Stress-dependent regulation of 13-lipoxygenases and 13-hydroperoxide lyase in olive fruit mesocarp

María N. Padilla, M. Luisa Hernández, Carlos Sanz, José M. Martínez-Rivas*

Department of Biochemistry and Molecular Biology of Plant Products, Instituto de la Grasa (CSIC), 41012-Sevilla, Spain

María N. Padilla: npadilla@ujaen.es

M. Luisa Hernández: mlhdez@cica.es

Carlos Sanz: carlos.sanz@ig.csic.es

*Corresponding author: Tel: +34-95-4611550; fax: +34-95-4616790.

E-mail address: mrivas@cica.es (J.M. Martínez-Rivas)

ABSTRACT

The effect of different environmental stresses on the expression and enzyme activity levels of 13-lipoxygenases (13-LOX) and 13-hydroperoxide lyase (13-HPL) and on the volatile compounds synthesized by their sequential action has been studied in the mesocarp tissue of olive fruit from the Picual and Arbequina cultivars. The results showed that temperature, light, wounding and water regime regulate olive *13-LOXs* and *13-HPL* genes at transcriptional level. Low temperature and wounding brought about an increase in LOX and HPL enzyme activities. A very slight increase in the total content of six straight-chain carbons (C6) volatile compounds was also observed in the case of low temperature and wounding treatments. The physiological roles of 13-LOXs and 13-HPL in the olive fruit stress response are discussed.

Keywords: Olea europaea; Oleae; Olive; Abiotic stress; Gene expression; Enzyme activity; Volatile compounds; Lipoxygenase; Hydroperoxide lyase

1. Introduction

The group of compounds formed by six straight-chain carbons (C6) aldehydes, alcohols and their esters synthesized through the 13-hydroperoxide lyase (13-HPL) branch of the lipoxygenase (LOX) pathway are known as "green leaf volatiles" (GLVs). The amount of GLVs is low in intact and healthy plant tissues. However, GLVs are formed rapidly when the tissues are disrupted. GLVs are important molecules involved in plant defense against microorganisms, in attracting predator upon herbivore attack, and also as signal compounds within and between plants to induce transcripts of several defense related genes (Matsui, 2006; Stumpe and Feussner, 2006). GLVs are produced through the LOX pathway by the consecutive action of several enzymes. First, polyunsaturated fatty acids are hydrolyzed from lipids by different types of acyl-lipid hydrolases. Afterwards, LOX catalyzes the stereospecific oxidation of the polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene structure such as linoleic (LA) and linolenic (LnA) acids. Plant LOXs are ubiquitous, encoded by multigene families and are classified with respect to their positional specificity of fatty acid oxygenation, which can occur either at C9 (9-LOX) or at C13 (13-LOX) of the hydrocarbon backbone in case of a C18 fatty acid (Mosblech et al., 2009). The 13-hydroperoxides produced by 13-LOX are subsequently cleaved by 13-HPL into C6 aldehydes and C12 oxoacids. 13-HPLs belong to a subfamily of cytochrome P450s (CYP74B) that, unlike other P450 enzymes, do not require molecular oxygen nor NAD(P)H dependent cytochrome P450-reductase as cofactors, using polyunsaturated fatty acid hydroperoxides as both substrate and oxygen donor (Gigot et al., 2010). C6 aldehydes can then undergo reduction by alcohol dehydrogenases (ADH) to form C6

alcohols that can finally be transformed into the corresponding esters by means of an alcohol acyltransferase (AAT) (Schwab et al., 2008).

GLVs are also the most important compounds of the virgin olive oil (VOO) aroma from either a quantitative or a qualitative point of view. These C6 volatile compounds are synthesized *de novo* when enzymes and substrates meet as tissues are disrupted during VOO processing. The participation of the 13-HPL branch of the LOX pathway in the biosynthesis of the C6 volatile compounds mainly responsible for VOO aroma has been previously demonstrated (Olias et al., 1993). In olive, four *LOX* genes have been isolated and characterized to date: two *LOX* genes encoding isoforms which show strictly 13-LOX activity and possibly chloroplast localization (*Oe1LOX2* and *Oe2LOX2*, Padilla et al., 2009) and two *LOX* genes encoding putative cytosolic isoforms that exhibit mainly 9-LOX activity, forming both 9- and 13-hydroperoxides from linoleic acid in a ratio of 2:1 (*Oe1LOX1*, Palmieri-Thiers et al., 2009) and 4:1 (*Oe2LOX1*, Padilla et al., 2012). *Oe2LOX2* showed an increase in its transcript level in mesocarp during olive fruit development and ripening, with a maximum at turning stage that coincides with an increase in the synthesis of volatile compounds present in VOO. On the contrary, *Oe1LOX2* exhibited constant expression levels. These results indicate a major involvement of the *Oe2LOX2* gene in the biosynthesis of VOO volatile compounds (Padilla et al., 2009). In addition, different LOX isoforms have been purified from olive fruits (Salas et al., 1999; Lorenzi et al., 2006) and callus (Williams and Harwood, 2008), both soluble and membrane-bound. On the other hand, only one olive *HPL* gene has been cloned and characterized so far (*OeHPL*, Padilla et al., 2010). The *OeHPL* gene codes for a HPL protein with a strict specificity for 13-hydroperoxides, putative chloroplast localization and it is expressed in olive fruit mesocarp displaying a slight, though significant, maximum at the onset of ripening.

This slight maximum preceded the fruit developmental stage which gives rise to oils with the highest contents of C6 aldehydes (Padilla et al., 2010). Besides, the native membrane-bound HPL enzyme has been purified from olive fruit (Salas and Sánchez, 1999).

Different plant LOX isoforms may have different physiological roles. Antisense studies in potato (León et al., 2002) and *Nicotiana attenuata* (Allmann et al., 2010) have shown evidences of a metabolic interaction between a specific 13-LOX isoform and 13-HPL for the production of GLVs. These findings are consistent with the co-localization of the 13-LOX isoform LOX H1 and the 13-HPL in the stromal part of the thylakoids reported in potato chloroplasts (Farmaki et al., 2007). LOX and HPL expression in plants is regulated throughout development and in response to stress. In particular, the effect of temperature on LOX and HPL transcript and activity levels has been studied during postharvest storage of fruits such as guava (González-Aguilar et al., 2004), kiwi (Zhang et al., 2006), peach (Zhang et al., 2011), tomato (Bai et al., 2011) and banana (Yang et al., 2011). However, very scarce information is available for fruits during development and ripening before harvesting. In contrast to the effect of light or water deficit where the available information is very scanty, the effect of wounding has been very much studied in plant leaves and fruits showing the involvement of LOX and HPL in the response to stress in wounded tissues (Howe and Schilmiller, 2002). In the case of olive fruit, wounding caused during harvesting and transport, or due to olive fruit infestation by olive fly can modify VOO volatile composition. In particular, a reduction in the total volatile compounds, especially *trans*-2-hexenal, in oils extracted from olive fruit infected by olive fly has been reported (Tamendjari et al., 2004). Furthermore, several studies have shown that different water regimes could also affect VOO volatile

content and composition (Gómez-Rico et al., 2006; Servili et al., 2007; Stefanoudaki et al., 2009; Dabbou et al., 2011).

With the aim of determining the environmental factors that regulate GLVs formation in olive fruit mesocarp, we have investigated in the present work the effect of low and high temperature, darkness, wounding, and water regime on 13-LOXs and 13-HPL in mesocarp tissue of the main cultivars grown in Spain for VOO extraction, Picual and Arbequina.

2. Results and discussion

2.1. Temperature regulation of olive 13-lipoxygenases and 13-hydroperoxide lyase

To investigate the effect of temperature on the olive *13-LOXs* and *13-HPL* transcript levels in olive fruit mesocarp from Picual and Arbequina cultivars, olive branches holding olive fruit with 28 weeks after flowering (WAF) (turning stage) were incubated at low (15 ºC) and high (35 ºC) temperature with a 12 h light / 12 h dark cycle, for 24 h. When olive fruit of the Picual cultivar were incubated at 15 ºC, a strong and transient increase in *Oe1LOX2* and *OeHPL* gene expression levels and a slight increase for *Oe2LOX2* was observed, reaching a maximum after 6 h of incubation, and then, decreasing to initials levels (Fig.1). In the case of Arbequina cultivar, the expression levels of the three genes were slightly up-regulated with a maximum at 3-6 h of treatment. The same expression patterns have been described in both cultivars, respectively, for a 9-LOX gene (*Oe2LOX1*; Padilla et al., 2012). The transient increase of transcript levels of a 13-LOX in response to low temperatures has also been reported in leaves of maize and *Caraganata jubata* plants incubated at 4 ºC, for the *ZmLOX10* (Nemchenko et al., 2006) and *CjLOX* (Bhardwaj et al., 2010) genes, respectively. In the same way, bean seeds germinated at 4 ºC showed an increase in the level of expression of a *LOX2* gene (Porta et al., 1999). In contrast, to our knowledge, no increase in *13-HPL* transcripts in response to low temperature has been reported so far in leaves or *in planta* ripening fruits.

