL1691Contig1	1.53	0.17	AT1G11580

no annotation available	aCL6Contig2	1.53	0.46	
DNA helicase-like	aC02023C11SK_c	1.53	2.81	AT5G35970
expressed protein	aCL9415Contig1	1.53	0.74	AT3G12685
aspartyl protease family protein	aCL1990Contig1	1.53	0.46	AT5G37540
CPRD2 protein	aCL1084Contig1	1.53	2.81	AT4G20820
Cinnamate 4-hydroxylase CYP73	aCL959Contig1	1.53	0.00	AT2G30490
expressed protein	aCL4845Contig1	1.52	2.81	AT3G52910
SPX (SYG1/Pho81/XPR1) domain-containing protein	aCL8920Contig1	1.52	0.00	AT2G26660
no annotation available	aC08010B11SK_c	1.52	4.32	
Hydroxyisourate hydrolase (glycosyl hydrolase family 1)	aCL3841Contig1	1.52	2.81	AT1G02850
no annotation available	aCL2956Contig1	1.52	1.78	
Aquaporin	aCL58Contig7	1.52	0.00	AT4G00430
Plasma intrinsic protein 2,2	aCL1621Contig2	1.52	0.00	AT2G37170
expressed protein, NuLL	aCL82Contig1	1.51	1.06	AT4G32020
Cinnamyl alcohol dehydrogenase	aCL1474Contig1	1.51	0.00	AT5G19440
Wts2L	aCL818Contig1	1.51	0.17	AT2G47140
Sesquiterpene synthase (Valencene synthase)	aC06019E08SK_c	1.51	0.00	AT5G23960
Inositol-3-phosphate synthase	aCL25Contig1	1.51	0.00	AT2G22240
Homocysteine S-methyltransferase 3	aCL373Contig1	1.51	0.00	AT3G22740
Calcium-dependent protein kinase 8 (CDPK19). Strong similarity to CPK7	aCL3679Contig1	1.51	0.57	AT5G19450
Sugar transport protein 14	aCL7536Contig1	1.51	0.17	AT1G77210
thioredoxin family protein	aC18019E01Rv_c	1.51	2.81	AT1G08570
Putative glycine hydroxymethyltransferase	aCL7087Contig1	1.50	0.92	AT5G26780
no annotation available	aC06007C08SK_c	1.50	0.00	
PHD finger family protein	aCL4634Contig1	1.50	4.32	AT3G52100
Cinnamoyl-CoA reductase-like protein	aCL8119Contig1	1.50	0.00	AT4G30470
expressed protein	aCL8860Contig1	1.50	0.46	AT3G21200
no annotation available	aCL39Contig3	1.50	0.17	
expressed protein	aC31103F04EF_c	1.50	0.74	AT2G01050
CBL-interacting protein kinase 12 (CIPK12)	aCL5678Contig1	1.49	0.00	AT4G18700
Xylem cysteine proteinase 1 precursor	aCL6325Contig1	1.49	0.00	AT4G35350
no annotation available	aC03005D04SK_c	1.49	1.36	
NAC family protein (ANAC002)	aCL943Contig3	1.49	0.00	AT1G01720
myb family transcription factor	aCL5971Contig1	1.49	0.00	AT5G17300
Rho-GTPase-activating protein-like	aCL9082Contig1	1.49	0.00	AT4G35750
Putative CONSTANS-like B-box zinc finger protein	aCL5350Contig1	1.49	0.57	AT4G38960
calcium-binding EF hand family protein	aC08007C09SK_c	1.48	0.17	AT3G10300
MADS-box protein (AGL62)	aCL8174Contig1	1.48	0.74	AT5G60440
AT-rich element binding factor 3	aCL3481Contig1	1.48	0.00	AT1G01360
no annotation available	aC08007A08SK_c	1.48	3.23	
Putative auxin-regulated protein	aCL1903Contig2	1.48	0.74	AT3G60690
hydrolase, alpha/beta fold family protein	aC18005G11Rv_c	1.48	1.06	AT3G09690
Inositol-3-phosphate synthase	aC31301D12EF_c	1.48	0.00	AT2G22240
glycosyltransferase family protein 1	aC03003F06SK_c	1.48	1.36	AT4G01210
no annotation available	aC07012E11SK_c	1.48	0.57	
expressed protein	aCL1583Contig1	1.48	0.57	AT1G54680
expressed protein	aCL375Contig2	1.48	0.17	AT2G46080
Ribonuclease 2 precursor	aCL3877Contig1	1.48	2.81	AT2G39780
no annotation available	aC31302G05EF_c	1.47	0.46	
ABC1 family protein	aCL9230Contig1	1.47	0.00	AT1G71810
Putative ripening-related bZIP protein	aCL3553Contig1	1.47	0.00	AT1G45249

histone H4	aCL1120Contig1	1.47	0.92	AT5G59690
Peripheral-type benzodiazepine receptor	aCL1112Contig2	1.47	0.74	AT2G47770
Anthranilate N-benzoyltransferase	aC18024F04Rv_c	1.47	4.32	AT5G48930
Limonoid UDP-glucosyltransferase	aC08034F10SK_c	1.47	1.06	AT3G21560
NBS-LRR resistance-like protein B8	aC20002B03SK_c	1.47	0.00	AT3G14460
Methylthioribose kinase	aCL3452Contig2	1.47	0.00	AT1G49820
octicosapeptide/Phox/Bem1p (PB1)	aC34205A08EF_c	1.46	0.74	AT1G70640
VQ motif-containing protein	aCL1812Contig2	1.46	0.00	AT3G56880
Similarity to transcription regulator	aCL3613Contig1	1.46	4.32	AT5G18230
Starch branching enzyme	aC02008F01SK_c	1.46	0.00	AT5G03650
Amine oxidase	aC01017A05SK_c	1.46	0.17	AT4G12290
Galactinol synthase	aCL381Contig1	1.46	0.17	AT1G56600
heat shock transcription factor family protein	aC31704F01EF_c	1.46	0.74	AT5G45710
Expressed protein	aCL2075Contig1	1.46	0.17	AT5G19160
Pectinesterase	aCL2737Contig1	1.46	1.06	AT5G53370
Chitinase CHI1	aCL371Contig2	1.46	0.17	AT3G54420
bZIP transcription factor, putative (bZIP69)	aCL816Contig1	1.45	1.36	AT1G06070
Callose synthase 1 catalytic subunit	aCL6983Contig1	1.45	0.74	AT1G05570
no annotation available	aC01008D06SK_c	1.45	0.00	
Lecithine cholesterol acyltransferase-like protein	aCL558Contig1	1.45	1.36	AT1G27480
Catalase	aC31003C03EF_c	1.45	0.00	AT1G20620
expressed protein	aC31703H01EF_c	1.45	1.06	AT2G38780
no annotation available	aCL8020Contig1	1.45	1.36	
thiamin pyrophosphokinase	aCL410Contig2	1.45	3.23	AT1G02880
chorismate mutase, chloroplast (CM1)	aC04013E01SK_c	1.45	0.46	AT3G29200
WRKY6	aCL1201Contig2	1.45	2.81	AT1G62300
Glucose-1-phosphate adenylyltransferase small subunit 2	aCL5827Contig1	1.45	0.17	AT5G48300
Glucosyltransferase NTGT2	aCL678Contig4	1.45	0.00	AT1G05530
Similarity to auxin-independent growth promoter	aCL3382Contig1	1.44	0.92	AT5G65470
expressed protein	aCL4109Contig1	1.44	1.78	AT3G21190
no annotation available	aCL7161Contig1	1.44	0.74	
Permease 1	aCL1822Contig1	1.44	1.06	AT5G62890
expressed protein	aC07007G05SK_c	1.44	0.00	AT4G22000
ROPGEF1	aC31805A07EF_c	1.44	0.17	AT4G38430
octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	aC31501D04EF_c	1.44	0.00	AT4G05150
Oxylase-like protein	aCL4163Contig1	1.44	0.17	AT3G19000
Nucleotide sugar epimerase-like protein	aCL1692Contig1	1.44	0.57	AT4G30440
S-adenosyl-L-methionine synthetase 1	aCL414Contig2	1.43	0.17	AT3G17390
sulfate transporter	aC19008G08T7_c	1.43	0.00	AT4G02700
vacuolar sorting receptor	aC06001G12SK_c	1.43	0.74	AT1G30900
potassium channel tetramerisation domain-containing protein	aC05054G10SK_c	1.43	1.06	AT5G41330
no annotation available	aCL6647Contig1	1.43	1.06	
protein kinase family protein	aCL9409Contig1	1.43	4.32	AT5G47740
no annotation available	aC05070A03SK_c	1.43	0.17	
no annotation available	aC07001C09SK_c	1.43	0.74	
Putative beta-1,3-glucanase (glycosyl hydrolase family 17)	aCL2349Contig1	1.43	0.74	AT2G16230
A.thaliana gene induced upon wounding stress	aC31809E04EF_c	1.43	0.74	AT4G24220
Putative bacterial blight resistance protein LRR	aC31006A01EF_c	1.43	1.36	AT3G49670
no annotation available	aC01013F10SK_c	1.43	0.17	
Starch phosphorylase type H	aC08037B01SK_c	1.43	1.36	AT3G46970

Alkaline alpha galactosidase II	aC34008C07EF_c	1.42	0.57	AT3G57520
Clathrin binding protein-like	aCL3017Contig1	1.42	1.36	AT5G11710
Pectin methylesterase	aCL4116Contig2	1.42	0.46	AT5G53370
Similarity to transporter protein	aCL7628Contig1	1.42	0.00	AT3G25410
Sulfate transporter 2	aCL5087Contig1	1.42	1.36	AT1G22150
Flavonoid-3'-hydroxylase	aCL893Contig1	1.42	1.36	AT5G07990
expressed protein	aCL9316Contig1	1.42	0.74	AT5G02580
expressed protein	aCL37Contig1	1.42	1.36	AT1G05340
no annotation available	aCL4149Contig1	1.42	4.32	
no annotation available	aC16012C10SK_c	1.42	4.32	
Putative O-methyltransferase	aC02001E07SK_c	1.41	0.74	AT4G02405
Carboxypeptidase type III	aC31806B06EF_c	1.41	0.46	AT3G10410
Serine/threonine specific protein kinase-like	aC31701B09EF_c	1.41	3.23	AT5G15080
Chloride channel protein CLC-c	aCL8241Contig1	1.41	4.32	AT5G49890
copine-related	aCL4759Contig1	1.41	3.23	AT5G14420
SOS2-like protein kinase (CIPK6)	aCL717Contig1	1.41	0.46	AT4G30960
no annotation available	aCL8147Contig1	1.40	3.23	
Reductase 2	aC31004B03EF_c	1.40	0.57	AT1G59950
expressed protein	aC03004F01SK_c	1.40	0.74	AT5G67390
expressed protein	aCL5420Contig1	1.40	0.57	AT4G15545
Peroxidase precursor	aCL36Contig2	1.40	0.74	AT4G21960
expressed protein	aCL8195Contig1	1.40	0.74	AT1G73390
Glucosyl transferase	aCL1582Contig1	1.40	0.17	AT3G11340
Catalase	aCL63Contig3	1.40	0.00	AT4G35090
Heat shock factor protein HSF24	aCL432Contig1	1.40	0.00	AT4G36990
no annotation available	aC18015B09Rv_c	1.40	2.81	
Putative carbonyl reductase	aC31710C05EF_c	1.40	1.36	AT3G61220
Acid invertase (Glycosyl hydrolases family 32)	aCL2322Contig1	1.40	0.00	AT1G12240
disease resistance protein (TIR-NBS-LRR class)	aC31704B11EF_c	1.40	4.32	AT3G44400
no annotation available	aCL1329Contig1	1.39	2.81	
Histone H3.3	aCL1455Contig1	1.39	0.17	AT5G10980
no annotation available	aC07011B02SK_c	1.39	1.36	
no annotation available	aCL8567Contig1	1.39	1.06	
SAC1-like protein AtSAC1b	aCL36Contig1	1.39	1.78	AT3G51460
no annotation available	aKN0AAI1CD03FM2_c	1.39	4.32	
Salt tolerance zinc finger protein	aCL146Contig3	1.39	0.92	AT1G27730
Purple acid phosphatase-like	aCL4001Contig1	1.39	3.23	AT3G10150
Clavaminate synthase-like protein	aCL7936Contig1	1.39	0.74	AT3G21360
Guanylate kinase-like protein	aCL2233Contig1	1.39	0.74	AT3G57550
Endoxyloglucan transferase	aCL156Contig1	1.39	0.74	AT1G14720
Callose synthase catalytic subunit-like protein	aC18005G07Rv_c	1.39	1.06	AT5G13000
expressed protein	aCL5426Contig1	1.39	1.36	AT4G36630
expressed protein	aC02002C08SK_c	1.39	0.46	AT1G31940
Hydroquinone glucosyltransferase	aC16012D02SK_c	1.39	2.81	AT4G01070
no annotation available	aCL9308Contig1	1.39	2.81	
Putative hexose transporter	aC20008H02SK_c	1.38	0.74	AT4G35300
expressed protein	aCL2430Contig1	1.38	2.81	AT1G78110
Phosphoenolpyruvate carboxylase 2	aCL4972Contig1	1.38	0.92	AT1G53310
Cystatin-like protein	aCL1198Contig1	1.38	1.06	AT5G47550
NAC domain protein NAC19	aCL35Contig3	1.38	0.74	AT1G52890
Aluminum-induced protein	aC31305F09EF_c	1.38	1.36	AT5G19140
Profilin-3	aCL1323Contig1	1.38	0.92	AT4G29340

