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

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Footnotes:

Abstract

Terpenoid volatiles are isoprene compounds emitted by plants that act as signals for communication with the environment. In addition to their function in repelling herbivores and attracting carnivorous predators in green tissues, the primary function of terpenoid volatiles released from fruits is the attraction of seed-dispersing animals. Orange fruit mainly 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*. Metabolic diversion due to the manipulation of monoterpene biosynthesis could have induced changes in fruit morphology and/or biochemistry that might explain this response; however, we demonstrate here that the downregulation of D-limonene in orange peels did not lead to pleiotropic alterations in fruit development. A global gene expression analysis of transgenic fruits expressing antisense vs. empty vector constructs revealed that the downregulation of D-limonene upregulates genes involved in the innate immunity response. This coincided with an increase in the accumulation of a jasmonate precursor in orange peels, which may explain the resistance to necrotrophic fungi observed in antisense fruits. In nature, D-limonene levels increase in the orange fruit once the seeds are fully viable. The inverse correlation between the increase in D-limonene content and the decrease in jasmonate-mediated defense response suggests that D-limonene acts as an attractant for microorganisms that are likely to be involved in facilitating access to the pulp for seed dispersal frugivores.

Introduction

Plants are sessile organisms that produce and emit a vast array of volatile organic compounds (VOCs) to allow communication both between distal parts of the same plant and with other plants in the surrounding biotic environment. 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). They are also known as isoprenoids because 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. This process gives 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 then be stored in specific organs or tissues where they would be most effective in defense responses, such as in 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). It has been well documented through genetic engineering experiments 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, thus protecting 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 could act synergistically, as in conifer resin, for simultaneous protection against pests and pathogens (Phillips and Croteau, 1999). Although fatty acid degradation products (such as methyl jasmonate) and phenylpropanoids (such as methyl salicylate) 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 defense against microorganisms in planta.

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 (Goff and Klee, 2006; Rodríguez, et al., 2011b).

Sweet orange (*Citrus sinensis* (L) Osb.) is a perennial tree species that is exposed to recurrent biotic and abiotic challenges during 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 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; this is 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.

To examine the biological role of D-limonene, we manipulated oil gland chemistry by 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 *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* was restored after AS fruits were supplemented with D-limonene but not other monoterpene alcohols, our results indicated 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 due to fungal decay during fruit storage at ambient temperatures. *P. digitatum* is considered to be a specialist pathogen of citrus fruits that efficiently infects the peel through injuries where 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 make searching for alternatives, such as the generation of citrus trees with fruits that are genetically resistant to the pathogen, desirable.

In this work, to better understand the mechanism underlying the constitutive resistance to *P. digitatum* that was observed in AS orange fruits, we have analyzed the pattern of fruit growth, the morphological and biochemical developmental characteristics, performed global gene expression analysis using a 20K citrus microarray, and examined the possible involvement of hormone signals, such as those that may be elicited by salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) in the fruit peel in response to the fungus. We report here that 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 jasmonates, establishing for the first time a correlation between volatile terpene content and decline of JA-mediated defense responses in a fleshy fruit, thus facilitating necrotrophic 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 the flavedo is more resistant to *P. digitatum* infection than the albedo (inner white area without oil glands), the latter being the entrance for fungal colonization (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 was partially peeled off and the remaining fruit was left on the bench at room temperature to facilitate germination of ubiquitous fungal spores. While EV control fruits were infected by several fungi by the 3rd day after peeling, samples from AS3 and AS7 antisense lines started to become infected to a much lower extent by the 7th 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 (Fig. 1 for Navelina; results not shown for Pineapple). During the first week after peeling, fungal infection was exclusively restricted to wounded flavedo areas (Fig. 1 A and B), and resistance was linked to very low D-limonene levels in the oil glands of AS transformants (Fig. 1, C and D). 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 main inducers of fungal germination and growth.

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 orange fruit flavedo (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 Navelina and Pineapple oranges were estimated. As shown in Fig. 2A and B, as well as Supplemental Fig. S2A and B, oil glands were comparable in number and diameter in AS and EV fruits, illustrating that oil glands increase in size as fruit grows. Moreover, peel thickness was also similar between AS and EV samples at the different developmental stages analyzed (Fig. 2C and Supplemental Fig. S2C).

We then tested whether 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 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). Degreening of 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 Pineapple orange (Supplemental Fig. S4). In addition to this, the percentage of individual xanthophylls and carotenes remained basically at 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 decreases in monoterpene accumulation; thus, other factors must be responsible for the increased disease resistance found in the peel of D-limonene antisense plants.

