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Engineering D-limonene synthase down-regulation in orange fruit induces resistance against the fungus *Phyllosticta citricarpa* through enhanced accumulation of monoterpene alcohols and activation of defence

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SUMMARY

Terpene volatiles play an important role in the interactions between specialized pathogens and fruits. Citrus black spot (CBS), caused by the fungus *Phyllosticta citricarpa*, is associated with crop losses in different citrus-growing areas worldwide. The pathogen may infect the fruit for 20–24 weeks after petal fall, but the typical hard spot symptoms appear when the fruit have almost reached maturity, caused by fungal colonization and the induction of cell lysis around essential oil cavities. D-Limonene represents approximately 95% of the total oil gland content in mature orange fruit. Herein, we investigated whether orange fruit with reduced D-limonene content in peel oil glands via an antisense (AS) approach may affect fruit interaction with *P. citricarpa* relative to empty vector (EV) controls. AS fruit showed enhanced resistance to the fungus relative to EV fruit. Because of the reduced D-limonene content, an over-accumulation of linalool and other monoterpene alcohols was found in AS relative to EV fruit. A global gene expression analysis at 2 h and 8 days after inoculation with *P. citricarpa* revealed the activation of defence responses in AS fruit via the up-regulation of different pathogenesis-related (PR) protein genes, probably as a result of enhanced constitutive accumulation of linalool and other alcohols. When assayed *in vitro* and *in vivo*, monoterpene alcohols at the concentrations present in AS fruit showed strong antifungal activity. We show here that terpene engineering in fruit peels could be a promising method for the development of new strategies to obtain resistance to fruit diseases.

Keywords: CBS, D-limonene, genetic engineering, *Guignardia citricarpa*, linalool, *Phyllosticta citricarpa*.

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INTRODUCTION

Volatile organic compounds (VOCs) are widely emitted from all plant organs, and fruit VOCs are of great importance to plant fitness because of their function in attracting seed dispersers and repellence/resistance to insect herbivores and pathogens (Dudareva and Pichersky, 2008). From a biological viewpoint, the accumulation (or *de novo* formation) of volatile terpenes at a certain stage of fruit maturation may correspond to different functions related to plant defence and/or to the attraction of specialized beneficial organisms, such as parasitoids and/or seed disseminators. In this way, some terpenes may have a defensive role in green fruits, thus protecting the immature seeds (Cascone *et al.*, 2015; Maffei, 2010). When the fruit is fully ripe, the accumulation of other volatiles could be associated with the attraction of seed dispersers and the repellence of seed predators (Leonhardt *et al.*, 2014; Nieuwenhuizen *et al.*, 2010; Rodríguez *et al.*, 2011). In citrus, VOCs accumulate abundantly in specific cavities, such as the oil glands, present in all organs except for the roots. The oil glands of fruit flavedo (peel) are rich in D-limonene, which is the main VOC in all citrus fruits, accounting for 95% of total oil gland content in mature orange fruit.

Citrus black spot (CBS), caused by the fungus *Phyllosticta citricarpa* (McAlpine) van der Aa (teleomorph: *Guignardia citricarpa* Kiely), is the most important fungal disease affecting citrus trees worldwide. The disease causes fruit blemishes and premature fruit drop, leading to yield loss only in highly favourable climates. However, the juice quality remains unaffected (Kotzé, 1981, 2000). CBS has been detected in different citrus-growing areas of Africa, Oceania, Asia and the Americas. However, the disease is absent from some Mediterranean climate regions, including Spain, Greece and Italy in Europe and California in the USA (EFSA, 2014).

CBS affects the peel of almost all types of citrus fruit, especially oranges [*Citrus sinensis* (L.) Osb.], mandarins (*Citrus*

reticulata Blanco) and lemons [*Citrus limon* (L.) Burm.]. The only citrus species considered to be resistant or tolerant to CBS are sour oranges (*Citrus aurantium* L.) and their hybrids, and 'Tahiti' limes (*Citrus latifolia* Tan.). CBS lesions on fruit are classified into six types: false melanose, hard spot, freckle spot, virulent spot, cracked spot and lacy spot (Kotzé, 1981, 2000; da Silva *et al.*, 2016a,b). Hard, freckle and virulent spot symptoms appear later in the season, when the fruit is almost mature, and are the consequence of fungal-induced cell lysis and collapse around oil cavities (Marques *et al.*, 2012). As a result of the long incubation period after inoculation for the development of the different types of lesion (Frare, 2015), the study of this plant–pathogen interaction has been burdensome.

Phyllosticta citricarpa produces ascospores and conidia as infective propagules. Ascospores are formed on the citrus leaf litter, whereas conidia, produced in pycnidia, are formed on fruit, dead twigs and the leaves of the tree, as well as on leaf litter (Kotzé, 2000). The ascospores are spread mainly by wind and are important to the introduction of CBS into new areas (Fourie *et al.*, 2013; Kotzé, 1981; McOnie, 1964). The conidia are washed down to the adjacent fruit, twigs and leaves, and are influential in CBS epidemics in regions with higher rainfall, such as Brazil and Ghana (Brentu *et al.*, 2012; Spósito *et al.*, 2011). CBS is mainly controlled by fungicide sprays during the critical period of fruit infection (Schutte *et al.*, 1997, 2003; da Silva *et al.*, 2016b). In 2015, CBS control in the São Paulo citrus belt alone represented an estimated cost of approximately 57 million dollars for the Brazilian citrus industry (da Silva *et al.*, 2016a).

Currently, the search for alternative solutions to the use of chemical fungicides is of major interest. As such, several studies have focused on attempts to use VOCs or plant essential oils (EOs) for the control of fungal growth and development to maintain the fruit quality during storage (Text S1, see Supporting Information). In parallel, in fruit that accumulates natural VOCs at high concentrations, it is important to understand the mode of action of natural VOCs in the interaction with fruit pathogens. The generation of mutants or transgenic plants with altered scent profiles is essential to perform such studies.

Previously, we have generated transgenic orange plants with a D-limonene synthase transgene in the antisense (AS) configuration in which D-limonene production was reduced by up to 100-fold in the fruit peel (flavedo) when compared with that of empty vector (EV) controls (Rodríguez *et al.*, 2011, 2014). When pure D-limonene was added to the peel of AS fruit, the attraction effect on the medfly (*Ceratitis capitata* (Wied.)) and the susceptibility to *Penicillium digitatum* (Pers.) Sacc. were restored, confirming the role of this VOC in the attraction of the male medfly and the stimulatory effect on *Penicillium digitatum* growth, respectively. In addition, global gene expression analysis using a microarray comparing AS and EV intact fruit revealed a constitutive activation of the immune response in AS fruit (Rodríguez *et al.*, 2014).

In this study, to assess the role of D-limonene and other altered terpene volatiles in the interaction of *P. citricarpa* with AS and EV orange fruit, we established an effective and reliable protocol to inoculate and follow the fungal infection process in detached orange fruit. Moreover, we investigated the transcriptome of challenged fruit at different time points after inoculation using a citrus probe microarray. In addition, the potential effects of the different VOCs showing consistently altered concentrations in AS vs. EV fruit against *P. citricarpa* were assayed using pure compounds alone or combined at real fruit concentrations through *in vitro* assays. We report here that the activation of different defence pathways, including the up-regulation of several pathogenesis-related (PR) protein genes 2 h after inoculation, were tightly associated with the resistance response to *P. citricarpa* in AS fruit, probably because of the constitutively increased levels of monoterpene alcohols.

RESULTS

Phyllosticta citricarpa induces visible symptoms in EV detached fruits

In this study, symptoms were observed reliably on detached EV orange fruit inoculated with *P. citricarpa* only 28 days after inoculation in the laboratory with a highly concentrated conidial suspension. *Phyllosticta citricarpa* caused infection and more pronounced fruit damage on EV relative to AS1 and AS7 'Navelina' orange fruit (Fig. 1) on which fungal growth and symptom development were considerably retarded. Analyses showed that the lesion areas in AS1 and AS7 fruit were 2.3 and 2 mm^2, respectively, compared with 5.1 mm^2 in EV fruit (Fig. 1a). This resistance phenotype was reproducible and independent of the AS transgenic line or the orange cultivar used (Figs S1 and S2, see Supporting Information). In general, lesions on EV fruit resembled those typical of an early stage of hard spot symptoms, with a dark brown rim (sometimes surrounded by the presence of green or reddish tissue) with slight depressions and 2–4-mm-diameter shallow circular lesions (Fig. 1b). In some cases, the pycnidia could be seen inside the spot 50 days after challenge by inoculation (Fig. 1c). Although most AS fruit inoculation points did not show symptoms, when present, the typical lesions had a greenish rim around the spot without the development of more severe symptoms (Figs 1, S1 and S2). Control EV and AS fruit treated with a saline solution showed no symptoms (Fig. S3, see Supporting Information).

Fifteen days after inoculation, real-time polymerase chain reaction (PCR) showed that the concentration of fungal DNA was approximately six-fold higher in EV fruit than in AS fruit (Fig. 2), indicating that *P. citricarpa* colonized a larger area in EV than in AS fruit.

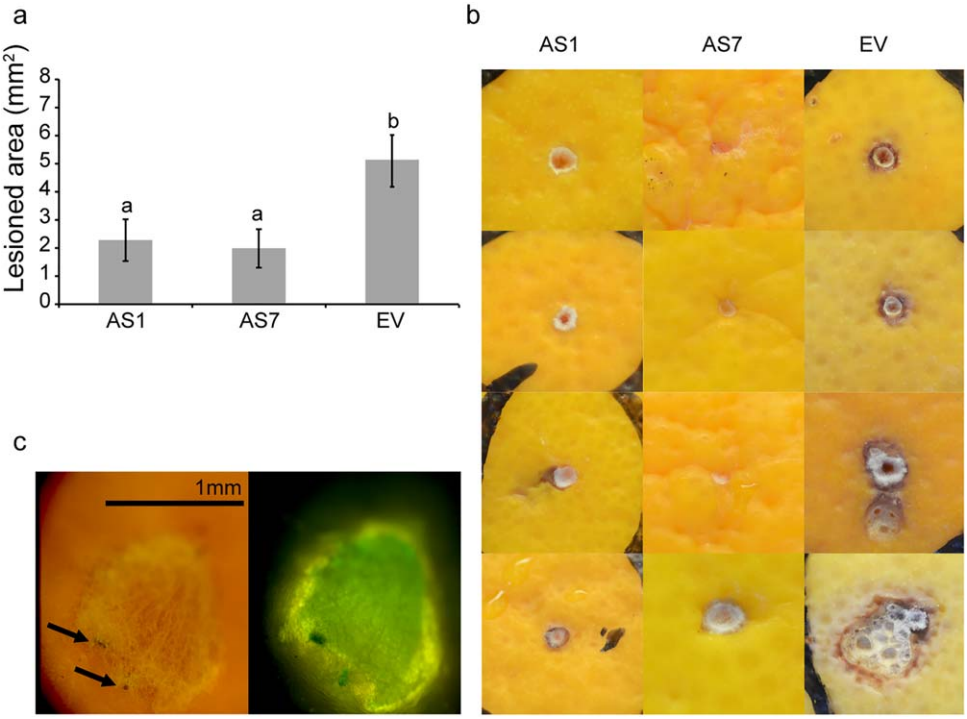

Fig. 1 *Phyllosticta citricarpa* infection in 'Navelina' transgenic antisense (AS) and empty vector (EV) control fruit in November. (a) Average measurements of the lesioned area (mm^2) at 28 days after inoculation in mature fruit of EV and AS lines. Data are presented as the means ± standard error (SE) and are derived from at least 20 fruit per line. Different letters indicate significant differences at $P < 0.05$ using Fisher's protected least significant difference (LSD) test. (b) Different disease severity in EV control fruit vs. AS fruit at 28 days after inoculation. (c) A lesion with growing mycelia and pycnidia inside (arrows) under normal and UV light using a magnifying glass.

