Citrus phenylpropanoids and defense against pathogens. Part II: Gene expression and metabolite accumulation in the response of fruits to *Penicillium digitatum* infection

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Abstract

The effect of infection of *Citrus sinensis* (var. Navelina) fruits with *Penicillium digitatum* was studied at gene expression and metabolite levels. In this study, expression of genes involved in the phenylpropanoid pathway was studied in the flavedo (outer colored part of the peel) and albedo (inner white part) in response to pathogen infection. Results of the time-course experiment showed that maximal expression of 10 out of 17 phenylpropanoid genes analyzed occurred at 48 h post-inoculation, when decay symptoms started to appear, and mRNA levels either kept constant or decreased after 72 h post-inoculation. To further investigate the putative involvement of the phenylpropanoid pathway in the defense of citrus fruit, changes in the metabolic profile of both tissues infected with *P. digitatum* was studied by means of HPLC-PDA-FD. Metabolite accumulation levels along the time course suggest that flavanones, flavones, polymethoxylated flavones and scoparone are induced in citrus fruit in response to *P. digitatum* infection, although with different trends depending on the tissue.

Keywords

Albedo; *Citrus sinensis*; defense; flavedo; gene expression; infection; metabolic profiling; oranges; *Penicillium digitatum*; phenylpropanoids; polymethoxylated flavones; scoparone

1. Introduction

Penicillium digitatum is one of the most important postharvest diseases in citrus fruit. Currently, control of postharvest fungi is performed by the widespread use of synthetic fungicides, because they act quickly and effectively. However, the emergence of resistant strains and the growing public concern over health and environmental risks associated with the high use of pesticides in fruits have resulted in a high interest in developing alternative methods of control.

The peel of citrus fruits is a rich source of flavanones and many polymethoxylated flavones (PMFs), some of which are considered specific of citrus fruits such as naringenin and hesperidin (glycoside flavanones) and PMFs (A. M. Ortuño, Arcas, Benavente-García, & Del Río, 1999). The most important PMFs in citrus are tangeretin, sinensetin and heptamethoxyflavone (Nogata, Sakamoto, Shiratsuchi, Ishii, Yano, & Ohta, 2006). The concentration of these compounds is abundant in the peel of citrus fruit, whereas in the edible part of the fruit the levels are lower (Goulas & Manganaris, 2012; Lafuente, Ballester, Calejero, Zacarías, & González-Candelas, 2011). The concentration of phenylpropanoids depends on the citrus variety, fruit growth stage, and ripening degree of full size fruit (A. M. Ortuño, Arcas, Benavente-García, & Del Río, 1999).

Several studies have reported that flavonoids and PMFs are naturally synthesized by the fruit and may act as phytoanticipins and be involved in the natural defense of citrus fruit against pathogen infection (Arcas, Botía, Ortuño, & Del Río, 2000; Del Río, Gómez, Báidez, Arcas, Botía, & Ortuño, 2004; A. Ortuño, Báidez, Gómez, Arcas, Porras, García-Lidón et al., 2006). Moreover, citrus fruit can accumulate compounds, such as the coumarin scoparone, in

response to a pathogen attack that act as phytoalexins in the defense response of citrus fruit (Del Río, Gómez, Báidez, Arcas, Botía, & Ortuño, 2004; H. G. Kim, Kim, Lee, Park, Jeong, Kim et al., 2011; Kuniga & Matsumoto, 2006; A. Ortuño, Díaz, Alvarez, Porras, García-Lidón, & Del Río, 2011), which are also induced in response to elicitor treatments (Arcas, Botía, Ortuño, & Del Río, 2000; Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted; J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991).

A recent study has pointed out the involvement of isoprenoid, alkaloid and phenylpropanoid biosynthetic genes in the transcriptomic response of citrus fruit to *P. digitatum* infection (Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010). The first enzyme in the biosynthesis of phenylpropanoids is phenylalanine ammonia-lyase (PAL) and its involvement in the defense of citrus fruit against biotic and abiotic stresses has been previously reported (Ballester, Lafuente, & González-Candelas, 2006; Sánchez-Ballesta, Zacarías, Granell, & Lafuente, 2000). Moreover, some recent studies have addressed the importance of other genes involved in the phenylpropanoid pathway, such as O-methyltransferases (*OMTs*) and peroxidases (*POX*), in the induction of resistance of citrus fruits (Ballester, Lafuente, Forment, Gadea, De Vos, Bovy et al., 2011; Hershkovitz, Ben-Dayan, Raphael, Pasmanik-Chor, Liu, Belausov et al., 2011). However, the role of flavonoid-related genes in the defense reaction against pathogens in citrus fruit is poorly understood. There is also an important lack of knowledge about the metabolic pathway of phenylpropanoids and flavonoids and of its regulatory network in citrus fruit. Therefore, the objective of this work was to examine the changes in the expression of phenylpropanoid genes and of principal flavonoids occurring in

