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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).

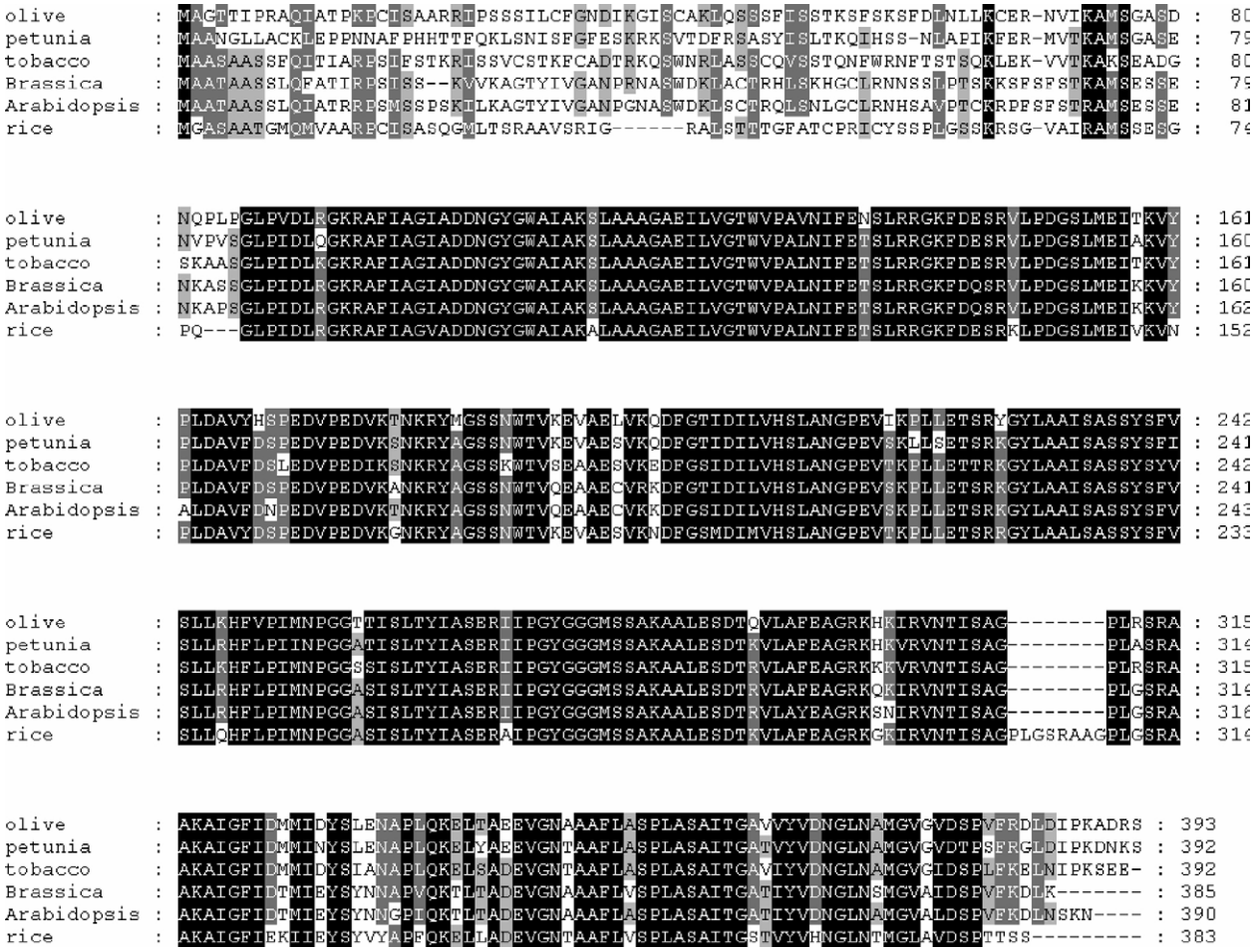

Fig. 1. The amino acid alignment of deduced enoyl-ACP reductase polypeptides from plants. One letter code was used. Dashes have been introduced to optimize sequence alignment. Identical or similar amino acids are black or grey shaded, respectively. Numbers to the right represent the position of the amino acids. Olive (accession number AAL93621), petunia, (acc. numb. CAA05879), tobacco (acc. numb. CAA74176), *Brassica* (acc. numb. CAC41366), *Arabidopsis* (acc. numb. NP565331) and rice (acc. numb CAA05816).