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

With the aim of understanding 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 two EV control lines. We found differential gene expression in the AS3 line, with 82.9% of genes upregulated and 17.1% downregulated (Supplemental Table SIA). Among the genes deregulated in the AS7 line, 93% were upregulated while only 7% were downregulated (Supplemental Table SIB). Common genes from both AS lines showing at least a 1.6-fold expression change vs. EV lines were identified as differentially expressed for further analysis (Table I). To elucidate the key processes that were altered in AS fruits, functional enrichment categories for the full set of differentially expressed genes were searched (Fig. 4). Based on gene ontology (GO) terms, genes downregulated in the flavedo of AS fruits were mainly involved in biological processes associated with secondary metabolism (Fig. 4; Supplemental Fig. S6). Antisense downregulation of the D-limonene synthase gene was found to reduce the transcription of nine genes; four of these genes encode enzymes that are potentially 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 times in AS vs. EV samples in the microarray analysis (Fig. 5A) and 5 and 8 times in qRT-PCR analyses (Fig. 6). Although a homolog of a *CHS* (Chalcone synthase) gene was upregulated in both AS lines, an *OMT1* homolog was clearly downregulated (Fig. 5A; Table I).

Conversely, the biological processes that were over-represented in the AS flavedo compared with the EV controls were mainly associated with defense responses against biotic and abiotic stresses (Fig. 4; Supplemental Fig. S6). At least half of the 59 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 (Table I). Several genes coding for calcium-binding proteins, including at least one calmodulin, were also upregulated in the AS lines. This calmodulin-like protein gene was confirmed to be upregulated in further qRT-PCR analysis (Fig. 6). Calcium signals are sensed by calcium-dependent protein kinases (CDPK). Together with mitogen-activating protein kinases (MAPK), these kinases 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, were found to be only slightly upregulated (more than 1.5-fold) in one or both of the AS lines (Supplemental Tables SIA and SIB); the citrus homolog of the early-response *YLS9* gene (also known as *NHL10*) (Zipfel, et al., 2004) was found to be upregulated about two times in AS3 and AS7 plants (Table I). It has also been shown that several CPKs strongly induce *YLS9* (Boudsocq, et al., 2010). Our results suggest 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 also found to be strongly induced in both AS lines (Fig. 5B; Table I).

The target genes of these signaling cascades included Zn finger (CCCH-type), ERF/AP2 and MYB family transcription factors, which were found to be upregulated in both AS lines (Fig. 5B; Table I; Supplemental Tables SIA and SIB). Citrus homologs of MYB6 and ATERF6 were confirmed to be constitutively upregulated in the flavedo of both AS lines by qRT-PCR analyses (Fig. 6). A putative WRKY6 transcription factor was found to be induced more than four-times, but this was only observed in line AS7 (Supplemental Table SIB). Moreover, several "no apical meristem (NAC domain)" genes were upregulated about two-fold in both lines (Supplemental Tables SIA and SIB).

Additionally, it is noteworthy that the expression of phenylpropanoid biosynthetic genes, such as a putative *CHS* (Chalcone synthase) and *PAL1* (Phenylalanine ammonia-lyase 1), and other defense related genes, such as *LTP1* (nonspecific lipid transfer protein 1) and *NBS-LRR* (nucleotide-binding site–leucine-rich repeat) genes, were highly induced in the AS lines. *LTP1* and *PAL1* were confirmed to be upregulated by 4- to 7-fold in subsequent qRT-PCR analyses (Fig. 6), suggesting their involvement in the induction of disease resistance responses in AS citrus fruits (Table I; Fig. 5B; Fig. 6). Regarding cell wall organization and biogenesis, several homologs of cellulose synthase and other xyloglucan endotransglycosylase genes were found to also be upregulated in both AS3 and AS7 fruits (Supplemental Tables SIA and SIB). Genes for other enzymes putatively involved in starch biosynthesis or electron transport were upregulated as well (Supplemental Tables SIA and SIB; Table I). Overall, these results indicated that terpene downregulation activates constitutive defense responses in the fruit flavedo.

Downregulation of D-limonene and related terpenes triggered accumulation of JA in orange peels upon wounding

Disease resistance in plants is also regulated by several phytohormones including SA, JA and ethylene (ET) (Glazebrook, 2005). Microarray gene expression analysis of the differentially expressed genes did not reveal any clear biological process related to SA or JA metabolism in transgenic fruits. However, negative evidence is not surprising when working with standard tools that are not designed for poorly annotated organisms (Romero, et al., 2012).

