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Additional Information

Ethylene biosynthesis and perception during ripening of loquat fruit (*Eriobotrya japonica* Lindl.)

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Short title: Ethylene metabolism in loquat fruit

Abstract

In order to gain insights into the controversial ripening behavior of loquat fruits, in the present study we have analyzed the expression of three genes related to ethylene biosynthesis (*ACS1*, *ACO1* and *ACO2*), two ethylene receptors (*ERS1a* and *ERS1b*), one signal transduction component (*CTR1*) and one transcription factor (*EIL1*) in peel and pulp of loquat fruit during natural ripening and also in fruits treated with ethylene (10 µl L^{-1}) and 1-MCP (10 µl L^{-1}), an ethylene action inhibitor. In fruits attached to or detached from the tree, a slight increase in ethylene production was detected at the yellow stage, but the respiration rate declined progressively during ripening. Accumulation of transcripts of ethylene biosynthetic genes did not correlate with changes in ethylene production, since the maximum accumulation of *ACS1* and *ACO1* mRNA was detected in fully coloured fruits. Expression of ethylene receptor and signaling genes followed a different pattern in peel and pulp tissues. After fruit detachment and incubation at 20 ºC for up to 6 days, *ACS1* mRNA slightly increased, *ACO1* experienced a substantial increment and *ACO2* declined. In the peel, these changes were advanced by exogenous ethylene and partially inhibited by 1-MCP. In the pulp, 1-MCP repressed most of the changes in the expression of biosynthetic genes, while ethylene had almost no effects. Expression of ethylene perception and signaling genes was barely affected by ethylene or 1-MCP. Collectively, a differential transcriptional regulation of ethylene biosynthetic genes operates in peel and pulp, and support the notion of non-climacteric ripening in loquat fruits. Ethylene action, however, appears to be required to sustain or maintain the expression of specific genes.

Keywords: ethylene, gene expression, loquat, 1-MCP, postharvest, ripening.

Introduction

Fruit ripening is a complex developmentally regulated process, culminating the life-span of the fruit which is accompanied by profound biochemical and molecular transformations, such as loss of texture, accumulation of sugar and reduction of acids, changes in aroma volatiles, degradation of chlorophyll and accumulation of coloured pigments, among others (Gapper et al., 2013; Osorio and Fernie, 2014). The involvement of ethylene in the regulation of fruit ripening has been long recognized and extensively investigated over the years in a large number of crops (Pech et al., 2012; Liu et al., 2015). Based on the presence or absence of an increase in the rate of respiration and an autocatalytic ethylene burst at the onset of ripening, fleshy fruits have been categorized as climacteric and non-climacteric, respectively (McMurchie et al., 1972; Hiwasa-Tanase and Ezura, 2014). Ethylene biosynthesis, signaling and perception in climacteric-like fruits have been widely studied, becoming tomato fruit the model plant system (Cara and Giovannoni, 2008; Klee and Giovannoni, 2011; Liu et al., 2015). The conversion of SAM (S-adenosyl-L-methionine) to ACC (1-aminocyclopropane-1-carboxylic acid), catalysed by the enzyme ACC synthase (ACS) is the first committed step of ethylene biosynthesis and considered the rate limiting step of the pathway. Then, ACC is oxidized to ethylene by ACC oxidase (ACO), an iron and ascorbate-dependant dioxygenase (reviewed in Grierson, 2014). One of the major features of climacteric fruit is the presence of the so-called System-2 which operates at the onset of ripening to induce autocatalytic ethylene production. By contrast, non-climacteric fruits only produce ethylene by the so-called System 1, which is negatively regulated by ethylene, and responsible for the low levels of ethylene produced by vegetative tissues and non-climacteric fruits (reviewed by Pech et al., 2012; Hiwasa-Tanase and Ezura, 2014). The molecular mechanism operating in the autocatalysis of ethylene (System 2) involves the stimulation by ethylene of specific *ACS* genes that are induced at the beginning of the ripening process. In tomato, for example, *LeACS2* and *LeACS4* (System 2) transcripts accumulate before initial signals of colour development and are up-regulated by ethylene, whereas *LeACS1* and *LeACS6* (System 1) are down-regulated by the hormone (Nakatsuka et al., 1998; Cara and Giovannoni, 2008).

In the ethylene signal transduction pathway, the hormone binds to a receptor that may belong to two subfamilies, ETR and ERS, that are thought to negatively regulate the responses to ethylene in plants (Cara and Giovannoni, 2008; Klee and Giovannoni, 2011). However, analysis of the expression of ethylene receptor genes during ripening in many fruits does not follow a pattern consistent with the predicted model, indicating that more complex molecular mechanisms are operating in the regulation of ethylene perception during fruit ripening (Agarwal et al., 2012; Liu et al., 2015). Ethylene receptors physically interact with the CONSTITUTIVE TRIPLE RESPONSE KINASE 1 (CTR1) and downstream CTR1, ETHYLENE INSENSITIVE ELEMENTS (EINs) play a role by positively regulating other components (EILs) that function as transcriptional regulators of ethylene responsive genes (Klee and Giovannini, 2011; Lui et al., 2015).

Loquat (*Eryobotrya japonica* L.) belongs to the Rosaceae family, is native of Southern China and is nowadays cultivated in more than 20 countries being China and Spain the main producers. Although loquat fruits have been classified as non-climacteric because mature fruits produce low and constant amounts of ethylene (Blumenfeld, 1980; Cai et al., 2006), other studies have questioned this assumption. In fruit of several loquat cultivars a climacteric-like increase in ethylene production and respiration rate are coincident with the change in peel colour, suggesting a climacteric ripening pattern (Hamauzu et al., 1997; Amoros et al., 2003; Jiang et al., 2011). Other authors only reported increases in ethylene production but not in the respiration rate (Undurraga et al., 2011). In a recent study, we found a significant increment in ethylene production in on-tree fruit but the application of the ethylene-releasing compound, ethephon, did not induce an autocatalytic enhancement of the ethylene production rate. Moreover, it was also suggested that a decline in the concentration of gibberellins, auxins and cytokinins may be required to allow the ripening process to proceed (Reig et al., 2016). Jiang et al., (2011) have reported a climacteric-like peak in ethylene production and in the accumulation of *EjACO2* transcripts in 'Luoyangqing' loquat, but the maximum expression of the *EjACS1* gene was detected in over-mature fruit. However, no significant changes in ethylene production and in the expression of ethylene biosynthetic genes were detected in detached fruits. It seems,

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then, that the discrepancies in the non-climacteric behavior of loquat fruits based on the rate of ethylene emission are not always consistent with other characteristic climacteric-like ripening responses.