To check if the observed increases in transcript levels were accompanied of increases in the corresponding enzyme activity levels, crude extracts from mesocarp of

olive fruit of both cultivars incubated at 15 ºC were obtained, and the LOX and HPL activity levels were determined. As shown in Fig. 2A, an increase in the LOX and HPL activity levels was detected, which correlates with the increase of expression of the corresponding genes observed during the first 6 h of treatment. However, not all the detected increase in LOX activity can be necessarily attributed to 13-LOX, since crude extracts obtained from olive fruit mesocarp exhibit both 9- and 13-LOX activity in a approximately 60:40 ratio (Olias et al., 1993).

In the same way, to verify if the observed increases in expression and activity levels of olive 13-LOXs and 13-HPL brought about the corresponding increase in the content of the main volatile compounds (C6), homogenates from mesocarp of olive fruit of both cultivars incubated at 15 ºC were obtained to analyze volatile compounds (Fig. 2B). No significant differences were found in the volatile contents along time except for the C6 volatile compounds generated from LA in cultivar Picual, which showed a slight significant increase after 6 h.

Several factors can contribute to explain this discrepancy between transcript, activity and volatiles levels. Firstly, the participation of other *LOX* genes with 13-LOX activity and/or the existence of post-transcriptional regulatory mechanisms cannot be ruled out. On the other hand, previous studies modifying the 13-LOX activity load during the oil extraction process have shown that 13-LOX activity could be a limiting factor for the synthesis of the volatile fraction, this limitation being significantly higher in Picual cultivar that in Arbequina, in line with the lowest content of volatile compounds in the oils obtained from the former (Sánchez-Ortiz et al., 2012b). In addition, 13-LOX isoenzymes could compete with 9-LOX isoenzymes for LA and LnA as substrates. In fact, it has been shown that the expression levels of a 9-LOX gene (*Oe2LOX1*) transiently increase when olive fruit from both cultivars were incubated at

15 ºC (Padilla et al., 2012). Not only 13-LOX enzyme activity, but also polyunsaturated fatty acids acting as substrates have been suggested as limiting factors for the biogenesis of the VOO aroma in Picual and Arbequina cultivars (Sánchez-Ortiz et al., 2007). Finally, the involvement of non-enzymatic lipid peroxidation has also been proposed (Yilmaz et al., 2001).

Regarding a possible physiological explanation for the increase of LOX transcript and activity level when plants are exposed to low temperature, LOX-mediated lipid peroxidation has been proposed as a source of active oxygen species, particularly under stress conditions (Blokhina et al., 2003). In vegetative tissues such as roots, increased LOX activity and up-regulation of transcripts in response to low temperature have been associated with chilling tolerance (Lee et al., 2005).

Unlike low temperature, the incubation of olive fruit at high temperature (35 ºC) caused a decrease in the expression levels of the three genes studied in both cultivars (Fig. 3). A similar expression pattern has been described in Picual and Arbequina cultivars for a 9-LOX gene (*Oe2LOX1*; Padilla et al., 2012). Furthermore, a decrease in the *LOX* gene expression level has been reported in banana fruit incubated at 30ºC (Yang et al., 2011). High temperature could affect not only the transcriptional level of the mentioned genes, but also the activity level of some of the encoded proteins. In fact, it has been shown that after 30 min *in vitro* incubation of purified recombinant proteins at 35 ºC, Oe1LOX2 and Oe2LOX2 exhibit only about 40% of the initial activity, whereas OeHPL maintains 100% activity level (Padilla et al., 2012).

2.2. 13-lipoxygenases and 13-hydroperoxide lyase genes from olive are repressed by darkness

In order to study the effect of darkness on the *Oe1LOX2*, *Oe2LOX2* and *OeHPL* gene expression levels in olive fruit mesocarp from Picual and Arbequina cultivars, olive branches were incubated at 25 ºC in the darkness for 24 h. A reduction in the transcript levels of the three genes studied was observed, especially in Picual during the first 3 h of treatment (Fig. 4). A maize 13-LOX gene (*ZmLOX10*; Nemchenko et al., 2006) has been reported to exhibit the characteristics of a circadian-regulated gene since it maintains the same cyclic expression pattern when the plants were transferred from a 12 h light / 12 h dark photoperiod into constant darkness. On the contrary, the decrease of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* transcript levels observed when olive fruit were shifted to darkness indicates a light-dependent transcriptional regulation of these three genes, as it was previously described for an olive 9-LOX gene (*Oe2LOX1*; Padilla et al., 2012).

2.3. Regulation of olive 13-lipoxygenases and 13-hydroperoxide lyase in response to wounding

To study the effect of wounding on the *13-LOXs* and *13-HPL* genes expression in olive fruit mesocarp, olive branches of Picual and Arbequina cultivars were incubated at standard conditions, except that olive fruit were subjected to mechanical damage with pressure using forceps with serrated tips. *Oe1LOX2* and *OeHPL* transcript levels were transiently increased after wounding, especially in Arbequina cultivar, reaching a maximum after 1 h incubation and then, decreasing rapidly to initial levels (Fig. 5). On the contrary, *Oe2LOX2* expression level was not altered by wounding. Based on the

high degree of sequence similarity and expression pattern of these two olive *13-LOX* genes to those previously well characterized in potato (Royo et al., 1996) and tomato (Heitz et al., 1997), it has been proposed that *Oe1LOX2* should be wound-inducible and involved in the synthesis of jasmonic acid, whereas *Oe2LOX2* could be implicated in the generation of volatile compounds (Padilla et al., 2009). The present data support this hypothesis. On the other hand, the induction of the *13-HPL* gene in wounded leaves has been reported for several plants like Arabidopsis (Bate et al., 1998), potato (Vancanneyt et al., 2001), and *Nicotiana attenuata* (Halitschke et al., 2004), but no information was available in wounded fruits.

The observed increase of *Oe1LOX2* and *OeHPL* expression levels caused by mechanical damage was accompanied of increases in the corresponding enzyme activity levels. As shown in Figure 6A, crude extracts from mesocarp of wounded olive fruit of Arbequina cultivar exhibit an increase in both LOX and HPL activities, which is parallel to the increase of transcript levels of the corresponding genes detected during the first 3 h after treatment. Nevertheless, given that crude extracts obtained from olive fruit mesocarp show both 9- and 13-LOX activity in a 60:40 ratio (Olias et al., 1993), 13-LOX isoforms are not necessarily the exclusive responsible for the detected increase in LOX activity. In fact, a transient increase in response to wounding has been reported for a 9-LOX gene (*Oe2LOX1*) in both cultivars (Padilla et al., 2012). Similar increases in LOX and HPL activity have also been observed in wounded leaves of rice seedlings (Wang et al., 2008) and rough lemon (Gomi et al., 2003), respectively.

Likewise, to check if the detected increases in transcript and activity levels of olive 13-LOXs and 13-HPL produced the corresponding increase in the content of the C6 volatile compounds, homogenates from mesocarp of wounded olive fruit of Arbequina

cultivar were obtained to analyze volatile compounds. A significant increase in the total content of C6 volatile compounds generated from LnA was observed at 0.5 h (Fig. 6B).

As mentioned above, the divergence between transcript, activity and volatiles levels could be related to different factors such as the participation of other *LOX* genes with 13-LOX activity, the possible occurrence of post-transcriptional regulatory mechanisms, the limitation of 13-LOX activity (Sánchez-Ortiz et al., 2012b) and polyunsaturated fatty acids acting as substrates (Sánchez-Ortiz et al., 2007) for the synthesis of the volatile fraction, the competition of 9-LOX isoenzymes with 13-LOX isoenzymes for LA and LnA acids as substrates (Padilla et al., 2012), as well as the contribution of non-enzymatic lipid peroxidation (Yilmaz et al., 2001).

2.4. Effect of water regime on 13-lipoxygenases and 13-hydroperoxide lyase genes from olive

The induction of *LOX* genes in hypocotyls of plants subjected to water deficit such as soybean (Bell and Mullet, 1991) or common bean (Porta et al., 1999) has been previously described. In addition, the transcript abundance of two *LOXs* and a *HPL* gene was up-regulated significantly by water deficit in grape berries during development and ripening (Deluc et al., 2009). For those reasons, the effect of two different water regimes (natural rainfall and additional irrigation) on the olive *13-LOXs* and *13-HPL* transcript levels has also been studied in olive fruit mesocarp from Picual and Arbequina cultivars. Similar expression levels of *Oe1LOX2* and *OeHPL* genes were detected for both cultivars in the two water regimes (Fig. 7), as it was reported for an olive 9-LOX gene (*Oe2LOX1*; Padilla et al., 2012). In contrast, a higher expression

level was observed for the *Oe2LOX2* gene when both cultivars were grown with natural rainfall only. This increase could be related to the alteration of the functionality of the cell membranes. It has been shown that water deficit increases LOX activity and the hydroperoxide content of the lipid ester fraction, leading to changes in membrane fluidity and permeability, ultimately giving rise to dysfunctioning of the lipid bilayer (Maccarrone et al., 1995).