88 citrus fruit in response to *P. digitatum* infection and compare these changes with
89 those that take place in response to an elicitor treatment that triggers induced
90 resistance (Ballester, Lafuente, De Vos, Bovy, & González-Candelas,
91 submitted; Ballester et al., 2011). The study has been performed in the outer
92 (flavedo) and the inner white (albedo) parts of the peel since both tissues show
93 different susceptibility to infection and distinctive phenylpropanoid metabolite
94 profiles.
95
96 **2. Materials and methods**
97 *2.1. Plant and fungal material*
98 Navelina orange fruits (*Citrus sinensis* L. Obseck) were harvested from adult
99 trees grown in a commercial orchard in Lliria (Valencia, Spain) under normal
100 cultural practices and before any commercial postharvest treatment was
101 applied. Freshly harvested fruits were surface-sterilized with a 5% commercial
102 bleach solution for 5 min, rinsed with tap water, and dried at room temperature
103 until next day.
104 *Penicillium digitatum* (Pers.:Fr.) isolate PHI-26 (López-García, González-
105 Candelas, Pérez-Payá, & Marcos, 2000) was used in this study to infect the
106 fruits. Spore suspensions were prepared from 7 days old cultures on potato
107 dextrose agar incubated in the dark at 24 ºC. Spores were scrapped off from
108 the agar with a sterile spatula, transferred to sterile water, and the mycelia
109 fragments were removed by filtration through a nylon mesh. The concentration
110 of the spore suspension was determined with a haemocytometer and adjusted
111 to 10^6 conidia mL^{-1} by dilution with sterile water.
112

2.2. Orange inoculation with P. digitatum

Fruit inoculation with *P. digitatum* was conducted as described previously by Ballester, Lafuente, and González-Candelas (2006) with minor modifications. Fruits were wounded with a sterilized needle (5 mm in depth) and immediately inoculated by adding 10 µL of *P. digitatum* conidia suspension adjusted to 10^6 conidia mL^{-1} in order to synchronize the infection process (Sample I). Three replicates of 5 infected fruit with 12 wounds per fruit were placed on plastic boxes and incubated at 20 ºC and 90-95% relative humidity for 72 h. Wounded fruits that were mock-inoculated with 10 µL of sterile water (sample W) and also intact non-wounded fruits (sample NT) were used as controls. At either 24, 48 and 72 h post-inoculation (hpi), discs of 5 mm in diameter around the point of inoculation were sampled using a cork borer. Flavedo (F,) and albedo (A) tissues were separated with a scalpel, immediately frozen in liquid nitrogen, ground to a fine powder with a coffee mill, and stored at -80 °C until use for RNA isolation or phenolic compound extraction.

2.3. RNA extraction and Northern blot analysis

Total RNA was isolated from frozen tissues as described previously by Ballester, Lafuente, and González-Candelas (2006). RNA concentration was measured spectrophotometrically and the integrity was verified by agarose gel electrophoresis and ethidium-bromide staining.

Northern blot analysis, including cDNA synthesis and labeling, hybridization, quantification and normalization using 26S rDNA *C. sinensis* probe, was performed according to Ballester, Lafuente, and González-Candelas (2006). With few exceptions, for each gene, a value of 1.0 was assigned to the

normalized signal of non-treated flavedo (FNT) and the expression level of the rest of the samples was referred to it. After striping the blots, they were hybridized using the 28S rDNA *P. digitatum* probe. Probe design for the 17 phenylpropanoid genes analyzed has been described previously (Ballester et al., 2011).

2.4. Extraction of phenolic compounds and HPLC-PDA-FD analysis

Phenolic compounds were extracted from frozen ground flavedo and albedo tissues as described previously (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010; Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted). Standards used were the same as described in Ballester, Lafuente, De Vos, Bovy, and González-Candelas (submitted). Each result is the mean of at least two biological replicates ± standard deviation (SD).

