Interrelation between ABA and phospholipases D, C and A_2 in early responses of citrus fruit to *Penicillium digitatum* infection

María T. Lafuente[1,*], Ana-Rosa Ballester[1], Nely Holland[1,2], Jordi Cerveró[1], Paco Romero[1]

[1]Department of Food Biotechnology, Instituto de Agroquímica y Tecnología de Alimentos, (IATA-CSIC), Av. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[2]Current address: Departamento de Nutrição – CCS – Universidade Federal do Rio Grande do Norte, CEP: 59012-570 – Natal – RN, Brazil

*Corresponding author: María T. Lafuente, mtlafuente@iata.csic.es

Telephone: +34-963900022; FAX: +34-963636301

Ana Rosa Ballester: ballesterar@iata.csic.es

Nely Holland: nely@ufrnet.br

Jordi Cerveró: Jordi_ce_mo@hotmail.com

Paco Romero: promero@iata.csic.es

ABSTRACT

We investigated whether phospholipases play a role in citrus fruit susceptibility to be infected by *Penicillium digitatum,* and whether a connection exists between hormone abscisic acid (ABA) and phospholipases in the citrus fruit–*P. digitatum* interaction. Changes in both the activity of enzymes PLD, PLC and PLA_2 and the expression of a set of genes encoding them in response to infection in Navelate (*Citrus sinensis* (L.) Osbeck) orange and its ABA–deficient mutant Pinalate, which is less resistant to infection, were compared. The results showed the activation of PLD and PLC in infected Navelate fruit before disease development, and this activation was attenuated in the mutant, which suggests that both enzymes play a protective role in citrus fruit to cope with *P. digitatum* infection and the participation of ABA in their regulation. The transcriptional analyses further demonstrated a differential activation of various phospholipases–encoding genes by the fungus. Of the *CsPLD* genes (***CsPLDα, CsPLDβ, CsPLDδ, CsPLDγ, CsPLDζ***), the fungus had a stronger effect on ***CsPLDγ*** and ***CsPLDζ***. This is the first report to suggest the participation of a ***PLDζ*** isoform in the plant–microbe interaction, and to indicate that this gene may be modulated by ABA in response to infection. The results also revealed that the *CsPLC* isoforms encoding both non–specific PLC (NPC) and phosphoinositide–specific PLCs (PI–PLC) may participate in the citrus fruit–*P. digitatum* interaction, and that ABA action occurs upstream of *CsPI-PLC* gene activation in infected citrus fruit. The changes induced by the fungus in PLA_2 activity and gene expression were less relevant.

KEYWORDS

Abscisic acid–deficiency; fungal disease; green mold; phospholipase isoforms; resistance to infection.

45 1. INTRODUCTION

46 The rot rates of citrus fruit loss during postharvest handling and storage leads to major
47 economic losses. Postharvest green mold disease caused by the necrotrophic fungus *P.*
48 *digitatum* (Pers.:Fr.) Sacc. is the most serious infection for the citrus fruit grown under
49 Mediterranean conditions. The use of chemical fungicides is a widespread method to prevent
50 infections by this pathogen, although the demand for safe and effective alternative control
51 methods (Palou et al., 2016) is growing due to consumers' safety concerns and the ability of
52 *P. digitatum* to become resistant against common fungicides (Sánchez–Torres and Tuset,
53 2011). Therefore, it is necessary to better understand the mechanisms of citrus fruit resistance
54 against this fungus.

55 Upon pathogen attack, infected plant cells induce signalling molecules to initiate
56 mechanisms in surrounding cells to reduce pathogen spread. Significant progress has been
57 made in the characterisation of the enzymes and genes participating in oxylipins biosynthesis
58 in plant and fruit defence responses against pathogens (Blée, 2002; Wasternack and Hause,
59 2013). However, a potential regulator site, the initial hydrolysis step that mobilises fatty acid
60 precursors from membrane lipids, has been less characterised. Such mobilisation may be
61 catalysed by phospholipases A_2 (PLA_2) (Dhondt et al., 2000), which can act in coordination
62 with phospholipases D (PLD) and C (PLC) in defence signalling (Wang, 2005; Meijer and
63 Munnik, 2003; Hong et al., 2016). PLD hydrolyses the phosphodiester bond on the head
64 group side of phospholipids to produce soluble head groups and second lipid messenger
65 phosphatidic acid (PA), which, in turn, can be degraded by PLA_2 (Kirik and Mudgett, 2009).
66 On the other hand, PLC hydrolyses the phosphodiester bond on the glycerol side of
67 phospholipids to produce a phosphorylate head group and diacylglycerol (DAG), which is a
68 well–documented lipid messenger in animals. However, its signalling function in plants is

still an open question. Intracellular PLCs in plants can be divided into non–specific PLCs (NPC), which act on common phospholipids like phosphatidylcholine (PC), and phosphatidylethanolamine (PE) and phosphoinositide–specific PLCs (PI–PLC), which hydrolyse phosphoinositides.

Numerous reports indicate that the activity of enzymes PLD, PLC and PLA_2 rises in response to diverse abiotic and biotic stresses, and that different isoforms of the genes encoding these enzymes possess distinguishable subcellular localisation and functions in plants and fruit (Ryu, 2004; Alférez et al., 2008; Romero et al., 2013, 2014; Hong et al., 2016; Shuai et al., 2020). Many studies demonstrate the involvement of different gene isoforms in plant responses defences against pathogens (Zhao et al., 2013; Hong et al., 2016), and how phospholipases are widely distributed in diverse organisms, including pathogenic fungi (Ghannoum, 2000; Hong et al., 2016). Fewer studies are available about fruit crops infected by phytopathogenic fungi, and they focus mostly on the activity of enzymes rather than in the expression of the different genes encoding them (Yi et al., 2008; Sun et al., 2011; Zhang et al., 2018; Chen et al., 2020; Shuai et al., 2020). Information about the putative involvement of phospholipases in citrus fruit susceptibility to be infected by *P. digitatum* is limited. An increase in the incidence of natural disease was observed in Fallglo tangerine when treated with a specific inhibitor of PLA_2 to investigate the involvement of this enzyme in peel pitting development (Alférez et al., 2008). In view of this result, those authors performed further pharmacological experiments to examine the effect of this inhibitor and of different phenolics that may inhibit PLA_2, as well as the effect of two compounds that may inhibite PLC and PLD activities, on natural Navel orange infection (Alférez et al., 2008). Their work found that all the inhibitors increased natural disease incidence. Thereafter, Alférez et al., (2012) demonstrated that the specific inhibitor of PLA_2 increased disease when Fallglo tangerine

93 was inoculated with *P. digitatum*. They also suggested that the increase in both PLA_2 activity
94 and ***CsPLA2α*** and ***CsPLA2β*** gene expression in response to blue light treatment was
95 associated with reduced citrus postharvest decay, whereas the repression of ***CsPLDα*** and
96 ***CsPLDβ*** by red light was associated with increased decay (Alférez et al., 2012). It is
97 noteworthy that ABA plays a protective role against *P. digitatum* in citrus fruit (Lafuente et
98 al., 2019), and that ABA action appears to take place upstream of phospholipase activation
99 in dehydrated citrus fruit (Romero et al., 2013). Moreover in citrus fruit subjected to mild
100 stress, the connection between phospholipases A_2 and D and the ABA signal has been shown
101 (Romero et al., 2014). According to the above information, there is a gap in knowledge about
102 how ABA and infection itself regulate the expression and activity of phospholipases PLA_2,
103 PLD and PLC, incluiding isoforms that have never been asssociated with this process.
104 Therefore, the effect of citrus fruit infection by *P. digitatum* on the activity of these
105 phospholipases and on the gene expression of different isoforms, and the putative role of
106 ABA in their regulation, were studied in the present work. In previous research, we
107 demonstrated the limitations of pharmacological experiments to investigate the involvement
108 of ABA in citrus fruit response to infection (Lafuente et al., 2019). As explained in that work,
109 we assayed the effect of three inhibitors of ABA biosynthesis on the resistance of citrus fruits
110 to be infected by this pathogen: Tungstate, which inhibits the last ABA biosynthesis step by
111 inhibiting ABA aldehyde oxidase; 2) nordihydroguaiaretic acid (NDGA), an inhibitor of 9–
112 cis epoxycarotenoid dioxygenase, which is a key enzyme in the biosynthesis of ABA in citrus
113 fruit; and 3) norflurazon (NFZ), which inhibits phytoene desaturase at the beginning of
114 carotenoid biosynthesis. Unfortunately, both tungstate and NDGA had a clear effect on
115 reducing the in vitro growth of *P. digitatum* and, therefore, affected to the fungal viability
116 and capability to infect the fruit. On the other hand, NFZ induced peel damage, which should

117 favor disease. Therefore, **the three inhibitors should be ruled out'**. However, employing a
118 yellow mutant of Navelate (*Citrus sinensis* (L.) Osbeck) orange, named Pinalate, which
119 contains low ABA levels (Rodrigo et al., 2003; Rodrigo et al., 2019; Romero et al., 2019),
120 has been a valuable tool to gain better knowledge about the role of the hormone on fruit
121 infection (Lafuente et al., 2019). Hence in this work, we compared the regulation of
122 phospholipases in Navelate and Pinalate sweet oranges in response to infection. The
123 protective role of different phospholipase-encoding genes against infection has been widely
124 proposed (Hong et al., 2016), but contrasting results have been found in fruit as increased
125 phospholipases activity or gene expression has been proposed to form part of defence
126 responses (Shuai et al., 2020), and to also favour membrane deterioration and disease
127 development (Yi et al., 2008; Zhang et al., 2018; Chen et al., 2020). In the latter, increased
128 phospholipases activities were concomitant with disease development. So in the present
129 study, we concentrated on examining early citrus fruit responses to infection by *P. digitatum*
130 that occur before disease development to further understand the role of phospholipases and
131 their interplay with ABA in citrus fruit resistance to infection.
132
133 ## 2. MATERIAL AND METHODS
134 ### *2.1. Fungal and fruit material*
135 *Penicillium digitatum* (Pers.:Fr.) Sacc isolate Pd1 (CECT 20795) (Marcet-Houben et
136 al., 2012) was used to inoculate fruit. The conidial suspension was prepared in sterile distilled
137 water from 7-day-old cultures grown on potato dextrose agar (PDA) (Thermo Fisher
138 Scientific, Wilmington, DE, USA) at 24 ºC, whose concentration was measured with a
139 haemocytometer and adjusted to 10^4 conidia mL^{-1} as previously described by Ballester et al.
140 (2006).