3. Concluding remarks

In the present work, the transcriptional regulation of olive *13-LOXs* and *13-HPL* genes by temperature, light, wounding and water regime has been shown. In addition, a significant increase in LOX and HPL enzyme activities has been also detected in the case of low temperature and wounding stresses. However, only a very slight increase in the total content of C6 volatile compounds was observed for both treatments. Our results also support the previous hypothesis that Oe1LOX2 could be involved in the synthesis of jasmonic acid, whereas Oe2LOX2 can be implicated in the formation of GLVs in combination with OeHPL. This research represents an important step towards the understanding of the factors involved in the regulation of the synthesis of GLVs in fruits. In the case of olive, this knowledge will allow the development of molecular markers to be used in the marker-assisted selection of new cultivars with improved VOO aroma.

4. Experimental

4.1. Plant material and stress treatments

Olive (*Olea europaea* L.) trees cv. Picual and Arbequina were grown in the experimental orchards of Instituto de la Grasa, Seville (Spain), with drip irrigation and fertirrigation from the time of full bloom to fruit maturation. In the case of no irrigation, the olive trees received only natural rainfall. For the stress treatments, branches with about 100 olive fruit at 28 WAF (turning stage) were collected from Picual or Arbequina olive trees and incubated in a growth chamber at 25 ºC with a 12 h light/12 h dark cycle to mimic physiological conditions of the tree. The light intensity was 11.5 µmol m^{-2} s^{-1}. With these standard conditions, no changes in gene expression levels were detected in the mesocarp of the olive fruit. For stress treatments, standard conditions were modified depending on the effect studied. For low and high temperature experiments, the branches containing the olive fruit were incubated at 15 or 35 ºC, respectively, at the standard light intensity. To assess the effect of the darkness, light was turned off and the standard temperature was maintained. To study the effect of wounding, the whole surface of the olive fruit was mechanically damaged affecting mesocarp tissue, with pressure at zero time using forceps with serrated tips. The zero time of each experiment was selected 2 h after the beginning of the light period to preserve the natural photoperiod day/night of the olive fruit. For RNA isolation, olive mesocarp tissues were sampled, frozen in liquid nitrogen and stored at -80 ºC. In the case of crude extracts preparation or analysis of volatile compounds, fresh mesocarp tissue was always used.

4.2. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA isolation was performed from 1-2 g of frozen mesocarp tissue from different olive fruit as described by Hernández et al. (2005). RNA quality verification, removal of contaminating DNA, and cDNA synthesis were carried out according to Hernández et al. (2009).

Gene expression analysis was performed by qRT-PCR using a Mx3000P^{TM} real-time PCR System and the "Brilliant® SYBR® Green Q-PCR Master Mix (Stratagene, La Jolla, USA) as previously described (Hernández et al., 2009). Primers for gene-specific amplification (Padilla et al., 2009; 2010) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The specificity of the PCR amplification and the presence of primer dimers was monitored by melting curve analysis following the final step of the PCR, and beginning at 55 ºC through 95 ºC, at 0.1 ºC s^{-1}. Additionally, PCR products were also checked for purity by agarose gel electrophoresis. PCR efficiencies (*E*) of all primers were calculated using dilution curves with eight dilution points, two-fold dilution, and the equation $E = [10^{(-1/slope)}] - 1$. The housekeeping olive ubiquitin2 gene (*OeUBQ2*, AF429430) was used as an endogenous reference to normalize. The real-time PCR data were calibrated relative to the corresponding gene expression level at zero time for each treatment and cultivar. In both cases, the $2^{-\Delta\Delta Ct}$ method for relative quantification was followed (Livak and Schmittgen, 2001). The data are presented as means ± standard deviation (SD) of three reactions performed in different 96-well plates, each having two replicates in each plate.

4.3. Crude extracts preparation from olive fruit mesocarp

Crude extracts to measure LOX activity were obtained by grinding 4 g of fresh mesocarp tissue from different olive fruit in 16 ml of 100 mM sodium phosphate buffer (pH 6.7) containing 0.1 % Triton X-100, 1 mM EDTA, 0.1 mM benzamidine, 5 mM α-aminocaproic acid, 0.1 mM phenylmethylsulfonyl fluoride, and 5 % PVPP. For HPL activity assay, crude extracts were obtained by grinding the same amount of mesocarp tissue in 20 ml 50 mM HEPES-NaOH (pH 7.5), 20 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM $Na_2S_2O_5$, 7 mM DTT, 0.1 % ascorbate, 0.5 % Triton X-100, and 12.5 % PVPP. In both cases, grinding was carried out in two 1 min periods with an Ultraturrax homogenizer. The homogenates were filtered under vacuum through Miracloth and centrifuged at 27000*g* for 20 min at 4 ºC. The supernatant was centrifuged again at 10000*g* for 10 min at 4 ºC and used as crude extract.

4.4. LOX and HPL activity assay

Monitoring the conjugated diene formation, that occurs during the hydroperoxidation of the fatty acids, was used as approach to measure the level of LOX activity in olive fruit crude extracts as it has been also used previously in different publications on olive fruit (Olías et al., 1993; Salas et al., 1999; Lorenzi et al., 2006; Williams and Harwood, 2008, Patui et al., 2010) and other fruits (Gonzalez-Aguilar et al., 2004: Zhang et al., 2006; Bai et al., 2011). *In vitro* LOX activity was determined spectrophotometrically by continuously monitoring at 234 nm the formation of the conjugated diene. The

standard assay mixture consisted of 1.5 ml of 100 mM sodium phosphate buffer (pH 6.5), 25 µl of substrate solution (10 mM LnA, 0.85 % Tween-20), and the amount of enzyme solution (2-25 µl) giving rise to a slope no higher than 1 dA_{234} min^{-1}. One unit (U) of LOX activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of product min^{-1}.

In vitro HPL activity from crude extracts could not be tested using the coupled HPL/alcohol dehydrogenase (ADH) method according to Vick (1991) due to strong inhibition of ADH by components in the olive crude extracts (Sánchez-Ortiz et al., 2012a). To circumvent this problem, an approach for measuring the HPL activity in olive fruit crude extracts was chosen by monitoring the decrease of A_{234} due to the disruption of the conjugated diene chromophore of the substrates at 25 °C, which has been previously described in other publications on olive fruit (Olías et al., 1993; Salas and Sanchez, 1999; Luaces et al., 2007; Patui et al., 2010). The 1.5 ml standard assay mixture consisted of 100 mM sodium phosphate buffer (pH 8.0), 8 µl of 10 mM substrate solution and 10-20 µl of enzyme solution. One unit (U) of HPL activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of product min^{-1}. The 13-hydroperoxy isomer from the LnA utilized as substrate was prepared using soybean LOX according to the method of Hamberg and Samuelsson (1967).

4.5. Analysis of volatile compounds

The analysis of volatile compounds was carried out through the fruit homogenate approach according to Riley and Thompson (1998) modified by Sanchez-Ortiz et al. (2012a). For this purpose, 4 g of fresh olive fruit mesocarp was homogenized with 8 mL

of distilled water by means of an Ultraturrax at 24000 rpm for 1 min. After an equilibrium period of 5 min at 25 °C, homogenate aliquots of 1.5 mL were taken into 10 mL vials containing 1.5 mL of a saturated $CaCl_2$ solution that halts enzymatic changes which might induce quantitative and qualitative alterations in the samples' volatile profile following the olive mesocarp homogenization. Then vials were sealed and stored at −18 °C until analysis. Homogenate samples were conditioned to room temperature and then placed in a vial heater at 40 °C. After 10 min of equilibrium time, volatile compounds from headspace were adsorbed on a solid-phase microextraction (SPME) fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA) according to Luaces et al. (2003). The sampling time was 50 min at 40 °C, and it was carried out in triplicate. Desorption of volatile compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles were analyzed using a HP-6890 gas chromatograph equipped with a DB-Wax capillary column (60 m × 0.25 mm i.d., film thickness 0.25 μm; J&W, Scientific, Folsom, CA, USA). Operating conditions were as follows: N_2 as carrier, gas injector and detector at 250 °C, column was held for 6 min at 40 °C and then programmed at 2 °C min^{-1} to 128 °C. Quantification was performed using individual calibration curves for each identified compound by adding known amounts of different compounds to re-deodorized high oleic sunflower oil. Compound identification was carried out on a HRGC-MS Fisons series 8000 equipped with a similar stationary phase column and two different lengths, 30 and 60 m, matching against the Wiley/NBS Library, and by GC retention time against standards. Main volatile compounds were clustered according to the polyunsaturated fatty acid origin into different classes. Quantitative data for every volatile class is the sum of the content of the following compounds, showing their Kovats indices in square brackets. C6/LnA compounds: (E)-hex-3-enal [1137], (Z)-hex-3-enal [1156], (Z)-hex-2-enal [1218], (E)-

hex-2-enal [1233], (E)-hex-3-enol [1364], (Z)-hex-3-enol [1383], and (E)-hex-2-enol [1399]. C6/LA compounds: hexanal [1074] and hexan-1-ol [1355].

