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Resistance to pathogens in terpene down-regulated orange fruits inversely correlates with the accumulation of D-limonene in peel oil glands

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Volatile organic compounds (VOCs) are secondary metabolites acting as a language for the communication of plants with the environment. In orange fruits, the monoterpene D-limonene accumulates at very high levels in oil glands from the peel. Drastic down-regulation of D-limonene synthase gene expression in the peel of transgenic oranges harboring a D-limonene synthase transgene in antisense (AS) configuration altered the monoterpene profile in oil glands, mainly resulting in reduced accumulation of D-limonene. This led to fruit resistance against *Penicillium digitatum* (Pd), *Xanthomonas citri* subsp. *citri* (Xcc) and other specialized pathogens. Here, we analyze resistance to pathogens in independent AS and empty vector (EV) lines, which have low, medium or high D-limonene concentrations and show that the level of resistance is inversely related to the accumulation of D-limonene in orange peels, thus explaining the need of high D-limonene accumulation in mature oranges in nature for the efficient attraction of specialized microorganism frugivores.

Higher plants produce a wide diversity of chemical compounds, traditionally known as secondary metabolites; many of them are volatiles that defend them against herbivores and pathogens and influence the feeding behavior of pollinators, seed dispersers, and herbivore predators.¹⁻⁵

These secondary metabolites, including terpenoids, offer great potential for biotechnological applications, mainly with the aim of achieving resistance to pest and pathogens in crops. An improvement of our knowledge beyond general phytochemical cataloging of these compounds is needed, by performing specific experiments raised to identify their mode of action within the plant and on plant interactions with other organisms.⁶

Plants with either down- or up-regulated volatile isoprenoid synthesis are excellent tools to dissect the biological role of specific plant VOCs. In the same way that the up-regulation of some terpenoids have been associated generally with plant defense properties,⁷⁻¹¹ the downregulation of their production may sometimes reduce the susceptibility to specific pests or microorganisms, as it has been shown in plants such as tobacco or poplar.^{12,13}

In a previous report, we showed that transgenic oranges (*Citrus sinensis* L. Osb. cv. Navelina and Pineapple) accumulating highly reduced levels of the monoterpene D-limonene in the fruit peel became resistant to the bacterium *Xanthomonas citri* subsp *citri* (Xcc), to the fungus *Penicillium digitatum* (Pd) and to other specialized fungi.^{5,14,15} D-limonene synthase down-regulation was associated with constitutive upregulation of genes involved in plant innate immune response to pathogens and to increased accumulation of jasmonic acid upon challenge by the pathogen.⁵

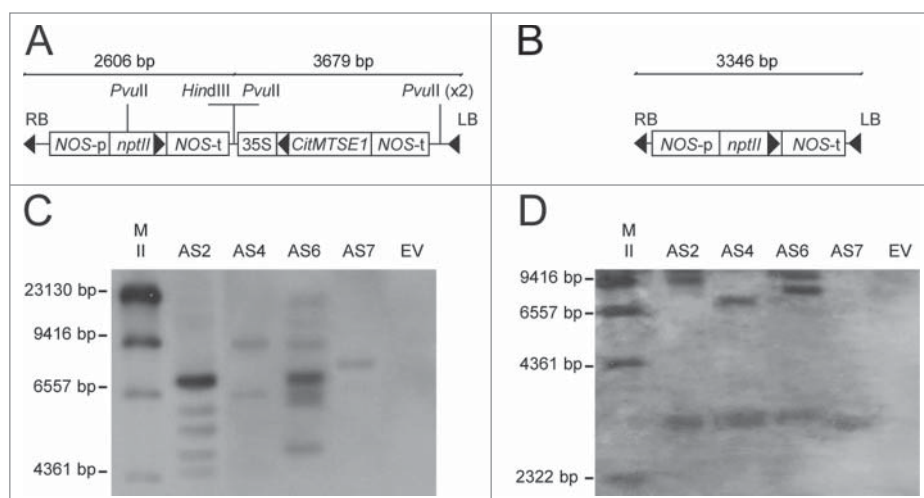


Figure 1. Molecular analysis of DNA isolated from orange leaves of antisense (AS) and empty vector control (EV) Navelina sweet orange transgenic plants. **(A, B)** Map of the T-DNA region of the binary vector used to transform AS **(A)** and EV **(B)** plants. LB, left T-DNA border region; RB, right T-DNA border region; *nptII*, neomycin phosphotransferase II transgene conferring kanamycin resistance, under the control of the nopaline synthase (*NOS*) promoter and terminator regions; *CitMTSE1*, limonene synthase gene in antisense orientation under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and the *NOS* terminator. **(C, D)** Southern blot analysis of independent AS transgenic lines (AS2, AS4 and AS6 and AS7) and the EV control line. The DNA was digested with the enzymes *HindIII* for testing loci number integrations **(C)** or *PvuII* for assessing integrity of the D-limonene transgene **(D)**. The 35S promoter was used as a probe. M: DNA molecular weight marker II from Roche Applied Science.