The inhibitor of ethylene action, 1-methylcyclopropene (1-MCP) has been proved to be highly effective in delaying many ripening-associated processes and in maintaining fruit quality in a number of climacteric fruits and also to evaluate the involvement of ethylene in several developmental processes in climacteric and non-climacteric fruit (Watkins, 2008; Li et al., 2016). Application of 1-MCP to loquat fruits has been demonstrated to alleviate chilling-injury symptoms, such as pulp browning and also reduced leatheriness and mealiness (Cai et al., 2006; Wang et al., 2010). Chilling induced the expression of ethylene receptor genes and 1-MCP suppressed this response, suggesting that ethylene is involved in the regulation of chilling injury in loquat fruit. Moreover, 1-MCP also reduced other postharvest physiological disorders and prevented the induction of polyphenol oxidase, lipoxygenase, lignin and malonaldehyde accumulation and other oxidative processes (Cai et al., 2006; Cao et al., 2009; Liguori et al., 2015) indicating that the effect of 1-MCP on cold stored loquat fruits depends on the applied concentration and the cultivar, but in general, 1-MCP delays deterioration, water loss, reduction in total acidity and chilling incidence. Moreover, a significant reduction in the decay caused by the fungus *Colletotrichum acutatum* has been also observed (Cao et al., 2010). Collectively, these results may indicate that the distinction between climacteric versus non-climacteric fruits may be a simplification and even if the ripening processes of loquat fruit may not evolve significant increases in ethylene production, the hormone may play a role in several developmental processes as well as in the responses of the fruits to stress conditions. In other climacteric fruits, such as grapes, strawberry and citrus, application of 1-MCP has revealed that ethylene is also involved in the regulation of different ripening-related processes and in some instances in changes in the transcript levels of ethylene receptors and ethylene responsive genes (Trainnoti et al., 2005; Iannetta et al., 2006: Chervin et al., 2005 and 2008; Rodrigo and Zacarías, 2007; Villarreal et al., 2010; Alos et al., 2014).

Despite the controversial non-climacteric behavior of loquat fruit, a detailed transcriptional analysis of the regulation of ethylene biosynthesis, perception and signaling during ripening of loquat fruits has not been accomplished yet. Hence, the objective of the present work was to analyze the changes in the expression of three genes related to ethylene biosynthesis (*ACS1*, *ACO1* and *ACO2*), two ethylene receptor (*ERS1a* and *ERS1b*), one signaling component (*CTR1*) and one transcription factor (*EIL1*) in peel and pulp tissues during natural ripening and also after fruit detachment, and to determine the relationship between the transcript levels and the changes in ethylene production. In order to provide new evidence on the non-climacteric behavior of loquat, the effect of exogenous ethylene and the ethylene action inhibitor, 1-MCP, on the expression of the above-mentioned genes in fruit peel and pulp was also analyzed.

Materials and Methods

Plant material and treatments

Loquat fruits (*Eriobotrya japonica* Lindl. cv. Algerie) were harvested from six adult (20-25-year-old) trees grown in a commercial orchard located in Callosa d'En Sarriá, (Alicante, Spain). Trees were budded onto loquat seedling rootstocks, grown in a loamy-clay soil, pH 7.5-8.0, planted 4 x 4 m apart, with drip irrigation (two drippers per tree), and pruned to a vase shape. Fertilization, annual pruning, thinning, as well as pest and disease management were carried out in accordance with standard commercial practices.

To determine the respiration rate and ethylene production from loquat fruits attached to the tree, two fruiting shoots per tree bearing at least three fruits each were randomly selected from 6 trees. The entire shoots with the attached fruits were sealed in hermetic 3-L plastic bottles with a 1.5 cm-diameter rubber stopper (septum). After 2-3 h, triplicate 1 mL air samples were withdrawn with a hypodermic syringe through the septum for ethylene analysis and three syringes were taken for CO_2 determination. Afterwards, the bottle was removed, and fruit colour measured. The procedure was used for fruits at five ripening stages: mature-green (MG), when

fruit reached 90% of final size (colour growth stage 709 on the BBCH-scale); colour break (BK), stage 801; yellow colour (Y), stage 803; and two coloured fruits (FC1 and FC2) corresponding to stages 807 and 809, respectively, on the BBCH scale (Martínez-Calvo et al., 1999).

In a separate experiment, MG, BK and FC2 fruits (at the stage 801, 803, and 809, respectively) were harvested, delivered to the laboratory and selected for uniformity and the absence of any lesion or injury. To determine the rate of ethylene production, fruits at the three ripening stages were incubated in 1.5-L sealed jars at 20 ºC. After 1-1.5 h, triplicate samples of 1 mL air of the headspace were withdrawn with a hypodermic syringe to analyse ethylene production by gas chromatography. Afterwards, peel and pulp were carefully separated with a scalpel, immediately frozen in liquid nitrogen, ground to a fine powder and stored at -80ºC until RNA extractions were performed.