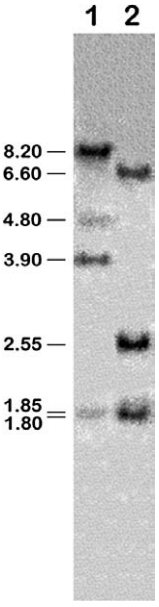

Fig. 2. Southern blot of olive genomic DNA probed with *ENR* cDNA. Each lane contains 4 μg genomic DNA digested with *Hin*dIII (lane 1) or *Pst*I (lane 2). Numbers on the left indicate molecular size markers in kb.

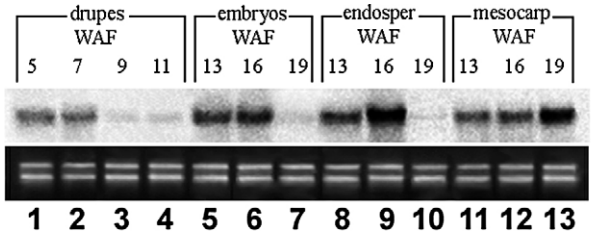

Fig. 3. Expression of *ENR* gene and mRNA accumulation during olive fruit development. Total RNA was extracted from young drupes, embryos, endosperms and mesocarps. Each lane contains 10 μg of total RNA. Drupe lanes: RNA from drupes of 5 (1–2 mm in diameter drupes), 7 (3–4 mm), 9 (5–8 mm) and 11 WAF (9–11 mm). Embryo lanes: RNA from 13 (early torpedo), 16 (early–mid torpedo) and 19 WAF (mid–late torpedo). Endosperm lanes: RNA from endosperms of 13, 16 and 19 WAF. Mesocarp lanes: RNA from mesocarps of 13, 16 and 19 WAF. Equivalent amount of RNA were loaded onto a gel and stained to evaluate equal loading (lower panel).

2.3. Expression of ENR gene in leaves and during flower development

In situ hybridization was used to study the spatial and temporal pattern of expression of the *ENR* in leaves and during flower development. A sense probe was used as a control for

the background levels of hybridization signal (Fig. 4). The results were constant in numerous samples analyzed. Young leaves were transversally sectioned and hybridized to an antisense probe. *Enoyl-ACP reductase* transcripts were detected in developing leaves mainly in the palisade cells and vascular bundles, while the gene was also expressed, albeit at much lower levels, in spongy parenchyma cells (Fig. 4).

In order to study spatial and developmental regulation of *ENR* gene expression prior to fertilization, ovaries and anthers from different sized flower buds (1.5, 2.5 and 3.5 mm) were dissected out and collected. In anthers, the highest hybridization signal was detected in vascular bundles throughout the development of the anthers. Nevertheless, the antisense riboprobe gave high signal to the early maturing/pollen grain (Fig. 4). Later in developing anthers, *enoyl-ACP reductase* mRNA was prominent in the tapetum. The tapetal signal peaks at 3.5 mm bud length immediately prior to cell lysis, while the *enoyl-ACP reductase* mRNA is highly expressed in early developing pollen grains and continue to have a signal albeit at low levels, at 3.5 mm bud length, shortly before anthesis. A lower level of *ENR* gene expression was detected in the female organs of the bud (Fig. 4). The *ENR* mRNA was localized in the nucellus surrounding the embryo sac, vascular bundles and transmitting tissue in an equivalent mode through the ovary development (Fig. 4). It is interesting to note that both the vascular tissues of leaves and anthers express the *ENR* gene at particularly high levels.

