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Rapid and Transient Induction of a Parsley Microsomal Δ12 Fatty Acid Desaturase mRNA by Fungal Elicitor[1]

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Treatment of cultured parsley (*Petroselinum crispum* L.) cells with a structurally defined peptide elicitor (Pep25) of fungal origin has previously been shown to cause rapid and large changes in the levels of various desaturated fatty acids. We isolated two distinct parsley cDNAs sharing high sequence similarity with microsomal ω-6 fatty acid desaturases (FADs). One of them was functionally identified as a Δ12 FAD by expression in the yeast *Saccharomyces cerevisiae*. Two dienoic fatty acids, hexadecadienoic and linoleic, which were not detectable in control cells, together constituted up to 12% of the total fatty acids in the transformed yeast cells. Δ12 FAD mRNA accumulated rapidly and transiently in elicitor-treated parsley cells, protoplasts, and leaves. These and previous results indicate that fatty acid desaturation is an important early component of the complex defense response of parsley to attempted fungal infection.

Plants have evolved highly complex and efficient defense mechanisms to cope with the numerous potential pathogens surrounding them in their environment. Successful defense requires both the capacity of the challenged plant cell to rapidly perceive the invading organism and an efficient means by which to mobilize all available metabolic resources that may contribute to its impairment (Kombrink and Somssich, 1995). Studies using diverse plant pathosystems have demonstrated that transcriptional activation of numerous genes is one important feature of the plant's defense response (Hahlbrock and Scheel, 1989; Dixon and Harrison, 1990; Alexander et al., 1994; Somssich, 1994). Many of these genes encode enzymes involved in the formation of a large variety of defense-related compounds. Several of these compounds have been shown to be either antimicrobially active or involved in the reinforcement of the cell wall. Others may have a role in intracellular signal transduction cascades that activate defense-related genes or in intercellular signaling that alerts neighboring cells to imminent danger.

Genes encoding phenylpropanoid-biosynthetic enzymes have been shown to be greatly stimulated by a large number of pathogens (Hahlbrock and Scheel, 1989; Nicholson and Hammerschmidt, 1992; Hahlbrock et al., 1995; Douglas, 1996; Smith, 1996). The resulting products serve numerous functions, including reinforcement of the preexisting structural barrier (e.g. cell wall modification), signaling (e.g. generation of salicylic acid), and direct defense (e.g. formation of low-molecular-weight antimicrobial substances termed phytoalexins). Other genes with infection-induced expression in various plant species include those encoding glucanases, chitinases, peroxidases, proteinase inhibitors, and enzymes of the shikimate pathway (van Loon et al., 1994; Herrmann, 1995; Kombrink and Somssich, 1995).

The nonhost interaction of parsley (*Petroselinum crispum* L.) with the soybean (*Glycine max*) pathogenic fungus *Phytophthora sojae* results in a strong local resistance response that very efficiently limits pathogen ingress (Jahnen and Hahlbrock, 1988). Treatment of suspension-cultured parsley cells with a structurally defined peptide elicitor (Pep25) from this fungus closely mimics the infection-induced plant defense response and thus greatly facilitates studies of the molecular mechanisms governing this response (Nürnberger et al., 1994; Hahlbrock et al., 1995). Numerous elicitor-responsive parsley genes encoding various enzymes of both primary and secondary metabolism have been characterized and the corresponding mRNAs have been shown to massively accumulate both in elicitor-treated cells and locally around fungus-infected leaf tissue (Schmelzer et al., 1988; Somssich et al., 1989; Kawalleck et al., 1995; Reinold and Hahlbrock, 1996).

Recently, we demonstrated that treatment of parsley cells with the Pep25 elicitor also induced large changes in the levels of unsaturated fatty acids and that these changes immediately followed rapid, transient accumulation of an mRNA encoding a plastid-localized ω-3 FAD. Induction of this mRNA was equally rapid and transient in fungus-infected parsley leaves and resulted in the highly localized accumulation of ω-3 FAD mRNA at infection sites (Kirsch et al., 1997).