4.5. Statistical analysis

Data for enzyme activity and volatile compounds were statistically evaluated using Statgraphics Plus 5.1 (Manugistic Inc., Rockville, MD). Analysis of variance (ANOVA) was applied and comparison of means was done by the Student-Newman-Keuls/Duncan test at a significance level of 0.05.

Acknowledgments

This work was supported by research projects AGL2005-03959 and AGL2008-00258 from Programa Nacional de Recursos y Tecnologías Alimentarias and 2006 7 0I 055 from Programa I3 (MEC-CSIC), both funded by the Spanish Government, and P06-AGR-02151 and P09-AGR-04516 from Incentivos a Proyectos de Investigación de Excelencia funded by the Andalusian Government. M.N.P. and M.L.H. were recipients of FPI fellowships from Junta de Andalucía.

Figures legends

Fig. 1. Effect of low temperature on the relative expression levels of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* genes in the mesocarp tissue from Picual and Arbequina cultivars. Olive tree branches with about 100 olive fruit (28 WAF) were incubated using standard conditions except that the temperature was 15 ºC. Relative expressions levels at different times were determined by qRT-PCR using the expression level of the corresponding gene at zero time as calibrator.

Fig. 2. Effect of low temperature on the LOX and HPL activity levels in the mesocarp tissue (**A**) and on the content of C6 volatile compounds synthesized from LnA (black bars) or LA (white bars) in homogenates obtained from mesocarp tissue (**B**), corresponding to Picual and Arbequina cultivars. Olive tree branches with about 100 olive fruit (28 WAF) were incubated using standard conditions except that the temperature was 15 ºC. At the indicated times, crude extracts and homogenates were obtained from the corresponding mesocarp tissues and used to determine LOX and HPL activity and volatile composition, respectively. Values for each enzymatic activity or

volatile class with different letters within each olive cultivar are significantly different ($P \leq 0.05$). Each class of volatile compounds comprises compounds listed in Experimental section.

Fig. 3. Effect of high temperature on the relative expression levels of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* genes in the mesocarp tissue from Picual and Arbequina cultivars. Olive tree branches with about 100 olive fruit (28 WAF) were incubated using standard conditions except that the temperature was 35 ºC. Relative expressions levels at different times were determined by qRT-PCR using the expression level of the corresponding gene at zero time as calibrator.

Fig. 4. Effect of darkness on the relative expression levels of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* genes in the mesocarp tissue from Picual and Arbequina cultivars. Olive tree branches with about 100 olive fruit (28 WAF) were incubated at 25 ºC under darkness conditions for 24 h. Relative expressions levels at different times were determined by qRT-PCR using the expression level of the corresponding gene at zero time as calibrator.

Fig. 5. Effect of wounding on the relative expression levels of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* genes in the mesocarp tissue from Picual and Arbequina cultivars. Olive tree branches with about 100 olive fruit (28 WAF) were incubated using standard conditions except that olive fruit were mechanically damaged at zero time. At the indicated times,

relative expressions levels were determined by qRT-PCR using the expression level of the corresponding gene at zero time as calibrator.

Fig. 6. Effect of wounding on the LOX and HPL activity levels in the mesocarp tissue (A) and on the content of C6 volatile compounds synthesized from LnA (black bars) or LA (white bars) in homogenates obtained from mesocarp tissue (B) corresponding to Arbequina cultivar. Olive tree branches with about 100 olive fruit (28 WAF) were incubated using standard conditions except that olive fruit were mechanically damaged at zero time. At the indicated times, crude extracts and homogenates were obtained from the corresponding mesocarp tissues and used to determine LOX and HPL activity and volatile composition, respectively. Values for each enzymatic activity or volatile class with different letters are significantly different ($P \leq 0.05$). Each class of volatile compounds comprises compounds listed in Experimental section.

Fig. 7. Effect of water regime on the relative expression levels of *Oe1LOX2*, *Oe2LOX2* and *OeHPL* genes in the mesocarp tissue of Picual and Arbequina cultivars cultivated with irrigation or natural rainfall. Relative expression levels were determined by qRT-PCR using the expression level of the corresponding gene in 12 WAF mesocarp tissue from irrigated Picual as calibrator.

References

Allmann, S., Halitschke, R., Schuurink, R.C., Baldwin, I.T., 2010. Oxylipin channeling in *Nicotiana attenuata*: lipoxygenase 2 supplies substrates for green leaf volatile production. Plant Cell Environ. 33, 2028-2040.

Bai, J., Baldwin, E.A., Imahori, Y., Kostenyuk, I., Burns, J., Brecht, J.K., 2011. Chilling and heating may regulate C6 volatile aroma production by different mechanisms in tomato (*Solanum lycopersicum*) fruit. Postharvest Biol. Technol. 60, 111-120.

Bate, N.J., Sivasankar, S., Moxon, C., Riley, J.M.C., Thompson, J.E., Rothstein, S.J., 1998. Molecular characterization of an Arabidopsis gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. Plant Physiol. 117, 1393-1400.

Bell, E., Mullet, J.E., 1991. Lipoxygenase gene expression is modulated in plants by water deficit, wounding, and methyl jasmonate. Mol. Gen. Genet. 230, 456-462.

Bhardwaj, P.K., Kaur, J., Sobti, R.C., Ahuja, P., S., Kumar, S., 2011. Lipoxygenase in *Caragana jubata* responds to low temperature, abscisic acid, methyl jasmonate and salicylic acid. Gene 483, 49-53.

Blokhina, O., Virolainen, E., Fagerstedt, K., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany 91, 179-194.

Dabbou, S., Brahmi, F., Selavaggini, R., Chehab, H., Dabbou, S., Taticchi, A., Servili, M., Hammami, M., 2011. Contribution of irrigation and cultivars to volatile profile and sensory attributes. Int. J. Food Sci. Technol. 46, 1964-1976.

Deluc, L.G., Quilici, D.R., Decendit, A., Grimplet, J., Wheatley, M.D., Schlauch, K.A., Merillon, J.M., Cushman J.C., Cramer, G.R., 2009. Water deficit alters differentially metabolic pathways affecting important flavour and quality traits in grape berries of Cabernet Sauvignon and Chardonnay. BMC Genomics 10, 212.

Farmaki, T., Sanmartín, M., Jiménez, P., Paneque, M., Sanz, C., Vancanneyt, G., León, J., Sánchez-Serrano, J.J., 2007. Differential distribution of the lipoxygenase pathway enzymes within potato chloroplasts. J. Exp. Bot. 58, 555-568.

Gigot, C., Ongena, M., Fauconnier M.-L., Wathelet, J.-P., Du Jardin, P., Thonart, P., 2010. The lipoxygenase metabolic pathway in plants: potential for industrial production of natural green leaf volatiles. Biotechnol. Agron. Soc. Environ. 14, 451-460.

Gómez-Rico, A., Salvador, M.D., Greca, M.L., Fregapane, G., 2006. Phenolic and volatile compounds of extra virgin olive oil (*Olea europaea* L. cv. Cornicabra) with regard to fruit ripening and irrigation management. J. Agric. Food Chem. 54, 7130-7136.

Gomi, K., Yamasaki, Y., Yamamoto, Y., Akimitsu, K., 2003. Characterization of a hydroperoxide lyase gene and effect of C_6-volatiles on expression of genes of the oxylipin metabolism in Citrus. J. Plant Physiol. 160, 1219-1231.

González-Aguilar, G.A., Tiznado-Hernández, M.E., Zavaleta-Gatica, R., Martínez-Téllez, M.A., 2004. Methyl jasmonate treatments reduce chilling injury and activate the defense response of guava fruits. Biochem. Biophys. Res. Commun. 313, 694-701.

Halitschke, R., Ziegler, J., Keinänen, M., Baldwin, I.T., 2004. Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata*. Plant J. 40, 35-46.

Hamberg, M., Samuelsson, B., 1967. On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxygenase. J. Biol. Chem. 242, 5329–5335.

Heitz, T., Bergey, D.R., Ryan, C.A., 1997. A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol. 114, 1085–1093.

Hernández, M.L., Mancha, M., Martínez-Rivas, J.M., 2005. Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (*FAD2*) from olive. Phytochemistry 66, 1417-1426.

Hernández, M.L., Padilla, M.N., Mancha, M., Martínez-Rivas, J.M., 2009. Expression analysis identifies *FAD2-2* as the olive oleate desaturase gene mainly responsible for the linoleic acid content in virgin olive oil. J. Agric. Food Chem. 57, 6199-6206.