3. Discussion

In order to follow up the spatial and developmental regulation of the lipid formation at transcriptional level a number of genes encoding for enzymes involved in olive lipid biosynthesis and modification have been isolated. Central to this, in higher plants the core fatty acid synthesis multi-enzyme complex consists of different components at equivalent morality. ENR is a component in FAS activity and therefore studying its spatial and developmental regulation we could study the FAS regulation at transcriptional level. However, the possibility of individual FAS components be encoded by gene families, of which distinct members are tissue-specific and developmentally regulated, cannot be excluded. Nevertheless, recent results have shown that genes encoding for different components of the FAS complex are expressed at constant molar ratios but different absolute levels during *Brassica* embryogenesis [24].

A rapeseed cDNA probe encoding an *ENR* gene was used as a probe to isolate the homologous gene from 13-WAF olive fruit cDNA library [14]. At this stage of development, the fruits are green, fully photosynthetically active and are synthesizing predominately thylakoid membrane lipids [7], while the accumulation of lipids is just beginning [2,27]. Based upon its sequence similarity, the olive gene encodes an ENR polypeptide possessing a transit peptide targeting the mature enzyme in the stoma of the plastids. As expected the deduced polypeptide from olive has high homology to the *ENR* genes isolated from different plants. The pattern of Southern hybridization showed that a single gene should be present per haploid genome. One copy of *ENR* gene per haploid genome has been also reported in other plants [5].

The high levels of olive *ENR* gene expression found in young photosynthetically active fruits (5–7 WAF) (Fig. 3) and in palisade cells of leaves (Fig. 4) are correlated with thylakoid membrane synthesis in such tissues. Other transcripts involved in lipid biosynthesis such as *Δ9 desaturase*, *FAD7* and *CYTb5* have been found to accumulate at high levels in olive young drupes [12,19,25]. During this period, (5–9 WAF) the size of the fruit is enlarged by fivefold and remains almost the same in size thereafter. *ENR* transcripts are barely detected in 9–11 WAF drupes. During this period, mesocarp RNA contributes the major part of total RNA [12] in drupes.

A bimodial shape of *ENR* mRNA accumulation is detected as mesocarp growth proceeds. High levels of *ENR* transcripts are accumulated at 13 or 16 WAF mesocarp tissues, with maximum levels at 19 WAF. This second peak of transcript accumulation coincides with the onset of lipid storage. Therefore, the first peak of *ENR* transcript accumulation at 5 or 7 WAF is to furnish the cell with lipids mostly used for thylakoid membranes while the second peak is to provide lipids for deposition. The same bimodial pattern of transcript accumulation was also found for *Δ9 desaturase* [12], the enzyme that catalyzes the production of the most concentrated fatty acid in olive stored lipids. It is anticipated that both genes are coordinately up-regulated at two distinct periods in mesocarps: during early stages of drupe development and during mesocarp growth and later, during active lipid deposition.

Seed tissues, at early and mid torpedo stages of development accumulate high levels of *ENR* transcripts. Maximum mRNA levels were detected at 16 WAF and thereafter, at mid to late torpedo stage, were barely seen. Since, olive *Δ9 desaturase* transcripts have the same pattern of accumulation while *FAD7* mRNAs were almost absent during these stages of seed development [12,25], it is anticipated that *ENR* expression provides catalytic activities mostly for lipid storage in seed tissues.

The expression of *ENR* gene in *Brassica* [10] is also characterized by an up-regulation during seed development. The linear increase in early development is followed by rapid decrease to almost undetectable levels of *ENR* transcripts. However, the ENR polypeptide levels increase during early development but remain almost at same level, at later stages of development coinciding with the lipid deposition [9].