141 We harvested 150 mature Navelate (*Citrus sinensis* (L.) Osbeck) orange fruit and 150
142 of its spontaneous ABA–deficient mutants (Pinalate) in January from the trees grown in
143 experimental orchards according to the normal cultural practices of **the 'The Spanish Citrus**
144 **Germplasm Bank' at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada,**
145 Valencia, Spain). Fruit diameters were 7.0±0.1 cm (Navelate) and 6.9±0.1 (Pinalate). Internal
146 maturity indices were 6.79±0.09 (Navelate) and 6.82±0.07 (Pinalate). The external color
147 index (a/b) of Navelate fruit was 0.61±0.04 and that of the yellow ABA–deficient mutant was
148 0.12±0.04. Oranges of both cultivars were immediately delivered to the laboratory, surface–
149 sterilised with commercial bleach for 5 min, rinsed with abundant tap water and dried at room
150 temperature for 2 h as previously described by Ballester et al. (2010), and then sorted into
151 two groups.

152 The fruit from the first group were immediately inoculated with 10 µL of the *P.*
153 *digitatum* conidial suspension, and those of the second group with the same volume of water
154 (control fruit). The oranges in each group were sorted into two subgroups. The first subgroup
155 was used to determine disease evolution and contained three replicates of five fruit each. The
156 second subgroup was used to periodically determine changes in the activities and expression
157 levels of the genes encoding the enzymes PLD, PLC, and PLA_2 on flavedo discs (7 mm in
158 diameter) taken around the inoculation site. Determinations were made in flavedo samples
159 rather than in whole peel because ABA deficiency in the Pinalate mutant was evident mainly
160 in flavedo. This subgroup was made up of three replicates of at least five fruit per sampling
161 period, and a minimum of eight discs per fruit were used in each replicate (40 discs). Discs
162 were immediately frozen, homogenised in liquid nitrogen and kept at –80 ºC for later
163 analyses. All the fruit were stored at 20 ºC and 90–95% relative humidity (RH), and the discs

164 from freshly harvested fruit, and from the fruit kept under this experimental condition for 1,
165 2 and 3 d were used for the analyses.
166
167 *2.2. Fruit inoculation and disease severity determination*
168 Fruit were inoculated by wounding peel with a flame sterilised needle (4 mm depth).
169 Ten microlitres of the conidial suspension adjusted to 10^4 conidia mL^{-1} were used to inoculate
170 each wound, and the wounds in the control fruit were inoculated with the same volume of
171 water. Four and eight inoculations per fruit were performed on the equatorial axis of each
172 fruit to follow disease severity evolution and collect flavedo samples for later analyses,
173 respectively.
174 Disease severity was determined by measuring the lesion diameter (cm) of the fruit
175 macerated zone in two perpendicular directions with a flexible ruler and then calculating the
176 lesion area. Determinations were made daily in the inoculated Navelate and Pinalate oranges
177 stored in plastic boxes in the dark at 20 °C and 90–95% RH. Three replicate samples of five
178 fruit with four equidistant wounds in the equatorial zone per fruit were used.
179
180 *2.3. Determination of fruit size, color, maturity index and ABA content in flavedo*
181 Fruit size, colour and maturity index at harvest time were determined in three
182 replicates of 10 fruit as previously described by Lafuente et al. (2014). The color index was
183 expressed as the *a/b* Hunter ratio, which is classically used for color index determinations in
184 citrus fruit. The a and b values were determined with a Minolta CR–300 Chromameter
185 (Konica Minolta Inc, USA) with a measuring area of 8 mm at three locations around the
186 equatorial plane of fruit (Lafuente et al., 2014). This ratio is negative for green fruit and
187 positive for orange fruit. The internal maturity index was assessed by measuring acidity and

soluble solids content (°Brix) in pulp. The soluble solids content was determined from fruit juice by an Atago/X–1000 digital refractometer (Atago Co. Ltd., Tokyo, Japan). The acid content was titrated with 0.1 N NaOH using phenolphthalein as an indicator as described by Lafuente et al. (2014). The maturity index was calculated by dividing the °Brix of the extracted juice by its acid content.

The ABA content was determined in representative homogenised frozen flavedo samples taken from freshly harvested Navelate and Pinalate fruit as reported by Lafuente et al. (2019). Three biological replicates containing five fruit each were used. Briefly, 1 g of fresh weight frozen tissues was extracted with 80% acetone containing 0.5 g L^{-1} citric acid and 0.1 g L^{-1} butylated hydroxytoluene using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). Extracts were centrifuged at 13000 × *g* for 5 min, and supernatants were diluted with cold TBS (6.05 g L^{-1} Tris, 0.2 mg L^{-1} $MgCl_2$, and 8.8 g L^{-1} NaCl) at pH 7.8 to reach ABA concentrations that fell within the linear range of the ABA standard curve. The extracted flavedo samples were analysed in duplicate by an indirect ELISA method. To that end, an ABA-**4'**–BSA conjugate was synthesised as described by Weiler (1980) with the modifications proposed by Norman et al. (1988).

2.4. Analysis of activities PLD, PLA_2 and PLC

The activities of enzymes PLD, PLA_2, and PLC were determined in the homogenised frozen samples. To that end, 300 mg of the frozen flavedo tissue were extracted with 1.5 mL of the chilled extraction buffer reported by Cronjé et al., (2017) (1 mM EDTA, 100 mM Tris–HCl (pH 7.5), 2% PVPP, and sorbitol 0.15 M) using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). The homogenised extract of each sample was centrifuged at 13000

211 × *g* at 4 ºC for 5 min, and the supernatant was used to determine the activities of each enzyme
212 with specific kits, which provide all the necessary reagents for the analyses.

213 Total PLD activity was assayed with the Amplex™ Red Phospholipase D Assay Kit
214 (Invitrogen™, Reference A12219, Thermo Fisher Scientific) by continuously measuring
215 fluorescence up to 10 min using a microplate reader with the excitation and emission
216 detection fluorescence maxima set at 540 nm and emission detection at 590 nm, respectively.
217 In this enzyme–coupled assay, PLD activity was monitored indirectly using 10–acetyl–3,7–
218 dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. The
219 assay was run by incubating 100 µL of the supernatant of the extracted flavedo sample with
220 100 µL of a working solution, prepared as indicated by the manufacturer, which contains
221 Amplex Red reagent, L–α–phosphatidylcholine (lecithin), horseradish peroxidase and choline
222 oxidase. In a first step, PLD cleaves the substrate to yield choline and PA. Then choline is
223 oxidised by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of
224 horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry to generate
225 the highly fluorescent product, resorufin. For each point, the background fluorescence was
226 corrected by subtracting the values that derived from the no–PLD control. Reactions were
227 performed at 37 °C, protected from light, and enzymatic activity was expressed as the
228 increment in fluorescence units per kg and s ($\Delta U\ kg^{-1}\ s^{-1}$).

229 The PLC activity was assayed as described above for PLD activity, but using the
230 Amplex® Red Phospholipase C Assay Kit (Invitrogen™, Reference A12218). The assay was
231 performed by incubating 100 µL of the supernatant of the extracted sample with 100 µL of
232 the working solution, which was prepared by following the manufacturer's instructions. The
233 working solution in this assay kit contains Amplex Red reagent, lecithin, alkaline
234 phosphatase, horseradish peroxidase, and choline oxidase. As for PLD activity, the

enzymatic activity was expressed as the increment in fluorescence units per kg and s ($\Delta U\ kg^{-1}\ s^{-1}$).

Total PLA_2 activity (patatin and secretory PLA_2) was assayed with the $cPLA_2$ kit from Cayman Chemical Co (Reference 765021, Ann Arbor, Michigan, USA) following the method described by Alférez et al., (2008). The appropriate volume of the extracted flavedo sample was incubated with arachidonoyl Thio–PC as a substrate for 60 min to complete the hydrolysis of the arachidonoyl thioester bond. Then the released free thiols were detected by the addition of DTNB/EGTA [5.5' dithiobis (2-dinitrobenzoic acid) Ellman's reagent] and measuring the change in $A_{414\ nm}$ at the end of the incubation period by a plate reader spectrophotometer. Commercial bee venom was used as a standard positive control and spiking samples with increasing concentrations of PLA_2 standard allowed reaction linearity to be assessed. The reaction rate was determined using the DTNB extinction coefficient of 10.66 mM^{-1} and activity was expressed as kat kg^{-1}.

2.5. Total RNA extraction and expression analysis of genes CsPLD, CsPLC and $CsPLA_2$

The total RNA extraction and expression analyses of genes *CsPLD, CsPLC* and *$CsPLA_2$* were performed as previously described by Romero et al. (2020a). Briefly, the total RNA extracted from flavedo was treated with Ribonuclease-free DNAse (Thermo Fisher Scientific) by following the manufacturer's recommendations for removing genomic DNA contaminations. RNA quality and integrity were checked by agarose gel electrophoresis and GelRed staining (Biotium, Fremont, CA, USA), and its total concentration was spectrophotometrically determined. The cDNA from each sample was obtained from 2 µg of total RNA, while first-strand cDNA was synthetised using SuperScript III RT (Thermo Fisher Scientific) and Ribonuclease inhibitor (Thermo Fisher Scientific), as indicated by the

manufacturer. Gene-specific primer pairs (***CsPLDα, CsPLD β, CsPLDδ, CsPLDγ*** and *CsPLDζ; CsNPC3, CsPLC2* and *CsPI-PLC* **and *CsPLA2α, CsPLA$_2$β*** and *CsPAT1*) were designed with the DNAMAN 4.03 software (Lynnon BioSoft; https://www.lynnon.com/dnaman.html). Their forward and reverse sequences are summarised in Supplementary Table S1. Two reference genes (*CsACT* and *CsTUB*) were used for data normalisation. The cDNA amplification was monitored at 95 ºC for 10 s, 60 ºC for 5 s and 72 ºC for 10 s by employing SYBR Green 1 Master (Roche Diagnostics, Barcelona, Spain) and a LightCycler480 System (Roche Diagnostics). The gene expression analysis was performed by using the Relative Expression Software Tool (REST, http://rest.gene-quantification.info), and the expression levels for the flavedo samples taken from Navelate and Pinalate fruit were referred to that obtained in the flavedo of freshly harvested fruit from the respective cultivar. Values correspond to the mean of three biological replicates samples, with two technical replicates±standard error.

2.6. Statistical analysis

The statistical analyses were performed by the Statgraphics Plus 4.0 software (Manugistics, Inc., Rockville, MD, USA). The results are the means of three biological replicated samples±standard error. **A mean comparison using Tukey's test was made to** determine whether the mean values were significantly different at $P \leq 0.05$.

