Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in citrus fruit

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ABSTRACT

To get an insight into the mechanisms underlying resistance of citrus fruit against *Penicillium digitatum*, we have analyzed at the enzyme activity and gene expression levels the possible involvement of phenylalanine ammonia-lyase (PAL), peroxidase, β-1,3-glucanase and chitinase in the flavedo (outer colored part of the fruit peel) and albedo (the inner white part) in elicited fruit. As a tool to induce resistance, we inoculated oranges with *P. digitatum* and 1 day later fruits were exposed to a hot air treatment at 37ºC for 3 days. Our results showed that all enzyme activities increased in parallel with increased resistance, especially in the albedo, although the highest activities were generally found in the flavedo. Expression of the PAL encoding gene and that of the genes coding for the basic, rather than for the acidic, isoforms of the PR proteins were also induced in both tissues, but most remarkably in the albedo.

KEYWORDS: chitinase; citrus fruit; gene expression; glucanase; induced resistance; infection; *Penicillium digitatum*; peroxidase; phenylalanine ammonia-lyase; PR proteins; scoparone

INTRODUCTION

Green and blue mold rots, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively, are the most important postharvest diseases of citrus fruit grown under Mediterranean climate conditions. Both pathogens are necrotrophs that require wounds to enter the fruit through the flavedo. Although synthetic fungicides have a major role in reducing postharvest losses due to fungal decay, problems associated with their widespread use as well as the increasing awareness of their associated health and environmental risks have promoted the search for new and safer alternatives. Besides these concerns, the proliferation of resistant strains is a serious risk to the effectiveness of fungicides. Additionally, the number of wholesalers and final retailers that require produce with lower chemical residue levels than official requirements is growing steadily (WTO, 2009).

Increasing the fruit's natural defense capabilities through induction of resistance is one of the alternative strategies being investigated as a means to reduce the use of chemical fungicides during postharvest handling and storage of fruits and vegetables. In fully mature citrus fruit, increased resistance against *P. digitatum* infection can be achieved by application of physical (Kim et al., 1991; Ben Yehoshua et al., 1992; Rodov et al., 1992; Droby et al., 1993; Arcas et al., 2000), chemical (Porat et al., 2001; 2002; Venditti et al., 2005), or antagonistic microorganisms treatments (Arras, 1996; Fajardo et al., 1998; Droby et al., 2002). The efficacy of these treatments in eliciting induced resistance is variable, and in many instances depends on the maturity stage of the fruit. Although understanding the mechanisms underlying induced resistance would help to improve the development of this alternative strategy, our knowledge of the biochemical and molecular bases of induced resistance is very poor. In only a few studies have the induction of pathogenesis-related (PR) proteins,

synthesis of phytoalexins and β-1,3-glucanase and chitinase gene expression been analyzed in the context of induced resistance in citrus fruit, as discussed below.

UV irradiation, hot water and biocontrol yeast elicitation of fruit resistance is accompanied by induction of the PR proteins chitinase and β-1,3-glucanase (Porat et al., 1999; 2001). However, Fajardo et al. (1998) did not find induction of PR proteins in the flavedo of oranges treated with different biological derived elicitors and then inoculated with *P. digitatum*. Lignification and accumulation of phenolic compounds have also been associated with the resistance of citrus fruit to *P. digitatum* infection (1991; Angioni et al., 1998; 1999; Ortuño et al., 2006). The levels of the antifungal scoparone in the flavedo of citrus fruit rose to fungicidal levels in pathogen-challenged fruit that were subsequently subjected to a heat treatment, but pathogen infection did not promote such an increase (Kim et al., 1991). Induction of this citrus phytoalexin has also been observed in UV-irradiated fruit (Rodov et al., 1992) or after elicitation of resistance by antagonistic yeasts (Arras, 1996; Droby et al., 2002). Therefore, scoparone has been considered a good marker of induced resistance in citrus fruit.

PAL and peroxidase activities are induced in grapefruit after elicitation of resistance by UV irradiation (Droby et al., 1993) or by the biocontrol yeast *Candida oleophila* (Droby et al., 2002). PAL is the first enzyme in the phenylpropanoid pathway, from which scoparone and scopoletin are synthesized. On the other hand, peroxidases play a key role at a later stage in the pathway during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against multiple pathogens, and may alter the antioxidant ability of citrus fruit to cope with *Penicillium* infection (Ballester et al., 2006). In this regard, it is noteworthy that both enzymes have been suggested to play a role in the defense response of citrus fruit against *P. digitatum* (Ballester et al.,

2006), although their transcriptional regulation during development of induced resistance in citrus fruit remains unknown. In addition, previous studies indicate that the albedo is more susceptible to *P. digitatum* infection than the external flavedo (Kavanagh and Wood, 1967; Ballester et al., 2006), which suggests that defense responses triggered in the inner tissue should be more critical to resistance. Nevertheless, the responses of the albedo to elicitors that increase resistance to pathogen attack have barely been studied (1999; Venditti et al., 2005).