no annotation available	aC08019G01SK_c	1.38	2.81	
AMP-binding protein	aCL5216Contig1	1.38	0.17	AT5G16340
cysteine proteinase, putative / AALP protein (AALP)	aCL13Contig5	1.38	0.46	AT5G60360
Pyrophosphate-dependent phosphofructokinase beta subunit	aC06008H03SK_c	1.38	1.36	AT1G12000
shikimate kinase-related	aC08031D04SK_c	1.38	0.46	AT2G35500
NADC homolog	aC34205E07EF_c	1.38	3.23	AT2G01350
Two-component response regulator-like APRR5	aCL4006Contig1	1.38	1.36	AT5G24470
late embryogenesis abundant 3 family protein	aCL6Contig4	1.37	0.57	AT4G02380
FAD linked oxidase family protein	aCL3809Contig1	1.37	1.36	AT5G06580
glycosyl hydrolase family 3 protein	aCL3345Contig1	1.37	4.32	AT1G78060
CCAAT-box binding transcription factor subunit B (AHAP3) family	aCL1964Contig1	1.37	3.23	AT4G14540
zinc finger (C3HC4-type RING finger) family protein	aCL8939Contig1	1.37	0.74	AT5G57740
no annotation available	aC08006H05SK_c	1.37	1.78	
Metal tolerance protein C1	aC20001A04SK_c	1.37	0.92	AT2G47830
MipC	aCL165Contig1	1.37	0.57	AT5G60660
late embryogenesis abundant 3 family protein	aCL6Contig22	1.37	1.06	AT4G02380
basic helix-loop-helix (bHLH) protein (RAP-1)	aC04028A10SK_c	1.37	0.46	AT1G32640
expressed protein	aCL2009Contig1	1.37	1.36	AT1G27100
Tonoplast dicarboxylate transporter	aCL5Contig3	1.37	1.06	AT5G47560
haloacid dehalogenase-like hydrolase family protein	aC31501F12EF_c	1.37	1.06	AT2G41250
similar to glycosyl transferase family 48 protein	aCL2860Contig1	1.37	3.23	AT1G05570
copper chaperone (CCH)-related	aCL5712Contig1	1.36	0.17	AT5G63530
glyceraldehyde 3-phosphate dehydrogenase	aCL218Contig2	1.36	1.36	AT1G16300
no annotation available	aC04019C10SK_c	1.36	2.81	
late embryogenesis abundant 3 family protein	aCL6Contig5	1.36	0.74	AT4G02380
Putative membrane transporter	aCL854Contig2	1.36	0.46	AT2G43330
zinc finger (AN1-like) family protein	aCL511Contig7	1.36	1.78	AT2G27580
Thiazole biosynthetic enzyme, chloroplast precursor	aCL1030Contig1	1.36	2.81	AT5G54770
Low temperature-induced protein lt101.1	aC21001B11Rv_c	1.36	0.46	AT2G38905
Putative succinate dehydrogenase flavoprotein alpha subunit	aC08015F02SK_c	1.36	2.81	AT5G66760
no annotation available	aCL20Contig4	1.36	0.74	
MYB-like DNA-binding protein	aC06013H06SK_c	1.36	1.06	AT5G13820
Cinnamoyl-CoA reductase	aCL653Contig1	1.36	0.57	AT2G23910
ADP-glucose pyrophosphorylase small subunit	aC31305H08EF_c	1.36	1.36	AT5G48300
WRKY transcription factor NtEIG-D48	aCL2048Contig1	1.36	1.78	AT4G24240
expressed protein	aIC0AAA32DG11RM1_c	1.36	2.81	AT5G62950
Aquaporin PIP1.3	aCL58Contig3	1.36	0.17	AT4G00430
plectin-related	aCL5463Contig1	1.36	4.32	AT2G26770
Carbonic anhydrase	aCL871Contig1	1.35	2.81	AT3G01500
L-asparaginase	aCL506Contig2	1.35	0.74	AT3G16150
expressed protein	aCL1108Contig1	1.35	0.74	AT1G55340
expressed protein	aCL4644Contig1	1.35	1.06	AT1G67785
heat shock protein-related	aCL2011Contig1	1.35	2.81	AT5G57710
Flavonol synthase/flavanone 3-hydroxylase	aCL336Contig1	1.35	1.06	AT5G08640
expressed protein	aCL1118Contig2	1.35	1.36	AT1G50120
expressed protein	aCL7570Contig1	1.35	1.06	AT4G27020
no annotation available	aCL9431Contig1	1.35	2.81	
no annotation available	aCL1112Contig1	1.35	0.92	
expressed protein	aCL5894Contig1	1.35	4.32	AT1G21680

BZIP transcription facto	aCL3570Contig2	1.35	1.36	AT1G42990
Glutathione-conjugate transporter AtMRP4	aCL4731Contig1	1.35	1.78	AT2G47800
expressed protein	aC08033G08SK_c	1.35	2.81	AT2G44010
70kD heat shock protein	aC34105F11EF_c	1.34	0.74	AT2G32120
Phi-1 protein	aCL1406Contig1	1.34	1.36	AT4G08950
Peroxisomal (S)-2-hydroxy-acid oxidase	aCL2300Contig1	1.34	1.36	AT3G14420
lipid transfer protein 4 (LTP4)	aCL3Contig25	1.34	1.78	AT5G59310
Type IIIa membrane protein cp-wap13	aC08013B09SK_c	1.34	0.92	AT3G02230
calmodulin-7 (CAM7)	aCL13Contig1	1.34	4.32	AT3G43810
Kinase-like protein	aCL5503Contig1	1.34	1.36	AT5G63320
Cinnamyl-alcohol dehydrogenase 1	aCL1427Contig1	1.34	0.74	AT3G19450
Hydroxyproline-rich glycoprotein	aCL150Contig2	1.34	1.78	AT3G22440
Abscisic acid-induced-like protein	aC31106H02EF_c	1.34	1.36	AT5G50720
no annotation available	aC04035C12SK_c	1.34	1.36	
Putative histone H2A	aCL4564Contig1	1.34	4.32	AT5G02560
Anthraniloyal-CoA: methanol anthraniloyal transferase	aC31206H01EF_c	1.34	3.23	AT5G17540
no annotation available	aC04027F11SK_c	1.34	3.23	
expressed protein	aCL59Contig3	1.34	0.74	AT2G27830
C2 domain-containing protein	aCL2978Contig1	1.34	0.74	AT4G11610
no annotation available	aC02013H01SK_c	1.33	1.78	
Ser/Thr protein kinase (CIPK3)	aC06015C02SK_c	1.33	1.36	AT2G26980
Receptor-kinase isolog (LRR-RLK)	aC20007G06SK_c	1.33	1.78	AT1G60630
Expressed protein	aCL8735Contig1	1.33	0.74	AT2G31190
Early nodulin-like protein 2 precursor	aCL931Contig1	1.33	0.17	AT4G27520
UDP-glucosyltransferase HRA25	aCL7527Contig1	1.33	1.78	AT3G02100
no annotation available	aC31207F04EF_c	1.33	0.92	
no annotation available	aCL4356Contig1	1.33	1.36	
similar to phosphatidate cytidylyltransferase	aC31803H08EF_c	1.33	1.36	AT4G22340
glutathione S-transferase C-terminal domain-containing protein	aCL350Contig1	1.33	3.23	AT5G44000
no annotation available	aCL3756Contig1	1.33	1.78	
S-adenosyl-L-methionine:delta24-sterol-C-methyltransferase	aCL6040Contig1	1.33	1.06	AT5G13710
expressed protein	aCL266Contig1	1.33	1.36	AT5G51510
expressed protein	aCL4174Contig1	1.33	1.36	AT2G43780
nitrate transporter (NTP3)	aC06008E06SK_c	1.33	1.06	AT3G21670
acyl-(acyl-carrier-protein) desaturase / stearoyl-ACP desaturase (SSI2)	aCL8721Contig1	1.32	2.81	AT2G43710
chalcone synthase / naringenin-chalcone synthase	aC31807C06EF_c	1.32	3.23	AT5G13930
hydroxyproline-rich glycoprotein family protein,	aC06005F09SK_c	1.32	0.92	AT1G23040
Putative kinesin light chain	aC02010B05SK_c	1.32	1.06	AT1G27500
cytochrome b6f complex subunit (petM)	aCL42Contig1	1.32	1.78	AT2G26500
BZIP transcription factor protein	aCL3570Contig1	1.32	2.81	AT1G42990
Pyrophosphate-energized vacuolar membrane proton pump	aC32007A12EF_c	1.32	1.36	AT1G15690
Alcohol acyl transferase	aC06011E10SK_c	1.32	4.32	AT5G17540
Proteophosphoglycan 5	aCL5627Contig1	1.32	3.23	
Protein phosphatase-2C	aKN0AAP12YJ01FM1_c	1.32	3.23	AT3G62260
Putative ethylene response factor ERF3a (AP2 domain)	aCL1567Contig2	1.32	1.36	AT5G44210
delta-8 sphingolipid desaturase	aCL4105Contig1	1.32	0.92	AT2G46210
calcium-binding EF hand family protein	aCL26Contig5	1.32	1.06	AT5G39670
expressed protein	aC05133F03SK_c	1.32	1.06	AT1G10740
Ferritin-3, chloroplast precursor	aCL859Contig1	1.32	1.36	AT5G01600

expressed protein	aCL598Contig1	1.32	1.36	AT4G01670
Serine/threonine protein kinase	aC06005C08SK_c	1.32	1.78	AT5G47750
expressed protein	aC34009A08EF_c	1.32	0.74	AT4G02920
Cinnamoyl CoA reductase	aCL8388Contig1	1.32	1.36	AT1G15950
transducin family protein / WD-40 repeat family protein	aC04028G03SK_c	1.32	1.36	AT5G49430
no annotation available	aC02011B12SK_c	1.32	0.57	
harpin-induced family protein / HIN1 family protein	aCL1271Contig1	1.31	3.23	AT3G11660
Heat shock protein 101	aCL7935Contig1	1.31	3.23	AT1G74310
Tubulin beta-2 chain	aC31804C06EF_c	1.31	0.92	AT5G12250
expressed protein	aCL5508Contig1	1.31	1.36	AT5G13240
Putative peroxisomal membrane protein	aC08036G02SK_c	1.31	2.81	AT1G52870
Putative early light induced protein	aCL5Contig15	1.31	1.78	AT3G22840
expressed protein	aCL2576Contig1	1.31	1.78	AT5G18130
adhesin-related	aC31501F04EF_c	1.31	4.32	AT1G20970
Zinc finger, C3HC4 type	aCL128Contig2	1.31	2.81	AT5G22000
HyuC-like protein	aC31401C02EF_c	1.31	1.78	AT4G20070
Serine/threonine-protein phosphatase BSL3	aC18009G03Rv_c	1.31	2.81	AT2G27210
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	aCL90Contig4	1.31	1.36	AT5G17920
expressed protein	aCL225Contig1	1.31	1.36	AT2G27385
zinc finger (GATA type) family protein	aC31706D05EF_c	1.31	3.23	AT3G54810
copine-related	aCL57Contig2	1.31	1.06	AT5G14420
Putative inorganic pyrophosphatase	aCL3796Contig1	1.30	1.36	AT3G53620
no annotation available	aCL5494Contig1	1.30	2.81	
ABC transporter-like protein	aCL3233Contig1	1.30	3.23	AT5G02270
Hydroxyproline-rich glycoprotein	aC31604D08EF_c	1.30	1.36	AT3G22440
no annotation available	aC08007E09SK_c	1.30	2.81	
40S ribosomal protein S24	aCL3908Contig1	1.30	3.23	AT3G04920
4-coumarate:CoA ligase 2	aCL7230Contig1	1.30	1.36	AT1G51680
Glucose-6-phosphate/phosphate-translocator precursor	aCL2727Contig1	1.30	1.36	AT1G61800
Proline-rich protein	aCL1641Contig1	1.30	3.23	AT5G15780
zinc finger (C3HC4-type RING finger) family protein	aCL703Contig1	1.30	1.78	AT5G59550
Allene oxide synthase	aCL6896Contig1	1.30	2.81	AT5G42650
Cytochrome P450-like protein	aCL1058Contig1	1.30	2.81	AT3G56630
ARIADNE-like protein ARI7	aCL2811Contig1	1.30	2.81	AT2G31510
Glutamine synthetase	aCL86Contig1	1.29	2.81	AT5G37600
expressed protein	aCL5491Contig1	1.29	4.32	AT4G10080
Glycine dehydrogenase [decarboxylating]	aCL105Contig2	1.29	1.78	AT4G33010
expressed protein	aCL3774Contig1	1.29	1.06	AT2G05620
Putative callose synthase 1 catalytic subunit	aCL6722Contig1	1.29	3.23	AT4G04970
disease resistance-responsive protein-related	aC06008D06SK_c	1.29	2.81	AT1G58170
UDP-glucose glucosyltransferase	aC34108E04EF_c	1.29	0.74	AT4G34135
Plasma membrane H+ ATPase	aCL1735Contig1	1.29	1.36	AT2G24520
Aspartic proteinase 4	aCL958Contig1	1.29	1.36	AT1G11910
Histone H2B	aCL225Contig3	1.29	3.23	AT5G59910
Putative glutathione S-transferase T3	aCL803Contig1	1.29	3.23	AT2G29420
Putative receptor protein kinase LRR	aCL4613Contig1	1.29	2.81	AT2G26330
protein kinase family protein	aCL6258Contig1	1.29	1.36	AT5G60550
Nodulin-like protein	aCL696Contig1	1.28	0.74	AT1G80530
no annotation available	aC05808E03SK_c	1.28	1.36	
no annotation available	aIC0AAA94AE10RM1_c	1.28	4.32	