3. Results and discussion

Navelina oranges were inoculated with a *P. digitatum* conidia suspension at a high concentration in order to synchronize the infection in the inoculated wounds and to collect the tissue for the time-course experiment at the same stage of infection. Using 10^6 conidia mL^{-1}, 100% of the wounds were infected by the fungus by day 3 (data not showed). Both flavedo and albedo tissues around the inoculation point were separated and used for RNA isolation and phenolic compound extraction.

3.1. Involvement of the phenylpropanoid pathway in the response of citrus fruit to P. digitatum infection.

We have examined changes in the expression of 17 genes specifically related to the phenylpropanoid pathway during infection of citrus fruit by *P. digitatum* using Northern blot hybridization (Fig. 1). Results of the time-course experiment showed that the expression of 10 genes encoding PAL (*PAL1*), cinnamate 4-hydroxylase (*C4H1*), isoflavone reductase (*IRL1*), different O-methyl transferases (*COMT1*, *CCoAOMT1*, *CCoAOMT2*), cinnamyl alcohol dehydrogenases (*CAD2*, *CAD3*), sinapyl alcohol dehydrogenase (*SAD*) and peroxidase (*POX1*) were induced in the flavedo in response to *P. digitatum* infection when compared to control or mock-inoculated fruits. Maximum expression of most of them was observed by 48 hpi, when the first symptoms of decay started to appear. Thereafter, their expression levels either increased (*IRL1*), remained nearly constant (*C4H1*, *COMT1*, *CAD3* and *SAD*) or decreased (*PAL1*, *CCoAOMT1*, *CCoAOMT2*, *CAD2 and POX1*). The highest inductions with respect to control fruits were detected in *COMT1* and *PAL1,* with 92- and 20- fold inductions, respectively. These 10 genes were also induced in the albedo, with the exception of *IRL1*. Furthermore, 3 more genes encoding a second cinnamate 4-hydroxylase (*C4H2*) a flavanone 3-hydroxylase (*F3H*) and another O-methyl transferase (*COMT2*) were induced in the inner white peel tissue. Interestingly, maximum expression levels in the albedo were found by 72 hpi, 24 hr later than in the flavedo.

In a previous report we have shown that the expression of these genes also increased in oranges exposed to an elicitor treatment that reduced disease development when fruits were exposed to a subsequent pathogen infection

(Ballester et al., 2011). It is important to note that for the majority of the genes induction levels in response to *P. digitatum* infection were higher than those induced by the elicitor treatment. As an example, *PAL1* expression was induced 2- and 5-fold in the flavedo and albedo of elicited fruits, respectively, whereas inductions reached 18- and 13-fold in the flavedo and albedo of *P. digitatum* infected fruits.

The highest gene expression values were detected in the infected flavedo, although the highest relative inductions in response to pathogen invasion were observed in general in the albedo, with the exception of *PAL1* and *IRL1*. These results reinforce previous findings indicating that the flavedo is more resistant than the albedo to *P. digitatum* infection (Afek, Orenstein, Carmeli, Rodov, & Joseph, 1999; Ballester, Izquierdo, Lafuente, & González-Candelas, 2010; Ballester et al., 2011; Ballester, Lafuente, & González-Candelas, 2006).

The expression of the phenylpropanoid genes was also analyzed in wounded and mock-inoculated (W) and in non-treated (NT) control fruits (Fig. 1). Overall expression levels in NT fruits were low with minor changes along the storage period. However, in wounded fruits there was a transient induction in the expression of *PAL1*, *C4H1*, *CCoAOMT2* and *CAD3* in the flavedo and/or albedo after 24 h of wounding, but such wound-induced responses were clearly lower than in the infected fruit. These results are in agreement with previous reports that indicate that *PAL1* and *C4H1* are also wound-inducible (Betz, McCollum, & Mayer, 2001; Marcos, González-Candelas, & Zacarías, 2005)