Microarray analyses after inoculation with *P. citricarpa* show activation of a complex regulatory network up-regulating different defence pathways in AS fruits

We found that, at 2 h and 8 days after inoculation with *P. citricarpa*, 329 and 226 common genes, respectively, were up-regulated in both AS lines, having at least a two-fold expression change compared with EV lines, whereas 463 and 242 common genes, respectively, were down-regulated compared with EV lines (Tables S1 and S2, see Supporting Information).

A snapshot of the altered genes showed that, before and at 2 h post-inoculation, a higher number of genes categorized as phenylpropanoids and light-related genes were up-regulated, whereas some terpene, flavonoid and cell wall-related genes were down-regulated in AS fruit (Figs 3a and S4, see Supporting Information). Eight days after inoculation with *P. citricarpa*, terpene and flavonoid genes appeared to be mostly down-regulated, whereas some cell wall genes appeared to be up-regulated (Fig. 3b).

Based on gene ontology (GO) terms, and delving into the altered processes in AS fruit, the up-regulated genes in the peel of AS fruit at 2 h and 8 days after inoculation were primarily related to defence responses (Tables 1a and 2a; Figs S5 and S6, see Supporting Information). Amongst the up-regulated genes, we found different antifungal PR protein genes involved in defence which were highly up-regulated at 2 h after inoculation, such as those coding for different peptides belonging to the PR-6 proteinase inhibitor family (more than 12- and nine-fold up-regulated in AS1 and AS7, respectively) or PR-4 (more than three- and two-fold up-regulated in AS1 and AS7, respectively) in microarray analyses. These results were confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses to be more than two-fold up-regulated in both lines relative to EV lines (Table 1a; Fig. 4a). Moreover, an osmotin-like protein gene (*OSM34*) related to defence responses against bacteria and fungi was highly up-regulated at 2 h and 8 days after inoculation in microarray analyses and was confirmed by qRT-PCR (Tables 1a and 2a; Fig. 4a). Amongst other defence response-related genes,

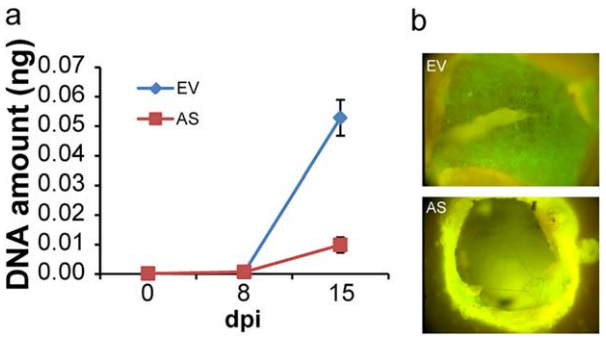

Fig. 2 (a) Quantification of *Phyllosticta citricarpa* DNA by real-time polymerase chain reaction (PCR) of inoculated fruit of antisense (AS) and empty vector (EV) plants at 0, 8 and 15 days post-inoculation (dpi). (b) Growth of the pathogen mycelium in EV and AS lesions 40 days after inoculation under UV light with a magnifying glass.

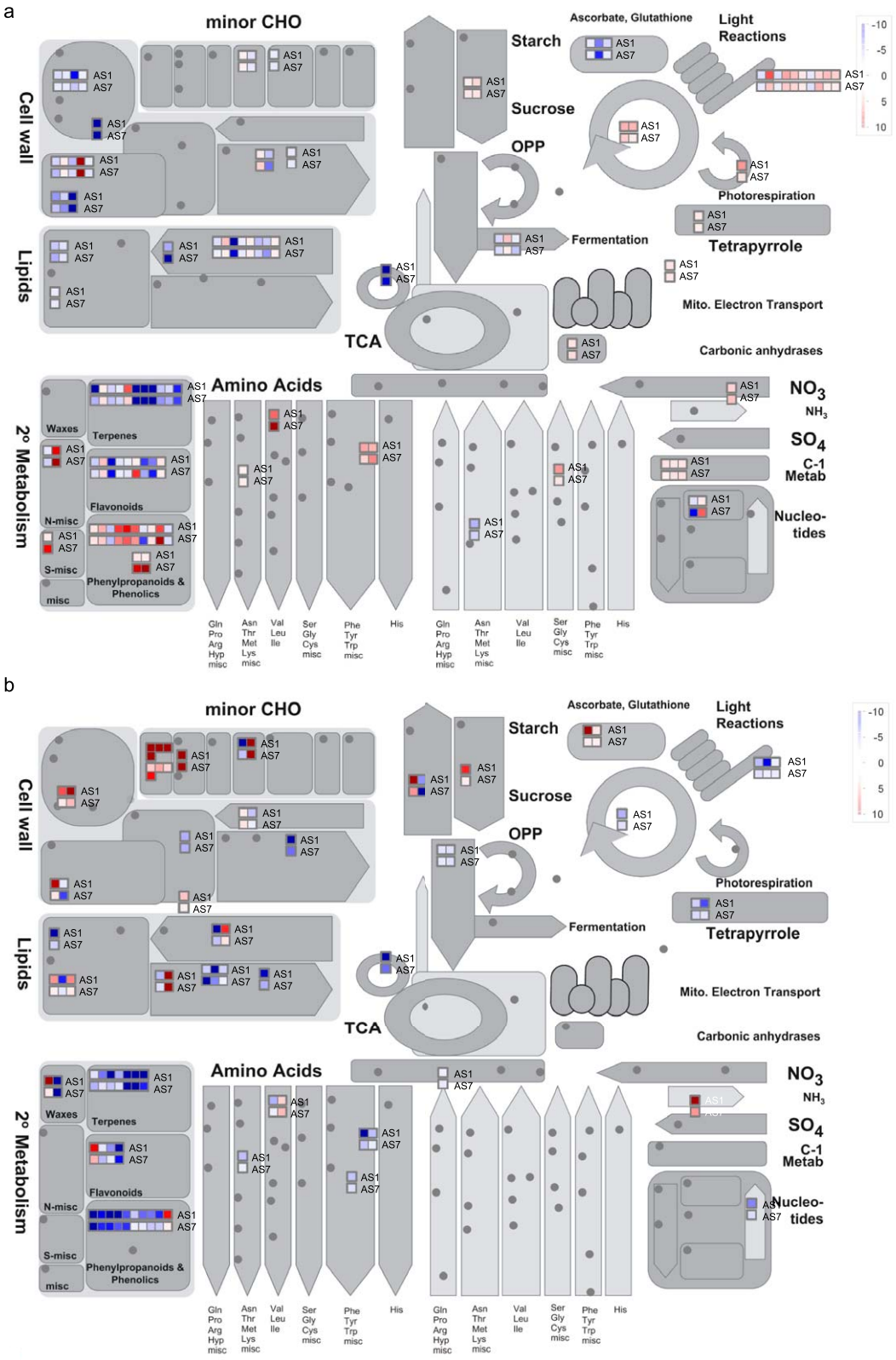

Fig. 3 MapMan metabolism overview showing commonly altered genes in antisense (AS) fruit on *Phyllosticta citricarpa* inoculation referenced to empty vector (EV) control fruit. (a) Modulated genes at 2 h post-inoculation. (b) Modulated genes at 8 days post-inoculation. The colour scale indicates fold changes ranging from −10 to 10 of up- and down-regulated genes in red and blue, respectively. Carbohydrates (CHO); oxidative pentose phosphate (OPP); tricarboxylic acid (TCA).

Table 1 Common deregulated genes in two independent transgenic antisense (AS) vs. empty vector (EV) plants at 2 h after *Phyllosticta citricarpa* inoculation.