Hydroxycinnamoyl CoA quinate transferase	aCL5400Contig1	1.28	3.23	AT5G48930
MYB Transcription factor (MYBR2)	aCL795Contig2	1.28	4.32	AT3G16350
Lectin-like protein kinase	aC08036G07SK_c	1.28	2.81	AT5G06740
Leaf protein	aC31601F12EF_c	1.28	2.81	AT5G21222
expressed protein	aC31802F07EF_c	1.28	3.23	AT5G53420
Putative allantoinase	aC31504H10EF_c	1.28	2.81	AT4G04955
rubber elongation factor (REF) family protein	aCL1Contig22	1.28	2.81	AT1G67360
Probable pyridoxin biosynthesis PDX1-like protein 2	aCL901Contig2	1.28	3.23	AT3G16050
Putative lipase	aCL2971Contig1	1.27	4.32	AT3G48460
Eukaryotic peptide chain release factor subunit 1-3	aCL351Contig1	1.27	3.23	AT3G26618
expressed protein	aC18007A06Rv_c	1.27	1.06	AT1G15780
similar to expressed protein	aCL5741Contig1	1.27	1.78	AT5G45030
ubiquitin-specific protease 3 (UBP3)	aCL8124Contig1	1.27	4.32	AT4G39910
no annotation available	aC01011D03SK_c	1.27	3.23	
COBRA protein precursor	aCL648Contig2	1.27	1.36	AT5G60920
nucleoporin interacting component family protein	aC02004G03SK_c	1.27	1.36	AT2G41620
no annotation available	aC04028G06SK_c	1.27	1.78	
Glucose-6-phosphate/phosphate-translocator precursor	aC06024H01SK_c	1.26	1.36	AT1G61800
expressed protein	aCL5778Contig1	1.26	3.23	AT1G07090
Glutamine synthetase	aCL109Contig1	1.26	1.78	AT5G37600
Purple acid phosphatase-like protein	aCL3990Contig1	1.26	1.78	AT3G20500
Putative mitochondrial ATP synthase	aIC0AAA53CB11RM1_c	1.26	4.32	AT3G52300
Chloroplast lipocalin	aC02003B07SK_c	1.26	4.32	AT3G47860
similar to leucine-rich repeat family protein	aC31304F02EF_c	1.26	3.23	AT1G78230
no annotation available	aC32106F08EF_c	1.26	3.23	
DEAD-Box RNA helicase-like protein	aCL4154Contig1	1.26	2.81	AT3G22330
COBRA-like protein 7 precursor	aCL1086Contig1	1.26	4.32	AT4G16120
no annotation available	aC02013F03SK_c	1.26	3.23	
Cellulose synthase	aC03001C04Rv_c	1.26	3.23	AT4G39350
galactosyltransferase family protein	aC32107E12EF_c	1.26	4.32	AT3G06440
L1 protein	aCL2360Contig1	1.25	2.81	AT3G63490
no annotation available	aC16015B08SK_c	1.25	4.32	
Beta 1,4 N-acetylglucosaminyltransferase	aC18019G01Rv_c	1.25	1.78	AT1G12990
similar to RNA recognition motif (RRM)-containing protein	aCL1191Contig1	1.25	3.23	AT4G00830
Putative ubiquinol--cytochrome-c reductase	aCL6255Contig1	1.25	1.78	AT1G15120
MYB-like DNA-binding protein	aCL5608Contig1	1.25	3.23	AT5G13820
expressed protein	aCL1553Contig1	1.24	1.36	AT1G80000
Putative carbonyl reductase	aCL4319Contig1	1.24	2.81	AT3G61220
nonspecific lipid transfer protein 1 (LTP1)	aCL155Contig1	1.24	4.32	AT2G38540
WRKY family transcription factor (SPF1)	aCL1506Contig1	1.24	3.23	AT2G38470
Histone deacetylase-like protein	aCL3308Contig1	1.24	3.23	AT5G61060
calcium-dependent protein kinase, putative / CDPK	aCL8407Contig1	1.24	2.81	AT3G56760
Putative GTP-binding protein	aC04026C08SK_c	1.23	4.32	AT1G06400
no annotation available	aC04016E12SK_c	1.23	4.32	
AAA-type ATPase-like protein	aCL580Contig2	1.23	3.23	AT5G17760
Flavonol sulfotransferase-like	aCL7291Contig1	1.23	4.32	AT2G03760
Putative membrane related protein CP5	aC01009H02SK_c	1.23	2.81	AT1G64720
expressed protein	aC31705E05EF_c	1.23	2.81	AT2G46550
expressed protein	aC04013G12SK_c	1.23	4.32	AT5G47680
no annotation available	aCL5268Contig1	1.22	4.32	

Trehalose-6-phosphate synthase	aC31403D05EF_c	1.22	3.23	AT1G68020
Glycine-rich RNA-binding protein	aCL5Contig23	1.22	4.32	AT2G21660
expressed protein	aC32201A09EF_c	1.22	2.81	AT1G11700
expressed protein	aCL1096Contig1	1.22	1.78	AT3G52610
CLB1	aCL2037Contig1	1.21	4.32	AT3G61050
no annotation available	aC19009F02T7_c	-8.17	1.78	
geranylgeranyl pyrophosphate synthase (GGPS1)	aCL960Contig1	-6.25	0.00	AT4G36810
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aC02013A08SK_c	-4.65	0.00	AT3G25820
annexin 4 (ANN4)	aC20005D02SK_c	-4.64	0.00	AT2G38750
Sesquiterpene synthase (germacrene-D synthase)	aCL4874Contig1	-4.03	0.00	AT5G23960
expressed protein	aC20001E01SK_c	-3.55	0.00	AT1G06720
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aCL2450Contig1	-3.48	0.00	AT3G25820
acetolactate synthase small subunit	aC16018D09SK_c	-3.18	0.00	AT2G31810
(E)-beta-ocimene/alpha-farnesene synthase activity	aCL2450Contig2	-3.15	0.00	AT4G16740
expressed protein	aCL1Contig8	-3.12	0.92	AT5G13220
flavonol 3-O-methyltransferase 1 / caffeic acid	aCL38Contig8	-3.01	0.00	AT5G54160
expressed protein	aCL2040Contig3	-2.89	0.00	AT1G19180
tryptophan synthase	aC31005F03EF_c	-2.84	0.00	AT5G28237
flavonol 3-O-methyltransferase 1 / caffeic acid	aCL4936Contig1	-2.71	0.18	AT5G54160
expressed protein	aCL132Contig1	-2.64	0.00	AT1G19180
glutamate receptor family protein (GLR3.3)	aCL3255Contig1	-2.48	0.00	AT1G42540
tryptophan synthase, beta subunit	aCL626Contig1	-2.37	0.46	AT5G28237
alcohol dehydrogenase	aC34109F01EF_c	-2.27	0.00	AT5G42250
allene oxide synthase (AOS)	aCL1628Contig2	-2.18	0.00	AT5G42650
Plastidic ATP/ADP transporter	aCL694Contig1	-2.16	0.00	AT1G80300
protein kinase family protein	aC05804D11SK_c	-2.10	0.46	AT3G51550
hydroperoxide lyase (HPL1)	aCL5513Contig1	-2.08	0.00	AT4G15440
early-responsive to dehydration protein-related	aCL7793Contig1	-1.99	0.18	AT4G22120
ABC transporter family protein	aCL862Contig1	-1.98	0.00	AT1G17840
potassium transporter (KUP1)	aC31804F08EF_c	-1.97	0.00	AT2G30070
esterase/lipase/thioesterase family protein	aCL4614Contig1	-1.95	0.00	AT5G22460
TIP1;3 | major intrinsic family protein	aCL824Contig2	-1.94	0.00	AT4G01470
COP9 signalosome complex subunit 1	aCL2021Contig1	-1.93	0.00	AT3G61140
Ribose-phosphate pyrophosphokinase 1	aCL2448Contig1	-1.92	0.00	AT2G35390
WD-40 repeat family protein	aCL7691Contig1	-1.87	0.00	AT1G58230
esterase/lipase/thioesterase family protein	aCL8342Contig1	-1.87	0.00	AT2G39420
DNAJ heat shock N-terminal domain-containing protein	aCL3788Contig1	-1.86	0.00	AT1G56300
annexin 4 (ANN4)	aC31701H09EF_c	-1.85	0.00	AT2G38750
ACC oxidase	aC31605B08EF_c	-1.85	0.00	AT1G05010
Putative dioxygenase	aCL8911Contig1	-1.84	3.23	AT4G15093
late embryogenesis abundant protein	aCL1327Contig1	-1.84	1.78	AT1G01470
O-methyltransferase family 2 protein	aCL3052Contig1	-1.82	0.00	AT4G35160
pentatricopeptide (PPR) repeat-containing protein	aC02025C07SK_c	-1.80	0.00	AT5G59600
flavonol 3-O-methyltransferase 1 / caffeic acid	aCL38Contig7	-1.78	0.00	AT5G54160
annexin 2 (ANN2)	aCL296Contig1	-1.77	0.00	AT5G65020
aldo/keto reductase	aC31810E10EF_c	-1.77	0.00	AT1G59950
expressed protein	aCL7402Contig1	-1.76	0.00	AT2G26070

ABC transporter family protein	aCL862Contig2	-1.73	0.00	AT1G17840
Nodulin-26	aCL824Contig1	-1.73	1.49	AT3G26520
GmCK2p	aCL395Contig1	-1.73	0.00	AT4G09760
O-methyltransferase family 2 protein	aCL4905Contig1	-1.72	0.00	AT4G35160
Anthranilate N-benzoyltransferase-like protein	aCL253Contig1	-1.71	0.18	AT5G01210
CYP82C1p	aCL1683Contig1	-1.70	0.46	AT4G31940
no apical meristem (NAM) family protein	aC19004C08T7_c	-1.69	0.18	AT3G04070
Putative exostoses	aCL8580Contig1	-1.68	0.00	AT5G04500
dehydration-responsive protein-related	aCL4197Contig1	-1.68	0.00	AT1G78240
1-deoxy-D-xylulose 5-phosphate synthase	aC31504D11EF_c	-1.67	0.00	AT4G15560
SET domain-containing protein	aC08026D10SK_c	-1.66	0.81	AT5G17240
pyridoxal-dependent decarboxylase family protein	aC04032H01SK_c	-1.63	0.00	AT1G27980
omega-3 fatty acid desaturase	aCL5Contig18	-1.63	0.18	AT5G05580
amino acid transporter family protein	aCL4668Contig1	-1.62	0.00	AT4G38250
no annotation available	aC01012H12SK_c	-1.61	4.32	
glycosyl hydrolase family 17 protein	aCL168Contig3	-1.60	0.00	AT2G27500
26.5 kDa class I small heat shock protein-like (HSP26.5-P)	aCL5468Contig1	-1.59	1.49	AT1G52560
SET domain-containing protein	aC08037B11SK_c	-1.59	0.00	AT5G17240
no annotation available	aC19003F01T7_c	-1.59	0.81	
similar to raffinose synthase family protein	aCL6554Contig1	-1.58	1.49	AT4G01970
protein kinase family protein	aC31502A04EF_c	-1.58	0.00	AT5G63940
no annotation available	aCL913Contig1	-1.58	0.00	
hypoxia-responsive family protein	aCL1469Contig3	-1.58	0.81	AT3G05550
C2 domain-containing protein	aCL61Contig1	-1.57	0.00	AT5G23950
Putative exostoses	aCL6247Contig1	-1.56	0.92	AT5G04500
Sphingosine-1-phosphate lyase	aCL4647Contig1	-1.56	0.81	AT1G27980
sugar transporter family protein	aC18005D05Rv_c	-1.56	3.23	AT5G13740
flavonol 3-O-methyltransferase 1 / caffeic acid	aCL38Contig2	-1.55	0.81	AT5G54160
basic helix-loop-helix (bHLH) family protein	aCL2806Contig1	-1.55	0.00	AT5G57150
no annotation available	aCL8Contig9	-1.55	2.81	
acetolactate synthase small subunit	aC05065C02SK_c	-1.54	0.18	AT5G16290
similar to protease inhibitor/seed storage/lipid transfer protein	aCL2501Contig1	-1.54	0.18	AT4G33550
expressed protein	aCL3940Contig1	-1.54	2.81	AT1G55230
mitochondrial substrate carrier family protein	aCL536Contig1	-1.54	1.78	AT2G22500
ABC transporter family protein	aC34101E02EF_c	-1.51	0.81	AT1G66950
no annotation available	aCL4456Contig1	-1.51	0.92	
photosystem I reaction center subunit XI	aCL2872Contig1	-1.50	3.23	AT4G12800
photoassimilate-responsive protein-related	aCL4667Contig1	-1.50	2.81	AT3G54040
expressed protein	aCL5398Contig1	-1.48	1.49	AT4G37300
Monooxygenase-like	aCL360Contig2	-1.47	1.06	AT5G11330
basic helix-loop-helix (bHLH) family protein, bHLH protein	aCL7106Contig1	-1.47	0.57	AT4G17880
protein kinase family protein	aCL5363Contig1	-1.47	1.49	AT1G77280
no annotation available	aC18013E09Rv_c	-1.46	0.00	
no annotation available	aC19007C04T7_c	-1.46	1.78	
HNH endonuclease domain-containing protein	aC01018D02SK_c	-1.45	0.18	AT2G23840
similar to sugar transporter family protein	aC34005E02EF_c	-1.45	1.49	AT5G13750
ABC transporter family protein	aCL387Contig3	-1.45	0.00	AT1G66950
flavonoid 3'-monooxygenase / flavonoid 3'-hydroxylase (F3'H)	aCL945Contig1	-1.44	0.81	AT5G07990
expressed protein	aCL1844Contig1	-1.44	0.92	AT3G04350
DNAJ heat shock N-terminal domain-containing protein	aCL5139Contig1	-1.44	3.23	AT3G14200