The objective of the present work was to analyze gene regulation and enzyme activities of PAL, peroxidase, β-1,3-glucanase and chitinase in flavedo and albedo tissues of oranges that had been previously inoculated with *P. digitatum* and 1 day later were cured for 3 days at 37 ºC (Kim et al., 1991). This treatment was selected because of its high reproducibility and efficacy in eliciting the induction of the phytoalexin scoparone, which, as mentioned before, is considered a good marker of induced resistance in citrus fruit.

MATERIALS AND METHODS

Plant and fungal material

Mature oranges (*Citrus sinensis* L. Obseck) from the cultivar 'Navelate' were harvested from adult trees grown at Lliria, Valencia, Spain and processed the same day. Fruit were surface-sterilized with a 5% commercial bleach solution for 5 min, extensively washed with tap water and allowed to dry at room temperature until next day.

Penicillium digitatum (Pers.: Fr.) Sacc. isolate PHI-26 used in this study was obtained from a rotten orange and cultured on potato dextrose agar (Difco, Detroit, USA) plates at 24ºC (López-García et al., 2000). Conidia from a 7-day-old culture were collected

by scraping them with a sterile spatula and transferred to sterile water. Conidia suspension was then filtered and the concentration determined with a haemocytometer.

Induction of resistance treatment

Fig. 1 represents a schematic diagram indicating tissue sampling and pathogen inoculation for each treatment. Fruit were wounded by making punctures (approximately 3 mm in depth) with a sterilized nail and inoculated with 10 μL of a *P. digitatum* conidial suspension adjusted to 10^5 conidia per mL. Inoculated fruit were arranged in plastic boxes and maintained at 90-95% relative humidity (RH) and 20ºC for 1 day to allow pathogen development. Then, fruit were heat-treated at 37ºC for 3 days under water-saturated conditions (curing) in order to stop the progress of the pathogen. After the heat treatment, fruit were maintained at 20ºC and 90-95% RH. At 3 days post-treatment (dpt), 7 days from the beginning of the experiment, peel tissue discs of 13 mm in diameter around the inoculation site were sampled by using a cork borer (Sample I). Control inoculations without pathogen were carried out by injecting 10 μL of sterile water and holding the fruit under the same conditions (Sample W). An additional control consisted of intact non-wounded fruit held at 20ºC for 1 day and then at 37ºC for 3 days (Sample C). With this experimental design we can asses independently the effect of the pathogen and the wound response. Moreover, tissue from non-treated fruit was obtained the first day of the experiment (Sample NT). Flavedo and albedo tissues were separated with a scalpel. Tissue discs obtained from 15 fruits (8 discs per fruit) were immediately frozen in liquid nitrogen, mixed and grounded to a fine powder with a coffee mill and stored at -80ºC until further analysis.

To asses the effectiveness of the induction of resistance treatment, disease susceptibility was analyzed both at the beginning of the experiment in non-treated fruit and at 3 dpt for the remaining treatments. Each fruit was punched at a distance of 0.5 cm from the previous wound or in the equatorial axis in fruit that had not been previously inoculated. Thereafter, 10 µL of a suspension of *P. digitatum* containing 10^4 conidia per mL were inoculated into each wound. This lower inoculum level was selected to avoid a very rapid disease progression that could mask differences among treatments. Inoculated fruit were arranged separately on plastic boxes and maintained at 90-95% RH and 20ºC for 6 days. The incidence (percentage of lesions) and the severity (maceration area, in cm^2) of the infection were assessed at 6 days post-inoculation (dpi). The experimental design consisted of 3 replicates of 5 fruits, with 4 wounds per fruit, for each treatment. To test the effect of the treatments, a one-way analysis of variance (ANOVA) was performed. Means were separated using the Tukey's Honestly Significant Difference test at $p<0.05$. The analysis was preformed with Statgraphics Plus 5.1 Software (Manugistics, Inc.).