In a third experiment, the effect of exogenous ethylene and of the ethylene action inhibitor 1-methylcyclopropene (1-MCP) on gene expression of loquat fruit was also evaluated. Yellow-coloured fruits (Y, growth stage 803 on the BBCH-scale) were delivered to the laboratory and selected for fruit size uniformity and free of any injury. Fruits were divided in three lots, containing three replicate samples of 20 fruits each. The first lot was incubated in an ethylene-free atmosphere (control fruits), and the second lot was incubated in an atmosphere of 10 μL L^{-1} ethylene in 25 L tanks at 20 ºC and 85-90% RH in the dark for up to 6 days (Rodrigo and Zacarías, 2007). The third lot of fruits was treated with 1 μL L^{-1} of the inhibitor of ethylene action, 1-methylcyclopropene (1-MCP) for 16 h and subsequently incubated in an air atmosphere at 20 ºC and 85-90% RH in the dark for up to 6 days. To avoid an excess of respiratory CO_2, $Ca(OH)_2$ powder was introduced in the tanks and fruits were ventilated every day. On day 0, 2 and 6 after the incubation peel and pulp tissues from 5 fruits were excised, frozen in liquid nitrogen, ground to a fine powder and stored at -80º C until RNA extractions were performed.

Fruit colour was analyzed by measuring the a and b Hunter coordinates in at least 20 fruits from each treatment. Three measurements were made at the equatorial area of the fruit using a

Minolta Chroma Meter CR-300 (Tokyo, Japan). Colour readings of *a* denote green when negative and red when positive, and *b* denote blue when negative and yellow when positive. Colour index of the fruit peel at different ripening stages was expressed as the *a*/*b* Hunter ratio, which is negative for green fruit, around zero for yellow fruit at colour break, and positive for orange coloured fruit (Stewart and Wheaton, 1972; Rodrigo et al., 2004).

Determination of ethylene production and respiration rate

For analysis of ethylene production, 1 mL replicate samples of the air of the headspace of the bottle containing the attached fruits or the sealed jars containing detached fruits, were withdrawn and analyzed for ethylene production in a Perkin-Elmer Autosampler gas chromatograph equipped with and activated alumina column and a flame ionization detector. Nitrogen was used as carrier gas at a flow rate of 45 mL min^{-1} and the column and detector temperatures were maintained at 100 ºC and 140 ºC, respectively. The concentration of carbon dioxide concentration in the air samples was determined in a Perkin-Elmer Autosampler gas chromatograph equipped with a Carbowax column and a thermal conductivity detector. Column and detector temperatures were maintained at 60 ºC and 140 ºC, respectively. Helium was used as carrier gas at a flow rate of 45 mL min^{-1}. In all cases, emission of ethylene and carbon dioxide are the mean of 3 replicates.

RNA extraction and cDNA synthesis

Total RNA from peel and pulp of loquat fruit was extracted according to Martínez-Fuentes et al., 2015). The purified RNA was measured by spectrophotometric analysis (ND1000 NanoDrop, Thermo Fisher Scientific, Madrid, Spain). Purity and yield of the total RNA isolated were estimated from the absorbance ratios $A_{260/230}$ and $A_{260/280}$, which indicate the contamination of the extracts by polyphenols/carbohydrates and proteins, respectively. Typically, 2 µL of each sample were used for these determinations.

The integrity of total RNA was verified by agarose gel electrophoresis stained with ethidium bromide and visualized under UV light. In addition, the integrity and quality of the purified

RNA were also analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, http://www.agilent.com/) according to the manufacturer's instructions (RNA Integrity Number (RIN) values). The absence of DNA contamination was checked by performing a no-reverse transcription assay which consisted of a PCR with each RNA sample using the *ACTIN* primers (Fu et al., 2012). No amplified products were detected which confirmed the purity of the RNA extracts. The transcripts present in 5 μg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 μL. One μL of a 3-fold diluted first-strand cDNA was used for each amplification reaction.

Gene expression analysis by real-time PCR

Gene expression analyses were performed following the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Quantitative real-time PCR was carried out on a LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions followed the manufacturer's instructions with some modifications. The PCR mix contained 1 μL of diluted cDNA, 5 μL of SYBR Green I Master Mix, 1 μL of 3 μM primer F and 1 μL of 3 μM primer R, being the final volume of 10 μL. The sequences of the primers (PSF purified, Isogen, The Netherlands) for the amplification of *ACO1*, *ACO2* and *ACS1* were obtained from Jiang et al., (2011), *ACTIN* from Fu et al., (2012); and *ERS1a*, *ERS1b*, *CTR1* and *EIL1* from Wang et al., (2010). The cycling protocol, for all genes, consisted of 10 min at 95 °C for pre-incubation, then 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data was acquired during the extension time with the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and were transformed into mRNA levels by using specific standard curves for all analysed genes.

The specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps followed by the sequencing of the amplicon. The expression levels relative to values of a reference sample were calculated using the Relative Expression Software Tool (REST, http://rest.gene-quantification.info; Pfaffl et al.,

2002). The reference sample was the expression value obtained for each gene on the pulp of the loquat fruits at the yellow stage which was arbitrarily given the expression value of 1. Results were the mean of at least 3 independent replicates.

Results

Evolution of respiration rate and ethylene production during on-tree loquat fruit development and ripening

To determine the potential climacteric-like ripening behavior of loquat fruit (*Eriobotrya japonica* Lindl, cv. Algerie), the evolution of the respiration rate and ethylene emission was determined on on-tree fruits at five ripening stages, corresponding to the phenological stages 709, 801, 803, 807 and 809 on the BBCH-scale (Fig. 1). Thus, one single shoot with 2-3 fruits attached to the tree was tingly sealed in a plastic container from which gas samples were collected to analyze the carbon dioxide and ethylene emission. Figure 1 shows changes in fruit colouration and the concomitant evolution of respiration and ethylene production. The rate of respiration was maximum (70 µL g FW^{-1} h^{-1}) in mature-green fruits (709 at the BBHC-scale) and declined progressively throughout maturation to reach values near 50% lower in full-coloured fruits (809 at the BBHC-scale) (Fig. 1C). The evolution of ethylene production followed a different trend than that of the respiration rate. Mature-green fruits attached to the tree evolved an ethylene emission of around 0.7 nL g FW^{-1} h^{-1} that increased to a maximum (1 nL g FW^{-1} h^{-1}) in yellow fruits (803 at the BBHC-scale). Then, ethylene production experienced a reduction of around 40% in mature coloured fruits (807 and 809 at the BBCH-scale, Fig. 1D).