3. RESULTS

3.1. Differences in ABA, and in the activity and gene expression of phospholipases between Navelate and Pinalate fruit

282 In this work, differences in PLD, PLC and PLA_2 activities, and in the expression of a
283 set of genes encoding them, were examined in the outer part of fruit peel of Navelate and its
284 mutant Pinalate cultivars because of their different ABA content (Fig. 1) and resistance to *P.*
285 *digitatum* infection (Lafuente et al., 2019).

286 The ABA content of the freshly harvested fruit was about 2–fold higher in Navelate
287 than in the mutant (Fig. 1A). Very few differences were found in the activity of enzymes
288 PLC and PLD between both cultivars at harvest time, but PLA_2 activity was about 1.7–fold
289 higher in the Navelate fruit (Fig. 1A). The expressions of all the PLD isoforms were lower
290 in the flavedo of the freshly harvested Navelate fruit (Fig. 1B). The biggest and smallest
291 differences between genotypes were found when comparing the accumulations of ***CsPLDα***
292 and *CsPLDδ* transcripts, respectively. Very few differences in the expression of the *CsNPC3*
293 and *CsPLC2* were also found, but the expression level of *CsPI-PLC* was much lower in the
294 freshly harvested Navelate fruit than in their mutant (Fig. 1B). Of the three genes encoding
295 PLA, major differences between both cultivars were found in the *CsPAT1* gene (3–fold lower
296 in the Navelate fruit). The $CsPLA_2\beta$ expression was 1.5–fold lower in Navelate and no
297 difference in the accumulation of $CsPLA_2\alpha$ transcripts between both cultivars was found (Fig.
298 1B).

299

300 *3.2. Changes in the activity of phospholipases in response to infection*

301 To examine the early responses to *P. digitatum* in citrus fruit, changes in both
302 phospholipases activities and gene expressions were determined for up to 3 d post–
303 inoculation (dpi). As shown in Supplementary Fig. S1, no disease symptom was observed in
304 Navelate fruit during this period, and disease was barely detected in the Pinalate fruit by day
305 3. Thereafter, disease increased in both cultivars and this increase was more marked in the

306 Pinalate fruit. This experimental period was also selected because previous results from our
307 group in citrus fruit have indicated that after 3 dpi, responses to infection by *P. digitatum* can
308 derive from either fruit or the fungus (Ballester et al., 2006), and phospholipases are
309 distributed in diverse organisms, including fungi (Ghannoum, 2000; Hong et al., 2016).

310 PLD and PLC activities remained almost constant in the control fruit, inoculated with
311 the water, of both cultivars during fruit storage (Fig. 2A–D). In contrast, they increased in
312 response to infection after 1 dpi (Fig. 2A–D). Very few differences between cultivars were
313 observed by 2 dpi, but the activity of both enzymes was greater by 3 dpi in Navelate (Fig. 2A
314 and 2C) than in the mutant Pinalate (Fig. 2B and 2D), with low ABA levels (Fig. 1A). During
315 this period, PLD activity increased by about 7– and 2–fold in the Navelate (Fig. 2A) and
316 Pinalate (Fig. 2B) fruit, respectively, compared to the freshly harvested fruit (0 dpi). A small
317 difference was observed when examining changes in PLC activity. As shown in Fig. 2C and
318 2D, PLC activity was about 1.5–fold higher in the Navelate than in Pinalate fruit by 3 dpi.

319 The trend of changes in PLA_2 activity was different (Fig. 2E and 2F). In Navelate, it
320 decreased in both the control and infected fruit up to 3 dpi (Fig. 2E). In this cultivar, few but
321 significant differences between the control and infected fruit were noticed by 2 dpi. During
322 this period, activity was greater in the fruit inoculated with the pathogen. The decreases
323 observed in PLA_2 activity in the Pinalate fruit were less marked (Fig. 2F). Moreover, the
324 differences between the control and infected fruit were erratic during fruit storage.

325

326 *3.3. Fruit infection on the expression of the PLD-encoding genes*

327 Different trends in the pattern of changes in the expression of most PLD–encoding
328 genes were observed in response to *P. digitatum* infection between the fruit from the Navelate
329 cultivar (Fig. 3) and its ABA–deficient mutant (Pinalate) (Fig. 4).

330 Of the five genes encoding PLD, major increases in response to infection in Navelate
331 were found in genes ***CsPLDγ*** and *CsPLDζ*, whose expression continuously increased in the
332 fruit inoculated with the pathogen, and was 10–and 2.7–fold higher, respectively, by 3 dpi in
333 the infected fruit than in the control fruit (Fig. 3). In an earlier infection stage (2 dpi), the
334 expression level of gene ***CsPLDγ*** was already 3.9–fold higher in the infected fruit than in their
335 respective control fruit. A good correlation was found between increased PLD activity and
336 the *CsPLDζ* expression levels in response to infection when considering both the Navelate
337 and Pinate fruit (R^2 = 0.755). With ***CsPLDγ***, a good correlation was found only for the
338 Navelate fruit (R^2 = 0.887). The expression of the other *CsPLD* isoforms transiently lowered
339 at 1 dpi in the infected Navelate fruit (Fig. 3). At that time, no significant difference in
340 *CsPLDδ* gene expression was found between the control and infected fruit, and the
341 expression of the ***CsPLDα*** and ***CsPLDβ*** isoforms was slightly higher in the control than in
342 the infected fruit (Fig. 3). After 1 dpi, the expression of these genes increased in the infected
343 Navelate fruit. However, significantly higher levels in the infected fruit appeared in the
344 expression of the ***CsPLDα***–encoding gene only by 2 dpi (Fig. 3). Enzyme activity did not
345 correlate with the expression levels of these three isoforms.
346 In Pinalate (Fig. 4), with lower ABA levels compared to Navelate (Fig. 1A), the
347 pattern of changes in the expression of gene ***CsPLDγ*** in response to infection was similar to
348 that found in Navelate. It increased during the incubation of the infected fruit at 20 °C. In this
349 cultivar, expression levels were about 2.3–and 6–fold higher in the infected than in the control
350 fruit by day 2 and 3, respectively (Fig. 4).
351 The *CsPLDζ* expression barely increased in response to infection in the Pinalate fruit
352 compared to the freshly harvested fruit (Fig. 4). By 2 and 3 dpi, its expression was higher in
353 the infected than the control fruit, but differences between both samples were much smaller

than they were in Navelate (Fig. 3). The expression of the other *CsPLD* isoforms (***CsPLDα, CsPLDβ, CsPLDδ***) sharply dropped during fruit infection in Pinalate (Fig. 4). Their expressions also lowered in the control fruit and were slightly lower than in the infected fruit by 2 dpi. However by 3 dpi, no significant difference was found for transcripts ***CsPLDα*** and ***CsPLDβ*** between the control and infected fruit, and the *CsPLDδ* expression level was slightly higher in the control than in the infected Pinalate fruit (Fig. 4).

3.4. Fruit infection on the expression of PLC-encoding genes

The expressions of genes *CsNPC3* (Fig. 5A) and *CsPI-PLC* (Fig. 5E) increased in the Navelate fruit in response to infection up to 2 dpi and, then, slightly decreased. The most marked increase in relation to the freshly harvested fruit was observed in the expression of gene *CsPI-PLC* (17–fold increase by 2 dpi). This gene was also up–regulated in the control fruit (Fig. 5E) but, by 2 dpi, its expression was about 2.5–fold higher in the infected than in the control fruit. By 2 dpi, the differences in expression levels of *CsNPC3* between the control and infected Navelate fruit (Fig. 5A) were bigger (10–fold). The *CsPLC2* expression sharply dropped after fruit detachment in both the control and infected Navelate fruit (Fig. 5C). By 1 dpi, no differences in gene expression between the control and infected fruit were found in this cultivar. Thereafter, the *CsPLC2* expression levels were higher in the fruit inoculated with *P. digitatum*.

The trend of changes in the expression of gene *CsNPC3* in response to infection in the ABA–deficient mutant (Fig. 5B) was similar to that of the Navelate fruit (Fig. 5A), although the decrease in its expression in the infected fruit from 2 to 3 dpi was much more marked in the mutant. In contrast, a marked difference was observed when examining changes in *CsPI-PLC* expression in Pinalate (Fig. 5F). In this mutant, *CsPI-PLC* was down–

regulated in both the control and infected fruit, and no relevant difference was found between both samples during fruit storage. The trend of changes in *CsNPLC2* in response to infection also differed in Pinalate (Fig. 5D). This gene was down-regulated after fruit detachment in the control fruit, whereas its expression remained by 1 dpi in the infected fruit and decreased thereafter. As a result, the expression of this gene was higher by 1dpi, and lower by 2 dpi in the infected than in the control fruit. No correlation between the activity of this enzyme and the expression of *CsPLC* isoforms was found in response to infection as R^2 was always lower than 0.35.

3.5. Fruit infection on the expression of PLA-encoding genes

An examination of the transcriptional changes in the three genes encoding PLA_2 revealed that the expression levels of all the genes lowered during storage at 20 ºC in the control Navelate and Pinalate fruit, and about one half expression level was achieved compared to the freshly harvested fruit (0 dpi) in both cultivars by 3 dpi (Fig. 6). The expression of *CsPLA*$_2\alpha$ also lowered in the infected Navelate fruit, but this decrease was slower and resulted in slightly higher expression levels in the infected than in the control fruit by 1 and 2 dpi (Fig. 6A). A rapid (1 dpi) increase was observed in the expression of this gene in response to infection in the mutant (Fig. 6B). This increase was minor and transitory, but significant higher transcript accumulation was found in the infected fruit *versus* its control by 1 dpi. This difference did not remain thereafter and, by 3 dpi, *CsPLA*$_2\alpha$ gene expression was higher in the control fruit (Fig. 6B). Differences in *CsPLA*$_2\beta$ expression were also small in both cultivars, but the accumulation of this transcript was significantly higher in the infected Navelate fruit by 2 dpi, and also by 2 and 3 dpi in the mutant (Fig. 6C and 6D). *CsPAT1* expression sharply decreased in the control and infected Pinalate fruit (Fig. 6F). No

significant effect of infection was observed compared to the control samples in this cultivar. In the Navelate fruit (Fig. 6E), significant differences were found only by 3 dpi (1.5–fold higher in the infected fruit). Of these three genes, the highest correlation between activity and expression levels in response to infection was found for *CsPLA$_2\alpha$* (R^2 = 0.655). The correlation of the other two isoforms was below 0.1. As the pattern of changes in the expression levels of *CsPLA$_2\beta$* and *CsPAT1* between the Navelate and mutant fruit were clearly different, we cannot rule out the involvement of ABA in their regulation during fruit infection in citrus fruit.