Howe, G.A., Schilmiller A.L., 2002. Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. 5, 230-236.

Lee, S.H., Ahn, S.J., Im, Y.J., Cho, K., Chung, G.-C., Cho, B.-H., Han, O., 2005. Differential impact of low temperature on fatty acid unsaturation and lipoxygenase activity in figleaf gourd and cucumber roots. Biochem. Biophys. Res. Commun. 330, 1194-1198.

León, J., Royo, J., Vancanneyt, G., Sanz, C., Silkowski, H., Griffiths, G., Sánchez-Serrano, J.J., 2002. Lipoxygenase H1 gene silencing reveals a specific role in supplying fatty acid hydroperoxides for aliphatic aldehyde production. J. Biol. Chem. 277, 416-423.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25, 402-408.

Lorenzi, V., Maury, J., Casanova, J., Berti, L., 2006. Purification, product characterization and kinetic properties of lipoxygenase from olive fruit (*Olea europaea* L.). Plant Physiol. Biochem. 44, 450-454.

Luaces, P., Perez, A.G., Sanz, C., 2003. Role of olive seed in the biogenesis of virgin olive oil aroma. J. Agric. Food Chem. 51, 4741−4745.

Luaces, P., Sanz, C., Perez, A.G., 2007. Thermal stability of lipoxygenase and hydroperoxide lyase from olive fruit and repercussion on olive oil aroma biosynthesis. J. Agric. Food Chem. 55, 6309−6313.

Maccarrone, M., Veldink, G.A., Agrò, A.F., Vliegenthart, J.F.G., 1995. Modulation of soybean lipoxygenase expression and membrane oxidation by water deficit. FEBS Lett. 371, 223-226.

Matsui, K., 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant Biol. 9, 274-280.

Mosblech, A., Feussner, I., Heilmann I., 2009. Oxylipins: Structurally diverse metabolites from fatty acid oxidation. Plant Physiol. Biochem. 47, 511-517.

Nemchenko, A., Kunze, S., Feussner, I., Kolomiets, M., 2006. Duplicate maize 13-lipoxygenase genes are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection, and hormonal treatments. J. Exp. Bot. 57, 3767-3779.

Olías, J.M., Pérez, A.G., Rios, J.J., Sanz, C., 1993. Aroma of virgin olive oil: biogenesis of the green odor notes. J. Agric. Food Chem. 41, 2368-2373.

Padilla, M.N., Hernández, M.L., Pérez, A.G., Sanz, C., Martínez-Rivas, J.M., 2010. Isolation, expression, and characterization of a 13-hydroperoxide lyase gene from olive fruit related to the biosynthesis of the main virgin olive oil aroma compounds. J. Agric. Food Chem. 58, 5649-5657.

Padilla, M.N., Hernández, M.L., Sanz, C., Martínez-Rivas, J.M., 2009. Functional characterization of two 13-lipoxygenase genes from olive fruit in relation to the biosynthesis of volatile compounds of virgin olive oil. J. Agric. Food Chem. 57, 9097-9107.

Padilla, M.N., Hernández, M.L., Sanz, C., Martínez-Rivas, J.M., 2012. Molecular cloning, functional characterization and transcriptional regulation of a 9-lipoxygenase gene from olive. Phytochemistry 74, 58-68.

Palmieri-Thiers, C., Canaan, S., Brunini, V., Lorenzi, V., Tomi, F., Desseyn, J.L., Garscha, U., Oliw, E.H., Berti, L., Maury, J., 2009. A lipoxygenase with dual

positional specificity is expressed in olives (*Olea europaea* L.) during ripening. Biochim. Biophys. Acta 1791, 339-346.

Patui, S., Braidot, E., Peresson, C., Tubaro, F., Mizzau, M., Rabiei, Z., Conte, L., Macri, F., Vianello, A., 2010. Lipoxygenase and hydroperoxide lyase activities in two olive varieties from Northern Italy. Eur. J. Lipid Sci. Technol. 7, 780-790.

Porta, H., Rueda-Benítez, P., Campos, F., Colmenero-Flores, J.M., Colorado, J.M., Carmona, M.J., Covarrubias, A.A., Rocha-Sosa, M., 1999. Analysis of lipoxygenase mRNA accumulation in the common bean (*Phaseolus vulgaris* L.) during development and under stress conditions. Plant Cell Physiol. 40, 850-858.

Riley, J.C.M., Thompson, J.E., 1998. Ripening-induced acceleration of volatile aldehyde generation following tissue disruption in tomato fruit. Physiol. Plant. 104, 571-576.

Royo, J., Vancanneyt, G., Pérez, A.G., Sanz, C., Störmann, K., Rosahl, S., Sánchez-Serrano, J.J., 1996. Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J. Biol. Chem. 271, 21012–21019.

Salas, J.J., Sánchez, J., 1999. Hydroperoxide lyase from olive (*Olea europaea*) fruits. Plant Sci. 143, 19-26.

Salas, J.J., Williams, M., Harwood, J.L., Sánchez, J., 1999. Lipoxygenase activity in olive (*Olea europaea*) fruit. J. Am. Oil Chem. Soc. 76, 1163-1169.

Sánchez-Ortiz, A., Pérez, A.G., Sanz, C., 2007. Cultivar differences on nonesterified polyunsaturated fatty acid as a limiting factor for biogenesis of virgin olive oil aroma. J. Agric. Food Chem. 55, 7869-7873.

Sánchez-Ortiz, A., Romero-Segura, C, Gazda, V.E., Graham, I.A., Sanz, C., Pérez, A.G., 2012a. Factors limiting the synthesis of virgin olive oil volatile esters. J. Agric. Food Chem. 60, 1300-1307.

Sánchez-Ortiz, A., Romero-Segura, C, Sanz, C., Pérez, A.G., 2012b. Synthesis of volatile compounds of virgin olive oil is limited by the lipoxygenase activity load during the oil extraction process. J. Agric. Food Chem. 60, 812-822.

Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E., 2008. Biosynthesis of plant-derived flavor compounds. Plant J. 54, 712-732.

Servili, M., Esposto, S., Lodolini, E., Selvaggini, R., Taticchi, A., Urbani, S., Montedoro, G., Serravalle, M., Gucci, R., 2007. Irrigation effects on quality, phenolic composition, and selected volatiles of virgin olive oils cv. Leccino. J. Agric. Food Chem. 55, 6609-6618.

Stefanoudaki, E., Williams, M., Chartzoulakis, K., Harwood, J.L., 2009. Effect of irrigation on quality attributes of olive oil. J. Agric. Food Chem. 57, 7048-7055.

Stumpe, M., Feussner, I., 2006. Formation of oxylipins by CYP74 enzymes. Phytochem. Rev. 5, 347–57.

Tamendjari, A., Angerosa, F., Bellal, M.M., 2004. Influence of *Bactrocera oleae* infestation on olive oil quality during ripening of *Chemlal* olives. Ital. J. Food Sci. 16, 343-354.

Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castañera, P., Sánchez-Serrano, J.J., 2001. Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. Proc. Nat. Acad. Sci. 98, 8139-8144.

Vick, B.A., 1991. A spectrometric assay for hydroperoxide lyase. Lipids 26, 315-320.

Wang, R., Shen, W., Liu, L., Jiang, L. Liu, Y., Su, N., Wan, J., 2008. A novel lipoxygenase gene from developing rice seeds confers dual position specificity and responds to wounding and insect attack. Plant Mol. Biol. 66, 401-414.