3.2. Quantification of phenolic compounds in healthy Navelina fruits

Flavonoids were isolated from the flavedo and albedo of healthy Navelina fruits and analyzed by HPLC coupled to a PDA and a FD (Fig. 2 and Table 1). The flavanone hesperidin was the most abundant flavonoid in the flavedo of non-treated Navelina oranges, followed by other flavanones such as narirutin and didymin, and the flavones isorhoifolin and diosmin. The external tissue also contained high amounts of the PMFs sinensetin, tetramethyl-O-scutellarein, heptamethoxyflavone, tangeretin, and nobiletin, as well as lower amounts of other PMFs. The albedo of healthy oranges contained similar levels of hesperidin and higher levels of narirutin and didymin, whereas levels of all PMFs were always lower than in the external tissue. Among the PMFs, the amount of sinensetin was the highest and that of hexamethyl-O-gossypetin the lowest in this tissue. These results are in concordance with the abundance of flavonoids reported in the peel of other citrus fruit (Nogata, Sakamoto, Shiratsuchi, Ishii, Yano, & Ohta, 2006), being the levels observed in Navelina higher than those found in Navelate oranges (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted). The amounts of almost all the identified phenylpropanoid and flavonoid compounds decreased slightly or were constant during fruit storage at 20 ºC in both flavedo and albedo tissues (Fig. 3). In general, the major decline in the levels of these metabolites was detected after 48 h of storage.

3.3. Metabolic profiling in P. digitatum-infected oranges

The accumulation of PMFs along the development of *P. digitatum* infection was different in the flavedo and albedo tissues (Fig. 3A). They slightly decreased, in general, at early stages of infection in the flavedo but increased thereafter.

Thus, by 72 hpi the level of PMFs were higher in the flavedo of infected fruits than in either control or wounded fruits. This response of Navelina flavedo tissue to *P. digitatum* infection agrees with that observed in *Citrus unshiu* Marc. fruit peel infected with the same pathogen (H. G. Kim et al., 2011). Levels of PMFs also decreased in this cultivar at the early stages of infection and showed a transient increase thereafter. The late increase in PMFs indicates that their synthesis mainly occurs after major increases in the expression of flavonoid genes were detected (48 hpi) (Fig. 1).

A different pattern of PMFs accumulation was observed in the albedo of infected fruits (Fig. 3A). In general, the levels of the PFMs decreased in infected fruits in the internal tissue. However, the levels of isosinensetin and hexamethyl-O-gossypetin rose drastically at the latest stages of *P. digitatum* infection. Only the pattern of tangeretin accumulation, which increased transiently by 48 hpi, was similar in both tissues. Thus, the overall pattern of flavonoids accumulation in the albedo did not parallel the induction of phenylpropanoid genes. Although wounding also led to an increase in the levels of PMFs in the flavedo by 48 h, the major metabolite content in the flavedo was observed at 72 hpi in infected fruits. Moreover, *in vitro* studies have revealed that some PMFs, such as nobiletin and tangeretin, are able to reduce the radial growth of *P. digitatum* (Arcas, Botía, Ortuño, & Del Río, 2000; A. Ortuño et al., 2006), being even more active against other citrus pathogens such as *Phytophthora citrophthora* (Del Río, Gómez, Báidez, Arcas, Botía, & Ortuño, 2004). Therefore, results of the present work reinforce the idea that these compounds may act as fungitoxins to control pathogen infection.

It is well known that O-methylation is an important step in the synthesis of PMFs (Ibrahim, Bruneau, & Bantignies, 1998), however the key genes involved in their synthesis are still unknown. Results of the present work show that genes encoding the O-methyl transferases *COMT1*, *CCoAOMT1* and CCoAOMT2 may be relevant in this process since their induction preceded the most important increases in the synthesis of PMFs in the flavedo of infected fruits. Nevertheless, global results indicate that the induction of other genes may be important since some PMFs increased in the flavedo but not in the albedo though the expression of these genes increased in both tissues.

HPLC analysis also revealed the presence of other phenylpropanoid and flavonoid metabolites in infected orange fruits with a similar pattern of accumulation in both tissues (Fig. 3B). The levels of 3 metabolites, chlorogenic acid, didymin and scoparone, increased during the development of the pathogen. The results obtained for chlorogenic acid are in agreement with previous results obtained in tomato fruit, in which the levels of this compound increased in response to *Alternaria alternata* infection acting as a phytoanticipin that was able to inhibit spore germination of the pathogen in *in vitro* assays (Ruelas, Tiznado-Hernández, Sánchez-Estrada, Robles-Burgueño, & Troncoso-Rojas, 2006). These findings suggest that cinnamic acid derivatives might participate in the defense response of citrus fruit against pathogen infection.

