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Immunocytological localization of two plant fatty acid desaturases in the endoplasmic reticulum

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Abstract The subcellular location of two integral membrane-bound fatty acid desaturases (Fads), Fad2 and Fad3, was elucidated by immunofluorescence microscopic analyses of tobacco suspension cells transiently transformed with different epitope-tagged versions of the enzymes. Both myc- or hemagglutinin-tagged Fad2 and Fad3 localized to the same region of the endoplasmic reticulum (ER), as evidenced by their co-localization with the ER lumenal protein calreticulin. Results from differential permeabilization experiments revealed that the N-termini of both epitope-tagged Fad2 and Fad3 were exposed on the cytosolic side of ER membranes. These data define the subcellular location and topological orientation of plant desaturases in ER membranes. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Fatty acid desaturase; Endoplasmic reticulum; Subcellular localization; Immunofluorescence microscopy

1. Introduction

Many of the storage oils derived from plant seeds are enriched in polyunsaturated fatty acids, such as linoleic (C18:2) and linolenic acids (C18:3). These fatty acids are an essential part of human diet, serving as precursors for the synthesis of important structural and functional lipids including sphingolipids and eicosanoids. Enzymatic activities capable of synthesizing polyunsaturated fatty acids, i.e. desaturases, were initially measured in microsomal and plastidial preparations of various plant tissues [1,2]. However, with the exception of one plastidial enzyme [3], the identity of the enzymes was obscured by difficulties in purifying them using conventional biochemical methods. As an alternative approach to identifying fatty acid desaturases (Fads), Somerville and coworkers [4] employed a genetic–biochemical selection scheme to isolate several *Arabidopsis* mutants defective in various aspects of fatty acid desaturation. Genes encoding these Fads were subsequently identified by map-based cloning or insertional mutagenesis [4,5]. Fad genes have since been cloned from a number of different plant species, revealing a high degree of conservation among their deduced amino acid sequences [6].

The availability of cloned Fad genes has permitted a more thorough analysis of enzyme structure–function relationships. For instance, expression of plant Fads in a heterologous host, such as yeast, has commonly been used to verify enzyme activity and explore substrate–product relationships [7,8]. However, relatively little is known about the subcellular sorting, localization, and assembly of Fads in plant cells, largely due to the lack of monospecific antibodies.

To overcome this problem, we developed an epitope-tagging scheme that allowed us to immunologically distinguish transiently expressed Fad2 and Fad3 in plant suspension-cultured cells. The Fad2 and Fad3 enzymes catalyze the production of linoleic and linolenic acids, respectively, and play a central role in production of polyunsaturated fatty acids in plant storage oils [9]. The results presented in this study provide unequivocal evidence for the localization of both desaturases in the endoplasmic reticulum (ER). Additional evidence is presented that indicates that the N-termini of Fad2 and Fad3 are exposed on the cytosolic side of the ER membrane, in agreement with proposed models for the catalytic role of these enzymes in plant fatty acid biosynthesis.

2. Materials and methods

2.1. Plasmid construction

The vector pKS-myc was constructed for fusing a single copy of the myc epitope tag [10] to the N-terminus of a passenger protein by annealing synthetic oligonucleotides Myctop (5′-AGCTTGAATTCCCATGGCTGAACAAAAGTTGATTTCTGAAGAAGATTTGGCTAGCGGTCTGCA-3′) and Mycbot (5′-GACCGCTAGCCAAATCTTCTTCAGAAATCAACTTTTGTTCAGCCATGGGAATTCA-3′) then subcloning them into the *Hin*dIII/*Pst*I sites of pBluescript KS− (Stratagene). The oligonucleotides encoded, from 5′ to 3′, *Hin*dIII, *Eco*RI, and *Nco*I sites, an initiator methionine codon, an alanine spacer codon, the myc epitope sequence (EQKLISEEDL), and an *Nhe*I site for fusion to the 5′ end of the passenger protein open reading frame (ORF).