Extraction and quantification of scoparone

Phenolic compounds were extracted from 100 mg of tissue in 2 mL tubes containing five 1.2 mm steel beads and 500 µL of 80% methanol with the aid of a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). Samples were extracted twice at 4ºC for 1 min, centrifuged at 11600 × *g* for 10 min at 4ºC to eliminate cell debris and the supernatants were filtered through 0.45 µm polytetrafluoroethylene (PTFE) Millipore filters. Samples were immediately analyzed by HPLC using a Waters liquid chromatography system equipped with a Waters 600 quaternary pump, a Waters 474 fluorescence detector, a Waters 2996 photodiode array detector and the Empower

Software (Waters). A Luna C18 (Phenomenex, Inc.) column (250 x 4.6 mm, 5 µm) coupled to a guard column µBondapak C18 (Waters) (10 µm) was used and phenolic compounds were separated at 35ºC using a binary gradient of water (A), brought to pH 2.5 with phosphoric acid, and acetonitrile (B). The initial solvent composition consisted of 99% A and 1% B. The solvent composition changed in a linear gradient to 70% A and 30% B during 60 min. During the next 45 min (105 min running time), the solvent composition was changed to 1% A and 99% B, and then kept for 10 min. Initial conditions were reestablished in 1 min and the column was reequilibrated for 24 min before the next injection. The flow rate was 0.8 mL per min and the injection volume was 20 µL. Phenolics were detected by fluorescence at excitation and emission wavelengths of 313 and of 405 nm, respectively, and by setting the photodiode array detector to scan from 200 to 400 nm. Scoparone was identified by comparison of the spectrum and retention time with an authentic standard and quantified by peak area comparison using a standard curve. Each result is the mean of 3 replicate samples. Tukey's test was performed to identify significant differences between samples at $p<0.05$.

RNA extraction and Northern analysis

Total RNA was isolated from the flavedo and the albedo tissues according to Ballester et al. (2006). RNA (10 µg per lane) was electrophoresed through a formaldehyde gel and blotted onto a Hybond-N^+ membrane (GE Healthcare). cDNA probes were labeled with $\alpha(^{32}P)$-dATP by using the Strip-EZ™ PCR kit (Ambion, Inc.). Blots were hybridized overnight at 42ºC in Ultrahyb™ Ultrasenstive Hybridization buffer (Ambion, Inc.) and then washed once at 55ºC for 15 min in 2X SSC and 0.1% SDS (w/v) and twice in 0.1X SSC and 0.1% SDS (w/v) at 55ºC for 30 min. Blots were

quantified by exposure to a BAS MP 2040 Imaging Plate (Fuji Photo Film Co., LTD.), scanned with a FLA-3000 laser scanner (Fuji Photo Film Co., LTD.) and quantified with the software ImageGauge V4.0 (Science Lab 98, Fuji Photo Film Co., LTD.). After stripping off the probe following the instructions in the Strip-EZ^{TM} PCR kit, membranes were rehybridized with different probes. Hybridization signals were normalized according to the *C. sinensis* 26S rRNA signal intensity in each sample.

Probes used for Northern hybridization were obtained from different cDNA libraries generated previously in our group. A full-length cDNA clone, *FPal1* (GeneBank Acc. No. AJ238753), was isolated from the flavedo of 'Fortune' mandarins stored at 1ºC (Sánchez-Ballesta et al., 2000). A full-length cDNA encoding an acidic class III β-1,3-glucanase (*CrglcQ*, Acc. No. AY971953) was isolated from the same library (Sánchez-Ballesta et al., 2006). A cDNA corresponding to the basic β-1,3-glucanase *gns1* gene isolated by McCollum et al. (1999) (Acc. No. AJ000081) was obtained from the RindPdig24 cDNA library, which is derived from 'Clemenules' mandarins infected with *P. digitatum* (Acc. No. CX298651) (Forment et al., 2005). These two β-1,3-glucanase encoding genes shared an 82% identity at the amino acid level. Two different cDNAs encoding a basic extracellular peroxidase (Acc. No. CX297694, designated *pox1*) that shares a 70% identity with the *Citrus maxima POD1* gene and an acidic one (Acc. No. CX299378, designated *pox2*) were isolated from the cDNA libraries RindPdig24 and FlavCurFr1, respectively. The latter library was obtained from heat-treated 'Fortune' mandarins (Forment et al., 2005). Comparison of the nucleotide sequences revealed that these two cDNAs shared low homology (less than 47%). Two different cDNAs encoding chitinases were isolated from the same two libraries. The first one (Acc. No. CX299099) was obtained from RindPdig24 and showed 99% identity with the basic chitinase encoding gene *chi1* isolated by Porat et

al. (2001) (Acc. No. AF090336). The second chitinase gene, designated *chi2*, was obtained from FlavCurFr1 (Acc. No. CX298387) and shares 95% identity with a class II acidic chitinase gene isolated by Osswald et al. (1994). The *C. sinensis* 26S rDNA (Acc. No. AJ969115) and the *P. digitatum* 28S rDNA (Acc. No. AJ969116) probes were obtained previously by Ballester et al. (2006).