Here we report that treatment of suspension-cultured cells or leaves of parsley with the Pep25 elicitor also rapidly induces the expression of other FAD or FAD-like genes. We present functional data showing that one of several parsley cDNAs analyzed encodes a microsomal Δ12 FAD, an enzyme catalyzing the conversion of oleic acid (18:1) to linoleic acid (18:2). These results further substantiate our previous findings that changes in the metabolism

[1] This paper is dedicated to Professor Clarence A. Ryan on the occasion of his 65th birthday.

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Abbreviations: FAD, fatty acid desaturase; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

of unsaturated fatty acids may have an important role in the defense response of parsley.

MATERIALS AND METHODS

Plant Material and Elicitor Treatment

Cultured parsley (*Petroselinum crispum* L.) cells were grown in the dark for 6 d under the conditions described previously by Kombrink and Hahlbrock (1986). The same cells were used for the isolation of protoplasts (Dangl et al., 1987). Parsley leaves were harvested from 5-month-old greenhouse-grown plants. Oligopeptide elicitor (Pep25) was added as an aqueous solution (Nürnberger et al., 1994) to cultured cells or protoplasts (final concentration 0.5 μg/mL) or by pressure infiltration (1 μg/mL) via the stomata into nondetached leaves using a 1-mL syringe.

Cloning of ELI12 and Δ12 FAD cDNAs

A specific primer (5′-ACGGTCTGAGTAAAGTGGGC-3′), derived from the partial ELI12 cDNA (Somssich et al., 1989) in combination with the T3 primer and an existing parsley λ-ZAP cDNA library (Korfhage et al., 1994) as a template, was used in a standard PCR-based approach to isolate the missing 5′ coding region of ELI12. Two fragments were generated. One fragment (724 bp) had a sequence 100% identical to the partial ELI12 cDNA within a 244-bp overlapping region. A common unique internal *Xho*I restriction site was used to construct an ELI12 cDNA containing the entire coding sequence. The sequence of the second PCR fragment (754 bp) was similar but distinct from ELI12. Therefore, a specific primer (5′-TCTGAGCTCCAGTCTGTTGC-3′) was also synthesized for this PCR fragment and used in combination with the universal primer and the parsley λ-ZAP cDNA library for a second round of PCR amplification, which resulted in the generation of a 755-bp fragment corresponding to the 3′ coding region of this cDNA. The 5′ and 3′ PCR fragments were identical in sequence within an overlapping region of 174 bp. The fragments were fused in-frame via a common internal *Sph*I restriction site to generate a cDNA designated Δ12 FAD, encompassing the complete coding region. For sequencing, all PCR fragments were subcloned into the vector pCR-Script (Stratagene).

Growth and Fatty Acid Analysis of Transformed Yeast Cells

For expression in yeast the parsley cDNAs were cloned behind a constitutive *ADH1* gene promoter in the yeast-*Escherichia coli* shuttle vector pVT102-U (Vernet et al., 1987). These constructs were used to transform *Saccharomyces cerevisiae* strain YM954 (*MATα ura3-52 his3-200 ade2-101 lys2-801 leu2⁻ trp1-901can^r gal4Δ542 gal80Δ338* [Wilson et al., 1991]; a gift of Dr. S. Fields) by the LiOAc method (Soni et al., 1993). Viable yeast cells were selected on minimal medium lacking uracil. Unless stated otherwise, 5 mL of minimal medium (2% Glc, 0.67% yeast N_2 base, with appropriate auxotrophic supplements) were inoculated with a single colony and the yeast cells were allowed to grow for 41 h at 20°C. Cells were harvested by centrifugation and the pellet was lyophilized and subsequently treated with 1 mL of methanolic HCl. Preparation of the fatty acid methyl esters and GC analysis were performed as described by Kirsch et al. (1997). The fatty acid methyl esters were identified by comparison of their retention times with those of authentic standards.

RNA Isolation and Analysis

RNA was isolated from cultured parsley cells, protoplasts, or leaves using a kit (Total RNA, Qiagen, Hilden, Germany). Approximately 20 μg of RNA per lane was denatured and separated in 1.2% (w/v) formaldehyde-agarose gels. The RNA was transferred to Hybond-N nylon membranes (Amersham) and cross-linked by UV irradiation. Prehybridization and hybridization conditions were as previously reported (Kawalleck et al., 1992). Hybridization signals were quantified by a phosphor imager using the Storm System hardware and Image Quant software (Molecular Dynamics, Krefeld, Germany).