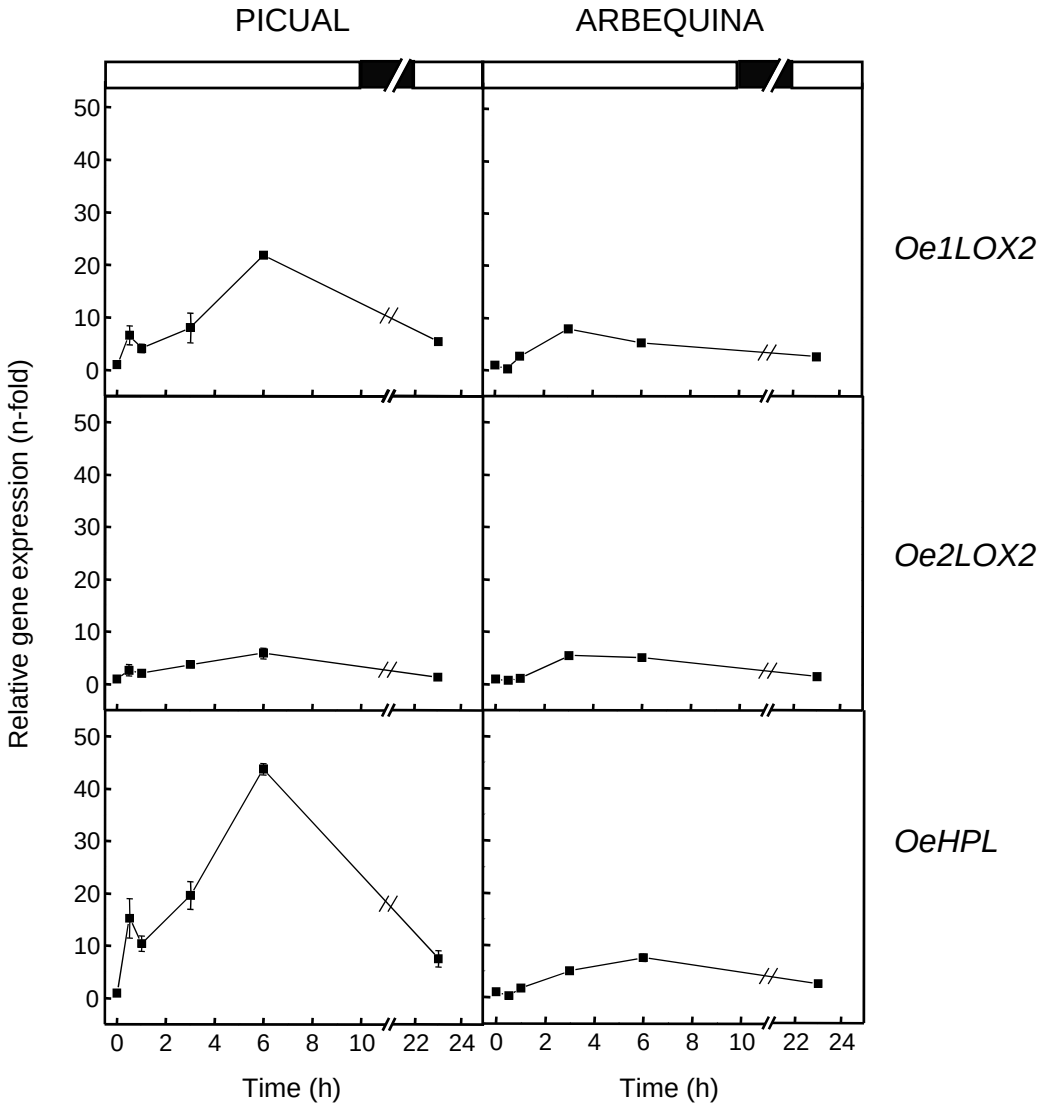
Williams, M., Harwood, J.L., 2008. Characterisation of lipoxygenase isoforms from olive callus cultures. Phytochemistry 69, 2532-2538.

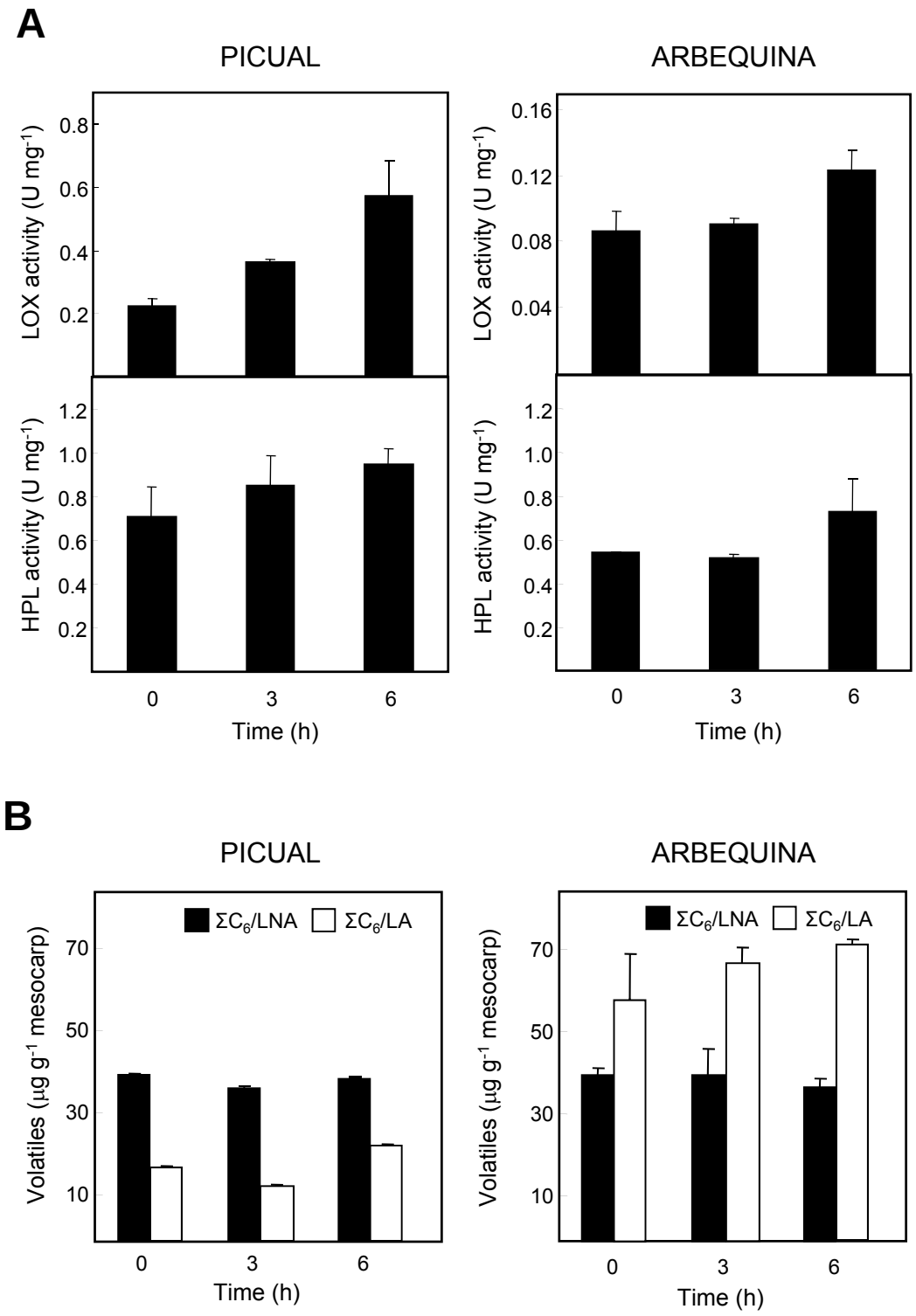
Yang, X., Song, J., Fillmore, S., Pang, X., Zhang, Z., 2011. Effect of high temperature on color, chlorophyll fluorescence and volatile biosynthesis in green-ripe banana fruit. Postharvest Biol. Technol. 62, 246-257.

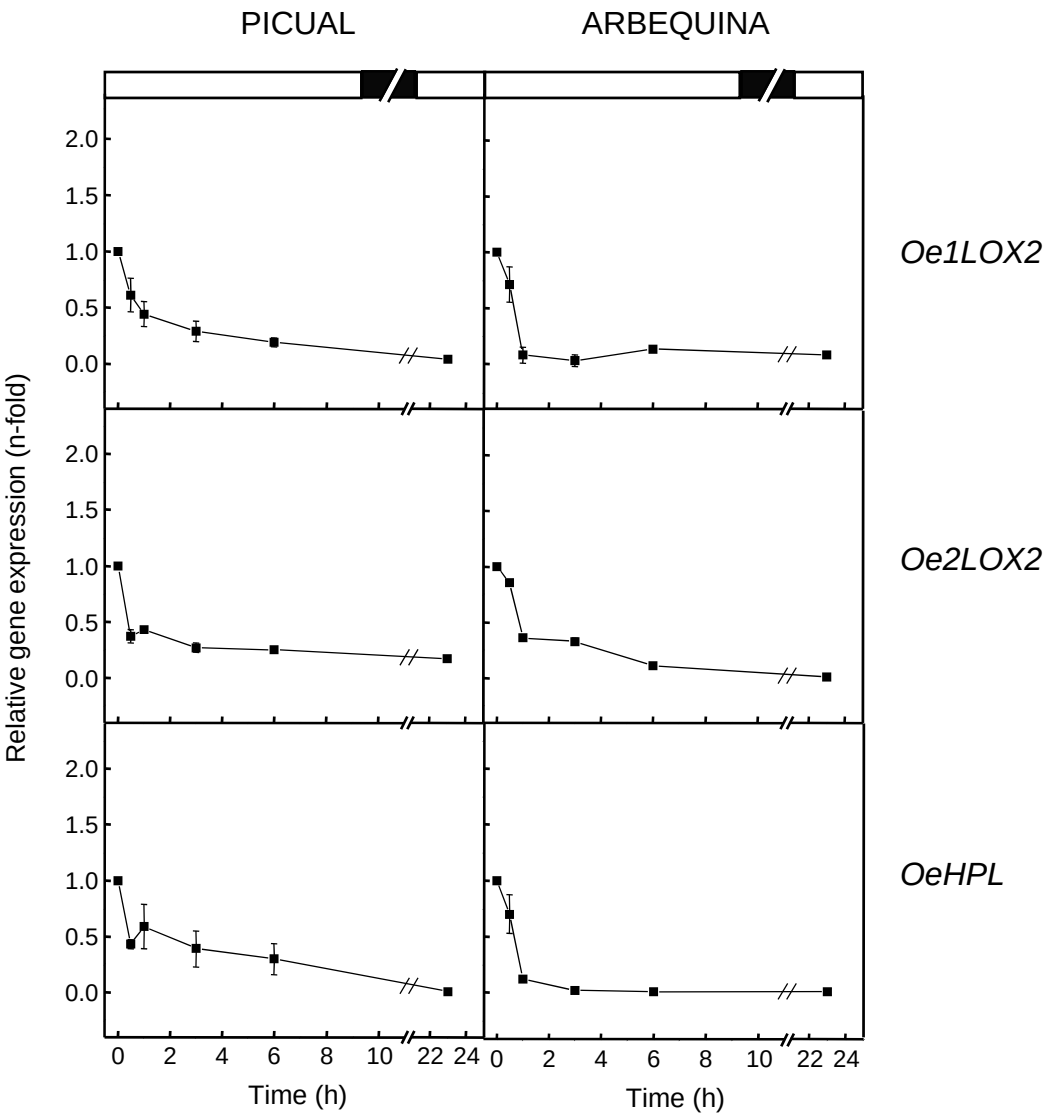
Yilmaz, E., Tandon, K.S., Scott, J.W., Baldwin, E.A., Shewfelt, R.L., 2001. Absence of a clear relationship between lipid pathway enzymes and volatile compounds in fresh tomatoes. J. Plant Physiol. 158, 1111-1116.