Description	Agilent probe	Fold change AS1	Fold change AS7	Most similar Arabidopsis thaliana (Ath) gene
(a) Differentially up-regulated genes				
Defence response				
Closely related 12-oxophytodienoic acid reductases	UF_Rut_AF_219875	2.7	3.9	AT1G76690
Putative phenylalanine ammonia-lyase 2 (PAL2)	UF_Rut_AF_211541	8.4	6.5	AT3G53260
Pathogenesis-related proteinase inhibitor family (PR-6)	UF_Rut_AF_204618	2.4	4.0	AT2G38870
Pathogenesis-related proteinase inhibitor family (PR-6)	UF_Rut_AM_240654	5.1	4.1	AT5G43580
	UF_Rut_AF_238137	12.1	9.2	
Lipoxygenase 4 (LOX4)	UF_Rut_AF_213588	2.6	2.5	AT1G72520
MYB domain protein 3 (MYB3)	UF_Rut_AF_224101	10.9	3.2	AT1G22640
Wound-responsive family protein	UF_Rut_AF_200464	5.3	4.4	AT4G10270
Lipoxygenase 2 (LOX2)	UF_Rut_AF_214342	2.1	3.7	AT3G45140
Transparent testa 4 (TT4)	UF_Rut_AF_222956	2.1	7.3	AT5G13930
Phenylalanine ammonia-lyase 1 (PAL1)	UF_Rut_AF_207822	7.2	5.6	AT2G37040
Encodes an aromatic aldehyde synthase (AtAAS)	UF_Rut_AF_223469	8.6	13.1	AT2G20340
Nudix hydrolase homologue 8 (NUDT8)	UF_Rut_AF_202004	2.4	6.4	AT5G47240
NADH dehydrogenase-like complex n (NDHN)	UF_Rut_AF_212832	3.4	2.1	AT5G58260
Phospholipase A 2A (PLA2A)	UF_Rut_AF_210848	3.3	2.0	AT2G26560
Disease resistance family protein/LRR family protein	UF_Rut_AF_208645	2.3	3.1	AT2G34930
Myo-inositol-1 phosphate synthase 2 (MIPS2)	UF_Rut_AF_206976	2.1	2.0	AT2G22240
Respiratory burst oxidase homologue B (RBOHB)	UF_Rut_AF_205184	3.7	4.1	AT1G09090
Homologue of carrot EP3-3 chitinase (EP3)	UF_Rut_AF_230447	2.1	4.0	AT3G54420
	UF_Rut_AF_223539	4.4	7.2	
	UF_Rut_AF_215892	2.9	7.6	
Receptor-like protein 47 (RLP47)	UF_Rut_AF_218978	2.6	2.5	AT4G13810
Disease resistance protein (TIR-NBS-LRR class) family	UF_Rut_AF_202269	2.7	2.0	AT4G11170
Tryptophan synthase alpha chain (TSA1)	UF_Rut_AF_218241	4.7	3.3	AT3G54640
Phosphoribulokinase (PRK)	UF_Rut_AF_211316	4.4	3.5	AT1G32060
Beta carbonic anhydrase 1 (CA1)	UF_Rut_AF_216944	2.6	3.0	AT3G01500
Osmotin 34 (OSM34)	UF_Rut_AF_208165	7.1	5.6	AT4G11650
	UF_Rut_AF_200510	6.8	3.5	
	UF_Rut_AF_203810	3.5	3.4	
Disease resistance protein (TIR-NBS-LRR class) family	UF_Rut_AF_217577	2.7	2.2	AT5G41750
ATP synthase delta-subunit gene (ATPD)	UF_Rut_AF_219056	4.0	3.2	AT4G09650
Pathogenesis-related 4 (PR-4)	UF_Rut_AF_204052	3.4	2.1	AT3G04720
Rubisco activase (RCA)	UF_Rut_AM_239919	4.3	2.2	AT2G39730
R2R3 transcription factor MYB108-like protein	UF_Rut_AF_231958	2.9	8.6	AT3G06490
Beta-1,3-glucanase 3 (BG3)	UF_Rut_AF_209159	5.5	6.5	AT3G57240
Phenylpropanoid metabolic process				
MYB domain protein 58 (MYB58)	UF_Rut_AF_235243	4.8	5.1	AT1G16490
Cinnamoyl CoA reductase 1 (CCR1)	UF_Rut_AF_220847	2.5	2.8	AT1G15950
Putative phenylalanine ammonia-lyase 2 (PAL2)	UF_Rut_AF_211541	8.4	6.5	AT3G53260
Laccase 7 (LAC7)	UF_Rut_AF_205689	2.2	10.7	AT3G09220
	UF_Rut_AF_201633	2.0	2.6	
	UF_Rut_AF_213212	2.1	2.8	
	UF_Rut_AF_229484	2.2	2.9	
Laccase 3 (LAC3)	UF_Rut_AF_201499	2.5	9.0	AT2G30210
MYB domain protein 3 (MYB3)	UF_Rut_AF_224101	10.9	3.2	AT1G22640
O-Methyltransferase 1 (OMT1)	UF_Rut_AF_228799	6.9	9.7	AT5G54160
Transparent testa 4 (TT4)	UF_Rut_AF_222956	2.1	7.3	AT5G13930
Phenylalanine ammonia-lyase 1 (PAL1)	UF_Rut_AF_207822	7.2	5.6	AT2G37040
Flavonol synthase 1 (FLS1)	UF_Rut_AF_218377	2.5	2.1	AT5G08640
(b) Differentially down-regulated genes				
Monoterpenoid biosynthetic process				
Terpene synthase-like sequence-1,8-cineole (TPS23)	UF_Rut_AF_216868	−3.1	−4.5	AT3G25820
Terpene synthase-like sequence-1,8-cineole (TPS27)				AT3G25830
D-Limonene synthase	UF_Rut_AF_234309	−11.6	−14.0	
Gamma-terpinene synthase	UF_Rut_AM_242150	−4.8	−12.3	

Table 1 *Continued*

Description	Agilent probe	Fold change AS1	Fold change AS7	Most similar Arabidopsis thaliana (Ath) gene
Gamma-terpinene synthase	UF_Rut_AF_220567	−3.1	−4.3	
D -Limonene synthase	UF_Rut_AF_232468	−28.7	−31.4	
D -Limonene synthase	UF_Rut_AF_221861	−3.5	−6.6	
Phenylpropanoid biosynthetic process				
Aldehyde dehydrogenase 2C4 (ALDH2C4)	UF_Rut_AF_201170	−2.2	−3.8	AT3G24503
Unfertilized embryo sac 10 (UNE10)	UF_Rut_AF_226767	−3.4	−4.3	AT5G66230
Downy mildew resistant 6 (DMR6)	UF_Rut_AF_219806	−7.1	−4.7	AT5G24530
O-Methyltransferase 1 (OMT1)	UF_Rut_AF_218678	−6.4	−3.1	AT5G54160
Ribonuclease 1 (RNS1)	UF_Rut_AF_210169	−2.5	−4.0	AT2G02990
Hydroxycinnamoyl-CoA: Shikimate hydroxycinnamoyl transferase (HCT)	UF_Rut_AF_211683	−2.6	−7.1	AT5G48930
Flavonol synthase 1 (FLS1)	UF_Rut_AF_214060	−2.4	−2.6	AT5G08640
Cytochrome P450 75B1 (CYP75B1)	UF_Rut_AF_221464	−5.8	−7.7	AT5G07990
	UF_Rut_AF_212713	−4.7	−11.4	
Response to oxidative stress and cutin biosynthetic process				
Glycerol-3-phosphate sn-2-acyltransferase 8 (GPAT8)	UF_Rut_AF_201598	−2.7	−2.6	AT4G00400
Glycerol-3-phosphate sn-2-acyltransferase 6 (GPAT6)	UF_Rut_AF_236998	−3.7	−4.5	AT2G38110
Long-chain acyl-CoA synthetase 2 (LRD2)	UF_Rut_AF_213219	−20.8	−9.1	AT1G49430
Permeable leaves3 (PEL3)	UF_Rut_AF_204169	−5.6	−4.8	AT5G23940
Peroxidase superfamily protein	UF_Rut_AF_224659	−9.1	−5.5	AT1G71695
Peroxidase 56 (PRX56)	UF_Rut_AF_224679	−7.9	−4.4	AT5G15180
Purple acid phosphatase 17 (PAP17)	UF_Rut_AF_215531	−3.9	−68.8	AT3G17790
Glutathione peroxidase 2 (GPX2)	UF_Rut_AF_203015	−3.2	−2.4	AT2G31570
Peroxidase superfamily protein	UF_Rut_AF_200596	−3.0	−2.6	AT4G33420
Superoxide dismutase 1 (SOD1)	UF_Rut_AF_207035	−3.9	−12.5	AT1G08830
Peroxidase 52 (PRX52)	UF_Rut_AF_231185	−11.9	−15.2	AT5G05340
Peroxidase 53 (PRX53)	UF_Rut_AF_220918	−3.6	−2.5	AT5G06720
Peroxidase superfamily protein	UF_Rut_AF_234643	−5.0	−2.2	AT5G06730
Root hair specific 19 (RHS19)	UF_Rut_AF_214336	−6.9	−4.4	AT5G67400

a putative phenylalanine ammonia-lyase 2 (*PAL2*) was found to be up-regulated by more than eight- and six-fold in AS1 and AS7, respectively, at 2 h after inoculation, but highly down-regulated at 8 days after inoculation (Tables 1a and 2b; Fig. 4a,d). In addition, two lipoxygenase genes (*LOX2* and *LOX4*) and different R2R3-MYB transcription factor genes were up-regulated by more than two-fold in both AS lines relative to EV controls at 2 h and 8 days after inoculation (Table 1a; Fig. 4a,c). The two other main genes related to different hormone stimuli, a member of the AP2/ERF transcription factor family (*DREB*) and the jasmonate-zim-domain protein 10 (*JAZ10*), were highly up-regulated at 8 days after inoculation in both AS lines and confirmed by qRT-PCR (Table 2a; Fig. 4c).

We found genes mainly related to terpenoid metabolism amongst the genes down-regulated at 2 h and 8 days after inoculation (Tables 1b and 2b; Figs S7 and S8, see Supporting Information). Some monoterpene synthase genes that were required for volatile terpenoid biosynthesis were highly down-regulated at both time points, including a D-limonene synthase gene (*LS*), which was more than 30- and six-fold repressed in microarray and qRT-PCR analyses, respectively, in both AS1 and AS7 lines (Tables 1b and 2b; Fig. 4b,d). In addition, different sesquiterpene synthase genes were repressed at both time points, such as a β-caryophyllene synthase gene that was down-regulated more than 10-fold in both lines in microarray analyses and confirmed by qRT-PCR (Table 2b; Fig. 4b,d). We also found, and confirmed by qRT-PCR 2 h after inoculation, different genes related to the cutin biosynthetic process, such as the acyl-CoA synthetase gene (*COA*), which was down-regulated 21- and nine-fold in AS1 and AS7, respectively, and other genes, such as a cytochrome P450 gene (*CYT*), which showed reduced expression in both AS lines relative to EV controls (Table 1b; Fig. 4b). In addition, genes of the carotenoid biosynthetic process were also down-regulated 8 days after inoculation, including a β-carotene hydroxylase *2* (*BCAROT*) gene that was repressed six- and three-fold in AS1 and AS7, respectively; these results were also confirmed by qRT-PCR (Table 2b; Fig. 4d).

Terpene VOC content changes before and after inoculation in both AS and EV fruit peels

We observed that, before inoculation, the D-limonene content in AS fruit was very low relative to that of EV fruit [around 1100 vs. 22000 μg/g fresh weight (FW), respectively; Fig. 5a], whereas the contents of linalool, nerol, geraniol and β-citronellol monoterpene

Table 2 Common deregulated genes in two independent transgenic antisense (AS) vs. empty vector (EV) plants at 8 days after *Phyllosticta citricarpa* inoculation.