The *Arabidopsis thaliana FAD2* cDNA was obtained from the Arabidopsis Biological Resource Center (clone pF2A, stock number CD3-89; [11]). The coding region was amplified using *Pfu* polymerase (Stratagene) and primers FAD2top (5′-GGGCTAGCATGGGTGCAGGTGGAAGA-3′) and FAD2bot (5′-GGCTGCAGTCATAACTTATTGTTGTACCAGTACACA-3′). These primers introduced an *Nhe*I site just before the start codon and *Pst*I site just after the stop codon. The resulting PCR product was subcloned into pCRScript-CAM (Stratagene) to generate the vector pCRsc-FAD2. The *FAD2* ORF was fused in-frame to the myc epitope sequence by transferring an *Nhe*I/*Pst*I fragment from pCRsc-FAD2 to the *Nhe*I/*Pst*I sites of pKS-myc. The fusion sequence was then transferred to the plant expression vector pRTL2 [12] as an *Nco*I/*Sac*I fragment.

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Abbreviations: BY-2, Bright Yellow 2; Fad, fatty acid desaturase; HA, hemagglutinin; MS, Murashige–Skoog; ORF, open reading frame; PBS, phosphate-buffered saline, pH 7.4

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A second epitope-tagging vector, namely pKS-HA, was constructed for fusion of the *Brassica napus FAD3* ORF (submitted) to the hemagglutinin (HA) epitope tag [10]. Briefly, annealed oligonucleotides encoding *Hin*dIII, *Eco*RI, and *Nco*I sites, an initiator methionine codon, an alanine spacer codon, the HA epitope sequence (YPYDVPDYA), and an *Nhe*I site for fusion to passenger sequences were cloned into the *Hin*dIII/*Pst*I sites of pBluescript KS−. The *FAD3* ORF was modified by PCR to introduce an *Nhe*I site just before the start codon then fused in-frame to the HA epitope tag sequence by subcloning into the *Nhe*I/*Pst*I sites of pKS-HA. The HA-tagged *FAD3* ORF was transferred to pRTL2DN/S [12,13] as a *Kpn*I/*Xba*I fragment.

To construct a HA-tagged version of Fad2 and a myc-tagged version of Fad3, the epitope tags on myc-FAD2 and HA-FAD3 were replaced by interchanging *Sma*I/*Nhe*I fragments between the two vectors. All clones generated by PCR or synthetic oligo modification were verified by TaqCycle automated sequencing (Applied Biosystems).

2.2. Plant cell culture

Tobacco (*Nicotiana tabacum* cv. Bright Yellow 2) (BY-2) suspension culture cells were grown in Murashige–Skoog (MS) medium (Life Technologies) at 26°C in the dark with an orbital shaker at 150 rpm as described previously [14].

2.3. Transient transformations

For transient transformation, cells were harvested 4 days after subculture by centrifugation and resuspended in an equal volume of either 1× transformation medium (MS medium without 2,4-D, plus 250 mM sorbitol and 250 mM mannitol) or, for differential permeabilization experiments (see below), MS medium [13,14]. Transformation of BY-2 cells was performed with a Biolistic Particle Delivery System (Bio-Rad) using 10 mg of plasmid DNA precipitated onto M-17 tungsten particles [13]. Transient co-transformations were performed as described above except that 5 mg of each DNA construct was precipitated on M-17 tungsten particles. Following bombardment, cells were held in covered Petri dishes at 26°C in the dark for 20–24 h and then processed for indirect immunofluorescence microscopy.

2.4. Immunofluorescence microscopy

Biolistically bombarded BY-2 cells were fixed for 1 h in 4% (w/v) formaldehyde (Ted Pella, Inc.) prepared in a final concentration of 0.5× transformation medium. Fixed cells were washed several times in phosphate-buffered saline pH 7.4 (PBS) and then incubated in 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co.) in PBS for 2 h at 30°C. After several washes in PBS, plasma and organellar membranes were permeabilized by incubating cells in 0.3% (v/v) Triton X-100 (Sigma) in PBS for 15 min at room temperature [13]. For experiments designed to demonstrate the topological orientation of transiently expressed epitope-tagged Fad2 and Fad3 in membranes, bombarded cells were fixed in 2% (w/v) formaldehyde in 50 mM K-phosphate (pH 7.2) as described previously [13]. Fixed cells were treated with pectolyase as described above, then differentially permeabilized with digitonin (25 μg/ml) (Sigma) rather than Triton X-100 to perforate the plasma membrane but not organelle membranes.