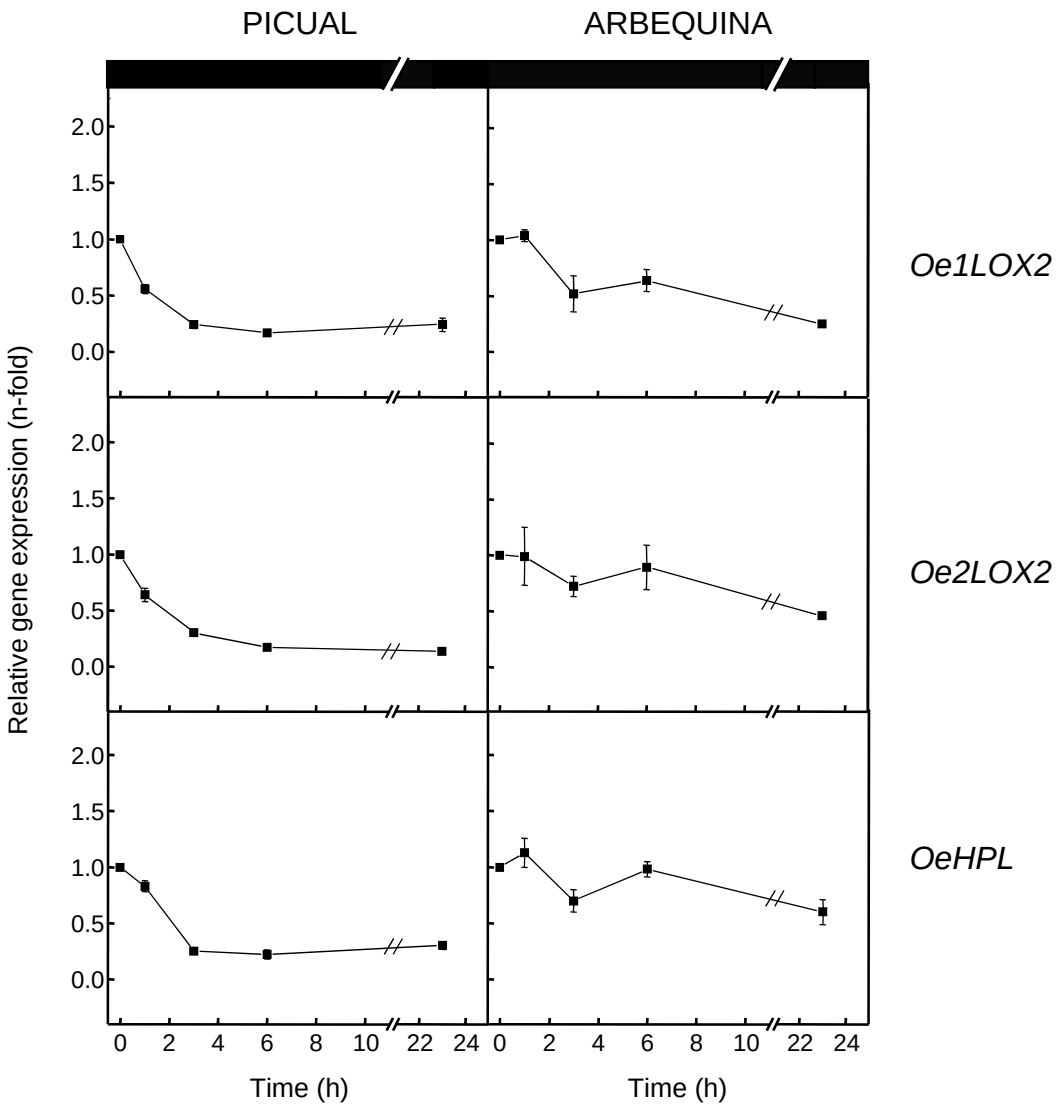
Zhang, B., Chen, K., Bowen, J., Allan, A., Espley, R., Karunairetman, S., Ferguson, I., 2006. Differential expression within the LOX gene family in ripening kiwifruit. J. Exp. Bot. 57, 3825-3836.

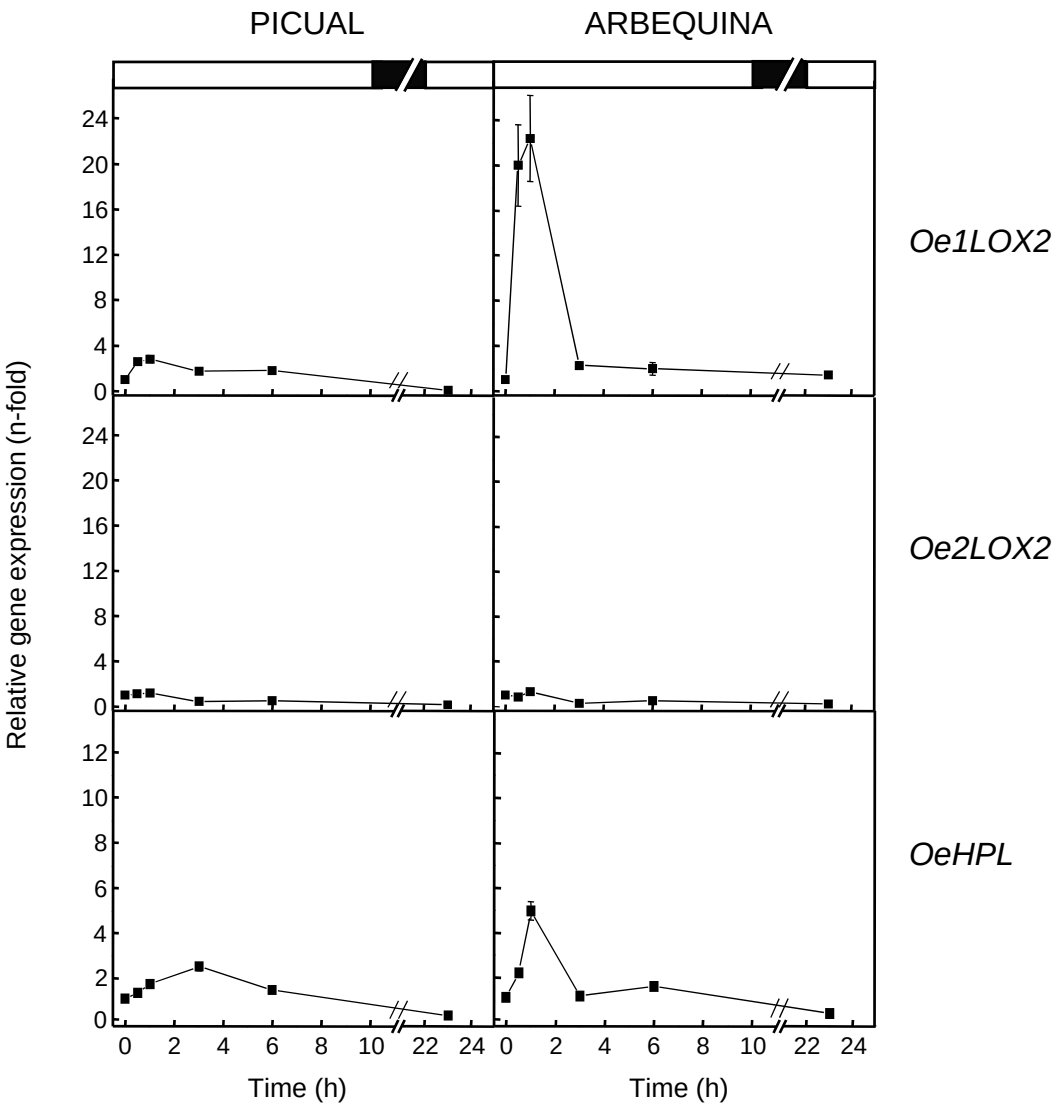
Zhang, B., Xi, W.-P., Wei, W.-W., Shen, J.-Y., Ferguson, I., Chen K.-S., 2011. Changes in aroma-related volatiles and gene expression during low temperature storage and subsequent shelf-life of peach fruit. Postharvest Biol. Technol. 60, 7-16.