Description	Agilent probe	Fold change AS1	Fold change AS7	Most similar Arabidopsis thaliana (Ath) gene
(a) Differentially up-regulated genes				
Defence response				
Jasmonate-zim-domain protein 10 (JAZ10)	UF_Rut_AF_223890	25.8	6.3	AT5G13220
Salt tolerance zinc finger (STZ)	UF_Rut_AF_221957	9.0	2.4	AT1G27730
WRKY DNA-binding protein 40 (WRKY40)	UF_Rut_AF_211024	6.4	2.2	AT1G80840
3-Ketoacyl-CoA synthase 11 (KCS11)	UF_Rut_AF_205609	7.4	2.6	AT2G26640
Elicitor-activated gene 3-2 (ELI3-2)	UF_Rut_AF_202422	7.9	2.1	AT4G37990
Encodes AP2C1. Belongs to clade B of the PP2C superfamily	UF_Rut_AF_223352	30.5	2.8	AT2G30020
Galactinol synthase 1 (GOLS1)	UF_Rut_AF_232379	99.9	3.6	AT2G47180
Raffinose synthase 5 (RS5)	UF_Rut_AF_202255	12.1	4.7	AT5G40390
AT-hook motif nuclear-localized protein 20 (AHL20)	UF_Rut_AF_220787	13.3	5.7	AT4G14465
Vitamin C defective 2 (VTC2)	UF_Rut_AF_221748	2.6	2.0	AT4G26850
Unknown protein	UF_Rut_AF_219552	55.9	4.3	AT1G15010
Methionine sulfoxide reductase A4 (MSRA4)	UF_Rut_AF_202442	2.8	2.2	AT4G25130
Soluble *N*-ethylmaleimide-sensitive factor adaptor protein 33 (SNP33)	UF_Rut_AF_202031	6.1	2.1	AT5G61210
Orthologue of sugar beet HS1 PRO-1 2 (HSPRO2)	UF_Rut_AF_204783	7.0	3.5	AT2G40000
Glutamate dehydrogenase 2 (GDH2)	UF_Rut_AF_213834	57.5	5.2	AT5G07440
AZI3	UF_Rut_AF_226184	265.7	9.6	AT4G12490
Eukaryotic aspartyl protease family protein	UF_Rut_AF_208509	7.2	2.2	AT1G03220
	UF_Rut_AF_213896	7.5	2.9	
Class I glutamine amidotransferase-like superfamily protein	UF_Rut_AF_235909	2.8	2.1	AT2G23970
Dehydration response element-binding protein 26 (DREB26)	UF_Rut_AF_233093	51.4	5.8	AT1G21910
Soluble epoxide hydrolase (SHE)	UF_Rut_AF_222983	25.0	20.2	AT2G26740
Spatula (SPT) (bHLH protein family)	UF_Rut_AF_202807	2.4	2.3	AT4G36930
Respiratory burst oxidase homologue D (RBOHD)	UF_Rut_AF_233889	8.1	3.5	AT5G47910
Ca^{2+}-binding protein 1 (CP1)	UF_Rut_AF_217281	73.5	6.4	AT5G49480
Heat shock protein 70-1 (HSC70)	UF_Rut_AF_214933	32.1	4.7	AT5G02500
Thiamine4 (THI4)	UF_Rut_AF_237296	12.8	2.1	AT5G54770
Chaperone DNAJ-domain superfamily protein DNAJ11 (J11)	UF_Rut_AF_226782	11.2	3.2	AT4G36040
Natural resistance-associated macrophage protein 3 (NRAMP3)	UF_Rut_AF_212254	2.1	2.4	AT2G23150
Ist1-like 6 (ISTL6)	UF_Rut_AF_203830	7.2	2.8	AT1G13340
Osmotin 34 (OSM34)	UF_Rut_AF_208165	12.3	5.6	AT4G11650
(b) Differentially down-regulated genes				
Phenylpropanoid metabolic process				
Beta-carotene hydroxylase 2 (BETA-OHASE 2)	UF_Rut_AF_201773	−5.9	−2.7	AT5G52570
Copper response defect 1 (CRD1)	UF_Rut_AF_236880	−3.4	−3.0	AT3G56940
O-Methyltransferase 1 (OMT1)	UF_Rut_AF_224138	−7.5	−3.6	AT5G54160
	UF_Rut_AF_227844	−6.8	−4.0	
Chlg (G4)	UF_Rut_AF_221059	−6.8	−2.6	AT3G51820
Cinnamoyl-CoA reductase	UF_Rut_AF_218832	−5.9	−3.7	AT5G14700
UDP-glucosyl transferase 72e1 (UGT72E1)	UF_Rut_AF_212020	−8.5	−7.5	AT3G50740
4-Coumarate:CoA ligase 1 (4CL1)	UF_Rut_AF_222314	−6.7	−7.3	AT1G51680
	UF_Rut_AF_208019	−23.2	−21.2	
	UF_Rut_AF_203973	−7.7	−8.1	
Hydroxycinnamoyl-CoA: Shikimate hydroxycinnamoyl transferase (HCT)	UF_Rut_AF_229474	−3.7	−2.1	AT5G48930
	UF_Rut_AF_210057	−20.1	−14.4	
Aliphatic suberin feruloyl-transferase (HHT)	UF_Rut_AF_235744	−14.1	−3.1	AT5G41040
Caffeoyl coenzyme A *O*-methyltransferase 1 (CCOAOMT1)	UF_Rut_AF_207026	−6.2	−2.3	AT4G34050
Isoflavone reductase, putative	UF_Rut_AF_225849	−15.9	−8.0	AT1G75280
Purple acid phosphatase 17	UF_Rut_AF_215531	−7.8	−28.2	AT3G17790
NAD(P)H dehydrogenase 18 (NDH18)	UF_Rut_AF_226658	−10.8	−3.0	AT5G43750
Transparent testa 4 (TT4)	UF_Rut_AF_230294	−2.5	−4.2	AT5G13930
Phenylalanine ammonia-lyase 1 (PAL1)	UF_Rut_AF_225740	−14.6	−7.8	AT2G37040
	UF_Rut_AF_213548	−6.5	−6.0	
Myo-inositol-1-phosphate synthase 2 (MIPS2)	UF_Rut_AF_226911	−10.5	−3.9	AT2G22240
	UF_Rut_AF_223219	−6.0	−4.2	
Putative phenylalanine ammonia-lyase 2 (PAL2)	UF_Rut_AF_207502	−32.1	−6.6	AT3G53260

Table 2 *Continued*

Description	Agilent probe	Fold change AS1	Fold change AS7	Most similar Arabidopsis thaliana (Ath) gene
Peroxidase superfamily protein	UF_Rut_AF_234643	−8.2	−3.1	AT5G06730
Peptidyl-prolyl *cis–trans* isomerases	UF_Rut_AF_224345	−3.0	−2.1	AT4G17070
Terpenoid biosynthetic process				
Beta-carotene hydroxylase 2 (BETA-OHASE 2)	UF_Rut_AF_201773	−5.9	−2.7	AT5G52570
ABA deficient 2 (ABA2)	UF_Rut_AF_234004	−3.4	−2.0	AT1G52340
Terpene synthase-like sequence-1,8-cineole (TPS27)				
D-Limonene synthase	UF_Rut_AF_234309	−166.5	−50.2	AT3G25830
Gamma-terpinene synthase	UF_Rut_AF_220567	−18.1	−6.1	
D-Limonene synthase	UF_Rut_AF_232468	−356.6	−58.6	
Sesquiterpene synthase				
(*E*)-beta-farnesene synthase	UF_Rut_AF_211943	−4.6	−2.7	AT5G23960
(*E*)-beta-caryophyllene synthase	UF_Rut_AF_236976	−9.6	−15.5	
Terpene synthase	UF_Rut_AF_214516	−15.2	−12.2	
Germacrene synthase	UF_Rut_AF_218533	−2.2	−2.0	
Terpene synthase-like sequence-1,8-cineole (TPS23)	UF_Rut_AF_223480	−23.9	−3.8	AT3G25820

alcohols were higher in AS fruit (192, 54.5, 21.5 and 87.5 μg/g FW, respectively) than in EV controls (78, 3.5, 3.4 and 5.5 μg/g FW, respectively; Fig. 5b).

Two hours after inoculation, there was a great increase in linalool content in AS fruit (from 192 to 700 μg/g FW), but only a slight increase in EV fruit (from 78 to 102 μg/g FW). The other monoterpene alcohol compounds decreased slightly and D-limonene remained almost at the same level (Fig. 5).

Eight days after inoculation, all the compounds decreased, including D-limonene, in both AS and EV fruit, although monoterpene alcohols remained higher in AS fruit than in EV controls (Fig. 5).

The addition of monoterpene alcohols to a mixture of terpene VOCs at concentrations resembling those present in AS fruit reveals an inhibitory effect of these compounds on *P. citricarpa* growth *in vitro*

To confirm whether the differential accumulation of VOCs in AS lines was, at least in part, responsible for the observed resistance to *P. citricarpa*, we next attempted to study the effect of some of these terpenes in different *in vitro* assays, both as pure compounds and in mixtures. We decided to focus on D-limonene and the alcohols nerol, β-citronellol, linalool and geraniol, as they were the compounds showing the greatest relative differences between AS and EV fruit.

When added at concentrations found in either mature AS or EV peels (in November), D-limonene had a marked inhibitory effect on the growth of *P. citricarpa* mycelium *in vitro* (Fig. 6a). Unexpectedly, the growth inhibition was 79% using the concentrations of D-limonene found in EV peels, compared with 14% using the concentrations present in AS peels. All the terpene alcohols assayed also had a pronounced inhibitory effect on *P. citricarpa* growth at almost all concentrations tested, but higher at AS rather than EV doses (Fig. 6a). The highest growth inhibition rates of 68% and 54% were obtained with linalool and β-citronellol concentrations, respectively, from AS fruit. Conversely, the concentrations of these products found in EV fruit reduced the pathogen growth by less than 25%. Nerol and geraniol inhibited growth by 30% and 28%, respectively, at AS fruit concentrations, compared with 6% and 8% of inhibition, respectively, at EV fruit concentrations (Fig. 6a).

We next tested terpene mixtures prepared following the composition of AS and EV fruit, in combinations of D-limonene with two, three or four alcohols. The most potent inhibition was observed in the mixture of compounds D-limonene, nerol, β-citronellol and linalool of AS vs. EV fruit, and the addition of geraniol to the mixture had only a slightly higher inhibitory effect for AS vs. EV peels (Fig. 6b). When linalool was not added to the mixtures, the concentration of the compounds D-limonene, nerol and β-citronellol in EV fruit produced an inhibitory effect on the growth of *P. citricarpa* comparable with that of AS fruit (Fig. 6b). Overall, these results indicated that linalool in AS fruit was the main compound responsible for the resistance to challenge inoculation, although the other alcohols exerted synergistic effects, especially at AS concentrations. Conversely, the very high D-limonene concentration of EV fruit was more detrimental than the concentration in AS peels for the fungus, although the D-limonene effects were apparently counteracted by a high concentration of linalool and other alcohols in AS peels.