4. DISCUSSION

The involvement of genes encoding phospholipases in plant defence against pathogen attack has been widely demonstrated (Hong et al., 2016), while phospholipases may positively or negatively affect the outcome of fruit–microbe interactions in fruit crops (Yi et al., 2008; Zhang et al., 2018; Chen et al., 2020; Shuai et al., 2020). For the reasons indicated in the Introduction in this work, we focused on the early responses induced by *P. digitatum* in citrus fruit before the tissue degradation caused by the pathogen became evident in the Navelate fruit and was negligible in its mutant (Supplementary Fig. S1). Moreover from previous results obtained with citrus fruit infected by *P. digitatum* (Ballester at al., 2006), it would be reasonable to think that in early infection stages, the contribution of fruit enzymes/genes prevails over that of the fungus. Different pieces of evidence also suggest the interplay between ABA signalling with phospholipase gene expression in citrus fruit subjected to abiotic stress (Romero et al., 2014 and 2020b), and reveal that ABA plays a protective role against *P. digitatum* in this fruit crop (Lafuente et al., 2019). For these reasons, we also examined whether the effect of the pathogen on phospholipases activity and gene

426 expression would differ between the Navelate oranges and fruit of their mutant Pinalate,
427 which has low ABA levels and is partially insensitive to this hormone (Romero et al., 2012).
428 Our results showed that, at harvest time, PLD and PLC activities (Fig. 1A) and the
429 expression levels of most of the genes encoding them (Fig. 1B) were similar or slightly lower
430 in the Navelate fruit, while the expression of the *CsPI-PLC* isoform was much lower.
431 However, PLA_2 activity was greater in the Navelate fruit (Fig. 1A) and the expressions of
432 isoforms *CsPLA$_2\beta$* and *CsPAT1* were lower (Fig. 1B). Such differences might be related to
433 the genotype, but also to the stressful environmental factors to which fruit are exposed when
434 they grow on trees, in which ABA plays a relevant role (Romero et al., 2013, Alferez et al.,
435 2020). A plausible explanation for the different trend in the pattern of changes between PLA_2
436 activity and the expression of genes encoding them when comparing both genotypes would
437 be the mutant's reduced ability to perform post–transcriptional modifications of PLA_2.
438 Moreover, other *CsPLA$_2$* isoforms might contribute to the greater activity noted in the
439 Navelate fruit.
440 Our results indicated that changes in both activity (Fig. 2) and gene expression (Fig.
441 3–6) in response to *P. digitatum* infection can be partially attenuated in the mutant. Therefore,
442 we cannot rule out the possibility of ABA participating and somehow controlling the
443 activation of specific phospholipases in response to infection in citrus fruit. Likewise, our
444 results revealed the activation of phospholipases in response to infection prior to disease
445 symptom development, and this activation was attenuated in the mutant, which is less
446 resistant to infection. Thus they suggest that: 1) activation of phospholipases should be a
447 protective mechanism in citrus fruit to cope with *P. digitatum* infection, which agrees with
448 previous findings from pharmacological experiments showing that an inhibitor of PLC activity

449 (neomycin) favours natural infection in this crop (Alférez et al; 2008); 2) this defence response
450 might be regulated by ABA.

451 Our global results indicated that the relevance of phospholipases in the citrus fruit–*P.*
452 *digitatum* interaction varied among specific phospholipases, and that PLD and PLC could
453 play major roles in citrus fruit resistance to *P. digitatum* compared to enzyme PLA_2. This
454 statement is based on a few facts: both PLD and PLC activities and the expression levels of
455 some PLD and PLC isoforms markedly increased in response to infection in the Navelate
456 fruit; PLA_2 activity did not increase; minor differences between the infected and control fruit
457 in the activity of this enzyme and in the expression of its related genes appeared compared
458 to other enzymes.

459 According to the results herein presented, ***CsPLDγ*** may be a key *CsPLD* isoform in
460 protecting citrus fruit against the pathogen as its induction in response to infection in both
461 the Navelate and Pinalate fruit was greater than that of other *CsPLD* isoforms. Interestingly,
462 Alférez et al., (2012) proposed a role for this gene in citrus fruit resistance against *P.*
463 *digitatum* infection because treating Fallglo tangerine with red light lowered ***CsPLDγ***
464 expression and promoted decay. The involvement of ABA in the regulation of this gene
465 would appear barely relevant, although its expression was about 10–fold and 6–fold higher in
466 the infected than in the control Navelate and Pinalate fruit, respectively, by 3 dpi (Fig. 3 and
467 4). Our results also showed that *P. digitatum* induced a marked increase (2.7–fold) in *CsPLDζ*
468 expression levels in response to infection in Navelate (Fig. 3), with a good correlation
469 between the induction of these isoforms and PLD activity in response to *P. digitatum* in both
470 cultivars. As far as we know, this is the first report to suggest the participation of *CsPLDζ* in
471 plant defence against phytopathogenic fungi. The increased expression of this gene was
472 higher in the Navelate (Fig. 3) than in the Pinalate fruit (Fig. 4). Therefore, the comparative

473 transcriptional analysis of the control and infected Navelate and Pinalate fruit suggested that
474 infection favoured the phospholipid–derived signalling mediated by ***CsPLDγ*** and *CsPLDζ* in
475 citrus fruit, and that ABA could modulate *CsPLDζ* expression to some extent, while the
476 connection between ABA and the ***CsPLDγ*** isoform in response to infection was less clear.
477 Therefore, increases in the expression of these genes agree with the role of phospholipids
478 acting as rich sources for signalling messengers in plant defence (Wang, 2001; Meijer and
479 Munnik, 2003). ***PLDζ*** may lead to DAG production. Moreover, this gene and the ***PLDγ***
480 isoform may play a role in basal PA accumulation (Zhao et al., 2011; Hong et al., 2016).
481 Therefore, the results in the present work encourage future research to further understand
482 whether PA and DAG act in citrus fruit defence against *P. digitatum*.

483 The transcriptional analysis revealed that isoforms *CsPLDδ* **and the** ***CsPLDβ*** were
484 **barely relevant in citrus fruit defence against *P. digitatum*, although the *PLDδ* gene has been**
485 **related to fungal attack in different plants** (Hong et al., 2016). Likewise, the involvement of
486 ***CsPLDα,*** which has been proposed to play a role in the plant microbe–interaction and in
487 resistance against pathogens (Hong et al., 2016), and in lesser citrus fruit resistance against
488 *P. digitatum* when exposed to red light, was less relevant than that of isoforms ***CsPLDγ*** and
489 *CsPLDζ* in the citrus fruit-*P. digitatum* interaction. However, we cannot rule out its protective
490 role as significantly higher (2–fold) transient expression values were found in the infected
491 than in the control Navelate fruit by 2 dpi (Fig. 3), and this difference was smaller in the
492 mutant (Fig. 4). This result also suggests that the expression of this gene depends on ABA,
493 at least partially.

494 The results of this research work also showed, for the first time, the activation of PLC
495 (Fig. 2) in the citrus fruit–*P. digitatum* interaction, and that the gene isoforms encoding both
496 NPC and PI–PLC may participate in this interaction (Fig. 5). These findings fall in line with

497 those found in banana fruit infected with anthracnose (Shuai et al., 2020). No correlation
498 between changes in PLC activity and the expression of the selected *CsPLC* isoforms was
499 found, which should be related partially to the transient induction of these genes by the
500 fungus. By considering that differences in PLC activity and in the expression levels of the
501 *CsPLC* isoforms between the control and infected flavedo samples were more marked in the
502 Navelate than in the ABA–deficient mutant, it would appear that PLC plays a defensive role
503 in citrus fruit against *P. digitatum*. Accordingly, it is noteworthy that genetic evidence for
504 *PI-PLC* isoforms's role in disease resistance has been obtained for tomato (Vossen et al.
505 2010) and Arabidopsis PLC2 (D'Ambrosio et al. 2017). Our data in citrus fruit strongly
506 suggest a role for ABA in the up–regulation of the *CsPI-PLC* isoform in response to infection
507 caused by *P. digitatum* as its expression sharply rose in the infected Navelate fruit (Fig. 5E)
508 and did not alter at all in Pinalate (Fig. 5F). This agrees with a previous report which indicated
509 that *PLC* genes are induced upon ABA treatment in plants (Liu et al., 2006; Pokotylo et al.,
510 2014). So it would appear that ABA action takes place upstream of *CsPI-PLC* gene activation
511 in infected fruit.

512 Previous research into Fallglo tangerine fruit suggests the involvement of PLA_2
513 activity and genes $CsPLA_2\alpha$ and $CsPLA_2\beta$ in citrus fruit defence against *P. digitatum*. These
514 statements were based on some results which have shown that an inhibitor of the enzyme
515 increases disease, and that blue light increases the expression of both genes and elicits
516 resistance against the pathogen (Alférez et al., 2012). However, the changes in PLA_2 activity
517 and gene expression were not studied during fruit infection. Our results in both the Navelate
518 and Pinalate fruit supported this idea, but indicated that the participation of this enzyme in
519 protecting citrus fruit was less relevant than that of enzymes PLD and PLC. Thus small or
520 inconsistent differences in PLA_2 enzyme activity between the control and infected fruit were

521 detected (Fig. 2). Similarly, differences in *CsPLA$_2\alpha$* and *CsPLA$_2\beta$* expressions between the
522 infected and control fruit were low, but slightly bigger at some sampling points in the infected
523 fruit (Fig. 6). We also examined changes in *CsPAT1* expression as this gene can display PLA$_2$
524 activity. Our results indicate, however, it participated less in citrus fruit defence against *P.*
525 *digitatum* infection (Fig. 3). Previous works have proposed that ABA action lies upstream of
526 *PLA$_2$* gene activation (Romero et al., 2013 and 2014) and indicated an interplay between the
527 PLA$_2$ and PLD-encoding genes in citrus fruit response to abiotic stress (Romero et al., 2013
528 and 2020b). Given the mild effect of infection on the expression of the PLA$_2$-encoding genes
529 in both the Navelate and Pinalate fruit, these statements cannot be extrapolated to the
530 response to infection in this horticultural crop.
531
532 5. CONCLUSIONS
533 In short, we showed the involvement of different phospholipases in early citrus fruit
534 responses to *P. digitatum* infection. As summarised in the scheme in Fig. 7, PLD and PLC
535 activities increased in the infected fruit prior to disease symptoms development, and these
536 increases were attenuated in the ABA-deficient mutant fruit, which was less resistant to
537 infection. Therefore, our results indicated that the activation of these enzymes in the infected
538 fruit was an early citrus fruit response to cope with pathogen attack stress, and that these
539 defence responses could be partially regulated by ABA. Our results also help to unravel the
540 lesser participation of PLA$_2$ activity in early defence responses. These results were also
541 supported by the transcriptional analyses, which also showed the differential activation of
542 several PLD-, PLC- and PLA-encoding genes in citrus fruit in response to infection by
543 necrotrophic fungus *P. digitatum*. The differences in the expression levels of the PLA$_2$-
544 encoding genes between the infected and control fruit inoculated with water were smaller