CER1 protein, putative (WAX2)	aCL2973Contig1	-1.43	1.78	AT5G57800
beta-ketoacyl-CoA synthase family protein	aC32009D10EF_c	-1.43	0.92	AT5G04530
CER1 protein, putative (WAX2)	aC06013B05SK_c	-1.43	0.92	AT5G57800
flavonoid 3'-monooxygenase / flavonoid 3'-hydroxylase (F3'H)	aCL9318Contig1	-1.42	1.78	AT5G07990
AAA-type ATPase family protein	aCL6434Contig1	-1.41	4.32	AT3G24530
respiratory burst oxidase protein C (RbohC)	aCL503Contig1	-1.41	0.92	AT5G51060
CER1 protein, putative (WAX2)	aCL8782Contig1	-1.40	3.23	AT5G57800
ammonium transporter 1	aCL630Contig1	-1.40	3.23	AT4G13510
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aCL7446Contig1	-1.39	2.81	AT3G11340
expressed protein	aCL2976Contig1	-1.39	0.46	AT2G47485
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aC02002B09SK_c	-1.38	4.32	AT3G11340
haloacid dehalogenase-like hydrolase family protein	aCL8075Contig1	-1.37	3.23	AT5G59480
bZIP transcription factor family protein	aC08036D08SK_c	-1.37	1.78	AT1G75390
no annotation available	aCL131Contig4	-1.35	4.32	
no annotation available	aCL3Contig24	-1.34	4.32	
40S ribosomal protein S24 (RPS24A)	aCL1696Contig1	-1.33	3.23	AT3G04920
disease resistance protein (TIR-NBS-LRR class)	aC05076B08SK_c	-1.33	1.49	AT4G19520
Carbonic anhydrase	aCL3993Contig1	-1.32	2.81	AT5G14740
WRKY family transcription factor	aCL9018Contig1	-1.29	3.23	AT5G13080
phytosulfokines-related	aCL499Contig1	-1.28	3.23	AT3G44735
no annotation available	aC02003A12SK_c	-1.28	4.32	

B) Differentially expressed genes in the AS7 line

Description	**Citrus unigene**	**fold-change (real)**	**q-value(%)**	**Most similar Ath gene**
no annotation available	aC05807D09SK_c	13.55	0.00	
no annotation available	aC08007E01SK_c	9.66	0.00	
Sugar transporter family protein	aCL854Contig1	4.68	0.70	AT2G43330
monooxygenase, putative (MO2)	aCL54Contig2	4.27	0.00	AT4G38540
transducin family protein / WD-40 repeat family protein	aCL5544Contig1	4.23	0.70	AT3G06880
no annotation available	aC19001H06T7_c	4.22	0.00	
WRKY6	aCL1201Contig2	4.18	0.00	AT1G62300
Sodium-inducible calcium-binding protein (ACP1)	aCL1345Contig2	3.97	0.00	AT5G49480
no annotation available	aCL1714Contig1	3.83	0.00	
Putative CCR4-associated factor (CAF1)	aCL206Contig1	3.61	0.00	AT5G22250
zinc finger (CCCH-type) family protein (CZF1)	aC31603G11EF_c	3.53	0.00	AT2G40140
CYP81E8	aCL866Contig1	3.49	0.00	AT4G37370
nonspecific lipid transfer protein 1 (LTP1)	aCL4Contig13	3.46	0.00	AT2G38540
Encodes a member of the ERF subfamily B-3 of ERF/AP2 (ATERF-6)	aCL337Contig1	3.43	0.00	AT4G17490
Protein phosphatase 2C, putative / PP2C	aCL683Contig1	3.41	0.00	AT2G30020
no annotation available	aC19008H07T7_c	3.39	0.70	
geranylgeranyl pyrophosphate synthase (GGPS1)	aCL960Contig1	3.38	0.00	AT4G36810
cytochrome P450 71B10	aCL3183Contig1	3.29	0.00	AT5G57260
WRKY family transcription factor, AR41	aCL775Contig1	3.02	0.00	AT4G23810
calmodulin, putative, similar to calmodulin NtCaM13	aCL535Contig3	2.93	0.00	AT3G10190
cyclic nucleotide-regulated ion channel (CNGC2)	aCL5832Contig1	2.90	0.00	AT5G15410
basic helix-loop-helix (bHLH) family protein	aCL2806Contig1	2.86	0.70	AT5G57150
multidrug resistant (MDR) ABC transporter	aCL5595Contig1	2.81	3.56	AT3G62150

FAD-binding domain-containing protein	aCL246Contig2	2.80	0.70	AT4G20820
no annotation available	aCL2819Contig1	2.80	0.00	
ChaC-like family protein	aC05802B02SK_c	2.79	0.00	AT4G31290
zinc finger (AN1-like) family protein	aCL511Contig7	2.77	0.00	AT2G27580
WRKY family transcription factor (SPF1)	aCL1506Contig1	2.76	0.00	AT2G38470
sulfate transporter	aC19008G08T7_c	2.76	0.00	AT4G02700
cytochrome P450 family protein	aCL3370Contig1	2.74	3.25	AT3G26310
glutathione S-conjugate ABC transporter (MRP2)	aC18021E02Rv_c	2.70	3.56	AT2G34660
no annotation available	aC19006C07T7_c	2.62	3.25	
expressed protein	aCL1Contig8	2.59	0.00	AT5G13220
no annotation available	aCL8681Contig1	2.57	0.70	
no annotation available	aCL50Contig2	2.56	0.43	
non-symbiotic hemoglobin 1 (HB1) (GLB1)	aC16013D01SK_c	2.55	0.70	AT2G16060
sigA-binding protein	aCL4628Contig1	2.49	0.70	AT3G56710
FAD-binding domain-containing protein	aCL246Contig1	2.43	0.70	AT2G34790
serine-glyoxylate aminotransferase-related	aCL1561Contig3	2.42	3.56	AT2G13360
sulfate transporter (ST1)	aC34107H08EF_c	2.39	0.00	AT3G51895
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aCL7446Contig1	2.38	3.07	AT3G11340
potassium channel tetramerisation domain-containing protein	aC05054G10SK_c	2.35	0.70	AT5G41330
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aC02002B09SK_c	2.35	3.25	AT3G11340
R2R3-MYB family transcription factor (MYB73)	aCL693Contig1	2.34	0.70	AT4G37260
Probable adenylate kinase 2 - chloroplast precursor	aCL3168Contig1	2.33	4.30	AT5G47840
expressed protein	aCL6840Contig1	2.26	0.00	AT3G52740
similar to nucellin protein, putative	aC04016D07SK_c	2.26	0.70	AT4G33490
3-oxo-5-alpha-steroid 4-dehydrogenase family protein	aC01011F03SK_c	2.26	0.70	AT5G16010
Cytochrome P450-like protein	aC16013D12SK_c	2.25	0.70	AT4G31940
no annotation available	aCL2956Contig1	2.25	2.05	
no annotation available	aC05065F06SK_c	2.24	1.09	
ABC transporter family protein	aCL5223Contig1	2.14	4.30	AT5G09930
expressed protein	aCL8468Contig1	2.13	0.00	AT1G69760
heavy-metal-associated domain-containing protein	aC31605A08EF_c	2.12	0.70	AT5G48290
Salt tolerance zinc finger protein	aCL146Contig3	2.12	0.00	AT1G27730
disease resistance protein (NBS-LRR class)	aCL5233Contig1	2.12	2.16	AT3G14460
vacuolar processing enzyme gamma	aCL554Contig1	2.11	0.00	AT4G32940
no annotation available	aC31806D03EF_c	2.10	3.56	
ankyrin repeat family protein	aC31704F08EF_c	2.09	0.00	AT1G07710
no annotation available	aC31807H02EF_c	2.09	0.00	
expressed protein	aC08031A08SK_c	2.08	3.25	AT5G41110
aldehyde dehydrogenase, putative (ALDH)	aC18012D10Rv_c	2.08	3.25	AT1G44170
expressed protein	aCL6679Contig1	2.08	3.56	AT1G59710
Transcription factor WRKY1	aCL2927Contig1	2.07	0.00	AT1G80840
zinc finger (AN1-like) family protein	aCL511Contig2	2.04	0.70	AT2G27580
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aC02002E10SK_c	2.04	0.70	AT3G02100
Similar to metallo-beta-lactamase family protein	aC08035F02SK_c	2.04	0.00	AT1G61010
similar to protein kinase family protein	aCL9409Contig1	2.03	0.00	AT5G47740
ERD1 protein - chloroplast precursor	aCL4690Contig1	2.03	0.00	AT5G51070
Anthranilate N-benzoyltransferase	aC18024F04Rv_c	2.03	3.25	AT5G48930
peptidase S41 family protein	aCL7817Contig1	2.02	0.70	AT3G57680
3-dehydroquinate dehydratase / shikimate dehydrogenase isoform 2	aC20009A07SK_c	2.02	0.00	AT3G06350
no annotation available	aCL1184Contig3	2.02	3.07	
nodulin MtN21 family protein	aCL8434Contig1	2.01	0.00	AT1G68170
no annotation available	aC05810F08SK_c	2.01	3.56	
expressed protein	aC19009D03T7_c	2.00	3.56	AT5G65960
late embryogenesis abundant 3 family protein	aCL6Contig4	2.00	0.70	AT4G02380

No apical meristem (NAM) family protein (NAC72)	aCL35Contig5	1.99	0.00	AT4G27410
no annotation available	aC08007C02SK_c	1.99	0.00	
no annotation available	aCL5242Contig1	1.98	3.56	
late embryogenesis abundant 3 family protein	aCL6Contig5	1.98	0.00	AT4G02380
calcium-binding EF hand family protein	aC08007C09SK_c	1.97	0.00	AT3G10300
no annotation available	aC31305G08EF_c	1.97	0.70	
no annotation available	aCL1329Contig1	1.97	0.00	
phenylalanine ammonia-lyase 1 (PAL1)	aCL1166Contig2	1.96	0.70	AT2G37040
UDP-glucoronosyl/UDP-glucosyl transferase family protein	aCL5570Contig1	1.95	3.56	AT2G36970
late embryogenesis abundant 3 family protein	aCL6Contig22	1.94	0.43	AT4G02380
chalcone synthase / naringenin-chalcone synthase	aCL27Contig2	1.94	0.00	AT5G13930
no annotation available	aC18001E11Rv_c	1.94	0.00	
C2 domain-containing protein, contains similarity to CLB1	aCL9264Contig1	1.93	1.09	AT5G47710
ChaC-like family protein	aCL283Contig1	1.91	0.00	AT4G31290
amino acid transporter family protein	aCL358Contig2	1.90	0.70	AT3G56200
strictosidine synthase family protein	aC31201B02EF_c	1.90	0.70	AT3G59530
2-nitropropane dioxygenase family / NPD family	aCL1003Contig1	1.90	1.09	AT5G64250
WD-40 repeat family protein / phytochrome A-related	aCL6446Contig1	1.90	0.43	AT1G53090
copper chaperone (CCH)-related	aCL4708Contig1	1.90	0.00	AT5G63530
ChaC-like family protein	aC31807B06EF_c	1.89	0.00	AT4G31290
cyclic nucleotide-regulated ion channel (CNGC2)	aC32102F03EF_c	1.88	0.00	AT5G15410
WRKY transcription factor NtEIG-D48	aCL2048Contig1	1.88	0.70	AT4G24240
expressed protein	aCL8222Contig1	1.88	0.00	AT3G61870
SYNC1 protein	aCL1665Contig1	1.87	0.00	AT2G25460
MYB Transcription factor (MYBR2)	aCL795Contig2	1.87	0.00	AT3G16350
cytochrome P450 monooxygenase	aCL4922Contig1	1.87	0.00	AT4G31940
no annotation available	aC03007D01SK_c	1.87	3.07	
Rubisco activase	aC05804A10SK_c	1.86	0.70	AT2G39730
no annotation available	aC31206E07EF_c	1.86	2.16	
F-box family protein (FBL3)	aC05056H07SK_c	1.86	0.00	AT5G01720
Putative ripening-related protein	aCL1973Contig2	1.85	0.70	AT5G02230
Lectin like protein	aCL2577Contig1	1.85	3.07	AT4G19840
no annotation available	aC31006C04EF_c	1.84	0.70	
no annotation available	aCL4787Contig1	1.83	0.00	
respiratory burst oxidase protein D (RbohD) / NADPH oxidase	aCL7567Contig1	1.82	0.00	AT5G47910
cellulose synthase family protein	aCL1355Contig1	1.82	0.00	AT2G32540
no annotation available	aC19003E02T7_c	1.82	0.70	
Ribulose bisphosphate carboxylase/oxygenase activase 1	aCL48Contig2	1.82	0.70	AT2G39730
similar to branched-chain amino acid aminotransferase 5 (BCAT5)	aCL3102Contig1	1.82	0.70	AT1G10070
no annotation available	aC04035C12SK_c	1.81	0.00	
MADS-box protein (AGL62)	aCL8174Contig1	1.81	3.25	AT5G60440
Homogentisate geranylgeranyl transferase	aCL895Contig3	1.81	0.00	AT2G18950
MYB70	aCL891Contig1	1.81	3.07	AT2G23290
calcium-binding EF hand family protein	aCL8972Contig1	1.81	2.16	AT1G05150
ATPDX2	aCL8162Contig1	1.80	0.70	AT5G60540
expressed protein	aC04027F02SK_c	1.80	3.25	AT3G07090
expressed protein	aCL5746Contig1	1.80	0.00	AT1G29950
epsin N-terminal homology (ENTH) domain-containing protein	aCL8521Contig1	1.79	0.70	AT5G35200
expressed protein	aCL59Contig3	1.79	0.00	AT2G27830
signal recognition particle 54 kDa protein	aCL4168Contig1	1.79	0.70	AT5G03940
S-receptor kinase-like protein 3	aC01014F11SK_c	1.79	0.70	AT1G11410
Alkaline/neutral invertase	aC02006E05SK_c	1.78	0.70	AT5G22510
eukaryotic translation initiation factor SUI1	aCL1184Contig4	1.78	0.00	AT5G54940