Fixed, permeabilized cells were processed for indirect immunofluorescence microscopy as described previously [15]. Primary antibody sources and concentrations used were as follows: mouse anti-myc epitope monoclonal antibody (1:400) (clone 9E10; Covance Research Products); rabbit anti-myc epitope affinity-purified (Protein A Sepharose) IgGs (1:500) (clone 9E10; Covance Research Products); mouse anti-HA epitope monoclonal antibody (1:300) (clone 12CA5; Boehringer Mannheim); mouse anti-α-tubulin monoclonal antibody (1:500) (clone DM 1A; Sigma); rabbit anti-castor bean calreticulin (1:500) (kindly provided by Sean Coughlan, Dupont, Wilmington, DE, USA) [16]. Fluorescent dye-conjugated secondary antibodies included goat anti-mouse Alexa Fluor 488 (1:1000); goat anti-rabbit Alexa Fluor 488 (1:1000) (Cedar Lane Laboratories Ltd.); goat anti-rabbit rhodamine red-X (1:500) (Jackson ImmunoResearch Laboratories Inc.).

Labeled cells were viewed using a Zeiss Axioskop 2 epifluorescence microscope (Carl Zeiss Inc.) with a Zeiss 63× Plan Apochromat oil immersion objective (Zeiss Inc.) and a Retiga 1300 CCD camera (Qimaging). All images shown were deconvolved and adjusted for brightness and contrast using Northern Eclipse 5.0 software (Empix Imaging Inc.), and then composed into figures using Adobe Photoshop 5.5 (Adobe Systems).

3. Results and discussion

To develop a specific, immuno-based method for characterizing the subcellular localization of plant desaturase enzymes, short peptide sequences encoding either the myc or HA epitope recognition motifs [10] were appended to the N-termini of *Arabidopsis* Fad2 and *Brassica* Fad3. The N-termini of the proteins were chosen as a suitable location for the epitope tag(s) for several reasons. First, preliminary data indicated that addition of an epitope tag to the N-termini of Fad2 or Fad3 did not significantly alter their overall structure since both epitope-tagged proteins exhibited similar enzymatic activities to their non-tagged counterparts when expressed in yeast (data not shown). Second, we speculated that since both desaturases lack a recognizable N-terminal hydrophobic signal sequence, addition of the epitope tag to the N-terminus of the protein would not likely affect targeting to the ER. Finally, the C-termini of Fad2 and Fad3 contain amino acid sequences similar to motifs that have been shown for some ER-resident membrane proteins to be critical for retention in the organelle [5].

Fig. 1 illustrates the results of double, indirect immunofluorescence staining of tobacco BY-2 suspension cells transiently transformed (via biolistic bombardment) with DNA encoding an epitope-tagged version of either *Arabidopsis* Fad2 or *Brassica* Fad3. Fig. 1A shows that in a representative cell expressing myc-tagged Fad2, a reticular immunofluorescence staining pattern was apparent in the perinuclear and cytosolic regions. This staining pattern was attributable to localization of myc-Fad2 in the ER, since it was superimposable upon the immunofluorescence staining pattern of endogenous ER calreticulin in the same cell (compare Fig. 1A,B). Similar co-localization results were observed when the staining patterns attributable to myc-Fad2 and ER lumenal binding protein (BiP) were compared (data not shown). To rule out the possibility that the myc peptide contributed to the localization of myc-Fad2 in the ER, the sorting of a HA-tagged Fad2 in BY-2 cells was examined. Fig. 1C,D shows the co-localization of expressed HA-Fad2 and endogenous calreticulin, indicating that the myc (and by inference the HA) epitope sequence does not possess ER targeting information. Analyses of cells expressing myc-tagged Fad3 (Fig. 1E) revealed that the protein also localized in ER, as evidenced by its co-localization with endogenous ER calreticulin (compare Fig. 1E,F). Furthermore, replacement of the myc epitope sequence on Fad3 with the HA tag did not alter localization to the ER, i.e. HA-Fad3 co-localized with endogenous ER calreticulin (data not shown). Taken together, these data provide convincing cytological evidence for subcellular localization of two plant Fads in the ER.