Ethylene production and expression of genes involved in ethylene biosynthesis, perception and signaling during loquat fruit ripening

Since on-tree ethylene production displayed a small increment in fruits at the yellow stage, we decided to select fruits at breaker, yellow and full-coloured stages to study the pattern of

expression of genes related with ethylene biosynthesis, perception and signaling; and the regulation of ethylene production during loquat fruit maturation. In this experiment, ethylene production was measured in fruits detached from the tree and incubated at 20 ºC. Results in Figure 2A show that ethylene production was slightly higher in yellow fruits than in breaker or coloured fruits. These data were consistent with those obtained in fruits attached to the tree, indicating a similar pattern and production of ethylene in both fruit detached or attached to the tree.

The expression of three genes involved in ethylene biosynthesis: *ACS1*, *ACO1* and *ACO2*; two ethylene receptors: *ERS1a*, *ERS1b; CTR1* which is involved in ethylene signal transduction and *EIL1*, which is a transcription factor that is involved in the ethylene primary response, was analyzed in peel and pulp of loquat fruits at BK, Y and FC stages. The expression levels of the genes involved in ethylene biosynthesis followed different patterns during ripening and in most cases also differed between both tissues. The profiling of *ACS1* mRNA accumulation was the only one similar between peel and pulp, since it remained stable until Y stage and then increased at the FC stage around 2- and 5-times in peel and pulp, respectively (Fig. 2B). *ACO1* was similarly expressed at the three ripening stages in the peel, while in the pulp it decreased at the Y stage to increase latter on and reached a maximum at the FC stage. Expression of *ACO2* slightly decreased in the peel during ripening, whereas in the pulp this gene displayed the highest mRNA abundance at the BK stage, decreasing at advanced ripening stages (Fig. 2B). Transcript levels of the ethylene receptors, perception and signaling genes are reported in Figure 3. It is interesting to indicate that the four genes analyzed followed a similar profile, although changes had different magnitude, in each tissue. In the peel, the maximum mRNA accumulation of *ERS1a, ERS1b, CTR1* and *EIL1* was attained at the BK stage and then progressively decreased. In contrast, in the pulp the lowest mRNA levels of these genes were detected in yellow fruits, and reached the maximum mRNA abundance in the pulp of coloured fruits (Fig. 3).

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Effects of ethylene and 1-MCP on the expression of ethylene biosynthetic genes in peel and pulp of loquat fruits

Ethylene biosynthetic genes, namely *ACS* and *ACO*, have been shown to be differentially regulated at the transcriptional level by developmental and external stimuli, such as ethylene itself. Hence, the application of exogenous ethylene or the inhibitor of its action, 1-MCP, has significant effects on the expression of the genes of its own biosynthesis (Nakatsuka et al., 1998; Watkins, 2008; Bouzayen et al., 2013). Therefore, in order to gain insights into the regulation of ethylene production during loquat fruit maturation, the effects of ethylene and 1-MCP on the expression of genes involved in ethylene biosynthesis, perception and signalling in peel and pulp of loquat fruits were further studied. Since yellow fruits (Y) displayed maximum ethylene production, this ripening stage was selected for ethylene and 1-MCP treatments. Fruits were incubated in an ethylene-free atmosphere (control fruits), or 10 μL L^{-1} of ethylene, or pre-treated for 16 h with 1 μL L^{-1} 1-MCP and then transferred to an ethylene-free atmosphere, and peel and pulp tissue collected for gene expression analysis after 2 and 6 days. Under air, *ACS1* slightly increased over the 6 days of treatment in the peel, but in the pulp experienced a 6-fold increment by day 6. Ethylene considerably stimulated accumulation of *ACS1* mRNA after 2 days in the peel and after 6 days in the pulp (Fig. 4). Treatment with 1-MCP partially overcame the effects of exogenous ethylene in expression of *ACS1*. The transcript levels of two genes encoding for ACC oxidase, *ACO1* and *ACO2*, followed opposite patterns, but for each gene the pattern was similar in both tissues. Expression of *ACO1* was stimulated in air and ethylene accelerated this effect, whereas 1-MCP substantially inhibited the accumulation of these transcripts. By contrast, accumulation of *ACO2* mRNA was higher in both peel and pulp of freshly-harvested fruits but dramatically declined after incubation in air (15-fold and 8-fold in peel and pulp, respectively). These reductions were slower in the peel of ethylene-treated fruits (Fig. 4). Thus, in the peel of loquat fruits, ethylene accelerated the changes in the expression of ethylene biosynthetic genes (*ACS1* and *ACO1*) occurring under air whereas the pulp appeared to be less responsive to ethylene. In both tissues, 1-MCP appeared to counteract the effect of exogenous ethylene.

Effects of ethylene and 1-MCP on the expression of genes involved in ethylene perception and signaling in peel and pulp of loquat fruits

The expression levels of two ethylene receptors: *ERS1a*, *ERS1b; CTR1*, an element involved in ethylene signal transduction and *EIL1*, a transcription factor participating in the ethylene primary response were also evaluated in peel and pulp of loquat fruits treated with ethylene and 1-MCP (Fig. 5). Upon storage at 20 ºC, *ERS1a* and *ERS1b* transcript abundance decreased in the peel of control fruits while *CTR1* and *EIL1* mRNA levels increased. The ethylene treatment induced a transient (day 2) accumulation of *ERS1a*, *ERS1b* and *CTR1* transcripts, reaching higher levels than in control fruits, that declined at day 6. The treatment with 1-MCP stimulated the accumulation of both *ERS1a* and *ERS1b* genes but did not affect that of *CTR1* and *EIL1*. Accumulation of *EIL1* transcript was similar in the peel of fruits subjected at the three different treatments (Fig. 5).

In the pulp, *ERS1a* transcript abundance was repressed by the 1-MCP treatment (a 3- and 2-fold reduction at day 2 and 6, respectively). *ERS1b* expression levels did not change during none of the treatments, while *CTR1* was slightly induced by ethylene and repressed by 1-MCP. On the other hand, *EIL1* was stimulated in air-treated fruits but transiently repressed by both ethylene and 1-MCP (Fig. 5).