Sequence Comparison

Sequences were compiled and analyzed using version 8.1 of the software package from the Genetics Computer Group (GCG, Madison, WI) (Devereux et al., 1984). For dendrogram creation, the GCG PileUp program was used to create multiple sequence alignments of FAD and FAD-like proteins from various organisms.

RESULTS

Cloning of a Putative Δ12 FAD cDNA

Using a cDNA encoding a plastid-localized ω-3 FAD and two partial cDNAs representing FAD-like genes, we recently demonstrated that the corresponding mRNAs were strongly induced in parsley cells upon treatment with the Pep25 elicitor (Kirsch et al., 1997). A closely related partial cDNA, previously isolated and designated ELI12 (Somssich et al., 1989), was found to share considerable sequence similarity with microsomal ω-6 FADs and has been used as a probe to isolate a gene (*FAD2* locus) encoding a microsomal ω-6 FAD gene from *Arabidopsis thaliana* (Kirsch et al., 1997). Thus, it was possible that ELI12 encoded an ω-6 FAD.

For unequivocal functional identification, a PCR-based approach was employed to obtain a cDNA containing the entire coding region. As a template we used a previously constructed library that was enriched for parsley cDNAs encoding elicitor-induced mRNAs (Korfhage et al., 1994). A PCR primer derived from the ELI12 cDNA sequence for extension toward the 5′ end enabled the isolation of two distinct PCR fragments. Sequence analysis revealed that one of them was identical within the overlapping region with ELI12 and extended on the 5′ side beyond the putative ATG start codon. The sequence of the second PCR fragment was similar but clearly distinct from that of ELI12

(62% nucleic acid sequence identity). A specific primer for this second fragment was therefore generated and used to obtain the missing 3′ coding region.

The complete coding regions of this new cDNA and the ELI12 cDNAs were 66 and 63% identical at the nucleotide and the deduced amino acid sequence levels, respectively. Both proteins share considerable sequence similarity with previously reported ω-6 FADs from other plant species. The similarity was somewhat greater for the new protein, which exhibited >75 and 71% identity with two functionally identified microsomal ω-6 FADs from soybean (*Glycine max*) and *A. thaliana* (Fig. 1), respectively. Inclusion of the two deduced parsley proteins in a tree representation of all known FAD and FAD-like sequences demonstrated the particularly close sequence relationship between the new protein and several authentic or putative microsomal ω-6 FADs (Fig. 2). DNA analysis suggested that the gene encoding the new protein was present in one to two copies per haploid parsley genome (data not shown).

Dienoic Fatty Acid Formation in Transformed Yeast Cells

The two cDNAs described above were cloned behind a constitutive *ADH1* gene promoter and transformed into yeast cells. It has previously been shown that *S. cerevisiae* transformed with the *A. thaliana FAD2* gene was capable of producing dienoic fatty acids (Covello and Reed, 1996; Kajiwara et al., 1996). GC analysis now demonstrated that yeast cells expressing the new parsley protein produced substantial amounts of hexadecadienoic (16:2) and 18:2 fatty acids (Fig. 3A). In contrast, these compounds were not formed by cells transformed with the parsley ELI12 cDNA (Fig. 3B) nor by control cells containing the empty vector (data not shown). In the latter case, the fatty acid profiles were essentially the same as those shown in Figure 3B. These results demonstrate that the newly isolated cDNA encodes a Δ12 FAD, whereas the functional identity of ELI12 remains open. Since the deduced Δ12 FAD protein does not contain an obvious signal sequence, the enzyme is assumed to be localized in the microsomal fraction of the cytosol.

The accumulation rates of dienoic fatty acids in the transformed yeast cells were strongly temperature-dependent, in accord with data reported by Covello and co-workers (1996), but in contrast to results obtained by Kajiwara et al. (1996). Decreasing the growth temperature of Δ12 FAD-expressing yeast cells from 30 to 20°C increased the amount of 16:2 and 18:2 fatty acids from 3% to more than 11% of the total fatty acids (Fig. 4).