The availability of different epitope tags on Fad2 and Fad3 allowed us to visualize the subcellular location of both desaturases when the enzymes were co-expressed in the same cell. As shown in Fig. 2, co-bombardment of HA-Fad2 and myc-Fad3 followed by immunostaining for each antibody revealed superimposable staining patterns. These results indicate that the HA and myc epitope tags can be used as convenient indicators of subcellular localization for separate, co-expressed proteins, which may be useful for future studies on desaturase targeting and assembly.

Sequence comparisons between the Fad2 and Fad3 enzyme families have revealed a number of conserved regions that represent important structural and/or functional domains

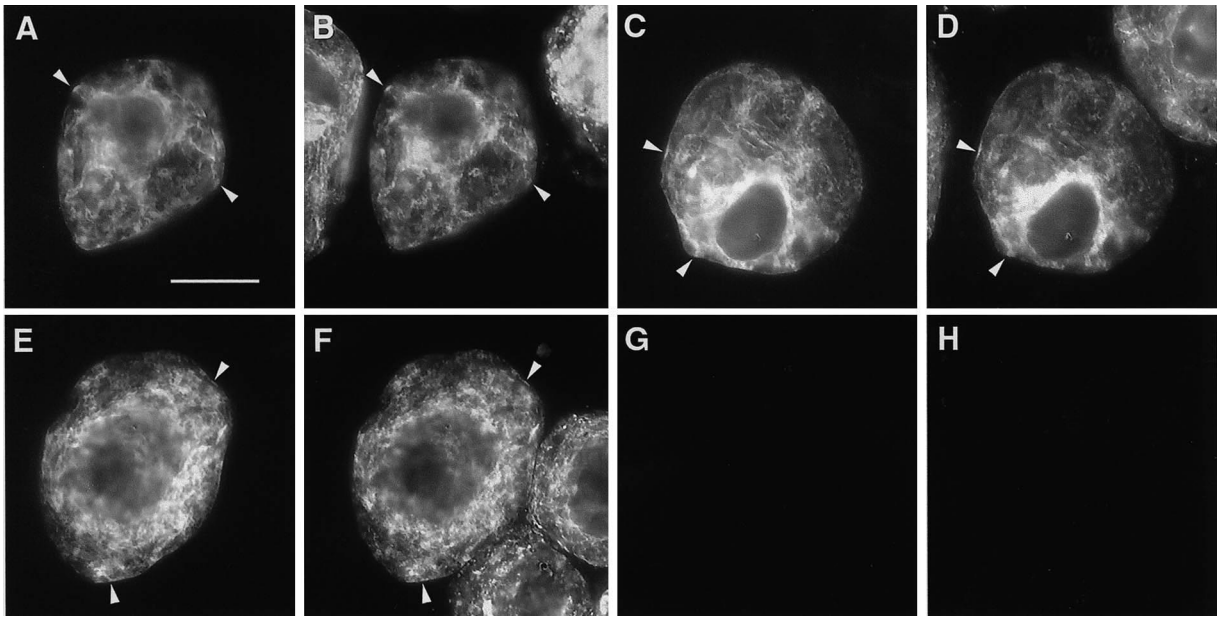

Fig. 1. Immunofluorescence localizations of transiently expressed, epitope-tagged Fad2 and Fad3 in tobacco BY-2 cells. Tobacco cells were biolistically bombarded with DNA encoding either epitope-tagged *Arabidopsis* Fad2 or *Brassica* Fad3, formaldehyde-fixed and then visualized by indirect immunofluorescence microscopy. The fluorescence staining patterns of two different proteins within the same group of BY-2 cells were obtained by virtue of two fluorescent dye-conjugated secondary antibodies that immunorecognize only one of the two sets of applied primary IgGs. The immunodetected proteins were then visualized using microscope filter sets designed specifically for the maximum emission wavelength of the dye-conjugated secondary antibodies employed. (A and B) Co-localization of myc-Fad2 (A) and calreticulin (B) in the ER of a transiently transformed cell. Arrows indicate regions of obvious co-localization. Note that endogenous BY-2 calreticulin staining with anti-calreticulin IgGs is apparent in all neighboring cells (see B, D and F). (C and D) Co-localization of HA-Fad2 (C) and calreticulin (D) in the ER of a transformed cell. (E and F) Co-localization of myc-Fad3 (E) and calreticulin (F) in the ER of a transformed cell. (G) Representative field of myc-Fad2-transformed cells showing the absence of fluorescence staining when primary antibodies (anti-myc IgGs) were omitted. (H) Absence of fluorescence staining in BY-2 cells mock-transformed with pRTL2. Bar in (A) = 10 μm.