Discussion

The maturation behaviour of loquat fruit has been classically categorized as non-climacteric (Kader, 2002). However, physiological and biochemical evidence has recently questioned this behaviour, suggesting that ripening in loquat may share responses to ethylene that are typical of both climacteric and non-climacteric fruits (Hirai et al., 1982; Amoros et al., 2003; Jiang et al., 2011). In order to shed light on this controversy, in the current work we have studied the transcriptional regulation of genes involved in ethylene biosynthesis (*ACS1, ACO1* and *ACO2*), perception (*ERS1a* and *ERS1b*) and signalling (*CTR1* and *EIL1*) in peel and pulp of loquat fruit and its relationship with the changes in ethylene production during natural ripening. Moreover,

the potential involvement of ethylene in the regulation of its own metabolism, that is also different between climacteric and non-climacteric fruits, has been addressed by analysing the expression of these genes in peel and pulp of fruit treated with ethylene and with the inhibitor of ethylene action, 1-MCP.

Analysis of ethylene production during maturation of loquat fruits (cv. Algerie) revealed a moderate and transient increase in yellow-coloured fruits (corresponding to the stage 803 on the BBCH-scale). This increment in ethylene emission was observed in both fruits attached to or detached from the tree (Figs. 1D and 2A), indicating that may be a general feature during the evolution of colour changes rather than an artefact or a wound-response. In fruits of other loquat cultivars, increases in ethylene production have been also detected, and although the magnitude of the increment was very variable, the ethylene upsurge was in most cases coincident with the transformation from chloroplast to chromoplast (Hamauzu et al., 1997; Amorós et al., 2003; Jiang et al., 2011; Reig et al., 2016). Based on these apparent climacteric-like increases in ethylene production several authors have suggested a climacteric ripening behavior in loquat fruits (Amoros et al., 2003; Jiang et al., 2011). However, the results obtained in the present work indicate that there is only a moderate increase in ethylene production in 'Algerie' loquat fruits at yellow stage, which is of minor relevance comparing the climacteric increment of other climacteric fruits (Kader, 2002; Pech et al., 2012; Hiwasa-Tanase and Ezura 2014), that may be then related to the physiological transition of the fruit to the ripe stage. Supporting this assumption is the fact that the rate of respiration declined progressively during the ripening process (at the FC2 stage it almost halved that of green fruit, Fig. 1C) in both fruits attached to or detached from the tree. Reig et al., (2016) also detected an important reduction in the respiration rate during colouration of loquat fruits treated or not with ethephon on the tree before colour-break. These two experiments that observed the lack of a climacteric-like increase in respiration, which is a classical and widely considered as an essential criterion for the categorization of the ripening behavior of a fruit (Kaber 2002; Pech et al., 2012), reinforce the idea that loquat fruit ripening follows non-climacteric patterns.

Based on the gene sequences of *ACS* (*EjACS1*) and *ACOs* (*EjACO1* and *EjACO2*) cloned by Liang et al., 2011) in 'Luoyangqing' loquat, we analyzed the expression of homologous genes in peel and pulp of 'Algerie' loquat at three maturation stages (Fig. 2). Interestingly, the accumulation of the transcript levels of the three genes did not follow a pattern of changes consistent with the evolution of ethylene production. The expression of *ACS1* was lower in both peel and pulp at the BR and Y stages, and sharply increased in FC fruits when ethylene production was lower than in fruits at earlier stages. These results are similar to those found during ripening of 'Loutangqing' loquat and suggest that the expression of *ACS1* may not be a limiting step in the regulation of ethylene production during ripening of loquat, which is contrary to what is observed in most climacteric-like fruits (Cara and Giovannoni, 2008; Grierson, 2014). A detailed transcriptomic analysis of the expression of the different *ACS* and *ACO* gene members during tomato fruit ripening revealed that the changes in ethylene production are mainly regulated by the preferential and coordinate expression of each gene at specific developmental stages (Klee and Giovannoni, 2011; Li et al., 2015). Thus, the potential involvement of other isoforms of *ACS* in the regulation of ethylene biosynthesis at different stages of the ripening in loquat cannot be discarded. The most important variation in the accumulation of *ACO1* and *ACO2* mRNA in the peel was a slight reduction of *ACO2* transcript levels in full coloured fruits coinciding with a reduction in ethylene production. Moreover, accumulation of *ACS1* and *ACO1* mRNA in the pulp reached their maximum whereas that of *ACO2* were low (Fig. 2). Thus, although the relative contribution of the peel and pulp to the production of ethylene by the whole fruit is unknown, the overall results suggest that the expression of *ACO2* may play an important role in the regulation of ethylene production at later stages of maturation of loquat fruits. Interestingly, *ACO1* and *ACO2* were more highly expressed in the pulp than in the peel at BK and FC stages and followed different expression patterns, suggesting a differential transcriptional regulation of ethylene biosynthesis in these tissues of the fruit (Fig. 2). Hence, the lack of a relationship between ethylene production and the induction of ethylene biosynthetic genes during loquat ripening supports the classification of

this fruit as non-climacteric, since in other climacteric fruits, such as apple, *ACS1* and *ACO1* are correlated with the ethylene climacteric burst (Yang et al., 2013).