Effects of Elicitor Treatment on Δ12 FAD mRNA Levels

Using nuclear run-on assays we previously demonstrated rapid elicitor-stimulated transcriptional activation of the *ELI12* gene (Somssich et al., 1989). Here, RNA-blot analysis was used to measure the effects of elicitor treatment on the Δ12 FAD mRNA levels in cultured parsley cells, protoplasts, and leaves. A 754-bp fragment from the 5′ portion of the Δ12 FAD cDNA was used as a probe in all of these experiments. This probe did not cross-hybridize with the other parsley FAD-like cDNAs under the conditions used. In cultured cells Δ12 FAD mRNA accumulated rapidly, strongly, and transiently upon elicitor treatment, with the highest levels occurring at 3 to 4 h; the mRNA level then declined markedly between 5 and 9 h, but increased again to give a second peak at 10 to 16 h (Fig. 5). A similar biphasic time course of mRNA accumulation has recently been observed for other elicitor-responsive parsley genes (O. Batz and K. Hahlbrock, unpublished results). However, the elicitor response pattern of Δ12 FAD mRNA was clearly distinct from that of the previously described plastidic ω-3 FAD mRNA, which was induced much more transiently (Fig. 5) (Kirsch et al., 1997).

The Pep25 elicitor induced the accumulation of Δ12 FAD mRNA in parsley protoplasts (Fig. 6A) and leaves (Fig. 6B). Inducibility in protoplasts was in agreement with the specific binding of Pep25 to sites on the plasma membrane and with the efficient triggering of various defense reactions in

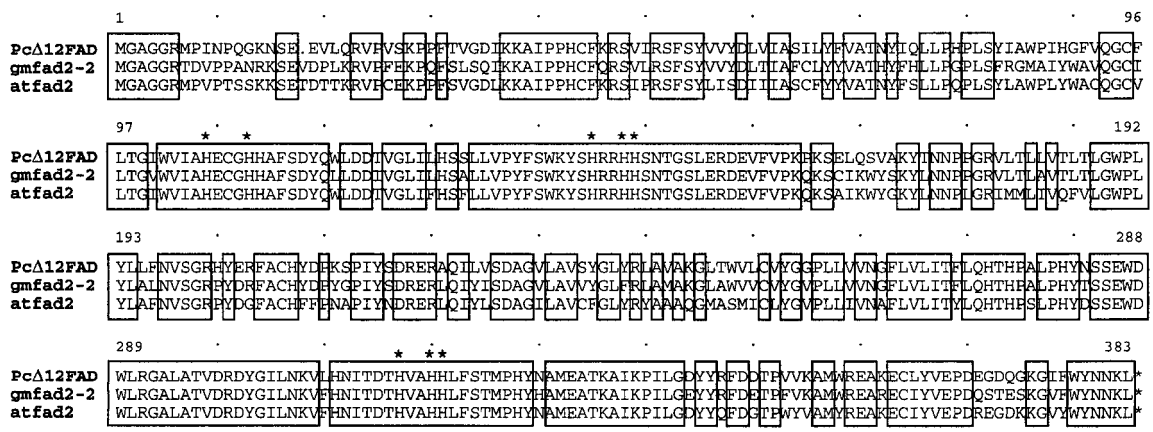

Figure 1. Amino acid sequence comparison of the parsley (Pc) Δ12 FAD and two functionally identified microsomal ω-6 FADs from soybean (gmfad2-2; accession no. L43921) and *A. thaliana* (atfad2; accession no. L26296). Identical amino acids are boxed. The eight invariant His's necessary for FAD function are indicated by asterisks (Shanklin et al., 1994).