545 than differences found in most PLD– and PLC–encoding genes. Among the distinct *CsPLD*
546 isoforms, the marked induction of genes ***CsPLDγ*** and ***CsPLDζ*** was remarkable. This work
547 also demonstrated, for the first time, the participation of a ***PLDζ*** gene in the plant–microbe
548 interaction, which suggests that changes in its expression in response to infection may be
549 modulated by ABA to some extent. Likewise, our results unraveled the notion that *P.*
550 *digitatum* induced marked increases in *CsNPC3* expression and in the *CsPI-PLC* isoforms,
551 and that ABA action appears to take place upstream of *CsPI-PLC* gene activation in infected
552 citrus fruit.
553

554 ## APPENDIX A: SUPPLEMENTARY DATA

555 The Supplementary Material related to this article can be found in the online version.
556

557 ## ACKNOWLEDGEMENTS

558 The technical assistance of R. Sampedro is gratefully acknowledged. We also thank
559 Dr. G. Ancillo (IVIA) for allowing us to use the Spanish Citrus Germoplasm Bank. This
560 work was supported by Projects AGL2014–55802–R, AGL2017–88120–R
561 (MCIU/AEI/FEDER, UE) and PROMETEOII/2014/027 (Generalitat Valenciana, Spain).
562

563 ## FUNDING

564 This work was supported by Projects AGL2014–55802–R, AGL2017–88120–R
565 (MCIU/AEI/FEDER, UE) and PROMETEOII/2014/027 (Generalitat Valenciana, Spain).
566

567 REFERENCES

568 Alférez, F., Liao, H–L., Burns, J.K., 2012., Blue light alters infection by *Penicillium*
569 *digitatum* in tangerines. Postharvest Biol. Technol. 63, 11–15. https://doi.org/10.1016/
570 j.postharvbio.2011.08.001

571 Alférez, F., Lluch, Y., Burns, J.K., 2008., Phospholipase A_2 and postharvest peel pitting in
572 citrus fruit. Postharvest Biol. Technol. 49, 69–76. https://doi.org/10.1016/
573 j.postharvbio.2008.01.010

574 Alferez, F., Wu, J., Graham, J.H., 2020., Phospholipase D (PLD) response to water stress in
575 citrus roots and leaves. Agronomy, 10, 45. https://doi.org/10.3390/agronomy10010045

576 Ballester, A.–R., Izquierdo, A., Lafuente, M.T., González–Candelas, L., 2010. Biochemical
577 and molecular characterization of induced resistance against *Penicillium digitatum* in
578 citrus fruit. Postharvest Biol. Technol. 56, 31–38. https://doi.org/10.1016/
579 j.postharvbio.2009.10.002

580 Ballester, A. R., Lafuente M.T., González–Candelas, L., 2006. Spatial study of antioxidant
581 enzymes, peroxidase and phenylalanine ammonia–lyase in the citrus fruit–*Penicillium*
582 *digitatum* interaction. Postharvest Biol. Technol. 39, 115–124.
583 https://doi.org/10.1016/j.postharvbio.2005.10.002Blée, E., 2002. Impact of phyto–
584 oxylipins in plant defense. Trends Plant Sci. 7, 315–322. https://doi.org/10.1016/S1360–
585 1385(02)02290–2

586 Chen, Y., Sun, J., Lin, H., Lin, M., Lin, Y., Wang, H. Hung,Y–C., 2020. Salicylic acid
587 treatment suppresses *Phomopsis longanae* Chi–induced disease development of
588 postharvest longan fruit by modulating membrane lipid metabolism. Postharvest Biol.
589 Technol. 164, 111168. https://doi.org/10.1016/j.postharvbio.2020.111168.

590 Cronjé, P.J.R., Zacarías, L., Alférez, F., 2017. Susceptibility to postharvest peel pitting in
591 Citrus fruits as related to albedo thickness, water loss and phospholipase activity.
592 Postharvest Biol. Technol. 123, 77–82. https://doi.org/10.1016/j.postharvbio.
593 2016.08.012

594 D'Ambrosio, J.M., Couto, D., Fabro, G., Scuffi, D., Lamattina, L., Munnik, T., Andersson,
595 M.X., Álvarez, M.E., Zipfel, C., Laxalt, A.M., 2017. Phospholipase C2 affects MAMP–
596 triggered immunity by modulating ROS production. Plant Physiol. 175, 970–981.
597 https://doi.org/10.1104/pp.17.00173

598 Dhondt, S., Geoffroy, P., Stelmach, B.A., Legrand, M., Heitz, T., 2000. Soluble
599 phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic
600 virus–infected tobacco leaves and is contributed by patatin–like enzymes. Plant
601 J. 23, 431–440. https://doi.org/10.1046/j.1365–313X.2000.00802.x
602 Ghannoum, M.A., 2000. Potential role of phospholipases in virulence and fungal
603 pathogenesis. Clin. Microbiol. Rev. 13, 122–143.
604 https://doi.org/10.1128/CMR.13.1.122–143.2000
605 Hong, Y., Zhao, J., Guo, L., Kim, S.–C., Deng, X., Wang, G., Zhang, G., Li, M., Wang, X.,
606 2016. Plant phospholipases D and C and their diverse functions in stress responses.
607 Progress Lipid Res. 62, 55–74. https://doi.org/10.1016/j.plipres.2016.01.002
608 Kirik, A., Mudgett, M.B., 2009. SOBER1 phospholipase activity suppresses phosphatidic
609 acid accumulation and plant immunity in response to bacterial effector AvrBsT.
610 Proc. Natl. Acad. Sci. U. S. A. 106, 20532–20537.
611 https://doi.org/10.1073/pnas.0903859106
612 Lafuente, M.T., Alférez, F., Romero, P., 2014. Postharvest ethylene conditioning as a tool to
613 reduce quality loss of stored mature sweet oranges. Postharvest Biol. Technol. 94, 104–
614 111. https://doi.org/10.1016/j.postharvbio.2014.03.011
615 Lafuente, M.T., Ballester, A.–R., González–Candelas, L., 2019. Involvement of abscisic acid
616 in the resistance of citrus fruit to *Penicillium digitatum* infection. Postharvest Biol.
617 Technol. 154, 31–40. https://doi.org/10.1016/j.postharvbio.2019.04.004
618 Liu, H.T., Liu, Y.Y., Pan, Q.H., Yang, H.R., Zhan, J.C., Huang, W.D., 2006. Novel
619 interrelationship between salicylic acid, abscisic acid, and PIP2–specific phospholipase
620 C in heat acclimation–induced thermotolerance in pea leaves. J. Exp. Bot. 57, 3337–
621 3347. https://doi.org/10.1093/jxb/erl098
622 Marcet–Houben, M., Ballester, A.–R., De la Fuente, B., Harries, E., Marcos, J.F., González–
623 Candelas, L., Gabaldón, T., 2012. Genome sequence of the necrotrophic fungus
624 *Penicillium digitatum*, the main postharvest pathogen of citrus. BMC Genomics 13, 646.
625 https://doi.org/10.1186/1471–2164–13–646
626 Meijer, H.J.G., Munnik, T., 2003. Phospholipid–based signaling in plants. Ann. Rev. Plant
627 Biol. 54, 265–306. https://doi.org/10.1146/annurev.arplant.54.031902.134748
628 Norman, S.M., Poling, S.M., Maier, V.P., 1988. An indirect enzyme–linked immunosorbent

assay for (+)–abscisic acid in *Citrus*, *Ricinus*, and *Xanthium* leaves. J. Agric. Food Chem. 36, 225–231. https://doi.org/10.1021/jf00079a056

Palou, L., Ali, A., Fallik, E., Romanazzi, G., 2016. GRAS, plant– and animal–derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. Postharvest Biol. Technol. 122, 41–52. https://doi.org/10.1016/j.postharvbio.2016.04.017

Pokotylo, I., Kolesnikov, Y., Kravets, V., Zachowski, A., Ruelland, E., 2014. Plant phosphoinositide–dependent phospholipases C: variations around a canonical theme. Biochimie, 96, 144–157. https://doi.org/10.1016/j.biochi.2013.07.004

Rodrigo, M.J., Lado, J., Alós, E., Alquézar, B., Dery, O., Hirschberg, J., Zacarías, L., 2019. A mutant allele of *ζ-carotene isomerase* (*Z-ISO*) is associated with the yellow pigmentation of the "Pinalate" sweet orange mutant and reveals new insights into its role in fruit carotenogenesis. BMC Plant Biol 19, 465. https://doi.org/10.1186/s12870–019–2078–2

Rodrigo, M.J., Marcos, J.F., Alferez, F., Mallent, M.D., Zacarías, L., 2003. Characterization of Pinalate, a novel *Citrus sinensis* mutant with a fruit–specific alteration that results in yellow pigmentation and decreased ABA content. J. Exp. Bot. 54, 727–738. https://doi.org/10.1093/jxb/erg083

Romero, P., Alférez, F., Establés–Ortiz, B., Lafuente M.T., 2020a. Insights into the regulation of molecular mechanisms involved in energy shortage in detached citrus fruit. Sci. Rep. 10, 1109. https://doi.org/10.1038/s41598–019–57012–7

Romero, P., Alférez, F., Lafuente M.T., 2020b. Involvement of phospholipases and sucrose in carbon starvation–induced non–chilling peel pitting in citrus fruit. Postharvest Biol. Technol. 169, 111295. https://doi.org/10.1016/j.postharvbio.2020.111295

Romero, P., Gandía, M., Alférez, F., 2013. Interplay between ABA and phospholipases A_2 and D in the response of citrus fruit to postharvest dehydration. Plant Phys. Biochem. 70, 287–294. https://doi.org/10.1016/j.plaphy.2013.06.002