The mechanism of ethylene perception and signaling is complex (Grierson 2014) and tomato fruit has become the model system to understand the role of ethylene in the regulation of fruit ripening (Klee and Giovanoni, 2011; Liu et al., 2015). The current model of ethylene signaling establishes that a reduced expression of transcripts and activity of ethylene receptors would increase the sensitivity to ethylene, whereas increased receptor levels would originate a reduction in ethylene sensitivity (Klee and Giovanoni, 2011). The changes in the expression of ethylene receptor genes during natural ripening of loquat fruit have not been previously studied. Our results showed a reduction of the mRNA levels of the receptors *ERS1a* and *ERS1b* in the peel after the breaker stage, suggesting that this tissue may become more sensitive to ethylene towards fruit senescence. Wang et al., (2010) have also analyzed the expression of *ERS1a* and *ERS1b* genes in chilling-stressed loquat fruit and concluded that ethylene appears to be involved in the response to cold. In the pulp, by contrast, accumulation of the *ERS1a* and *ERS1b* mRNAs increased in coloured fruit (Fig. 3) which may be indicative of a reduction in the sensitivity to ethylene once the fruit has completed full colouration. It is interesting to note that other genes analyzed involved in ethylene signaling, as *CTR1* and *EIL1*, followed an expression pattern similar to that of the receptor genes in both peel and pulp (Figs. 3 and 5). In strawberry, *CTR1* expression is induced before and after the white stage, and its down-regulation by virus induced gene silencing (VIGS) delayed ripening (Sun et al., 2013). Our results suggest a coordinate regulation of ethylene perception and signaling that may allow a more efficient ethylene action during loquat ripening. Moreover, these changes also reflect a differential transcriptional regulation of ethylene perception and signaling genes between peel and pulp during ripening of loquat fruits, similar to the ethylene biosynthetic genes.

Several reports have evidenced that the application of the ethylene action inhibitor, 1-MCP, to loquat fruit delayed several physiological processes, as internal browning, chilling injury symptoms, weight loss, reduced polyphenol-oxidase and lipoxygenase activities, electrolyte leakage and decay incidence (reviewed in Li et al, 2016) and maintained postharvest fruit

quality. Then, ethylene is thought to be involved in the regulation of these processes in loquat fruit (Wang et al., 2010; Liguori et al., 2015). In a previous study we have shown that application of 1-MCP stimulated and ethylene reduced endogenous ethylene emission in 'Algerie' loquat fruits (Reig et al., 2016), which are indicative of non-climacteric fruit ethylene responses. The expression of ethylene biosynthetic, perception and signaling genes has been analyzed in order to gain insights into the transcriptional regulation of ethylene metabolism in peel and pulp during fruit ripening (Figs. 4 and 5). After fruit detachment, ethylene experienced a transient increase (Reig et al., 2016) that is accompanied by an increment of *ACS1* and *ACO1* mRNAs, and a substantial reduction of that of *ACO2* in both peel and pulp (Figs. 2 and 4). Then, it seems that ethylene emission after fruit detachment may be regulated by a complex coordinated response between the different gene members of ACS and ACO family, as observed in order fruits (Chiriboga et al., 2013). Interestingly, application of exogenous ethylene only produced a transient enhancement of *ACS1* and *ACO1* mRNAs at day 2 in the peel and virtually no differences in the pulp respect to air-treated fruits (Figs. 2 and 4). Although in other processes a dose-dependent ethylene response has been shown (Cai et al., 2006), our results indicated a minor stimulation by ethylene of its biosynthetic genes, consistent with a non-climacteric behavior (Pech et al., 2012) which is also in agreement with our previous observation (Reig et al., 2016). Interestingly, the enhancement of ethylene production in 1-MCP-treated fruits appears to be determined by a higher accumulation of the three transcripts analyzed, respect to air-treated fruits. In the pulp, however, accumulation of the three mRNA was lower in fruits treated with 1-MCP than in the other treatments. One possible explanation to these results would be that ethylene may not be a triggering factor stimulating the expression of its biosynthetic genes but its action is required to maintain or sustain the relative expression of genes during maturation of detached fruits. A similar conclusion has been also postulated for the role of ethylene in other non-climacteric fruits, such citrus, in which ethylene appears to sustain the expression of carotenoid biosynthetic genes (Rodrigo and Zacarias, 2007) and other ethylene-regulated genes (Alos et al., 2014).

The effects of exogenous ethylene and 1-MCP on the expression of ethylene signaling and perception genes were variable between peel and pulp and, in general, did not show a pattern consistent with a clear involvement of ethylene in their regulation (Figs. 3 and 5). Ethylene provoked an early increment of the accumulation of the *ERS1a*, *ERS1b* and *CTR1* transcripts in the peel, respect to air-treated fruits, but 1-MCP produced variable effects on each transcript. The response to ethylene is similar to that observed in tomato fruits (Klee and Tieman, 2002), or apple (Yang et al., 2013), which are climacteric fruits, but also in the non-climacteric strawberry (Trainotti et al., 2005). In the pulp, 1-MCP reduced the accumulation of most transcripts, except for *ERS1b* (Fig. 5). Together, ethylene appears to play a minor role in the expression of ethylene receptor and signaling genes, probably sustaining the accumulation of some transcripts, and different mechanisms appear to operate in the regulation of the expression of these genes between peel and pulp of loquat fruits.

In summary, transcriptional analysis of genes related to ethylene biosynthesis, perception and signaling in peel and pulp indicate a non-climacteric like-maturation of loquat fruit. These results are consistent with the evolution of ethylene production and respiration rate in fruits attached to or detached from the tree. During fruit maturation, expression of the *ACO2* gene appears to be important in the regulation of ethylene production and most of the ethylene receptor and signaling genes were down-regulated. Application of 1-MCP reduced the expression of some genes, indicating that ethylene action may be required to sustain or maintain the expression of specific genes. Finally, differential transcriptional regulation of the genes analyzed appears to operate between peel and pulp of loquat fruits.

References

Agarwal, G., Choudhary, D., Singh, V.P., Arora, A. 2012. Role of ethylene receptors during senescence and ripening in horticultural crops. Plant Signal. Behav. 7, 1-20.

Alos, E., Distefano, G., Rodrigo, M.J., Gentile, A., Zacarias, L. 2014. Altered sensitivity to ethylene in 'Tardivo', a late-ripening mutant of Clementine mandarin. Physiol. Plant. 51, 507-521.

Amoros, A., Zapata, P., Pretel, M.T., Botella, M.A., Serrano, M. 2003. Physico-chemical and physiological changes during fruit development and ripening of five loquat (*Eriobotrya japonica* lindl.) cultivars. Food Sci. Technol. Int. 9, 43-51.

Blumenfeld, A. 1980. Fruit growth of loquat. J. Am. Soc. Hortic. Sci. 105, 747-750.

Bustin, S.A., Benes, V., Garson, J.A. Hellemansm J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611-622.