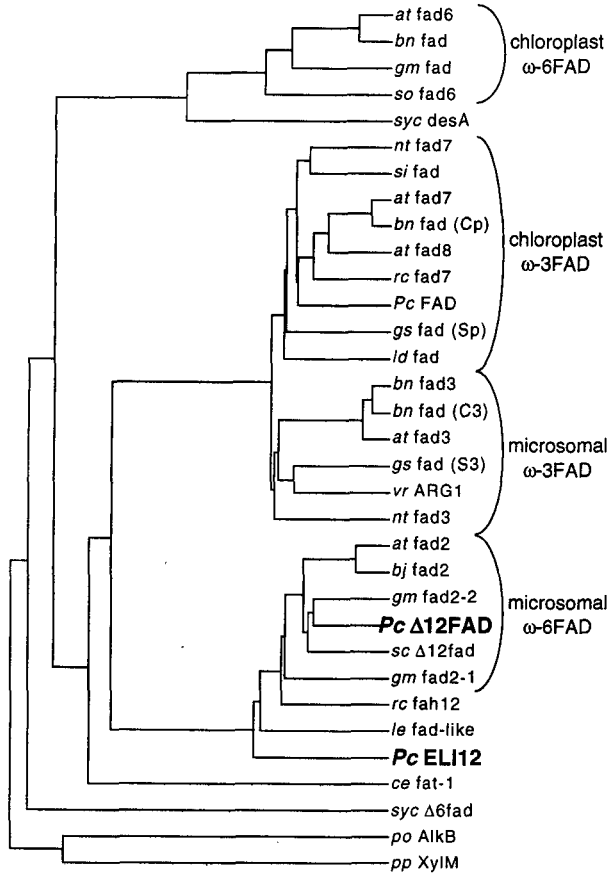

Figure 2. Dendrogram of FAD-like amino acid sequences from different organisms. Distances along the horizontal axes are proportional to sequence differences. Positions of the parsley ELI12 and Δ12 FAD proteins are highlighted in boldface. The putative localization and function of the various plant FADs are indicated on the right. The sequences were largely obtained from the GenBank database (accession numbers as given): *A. thaliana* (*at* fad6, U09503; *at* fad7, L22961; *at* fad8, D17578; *at* fad3, L22931; *at* fad2, L26296), *Brassica juncea* (*bj* fad2, X91139), *Brassica napus* (*bn* fad, L29214; *bn* fad [Cp], L22963; *bn* fad3, L01418; *bn* fad [C3], L22962), *Caenorhabditis elegans* (*ce* fat-1, U23523), *G. max* (*gm* fad, L29215; *gm* fad2-1, L43927; *gm* fad2-2, L43921), *Glycine soja* (*gs* fad [Sp], L22965; *gs* fad [S3], L22964), *Limnanthes douglasii* (*ld* fad, U17063), *L. esculentum* (*le* fad-like, X94944), *Nicotiana tabacum* (*nt* fad7, D79979; *nt* fad3, D26509), *P. crispum* (*Pc* fad; U75745), *Pseudomonas oleovorans* (*po* AlkB, J04618), *Pseudomonas putida* (*pp* XylM [Suzuki et al., 1991]), *Ricinus communis* (*rc* fad7, L25897; *rc* fah12, U22378), *Solanum commersonii* (*sc* Δ12fad, X92847), *Sesamum indicum* (*si* fad, U25817), *Spinacia oleracea* (*so* fad6, X78311), *Synechocystis* sp. (*syc* desA, X53508; *syc* Δ6fad, L11421), and *Vigna radiata* (*vr* ARG1, D14410).

the absence of the cell wall (Nürnberger et al., 1994). Induction in leaves was unlikely to be due to a wounding effect caused by the infiltration method because neighboring leaf sections infiltrated with water (Fig. 6B, lanes W) showed no increase in Δ12 FAD mRNA relative to untreated control leaves (Fig. 6B, lanes C). The more or less constitutively expressed ubiquitin mRNA was measured as a control for RNA loading on the gel.

DISCUSSION

Our results demonstrate the strong, rapid, and transient induction of Δ12 FAD mRNA in elicitor-treated parsley cells, and therefore extend previous observations that the metabolism of unsaturated fatty acids is strongly affected by this treatment, including the even more transient induction of an mRNA encoding a plastid-localized ω-3 FAD (Kirsch et al., 1997). The Δ12 FAD mRNA most likely encodes a microsome-localized enzyme, as concluded from the size of the deduced protein (M_r = 43,440), which is similar to that of other plant microsomal ω-6 FADs, and from the lack of any obvious N-terminal transit peptide that would be required for plastid targeting. Most importantly, we have shown that the enzyme catalyzes the formation of 16:2 and 18:2 fatty acids in yeast cells. This latter result not only verifies its functional identity as a Δ12 FAD, but also indicates that the membrane system of the yeast ER is a suitable environment for its catalytic activity. Thus, elicitor treatment in parsley induces mRNAs encoding both microsomal and plastid-localized fatty acid desaturases, strongly suggesting that the com-