Romero, P., Lafuente, M.T., Alférez, F., 2014. A transcriptional approach to unravel the connection between phospholipases A_2 and D and ABA signal in citrus under water stress. Plant Physiol. Biochem. 80, 23–32. https://doi.org/10.1016/j.plaphy.2014.03.014

659 Romero, P., Lafuente, M.T., Rodrigo, M.J., 2019. A sweet orange mutant impaired in
660 carotenoid biosynthesis and reduced ABA levels results in altered molecular responses
661 along peel ripening. Sci. Rep. 9, 9813. https://doi.org/10.1038/s41598-019-46365-8
662 Romero, P., Rodrigo, M.J., Alférez, F., Ballester, A.R., González-Candelas, L., Zacarías, L.,
663 Lafuente, M.T., 2012. Unravelling molecular responses to moderate dehydration in
664 sweet orange (*Citrus sinensis* L. Osbeck) by using a fruit-specific ABA-deficient mutant.
665 J. Exp. Bot. 63, 2753-2767. https://doi.org/10.1093/jxb/err461
666 Ryu, S.B., 2004. Phospholipid-derived signaling mediated by phospholipase A in plants.
667 Trends Plant Sci. 9, 229-235. https://doi.org/10.1016/j.tplants.2004.03.004
668 Sánchez-Torres, P., Tuset, J.J., 2011. Molecular insights into fungicide resistance in sensitive
669 and resistant *Penicillium digitatum* strains infecting citrus. Postharvest Biol. Technol.
670 59, 159-165. https://doi.org/10.1016/j.postharvbio.2010.08.017.
671 Shuai, L., Li, L., Sun, J., Liao, L., Duan, Z., Li, C., He, X., 2020. Role of phospholipase C in
672 banana in response to anthracnose infection. Food Sci. Nutr. 8, 1038-
673 1045. https://doi.org/10.1002/fsn3.1388
674 Sun, J., You, X., Li, L., Peng, H., Su, W., Li, C., He, Q., Liao, F., 2011.
675 Effects of a phospholipase D inhibitor on postharvest enzymatic browning and oxidative
676 stress of litchi fruit. Postharvest Biol. Technol. 62, 288-294. https://doi.org/10.1016/
677 j.postharvbio. 2011.07.001
678 Vossen, J.H., Abd-El-Haliem, A., Fradin, E.F., van den Berg, G. C., Ekengren, S.K., Meijer,
679 H.J., Seifi, A., Bai, Y., ten Have, A., Munnik, T., Thomma, B.P., Joosten, M.H., 2010.
680 Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family
681 members and the role of PLC4 and PLC6 in HR and disease resistance. Plant J. 62, 224
682 239. https://doi.org/10.1111/j.1365-313X.2010.04136.x
683 Wang, X., 2001. Plant Phospholipases. Ann. Rev. Plant Physiol. Plant Molec. Biol. 52, 211-
684 231. https://doi.org/10.1007/978-1-61779-600-5_8
685 Wang, X., 2005. Regulatory functions of phospholipase D and phosphatidic acid in plant
686 growth, development, and stress responses. Plant Physiol. 139, 566-573.
687 https://doi.org/10.1104/pp.105.068809
688 Wasternack, C., Hause, B., 2013. Jasmonates: Biosynthesis, perception, signal transduction
689 and action in plant stress response, growth and development. An update to the 2007

review in Annals of Botany. Annals Bot. 111, 1021–1058. https://doi.org/10.1093/aob/mct067

Weiler, E.W., 1980. Radioimmunoassays for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. Planta 148, 262–272. https://doi.org/10.1007/BF00380037

Yi, C., Qu, H.X., Jiang, Y.M., Shi, J., Duan, X.W., Joyce, D.C., Li, Y.B., 2008. ATP–induced changes in energy status and membrane integrity of harvested litchi fruit and its relation to pathogen resistance. J. Phytopathol. 156, 365–371. https://doi.org/10.1111/j.1439–0434.2007.01371.x

Zhang, S., Lin, Y., Lin, H., Lin, Y., Chen, Y., Wang, H., Shi, J., Lin, Y., 2018. *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.–induced disease development and pericarp browning of harvested longan fruit in association with membrane lipids metabolism. Food Chem. 244, 93–101. https://doi.org/10.1016/j.foodchem.2017.10.020

Zhao, J., Devaiah, S.P., Wang, C., Li, M., Welti, R., Wang X., 2013. Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. New Phytol. 199, 228–240. https://doi.org/10.1111/nph.12256

Zhao, J., Wang, C., Bedair, M., Welti, R., Sumner, L.W., Baxter, I., Wang, X., 2011. Suppression of phospholipase Dγs confers increased aluminium resistance in *Arabidopsis thaliana*. PloS one, 6(12), e28086. https://doi.org/10.1371/journal.pone.0028086

709 ## Figure Legends

710 Figure 1. Relative ABA content and activity of enzymes PLD, PLC and PLA_2 (A), and
711 relative expression levels of the *CsPLD–, CsPLC–* and *CsPLA*-encoding genes (B), in the
712 Navelate vs. the Pinalate fruit. Different letters within columns mean significant differences
713 **($p \leq 0.05$)** for data presented in panel A and for data presented in panel B. The error interval
714 indicates the standard error of the estimated mean value.

715

716 Figure 2. Changes in the activity of enzymes PLD (A, B), PLC (C, D) and PLA_2 (E, F) in
717 the flavedo of the Navelate (A, C, E) and Pinalate (B, D, F) oranges inoculated at a depth of
718 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were
719 inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 ºC. The error
720 interval indicates the standard error of the estimated mean value. Different letters mean
721 **significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar**
722 for the same storage period.

723

724 Figure 3. Changes in the expression of the genes encoding PLD in the flavedo of the Navelate
725 sweet orange inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}).
726 The control samples (○) were inoculated with 10 µL of water. After infection, fruit were
727 left in the dark at 20 ºC. The error interval indicates the standard error of the estimated mean
728 **value. Different letters mean significant differences ($p \leq 0.05$) between the infected and**
729 control fruit for the same storage period.

730

731 Figure 4. Changes in the expression of the genes encoding PLD in the flavedo of the Pinalate
732 mutant inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The

733 control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in
734 the dark at 20 ºC. The error interval indicates the standard error of the estimated mean value.
735 Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit
736 for the same storage period.
737
738 Figure 5. Changes in the expression of the genes encoding PLC in the flavedo of the Navelate
739 (A, C) and Pinalate (B, D) oranges inoculated at a depth of 4 mm (●) with 10 µL of *P.*
740 *digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water.
741 After infection, fruit were left in the dark at 20 ºC. The error interval indicates the standard
742 error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$)
743 between the infected and control fruit of the same cultivar for the same storage period.
744
745 Figure 6. Changes in the expression of the genes encoding PLA_2 in the flavedo of the
746 Navelate (A, C, D) and Pinalate (B, D, F) oranges inoculated at a depth of 4 mm (●) with 10
747 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL
748 of water. After infection, fruit were left in the dark at 20 ºC. The error interval indicates the
749 standard error of the estimated mean value. Different letters mean significant differences (p
750 ≤ 0.05) between the infected and control fruit of the same cultivar for the same storage period.
751
752 Figure 7. Schematic integration of the changes in phospholipases gene expression and
753 activities induced by *P. digitatum* into the Navelate and its ABA-deficient mutant Pinalate
754 before disease development. The effect of *P. digitatum* on phospholipases gene expression
755 is indicated by shape size: The bigger the size, the more marked the changes in the transcript
756 levels induced by the fungus. The genes within the smallest elipses were not differently

757 regulated between the infected and control fruit. The putative effect of ABA on
758 phospholipases gene expression was deduced by differences in the transcript levels between
759 Navelate and its ABA-deficient Pinalate fruit, and indicated by different proportions of
760 orange and yellow, respectively, in elipses/circles. A bigger orange area means more marked
761 induction in Navelate. Grey denotes no differences between both cultivars and, hence, no
762 effect of ABA on phospholipase transcript or activity regulation. The thickness of the black
763 arrows increases with the correlation between the gene expression of phospholipases
764 isoforms and their respective activities. The thicker green arrows indicate more involvement
765 of these activities in fruit response to infection. To visualise these colours, readers are
766 referred to the web version of this article.
767
768

Revised Fig. 1

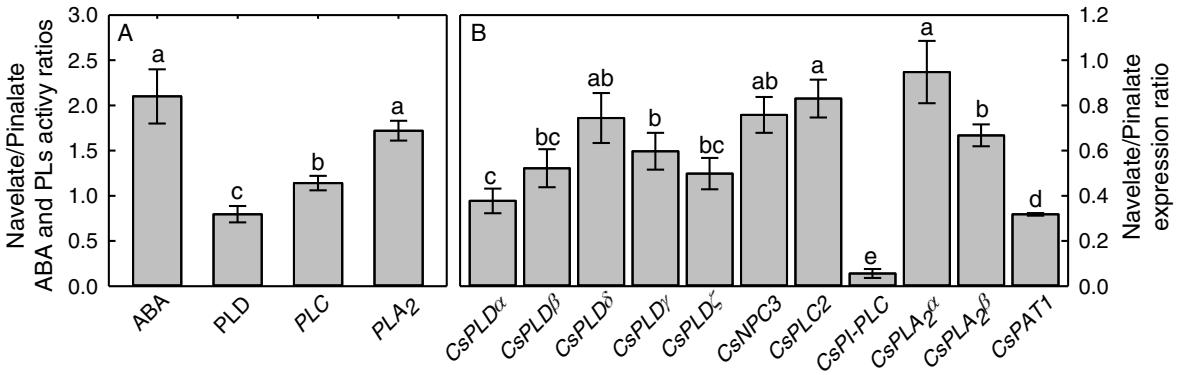


Figure 1. Relative ABA content and activity of enzymes PLD, PLC and PLA_2 (A), and relative expression levels of the *CsPLD-*, *CsPLC-* and *CsPLA*-encoding genes (B), in the Navelate vs. the Pinalate fruit. Different letters within columns mean significant differences $(p \leq 0.05)$ for data presented in panel A and for data presented in panel B. The error interval indicates the standard error of the estimated mean value.

