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Original article

Temporal and transient expression of olive enoyl-ACP reductase gene during flower and fruit development

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Abstract

Enoyl-ACP reductase is a catalytic component of the fatty acid synthetase (FAS) type II system in plants that is involved in the de novo fatty acid biosynthesis in plastids. A cDNA encoding an enoyl-ACP reductase responsible for the removal of the trans-unsaturated double bonds to form saturated acyl-ACP has been isolated from a library made from ripening fruits of *Olea europaea* L. The predicted protein contains 393 amino acid residues including a consensus chloroplast specific transit peptide. A strong homology was observed when olive enoyl-ACP reductase aligned with other plant sequences. Southern hybridization analysis revealed that enoyl-ACP reductase is encoded by a single gene in olives. Northern hybridization showed a transient expression of the enoyl-ACP reductase (*ENR*) gene at early stages of drupe (5–7 weeks after flowering, WAF), embryo and endosperm (13–16 WAF) while in mesocarp (13–19 WAF) the expression remained at high levels. In situ hybridization showed particularly prominent expression in the palisade and vascular tissue of young leaves, the tapetum, developing pollen grains and vascular tissue of anthers and to less extent in the embryo sac and transmitting tissue of the carpel. The distinctive spatial and temporal regulation of the *ENR* gene is consistent with major roles, not only in thylakoid membrane formation and fatty acid deposition, but also in the provision of precursor molecules for the biosynthesis of oxilipins that are important in plant tissues involved in transportation and reproduction.

Keywords: Development; Enoyl-ACP reductase; Gene expression; In situ hybridization; *Olea*

1. Introduction

Fatty acid synthetase (FAS) complex catalyzes the de novo biosynthesis of fatty acids. The complex uses acetyl-CoA as the primer moiety and malonyl-CoA as the elongator by the sequential addition of 2-carbon units to the growing acyl chain. The FAS complex in plants is localized exclusively in plastids [13,33]. It consists of eight freely dissociable polypeptides, each of which either acts as a carrier protein or catalyzes a separate enzymatic activity. This complex is termed "type II" FAS and is typical of prokaryotes and plants. The "type I" FAS system found in vertebrates and yeasts, is made up of either one or two large multifunctional proteins, respectively [20].

The components of the plastidial FAS complex are: acyl carrier protein (ACP), malonyl transacylase, acetyl transacylase, 3-hydroxyl-acyl-ACP synthetase, 3-keto acyl-ACP reductase, 3-hydroxy acyl-ACP dehydrase, enoyl-ACP reductase and the acyl-ACP thioesterase. Genetic complementation studies demonstrated that a single component of the plant FAS system, e.g. enoyl-ACP reductase can functionally replace its counterpart within the bacterial complex [16].

Molecular studies have firmly established that ACP [6,26], enoyl-ACP reductase [15,31] and 3-keto acyl-ACP reductase

Abbreviations: BCIP/NBT, 5-bromo-3-chloro-3-indolyphosphate/nitroblue tetrazolium; CTAB, cetyltrimethylammonium bromide; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; ENR, enoyl-ACP reductase; pfu, plaque forming units; SDS, sodium dodecyl sulfate; WAF, weeks after flowering.

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[31] are nuclear-encoded enzymes, and synthesized as precursor nascent polypeptides. The mature polypeptide is cleaved from the transit peptide, which directs translocation through the chloroplast membrane towards the stroma of these organelles. These results clearly support the endosymbiotic hypothesis of the prokaryotic nature of chloroplasts [29].

Enoyl-ACP reductase in the fatty acid elongation cycle catalyzes the reduction of the *trans*-2,3 double bond to saturated acyl chain. The protein has been purified from spinach leaves [30], avocado mesocarp [1] and rapeseeds [34]. In rapeseeds the enzyme is a homotetramer [32]. Amino acid sequence and two-dimensional Western blotting analysis suggested the presence of four isoforms of the enzyme in *Brassica* leaf and seed tissues [4,9]. Southern analysis showed that the tetraploid *Brassica napus* contains four genes [15] and in *Arabidopsis* is encoded by a single gene per haploid genome [5]. During seed development in *Brassica*, the enzyme is synthesized throughout the period of lipid biosynthesis and deposition [32]. Fatty acid biosynthetic genes are expressed at constant molar ratios but different absolute levels during *Brassica* embryogenesis [24].