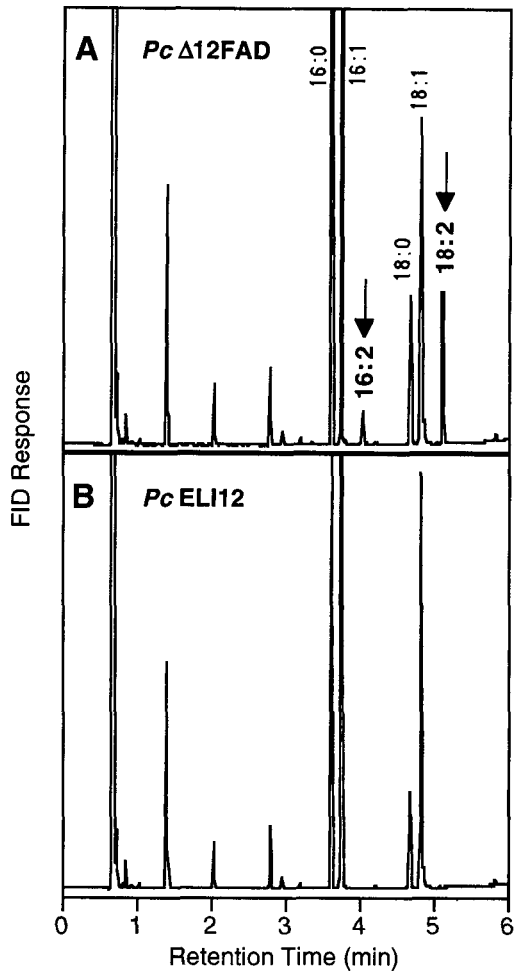

Figure 3. GC analysis of fatty acid methyl esters from yeast cells transformed with the parsley Δ12 FAD (A) or the parsley ELI12 cDNA (B). FID, Flame ionization detector.

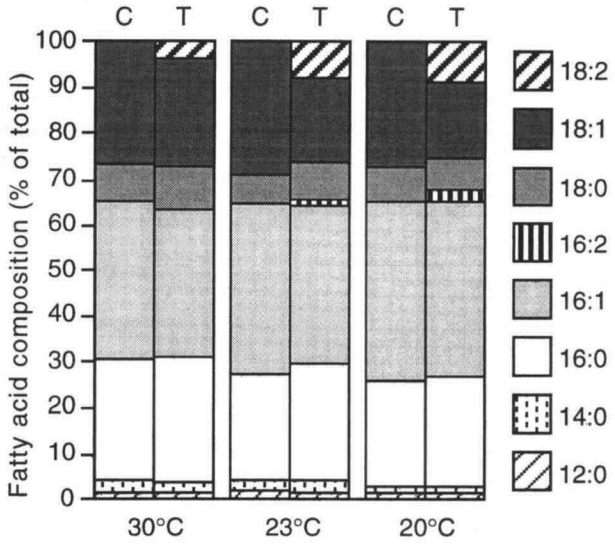

Figure 4. Effect of temperature on the accumulation of dienoic acids. The fatty acid composition relative to total fatty acid content is shown for yeast strains grown at the indicated temperature and containing either the empty vector (C) or the parsley Δ12 FAD cDNA (T). Fatty acids analyzed are indicated on the right. 18:0, stearate; 16:1, palmitoleate; 16:0 palmitate; 14:0, myristate; and 12:0, laurate.

plete fatty acid desaturation pathways are activated in each of the two compartments.

Two possible metabolic needs for the rapid formation of unsaturated fatty acids in elicitor-treated or infected plant cells are the compensation for losses caused by the elimination of lipid hydroperoxides from membranes damaged during the oxidative burst and by the conversion of 18:3 via the octadecanoid pathway to signal molecules such as jasmonates (Vick and Zimmermann, 1984; Tzeng and DeVay, 1993). Indeed, rapid formation of both H_2O_2 and jasmonate has frequently been observed in elicited parsley and other plant cells (Nürnberger et al., 1994; Blechert et al., 1995; Ellard-Ivey and Douglas, 1996; Jabs et al., 1997).