The cell-specific expression of the *ENR* gene in the different vegetative and reproductive tissues of olives was analyzed by in situ hybridization. Young expanding leaves showed the expected high levels of the *ENR* expression in thylakoid-rich photosynthetically active cell types, most notably in the palisade cells. However, there was also a consistently high signal associated with the vascular bundles of the leaf. The results demonstrate a surprising distribution of the gene activity, particularly in the two reproductive organs. A similar and

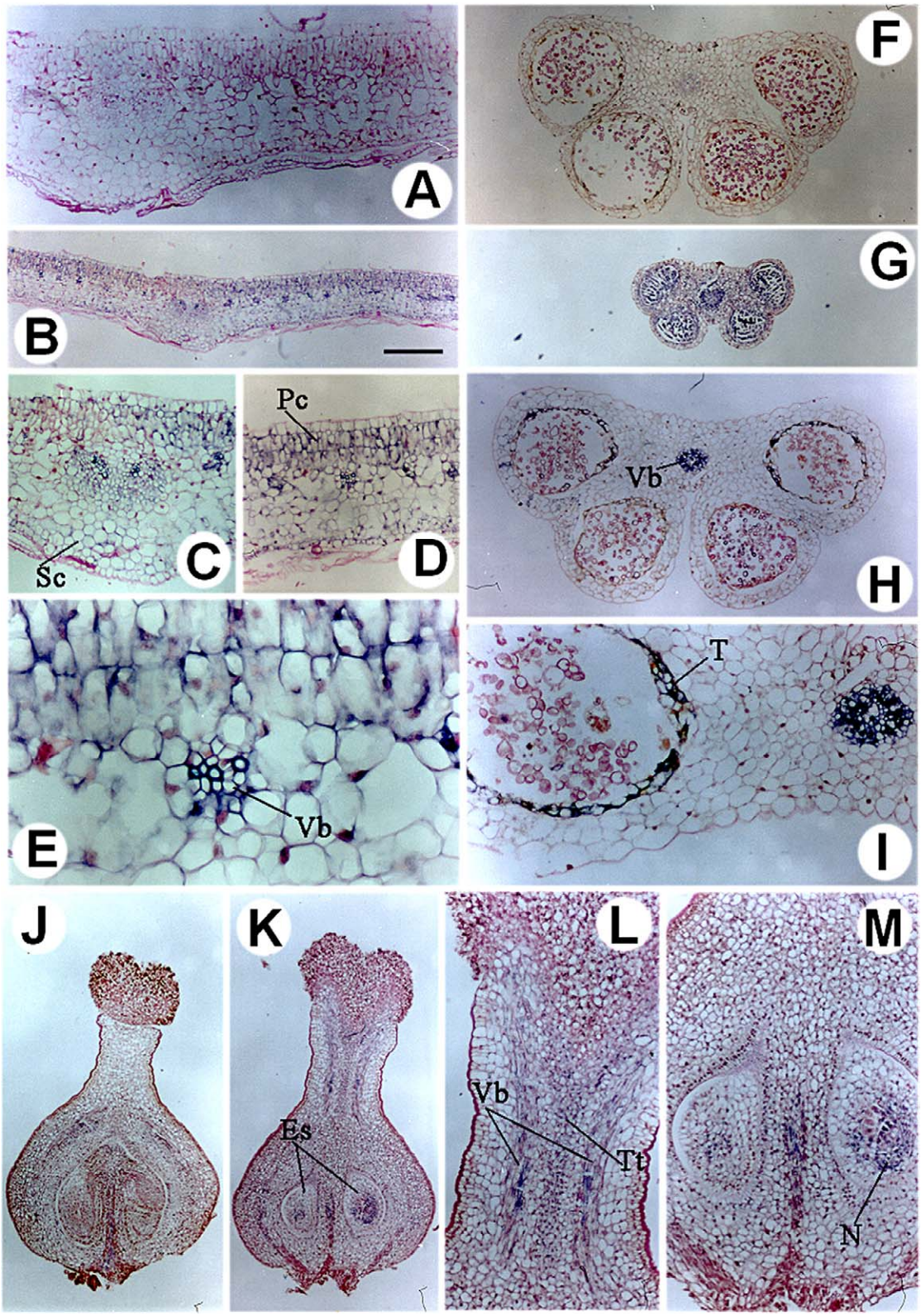

Fig. 4. In situ localization of *ENR* expression in young leaves (**A**–**E**) and developing anthers (**F**–**I**) and ovaries (**J**–**M**). mRNA in situ hybridization of *ENR* antisense (**B**–**E**, **G**–**I** and **K**–**M**) and sense (**A**, **F**, **J**). **C** (central leaf vein), **D** (leaf veins) and **E** (vascular bundles are higher magnification of the transverse section presented in **B**. Anthers and ovaries were dissected out from the developing olive flower buds. **F**, longitudinal section of 3.5-mm anthers; **G**, 1.5-mm; **H**, 3.5-mm buds. A higher magnification of H is shown in **I** showing vascular bundles, anther sacs and tapetum cells. **J** and **K** show medium longitudinal section of ovaries from 3.5-mm buds. **L** and **M**, higher magnification of the upper and lower part of the ovary in **K**. Es, embryo sac; N, nucelus; Pc, palisade cells; Sc, spongy cells; T, tapetum; Tt, transmitting tissue; Vb, vascular bundles. Scale bar for **B**, **F**–**H** and **J**–**K** equaled to 280 μm; **A**, **C**, **D**, **L** and **M** to 140 μm; **E** and **I** to 28 μm.

intense gene activity was profound in vascular bundles in both the female and male organs, particularly at the most active early stages of anther development. The leaf vascular cells are not the major sites of thylakoid membrane accumulation and in olive carpels and anthers, the vascular tissues are achlorophyllous and therefore completely lacking any photosynthetic membranes. We therefore presume that high levels of *ENR* gene expression in the vascular tissues are not associated with membrane or storage lipid biosynthesis. Several additional sites of the olive reproductive organs show *ENR* gene expression such as nucelus, embryo sac and transmitting tissue of the carpel and the tapetum of the anther.