Therefore, we concluded that D-limonene is required for specialized pathogens to establish infections in mature oranges. To assess whether different D-limonene concentrations in the fruit would affect infection rate and/or symptom intensity, we have now compared the responses to either Pd or Xcc inoculation displayed by transgenic oranges with very low and medium levels of D-limonene accumulation and EV transgenic oranges with very high levels of D-limonene accumulation (comparable to wild-type (WT) oranges).

Overexpression of the full-length cDNA from a D-limonene synthase gene from Satsuma mandarin (*CitMTSE1*) in antisense (AS) configuration in transgenic oranges generally resulted in a drastic reduction in the accumulation of D-limonene and increased amounts of monoterpene alcohols such as nerol, geraniol and citronellol in fruit peels.¹⁴ We have now identified independent transformants AS2, AS4 and AS6 harboring several insertions of the transgene (Fig. 1), which accumulated intermediate levels of these terpene compounds (Fig. S1).

Attempts to alter the concentration of D-limonene in citrus fruits may be counter-productive, as the modification of the flux of isoprenoids by metabolic engineering potentially risks the production of other isoprenoid derivatives and thus normal fruit growth and development. To test this possibility, we measured the number of oil glands and their size in green and mature peel of AS2, AS4, AS6 and EV fruits and found no significant differences between them (Fig. 2). Previously, we found no differences between fruit peel of AS lines with highly reduced levels of D-limonene and EV controls.⁵ Therefore, the decrease of D-limonene concentrations, either high or medium, did not cause morphological alterations or other pleiotropic effects in the AS transgenic fruits.

To compare the effect of medium, low and high (WT) levels of D-limonene accumulation on resistance to specialized pathogens of orange fruits, we chose AS6, AS7 and EV lines, respectively. Volatile terpene contents were analyzed by GC-MS as reported before for mature fruits of the 3 transgenic lines (Fig. 3).¹⁴ Challenge inoculations of Pd and Xcc were

performed as reported before.¹⁴ We observed that AS6 was resistant to both pathogens compared to the EV control line, but less than AS7, both in term of percentage of infected wounds and in symptom intensity (Fig. 4). The experiments were repeated with AS2 and AS4, obtaining results comparable to those of AS6 (data not shown). The resistance phenotype was co-related to the decrease in D-limonene concentration in the transgenic fruits. However, we cannot rule out that changes in the accumulation of other monoterpene compounds in peel oil glands or activation of defense responses derived from such constitutive changes may also contribute to the different levels of resistance observed in AS fruits.

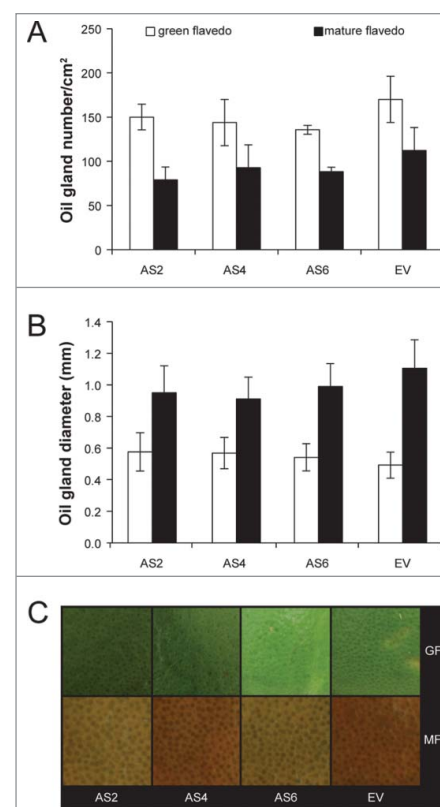


Figure 2. Characteristics of green (70 mm diameter) and mature (90 mm diameter) peels (flavedo) from AS and EV Navelina sweet orange fruits. **(A, B)** Oil gland number and size in green and mature flavedo, respectively. Data represent means \pm SE and are derived from analysis of 10 fruits per plant. No significant differences were found at $P \leq 0.05$ using Fisher's protected LSD test at each stage. **(C)** Magnification of oil glands in 4 cm² peel pieces of AS and EV fruits in green (GF) and mature flavedo (MF).