Revised Fig. 2

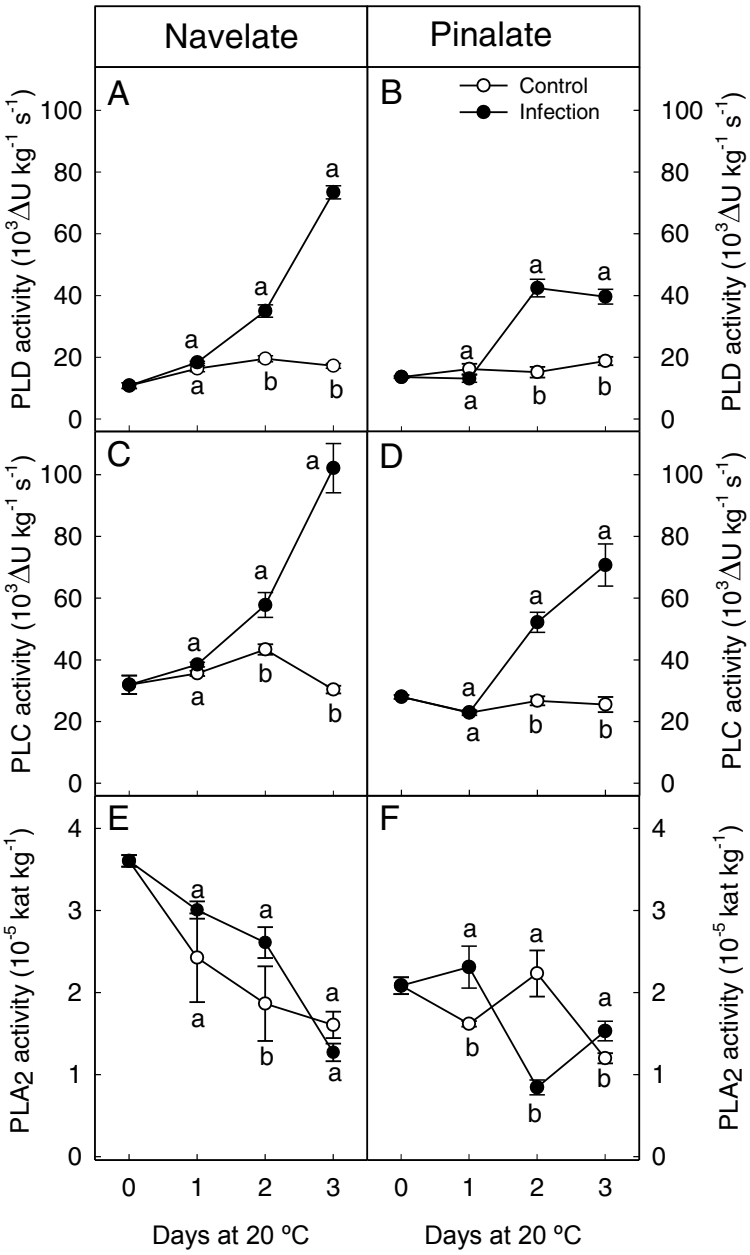


Figure 2. Changes in the activity of enzymes PLD (A, B), PLC (C, D) and PLA_2 (E, F) in the flavedo of the Navelate (A, C, E) and Pinalate (B, D, F) oranges inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 °C. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar for the same storage period.

Revised Fig. 3-v3

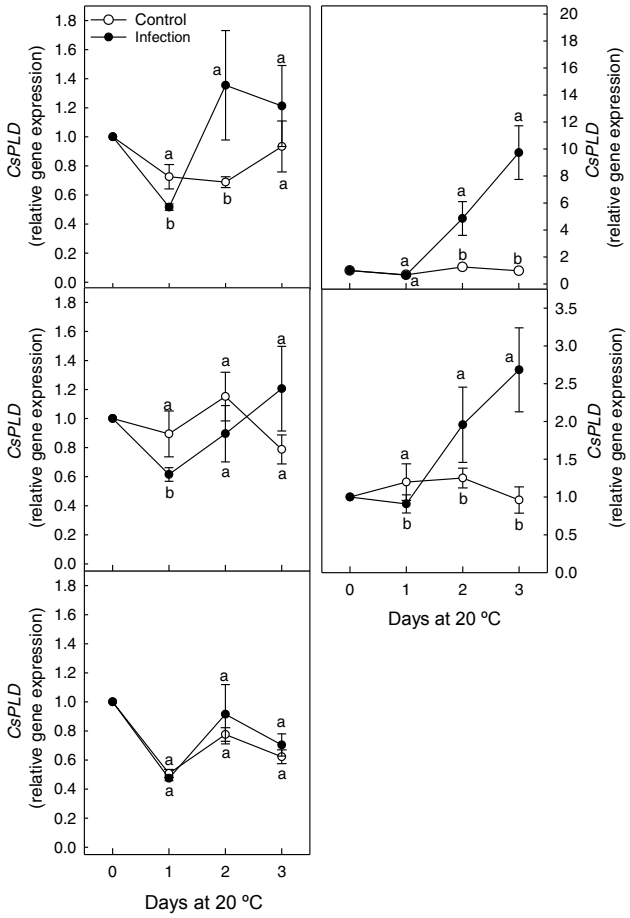


Figure 3. Changes in the expression of the genes encoding PLD in the flavedo of the Navelate sweet orange inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 ºC. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit for the same storage period.

Revised Fig. 4

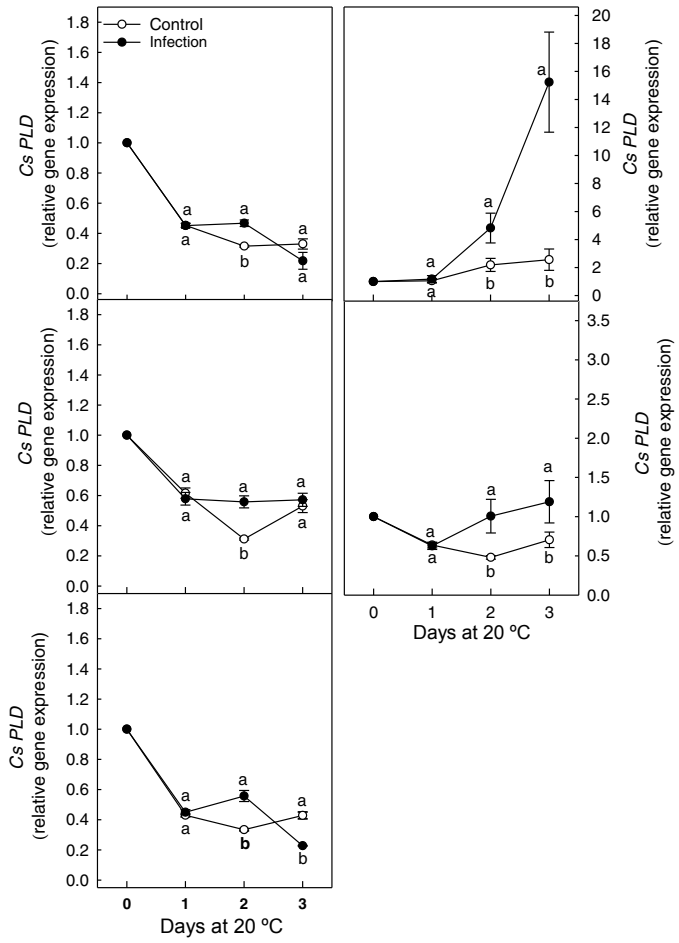


Figure 4. Changes in the expression of the genes encoding PLD in the flavedo of the Pinalate mutant inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 °C. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit for the same storage period.

Revised Fig. 5

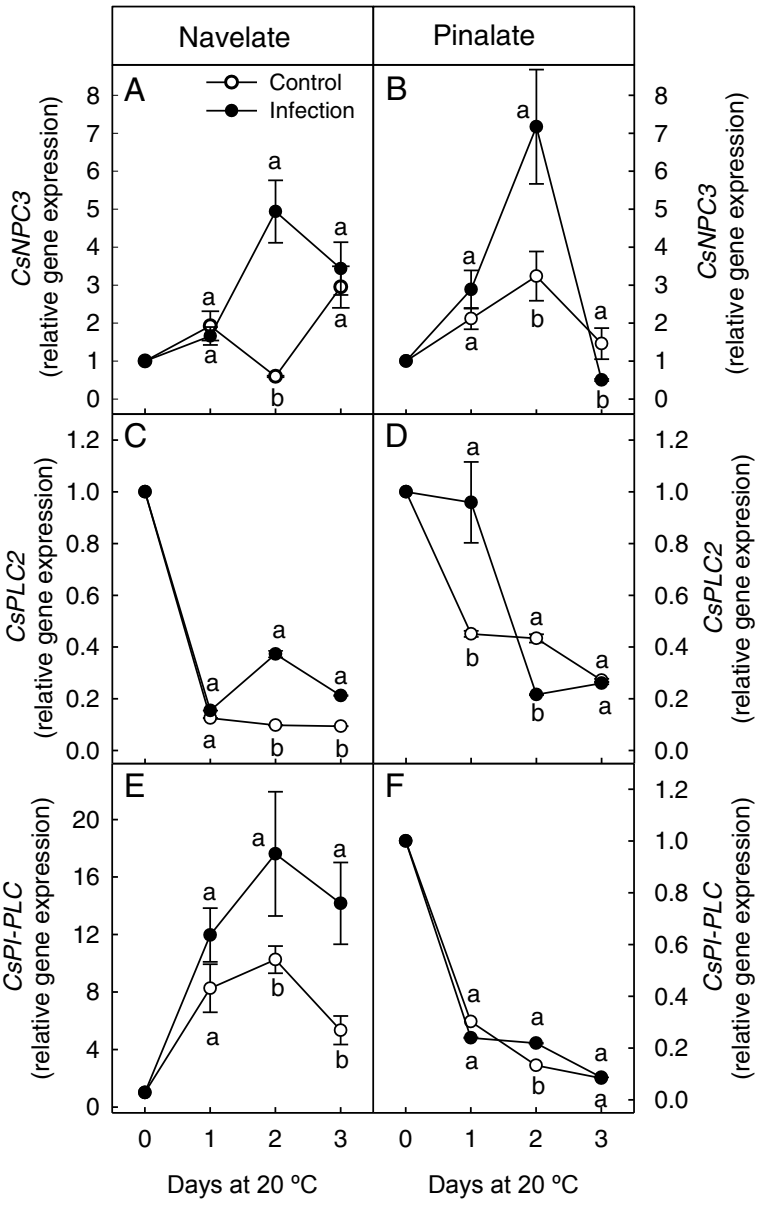


Figure 5. Changes in the expression of the genes encoding PLC in the flavedo of the Navelate (A, C) and Pinalate (B, D) oranges inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 ºC. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar for the same storage period.