Among the other flavonoids, only the concentration of diosmin in the flavedo, narirutin in the albedo, and didymin in both tissues increased by 72 hpi. Levels of these compounds were, in general, higher than the observed for the PMFs. However, whether any of these compounds has antimicrobial activity is not known. On the other hand, *in vitro* analyses have shown that PMFs are able to

284 reduce the *in vitro* growth of *P. digitatum* (Arcas, Botía, Ortuño, & Del Río,
285 2000; A. Ortuño et al., 2006). This inverse relation between flavanones,
286 flavones and PMFs levels present in the fruits and their susceptibility to the
287 fungus *P. digitatum* has been previously described by A. Ortuño, Díaz, Alvarez,
288 Porras, García-Lidón, and Del Río (2011). Flavones and PMFs are mainly
289 accumulated in the flavedo, whereas flavanones are mainly located in the
290 albedo. Taking into account the present results and the fact that the flavedo is
291 more resistant to *P. digitatum* infection, we may speculate that flavones and
292 PMFs confer the condition to the flavedo to be more resistant, whereas the
293 albedo, which contains lower levels of these compounds, is more prone to
294 infection.
295 Scoparone (6,7-dimethoxycoumarin) is the main phytoalexin associated with
296 resistance of citrus fruits against pathogens such as *P. digitatum* or *Botrytis*
297 *cinerea* (J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991; Kuniga &
298 Matsumoto, 2006; Venditti, Molinu, Dore, Agabbio, & D'Hallewin, 2005). High
299 levels of scoparone were observed in infected Navelina oranges, whereas this
300 compound was not detected in healthy fruit (Fig. 3B). The levels of scoparone
301 detected in the flavedo (14.8 µg g^{-1}) were higher than those detected in the
302 albedo (5.3 µg g^{-1}). However, these values are distant from the median effective
303 dose for the inhibition of germ tube elongation of *P. digitatum* spores (J. J. Kim,
304 Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991).
305 Interestingly, this compound was induced to a much higher level by elicitor
306 treatments that increased citrus fruit resistance against *P. digitatum* than by
307 fungal infection (Ballester, Lafuente, De Vos, Bovy, & González-Candelas,
308 submitted; J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991). Thus,

309 scoparone reached 90.5 µg g^{-1} in the flavedo of elicited Navelate oranges
310 (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted), a level
311 higher than the median effective dose that can explain the higher resistance of
312 the elicited tissue to *P. digitatum* infection. This coumarin has been considered
313 a good marker of induced resistance in citrus fruits due to its accumulation after
314 different elicitor treatments such as UV light (Rodov, Ben Yehoshua, Kim,
315 Shapiro, & Ittah, 1992) or antagonistic yeasts (Arras, 1996; Droby, Vinokur,
316 Weiss, Cohen, Daus, Goldschmidt et al., 2002). As mentioned before in elicited
317 fruits (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted),
318 there is a good correlation between *COMT1* expression and scoparone
319 induction, although a direct link between both is still lacking. We have shown
320 that the levels of 2 other fluorescent compounds, citrusnin A and drupanin
321 aldehyde, increased in response to the elicitor treatment (Ballester, Lafuente,
322 De Vos, Bovy, & González-Candelas, submitted). However, none of these
323 compounds have been detected in response to *P. digitatum* infection (data not
324 showed).
325 It is well known that the effectiveness of the defense response depends on the
326 timing and amplitude of the activation (Pozo, van Loon, & Pieterse, 2004). The
327 results of the present work indicate that the fruit is able to activate the defensive
328 barriers when the pathogen is recognized. However, the fruit is susceptible to
329 infection and the outcome of the interaction is the disease known as green
330 mold. So, such responses are not either quick or strong enough to deter the
331 development of *P. digitatum*. An active suppression of defense responses by *P.*
332 *digitatum* could also explain the failure of the fruit to contain the pathogen. This
333 suppression capability of *P. digitatum* has already been demonstrated for the

induction of PAL (Lisker, Cohen, Chalutz, & Fuchs, 1983) and reactive oxygen species (Macarisin, Cohen, Eick, Rafael, Belausov, Wisniewski et al., 2007) in response to *P. digitatum* infection. In this context it is interesting to note that although the expression of phenylpropanoid-related genes is induced to a higher level in response to *P. digitatum* infection than in elicited fruits (Ballester et al., 2011), the accumulation of most of the flavonoids analyzed is higher in elicited fruits (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, submitted), a fact that suggests that *P. digitatum* is able to downregulate the defense responses of the fruit. However, the mechanisms by which *P. digitatum* subverts citrus fruit defenses and whether this suppression is mediated by effectors is currently unknown.