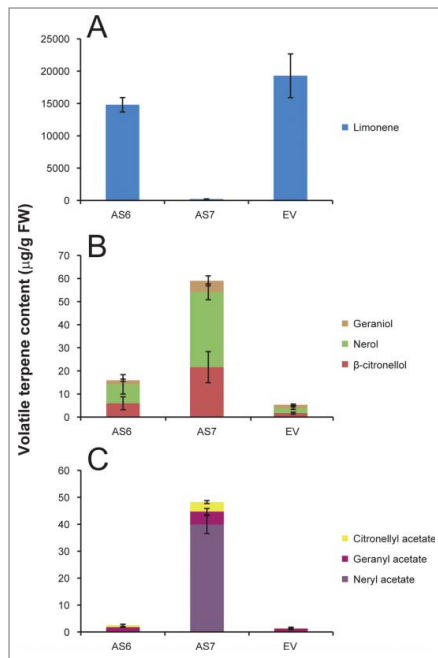


Figure 3. Comparison of the volatile terpene content ($\mu\text{g/g}$ FW) of mature flavedo from AS6, AS7 and EV transgenic fruits. (A) D-limonene; (B) monoterpene alcohols; (C) alcohol derivatives. Data represent means \pm SE and are derived from analysis of at least 5 fruits per plant. Quantification was achieved using calibration curves constructed for each volatile with linear regression equations of commercially available synthetic compounds. The volatiles which were not available commercially were quantified with standard curves obtained from similar structure available compounds.

Terpenoids represent one of the largest and diverse classes of metabolites in the plant kingdom and are involved in many physiological and ecological processes.¹⁶ Plants during their life cycles interact with a vast range of different microbial species. The ways by which plants recognize, coordinate and regulate the exchange of resources and information with the myriads of potentially interacting microbes are not yet completely understood.¹⁷

Metabolic engineering may create great opportunities to study the ecological importance of terpenoids in the interactions of plants with other organisms, including microbes.^{18,19} D-limonene accumulates at huge levels in mature oranges, representing more than 95% of total terpene compounds found in the oil glands from their fruit peel, and it is produced at a very high metabolic cost. We show here that reduced levels of

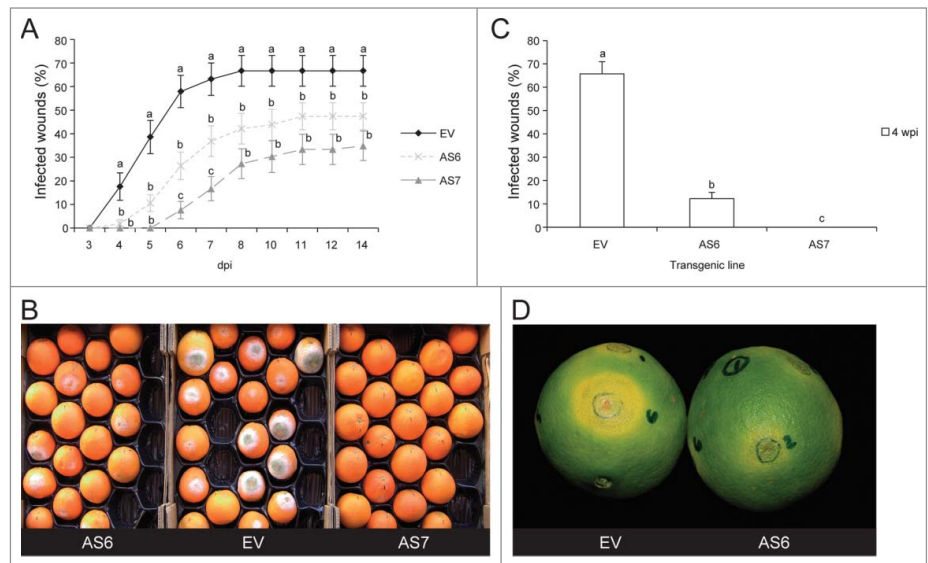


Figure 4. Transgenic expression of *CitMTSE1* in antisense orientation in orange fruits confers different levels of resistance against fungal and bacterial specialized pathogens. (A, B) Progress of the disease caused by the fungus *Penicillium digitatum* in mature orange fruits inoculated with 1×10^4 spores mL^{-1} . (A) Percentage of infected wounds in orange fruits from AS6, AS7 and EV lines. The results are the average \pm SEM ($n \geq 20$). dpi, days postinoculation. (B) AS and EV fruits at 6 dpi. (C, D) Progress of the disease caused by the bacterium *Xanthomonas citri* subsp. *citri* in green orange fruits inoculated with 10^6 CFU mL^{-1} . (C) Percentage of infected wounds in orange fruits from AS6, AS7 and EV lines at 4 weeks postinoculation (wpi). The results are the average \pm SEM ($n \geq 10$). (D) AS6 and EV fruits at 4 wpi. We repeated all experiments several times during 2 consecutive seasons and obtained similar results. For each time point, means with different letter are significantly different according to Fisher's Protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed data.

D-limonene as those found in AS6 fruits are sufficient to generate good levels of resistance against Pd and Xcc, though lower than those found in AS lines with very low concentrations of D-limonene. Therefore, high levels of D-limonene are required for efficient interactions of the fruit with specialized microorganisms, which may be involved in seed dispersal by vertebrate frugivores.

For biotechnological purposes, our results indicate that AS lines with the highest reduction of D-limonene concentrations in fruit peel would be more promising ones for generating field resistance against citrus pathogens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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