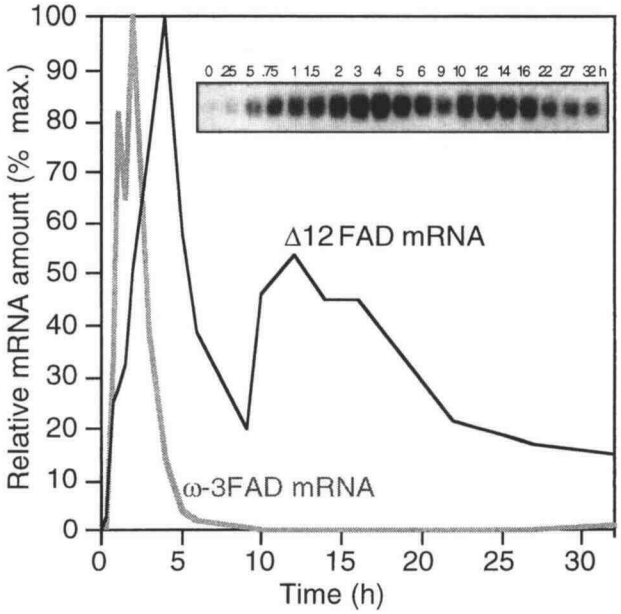

Figure 5. Time course of changes in the Δ12 FAD mRNA level in elicitor-treated parsley cells. The RNA blot (inset) was probed with a 754-bp fragment from the 5′ portion of the Δ12 FAD cDNA and the relative mRNA amounts were determined using a phosphor imager. For comparison, the previously reported changes in the relative amounts of the mRNA encoding a plastid-localized ω-3 FAD (data from Kirsch et al., 1997) are included.

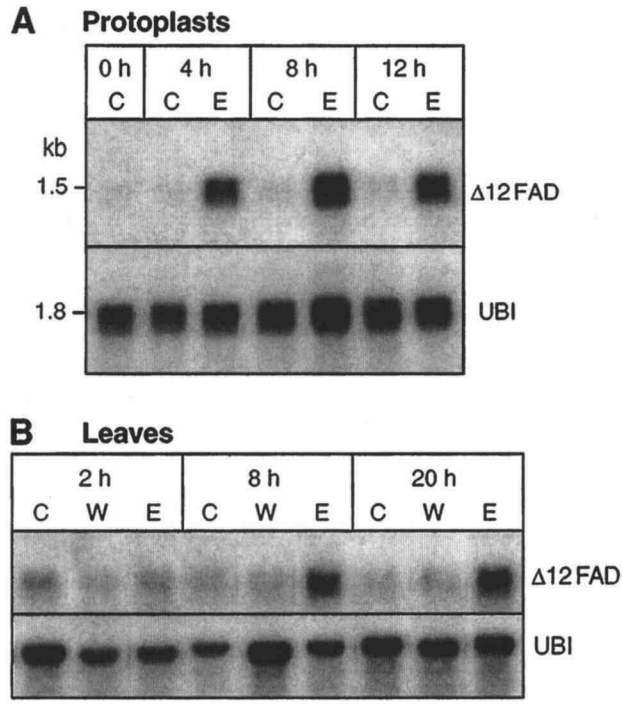

Figure 6. Changes in the Δ12 FAD mRNA levels in parsley protoplasts and leaves upon treatment with elicitor. A, Total RNA isolated at the indicated times from untreated control (C) or elicitor-treated (E) protoplasts. B, Total RNA isolated at the indicated times from control (C), water-infiltrated (W), or elicitor-infiltrated (E) leaves. The RNA blots were first probed with the 754-bp 5′ fragment of the Δ12 FAD cDNA and then reprobed with a ubiquitin cDNA (UBI) to monitor possible variations in RNA loading.

Likewise, the formation of lipid peroxides has been demonstrated in pathogen-infected plants or after fungal elicitor treatment (Adám et al., 1989; El-Moshaty et al., 1993; Rustérucci et al., 1996). Activation of such a rapid replenishment system in infected tissue would enable the release of unsaturated fatty acids from membranes without detrimental effects on the cellular metabolism. The occurrence of such a system in wounded tomato (*Lycopersicon esculentum*) plants could at least partially explain why a 15-fold increase in free 18:2 and 18:3 fatty acids contrasted with the lack of any detectable difference in the total fatty acid composition (Conconi et al., 1996). However, in view of the large amount of 18:3 present in the overall lipid pool of the cell, the need for increased expression of the desaturase genes in infected or wounded tissue is not obvious. One possible explanation may be that cells contain specialized lipid pools that may be specifically affected under these conditions; clearly, more work needs to be done to test this hypothesis.