Cai, C., Chen, K., Xu, W., Zhang, W., Li, X., Ferguson, I. 2006. Effect of 1-MCP on postharvest quality of loquat fruit. Postharvest Biol. Technol. 40, 155-162.

Cao, S., Zheng, Y. 2009. Effect of 1-methylcyclopropene on anthracnose rot cause by *Collecotrichum acutatum* and disease resistance in loquat fruit. J. Sci. Food Agric. 90, 2289-2294.

Cao, S., AHen, Y., Wnag, K., Rui, H. Shang, H.T., , S.S. 2010. The effect of 1-methylcyclopropene on chilling and cell wall metabolism in loquat fruit. J. Hortic. Sci. Biotech. 85, 147-153.

Cara, B., Giovannoni, J.J. 2008. Molecular biology of ethylene during tomato fruits development and maturation. Plant Sci. 175, 106-113.

Chervin, C., El-Kereamy, A., Roustan, J., Latché, A., Lamon, J., Bouzayen, M. 2004. Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. Plant Sci. 167, 1301-1305.

Chervin, C., Tira-Umphon, A., Roustan, J., Lamon, J., Latche, A., Bouzayen, M., El-Kereamy, A., Kanellis, A. 2005. Ethylene is required for the ripening of grape; Acta Hort. 689, 251-256.

Chervin, C., Tira-Umphon, A., Terrier, N., Zouine, M., Severac, D., Roustan, J. 2008. Stimulation of the grape berry expansion by ethylene and effects on related gene transcripts, over the ripening phase. Physiol. Plant 134, 534-546.

Chiriboga, M.A., Saladié, M., Giné Bordonaba, J., Recasens, I., Garcia-Mas, J., Larrigaudière, C. 2013. Effect of cold storage and 1-MCP treatment on ethylene perception, signalling and synthesis: influence on the development of the evergreen behaviour in 'Conference' pears." Postharvest Biol. Technol. 86, 212-220.

Fu, X., Kong, W., Peng, G., Zhou, J., Azam, M., Xu, C., Grierson, D., Chen, K. 2012. Plastid structure and carotenogenic gene expression in red-and white-fleshed loquat (*Eriobotrya japonica*) fruits. J. Exp. Bot. 63, 341-354.

Gapper, N.E., McQuinn, R.P., Giovannoni, J.J. 2013. Molecular and genetic regulation of fruit ripening. Plant Mol. Biol. 82, 575-91.

Grierson, D. 2014) Ethylene biosynthesis, in: Nath, P., Bouzayen, M., Matoo, A.K., Pech, J.C. (Eds), Fruit ripening: physiology, signaling and genomics. CAB International, Wallinford, pp. 178-192.

Hamauzu, Y., Chachin, K., Ding, C., Kuroaka, H. 1997. Differences in surface color, flesh firmness, physiological activity, and some components of loquat fruits picked at various stages of maturity. J. Japanese Soc. Hort. Sci. 65, 859-865.

Hirai, M. 1982. Accelerated sugar accumulation and ripening of loquat fruit. J. Jap. Soc. Hort. Sci. 65, 859-865.

Hiwasa-Tanase, K., Ezura, H. 2014. Climacteric and non-climacteric ripening, in: Nath, P., Bouzayen, M., Matoo, A.K., Pech, J.C. (Eds), Fruit ripening: physiology, signaling and genomics. CAB International, Wallinford, pp. 1-14.

Iannett,a P.P.M., Laarhoven, L., Medina-Escobar, N., James, E.K. McManus, M.T., Davies, H.V., Harren, F.J.M. 2006. Ethylene and carbon dioxide production by developing strawberries show a correlative pattern that is indicative of ripening climacteric fruit. Physiol. Plant. 127, 247-259.

Jiang, T., Wang, P., Yin, X., Zhang, B., Xu, C., Li, X., Chen, K. 2011. Ethylene biosynthesis and expression of related genes in loquat fruit at different developmental and ripening stages. Sci. Hort. 130, 452-458.

Kader, A.A. 2002. Biology and technology: an overview, in: Kader, A.A. (Ed.), Postharvest technology and horticultural crops. Agriculture and Natural Resources, University of California Publication 3311, pp. 39-48.

Klee, H.J., Giovannoni, J.J. 2011. Genetics and control of tomato fruit ripening and quality attributes. Annu. Rev. Genetics. 45, 41-59.

Klee, H.J., Tieman, D. 2002. The tomato ethylene receptor gene family: Form and function. Physiol. Plant. 115, 336-341.

Lafuente, M.T., Zacarias, L., Martínez-Téllez, M.A., Sanchez-Ballesta, M.T., Dupille, E. 2001. Phenylalanine ammonia-lyase as related to ethylene in the development of chilling symptoms during cold storage of citrus fruits. J. Agric. Food Chem. 49, 6020-6025.

Li, L., Lichter, A, Chalupowicz, D., Gamrasni, D., Goldberg, T., Nerya, O., Ben-Arie, R., Porat, R. 2016. Effects of the ethylene-action inhibitor 1-methylcyclopropene on postharvest quality of non-climacteric fruit crops. Postharvest Biol. Technol. 111, 322-329.

Liguori, G., Barone, E., Farina, V., Inglese, P. 2015. 1-Methylcyclopropene delays ripening and improves postharvest quality of white flesh loquat. Acta Hort. 1092, 153-158.

Liu, M., Pirrello, J., Chervin, C., Roustan, J.P., Bouzayen, M. 2015. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. Plant Physiol. 169, 2380-2390.

Martınez-Calvo, J., Badenes, M.L., Llacer, G., Bleiholder, H., Hack, H., Meier, U. 1999. Phenological growth stages of loquat tree (*Eriobotrya japonica* (Thunb) Lindl.). Ann. App. Biol. 134, 353-357.

McMurchie, E.J., McGlasson, W.B., Eaks, I.L. 1972. Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature 237, 235-236.

Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y., Inaba, A. 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, l-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol. 118, 1295-1305.

Osorio, S., Fernie, A.R. 2014. Fruit ripening: primary metabolism, in: Nath, P., Bouzayen, M., Matoo, A.K., Pech, J.C. (Eds.), Fruit ripening: physiology, signaling and genomics. CAB International, Wallinford, pp. 15-27.