These sites are not associated with the membrane lipid formation. Entomophilous species such as olives, have a lipid extracellular pollen coat and intracellular lipid storage bodies and the intense *ENR* expression during early stages of pollen grain development is mostly related to either membrane metabolism or storage of lipids. However, the most likely role of ENR activity in the reproductive and vascular tissues is the provision of fatty acids leading to the synthesis of oxylipin signaling molecules. Olive putative *FAD7* gene expression was also intense in these sites [25].

In view of the data presented here and with results obtained from other species [11,21,22] we suggest that while *ENR* expression in photosynthetically and storage tissues is associated with the fatty acid synthesis for membrane and storage lipids, its expression in achlorophyllous tissues is associated also with the systemic propagation of oxilipin signaling molecules, such as jasmonate. In conclusion, the *ENR* gene in olives is subject to complex pattern of spatial and temporal regulation. The major roles of the gene are (a) to furnish fatty acid for thylakoid membrane formation (b) to provide lipids for storage and (c) to provide precursor molecules for the biosynthesis of oxilipins. This later role is particularly detected in vascular and reproductive tissues.

4. Methods

4.1. Screening of Olea cDNA library

The cDNA library was constructed from mRNA extracted from 13 WAF olive drupes, *Olea europaea* L. as described by Haralampidis et al. [12], which was kindly provided by Dr. E. Heinz, University of Hamburg, Germany. At least 3×10^5 plaque forming units (pfu) of the cDNA library was screened under low-stringency conditions using [^{32}P]-labeled *Brassica napus enoyl-ACP reductase* cDNA as a probe. Hybridization was performed overnight according to Church and Gilbert [3] at 57 °C in 7% sodium dodecyl sulfate (SDS), 0.5 M Na_2HPO_4, 2 mM ethylenediaminetetracetic acid (EDTA), 1% bovine serum albumin. Nitrocellulose filters were washed twice with 5% SDS, 40 mM Na_2HPO_4, 1 mM EDTA at 57 and 52 °C for 15 min each, followed by four washes in 2% SDS, 40 mM Na_2HPO_4, 1 mM EDTA at 47 °C to room temperature. Positively hybridizing plaques were purified, and their plasmids were isolated according to the protocol described in the pBluescript II Phagemid kit (Stratagene).

4.2. Sequence analysis

DNA sequence analysis was carried out by the dideoxy termination method [28], using double strand as a template. The template was sequenced from both strands. The multiple sequence alignment of the deduced amino acid sequence was generated by Clustal X. The computing of possible membrane spanning segments was done by the method of Klein et al. [17]. The prediction of membrane associated helices was performed according to Eisenberg et al. [8].