within the proteins [6]. These include two long stretches of hydrophobic amino acids, predicted to span a lipid bilayer membrane at least four times, and three histidine-rich boxes, which coordinate iron atoms required for active-site chemistry. The identification of these domains, as well as consideration of Fad2 and Fad3 enzyme activity with respect to their interactions with hydrophobic fatty acid substrates and membrane-bound cytochrome proteins, has led to the development of a topological model for the orientation of Fad2 and Fad3 in ER membranes [17,18]. In this model, both enzymes are proposed to be anchored by their four membrane-spanning regions, with the histidine boxes and N- and C-termini facing the cytosolic side of ER membranes (Fig. 3).

To begin to directly test the proposed model for the topology of Fad2 and Fad3 in ER membranes, non-transformed and transiently transformed tobacco cells were differentially permeabilized using either Triton X-100 or digitonin, then processed for immunofluorescence microscopy. Fig. 4A–D shows results of control experiments with non-transformed cells. Triton X-100 perforates both plasma membranes and

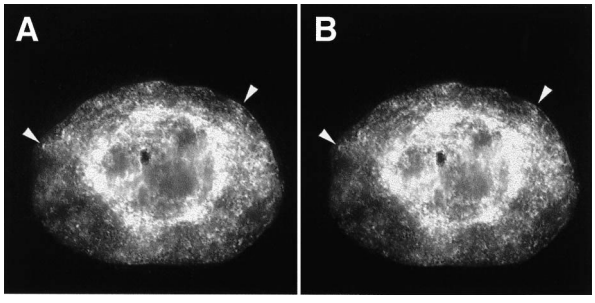

Fig. 2. Immunofluorescence co-localization of transiently expressed epitope-tagged Fad2 and Fad3 in the ER of tobacco cells. BY-2 cells were co-bombarded with DNA encoding HA-Fad2 and myc-Fad3 and then processed for indirect immunofluorescence microscopy. (A) Staining pattern attributable to HA-Fad2 (mouse anti-HA IgGs). (B) Same cell as in (A) stained for myc-Fad3 with rabbit anti-myc IgGs. Arrows indicate regions of obvious co-localization. Bar in (A) = 10 μm.

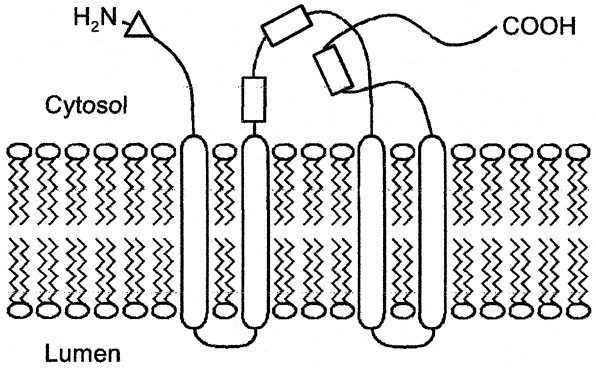

Fig. 3. Schematic representation of the predicted topology of Fad2 and Fad3 in ER membranes. The Fad2 and Fad3 proteins are anchored in the membrane by four hydrophobic spans, with a portion of the protein, including N- and C-termini and active-site histidine boxes (rectangles), exposed on the cytosolic side of the membrane [17,18]. The position of the myc or HA epitope tag at the N-terminus is denoted by a triangle.