PICUAL
ARBEQUINA
Relative gene expression (n-fold)
Oe1LOX2
Oe2LOX2
OeHPL
Time (h)
Time (h)
0
10
20
30
40
50
0
2
4
6
8
10
22
24

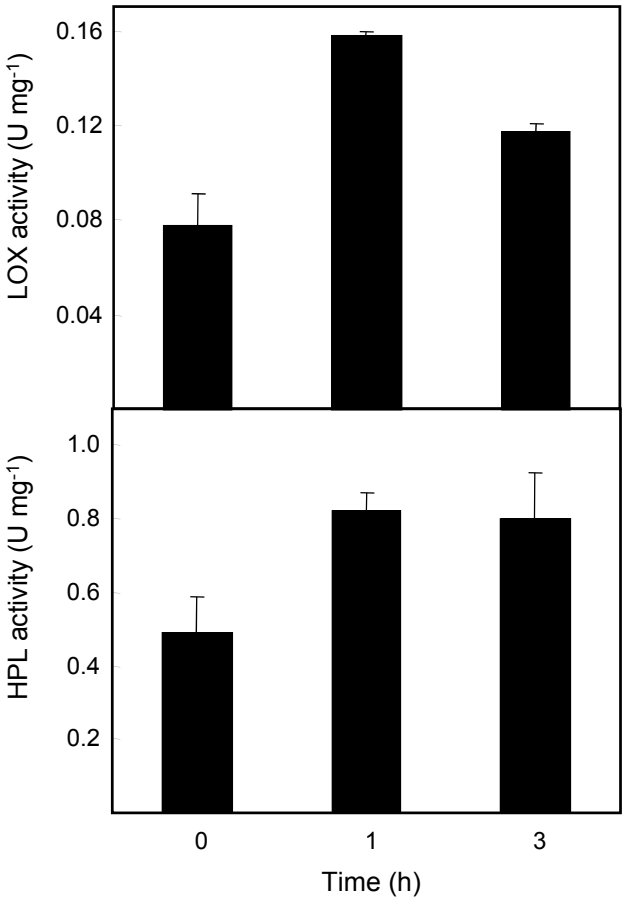

A
PICUAL
ARBEQUINA
LOX activity (U mg-1)
HPL activity (U mg-1)
0.8
0.6
0.4
0.2
0.16
0.12
0.08
0.04
1.2
1.0
0.8
0.6
0.4
0.2
0
3
6
Time (h)
B
PICUAL
ARBEQUINA
ΣC6/LNA
ΣC6/LA
Volatiles (μg g-1 mesocarp)
70
50
30
10
0
3
6
Time (h)


PICUAL
ARBEQUINA
Relative gene expression (n-fold)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
22
24
Time (h)
Oe1LOX2
Oe2LOX2
OeHPL

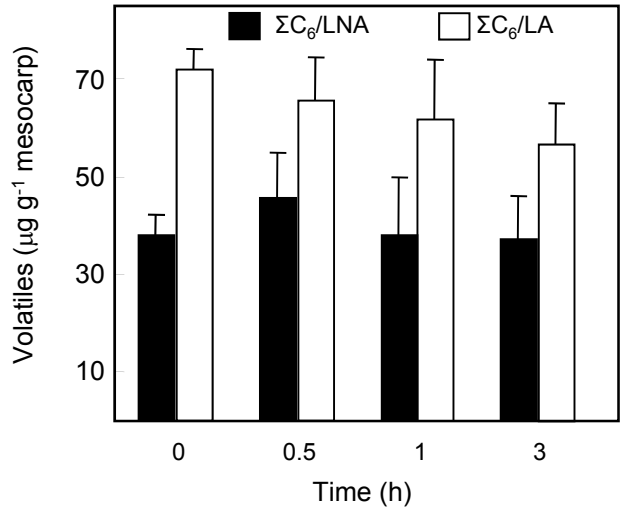

PICUAL
ARBEQUINA
Relative gene expression (n-fold)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
22
24
Time (h)
Oe1LOX2
Oe2LOX2
OeHPL

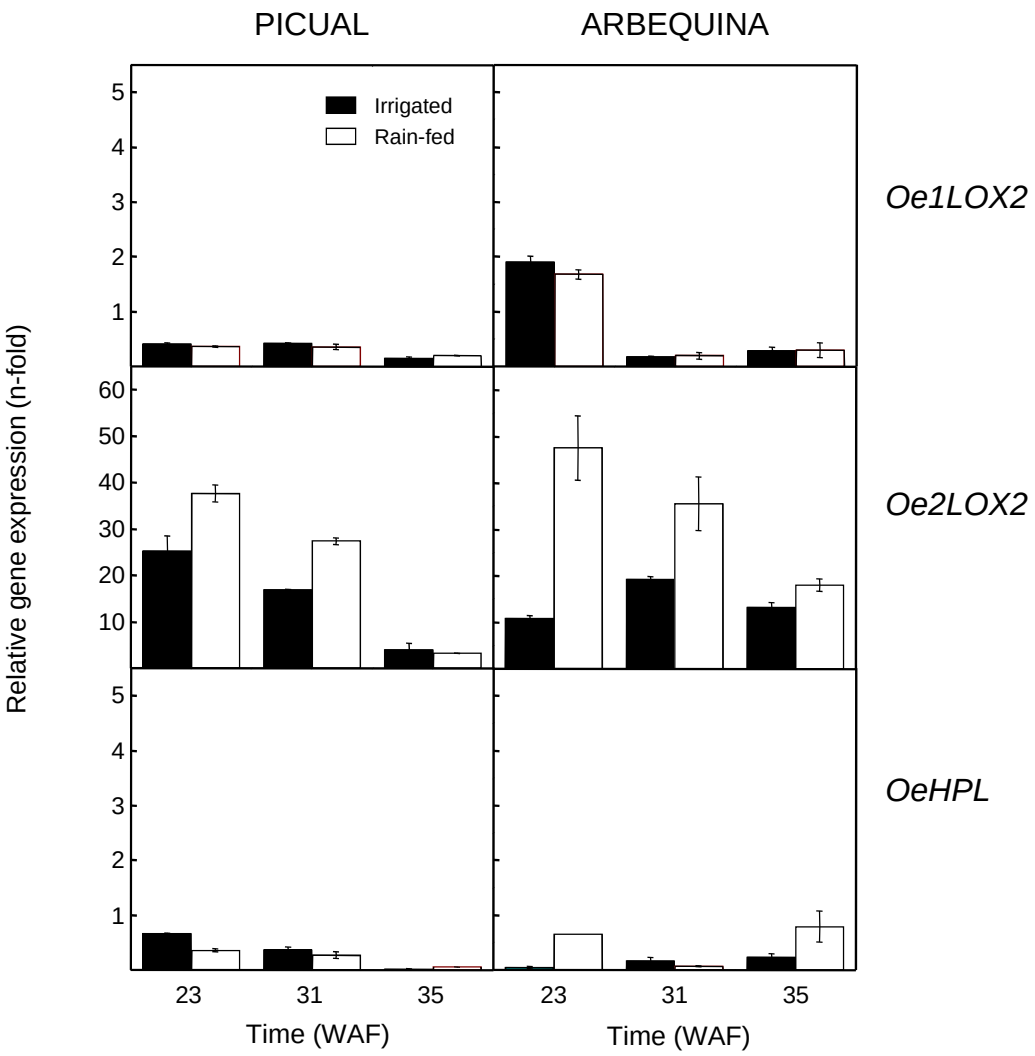

PICUAL
ARBEQUINA
Relative gene expression (n-fold)
Time (h)
Time (h)
Oe1LOX2
Oe2LOX2
OeHPL

A


ARBEQUINA
LOX activity (U mg-1)
0.16
0.12
0.08
0.04
HPL activity (U mg-1)
1.0
0.8
0.6
0.4
0.2
0
1
3
Time (h)

B


ΣC6/LNA
ΣC6/LA
Volatiles (μg g-1 mesocarp)
70
50
30
10
0
0.5
1
3
Time (h)


PICUAL
ARBEQUINA
Irrigated
Rain-fed
Relative gene expression (n-fold)
Oe1LOX2
Oe2LOX2
OeHPL
Time (WAF)
Time (WAF)
23
31
35