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Effect of different environmental stresses on the expression of oleate desaturase genes and fatty acid composition in olive fruit

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ABSTRACT

The regulation of microsomal and plastidial oleate desaturases by low and high temperature, darkness, and wounding was investigated. To this end, their gene expression levels and the fatty acid composition was determined in the mesocarp tissue of olive fruit from the Picual and Arbequina varieties subjected to the corresponding stress treatments. Firstly, a plastidial oleate desaturase from olive was cloned and its functional identity was confirmed by overexpression in *Escherichia coli*. The results showed that temperature and light regulate olive oleate desaturase genes at transcriptional level. However, no correlation between their expression levels and the linoleic acid content in microsomal and plastidial lipids was found. In addition, the involvement of microsomal but not plastidial oleate desaturases in the wounding response of olive fruit mesocarp is demonstrated. The fatty acid analysis revealed the appearance of palmitolinoleic acid only in microsomal lipids, reaching a maximum 3 h after wounding.

1. Introduction

Linoleic and α-linolenic acids, the two main polyunsaturated fatty acids in plant lipids, play key roles in plant metabolism as structural components of membrane lipids, as precursors of signaling molecules involved in plant development and stress response, and as storage compounds in the form of triacylglycerols (Ohlrogge and Browse, 1995; Weber, 2002).

In higher plants, the fatty acid biosynthesis starts in the plastids, yielding primarily palmitoyl-acyl carrier protein (ACP) and stearoyl-ACP by successive addition of two carbon atoms from acetyl-CoA (Harwood, 1996). Still in the plastid, most of the stearoyl-ACP is desaturated by the action of a soluble Δ9 stearoyl-ACP desaturase producing oleoyl-ACP, which is the main product of the plastidial fatty acid biosynthesis. The oleic acid is then incorporated into glycerolipids inside or outside plastids, and it can be further desaturated to linoleic, and then to α-linolenic acid by the consecutive action of Δ12 and Δ15 desaturases. Two set of these enzymes are present in plant cells, which differ in their cellular localization, lipid substrates, and electron donor system (Shanklin and Cahoon, 1998). The microsomal oleate desaturase (FAD2) and linoleate desaturase (FAD3) are located in the endoplasmic reticulum (ER), use phospholipids as acyl substrates and NADH, NADH-cytochrome b_5 reductase and cytochrome b_5 as electron donor system. In contrast, the plastidial oleate desaturase (FAD6) and linoleate desaturase (FAD7/8) are located in the chloroplast, use primarily glycolipids as acyl carriers and NAD(P)H, ferredoxin-NAD(P) reductase and ferredoxin as electron donor system.

Plant fatty acid desaturases are subjected to different kinds of regulation. Temperature is one of the main environmental factors regulating fatty acid desaturases. Low temperature increases polyunsaturated fatty acids content in plants, contributing to maintain the fluidity of biological membranes (Los and Murata, 1998). Although the molecular mechanism by which temperature regulates fatty acids desaturation is not well known, different hypothesis have been suggested. First of all, temperature could regulate desaturases genes expression levels, thus changing the protein amount. This effect has been observed for the Δ9 (Madi et al., 2003; Vega et al., 2004), Δ12 (Kargiotidou et al., 2008; Teixeira et al., 2009; Wang et al., 2004), and Δ15 (Gibson et al., 1994; Teixeira et al., 2010) desaturases in several plants. On the other hand, a second way of regulating desaturase activity by temperature is by post-transcriptional mechanisms, as it has been described for FAD3 in wheat roots (Horiguchi et al., 2000), for FAD8 in Arabidopsis leaves (Matsuda et al., 2005), and for the seed-specific FAD2-1 in soybean (Tang et al., 2005). Finally, temperature could regulate desaturase activity by varying the substrate availability. The regulatory role of oxygen has been observed in *in vivo* systems such as sycamore cells suspension cultures, where oxygen concentration in solution could be limiting for the desaturase (Rebeille et al., 1980). In sunflower seeds, it has been demonstrated that temperature

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regulates the FAD2 activity by means of two different mechanisms (García-Díaz et al., 2002): (i) a long-term direct effect mostly related to the low thermal stability of the enzyme (Martínez-Rivas et al., 2003; Sánchez-García et al., 2004), and (ii) a short-term indirect effect by which temperature determines the availability of oxygen, which, at the same time, causes reversible changes in the FAD2 activity level (Rolletschek et al., 2007).

Light is a second environmental factor affecting fatty acid desaturation. In photosynthetic tissues, a light-dependent increase of polyunsaturated fatty acids has been reported. In particular, an increase in linoleic and α-linolenic acids has been described in greening cucumber cotyledons exposed to light (Murphy and Stumpf, 1979), while in oat leaves (Ohnishi and Yamada, 1983) and Arabidopsis callus cultures (Brockman et al., 1990) light increases α-linolenic acid content. Nevertheless, little is known about the mechanism by which light regulates desaturases. Expression analysis of cotton *FAD2* gene (Kargiotidou et al., 2008), Arabidopsis (Nishiuchi et al., 1995) and wheat (Horiguchi et al., 1996) *FAD7*, as well as *FAD3* and *FAD8* genes in soybean cell cultures (Collados et al., 2006), revealed a transcriptional regulation by light. On the contrary, in soybean cell cultures, where *FAD7* gene transcription levels were not affected by darkness, a decrease in α-linolenic acid content was observed, indicating the presence of a post-translational regulatory mechanism (Collados et al., 2006).