DNA fragments used for probe labeling were obtained by PCR using the corresponding plasmids as templates and the primers FOR17 (5'-GTT TTC CCA GTC ACG AC-3') and REV17 (5'-CAG GAA ACA GCT ATG AC-3'). PCR conditions were 94ºC for 3 min, then 94ºC for 30 s, 56ºC for 45 s and 72ºC for 2 min for 35 cycles, followed by a final elongation step of 10 min at 72ºC. DNA fragments were purified with the High Pure PCR Product Purification kit (Roche Applied Sciences) and quantified by measuring the fluorescence with the Ribogreen® dye (Molecular Probe, Inc.).

Enzyme assays

All enzyme assays were determined from 3 replicate samples using a Hewlett Packard 8452A spectrophotometer connected to a thermostatized water bath. Crude extracts were obtained with a Mini Beadbeater 8 Cell Disruptor (Biospec Productor, Inc.) in 2 mL tubes containing five 1.2 mm steel beads. Samples were extracted twice at 4ºC for 1 min with a specific buffer and centrifuged at 11600 x *g* for 10 min at 4ºC to eliminate cell debris. The supernatant was used to analyze enzyme activities.

PAL, β-1,3-glucanase and chitinase activities were extracted from acetone powder. Five grams of frozen tissue were ground in 50 mL of acetone, previously chilled at -20ºC. The homogenate was filtered through a Buchner funnel, the residue washed

once with cold acetone and the resulting powder dried for 2 h at room temperature. The acetone powder was stored at -80ºC until use for enzymatic assays.

PAL (EC 4.3.1.24) activity was extracted as previously described by Ballester et al. (2006) from acetone powder. The reaction mixtures (2 mL) contained 660 µL of the partially purified enzyme and 200 µL of 100 mM L-phenylalanine. PAL activity was determined by measuring the absorbance of cinnamic acid at 290 nm over a period of 2 h at 40ºC. Enzyme activity was expressed as nmoles of cinnamic acid per g of acetone powder tissue per h.

Enzyme extraction and enzymatic assays for peroxidase (EC 1.11.1.7) activity were conducted as described by Ballester et al. (2006). Enzyme activity was expressed as the increase in absorbance at 470 nm per min per g of fresh weight tissue.

β-1,3-glucanase (EC 3.2.1.6) activity was measured using the colorimetric assay based on the hydrolysis of laminarin (Abeles and Forrence, 1970). The enzyme was extracted from 25 mg of flavedo or albedo acetone powder with 1.5 mL of 100 mM potassium phosphate buffer, pH 7.0, including 3% polyvinylpyrrolidone (PVPP). The reaction mixture contained 10 µL of the supernatant, 215 µL of 100 mM sodium acetate, pH 5.0, and 75 µL of 2% laminarin. The mixture was incubated at 50ºC for 1 or 2 h for the flavedo and albedo, respectively. The reaction was stopped by adding 600 µL of 1% dinitrosalicylic reagent and heating the tubes for 5 min at 100ºC. Absorbance of the samples was measured at 540 nm. A standard curve was constructed using glucose and the activity expressed as µmoles of glucose per h per g of acetone powder.

Chitinase (EC 3.2.1.14) activity assay was based on the protocol described by Fajardo et al. (1998) with minor modifications. Acetone powder (15 mg) was homogenized with 1.5 mL of 20 mM potassium phosphate buffer, pH 7.0, containing 3% PVPP and

the enzyme was extracted by cell disruption and centrifugation. The assay mixture contained 20 µL of the supernatant, 15 µL of 2 M sodium acetate buffer, pH 5.0, 150 µL of carboxymethyl-chitin-remazol brilliant violet and 415 µL of H_2O. Reaction mixtures were incubated at 37ºC for 15 min. The reaction was stopped by adding 150 µL of 1 N hydrochloric acid and the mixture was immediately cooled on ice for 10 min. Absorbance of samples was measured at 550 nm. Enzyme activity was expressed as the increase in absorbance at 550 nm per min per g of acetone powder.

Statistics

Enzyme activity values were the mean of 3 replicate samples ± SD. Data were further subjected to analysis of variance followed by Tukey´s test with Statgraphics Plus 5.1 Software (Manugistics, Inc.). Differences at $p<0.05$ were considered significant.

RESULTS

Induction of resistance in citrus fruit against *P. digitatum* and scoparone levels

Although previous reports have shown that the combination of pathogen challenge followed by a curing treatment elicited a high level of the phytoalexin scoparone in the peel of citrus fruit, nothing is known about the effectiveness of this treatment increasing fruit's resistance to a subsequent pathogen attack. Our results showed that this treatment (treatment I) significantly reduced both disease incidence and severity (Table 1). Wounded-cured fruit also showed a statistically significant, but lower, reduction in disease intensity. In contrast, fruit only subjected to the heat treatment at 37ºC for 3 days (C) showed the highest values of disease incidence and severity.