DC1 domain-containing protein	aCL2160Contig1	1.78	0.70	AT1G60420
protein kinase family protein (ORG-1)	aCL6565Contig1	1.78	0.70	AT5G53450
expressed protein	aC05133F03SK_c	1.78	0.70	AT1G10740
bromo-adjacent homology (BAH) domain-containing protein	aCL1560Contig1	1.77	3.25	AT1G68580
Callose synthase 1 catalytic subunit	aCL6983Contig1	1.77	3.25	AT1G05570
Putative serine/threonine kinase SRK2F	aC04002A03SK_c	1.76	0.00	AT4G40010
glycine-rich RNA-binding protein	aCL2713Contig1	1.76	2.16	AT5G04280
ANAC029 | no apical meristem (NAM)	aCL1571Contig1	1.75	3.56	AT1G69490
Ser-thr protein kinase	aCL5546Contig1	1.75	3.25	AT2G40270
esterase/lipase/thioesterase family protein	aCL5939Contig1	1.75	0.00	AT1G54570
Guanylate kinase-like protein	aCL2233Contig1	1.75	3.07	AT3G57550
F-box family protein	aCL4535Contig1	1.74	3.56	AT3G07870
amine oxidase family protein	aCL7309Contig1	1.74	3.07	AT1G65840
expressed protein	aCL1055Contig1	1.74	0.00	AT2G26530
no annotation available	aC31207F04EF_c	1.74	0.70	
translation initiation factor IF-2	aCL1666Contig1	1.73	0.00	AT1G17220
sugar transporter family protein	aC18005D05Rv_c	1.73	2.05	AT5G13740
chloroplast thylakoidal processing peptidase	aC20009E09SK_c	1.73	0.00	AT2G30440
expressed protein	aC34203C04EF_c	1.73	0.00	AT1G55480
Putative phosphatase	aCL4964Contig1	1.72	3.25	AT1G73010
no annotation available	aCL1802Contig1	1.72	3.25	
WD-40 repeat family protein	aC02012C02SK_c	1.71	3.07	AT5G67320
calcium-binding protein	aCL7914Contig1	1.71	0.70	AT1G21550
Putative allantoinase	aC31504H10EF_c	1.70	0.00	AT4G04955
Rieske (2Fe-2S) domain-containing protein	aCL1765Contig1	1.70	0.70	AT4G25650
sulfate adenylyltransferase 1 / ATP-sulfurylase 1 (APS1)	aCL438Contig2	1.70	0.00	AT3G22890
DNA helicase-like	aC02023C11SK_c	1.70	3.25	AT5G35970
histone H1-3 (HIS1-3)	aCL517Contig2	1.69	2.05	AT2G18050
expressed protein	aC01005C06SK_c	1.69	4.30	AT5G67370
mitogen-activated protein kinase kinase (MAPKK), putative (MKK9)	aCL2719Contig1	1.69	2.05	AT1G73500
remorin family protein	aCL1490Contig1	1.69	3.25	AT2G41870
phytochrome A signal transduction 1 (PAT1)	aCL5064Contig1	1.69	0.43	AT5G48150
expressed protein	aCL1081Contig2	1.69	0.43	AT3G19970
WRKY family transcription factor WRKY3/WRKY70	aCL6828Contig1	1.68	0.00	AT3G56400
Putative serine/threonine-specific protein kinase	aCL5214Contig1	1.67	4.30	AT2G33580
copper chaperone (CCH)-related	aCL2730Contig1	1.67	0.70	AT4G08570
no annotation available	aCL4637Contig1	1.67	0.70	
Peptidase family U7	aCL27Contig1	1.66	0.00	AT1G73990
transducin family protein / WD-40 repeat family protein	aC34008F06EF_c	1.65	0.00	AT3G06880
no annotation available	aCL101Contig2	1.65	0.00	
oxidoreductase, 2OG-Fe(II) oxygenase family protein	aC34009B09EF_c	1.65	0.00	AT4G10490
Glutamate decarboxylase 4a	aCL505Contig2	1.64	1.09	AT2G02010
expressed protein	aCL2400Contig1	1.64	0.00	AT1G32740
Fe-superoxide dismutase 1	aCL14Contig2	1.63	3.56	AT5G51100
Calcium-transporting ATPase 8 - plasma membrane-type	aC31103C11EF_c	1.63	0.70	AT3G21180
harpin-induced family protein (YLS9) / HIN1 family protein	aCL2389Contig2	1.63	0.00	AT2G35980
PDR12 | ABC transporter family involved in resistant to lead	aCL912Contig1	1.63	0.70	AT1G15520
amino acid transporter family protein	aCL1103Contig1	1.63	4.30	AT5G23810
basic helix-loop-helix (bHLH) protein (RAP-1)	aC04028A10SK_c	1.63	0.00	AT1G32640
NAC family protein (ANAC002)	aCL943Contig3	1.63	0.70	AT1G01720
expressed protein	aCL2976Contig1	1.63	4.30	AT2G47485
expressed protein	aCL8373Contig1	1.63	0.43	AT2G26310
Similarity to calmodulin-binding protein	aCL9279Contig1	1.63	3.25	AT5G62570
no annotation available	aC19005E05T7_c	1.62	4.30	

SAMT	aCL32Contig2	1.62	4.30	AT1G19640
ferredoxin, chloroplast (PETF)	aCL6164Contig1	1.62	3.25	AT1G60950
Allantoinase	aC34001D12EF_c	1.62	3.25	AT4G04955
no annotation available	aC08039B09SK_c	1.61	3.25	
pseudo-response regulator 5 (APRR5)	aCL4006Contig1	1.61	0.70	AT5G24470
AAA-type ATPase family protein	aCL2094Contig1	1.60	3.07	AT3G50930
Glutathione-conjugate transporter AtMRP4	aCL4731Contig1	1.60	0.00	AT2G47800
zinc finger (CCCH-type) family protein	aCL8680Contig1	1.60	0.00	AT3G55980
Putative AMP-binding protein	aC21001D05Rv_c	1.59	2.16	AT1G20510
10-hydroxygeraniol oxidoreductase	aCL85Contig3	1.59	0.70	AT4G37990
mitogen-activated protein kinase (MPK3)	aC31603C04EF_c	1.59	0.70	AT3G45640
no annotation available	aCL5472Contig1	1.58	0.70	
(PB1) domain-containing protein/(TPR)-containing protein	aCL9297Contig1	1.58	4.30	AT2G25290
Histone H3.3	aCL1455Contig1	1.57	0.00	AT5G10980
early-responsive to dehydration stress protein (ERD4)	aCL3691Contig1	1.57	0.70	AT1G30360
Trehalose-6-phosphate synthase	aC31403D05EF_c	1.57	0.00	AT1G68020
Receptor-protein kinase-like protein	aC05804D11SK_c	1.57	4.30	AT3G51550
Xyloglucan galactosyltransferase KATAMARI 1	aC08005B05SK_c	1.56	0.70	AT2G20370
Syntaxin 121	aCL706Contig1	1.56	3.25	AT3G11820
similar to mechanosensitive ion channel domain-containing protein	aCL4892Contig1	1.55	3.07	AT5G10490
calmodulin-7 (CAM7)	aCL13Contig1	1.54	0.70	AT3G43810
MATE efflux family protein	aCL312Contig2	1.54	0.70	AT3G21690
photosystem II reaction center W (PsbW) protein-related	aCL1139Contig1	1.54	3.07	AT2G30570
epsin N-terminal homology (ENTH) domain-containing protein	aC18009A10Rv_c	1.54	3.07	AT5G35200
drought-responsive family protein	aCL2867Contig1	1.53	3.25	AT5G26990
floral homeotic protein APETALA2 (AP2)	aC01019D09SK_c	1.53	3.56	AT4G36920
arogenate dehydrogenase	aCL5052Contig1	1.53	3.56	AT5G34930
no annotation available	aC32105H05EF_c	1.53	3.25	
no annotation available	aCL749Contig1	1.52	4.30	
hydrolase, alpha/beta fold family protein	aCL3417Contig1	1.52	3.56	AT4G24160
ABC transporter family protein	aCL5695Contig1	1.52	0.70	AT5G64840
Cinnamyl alcohol dehydrogenase	aCL1474Contig1	1.51	0.00	AT5G19440
Calmodulin-domain protein kinase isoform 7 (CPK7)	aC31501A07EF_c	1.51	0.70	AT5G12480
photosystem I reaction center subunit VI	aCL2388Contig1	1.51	0.70	AT1G52230
Aldo/keto reductase	aC31204A08EF_c	1.51	3.07	AT1G59960
chloride channel protein (CLC-c)	aCL8241Contig1	1.51	0.70	AT5G49890
disease resistance protein B8 (NBS-LRR class)	aC20002B03SK_c	1.51	2.16	AT3G14460
aldo/keto reductase	aCL2708Contig1	1.51	3.07	AT1G06690
(PB1) domain-containing protein/(TPR)-containing protein	aC34205A08EF_c	1.51	3.25	AT1G70640
no annotation available	aC08007G11SK_c	1.51	3.25	
no annotation available	aCL6488Contig1	1.51	3.07	
no annotation available	aC31808D06EF_c	1.51	2.05	
no annotation available	aC31109H05EF_c	1.50	2.05	
Tonoplast dicarboxylate transporter	aCL5Contig3	1.49	4.30	AT5G47560
expressed protein	aCL37Contig1	1.49	0.70	AT1G05340
elongation factor Tu family protein	aC02027E05SK_c	1.49	3.07	AT1G62750
2-oxoglutarate-dependent dioxygenase	aCL322Contig1	1.49	0.70	AT1G03400
cytochrome P450 98A3, putative (CYP98A3)	aCL627Contig1	1.49	2.05	AT2G40890
zinc finger (B-box type) family protein / salt-tolerance protein (STO)	aC31709D11EF_c	1.49	0.00	AT1G06040
lectin protein kinase	aC31504F06EF_c	1.49	3.56	AT4G04960
Kinase-like protein	aCL5503Contig1	1.48	0.00	AT5G63320
mitochondrial substrate carrier family protein	aCL4488Contig1	1.48	3.25	AT3G05290
aldehyde dehydrogenase (ALDH1a)	aCL4476Contig1	1.48	0.70	AT3G24503
no apical meristem (NAM) family protein	aCL8380Contig1	1.48	0.70	AT4G35580

