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Citrus phenylpropanoids and defence 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 coloured 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 analysed 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 defence 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.

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 (Ortuño, Arcas, Benavente-García, & Del Río, 1999). The most important PMFs in citrus are tangeretin, sinensetin and heptamethoxyflavone (Nogata et al., 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 (Ortuño et al., 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 defence of citrus fruit against pathogen infection (Arcas, Botía, Ortuño, & Del Río, 2000; Del Río et al., 2004; Ortuño 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 defence response of citrus fruit (Del Río et al., 2004; Kim et al., 2011; Kuniga & Matsumoto, 2006; Ortuño et al., 2011), which are also induced in response to elicitor treatments (Arcas et al., 2000; Ballester, Lafuente, De Vos, Bovy, & González-Candelas, 2013; 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 defence 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

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involved in the phenylpropanoid pathway, such as O-methyltransferases (*OMTs*) and peroxidases (*POX*), in the induction of resistance of citrus fruits (Ballester et al., 2011; Hershkovitz et al., 2011). However, the role of flavonoid-related genes in the defence 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 citrus fruit in response to *P. digitatum* infection and compare these changes with those that take place in response to an elicitor treatment that triggers induced resistance (Ballester et al., 2013, 2011). The study has been performed in the outer (flavedo) and the inner white (albedo) parts of the peel since both tissues show different susceptibility to infection and distinctive phenylpropanoid metabolite profiles.

2. Materials and methods

2.1. Plant and fungal material

Navelina orange fruits (*Citrus sinensis* L. Obseck) were harvested from adult trees grown in a commercial orchard in Lliria (Valencia, Spain) under normal cultural practises, before any commercial postharvest treatment was applied. Freshly harvested fruits were surface-sterilised with a 5% commercial bleach solution for 5 min, rinsed with tap water, and dried at room temperature until next day.

P. digitatum (Pers.:Fr.) isolate PHI-26 (López-García, González-Candelas, Pérez-Payá, & Marcos, 2000) was used in this study to infect the fruits. Spore suspensions were prepared from 7 days old cultures on potato dextrose agar incubated in the dark at 24 °C. Spores were scrapped off from the agar with a sterile spatula, transferred to sterile water, and the mycelia fragments were removed by filtration through a nylon mesh. The concentration of the spore suspension was determined with a haemocytometer and adjusted to 10^6 conidia ml^{-1} by dilution with sterile water.

2.2. Orange inoculation with P. digitatum

Fruit inoculation with *P. digitatum* was conducted as described previously by Ballester et al. (2006) with minor modifications. Fruits were wounded with a sterilised 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 synchronise 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 °C 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 et al. (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 labelling, hybridisation, quantification and normalisation using 26S rDNA *C. sinensis* probe, was performed according to Ballester et al. (2006). With few exceptions, for each gene, a value of 1.0 was assigned to the normalised 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 hybridised using the 28S rDNA *P. digitatum* probe. Probe design for the 17 phenylpropanoid genes analysed 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 et al., 2013). Standards used were the same as described in Ballester et al. (2013). 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 synchronise 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 hybridisation (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, three 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 h 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 two and fivefold in the flavedo and albedo of elicited fruits,