Scoparone concentration was determined in the flavedo and albedo tissues. As shown in Table 2, scoparone was not detected in non-treated fruit and its concentration was

very low in the flavedo and albedo of cured oranges. However, substantial amounts of scoparone were detected in the flavedo, and to a lower extent in the albedo, of infected-cured fruit. The level of scoparone in wounded-cured fruit also increased, but not as much as in response to the infection-curing treatment.

FPal1 gene expression and PAL activity in elicited fruit

The accumulation of *FPal1* mRNA and PAL activity were studied in the flavedo and albedo tissues of fruit subjected to the treatment that induced resistance (Fig. 2A). Hybridization with the *C. sinensis* ribosomal probe allowed normalization of *FPal1* gene expression values, whereas the lack of hybridization signal with the *P. digitatum* ribosomal probe indicated that the pathogen did not progress through the tissue in fruit that had previously been inoculated with the pathogen (treatment I). In control non-treated fruit, *FPal1* gene expression was only detected in the flavedo and the highest expression was found in infected-cured fruit. In the albedo, expression was only detected in this latter treatment. Thus, the observed induction with respect to non-treated fruit is more relevant in this inner tissue.

The pattern of PAL activity was similar in flavedo and albedo, although the activity in non-treated fruit was almost 10-fold higher in the flavedo (Fig. 2B). The highest activity was observed in both tissues in infected-cured fruit. PAL activity was 16-fold higher in the flavedo of these fruits as compared with that of non-treated oranges, whereas in the albedo the induction was 70-fold. PAL activity was also induced in response to the wounding-curing treatment, albeit to a lower extent, reaching a 6- and 15-fold induction as compared with non-treated fruit in the flavedo and albedo, respectively. On the other hand, curing did not cause any induction of PAL activity.

Peroxidase gene expression and activity in elicited fruit

The involvement of two novel different peroxidase genes previously isolated from 'Clemenules' mandarins infected with *P. digitatum* (*pox1*) and from heat-treated 'Fortune' mandarins (*pox2*) in the induction of resistance against *P. digitatum* in 'Navelate' oranges has been investigated. Northern analyses using both genes as probes revealed different patterns of expression (Fig. 3A). The accumulation of *pox1* and *pox2* mRNAs in the flavedo of non-treated fruit was higher than in the albedo. In both tissues, the highest level of *pox1* gene expression was detected in infected-cured fruit. Wounding and subsequent heat-treatment slightly induced the expression of *pox1* mRNA in the flavedo, but not in the albedo. However, the heat-treatment alone resulted in a lower accumulation of the transcript in the flavedo. On the other hand, *pox2* expression level decreased in both tissues in all treatments as compared with non-treated fruit.

Changes in soluble and insoluble peroxidase activities in the flavedo and albedo tissues are shown in Fig. 3B. The most noticeable difference observed between the two activities in both tissues is the low overall level of soluble peroxidase activity in the albedo, which in non-treated fruit was 8–fold lower than in the external tissue and 16-fold lower than its insoluble peroxidase activity counterpart. The trend of changes in soluble and insoluble peroxidase activities was similar in the flavedo. Curing alone did not significantly modify either activity. However, wounded-cured fruit showed an increase in both peroxidase activities, which reached the highest values in infected-cured fruit. In the albedo, the highest soluble peroxidase activity was also detected in infected-cured fruit, although it was 6-fold lower than in the external tissue of fruit subjected to the same treatment. On the other hand, insoluble peroxidase activity in the albedo remained quite unaltered in all treatments.

β-1,3-glucanase gene expression and activity in elicited fruit

The expression patterns of *gns1* and the larger *CrglucQ* transcript were similar (Fig. 4A). For both genes, the expression in the flavedo was higher than in the albedo, and the maximum induction was detected in infected-cured oranges in both tissues. However, the induction of *gns1* expression was higher than that of the larger *CrglucQ* transcript in both tissues. A lower induction of both genes was also observed in the flavedo of wounded-cured fruit, but their expression did not increase in the albedo.

The overall pattern of β-1,3-glucanase activity was similar in flavedo and albedo (Fig. 4B), although the activity in the flavedo of non-treated fruit was almost 5 times higher than in the underlying albedo. The highest activity was observed in cured fruit previously infected with *P. digitatum*. The induction level with respect to control non-treated fruit was slightly higher in the inner tissue, about 5-fold induction. A lower induction, 1.4-fold, was also found in both tissues in wounded-cured fruit, but heat-treatment alone did not induce β-1,3-glucanase activity.

Chitinase gene expression and activity in elicited fruit

The expression of two different chitinase genes isolated from 'Clemenules' mandarins infected with *P. digitatum* (*chi1*) or from heat-stressed 'Fortune' mandarins (*chi2*) was analyzed (Fig. 5A). Both genes showed different patterns of expression. The highest *chi1* transcript accumulation was observed in infected-cured oranges, being the expression in the albedo lower than in the flavedo. An important increase in *chi1* gene expression was also detected in the flavedo of wounded-cured fruit, but not in the albedo. However, *chi2* expression was only induced in the flavedo of wounded-

cured fruit. It is interesting to note that the expression of the two *chi* gene was negligible in the albedo of cured or wounded-cured fruit.