MybSt1	aCL973Contig1	1.48	2.05	AT5G47390
Glucosyl transferase	aCL1582Contig1	1.47	0.70	AT3G11340
Putative peroxisomal membrane protein	aC08036G02SK_c	1.47	0.70	AT1G52870
Hexose transporter	aCL605Contig2	1.47	0.70	AT5G26340
short-chain dehydrogenase/reductase (SDR) family protein	aCL818Contig1	1.47	0.70	AT2G47140
Putative CONSTANS-like B-box zinc finger protein	aCL5350Contig1	1.47	3.56	AT4G38960
calcium-binding EF hand family protein	aCL26Contig5	1.47	3.25	AT5G39670
ribulose bisphosphate carboxylase small chain 3B	aCL43Contig3	1.47	3.56	AT5G38410
VQ motif-containing protein	aCL1812Contig2	1.47	3.07	AT3G56880
short-chain dehydrogenase/reductase (SDR) family protein	aCL4319Contig1	1.47	0.00	AT3G61220
family II extracellular lipase 1 (EXL1)	aCL5964Contig1	1.46	1.09	AT1G75880
L-allo-threonine aldolase-related protein	aC08036F01SK_c	1.46	1.09	AT1G08630
Receptor-like protein kinase	aCL5636Contig1	1.46	0.70	AT5G48380
Encodes a plant small ubiquitin-like modifier (SUMO) E3 ligase	aCL5857Contig1	1.45	3.25	AT5G60410
Limonoid UDP-glucosyltransferase	aC08034F10SK_c	1.45	3.56	AT3G21560
Histidine amino acid transporter	aCL4429Contig1	1.44	3.07	AT5G40780
Serine hydroxymethyltransferase - mitochondrial precursor	aC31004H06EF_c	1.44	0.70	AT4G37930
ABA induced protein phosphatase 2C, PP2C	aCL5289Contig1	1.44	0.70	AT5G59220
expressed protein	aC08034B11SK_c	1.44	3.25	AT1G73650
expressed protein	aC31103F09EF_c	1.43	4.30	AT4G19400
Chloroplast photosystem II 10 kDa protein	aCL148Contig1	1.43	4.30	AT1G79040
nitrate transporter (NTP3)	aC06008E06SK_c	1.43	0.00	AT3G21670
myb family transcription factor	aCL3396Contig1	1.43	3.07	AT1G72650
no annotation available	aCL2116Contig1	1.43	3.56	
Cystathionine gamma synthase	aCL52Contig3	1.43	3.56	AT3G01120
Putative choline kinase	aC01005A10SK_c	1.43	3.25	AT2G26830
short-chain dehydrogenase/reductase (SDR) family protein	aC31710C05EF_c	1.43	4.30	AT3G61220
leucine-rich repeat family protein / protein kinase family protein	aC34003H08EF_c	1.42	3.07	AT3G14840
Granule-bound starch synthase 1	aCL60Contig1	1.42	3.25	AT1G32900
calmodulin-binding protein	aCL7706Contig1	1.42	1.09	AT2G24300
NAC domain protein NAC19	aCL35Contig3	1.42	3.07	AT1G52890
expressed protein	aCL407Contig1	1.42	3.25	AT3G52710
fatty acid elongase 3-ketoacyl-CoA synthase 1 (KCS1)	aCL5151Contig1	1.42	0.70	AT1G01120
Putative carbonyl reductase	aC34104H11EF_c	1.42	3.25	AT3G61220
cysteine proteinase, putative / AALP protein (AALP)	aCL13Contig5	1.42	0.00	AT5G60360
matrixin family protein	aCL258Contig1	1.42	0.70	AT1G24140
expressed protein	aCL375Contig2	1.42	4.30	AT2G46080
Oxygen evolving enhancer protein 1 precursor	aCL117Contig2	1.41	4.30	AT3G50820
no annotation available	aC01013F10SK_c	1.41	2.16	
no annotation available	aC08020F03SK_c	1.41	2.16	
ABC transporter family protein	aCL4569Contig1	1.41	2.05	AT1G65410
HSP70 family protein	aC34105F11EF_c	1.41	3.25	AT2G32120
expressed protein	aCL3829Contig1	1.41	3.25	AT2G25770
hydroxyproline-rich glycoprotein family protein	aCL4860Contig1	1.41	0.70	AT4G01050
no annotation available	aCL5627Contig1	1.41	3.07	
expressed protein	aCL3774Contig1	1.41	0.70	AT2G05620
copper transporter family protein	aCL1547Contig1	1.41	3.56	AT5G20650
expressed protein	aCL4348Contig1	1.41	3.07	AT4G28910
Sesquiterpene synthase, (α)-humulene	aCL6701Contig1	1.41	0.70	AT5G23960
no annotation available	aCL4390Contig1	1.41	3.07	
Putative peroxisomal membrane protein	aCL4716Contig1	1.40	0.70	AT1G52870
hydroxyproline-rich glycoprotein family protein	aCL4147Contig1	1.40	3.25	AT4G01050
protein transport protein-related	aC05076F06SK_c	1.40	3.25	AT5G27220

Ankyrin-like protein	aCL6038Contig1	1.40	2.05	AT3G12360
Allyl alcohol dehydrogenase	aCL160Contig1	1.40	3.25	AT5G16990
Putative phosphoglycerate mutase	aCL4870Contig1	1.39	4.30	AT1G22170
allene oxide synthase (AOS)	aCL6896Contig1	1.39	3.56	AT5G42650
no annotation available	aIC0AAA94AE10RM1_c	1.39	2.05	
Pyridoxine biosynthesis protein	aCL409Contig1	1.39	2.05	AT5G01410
Callose synthase catalytic subunit-like protein	aC18005G07Rv_c	1.39	4.30	AT5G13000
no annotation available	aC05808E03SK_c	1.38	3.07	
no annotation available	aC07011B02SK_c	1.38	3.25	
Putative phosphatase	aCL5757Contig1	1.38	0.70	AT2G32150
Fe-superoxide dismutase 1	aCL8011Contig1	1.38	4.30	AT5G51100
Putative 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 3	aCL214Contig1	1.38	4.30	AT1G22410
Putative ethylene response factor ERF3 subfamily B-1 (AP2 domain)	aCL1567Contig2	1.38	0.70	AT5G44210
MYB-like DNA-binding protein 1	aCL5608Contig1	1.38	0.70	AT5G13820
no annotation available	aCL3953Contig1	1.38	3.25	
MATE efflux family protein	aC04016G09SK_c	1.38	3.56	AT1G33090
expressed protein	aC03003E07SK_c	1.37	3.56	AT1G16720
Neutral invertase	aCL3327Contig1	1.37	3.25	AT1G56560
similar to bZIP family transcription factor (OBF4)	aCL7528Contig1	1.37	3.25	AT5G65210
expressed protein	aCL4644Contig1	1.37	3.25	AT1G67785
similar to ethylene-inducible ER33 protein	aCL4367Contig1	1.37	4.30	AT1G05710
no annotation available	aC16016F07SK_c	1.36	2.16	
Aldo/keto reductase AKR	aKN0AAA3BG09ZM1_c	1.36	4.30	AT1G60710
ATP-binding-cassette transporter (ABC1)	aCL4433Contig1	1.36	3.25	AT4G04770
Putative long chain acyl-CoA synthetase	aCL8561Contig1	1.36	4.30	AT2G04350
Alanine--glyoxylate aminotransferase 2 homolog 3	aCL5650Contig1	1.36	0.70	AT3G08860
leucine-rich repeat family protein	aC05146H12SK_c	1.36	3.25	AT5G48380
beta-fructosidase Glycosyl hydrolases family 32	aCL2322Contig1	1.35	2.16	AT1G12240
Dicyanin	aCL12Contig4	1.35	0.70	AT5G26330
expressed protein	aCL2009Contig1	1.35	3.07	AT1G27100
expressed protein	aC31009E09EF_c	1.34	2.16	AT1G62250
2-isopropylmalate synthase	aCL9432Contig1	1.34	0.70	AT1G18500
expressed protein	aCL2425Contig1	1.33	0.70	AT5G49820
S-adenosyl-L-methionine:delta24-sterol-C-methyltransferase	aCL6040Contig1	1.33	3.25	AT5G13710
2-nitropropane dioxygenase family	aCL1003Contig2	1.32	3.25	AT5G64250
Aspartic proteinase 4	aCL958Contig1	1.32	3.56	AT1G11910
Putative pantothenate kinase 4	aC16012A08SK_c	1.32	3.56	AT4G32180
no annotation available	aC34201H06EF_c	1.32	3.56	AT3G13480
BTB/POZ domain-containing protein	aCL1109Contig1	1.32	4.30	AT3G61600
inositol-3-phosphate synthase	aCL25Contig1	1.31	3.25	AT2G22240
expressed protein	aCL3642Contig2	1.31	3.07	AT4G38060
encodes a member of the ERF subfamily B-4 (AP2 domain)	aC16018F01SK_c	1.31	3.56	AT5G64750
expressed protein	aCL5699Contig1	1.31	3.25	AT2G48110
similar to epoxide hydrolase	aCL1400Contig1	1.30	4.30	AT5G53050
phosphatidate cytidylyltransferase family protein	aC01008H04SK_c	1.30	4.30	AT3G45040
axial regulator YABBY1 (YABBY1)	aC31702D12EF_c	1.29	4.30	AT2G45190
NADPH-dependent FMN reductase family protein	aCL4551Contig1	1.29	0.70	AT3G27890
cinnamoyl-CoA reductase-related	aCL653Contig1	1.29	3.25	AT2G23910
expressed protein	aCL9065Contig1	1.28	3.07	AT4G27390
expressed protein	aC31001D12EF_c	1.28	4.30	AT5G24690
expressed protein	aCL225Contig1	1.28	3.25	AT2G27385
adenylate kinase family protein	aCL903Contig2	1.26	3.56	AT2G37250
Beta-1 -3-glucanase precursor (glycosyl hydrolase family 17)	aCL20Contig7	1.26	3.25	AT3G57270
nitrogen fixation NifU-like family protein	aCL1518Contig1	1.25	3.56	AT5G49940

heat shock factor protein, putative (HSF6)	aCL800Contig1	1.25	4.30	AT5G62020
Sesquiterpene synthase (germacrene-D synthase)	aCL4874Contig1	-5.48	0.00	AT5G23960
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aC02013A08SK_c	-4.68	0.00	AT3G25820
expressed protein	aC20001E01SK_c	-3.13	0.00	AT1G06720
ATTPS-CIN | Encodes the monoterpene 1,8-cineole synthase. Highly similar to (R)-limonene synthase 1	aCL2450Contig1	-3.12	0.00	AT3G25820
no annotation available	aC01014H01SK_c	-2.86	0.92	
no annotation available	aCL8Contig9	-2.55	1.09	
TIP1;3 | major intrinsic family protein. Aquaporin	aC04032D05SK_c	-2.43	0.92	AT4G01470
flavonol 3-O-methyltransferase 1 / caffeic acid	aCL38Contig8	-2.41	2.16	AT5G54160
TIP1;3 | major intrinsic family protein. Aquaporin	aCL824Contig2	-2.40	2.16	AT4G01470
similar to caffeic acid O-methyltransferase	aCL4905Contig1	-2.32	0.92	AT4G35160
Sesquiterpene synthase, (-)-germacrene D synthase	aC32102B11EF_c	-2.30	0.43	AT3G14490
similar to caffeic acid O-methyltransferase	aCL3052Contig1	-2.27	1.09	AT4G35160
no annotation available	aKN0AAA2AC02ZM1_c	-2.26	1.09	
Alcohol dehydrogenase	aC34109F01EF_c	-2.20	0.00	AT5G42250
Monooxygenase-like	aCL360Contig2	-2.08	4.30	AT5G11330
NAD-dependent epimerase/dehydratase family protein	aCL2200Contig1	-1.81	3.07	AT3G23820
26.5 kDa class I small heat shock protein-like (HSP26.5-P)	aCL5468Contig1	-1.78	0.92	AT1G52560
no annotation available	aC03008B02SK_c	-1.78	3.07	
flavonoid 3'-monooxygenase / flavonoid 3'-hydroxylase (F3'H)	aCL9318Contig1	-1.66	3.25	AT5G07990
cytochrome P450 family protein, flavonoid 3',5'-hydroxylase	aCL796Contig1	-1.59	0.43	AT4G12300
Ras-related protein RGP1	aCL5312Contig1)	-1.53	4.30	AT5G47960
Extensin-like protein	aCL3701Contig1	-1.51	3.25	AT1G62510
Invertase inhibitor precursor	aCL7849Contig1	-1.49	3.07	AT1G47960
Pectinesterase PPE8B precursor	aCL1321Contig2	-1.47	0.92	AT3G43270
Cytosolic class II low molecular weight heat shock protein	aCL186Contig2	-1.34	2.16	AT5G12020
ankyrin repeat family protein	aIC0AAA47BE04RM1_c	-1.33	2.16	AT4G19660

Supplemental Table SIII. Primers designed for quantitative real-time RT-PCR to evaluate the expression of genes selected from microarray analyses and from jasmonic acid and terpenoid pathways.

Citrus unigene/Accesion number	Putative gene	Primer name	Primer sequence (5' – 3')	Primer amount[b] (μL)	Amplicon size (bp)
aC02013A08SK_c	Limonene synthase (LS)	qLS-F	AGTCACTGAATAGCAACTATACG	3	107
		qLS-R	CTGATCCAGAGGCTCGGTTA	0.5	
aCL4874Contig1	Germacrene-D synthase (GER)	qGER-F	AGAAGCGATGAAGAATCAAGTTA	3	100
		qGER-R	TAATGCAACCGTCATGTACTC	0.5	
aCL535Contig3	Calmodulin (CALMOD)	qCALMOD-F	CCTCCATTTAACGCTCAACC	3	141
		qCALMOD-R	TTCTCGATGAGTTTCATTTCCG	0.5	
aCL693Contig1	MYB73 Transcription Factor (MYB)	qMYB-F	GAGATGATAAGGAAAGAAGTGAGAAACTA	3	119
		qMYB-R	TCAGCCTTGTTAATCCCGATAC	0.5	
aCL1166Contig2	Phenylalanine ammonia-lyase 1 (PAL1)	qPAL1-F	TGTTACACAAAGAATGAACTTATTTACAAATC	3	101
		qPAL1-R	AGGGAGGACATTTCCACCTA	0.5	
aCL4Contig13	Nonspecific lipid transfer protein 1 (LTP1)	qLTP1-F	GTGATATGGACTCTCCTAGGT	0.5	102
		qLTP1-R	TATATGAGAATCGTGGCAAGGG	0.5	
aCL337Contig1	Ethylene response factor subfamily B-3 (ERF6)	qERF-F	CTCTGCATCTTCAGATCGTAG	0.5	101
		qERF-R	TATTAGTGGGTTGCGGTGTA	0.5	
CX289161	Actin	ACT-F	CATGAAGTGTGATGTGGATATTAG	0.5	106
		ACT-R	TGATTTCCTTGCTCATACGG	3	
aCL241Contig1	Lipoxygenase (LOX)	LOX-F	GCAACATTGCCACTGAAGATCCATC	2	103
		LOX-R	GTAGCTTGAATCTGGGAAGGGAAGG	2	
aCL5987Contig1	Allene oxide synthase (AOS)	AOS-F	GTTTCAGCTCGCTCCGTTAC	2	209
		AOS-R	TGGCAAATACGAGGTTGTGA	2	
aCL1411Contig1	Allene oxide ciclase (AOC)	AOC-F	GAAGGGTGACCGGTTTGAAGC	2	105
		AOC-R	ACGGCCAAGTACGTGTCTTCG	2	
aCL3569Contig1	Coronatine-insensitive 1 (COI1)	COI1-F	GGTCTTGTCTCGCAAAGAGG	2	115
		COI1-R	TGGCACCAATACACTCCAGA	2	
aCL1923Contig1	bHLHzip-type TF (MYC2)	MYC2-F	GGTGACCATGAGCTCCAACTG	2	172
		MYC2-R	GGCCGAAGAGAGATTTGGCTA	2	
acL6968Contig1	Defensin-like gene (PDF1.2)	PDF-F	CAGTGGCAGAAGCAAAACAA	2	136
		PDF-R	CCGGGGAAGTCGTAGTGGC	2	
aCL303Contig1	1-deoxyxylulose 5-phosphate synthase (DXS)	DXS-F2	CCATGAGGAAGACTTCGGGG	2	121
		DXS-R2	ACAGCCATACCAAGACCAGC	2	
aIC0AAA32AB11RM1_c	Geranyl diphosphate synthase (GDP)	GDP-F2	GACAAGAGAACTGGCCGTGA	2	100
		GDP-R2	AAGTGCACGCCTTGACTTTG	2	
aCL9351Contig1	Farnesyl diphosphate synthase (FDP)	FDP-F	GCTGCTGAATGATCCTGCATTT	2	103
		FDP-R	TAGCCCTCGGTTCAGCTTTC	2	
aCL960Contig1	Geranygeranyl diphosphate syntase (GGDP)	GGDP-F2	AGGAGGTGGGAGTGAGAAGG	2	144
		GGDP-R2	ACTTCCCTGAGCTTGAAGGC	2	