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Figure Captions

Fig. 1. Accumulation of mRNAs from phenylpropanoid and flavonoid genes in the flavedo and albedo of Navelina oranges during the time course experiment. Non-treated fruits (□), mock-inoculated wounded fruits (△) and infected with *P. digitatum* (●) were stored at 20 ºC for 0, 24, 48 and 72 h. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rDNA *C. sinensis* signal. Normalized values of mRNAs accumulation in arbitrary units are represented using the non-treated flavedo as a reference. Gene codes: (*FPal1*) phenylalanine-ammonia lyase, (*C4H1*) cinnamate 4-hydroxylase, (*C4H2*) cinnamate 4-hydroxylase, (*4CL*) 4-coumarate-Coa ligase, (*CitF3H*) flavanone 3-hydroxylase, (*IRL1*) isoflavone reductase, (*COMT1*) caffeic acid 3-O-methyltransferase 1, (*COMT2*) caffeic acid O-methyltransferase, (*COMT3*) catechol O-methyltransferase, (*CCoAOMT1*) caffeoyl-CoA O-methyltransferase, (*CCoAOMT2*) caffeoyl-CoA O-methyltransferase, (*CAD1*) cinnamyl alcohol dehydrogenase, (*CAD2*) cinnamyl alcohol dehydrogenase, (*SAD*) sinapyl alcohol dehydrogenase, (*POX1*) peroxidase, (*POX2*) peroxidase, *Citrus sinensis* 26S rDNA, and *Penicillium digitatum* 26S rDNA.

Fig. 2. HPLC-PDA chromatogram of flavonoids isolated from the healthy non-treated flavedo (A; FNT0) and albedo (B; ANT0). The character above the individual peaks indicates the compound number: (1) chlorogenic acid, (2) caffeic acid, (3) eriocitrin, (4) narirutin, (5) eriocitrin, (6) diosmin, (7) hesperidin, (8) scoparone, (9) didymin, (10) isosinensetin, (11) hexamethyl-O-scutellarein, (12) sinensetin, (13) hexamethyl-O-quercetagenin, (14) nobiletin, (15)

tetramethyl-O-scutellarein, (16) heptamethoxyflavone, and (17) tangeretin. The star * indicates that the scoparone was only detected in infected tissue.

Fig. 3. Concentrations (µg g^{-1} fresh weight) of the main flavonoids identified in the flavedo and albedo of Navelina orange fruits during the time course experiment. *P. digitatum* infected fruits (●), mock-inoculated fruits (△) and non-treated fruits (□) were stored immediately at 20 ºC for 0, 24, 48 and 72 h. (A) Concentration of the polymethoxylated flavones: isorhoifolin (ISO), hexamethyl-O-gossypetin (HMG), sinensetin (SNT), hexamethyl-O-quercetagetin (HMQ), nobiletin (NBT), tetramethyl-O-scutellarein (TMO), heptamethoxyflavone (HPM), tangeretin (TNG) in Navelina oranges. Values of TMO represent the area of the peak in the chromatogram in mAU s. (B) Concentration of chlorogenic acid (CHA), narirutin (NRT), hesperidin (HSP), didymin (DID), scoparone (SCO), cinnamic acid (CA), isorhoifolin (IRF), diosmin (DSM), eriocitrin (ERI) in orange fruits. Note the different scale for each compound. The results represent the mean of at least two biological replicates of 10 fruits each. Asterisc in HMQ indicates that values represent the area (mAU s) of the peak in the chromatogram.

Table 1. Contents (µg g^{-1} fresh weight) of the main flavonoids identified in the healthy flavedo and albedo tissues of *C. sinensis* var. Navelina orange fruits.