Elicitor-stimulated activation of FADs, probably together with related enzymes acting on lipid-bound substrates, may generate other fatty acid derivatives that could also act as endogenous signal molecules or be involved in the synthesis of polymers reinforcing preexisting cell walls, e.g. by local suberization. Indirect evidence for the occurrence of such reactions in parsley has been provided by histochemical studies indicating the accumulation of phenol-lipid polymers at fungal infection sites (Jahnen and Hahlbrock, 1988) and by the demonstration of rapid transcriptional activation by elicitors of two gene families, *ELI12* and *ELI7*, which encode FAD-like proteins (Somssich et al., 1989). However, although the deduced ELI7 and ELI12 proteins are 60% identical in sequence to various plant microsomal ω-6 FADs and to the castor bean oleate 12-hydroxylase (Takamiya-Wik, 1995; van de Loo et al., 1995), failure of yeast cells transformed with the respective cDNAs to accumulate 18:2 or hydroxylated fatty acids (Fig. 3B and C. Kirsch and I.E. Somssich, unpublished results) argues against their function as desaturases or hydroxylases. Nevertheless, their high sequence similarity to fatty acid-metabolizing enzymes and their strong, concomitant responsiveness to elicitor seem to indicate a role in pathogen defense closely related to that of ω-6 FAD and ω-3 FAD.

In elicited cultured parsley cells a biphasic mRNA accumulation pattern is observed for Δ12 FAD (Fig. 5). It cannot be concluded from the limited time points tested in this study that a biphasic induction behavior with somewhat altered temporal kinetics for Δ12 FAD occurs in plant tissue or in protoplasts. However, a similar pattern of expression has also been found for some other defense-related genes both in elicitor-treated cells and in fungus-infected parsley leaves (Reinold and Hahlbrock, 1996; O. Batz and K. Hahlbrock, unpublished data).

The relative timing of Δ12 FAD and ω-3 FAD mRNA accumulation suggests that transcriptional activation of the genes encoding plastidic FADs is much more transient than that of the genes encoding microsomal FADs. This general conclusion was recently supported by the observation that the mRNA accumulation pattern for a putative microsomal ω-3 FAD isoform (C. Kirsch and I.E. Somssich, unpublished data) was very similar to that shown here for the microsomal Δ12 FAD. Together, these results indicate coordinated gene regulation for FAD enzymes within but not between the respective compartments. That the most rapid elicitor-induced changes so far observed for an enzyme-encoding mRNA in parsley cells were those shown recently for the plastidic isoform of ω-3 FAD (Kirsch et al., 1997) may be related to the fact that chloroplasts are the major sites for fatty acid hydroperoxide metabolism, including the octadecanoid pathway (Bell et al., 1995; Blée and Joyard, 1996; Laudert et al., 1996), the products of which have important roles in signaling and therefore may be required particularly early in the defense response.

Very little is known about the molecular mechanisms regulating the rates of *FAD* gene transcription. Expression of some plant *FAD* and *FAD*-like genes has been shown to be affected by light, hormones, temperature, wounding, or infection (Gadea et al., 1996; Hamada et al., 1996, and refs. therein). However, with the exception of the light-responsive Arabidopsis *FAD7* gene promoter (Nishiuchi et al., 1995), *FAD* gene promoters have not been analyzed to our knowledge. The parsley cell culture system appears ideally suited for such studies and should allow one to pinpoint functionally important elicitor-response elements within the *FAD* gene promoters. Identification of *cis*-regulatory promoter regions and *trans*-acting factors could help us understand the mechanisms of differential regulation, e.g. of the plastidic and microsomal isoforms of ω-6 FAD and ω-3 FAD.

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The accession numbers for the sequences reported in this article are U86072 (Δ12 FAD) and U86374 (ELI12).

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