To gain more insight into the contrasting effects of D-limonene and alcohols on fungal growth, a dose–response curve was designed and performed for each compound based on the concentrations regularly found in AS and EV fruit peels. It is worth noting that all the compounds at low concentrations had a slight

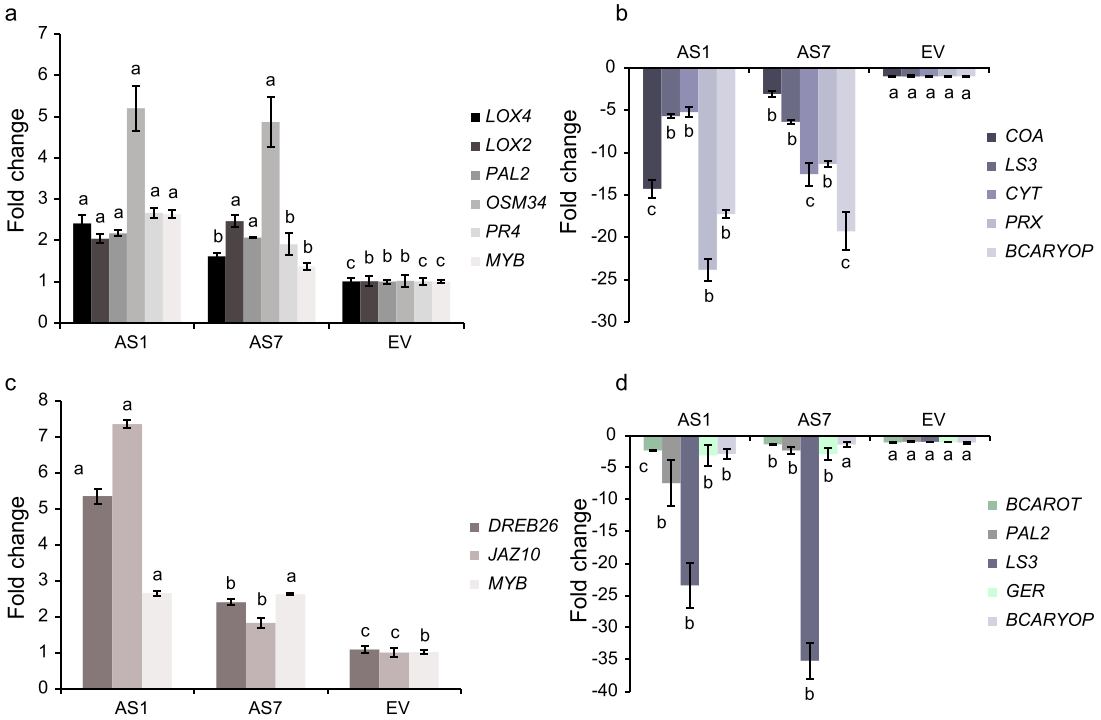

Fig. 4 Quantitative real-time polymerase chain reaction (PCR) analyses of genes deregulated in antisense (AS) fruit relative to empty vector (EV) control fruit after *Phyllosticta citricarpa* inoculation. (a, c) Differential expression of up-regulated defence response genes in EV control and AS fruit at 2 h (a) and 8 days (c) after *P. citricarpa* inoculation. (b, d) Differential expression of down-regulated genes in EV control and AS fruit at 2 h (b) and 8 days (d) after *P. citricarpa* inoculation. The genes were selected from the VirtualPlant microarray analyses: *Lipoxygenase 4 (LOX4)*, *Lipoxygenase 2 (LOX2)*, *Putative phenylalanine ammonia-lyase 2 (PAL2)*, *Osmotin 34 (OSM34)*, *Pathogenesis-related 4 (PR4)*, *MYB-type transcription factor (MYB)*, *Cinnamoyl CoA reductase 1 (COA)*, *Limonene synthase (LS3)*, *Cytochrome P450 (CYT)*, *Peroxidase (PRX)* and *(E)-beta-caryophyllene synthase (BCARYOP)* for deregulated genes at 2 h after inoculation; *Dehydration response element-binding protein 26 (DREB26)*, *Jasmonate-zim-domain protein 10 (JAZ10)*, *MYB*, *Beta-carotene hydroxylase 2 (BCAROT)*, *PAL2*, *LS3*, *Germacrene synthase (GER)* and *BCARYOP* for deregulated genes at 8 days after inoculation. Data are presented as the means ± standard error (SE). Fold change was calculated in relation to the EV control line at 2 h or 8 days after inoculation in each case, to which an arbitrary value of unity was assigned. Different letters indicate significant differences at $P < 0.05$ using Fisher's protected least significant difference (LSD) test. AS1 and AS7 refer to two independent AS transgenic lines.

stimulatory effect on the growth of *P. citricarpa*. However, all the compounds were detrimental to fungal growth above a certain concentration, which was highly variable depending on the specific terpene compound. Nevertheless, it was confirmed that D-limonene was more effective for growth inhibition at EV, rather than AS, concentrations, whereas the opposite occurred for the four alcohols, especially for linalool (Fig. 7).

Linalool supplementation inhibits pycnidial production of *P. citricarpa*, whereas D-limonene supplementation stimulates their formation, in orange leaf discs

To evaluate whether the increased accumulation of linalool may induce the resistance of mature orange peels to *P. citricarpa*, we attempted to add linalool to regular fruits before challenge by fungal inoculation. Linalool burned the fruit peel, even at low concentrations, precluding the possibility of performing such assays (Fig. S9, see Supporting Information). As an alternative, linalool addition at concentrations found in AS or EV green fruit was sufficient to completely inhibit pycnidial formation of *P. citricarpa* in leaf discs. The addition of D-limonene at concentrations present in EV green fruit produced a stimulatory effect on pycnidial production relative to that of control non-treated leaf discs (Fig. 8). These results suggest that linalool and perhaps the concentrations of other alcohols found in green fruit in June, 40 days after petal fall, may be sufficient to maintain *P. citricarpa* in a quiescent form in peels. In September, 150 days after petal fall, the linalool concentrations began to decline to levels below the threshold required for pathogen inhibition (Fig. S10, see Supporting Information), and may explain the expression of hard spot symptoms on fruit during or after the colour change. Under field conditions, the critical period of fruit infection occurred during the green stage of fruit development, from petal fall to 150–180 days later.

DISCUSSION

In this study, we have developed a practical and effective procedure of artificial inoculation using conidia of *P. citricarpa*

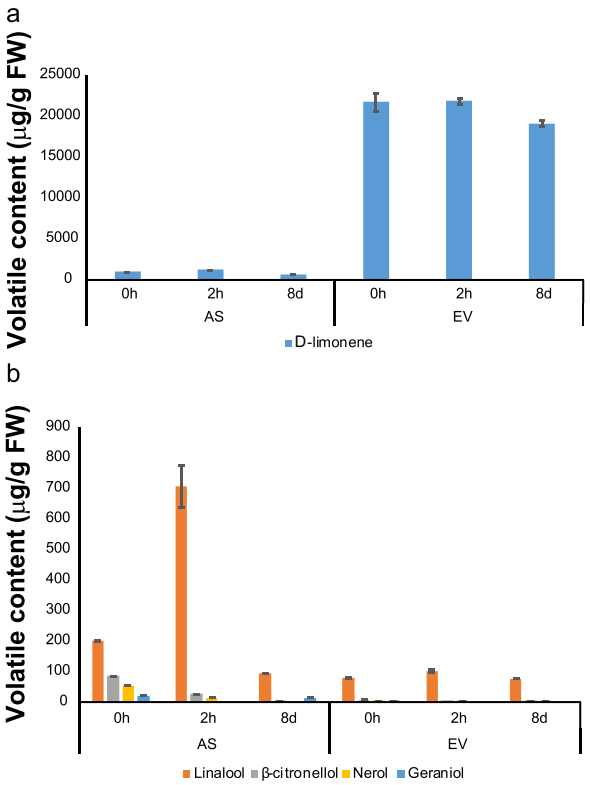

Fig. 5 Volatile content changes in µg/g fresh weight (FW) before (0 hours) and after (2 h or 8 days) inoculation with *Phyllosticta citricarpa* in antisense (AS) and empty vector (EV) control fruit. Data represent means ± standard error (SE) and are derived from at least 10 fruit per line. (a) D-limonene content. (b) Monoterpene alcohol content.

transformed with the reporter gene green fluorescent protein (GFP) as described by Figueiredo *et al.* (2010). This method proved to be feasible to compare and follow the process of pathogen infection and colonization in detached fruit of AS and EV lines. The method implemented here has been shown to be effective to induce black spot symptoms as early as 28 days after inoculation. Symptoms characterized by brown to dark necrotic lesions with pycnidia in and around the injury were associated with *P. citricarpa* colonization in detached and wounded fruit. These lesions are different from the six types of symptom commonly observed in fruit attached to citrus trees in orchards (da Silva *et al.*, 2016b). However, symptoms were associated with GFP fluorescence of *P. citricarpa* in the lesions of inoculated fruit and with the multiplication of *P. citricarpa* in infected fruit tissue. Thus, this *in vivo* assay using detached fruit was instrumental for our experiments and may also be used to investigate *P. citricarpa*–citrus interactions under laboratory conditions, and for screening sources of genetic resistance to this pathogen before performing field trials. Moreover, the incubation period needed to induce symptoms in detached orange fruit was less than 1 month, which was shorter than the 35–360 days observed by Frare (2015) to cause symptoms in non-injured and attached fruit of different sweet orange cultivars; thus, the shorter incubation period allows for reliable results to be obtained more rapidly.