Compound	**Concentration (µg g^{-1} fresh weight)**	
	Flavedo	**Albedo**
CINNAMIC ACID DERIVATIVES		
1 Chlorogenic acid	83.6 ± 22.1	31.2 ± 1.6
2 Caffeic acid	165.6 ± 28.8	nd
FLAVANONES		
3 Eriocitrin	nd	40.7 ± 18.0
4 Narirutin	125.1 ± 12.0	921.1 ± 42.9
7 Hesperidin	3856.6 ± 398.3	3201.1 ± 232.5
9 Didymin	108.0 ± 1.6	481.3 ± 23.3
FLAVONES		
5 Isorhoifolin	587.6 ± 5.3	nd
6 Diosmin	nd	142.5 ± 68.4
PMFs:		
10 Isosinensetin	26.2 ± 0.9	3.8 ± 0.0
11 Hexamethyl-O-gossypetin	3.7 ± 0.5	0.7 ± 0.1
12 Sinensetin	659.1 ± 1.1	102.3 ± 8.2
13 Hexamethyl-O- quercetagetin*	1370.8 ± 275.7	305.5 ± 15.7
14 Nobiletin	136.8 ± 3.4	22.6 ± 2.8
15 Tetramethyl-O-scutellarein	474.5 ± 13.0	92.4 ± 9.4
16 Heptamethoxyflavone	178.9 ± 6.3	54.5 ± 13.3
17 Tangeretin	136.4 ± 7.5	25.1 ± 2.6

* Values represent the area (mAU s) of the peak in the chromatogram.
nd: non-detected
Results represent the mean of at least two biological replicates ± SD.
Compound no. 8 is scoparone, not detected in healthy fruits.
PMFs: polymethoxylated flavones

Figure1

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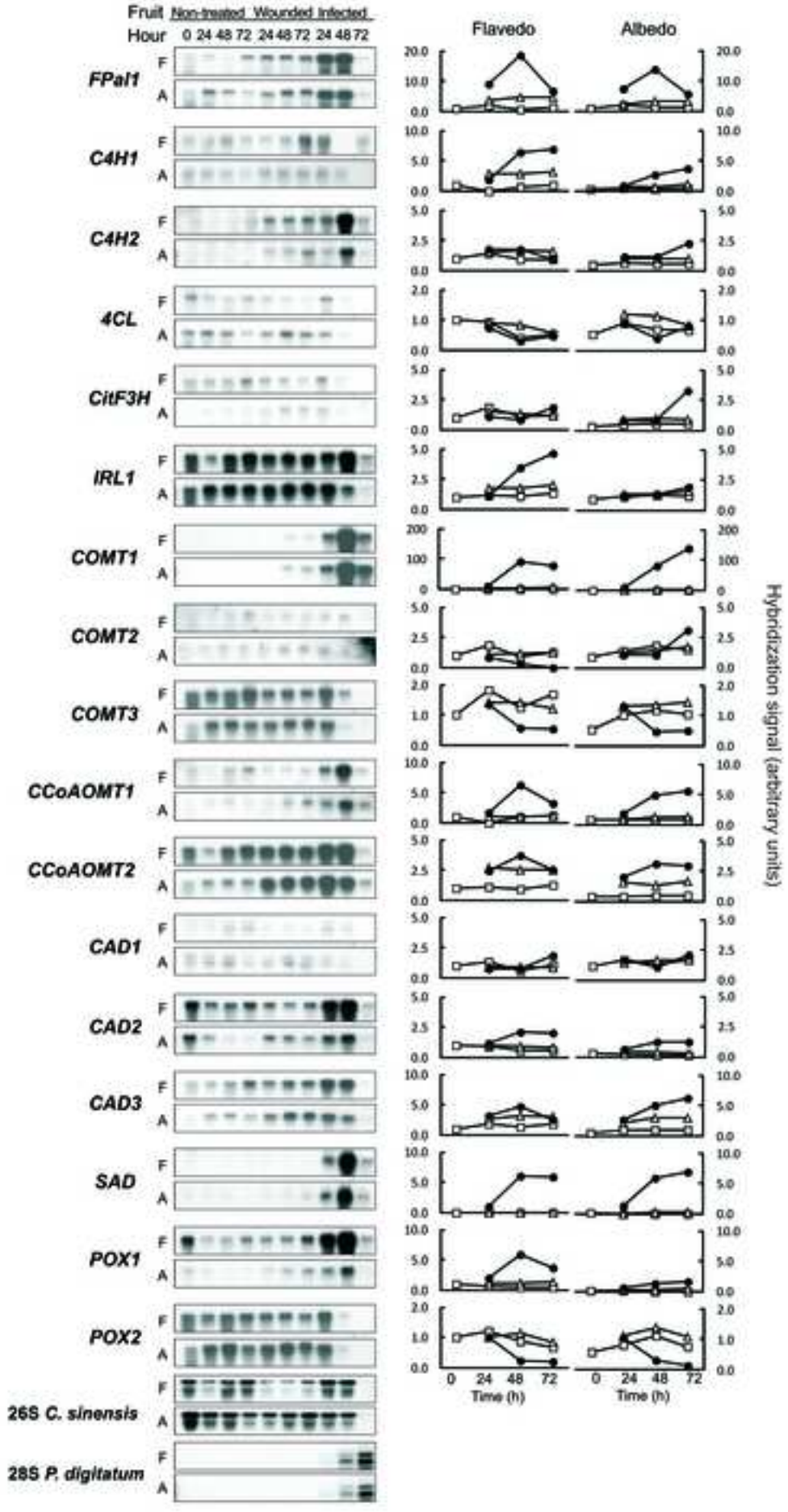