Chitinase was the only studied enzyme whose activity was higher in the albedo than in the flavedo of non-treated fruit (Fig. 5B). An increase in this activity was observed in the flavedo and albedo of infected-cured fruit, being higher in the flavedo. A slight increase was also found in the flavedo of wounded-cured fruit, but there were no significant differences in the albedo. On the other hand, curing itself did not induce the activity or even led to a slight decrease.

DISCUSSION

Increasing fruit's natural resistance to pathogens is one of the alternatives being explored in the effort to reduce the dependency on chemical fungicides. Although many efforts have been invested in the practical implementation of induced resistance in mature citrus fruit against *P. digitatum* infection, we have only gathered partial information on the mechanisms responsible for this process, as no comprehensive study has been conducted. Induced resistance has been associated with the *novo* synthesis of phytoalexins and induction of the PR proteins β-1,3-glucanase and chitinase in the flavedo of elicited fruits. However, a detailed study of induced resistance in citrus fruit including analysis of enzyme activities, genes and metabolites has not been addressed yet. Moreover, the possible role of the most susceptible albedo tissue in the induction of resistance remains to be investigated.

Kim et al. (1991) showed that the inoculation of mature lemons with *P. digitatum* followed 24 h later by a heat treatment increased the concentration of the phenolic compound scoparone to fungicide levels in the flavedo. However, the effectiveness of this treatment in eliciting increased resistance to a subsequent pathogen infection was

not investigated. The present study demonstrates that this treatment decreased the susceptibility of orange fruit to a subsequent *P. digitatum* infection (Table 1). The curative effect of high temperature in the form of either hot water dips or curing has been previously reported in citrus and tomato fruits (Brown et al., 1978; Lurie et al., 1997; Nafussi et al., 2001). In agreement with these findings, we have found that none of the 'Navelate' oranges inoculated with *P. digitatum* and 1 day later cured at 37ºC for 3 days under water-saturated conditions developed infection (data not shown). However, in spite of the high curative effect of this treatment, curing *per se* was not a preventive treatment, as cured fruit showed a higher disease incidence than non-treated fruit (Table 1). These results reinforce the idea that the curing treatment might be an eradicative tool for incipient infections, but also point out the higher susceptibility of cured fruit to a subsequent pathogen infection that may occur during handling of the fruit after the heat treatment. Likewise, the combination of wounding and high temperature may induce disease resistance at a close distance from the wounding site, but to a lower extent than the combination of pathogen challenge and curing. The fact that pathogen infected-cured fruit developed higher resistance than wounded-cured fruit suggests that elicitors produced either directly or indirectly by the pathogen are able to trigger a defense response strong enough to increase significantly the resistance of the fruit to a subsequent pathogen attack, although, as mentioned above, the fungus was not able to colonize the host tissue.

The present study shows that the highest activity of almost all the enzymes analyzed, was detected in the flavedo of infected-cured orange fruit, except for the chitinase activity, which was higher in the inner tissue. The same fact was observed at the gene expression level, as mRNA accumulation was higher in the flavedo of elicited oranges, but induction was higher in the albedo. These results reinforce the idea that

the albedo is less resistant to *P. digitatum* infection (Kavanagh and Wood, 1967; Afek et al., 1999; Ballester et al., 2006). However, it is noteworthy that this inner tissue showed the highest induction level of PAL, soluble peroxidase and β-1,3-glucanase activities. Therefore, it seems plausible to hypothesize that the activation of a combined group of defense responses in the albedo is necessary to deter *P. digitatum* infection.

PAL is the first committed enzyme in the pathway leading to the biosynthesis of phenolic compounds, such as scoparone, in higher plants. The involvement of PAL in the defense of citrus fruit against *Penicillium* has been suggested by examining changes in *PAL* gene expression in response to infection (Marcos et al., 2005; Ballester et al., 2006) or in PAL activity in fruit exposed to treatments such as UV light (Droby et al., 1993) and chemical or biological elicitors (Fajardo et al., 1998) that induce resistance against this pathogen. However, this is the first work reporting the expression pattern of this important gene during elicitation of induced resistance in citrus fruit. Our results showed that the highest levels of *FPa1l* gene expression and PAL enzymatic activity occurred in infected-cured fruit (Fig. 2). This induction might be directly related to the lower susceptibility of elicited oranges fruit against a subsequent *P. digitatum* infection. Among all the enzyme activities analyzed, PAL is the one that showed the largest induction in response to the treatments that increase the fruit's resistance. This induction was highest in the albedo, reaching a 70-fold induction in infected-cured fruit with respect to control non-treated fruit. Moreover, this result suggests that induction of PAL might be partially responsible for the highest scoparone levels found in the flavedo and albedo of infected-cured fruit (Table 2). Interestingly, wounding followed by the curing treatment led to a 13% and 33% reduction in disease incidence with respect to control and cured fruits,