D-Limonene is also present at high concentrations in green orange fruit of EV lines after anthesis (Fig. S10), a stage that is highly susceptible to CBS (Kotzé, 1981, 2000). We have demonstrated that AS fruits with considerably reduced content of D-limonene in the green and mature peel are more resistant to *P. citricarpa* relative to EV control fruit. Different investigations have postulated the inhibitory effect of EOs on the growth of the pathogen *P. citricarpa* (Duarte *et al.*, 2015; Lombardo *et al.*, 2016). A potent inhibitory effect of orange flavedo extracts on colonies cultured on potato dextrose agar (PDA) plates was attributed to the high concentrations of D-limonene present in the oil extract (Brodrick, 1971). In our study, assays with pure compounds performed *in vitro* showed that D-limonene used at concentrations found in mature EV fruit was more deleterious to the pathogen than D-limonene used at concentrations found in mature AS fruit. Thus, the resistance to CBS of mature AS fruit may be better explained by their higher constitutive accumulation of monoterpene alcohols, including linalool, β-citronellol, nerol and geraniol, as all of these were individually more effective in inhibiting pathogen growth at concentrations found in AS peel relative to EV peel. Moreover, experiments with mixtures of alcohol compounds indicated that linalool, at concentrations of mature AS fruit, seemed to be essential for fruit resistance to *P. citricarpa*. However, we cannot rule out the possibility that the change in the terpene profile in AS fruit could contribute to the resistance against *P. citricarpa* by disrupting the co-evolution in the *P. citricarpa*–citrus interaction, which may reduce the fitness of the fungus. All of these results show that several complex mechanisms are involved. Possibly, specialist pathogens (such as *P. citricarpa*) may recognize the specific blend of terpene volatiles of their hosts and use them to their own benefit to allow their survival and spread.

Recently, the EO of *Chenopodium ambrosioides* L., which is characterized mostly by its high content of oxygenated monoterpenes, such as linalool, has been shown to exhibit high inhibitory activity against *P. citricarpa* (Lombardo *et al.*, 2016). In general, aldehyde, alcoholic and phenolic compounds (such as thymol or carvacrol) have been reported to be more potent in the interaction with the microorganism's membrane proteins, although γ-terpinene and D-limonene have also been reported to produce membrane alterations at least *in vitro* (Cristani *et al.*, 2007; Di Pasqua *et al.*, 2006). Generally, it is considered that the action of VOCs on the membrane of microorganisms is dose dependent, although either stimulatory or inhibitory effects can be detected at certain concentrations, and even synergistic effects are found for specific blends of volatiles (Junker and Tholl, 2013).

With regard to their role in defence in citrus, oxygenated terpenes have been generally proven to possess strong antimicrobial

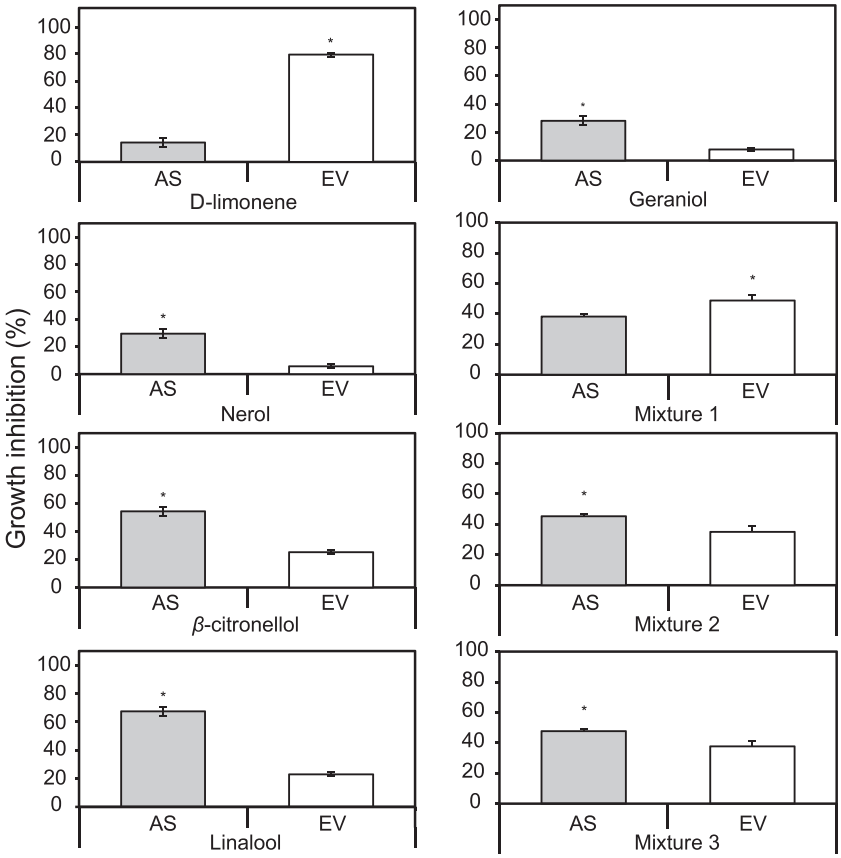

Fig. 6 Effect of different volatile compounds used at concentrations similar to those present in antisense (AS) and empty vector (EV) fruit peels on the percentage growth inhibition of *Phyllosticta citricarpa* in Petri dish assays. The effects of the pure compounds D-limonene, nerol, β-citronellol, linalool and geraniol (at 1135, 54.5, 87.5, 192 and 21.5 μg/g, respectively, for AS, and 22000, 3.5, 5.5, 78 and 3.4 μg/g, respectively, for EV) and the effects of different mixtures of the compounds on *P. citricarpa* growth were measured. Mixture 1, D-limonene + β-citronellol + nerol. Mixture 2, Mixture 1 + linalool. Mixture 3, Mixture 2 + geraniol. Treatments with asterisks were significantly different at $P < 0.05$ using Student's *t*-test.

properties. Shimada *et al.* (2014) studied the possible involvement of different oxygenated monoterpenes in resistance against the bacterium *Xanthomonas citri* ssp. *citri* (ex Hasse) Gabriel, Kingsley, Hunter & Gottwald and the fungus *Penicillium italicum* Wehmer. Amongst all the compounds analysed, linalool, citral, geraniol and β-citronellol showed the most potent effects, impeding the growth of these pathogens. When a linalool synthase gene from Satsuma mandarin (*C. unshiu* Marc.) was overexpressed in orange plants, the transgenic lines were more resistant to *X. citri* ssp. *citri* and showed the up-regulation of PR genes related to defence responses (Shimada *et al.*, 2017). Several studies focused on the action of microbial volatiles on the control of different fungi harmful for plants have suggested that VOCs may change gene expression and protein accumulation (Humphris *et al.*, 2002) or the activity of specific plant enzymes (Wheatley, 2002).

The transcriptomic analysis of orange fruit inoculated with *P. citricarpa* showed that the main biological processes up-regulated in AS fruit were related to defence responses to fungi and bacteria, which were already constitutively activated, at least in part, before inoculation with the pathogen, as a consequence of the genetic modification performed (Rodríguez *et al.*, 2014). Here, amongst the genes up-regulated at 2 h after inoculation, we found different PR genes whose homologues have been proven to be rapidly activated after elicitor treatments and to reduce the ability of the attacker to enter the host plant by different mechanisms (Hammond-Kosack and Jones, 1996; Sels *et al.*, 2008). We also found several *MYB* and *WRKY* transcription factor homologues up-regulated at 2 h and 8 days post-inoculation, respectively. For instance, *MYB108* and *MYB44* homologues, which were up-regulated in AS fruit, have been related to the enhanced tolerance to *Verticillium dahliae* Klebahn in cotton and to the resistance to *Penicillium simplicissimum* (Oudemans) Thom in Arabidopsis (Cheng *et al.*, 2016; Hieno *et al.*, 2016). A *WRKY40* homologue, which was highly up-regulated at 8 days after inoculation, has been proposed recently as a negative regulator of cell death (Lee *et al.*, 2017), thus potentially explaining, at least in part, the absence of hard necrotic lesions in AS fruit.

The phenylpropanoid pathway was also up-regulated in AS fruit at 2 h after inoculation. It is presumed that the activation of this pathway exerts a direct protective effect against pathogens (Ditt *et al.*, 2001). In AS fruit, different *PAL* homologues were highly up-regulated at 2 h after inoculation with *P. citricarpa*. In addition, homologues of other members downstream of the pathway also putatively related to defence responses against pathogens, such as *CHALCONE SYNTHASE*, *CINNAMOYL-COA*

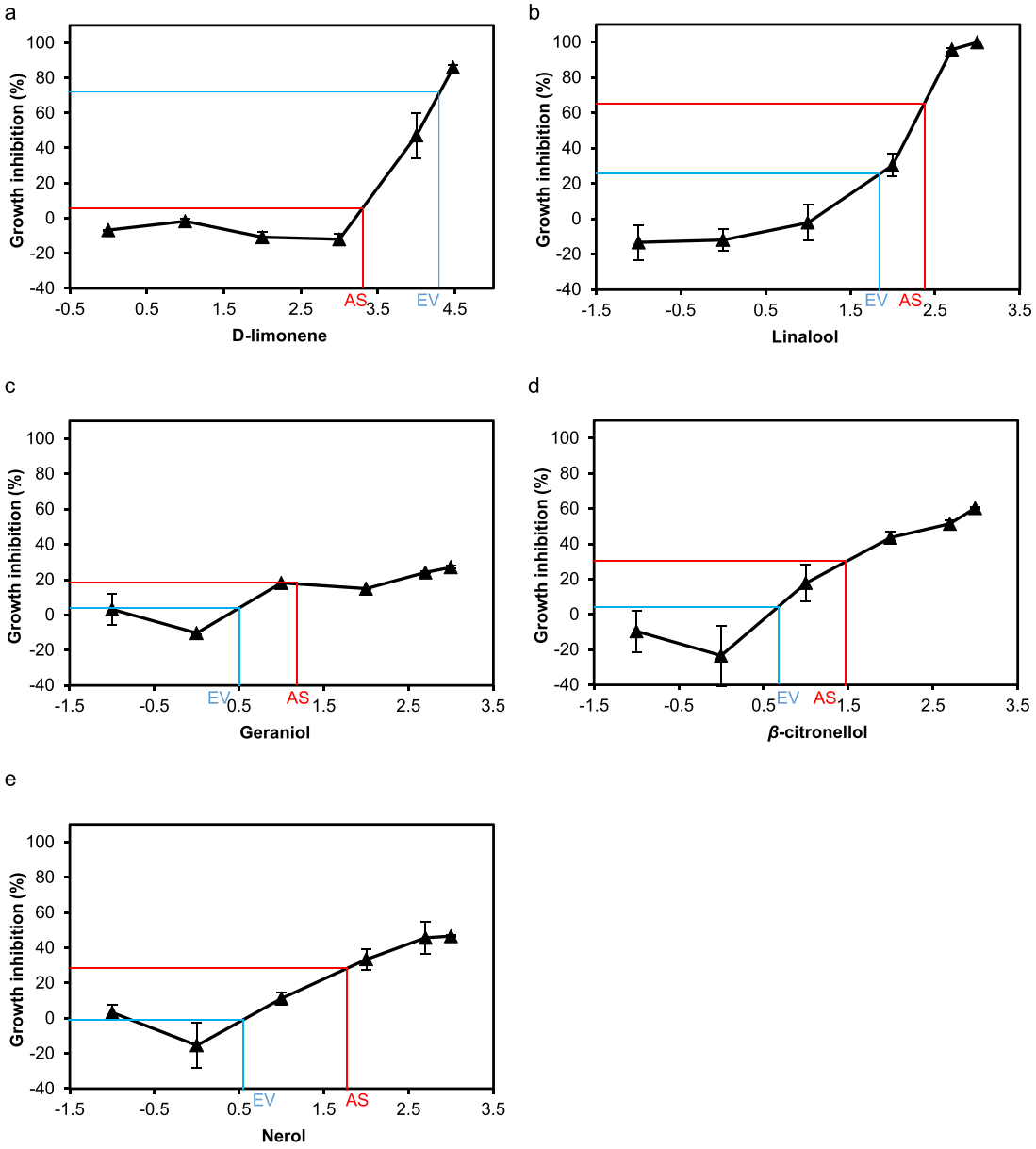

Fig. 7 Dose–response curves performed for volatile terpene compounds on *Phyllosticta citricarpa* growth. Concentrations of pure compounds are expressed as $\log_{10}$ of the concentration (in μg/mL). Normal values for mature antisense (AS) and empty vector (EV) fruit are represented as red and blue lines, respectively. (a) D-limonene. (b) Linalool. (c) Geraniol. (d) b-citronellol. (e) Nerol.