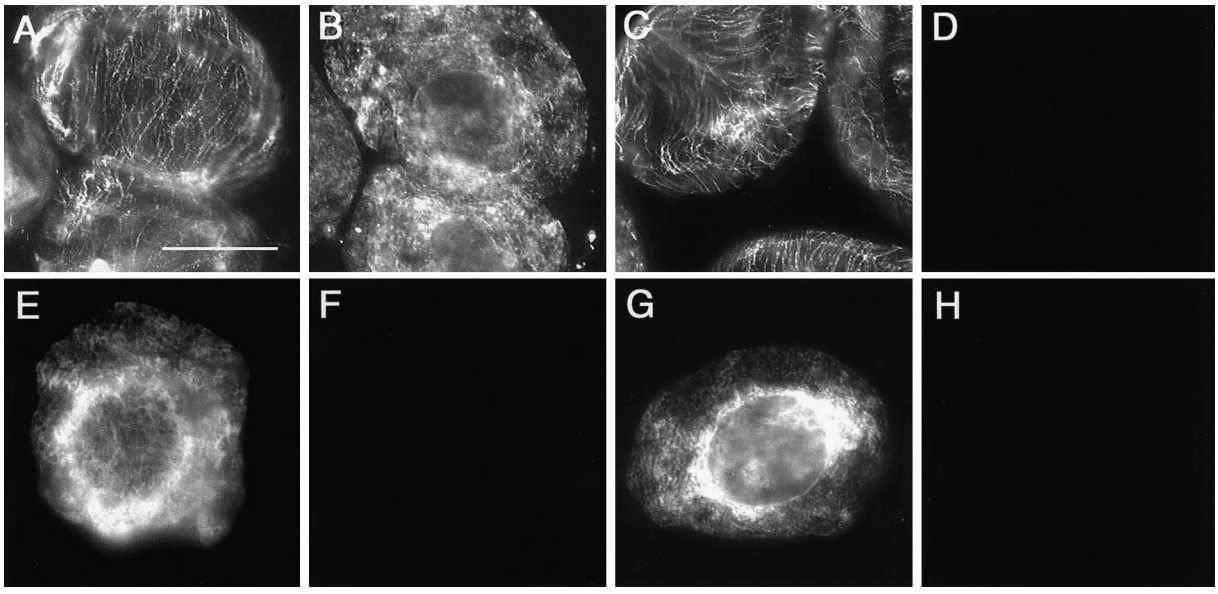

Fig. 4. Immunofluorescence images illustrating the membrane topology of transiently expressed myc-Fad2 and myc-Fad3 in differential permeabilized tobacco cells. Non-transformed (A–D) or transiently transformed (E–H) cells were fixed and permeabilized using either Triton X-100 (A and B) or digitonin (C–H), then processed for immunofluorescence microscopy. (A and B) Immunostaining of endogenous tubulin in cytosolic microtubules (A) and endogenous calreticulin in the lumen of the ER (B) in the same non-transformed, Triton X-100-permeabilized cells. (C and D) Immunostaining of cytosolic tubulin (C) but absence of calreticulin immunofluorescence (D) in the same digitonin-permeabilized cells. (E and F) Immunostaining of expressed myc-Fad2 in the ER (E), but absence of endogenous ER calreticulin (F) staining in the same digitonin-permeabilized cells. (G and H) Immunostaining of expressed myc-Fad3 (G) but absence of endogenous calreticulin staining (H) in the same digitonin-permeabilized cells. Bar in (A) = 10 μm.

internal cellular membranes and, thereby, allows for the immunodetection of cytosolic proteins, such as tubulin (Fig. 4A), and organelle lumenal/matrix proteins, such as calreticulin in the ER (Fig. 4B). Treatment with digitonin, however, selectively permeabilizes the plasma membrane, but not internal cellular membranes [13]. Consequently, only antigenic sites on the cytosolic side of organelle membranes are available to applied IgGs. Fig. 4C,D shows that cytosolic microtubules were immunostained in digitonin-permeabilized non-transformed cells (Fig. 4C), whereas ER lumenal calreticulin was not visualized in the same cells (Fig. 4D). Examination of cells transiently expressing myc-Fad2 and permeabilized with digitonin revealed that the protein was readily immunodetected in ER membranes, whereas lumenal calreticulin was not (Fig. 4E,F). Similar results were obtained for digitonin-permeabilized cells expressing myc-Fad3 (Fig. 4G,H), i.e. the myc epitope tag at the N-terminus of myc-Fad3 was immunodetected in digitonin-permeabilized cells indicating that this portion of the protein was exposed on the cytosolic, rather than lumenal, side of the ER membrane.

Taken together, the results presented in this paper provide unequivocal evidence for subcellular localization of Fad2 and Fad3 in the ER and define the topological properties of these enzymes in ER membranes. The availability of separate epitope tags on Fad2 and Fad3 will undoubtedly be valuable tools for future studies aimed at characterizing the assembly and regulation of each enzyme in plant cells.

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