4.3. Southern blot analysis with olive genomic DNA

Genomic DNA from *O. europaea* cv. Koroneiki young leaves was isolated by cetyltrimethylammonium bromide (CTAB) method [23]. The DNA was digested with restriction enzymes, fractionated on 0.8% agarose gel and transferred to nitrocellulose membranes as described [18]. Hybridization with [^{32}P]-labeled olive *enoyl-ACP reductase* cDNA probe was carried out under high stringency conditions according to Church and Gilbert [3] at 65 °C. Post-hybridization washes were performed starting from 65 °C as described above.

4.4. Total RNA isolation and analysis

The following olive tissues were harvested from field grown *O. europaea* cv. Koroneiki trees and frozen in liquid nitrogen: young leaves, developing drupes, embryos, endosperms and mesocarps. Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was isolated using phenol–chloroform extraction procedure [10]. RNA concentrations were determined spectrophotometrically and verified by ethidium bromide staining of agarose gels. The RNAs were electrophoresed on denaturing 1.4% agarose gel containing formaldehyde, transferred to nitrocellulose filters without treatment, and hybridized to [^{32}P]-labeled olive *enoyl-ACP reductase* cDNA. Hybridization was carried out overnight under the same conditions as described above for genomic Southern hybridization.

4.5. Microscopy and in situ hybridization

Ovaries and anthers dissected from developing buds, intact buds or young leaves of *O. europaea* cv. Koroneiki were fixed in solution containing 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer supplemented with 1 M NaCl (pH 7.4), followed by dehydration in ethanol series, and cleaning in ethanol/xylene series. After soaking in xylene and paraffin, they were embedded in paraffin. The paraffin blocks were sliced into 7–8 μm sections, mounted onto glass slides and incubated at 42 °C overnight.

Digoxygenin-11-UTP (Boehringer Mannheim) labeled sense and antisense RNA probes were transcribed using T7 and T3 polymerase and the promoter of pBluescript SK(-) (Stratagene), respectively. The coding strand provides a negative control probe for the specificity of the positive, i.e. antisense probe. The sense and antisense RNA probes were alkaline hydrolyzed to 150 nucleotide fragments and hybridized to paraffin-embedded fixed sections. The sections were hydrated by passing through ethanol series, treated with 1 μg ml^{-1} of proteinase K in 100 mM Tris–HCl (pH 7.5) 50 mM EDTA for 30 min at 37 °C and washed three times with sterile H_2O. Slides were incubated in 0.1 M triethanolamine in 0.25% (v/v) acetic anhydrite, pH 8–9 for 10 min and rinsed in 2 × SSC for 5 min, dehydrated through ethanol series and dried. The hybridization solution contained 50% formamide, 375 mM NaCl, 1.25 × Denhardt's solution, 12.5 mM Tris–HCl (pH 7.5) 1.25 mM EDTA, 87.5 mM dithiothreitol (DTT), 12.5% dextran sulfate and the probe at a concentration of 1 μg ml^{-1}. About 50 μl of hybridization mixture was added to each slide. Slides covered by coverslip were incubated for at least 12 h at 42 °C in a chamber containing water soaked paper. The samples were then washed four times for 10 min each, in 4 × SSC solution contained 5 mM DTT, and treated with 50 μg ml^{-1} RNase in 500 mM NaCl and 1 mM EDTA for 30 min at 37 °C. Slides were washed again with 500 mM NaCl and 1 mM EDTA solution supplemented with 5 mM DTT at 37 °C four times for 15 min each and then in 2 × SSC solution contained 1 mM DTT for 30 min at room temperature. Hybridization signal was detected using the alkaline phosphatase-conjugated antisera in conjunction with the color detection system of 5-bromo-3-chloro-3-indolyphosphate/nitroblue tetrazolium (BCIP/NBT). Sites of probe hybridization were detected as blue regions after color development. Sections were photographed with Olympus microscope using bright-field optics.

Acknowledgments

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