REDUCTASE 1 and *CAFFEATE O-METHYLTRANSFERASE 1* (*CHS*, *CCR1* and *OMT1*), were highly up-regulated (Bell *et al.*, 1984; Kawasaki *et al.*, 2006; Quentin *et al.*, 2009). In AS fruit, a putative member of the phenylpropanoid pathway, the *HYDROXYCINNAMOYL-CoA:SHIKIMATE HYDROXYCINNAMOYL TRANSFERASE (HCT)* gene, was down-regulated at 2 h post-inoculation. In *Medicago sativa*, the down-regulation of *HCT* promoted reduced lignin accumulation levels and a constitutive activation of defence responses as a result of impaired cell wall integrity (Gallego-Giraldo *et al.*, 2011). In these plants, an increase in defence responses in the absence of pathogen attack resulted in enhanced tolerance to subsequent fungal infection. It is noteworthy that some of the phenylpropanoid-related genes were down-regulated at 8 days after inoculation with *P. citricarpa*, including *PAL1*, *PAL2*, *HCT* and *OMT1*, which emphasizes the complex regulatory pathway at the transcriptional level following pathogen inoculation.

AS fruit also showed enhanced expression of genes putatively related to the jasmonic acid (JA) biosynthesis and signalling pathways. Among them, different homologues to *LIPOXYGENASE*

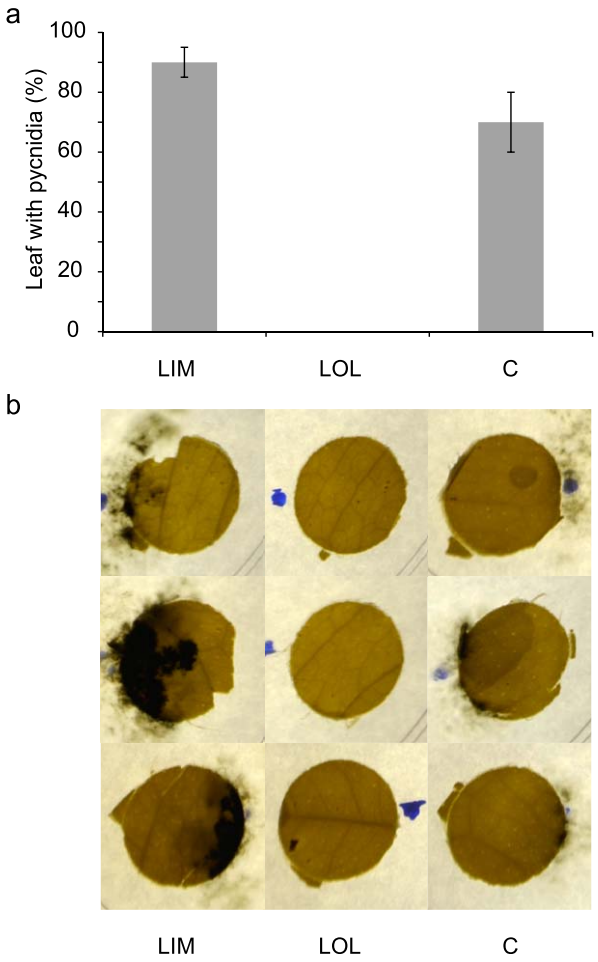

Fig. 8 *In vitro* pycnidial production of *Phyllosticta citricarpa* in empty vector (EV) leaves of orange fruit exposed to different concentrations of terpene volatiles that emulate those present in young green fruit 28 days after deposition. (a) Percentage of leaf with pycnidia exposed to 10 mg of D-limonene (LIM), 4 mg of linalool (LOL) and water as control (C). Data are presented as the percentage of the means ± standard error (SE) and are derived from at least 10 leaves per condition. (b) EV leaves showing the formation of pycnidia for the different assays.

(*LOX*) and *12-OXOPHYTODIENOATE REDUCTASE 2* (*OPR*) genes were up-regulated by more than two-fold in AS fruit at 2 h after inoculation with *P. citricarpa*. The increase in LOX activity has been related to pathogen resistance in other species in several ways. LOX may lead to the production of signal molecules, such as JA or methyl jasmonate, cause membrane damage or participate in the production of toxic volatile and non-volatile fatty acid-derived compounds (Hammond-Kosack and Jones, 1996). In addition, a *LOX2* homologue was found to be specifically down-regulated, whereas a *JAZ10* homologue was up-regulated, at 8 days post-inoculation. It has been shown that treatments with monoterpene VOCs up-regulate the expression of genes related to JA biosynthesis and of JAZ genes in Arabidopsis (Godard *et al.*, 2008). Likewise, treatments with JA highly up-regulate the production of monoterpenes in different plant species (Cheng *et al.*, 2007; Degenhardt *et al.*, 2010). For instance, the application of JA in rice plants induced the up-regulation of a linalool synthase and the accumulation of linalool via OsJAZ8. Moreover, transgenic rice plants overexpressing the linalool synthase gene and accumulating linalool at high levels were resistant to *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings, van den Mooter, Vauterin, Hoste, Gillis, Mew & Kersters, presumably because of the up-regulation of defence-related PR genes (Taniguchi *et al.*, 2014). Amongst these PR genes, the AK099973 PR gene showed 76% similarity to an EP3 chitinase protein, which was highly activated in AS plants at 2 h after inoculation with *P. citricarpa* (Table 1). In addition, other PR-related gene homologues of PR-6 and PR-4, a thaumatin-like protein gene (*OSM34*), some non-specific lipid transfer protein genes (such as *AZI3*) and other thaumatin-like PR protein genes were also up-regulated in AS orange peels at either 2 h or 8 days after inoculation (Tables 1, S1 and S2).

Furthermore, linalool has been shown to provide direct antibacterial and antifungal activities against a wide range of pathogens (Kotan *et al.*, 2007; Shimada *et al.*, 2014). In this study, both *in vitro* and *in vivo* assays support a fundamental role for the monoterpene alcohol linalool (and other monoterpene alcohols) in resistance against *P. citricarpa* in orange fruit. In citrus-growing areas affected by CBS, the high concentrations of linalool accumulated during the first months after petal fall could explain the absence of pathogen colonization and expression of hard freckle and virulent spot symptoms at these first fruit developmental stages. Generally, typical hard spot symptoms appear in orange fruit at the colour changes (October and May in the northern and southern hemispheres, respectively), which coincides with a strong decrease in linalool and other monoterpene alcohols. The high D-limonene concentration in fruit in the July–September period in the northern hemisphere may also contribute, at least in part, to the maintenance of the pathogen in a quiescent stage. A higher accumulation of linalool and other monoterpene alcohols in mature AS fruit in comparison with mature EV fruit may be suitable to control CBS symptoms not only under laboratory conditions, but also in citrus orchards. It would be interesting to determine how the overexpression of a linalool synthase alone, or in combination with other monoterpene alcohol synthases, in citrus plants influences the *P. citricarpa*–citrus interaction.

Terpene volatiles are important in the interaction of different plant organs with microbes in nature, in either antagonistic or cooperative interplays and at multiple trophic levels (Junker and Tholl, 2013). Therefore, the results shown here encourage us to establish field experiments in CBS-affected areas to assess whether these AS sweet orange trees may represent an alternative control method of one of the most important citrus diseases worldwide, leading to a future decrease in the use of chemical fungicides.

EXPERIMENTAL PROCEDURES

Plant material

Orange transformants used in this study have been generated previously, and trees are being grown currently at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Villarreal, Spain (39°56′40.4″N, 0°08′11.0″W and elevation of 67 m) (Rodríguez *et al.*, 2011). Transgenic AS (AS1, AS5, AS7, AS11) and EV control orange fruit (*Citrus sinensis*) cv. 'Navelina' and 'Pineapple' were harvested at the mature stage (November to February in the Mediterranean region).

Fungal inoculation assays

During the maturity season, 20–30 fruit per independent line (AS or EV) were harvested for each experiment. A total of 141 fruit of EV and 140 fruit of AS were inoculated. Spore suspensions were prepared from 21-day-old cultures of *P. citricarpa* on PDA (Difco, USA) incubated in the dark at 25 °C. Spores were suspended in 2 mL of sodium chloride aqueous solution (NaCl, 0.85 w/v) and removed from sporulating colonies with a sterile pipette. Suspensions were adjusted to 10^6 conidia/mL (similar to Perryman *et al.*, 2014)).

Oranges were wounded with a 1-mm-wide and 2-mm-deep sterile tip and were inoculated by injecting 5 μL of the spore suspension. Four different rind sites around the equator of each fruit were inoculated. As a control for each experiment, 5 μL of an aqueous solution (NaCl, 0.85 w/v) was used to inject AS and EV oranges. Inoculated fruit were kept in sealed plastic trays in a security chamber at 25 °C and 80% relative humidity in a P-2 laboratory.

Fruit were evaluated for the severity of symptoms by measuring the lesion area (in mm^2) without considering the point of inoculation (1 mm^2). Samples from the fruit infected with *P. citricarpa* placed on glass slides and details of the fruit were photographed with a Leica MZ 16 stereomicroscope or a magnifying glass, respectively, equipped with a GFP-Plus Fluorescence module and a Leica DFC490 camera (Leica Microsystems, Germany).