Figure2

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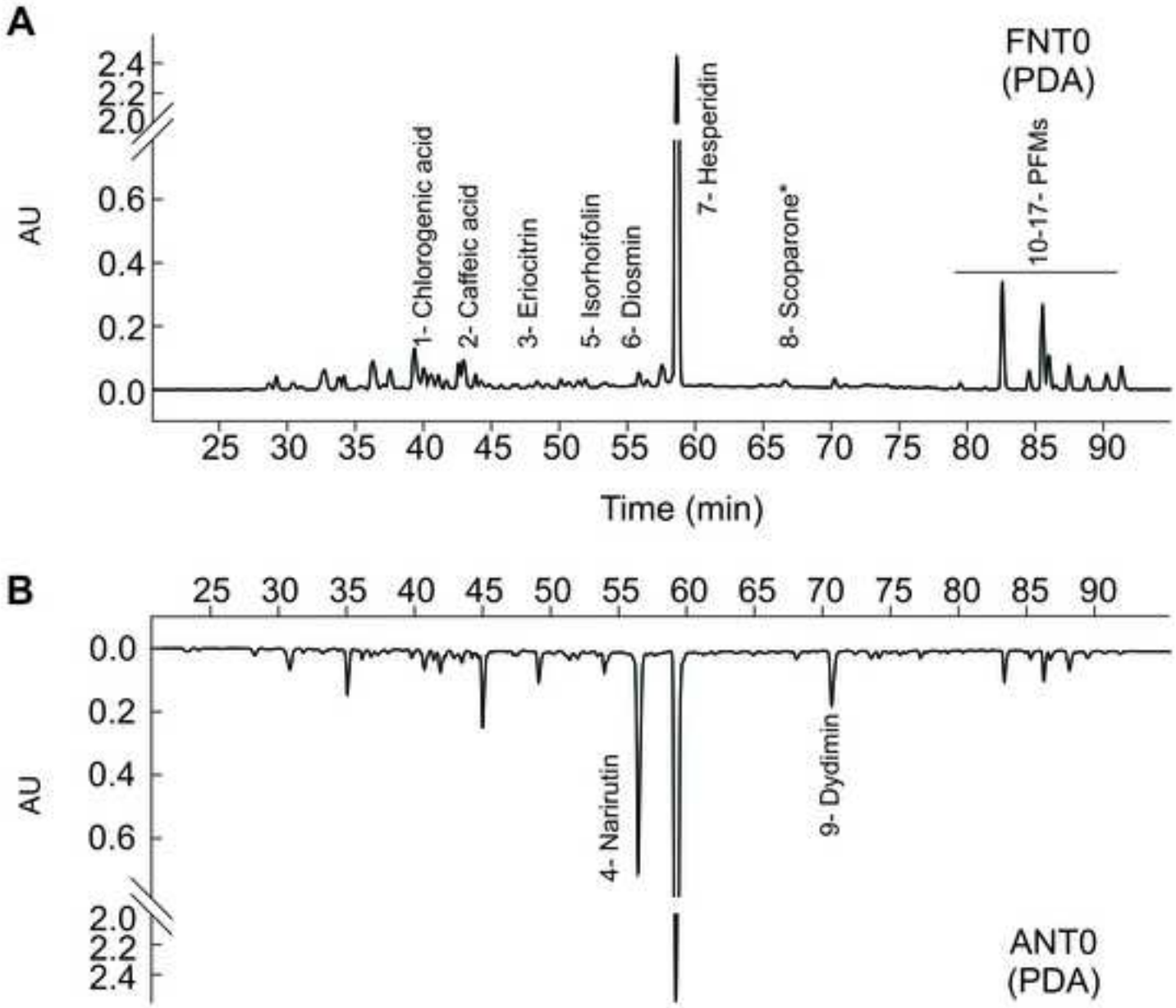

Figure3

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