respectively (Table 1). Although scoparone levels in the flavedo and albedo of wounded-cured fruit were 10 and 40 fold lower, respectively, than in the same tissues of infected-cured fruit (Table 2), disease incidence was only 10% higher than in infected-cured fruit and no significant differences in disease severity were observed (Table 1). This lack of correlation between disease resistance and scoparone levels, which has been classically considered a marker induced resistance in citrus fruit (Kim et al., 1991; Ben Yehoshua et al., 1992; Droby et al., 2002; Venditti et al., 2005), suggests that induced resistance must also rely on other factors.

Our results suggest that, besides PAL, soluble peroxidase might contribute to the beneficial effect of the infection-curing treatment reducing disease incidence of a subsequent infection as the highest activity was found in the flavedo and the albedo of infected-cured fruit. In concordance with this idea, soluble peroxidase activity also increased, but to a lower level, in response to wounding-curing, a treatment that also increased fruit's resistance. In this regard, it is noteworthy that peroxidase activity increases in citrus fruit elicited by UV irradiation (Droby et al., 1993) and in response to *P. digitatum* infection (Ballester et al., 2006). We cannot rule out the participation of insoluble peroxidase, which has been related to cell wall lignification, in the induced resistance, but this enzyme appears not to be as relevant as soluble peroxidase since no significant difference in this activity was found between wounded-cured fruit and the infected-cured ones in spite of the higher efficacy of the latter treatment. Overall changes in peroxidase activity seem to be more related to induction of *pox1* gene expression than to *pox2*, as the former gene, which codes for a basic isoform, parallels peroxidase activity.

β-1,3-glucanases and chitinases can potentially degrade the fungal cell wall, mainly composed by β-1,3-glucan and chitin (van Loon et al., 2006). PR proteins have a dual

cellular localization: acidic proteins are extracellular, whereas basic proteins are located into vacuoles. Generally, β-1,3-glucanase and chitinase basic isoforms have more inhibitory effect on pathogens than acidic isoforms (Joosten et al., 1995). Interestingly, we have found that the expression of *gns1*, *chi1* and *pox1*, all coding for basic isoforms, increased in response to the infection-curing treatment, whereas the acidic enzyme-encoding genes *chi2* and *pox2* were not induced. *CrglcQ*, coding for an acidic β -1,3-glucanase, showed a particular behavior, as treatments that led to an increase in disease resistance induced the accumulation of the larger transcript but had no effect on the smaller one. It is important to note that all the genes coding for basic isoforms were isolated from a cDNA library derived from mandarin fruits infected with *P. digitatum* and that their pattern of expression in response to the treatment that elicited fruit resistance was similar to that of changes in the activity of their respective enzymes. Therefore, they might play a role delaying fungal growth and decreasing the incidence of the infection caused by *P. digitatum* in citrus fruit. Chitinase and β-1,3-glucanase proteins were also induced in the flavedo of citrus fruit treated with a biocontrol yeast (Droby et al., 2002). It is also noteworthy that the expression of *gns1* increased in response *P. digitatum* infection, wounding, ethylene or to treatments that increase the resistance to *P. digitatum*, such as UV-irradiation, biocontrol agents, jasmonic acid or β-amino butyric acid (Porat et al., 2002).

In summary, this work presents a comprehensive approach to analyze simultaneously the biochemical and molecular mechanisms underlying induced resistance in citrus fruit against *P. digitatum*. Our data on gene expression and enzymatic activities in the two tissues that compose the citrus rind suggest that PAL and soluble peroxidase are involved in the induction of resistance of citrus fruit against *P. digitatum* infection. Moreover, basic β-1,3-glucanase and chitinase isoforms, but not their acidic

counterparts, may play a role increasing the fruit's defense capabilities. Finally, the response deployed by the albedo correlates better than that of the flavedo with the observed increase in disease resistance in citrus fruit, a fact that has been overlooked in previous studies.