Evaluation of volatile compounds on fungal growth *in vitro*

Mycelial growth

PDA medium was added to one side of a Petri dish, and a *P. citricarpa* mycelium disc of 0.5 cm in diameter of isolate LRS 25/98 was deposited on the medium surface. On the empty side of the plate, different concentrations of the compounds were added. Plates with dimethylsulfoxide (DMSO) were used as controls. The mycelial growth was evaluated as the mean diameter measured weekly and presented after 21 days. Plates were incubated at 25 °C, and at least 10 replicates per condition were performed. Experiments were conducted at least in duplicate.

Pycnidial production in citrus leaves

Water-agar medium (1.5%) was added to one side of a plate, and two EV orange 1-cm-diameter leaf discs, previously autoclaved, were placed on top. In the middle of the two leaf discs, a *P. citricarpa* mycelium disc of 0.5 cm in diameter of isolate LGMF6 was deposited (as in Baldassari *et al.*, 2009). On the other side of the plate, pure compounds were added. The plates were incubated at 25 °C for 30 days, and the presence of pycnidia was measured weekly. At least 10 replicates per condition were performed.

Extraction of volatiles and gas chromatography-mass spectrometry analysis

Flavedo pieces were ground in liquid nitrogen and frozen at −80 °C until extraction was performed, with pentane and 2-octanol as internal standards, as described previously (Rodríguez *et al.*, 2011).

RNA extraction

Total RNA was obtained from the peel of *P. citricarpa*-inoculated fruit using an RNeasy kit and cleaned up with an RNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions. RNA was treated with DNase I (RNase-Free DNase Set; Qiagen) for real-time qRT-PCR analysis and quantified using a NanoDrop spectrophotometer (NanoDrop 2000C; Thermo Scientific, USA).

Microarray analysis

Microarray assays were performed with the flavedo of two independent AS (AS1 and AS7) and EV transgenic 'Navelina' mature orange fruit, inoculated with *P. citricarpa*, comparing randomly selected AS vs. EV samples on the same slide with three biological replicates for each genotype. An Agilent Citrus (V4) Gene Expression 4x44K microarray, which contained 43 749 probes (60-mer oligonucleotides), was used for transcriptome analysis in a one-colour experimental design (Brazma *et al.*, 2001). Quality analysis for RNA, labelling, microarray hybridization, washing and analysis steps was performed as described previously (Gonzalez-Guzman *et al.*, 2012). Only features with ratio expression greater than two-fold and with $P < 0.05$ were selected after Student's *t*-test analysis with false discovery rate adjustment according to Benjamini and Hochberg (Gonzalez-Guzman *et al.*, 2012).

Functional classification of differentially expressed genes

VirtualPlant 1.3 (Katari *et al.*, 2010) and MapMan 3.6.0RC1 (Thimm *et al.*, 2004) software was used to categorize differentially expressed genes into GO categories according to their biological function.

Real-time qRT-PCR

A two-step RT-PCR was carried out to confirm the different expression of selected genes from microarray analyses. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, USA) following the manufacturer's instructions. Concentrations of primers were adjusted to 300 nM, and 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 40 ng of first-strand cDNA as template were used in a 20-μL reaction. Reactions were performed on MicroAmp Fast Optical 96-well reaction plates with barcode at 50 °C for 2 min, followed by 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a 7500 Fast Real-Time PCR system (Applied Biosystems). After PCR, a melting curve was generated to

confirm the absence of non-specific amplification. Fluorescence intensities were normalized against the ROX reference dye in each plate. A standard curve was generated with a five-fold dilution series of a cDNA sample and normalized to the citrus actin gene, as described previously (Rodríguez *et al.*, 2014), following the efficiency method (Pfaffl, 2001). Induction values of one-fold were assigned to the control sample at each time point. At least three independent biological replicates were used to quantify each transcript in each cDNA in AS and EV lines. Means ± standard error (SE) were calculated.

The selected genes for qRT-PCR from the global list of differentially expressed genes were *LOX4*, *LOX2*, *PAL2*, *OSM34*, *PR4*, *MYB*, *COA*, *LS3*, *CYT*, *LACS9* and *BCARYOP* for deregulated genes at 2 h, and *DREB26*, *JAZ10*, *MYB*, *BCAROT*, *PAL2*, *LS3*, *GER* and *BCARYOP* for deregulated genes at 8 days. The primers were designed with Primer3web v.4.0.0 software following the guidelines in Thornton and Basu (2011) based on the corresponding sequences available for the Agilent probes and compared with the sequences in the *Citrus sinensis* annotation project (http://citrus.hzau.edu.cn/orange/) (Table S1, see Supporting Information).

Statistical analysis

Data on lesion area, qRT-PCR and fungal growth inhibition were subjected to analysis of variance (ANOVA) using Statgraphics version 5.1 software (Manugistics, USA). Fisher's protected least significant difference (LSD) or Student's *t*-tests ($P < 0.05$) were carried out when appropriate to separate the means.

More details of these procedures are provided in Text S1.

Accession number

Microarray data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with the accession number GSE99295.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 *Phyllosticta citricarpa* infection in 'Navelina' antisense (AS) and empty vector (EV) control fruit in January. (a) Average measurements of the lesioned area (mm^2) at 28 days after inoculation of EV relative to AS1 and AS5 mature fruits. (b) Different disease severity in EV control fruit vs. AS fruit at 28 days after inoculation. (c) Microscopic green fluorescent protein (GFP) fluorescence of the strain of *P. citricarpa* used in the infection experiments under UV light. Data are presented as the means ± standard error (SE) and are derived from at least 20 fruit per line. Different letters indicate significant differences at $P < 0.05$ using Fisher's protected least significant difference (LSD) test.

Fig. S2 *Phyllosticta citricarpa* infection in 'Pineapple' transgenic antisense (AS) and empty vector (EV) control fruit in February. (a) Average measurements of the lesioned area (mm^2) at 28 days after inoculation of EV relative to AS11 mature fruits. (b) Different disease severity in EV control fruit vs. AS fruit at 28 days after inoculation. Data are presented as the means ± standard error (SE) and are derived from at least 20 fruit per line. Asterisk indicates significant difference at $P < 0.05$ using Student's *t*-test.

Fig. S3 Absence of lesions in a sodium chloride aqueous solution used as blank control in *Phyllosticta citricarpa* inoculation experiments in transgenic antisense (AS) and empty vector (EV) control 'Navelina' (a) and 'Pineapple' (b) fruit at 28 days after inoculation.

Fig. S4 MapMan metabolism overview showing commonly altered genes in regular antisense (AS) fruit relative to empty vector (EV) fruit without inoculation (referred to Rodríguez

et al., 2014). The colour scale indicates fold changes ranging from −6 to 6 of up- and down-regulated genes in red and blue, respectively.

Fig. S5 Hierarchical view of gene ontology (GO) biological categories significantly up-regulated in the flavedo of antisense (AS) fruit relative to empty vector (EV) control fruit at 2 h after inoculation with *Phyllosticta citricarpa*. The figure shows common differentially expressed genes in both AS lines relative to the EV control line. AS down-regulation of the D-limonene synthase gene causes the up-regulation of genes required for defence responses to fungi and bacteria and other phenylpropanoid metabolic processes. Significant categories (adjusted $P < 0.05$) are shown using colour scaling according to their significance level.

Fig. S6 Hierarchical view of gene ontology (GO) biological categories significantly up-regulated in the flavedo of antisense (AS) fruit relative to empty vector (EV) control fruit at 8 days after inoculation with *Phyllosticta citricarpa*. The figure shows common differentially expressed genes in both AS lines relative to the EV control line. AS down-regulation of the D-limonene synthase gene causes the up-regulation of genes required for defence responses to fungi and bacteria and other hormone stimuli. Significant categories (adjusted $P < 0.05$) are shown using colour scaling according to their significance level.

Fig. S7 Hierarchical view of gene ontology (GO) biological categories significantly down-regulated in the flavedo of antisense (AS) fruit relative to empty vector (EV) control fruit at 2 h after inoculation with *Phyllosticta citricarpa*. The figure shows common differentially expressed genes in both AS lines relative to the EV control line. AS down-regulation of the D-limonene synthase gene causes the down-regulation of genes required for monoterpenoid biosynthesis and other flavonoid metabolic processes. Significant categories (adjusted $P < 0.05$) are shown using colour scaling according to their significance level.

Fig. S8 Hierarchical view of gene ontology (GO) biological categories significantly down-regulated in the flavedo of antisense (AS) fruit relative to empty vector (EV) control fruit at 8 days after inoculation with *Phyllosticta citricarpa*. The figure shows common differentially expressed genes in both AS lines relative to the EV control line. AS down-regulation of the D-limonene synthase gene causes the down-regulation of genes required for mono- and sesquiterpenoid biosynthesis and other phenylpropanoid metabolic processes. Significant categories (adjusted $P < 0.05$) are shown using colour scaling according to their significance level.

Fig. S9 Exogenous application of the monoterpene linalool in oranges produces injuries in the peel. An incision was made in the peel of fruit simulating the inoculation process before the addition of 4.5 μg of the pure compound. (a, b) Front and lateral views, respectively. Photographs show the depressions produced in the peel and were taken 1 day after the addition of the compound.

Fig. S10 Volatile content changes during the maturation of antisense (AS) and empty vector (EV) fruit. The critical period of *Phyllosticta citricarpa* infection, in which it remains in a quiescent stage, ranges from June (just after petal fall) to September (just before colour change) in Mediterranean climatic conditions. (a) Linalool (μg/g). (b) Limonene (μg/g). (c) β-Citronellol (μg/g). (d) Nerol (μg/g). (e) Geraniol (μg/g). Blue and green lines correspond to AS and EV lines, respectively. Data are presented as the means ± standard error (SE) and are derived from at least five fruit per line.

Table S1 Differentially expressed genes in the mature flavedo of two independent transgenic antisense (AS) Navelina sweet orange plants [vs. two independent empty vector (EV) plants] 2 h after inoculation with *Phyllosticta citricarpa*.

Table S2 Differentially expressed genes in the mature flavedo of two independent transgenic antisense (AS) Navelina sweet orange plants [vs. two independent empty vector (EV) plants] 8 days after inoculation with *Phyllosticta citricarpa*.

Table S3 Primers designed for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) to evaluate the expression of genes selected from microarray analyses.

Text S1 (a) Specifications of *Phyllosticta citricarpa*. (b) Volatile organic compounds (VOCs) interfering with microorganisms. (c) Supplementary experimental procedures. (d) Supplementary references.