a. Oligonucleotides were designed using LightCycler Probe Design Software 1.0

b. Optimized amounts of a 5 μM oligonucleotide solution

6. GENERAL DISCUSSION AND OUTLOOK

6. General discussion and outlook

6.1. The language of volatiles

Plant volatile signaling in multitrophic interactions has become one of the more fascinating and fastest growing fields of research in recent years. Volatile organic compounds act as a language that plants use for their communication and interactions with the surrounding environment. These volatiles, released mainly from leaves, flowers, and fruits into the atmosphere and from roots into the soil, defend plants against herbivores and pathogens or provide a reproductive advantage by attracting pest predators, pollinators and seed dispersers (Pichersky and Gershenzon, 2002). Flowering species release diverse blends of volatile compounds from their flowers and fruits in addition to visual and tactile cues to attract pollinators and seed disseminators, thus ensuring reproductive success (Dudareva et al., 2006). Of the various plant organs, flowers in scented species and fleshy fruits produce the most diverse and the highest amount of volatile compounds, which peak when flowers are ready for pollination and fruits are fully mature (see Table I, Introduction). Volatile compounds emitted from fruits determine their overall aroma properties and taste, and consequently could play an important role in the attraction of animal seed dispersers and predators (Goff and Klee, 2006).

6.2. Fruit volatiles and seed dispersers

Fleshy fruits are made to be eaten (Schaefer and Ruxton, 2011). A fruit is the ripened ovary of a flowering plant that contains the seeds. Fleshy fruits seem to have evolved as a way of ensuring dispersal of larger seeds over longer distances than those normally achieved by abiotic means (Bolmgren and Eriksson, 2010). Although the primary biological function of fruits was to protect seeds, fleshy fruits evolved to attract frugivores. To do so, fruits have been selected during evolution for the manifestation of a number of characters, one of which is pleasant flavor. Fruit flavor affects animals' perception of a specific type of fruit. It is therefore important for the reproductive success of flowering plants (Lin et al., 2010). Fruits are the result of an evolutionary triad between plants, seed dispersers, and fruit predators like insects and microbes (which are the most abundant and ubiquitous frugivores but often the least obvious ones to humans). Seed dispersers and fruit predators impose conflicting selective pressures on the design of fleshy fruits. Ideally, fruits should be attractive to the former and at the same time repellent to the latter.

As outlined in this work, biochemistry rather than physical defense is the first barrier that plants use to mediate their interactions with fleshy fruit consumers. Plants produce a wide diversity of chemical compounds; many of them influence the feeding behavior of pollinators, seed dispersers, herbivores and their predators. Fruit biochemistry differs from the biochemistry of other plant organs (leaf, root) in that the fruit must balance the conflicting demands of defense against unwanted consumers and attraction of beneficial consumers (i.e., seed

dispersers and pollinators). When seeds are not yet able to germinate in a developing fruit, the fruit is termed unripe or immature. At this stage, any consumption of the fruit is detrimental to plant fitness; there is no need to attract but it is actually preferably to repel seed dispersers. Only when the seeds attain their ability to germinate, does the fruit become ripe or mature from the perspective of the plant. At this point, however, the fruit is not necessarily ripe from the perspective of a fruit-eating animal. Legitimate seed dispersers typically eat fleshy fruits as a nutritional reward for dispersing an enclosed seed(s). Although the balance between defense and attraction shifts towards increasing attraction during fruit ontogeny, fruit traits associated with ripening might also have evolved to screen out frugivores that do not disperse seeds. It is clear that fleshy fruit-bearing plants have evolved several characteristics to attract vertebrate seed dispersers. Whether these same characters also attract seed predators is a matter of discussion nowadays (Schaefer and Ruxton, 2011). In addition, separating vertebrate seed dispersers from vertebrate seed predators is not always easy; many vertebrates are both. Furthermore, insects and microorganisms, previously considered detrimental for fleshy fruits, may facilitate seed dispersal through positive interactions with vertebrates.

The various physiological and biochemical changes of fleshy fruits during maturation can thus be understood with reference to the fluctuations in the relative importance of defense and attraction. Remarkably, not all the changes induced during ripening evolved because they fulfill a communicative function; phylogenetic and physiological constraints should no be overlooked. However, because increased risk of seed death is associated with consumption of a not totally mature fruit, plants would clearly benefit from deterring unwanted fruit consumers by communicating to them. Consequently, fruit texture, color, smell, and taste are all important cues that can indicate the ripening stage of a fruit. As a rule of thumb, nutritional contents of fruit pulp increase during fruit ripening, the contents of deterrent secondary metabolites decrease, while the contents of pigments, aromatic compounds, and volatile flavor compounds increase, although there are many species-specific and component-specific variations to this pattern (Schaefer and Ruxton, 2011). Furthermore, a substance that is deterrent to one consumer does not necessarily deter another. In summary, attractive pulp constituents increase during ripening, whereas deterrent components decrease concomitantly; a pattern that reflects plants' shift in interest from protecting fruits when seeds are immature to promoting their consumption by seed dispersers once they are mature.

To gain insight on the role of volatile organic compounds from the peel of fleshy fruits in interactions with pests and microorganisms, sweet orange plants (*C. sinensis* L. Osb.) have been transformed with the full-length cDNA of a D-limonene synthase gene from satsuma mandarin (*C. unshiu* Marc.) in antisense (AS) or sense (S) orientation to down- or up-regulate, respectively, D-limonene expression in orange fruits. In contrast to the view that animal dispersers of seeds from fleshy fruit compete with microbes for food resources (Janzen, 1977; Cipollini and Levey, 1997), our data indicate that once a fruit has completely developed seeds, it advertises its condition to potential legitimate dispersers by inducing changes promoting the accumulation of specific terpene volatile signals, which also serve as a ripening signal to

specialized insects and microorganisms. In this way, they could indirectly increase seed dispersal by providing a nutritional benefit to vertebrates that eat insect-infested or pathogen-infected fruits (Cazetta et al., 2008). Dispersal could occur when the terpene-rich peel barrier is broken or soften, making the seeds more accessible to terrestrial mammals, or by releasing other volatiles that attract specialized vertebrates. This peel would otherwise be toxic or deterrent for seed-dispersing animals. It has been recently reported that the attraction of birds to heavily insect-infested trees is directly correlated with the emission of several specific terpene compounds (Mäntylä et al., 2008). Basic information on which volatiles attract which foragers and on the range that they are perceived is lacking. Given the high specificity of olfactory communication, it is possible that olfactory-guided seed dispersers and fruit predators use distinct volatiles to locate fruits. As such, olfactory communication might be much more specific than visual communication (Schaefer and Ruxton, 2011).

6.3. Fruit volatiles and insects

Understanding insect-plant volatile interactions is of interest not only from an ecological and evolutionary perspective but also for the development of novel crop protection strategies, for example by engineering or selecting crop plant variants for endogenous volatile-mediated repellency or less-attraction to insect pests.

In an ecological context, insects must be able to monitor ratios and blends of volatiles and also determine whether these emanate from their host plant (Bruce et al., 2005). The insect may recognize the correct blend against a background of physiologically active components that would be constantly emitted by non-host plants. In addition, learning behavior involving central processing can occur when a particular blend of volatiles becomes associated with a more abundant or more rewarding host (Bruce et al., 2005). We have shown here that D-limonene is used by *Ceratitis capitata* males as a necessary attractant to localize citrus fruits. Moreover, certain insects sequester or otherwise acquire host plant compounds and use them as sex pheromones or sex pheromone precursors. Other insects produce or release sex pheromones in response to particular host plant cues. By these means, host plants may be used by insects to regulate or mediate sexual communication, as in the case of *Ceratitis capitata* and citrus fruits (Landolt and Phillips, 1997). In preliminary studies in a field experiment that is being performed in a plot in Vila-real (Castellón) with D-limonene synthase AS and empty vector (EV) transgenic orange trees, we have found that EV control fruits are additionally more attractive to *C. capitata* females for oviposition, as these fruits presented much more medfly larvae than did AS fruits. Furthermore, medfly parasitoids were only found in control fruits (Rodríguez *et al*., unpublished results). This indicated that D-limonene was also used by medfly females to select oranges as oviposition substrates.

6.4. Fruit volatiles and microorganisms

The lack of basic knowledge on citrus molecular genetics and on the biology of citrus pathogens plus the difficulties of working with a genetically complex tree species make very difficult to produce new improved transgenic genotypes of real agricultural importance (Peña et al., 2008). There are citrus regions seriously threatened by diseases caused by fungi and bacteria. Looking for resistance against bacteria such as *Candidatus* Liberibacter spp. or *Xanthomonas citri subsp. citri* or against fungi such as *Phyllosticta citricarpa* or *Penicillium digitatum* is a major priority for the most important citrus industries in the world, including those from China, Brazil and U.S.A. Probably, the only opportunity for getting durable and sustainable resistance against these diseases could come from the incorporation of transgenes able to efficiently protect the most relevant and widely known scion and rootstock genotypes grown in these areas against bacteria/fungi and/or their vectors (Peña et al., 2008).

We have shown here that the down-regulation of D-limonene in orange peels leads to fruit resistance against *Penicillium digitatum* and *Xanthomonas citri* subsp*. citri* under laboratory conditions. These results encouraged us to study possible resistance of AS fruits to Citrus black spot (CBS), a fungal disease caused by *Phyllosticta citricarpa*, which has a high economic impact on citriculture, depreciating the commercial value of fruit intended for the fresh fruit market, reducing crop productivity due to premature fruit fall, and increasing considerably the costs of production. This disease occurs in many areas where citrus is cultivated, including Asia, Australia, South America, Southern Africa, Central America, the Caribbean region and more recently also North America (EPPO, 2009). It is a quarantine disease for the European Union. To investigate the role of D-limonene and related volatile organic compounds in the development of *P. citricarpa* in citrus hosts, AS orange fruits were challenged with this fungus in a P2 laboratory at the IVIA. Preliminary results showed that AS fruits were resistant to this pathogen compared to wild-type and EV control fruits (Rodríguez *et al*., unpublished data). Due to the importance of this disease in Brazil, Fundecitrus (Fundo de defesa da citricultura) has signed an agreement with the IVIA to import, propagate, plant and investigate the performance of the transgenic AS, S and EV trees and their fruits in the field in the states of São Paulo and Paraná. Brazil is an ideal place to perform such field challenge assays because *P. citricarpa* is epidemic in many citrus areas. Transgenic trees are being propagated and we are waiting for the approval of the CTNBio (Comissiao Técnica Nacional de Biosegurança), likely along this year, to establish the trials first in two localities of São Paulo State. Trees will be inoculated using symptomatic fruits to be located in the top of the trees and additionally using infected rotting leaves distributed in the soil. Infection and its symptomathology will be followed periodically by visual inspections.

Food plants and the fungi that colonize their fruiting parts (seeds and fruit) have been co-evolving for millennia. These fungal infections may result in various sorts of deterioration of seeds, ranging from decrease in germinability to complete spoilage. In our experience, the presence of impermeable seed coats in citrus seeds strongly prevents embryo infection and consequent deterioration. Even when the fruits (and their seeds) were completely infected by *P.*

digitatum, protected seeds were able to germinate and produce viable and fully normal plants after sowing them. In cases of severe infection of the seeds, there was a general decrease in the percentage of germination, but most of them were able to germinate at least under controlled greenhouse conditions (more than 50 % of the seeds were still viable). Conversely, peeled seeds were 100% deteriorated after *P. digitatum* infection (Peris *et al*., unpublished results), indicating that the seed coat may function as a barrier to microorganisms (Dalling et al., 2011) and perhaps also to frugivore predators (Paulsen et al., 2013).