Revised Fig. 6

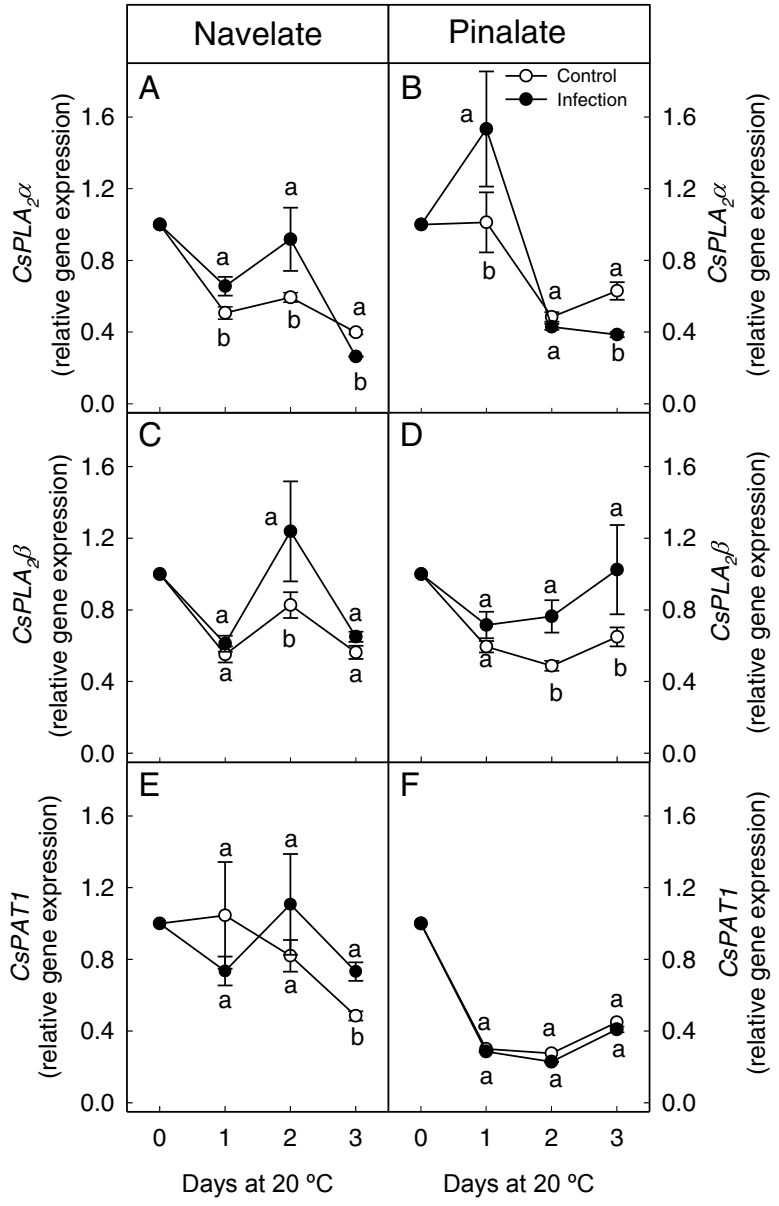


Figure 6. Changes in the expression of the genes encoding PLA_2 in the flavedo of the Navelate (A, C, D) and Pinalate (B, D, F) oranges inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^4 conidia mL^{-1}). The control samples (○) were inoculated with 10 µL of water. After infection, fruit were left in the dark at 20 ºC. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar for the same storage period.

Figure 7

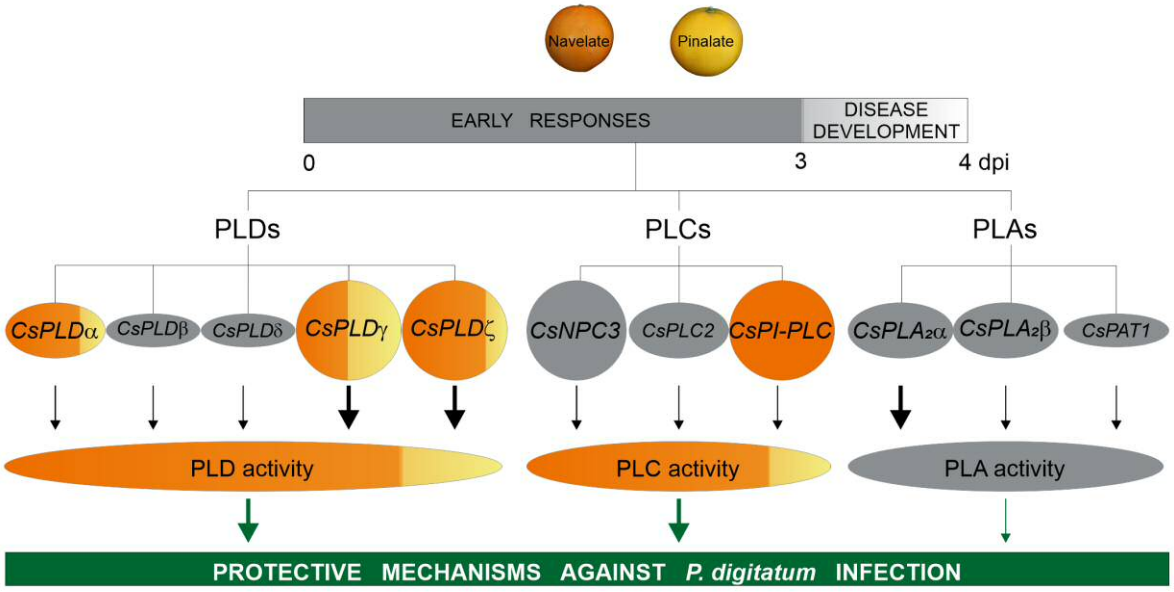
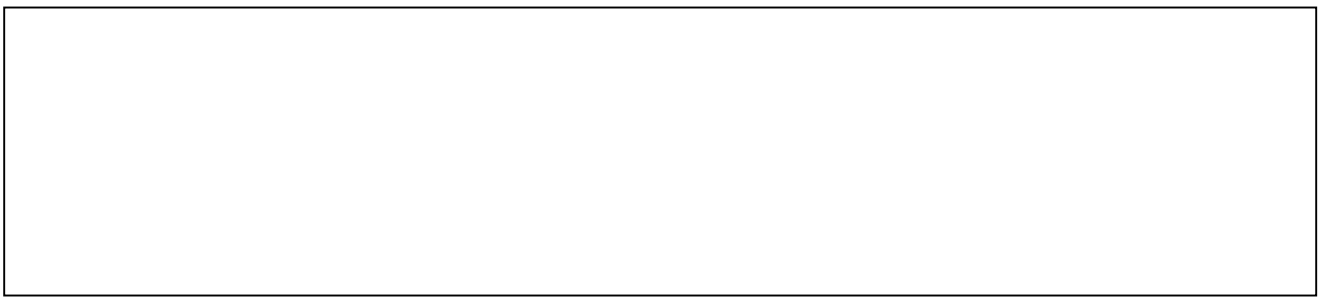


Figure 7. Schematic integration of the changes in phospholipases gene expression and activities induced by *P. digitatum* into the Navelate and its ABA-deficient mutant Pinalate before disease development. The effect of *P. digitatum* on phospholipases gene expression is indicated by shape size: The bigger the size, the more marked the changes in the transcript levels induced by the fungus. The genes within the smallest elipses were not differently regulated between the infected and control fruit. The putative effect of ABA on phospholipases gene expression was deduced by differences in the transcript levels between Navelate and its ABA-deficient Pinalate fruit, and indicated by different proportions of orange and yellow, respectively, in elipses/circles. A bigger orange area means more marked induction in Navelate. Grey denotes no differences between both cultivars and, hence, no effect of ABA on phospholipase transcript or activity regulation. The thickness of the black arrows increases with the correlation between the gene expression of phospholipases isoforms and their respective activities. The thicker green arrows indicate more involvement of these activities in fruit response to infection. To visualise these colours, readers are referred to the web version of this article.

Declaration of interests

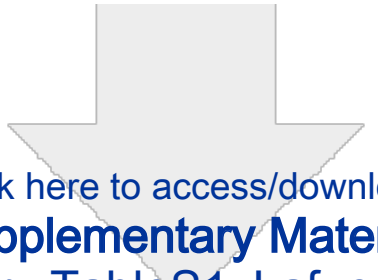
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Author statement:

M.T. Lafuente conceived the project and designed the experiments. A-R. Ballester, J. Cerveró, P. Romero, and M.T. Lafuente participated in the analysis of gene expression; and N. Holland, J. Cerveró and M.T. Lafuente in the analysis of the activities of the phospholipase enzymes. M.T. Lafuente wrote the manuscript, A.R. Ballester and P. Romero contributed to its improvement; and all the authors approved the final manuscript.

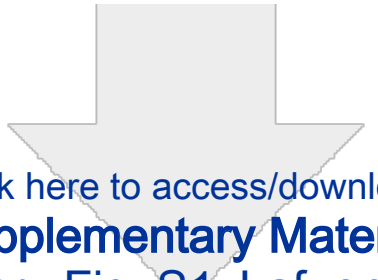


Click here to access/download

Supplementary Material

Supplementary TableS1_Lafuente et al.docx

Supplementary Material Fig. 1S



Click here to access/download

Supplementary Material

Supplementary Fig. S1_Lafuente et al.docx

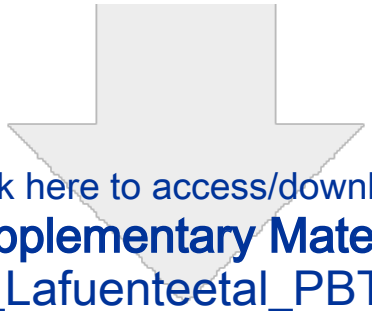
Revise MS with track changes

Revise MS with track changes including English corrections

Certificate corrections MS

Supplementary Material_revised references

Fig. 3 TIFF

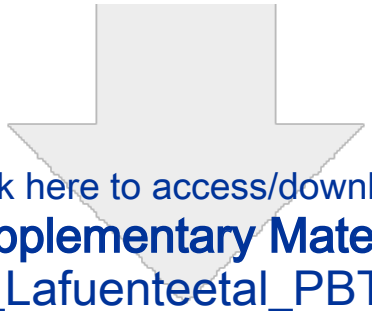


Click here to access/download

Supplementary Material

Figure3_Lafuenteetal_PBT_V3.TIF

Fig. 4 TIFF



Click here to access/download

Supplementary Material

Figure4_Lafuenteetal_PBT_V3.TIF