In addition to the response to temperature changes and light conditions, plants often encounter wounding and pathogen attack. It has been widely described the key role of linoleic and α-linolenic acids in plants defense response, mainly as precursors of signal molecules such as jasmonic acid (Farmer, 1994). Accordingly, the transient induction of the *FAD7* gene in response to wounding, with the corresponding increase of the α-linolenic acid content, has been observed in several plants (Hamada et al., 1996; Nishiuchi et al., 1997; Teixeira et al., 2010). In addition, the increase of *FAD2* expression in wounded leaves of purslane (Teixeira et al., 2009), the induction of the Δ9 stearoyl-ACP desaturase and *FAD2* genes in avocado fruits infected with *Colletotrichum* (Wang et al., 2004), and the rapid and transient induction of parsley *FAD2* and *FAD7* genes by a fungal elicitor (Kirsch et al., 1997a,b) have also been reported.

Unlike oilseeds or leaves, little information is available about the regulation of fatty acid desaturation in oil fruits. In olive (*Olea europaea* L.) fruit, oil accumulation takes place in two completely differentiated tissues: the mesocarp, with a major contribution to the final composition of the olive oil, and the seed, which is enclosed by a woody endocarp. Olive oil is one of the world's major edible oils and is highly enriched in oleic acid (55–83%), while linoleic acid account for 3.5–21% and α-linolenic acid for less than 1%. The relative proportions of these unsaturated fatty acids have a significant effect on olive oil quality. Elevated linoleic acid content has important consequences in the nutritional characteristics of the olive oil (Cunnane, 2003), and negatively affects the technological properties, such as the oxidative stability (Aparicio et al., 1999). The linoleic acid content depends mainly on the variety. Picual and Arbequina, the two varieties chosen in the present study, are characterized by a distinct linoleic acid percentage, low (4–5%) and moderate (11–14%), respectively. Environmental and culture conditions also affect the linoleic acid content of the olive oil. A slight decrease of the linoleic acid percentage is observed in oils obtained from olives grown in locations with higher ambient temperatures (Aguilera et al., 2005). On the other hand, although a number of effects of light on lipid synthesis and accumulation in olive fruit has been described (Sánchez, 1994), the effect of light on the fatty acid composition of olive oil has not been studied. Finally, wounding caused during olive fruit harvesting and transport, or due to olive fruit infestation by olive fly, is a third factor that can modify olive oil fatty acid composition. In particular, a slight increase in the linoleic acid content in olive fruit infected by olive fly has been described (Pereira et al., 2004; Tamendjari et al., 2004).

In olive, two genes encoding microsomal oleate desaturases (*OepFAD2-1* and *OepFAD2-2*) have been reported (Hernández et al., 2005), whereas only one gene corresponding to the plastidial oleate desaturase (*OeFAD6*) has been described (Banilas et al., 2005), but without confirming its functional identity. Expression analysis of these genes revealed that they are spatially and temporally regulated. In mesocarp tissue, *OepFAD2-1* and *OepFAD6* transcript levels were low compared with other tissues and they decreased during olive fruit development and ripening. In contrast, the *OepFAD2-2* expression level was high and it was increased at the beginning of the ripening process. According to these data, *OepFAD2-2* was identified as the gene mainly responsible for the linoleic acid content in the olive fruit mesocarp and, therefore, in virgin olive oil (Hernández et al., 2009).

In the present work, we have established the functional identity of an olive plastidial oleate desaturase gene. In addition, to determine the factors that regulate linoleic acid biosynthesis in olive fruit, we have investigated the effect of low and high temperature, darkness, and wounding on the expression levels of oleate desaturase genes, and on the fatty acid composition in olive fruit mesocarp from Picual and Arbequina varieties.

2. Results and discussion

2.1. Functional expression of olive FAD6 gene in Escherichia coli

An olive *FAD6* cDNA clone from the cultivar Koroneiki has been previously isolated and characterized (Banilas et al., 2005). However, its functional identity has not been established. In contrast, the functional identity of olive *FAD2-1* and *FAD2-2* cDNA clones have been demonstrated by functional expression in *Saccharomyces cerevisiae* (Hernández et al., 2005). Before starting the studies on the regulation of the expression of olive *FAD2* and *FAD6* genes, we decided to confirm the functional identity of the olive *FAD6* gene by functional expression in *E. coli*. To achieve that, a full-length cDNA clone corresponding to the olive plastidial oleate desaturase was isolated from the cultivar Picual and designated *OepFAD6*. The *OepFAD6* full-length cDNA clone, with size of 1571 bp, revealed an open reading frame (ORF) encoding a predicted protein of 443 amino acid residues, which corresponds to a calculated molecular mass of 51.2 kDa and pI of 9.1. This ORF was flanked by unique 5′- and 3′-untranslated regions of 109 and 112 bp, respectively, with a poly(A) tail at the 3′-end. Alignment of the deduced OepFAD6 amino acid sequence with that of the *FAD6* clone from olive cv. Koroneiki (Banilas et al., 2005) showed 98% identity. In addition, OepFAD6 exhibits a high degree of identity to the known plant FAD6 sequences (74–66%).

As previously reported for the olive *FAD6* gene from the cultivar Koroneiki (Banilas et al., 2005), the deduced OepFAD6 amino acid sequence revealed the eight histidines essential for desaturase activity, arranged in three boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (Shanklin and Cahoon, 1998), the four hydrophobic regions characteristic of membrane-bound desaturases (Shanklin et al., 1994), and the presence of an N-terminal extension of 65 amino acids with features of a chloroplast transit peptide. Southern blot analysis using genomic DNA isolated from the olive cultivar Picual revealed that one single copy of *OepFAD6* gene seems to be present in the olive genome (data not shown), as it was described for the cultivar Koroneiki (Banilas et al., 2005).

To confirm the functional identity of the *OepFAD6* gene, we have cloned the corresponding ORF, excluding the signal peptide