JA, 12-oxo-phytodienoic acid (OPDA), SA and ABA levels were quantified in the flavedo of AS and EV lines before and after wounding (which is required for green mold infection) to assess whether these defense signaling molecules were activated or repressed by monoterpene downregulation. Whereas low levels of JA were observed in AS fruits before wounding (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 fresh weight in the AS3 line and from 15 to 323 ng/g fresh weight in the AS7 line) (Fig 7A). A small decrease in JA levels was observed in the EV control samples before and after wounding (from 252 to 170 ng/g fresh weight), indicating that JA-mediated responses have a minimal effect in these fruits. Moreover, the EV controls accumulated much less JA than the AS samples after wounding (Fig 7A), indicating that wound-induced JA-signaling responses were enhanced in AS fruits. These results led us to analyze another important member of the octadecanoid pathway, OPDA. OPDA levels were about two times higher in AS samples compared to EV samples before wounding, but they dramatically decreased 2 h after wounding in both AS (from 1515 to 284 ng/g FW) and EV control lines (from 986 to 262 ng/g FW) (Fig. 7B). As OPDA is an important precursor of JA (Weber, et al., 1997), our results suggested that the downregulation of D-limonene and related terpenes in AS fruits induced the basal accumulation of OPDA; upon wounding, OPDA would be converted to JA, which is the most likely *in vivo* regulator of resistance against necrotrophic fungi in AS orange fruits. Conversely, high D-limonene contents in EV fruits could 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 wounding; however, SA reached much higher levels in EV samples (9-fold) compared to AS samples (6-fold in AS3) (Fig. 7C). The attenuated increases of SA in AS samples observed upon wounding 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). In spite of the general induction of defense responses against abiotic stress that was observed in AS lines using transcriptomic analysis (Fig. 4B), ABA levels were slightly reduced in AS samples when compared with EV controls, although they were strongly decreased in all samples after wounding (Fig. 7D). These results could be better explained by 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).

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Discussion

Terpenoids have been shown to be ecologically significant (Degenhardt, et al., 2003) due to various properties such as volatility, flavor/aroma, and toxicity; thus, they play important roles in plant defense, plant-to-plant communication, and pollinator attraction (Pichersky and Gershenzon, 2002). Transgenic plants with modified terpenoid production could contribute to fundamental studies aimed at understanding their function in plant/environment relationships (Aharoni, et al., 2005). The flavedo of orange fruits represents a valuable model for examining the interaction of terpene volatiles with the biotic environment because one specific monoterpene, D-limonene, accounts for more than 95% of the total terpenes in flavedo oil glands. Transgenic downregulation of a D-limonene synthase gene has led to a general and specific repression of monoterpene and sesquiterpene synthase gene expression and to a dramatic reduction in the levels of D-limonene and related terpenes in the orange peel. Consequently, fruits were more resistant to *P. digitatum*, other bacterial pathogens, and to a citrus pest (Rodríguez, et al., 2011a; Rodríguez, et al., 2011b). This biotic resistance was associated with reduced D-limonene concentration, as its supplementation to AS transgenic fruits restored disease susceptibility to the same levels as EV controls (Rodríguez, et al., 2011b).

However, other prominent isoprenoid compounds with defensive functions may have also been altered in transgenic orange peels. The overexpression and downregulation of a 1-deoxy-D-xylulose-5-phosphate synthase gene (*DXS*) in *Arabidopsis thaliana* affected the accumulation levels of various isoprenoids. Analysis of several transgenic lines showed that plants overexpressing *DXS* had increased levels of isoprenoids such as chlorophylls, tocopherols, carotenoids, ABA, and GA, and plants with suppressed levels of *DXS* had decreased levels of all of these compounds (Estevez, et al., 2001). Moreover, transgenic *Arabidopsis* plants expressing a linalool/nerolidol synthase from strawberry (*FaNES1*) displayed a growth reduction compared with wild-type plants grown under identical greenhouse conditions (Aharoni, et al., 2003). One of the possible explanations for the growth retardation phenotype in the transgenic lines was the depletion of the precursor pool in plastids, which may lead to reductions in the levels of essential isoprenoids. These include growth regulators and other vital components such as carotenoids, chlorophyll, and quinones. Similarly, another study has shown that transgenic tobacco plants engineered for high-level patchoulol biosynthesis via the MEP (plastidic) pathway were shorter in stature, exhibited lower leaf chlorosis, had lower pollen levels and less viable pollen along with reduced corolla pigmentation when compared to controls (Wu, et al., 2006). In contrast, transgenic orange fruits with strong D-limonene synthase downregulation did not show apparent growth retardation or changes in chlorophyll/carotenoid levels when compared to controls. Oil gland and peel morphology were also not affected by the drastically reduced D-limonene levels in AS peels. These results indicate that, in citrus fruit, neither a reduction in D-limonene nor its metabolic consequences cause morphological

alterations or other pleiotropic effects that may affect fruit resistance to fungal pathogens in transgenic plants.