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FIGURE LEGENDS

Fig. 1. Flow chart of the experimental design. Solid arrows indicate the temperature and duration of the incubation period. The induction of resistance treatment (panel A) consisted of fruit inoculated with *P. digitatum* and then incubated for 1 day at 20ºC before being transferred at 37ºC for 3 days to stop pathogen progress. At the end of this heat treatment, fruit were maintained at 20ºC. After 3 days of incubation at 20ºC (3 dpt), tissue samples were taken from 15 fruits and other 15 oranges were inoculated with *P. digitatum* to asses the effectiveness of the treatment. Infection was allowed to progress for 6 days, when disease incidence and severity were determined. Mock control fruit were wounded and inoculated with sterile water (panel B). Additional control non-wounded and heated fruit were also included (panel C). Control non-treated fruit (NT) were sampled at the beginning of the experiment (panel D). Letters in panels indicate when samples were taken for analysis. Inoculation with *P. digitatum* is indicated as Pdig.

Fig.. 2. Accumulation of *FPal1* mRNA (A) and PAL activity (B) in the flavedo and albedo of elicited 'Navelate' oranges. Intact oranges were incubated 1 day at 20ºC followed by 3 days at 37ºC (C). Wounded fruit were inoculated with either water (W) or a *P. digitatum* spore suspension (I) and incubated for 24 h at 20ºC and 3 days at 37ºC. At 3 days post-treatment peel tissue discs of 13 mm in diameter around the inoculation site were sampled by using a cork borer. Tissue from non-treated fruit (NT) was obtained the first day of the experiment. (A) Relative accumulation (R. A.) of *FPal1* mRNA in arbitrary units, using the non-treated flavedo tissue as a reference. Normalization was carried out with respect to the hybridization signal of the *C. sinensis* 26S rDNA probe. (B) Enzyme activity data represent the average of 3

replicates samples ± SD. Different letters in the same tissue indicate significant differences among treatments according to Tukey's test with a *p*-value of 0.05.

Fig. 3. Effect of the induction of resistance treatment on the accumulation of *pox1* and *pox2* mRNAs (A) and soluble and insoluble peroxidase activities (B) in flavedo and albedo of elicited 'Navelate' oranges. Intact oranges were incubated for 1 day at 20ºC and 3 days at 37 ºC (C). Wounded fruit were inoculated with either water (W) or a *P. digitatum* spore suspension (I) and incubated for 24 h at 20ºC and 3 d at 37ºC. At 3 days post-treatment peel tissue discs of 13 mm in diameter around the inoculation site were sampled by using a cork borer. Tissue from non-treated (NT) fruit was obtained the first day of the experiment. (A) Relative accumulation (R. A.) of *pox1* and *pox2* mRNAs in arbitrary units, using the non-treated flavedo tissue as a reference. Normalization was carried out with respect to the hybridization signal of the *C. sinensis* 26S rDNA probe. (B) Enzyme activity data represent the average of 3 replicates samples ± SD. Different letters in the same tissue indicate significant differences among treatments according to Tukey's test with a *p*-value of 0.05.

Fig. 4. Accumulation of *gns1* and *CrglucQ* mRNAs (A) and β-1,3-glucanase activity (B) in flavedo and albedo of elicited 'Navelate' oranges. Wounded fruit were inoculated with either water (W) or a *P. digitatum* spore suspension (I) and incubated for 1 day at 20ºC and 3 days at 37ºC. Intact oranges were maintained 1 day at 20ºC and 3 days at 37ºC (C). At 3 days post-treatment peel tissue discs of 13 mm in diameter around the inoculation site were sampled by using a cork borer. Tissue from non-treated fruit (NT) was obtained the first day of the experiment. (A) Relative accumulation (R. A.) of *gns1* and *CrglcQ* mRNAs in arbitrary units, using the non-

treated flavedo tissue as a reference. Normalization was carried out with respect to the hybridization signal of the *C. sinensis* 26S rDNA probe. (B) Enzyme activity data represent the average of 3 replicates samples ± SD. Different letters in the same tissue indicate significant differences among treatments according to Tukey's test with a *p*-value of 0.05.

Fig. 5. Effect of the induction of resistance treatment on the accumulation of *chi1* and *chi2* mRNAs (A) and chitinase activity (B) in flavedo and albedo of elicited 'Navelate' oranges. Intact fruit were maintained 1 day at 20ºC and 3 days at 37ºC (C). Wounded fruit were inoculated with either water (W) or a *P. digitatum* spore suspension (I) and incubated for 24 h at 20ºC and 3 d at 37ºC. At 3 days post-treatment peel tissue discs of 13 mm in diameter around the inoculation site were sampled by using a cork borer. Tissue from non-treated fruit (NT) was obtained the first day of the experiment. (A) Relative accumulation (R. A.) of *chi1* and *chi2* mRNAs in arbitrary units, using the non-treated flavedo tissue as a reference. Normalization was carried out with respect to the hybridization signal of the *C. sinensis* 26S rDNA probe. (B) Enzyme activity data represent the average of 3 replicates samples ± SD. Different letters in the same tissue indicate significant differences among treatments according to Tukey's test with a *p*-value of 0.05.