6.5. Fruit volatiles in multitrophic interactions

It is thought that vertebrate seed dispersers often reject fruits because of the presence of insects or microbes and/or the unpleasant taste caused by them. Nevertheless, in another scenario, insects can indirectly increase seed dispersal by providing a nutritional benefit to vertebrates that eat insect-infested fruits. As long as insects themselves haven't already killed or deteriorated the seeds, the presence of insects in fruits could also increase plant fitness by reducing seed predation (due to bitter tasting toxins) (Sallabanks and Courtney, 1992). More common is the finding that insect presence in seeds increases vertebrate seed predation. Several studies have found vertebrate seed predators selectively choosing seeds that contain insects, presumably for the same reasons that some vertebrate seed dispersers prefer insect-infested fruits (Sallabanks and Courtney, 1992).

Insects can promote fruit abortion by introducing fungi that cause plants to abort fruits. The introduction of microbes by insects may directly affect fruit and seed development. Moreover, microorganism may enter the fruit through wounds performed in the peel without the intervention of insects. Bacteria and fungi introduced into fruits can themselves make fruit pulps distasteful. However, by introducing microbes, insects may indirectly increase seed predation or dispersal if vertebrate seed predators/dispersers prefer microbe-infested mature fruits. Thus, microorganisms in the fruit exocarp or pulp can be either detrimental, neutral, or possibly even beneficial to plant fitness. The characteristic smell of rotting fruits attracts a large suite of animals including seed dispersers (Schaefer and Ruxton, 2011). In the case of citrus, a series of field experiments were performed to address whether fruit infected by fungi or bacteria is rejected or not by vertebrate frugivores, including small mammals and birds.

In a field trial at the IVIA, 150 mature fruits detached from clementine mandarin trees were numbered and arranged in the soil in random blocks to allow different frugivore agents to freely act on them. Approximately 46% of the fruits were attacked only by fungi or bacteria and none of these fruits was subsequently attacked by any other frugivore agent. When mammals or birds were implicated in the action on the detached fruits, 27% of the fruits eaten by mammals or birds had been previously attacked by fungi or bacteria while only 10% of the intact fruits were eaten by mammals or birds without previous action of microbes. This experiment was repeated with 180 numbered fruits in the clementine trees leaving them to freely fall down, obtaining similar results (41% of fruits attacked only by fungi or bacteria; 18% of fruits eaten by mammals or birds that had been previously attacked by fungi or bacteria; and 9.3% of fruits

eaten by mammals or birds without previous action of microbes). These results indicate that vertebrate frugivores did not show a preference for intact fruit but they ate either infected or uninfected fruits equally. We predict that similar cases will be found more commonly in the interactions among fleshy fruits, microorganisms and seed dispersers or predators in nature. The role of fruit volatiles in attracting animals in general and seed dispersers in particular is not well known. The various volatiles and gaseous plant hormones whose concentrations change during ripening may be informative cues or even signals of fruit quality to attract frugivores for seed dispersal.

6.6. Metabolic engineering of volatiles in relation to fruit flavor

Relevant information on fruit flavor compounds and their biosynthesis has been generated in recent years by using molecular and biochemical approaches. Albeit limited, the information gathered up to date regarding genes and metabolic pathways that generate fruit flavors has been crucial for the manipulation of aroma compounds in crops. Genes that directly influence fruit flavor formation have been identified for the main metabolic pathways in tomato, strawberry and melon fruits (Aharoni and Lewinsohn, 2010). Consequently, it has been possible to redirect and enhance the biosynthesis of fruit flavor compounds through modern biotechnological tools (Davidovich-Rikanati et al., 2007). Apart from this almost unexplored area, numerous attempts have been made to modify volatile content and emission formation in whole plants (basically *Arabidopsis*) via metabolic engineering to alter interactions with insect pests and their predators (Lücker et al., 2006; Aharoni et al., 2005).

The content and emission of volatiles in fruits, vegetables and herbs may have important influence on the performance of many plant species. As extensive breeding programs have been undertaken to maximize certain attributes of foodstuff – for example, overall yield (i.e. size), total solids, sugar content, or pigmentation – less attention have been devoted to enhancing or even maintaining volatile production. A result of this is that many current cultivars of domesticated plant species produce less volatiles than their wild relatives or earlier cultivars (Dudareva and Pichersky, 2008). The novel biotechnological advances can be used to restore the “lost” aromas of fruits.

Since markets of developed countries demand fresh fruit of increasing quality and with less agrochemical treatments, and also better and richer juices, more research is needed focused in understanding the genetic control of metabolic pathways regulating terpene/essential oil biosynthesis with the aim of improving fruit aroma. Apart from the sensations perceived by our taste such as sweetness, bitterness, sourness and saltiness, the unique “flavors” associated with food are contributed by aromas perceived through smell. Aroma is the description of sensations induced by volatile compounds via the olfactory bulb in the nose cavity and processed in the brain; it involves thousands of different volatiles providing various kinds of floral, fruity, minty, woody, mushroom, etc. sensations (Schwab et al., 2008). Although some aromas are prominently defined by a single molecule, most aromas consist of complex mixtures

of volatiles. The contribution of each component to the overall aroma of our foods differs according to their perception thresholds, synergism with, and masking of, other components.

Citrus fruits are appreciated for their organoleptic quality and health benefits, and each citrus type, such as orange, grapefruit, lemon or mandarin, has a unique and special flavor. The aroma of oranges is derived from a mixture of different volatiles, including alcohols, aldehydes, ketones, terpenes/hydrocarbons and esters. Most of the knowledge gained so far on aroma of the citrus fruit comes from studies with oranges, grapefruit and mandarins, mainly from analyzing the flavor of pasteurized juices rather than from fresh fruits or juices (Buettner and Schieberle, 2001; Perez-Cacho and Rouseff, 2008a; Perez-Cacho and Rouseff, 2008b). Recent studies have proposed that the pleasant aroma of orange juice is formed by a mixture of at least 36 different volatiles that include 14 aldehydes, 7 esters, 6 alcohols, 5 terpenes and 4 ketones (Perez-Cacho and Rouseff, 2008b).

Human perception of aroma is determined by sensory evaluation test panels. In order to assess the effect of modulating the D-limonene content (and related terpenes) in AS and S vs. EV orange fruits, we have conducted a sensory panel of orange juice aromas consisting of more than sixty untrained panelists in two consecutive years. Panelists were asked to select for aroma intensity and the preferred sample from two juices (the AS or S juice vs. the control juice). Panelists were instructed to inhale the vapors from a pair of vials and to choose the sample that they found more intense and then the preferred one. In these tests, panelists did not find differences in the intensity of AS vs. EV juices, neither they showed preference for any of them. However, when they were offered S vs. EV control juices, panelists found a “great” intensity in the juice aroma of S fruits and preferred it over the control juice (Rodríguez et al., unpublished results).

In another test, panelists were offered to select the preferred juice aroma among the three samples (AS, S and EV control). In this case, sensory evaluation tests comparing the three juices and rating them on a 9-point hedonic scale were performed. No significant differences were found between control and AS juices whereas panelists preferred the S over the control juice with a P=0.0001 (Rodríguez et al., unpublished results).

It is important to note that genetic engineering of fruit flavor is not restricted to the incorporation of new flavors or enhancing existing ones but also includes the removal of undesirable metabolites that generate “off-flavors”.

6.7. Plant volatile research

Transgenic plants with modified terpenoid profile can provide a valuable tool for studying the biosynthesis and regulation of these compounds and their ecological functions in plant-environment interactions (Yu and Utsumi, 2009). In general, the bioengineering of volatiles can be achieved either through the modification of existing pathways (e.g., upregulation of one or more steps or redirection of flux to a desirable compound by blockage of competing pathways) or by the introduction of new gene(s) or branchways normally not found in the host

plant. In contrast to metabolic engineering of volatiles from vegetative tissues to study the effect of altered emission profiles on insect and insect predator behavior, the impact of changes in floral and fruit aromas on insect or animal attraction has been scarcely investigated. Metabolic engineering often yield unpredictable results, highlighting our lack of comprehensive understanding of plant metabolic networks and their regulation, including our rudimentary knowledge concerning network organization, subcellular localization of enzymes involved, competing pathways, metabolic channeling, flux-controlling steps and possible feedback control. The widely reported negative effects of terpenoid engineering on plant growth and development could be overcome by better spatial and temporal control of transgene expression.

Recent use of large-scale sequencing, transcriptomic and metabolomic approaches in plant volatile research resulted in isolation of new biosynthetic genes and contributed to increase our understanding of the regulatory properties of the pathways involved in volatile formation (Aharoni and Lewinsohn, 2010). Moreover, the role of volatile profiles in host responses to pathogen infection and pest damage are essential to clarify the mechanisms of plant-pest-microbe interactions and to develop novel strategies for therapy. Furthermore, transgenic plants and fruits with altered volatile profiles and showing different responses to pathogens and pests may be used to elucidate volatile-mediated resistance mechanisms, as we have shown in this work.

The booming of functional genomics technologies that increase the resolution and coverage of genome, transcriptome, proteome, metabolome, as well as interactome analysis offers unprecedent ways for listing all the possible players involved in the regulation of plant volatile metabolism. The development of functional genomics is also a direct consequence of recent and forthcoming publication of the genome sequences of several fruit trees, leading to new perspectives in genomic research and in functional analysis of genes putatively involved in the production of volatiles, further providing insights into their regulation.

The challenge of producing safe agricultural products will remain at the forefront of scientific endeavors. Development of agricultural goods ensuring food safety, enhanced nutritional content, and with good agronomic qualities are requisites for the agriculture of the XXIth century, and research on plant-produced volatiles can play an important role to afford them. Despite the complexity of plant volatile production and their interaction with the environment, an integrated approach of laboratory- and field-based experiments can help delineate the intricate relationships of emitted volatiles as chemical cues for other plants, insects, microbes and vertebrates. Moreover, the use of mutants or transgenic plants (sense, antisense or RNAi-silenced) could help to determine the key compounds involved in such multitrophic interactions.

Little is known about the function of individual compounds emitted by plants in their interactions with the biotic environment in nature. The signaling cascades involved in perception of volatile compounds by the receptors and/or induction of their biosynthesis in the emitters are still unclear, and the evolutionary and ecological relevance of plant volatile emission and perception awaits further investigation. The identification of key compounds involved in volatile-

induced plant defenses against microorganisms, as well as in insect attraction and their effects on insect behavior in field studies, will greatly contribute to target selection for detailed studies of such form of communication. Major challenges lay in the development of novel model systems and in the ecological realism of experimental settings since very few studies performed to date were carried out under natural conditions. However, both approaches have limitations when afforded separately: the laboratory experiment (e.g. ex situ or in vitro) does not truly reflect the system as a whole and removes the sample out of its contextual environment, whereas the field experiment (e.g., in situ or ambient) may not yield detailed information, incorporates many variables, or may simply be impractical to study (Beck, 2012).

The knowledge derived from this work could be used for the development of increased pest repellence and disease resistance, improved processing of fruit products and enhanced organoleptic properties of fruit crops.

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7. CONCLUSIONS

7. Conclusions

1. It has been proven that it is feasible to modulate the accumulation of D-limonene and related terpenes in the peel of sweet orange fruits by genetic engineering. Either up- or down-regulation of D-limonene synthase in orange peels did not lead to adverse pleiotropic alterations in fruit development and biochemistry, neither in juice/pulp quality. Down-regulation of D-limonene in AS lines led to the accumulation of less monoterpene and sesquiterpene hydrocarbons, while increased the production of monoterpene alcohols and some esters.

2. This metabolic modification represents a promising method for developing broad-spectrum resistance to pests. Males of an important citrus pest, the medfly, were more attracted to EV control than to AS fruits suggesting that less D-limonene accumulation in AS fruits might decrease the mating success of medfly in the field and, consequently, the oviposition response of the females.

3. This metabolic modification represents a promising method for developing broad-spectrum resistance to pathogens in fleshy fruits. AS fruits were more resistant to fungal and bacterial diseases when inoculated either with *P.digitatum* or *X.citri* subsp. *citri*, respectively. The use of metabolic engineering of volatile organic compounds to induce resistance against biotic agents represents an alternative technology to the use of expensive and highly toxic fungicides, bactericides, and pesticides.

4. A large-scale gene expression analysis showed that defense cascades were activated in AS orange fruits while genes related to secondary metabolism were down-regulated. The results indicated that the activation of MAPK cascades together with the upregulation of TFs related to defense responses in plants and the accumulation of the sweet orange homologs of *AOS* and *MYC2* after wounding in AS fruits, could contribute to the strong resistance response against necrotrophic pathogens through the activation of the JA metabolism. Results suggested that induction of defense-related genes was able to prime fruits for JA-mediated defense upon challenge inoculation.

5. Upon maturation or ripening, terpene metabolism is usually altered in many economically important fruit crops. Our results suggest that altering the levels of certain abundant terpenes in the peel of mature fruits through genetic engineering of crop-specific terpene synthase/s may be used as a strategy to generate resistance to pests and pathogens to other fruit crops.

6. It seems that S lines could accumulate D-limonene to maximum levels that would not compromise cell and plant viability. Oil glands synthesize and accumulate near-saturating concentrations of D-limonene and overexpression of limonene synthase was not able to increase further the large amount of D-limonene occurring in oil glands.

7. The effects of D-limonene downregulation in the flavedo of AS orange fruits on medfly attraction and fungal and bacterial infections strongly indicate that the high accumulation of this monoterpene in the peel of citrus EV control and S fruits is used for informing to specialized insects and microorganisms, and also likely to potential legitimate dispersers, that the fruit has completely developed seeds and it is ready for consumption.

8. Transgenic plants with modified terpenoid production could contribute to fundamental studies aimed at understanding their function in fruit-frugivore relationships.