Microarray-mediated transcriptional profiling has proven to be successful for identifying constitutively activated defense signaling pathways in the flavedo of AS citrus fruit. 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 would phosphorylate transcription factors belonging to the MYB, WRKY, ERF/AP2, NAC domain, and Zn finger (CCCH-type) families, which are likely to participate in defense (Wang, et al., 2008; Guo, et al., 2009; Birkenbihl and Somssich, 2011). The concurrent upregulation of genes for these transcription factor families and at least one disease resistance/LRR family protein gene further supports the close association between reduced D-limonene levels and activation of prominent defense responses (Chen, et al., 2002). Transcription factors would subsequently activate other genes coding for proteins that are either antimicrobial themselves or that catalyze the production of antimicrobial compounds such as LTP1, which is predicted to be a member of the PR-14 pathogenesis-related protein family. Concerning their role in plant defense, 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). 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) used a 12K citrus cDNA microarray to study the transcriptional changes in elicited fruits. Elicitation consisted of inoculation with the fungus followed by a curing treatment one day later (37 ºC for 3 days with high relative humidity) 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 treatment would likely cause the upregulation of many stress-response 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 *PAL1*, together with *CHS*, was constitutively activated in the flavedo of D-limonene antisense plants. 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. Regarding the ET signaling pathway, we did not observe *ACO* upregulation in AS and EV orange fruits, although we acknowledge the possibility that ET signaling could be activated as a late response.

JA is a key hormone involved in direct and indirect defense against herbivores. Application of JA or related members of the octadecanoid pathway to plant leaves mediates the induction of volatiles, increases the level of certain toxins and upregulates defense gene

expression (Baldwin, 1998; Dicke, et al., 1999; Thaler, 1999). Many of the volatiles induced in response to mechanical damage or herbivory are fatty acid degradation products that share precursors with jasmonates. It has also been shown that JA treatment induced the biosynthesis of the homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (Koch, et al., 1999). In forest gymnosperm trees, jasmonate treatments induced resin duct formation and the upregulation of terpene synthase genes as well as terpenoid accumulation; these terpenoids were similar to those induced by weevil feeding, although they had less complex profiles (Martin, et al., 2002; Miller, et al., 2005). Moreover, in response to herbivore damage, lima bean plants emit specific homo- and monoterpenoid volatiles (not induced by wounding) that activate the JA signaling pathway and multiple defense genes not only in the damaged plant but also in neighboring plants (Arimura, et al., 2000). It has also been shown that, conversely, jasmonate-deficient plants have reduced direct and indirect defenses against herbivores (Thaler, et al., 2002; van Poecke and Dicke, 2002). Although there are results suggesting the involvement of JA-related volatiles in disease protection (Croft, et al., 1993), to our knowledge, the relationship between terpenoids and the activation of JA-mediated defenses against pathogens has not been previously reported. The downregulation of a D-limonene synthase in orange flavedo triggers innate immunity defense cascades linked to the activation of the jasmonate signaling pathway and consequent strong resistance to necrotrophic 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, another group of secondary metabolites previously identified as important volatiles involved in the avoidance of herbivory damage in Brassicaceae, are required for the plant defense response against fungal and bacterial pathogens in leaves; specifically, they 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).

The relative content of jasmonate and other members of its biosynthetic pathway, mainly JA and OPDA, is dynamic and changes over time during development and under stress depending on the plant type; this allows the cell to effectively fine-tune its gene expression (Weber, et al., 1997). Moreover, both OPDA and JA are biologically active and both are defense response regulators (Stintzi, et al., 2001). In AS orange flavedo, OPDA constitutively accumulates at high levels while JA is almost undetectable; upon wounding, OPDA levels strongly decrease and JA levels increase. Because AS fruits exhibit broad resistance to different pathogens (Rodríguez, et al., 2011b); this work) that either require or do not require wounding to establish infections, it may also be possible that JA and OPDA would be active against different pathogens and/or under distinct plant-pathogen 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 the mature flavedo 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 peels. 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 Navelina and Pineapple sweet orange plants (*Citrus sinensis* L. Osbeck) at different developmental stages (August and December; see Supplemental Fig. 1) were harvested over two 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 equators 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) wounding 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 over two consecutive fruiting seasons.

Fungal assays.

Ten fruits were used for each experiment. Fruits were harvested monthly during a five-month period for two consecutive years. Fruits were partially peeled and put into plastic trays for

natural infection by fungi. Observations were made daily for the appearance and progress of symptoms. Samples placed on slides for microscopic identification were obtained from fungi-infected fruits.

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 Figure 2 and Supplemental Fig. S3 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 (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 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 six 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", which covers GO categories such as 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 and sesquiterpenoid 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*, MYB44 transcription factor; *PAL1*, Phenylalanine ammonia-lyase 1; *LTP1*, Nonspecific lipid transfer protein 1; and *ERF*, Ethylene response factor (ATERF-6).

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. The primers were designed based on the corresponding sequences available in the database of the CFGP (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB). 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. 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. 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 independent lines (transgenic AS and EV control). 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 d6-IAA, d6-ABA, d6-SA, d5-OPDA 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).

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