Pech, J.C., Purgato, E., Bouzayen, M., Latche, A. 2012. Ethylene and fruit ripening. Annu. Plant Rev. 44, 275-304.

Pfaffl, M.W., Horgan, G.W., Dempfle, L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nuc. Acid Res. 30, e36.

Reig, C., Martinez-Fuentes, A., Mesejo, C., Rodrigo, M.J., Zacarias, L., Agusti, A. 2016. Loquat fruit lacks a ripening-associated autocatalytic rose in ethylene production. J. Plant Growth Regul. 35, 232-244.

Rodrigo, M.J., Marcos, J.F., Zacarías, L. 2004. Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L) during fruit development and maturation. J. Agric. Food Chem. 52, 6724-6731.

Rodrigo, M.J., Zacarías, L. 2007. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. Postharvest Biol. Technol. 43, 14-22.

Stewart, I., Wheaton, T.A. 1972. Carotenoids in citrus. Their accumulation induced by ethylene. J. Agric. Food Chem. 20, 448-449.

Sun, J.H., Luo, J.J., Tian, L., Li, C.L., Xing, Y., Shen, Y.Y. 2013. New evidence for the role of ethylene in strawberry fruit ripening. J. Plant Growth Regul. 32, 461-470.

Trainotti, L. Pavanello, A., Casadoro, G. 2005. Different ethylene receptors show an increased expression during the ripening of strawberries: does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits? J. Exp. Bot. 56, 2037-2046.

Undurraga, P., Olaeta, J.A., Cancino, C. 2011. Ethylene, enzymatic and respiratory patterns evolution in loquat (*Eriobotrya japonica* (Thumb.) Lindl.) cv. Golden Nugget in the last four sequential stages of maturation. Chil. J. Agric. Res. 71, 530-535.

Villarreal, N.M., Bustamante, C.A., Civello, P.M., Martínez, G.A. 2010. Effect of ethylene and 1-MCP treatments on strawberry fruit ripening. J. Sci. Food Agric. 90, 683-669.

Wang, P., Zhang, B., Li, X., Xu, C., Yin, X., Shan, L., Ferguson, I., Chen, K. 2010. Ethylene signal transduction elements involved in chilling injury in non-climacteric loquat fruit. J. Exp. Bot. 61, 179-190.

Watkins, C.R. 2008. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. Biotechnol Adv. 24, 389-409.

Yang, X., Song, J., Campbell-Palmer, L., Fillmore, S., Zhang, Z. 2013. Effect of ethylene and 1-MCP on expression of genes involved in ethylene biosynthesis and perception during ripening of apple fruit. Postharvest Biol. Technol. 78, 55-66.

Figure legends

Figure 1. External appearance (A) and colour index of the peel (B), and ethylene production (C) of loquat fruit (*Eriobotrya japonica* Lindl cv. Algerie) attached to the tree. Fruits at five ripening stages were selected: mature green (MG), breaker (BK), yellow (Y) and two coloured stages (FC1 and FC2), corresponding to the BBCH-scale indicated in the x-axis. Peel colour index is expressed as *a*/*b* Hunter ratio and data are the mean ± S.E. of 20 fruits. Respiration rate and ethylene production are the mean ± S.E of 3 replicate measurements.

Figure 2. Ethylene production of loquat fruits (*Eriobotrya japonica* Lindl cv. Algerie) detached from the tree (A), and expression of the ethylene biosynthetic genes: *ACC synthase 1* (*ACS1*), *ACC oxidase 1* (*ACO1*) and *ACC oxidase 2* (*ACO2*) in peel (left panels) and pulp (right panels) of loquat fruits at the breaker (BK), yellow (Y) and full coloured (FC) ripening stages. These ripening stages correspond to the stage 801, 803 and 807 of the BBCH-scale. Peel and pulp tissues used for gene expression were collected from the same fruits used for ethylene analysis. An arbitrary value of 1 was assigned to the value of expression obtained in the pulp of fruits at the yellow stage. The data are means ± S.E of at least 3 replicates.

Figure 3. Expression of ethylene perception (*ERS1a*, *ERS1b*) and signaling (*CTR1* and *EIL1*) genes in peel (left panels) and pulp (right panels) of loquat fruits (*Eriobotrya japonica* Lindl cv. Algerie) at the breaker (BK), yellow (Y) and full coloured (FC) ripening stages. These ripening stages correspond to the stage 801, 803 and 807 of the BBCH-scale. Peel and pulp tissues used for gene expression were collected from the same fruits used for ethylene analysis in Fig. 2. An arbitrary value of 1 was assigned to the value of expression obtained in the pulp of fruits at the yellow stage. The data are means ± S.E of at least 3 replicates.

Figure 4. Effect of ethylene (10 µL L^{-1}) and 1-MCP (1 µL L^{-1}) on the expression of ethylene biosynthesis genes: *ACC synthase 1* (*ACS1*), *ACC oxidase 1* (*ACS1*), *ACC oxidase 2* (*ACO2*) in peel (left panels) and pulp (right panels) of loquat fruit (*Eriobotrya japonica* Lindl cv. Algerie). Fruits were treated at the yellow stage, corresponding to 803 of the BBCH-scale. An arbitrary value of 1 was assigned to the value of expression obtained in the pulp of fruits at the yellow stage. The data are means ± S.E of at least 3 replicates.

Figure 5. Effect of ethylene (10 µL L^{-1}) and 1-MCP (1 µL L^{-1}) on the expression of ethylene perception (*ERS1a*, *ERS1b*) and signaling (*CTR1* and *EIL1*) genes in peel (left panels) and pulp (right panels) of loquat fruit (*Eriobotrya japonica* Lindl cv. Algerie). Fruits were treated at the yellow stage, corresponding to 803 of the BBCH-scale. An arbitrary value of 1 was assigned to

the value of expression obtained in the pulp of fruits at the yellow stage. The data are means ±
S.E of at least 3 replicates.

Figure 1

Figure 2

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