The isolated cDNA encoding for enoyl-ACP reductase in olives was used as probe in order to investigate the regulation of gene expression during flower and fruit development. The mRNA levels were determined on developing seed and mesocarp tissues showing temporal specificity of gene expression and transcript accumulation during fruit growth. By in situ hybridization the mRNAs were detected in different cell types of leaves and developing buds indicating a spatial and transient regulation of *enoyl-ACP reductase* gene expression.

2. Results

2.1. Isolation and characterization of an olive plastidial ENR gene

The olive drupe cDNA library was screened using a full-length *Brassica napus* microsomal *ENR* cDNA as a hybridization probe. The longest cDNA was selected and sequenced revealing a single 1182-nucleotide open reading frame encoding a predicted protein of 393 amino acid residues [14]. An alignment of predicted amino acid sequences reveals that the olive sequence contains an N-terminal domain with the characteristic features of a plastidial stroma targeting peptide. The putative cleavage site at Ala 74 is a rather conservative residue among the plant deduced enoyl-ACP reductases (Fig. 1). Hydropathy analysis determined at least three putative membrane spanning helices [14].

A BLASTX search showed a considerable identity (70–77%) of the olive cDNA with *enoyl-ACP reductase* genes from other plant species (Fig. 1). However, this identity is underestimated since it takes into consideration the putative transit peptides. The putative mature enoyl-ACP reductase from olive showed the highest identity to Petunia (92%) and less to tobacco (88%), cauliflower (87%), *Arabidopsis* (86%) and rice (80%).

Southern blot analysis showed four or three major hybridizing bands after digestion with *HindIII* or *PstI*, respectively (Fig. 2). Since within the *enoyl-ACP reductase* cDNA there are restriction sites for both endonucleases (data not shown) and the gene may contain a large number of introns as has been determined for *Arabidopsis* and *Brassica* [5], it is reasonable to conclude that the olive diploid genome contains most likely two *enoyl-ACP reductase* genes.

2.2. Expression of enoyl-ACP reductase gene during fruit development

To investigate the temporal expression and developmental accumulation of *enoyl-ACP reductase* transcripts during fruit development, total RNA was isolated from olive drupes, embryos, endosperms and mesocarps at different times (weeks after flowering, WAF). Since early globular and heart stages of olive zygotic embryos could not be excised without injury, RNA was isolated from small intact drupes grouped according to their age up to 11 WAF. Drupes of 5, 7, 9 or 11 WAF have sizes of 1–2, 3–4, 5–8 or 9–11 mm in length, respectively. During this period the embryo passes through the globular, heart and heart-torpedo stages. Beginning from 13 WAF, embryos (early torpedo stage), endosperms and mesocarps were dissected out and collected. At 16 and 19 WAF embryos are at early mid and mid torpedo stages, respectively, while at 22 WAF embryos reach late torpedo stage.

In young drupes (5 WAF) containing globular stage embryos, the *enoyl-ACP reductase* gene expression was high (Fig. 3). Transcript accumulation remained almost at the same level in 7 WAF drupes containing heart stage embryos followed by a rapid decline at 9 WAF drupes and even further at 11 WAF drupes. *Enoyl-ACP reductase* gene expression was high at early torpedo stage embryos and the corresponding endosperms (13 WAF) and transcript accumulation reaches maximum level in early mid torpedo embryos (16 WAF) and the corresponding endosperms. Accumulation declines when embryos and the corresponding endosperms are passing from the mid to late torpedo and maturation stages, where enoyl-ACP reductase mRNA is barely detectable (Fig. 3).

In contrast, the pattern of *enoyl-ACP reductase* gene expression in mesocarp is quite distinct from that found in embryos and endosperms. Transcription is high at 13 WAF and remains almost constant up to 16 WAF. As growth proceeds (19 WAF), *enoyl-ACP reductase* transcripts accumulate in the mesocarp at even higher rates (Fig. 3).

Densidometric analysis has shown that at 13 WAF *ENR* transcript accumulation is almost equivalent in embryos endosperms and mesocarps. However, at 19 WAF *ENR* transcript accumulation is barely detected in embryos and endosperms, whereas in mesocarps it reaches maximum accumulation (data not shown). RNA from the same stages of development was loaded on a gel and stained showing that there was no experimental degradation or sample overloading in any of the lanes (Fig. 3).