The GmFAD7 gene family from soybean: identification of novel genes and tissue-specific conformations of the FAD7 enzyme involved in desaturase activity

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Abstract

The FAD7 gene encodes a ω3 fatty acid desaturase which catalyses the production of trienoic fatty acids (TAs) in plant chloroplasts. A novel GmFAD7 gene (named GmFAD7-2) has been identified in soybean, with high homology to the previously annotated GmFAD7 gene. Genomic sequencing analysis together with searches at the soybean genome database further confirmed that both GmFAD7 genes were located in two different loci within the soybean genome, suggesting that the soybean ω3 plastidial desaturase FAD7 is encoded by two different paralogous genes. Both GmFAD7-1 and GmFAD7-2 genes were expressed in all soybean tissues examined, displaying their highest mRNA accumulation in leaves. This expression profile contrasted with GmFAD3A and GmFAD3B mRNA accumulation, which was very low in this tissue. These results suggested a concerted control of plastidial and reticular ω3 desaturase gene expression in soybean mature leaves. Analysis of GmFAD7 protein distribution in different soybean tissues showed that, in mature leaves, two bands were detected, coincident with the higher expression level of both GmFAD7 genes and the highest 18:3 fatty acid accumulation. By contrast, in seeds, where FAD7 activity is low, specific GmFAD7 protein conformations were observed. These GmFAD7 protein conformations were affected in vitro by changes in the redox conditions of thiol groups and iron availability. These results suggest the existence of tissue-specific post-translational regulatory mechanisms affecting the distribution and conformation of the FAD7 enzymes related with the control of its activity.

Key words: Desaturase, FAD7, fatty-acid, gene expression, plastid, protein conformation, soybean.

Introduction

Fatty acids are the major constituents of membrane lipids. In plants, fatty acids are synthesized de novo in the stroma of plastids through a complex series of condensation reactions to produce either C16 or C18 fatty acids (Browse and Somerville, 1991). These fatty acids are then incorporated into the two glycerolipid synthetic pathways that exist in plants. In the so called ‘prokaryotic pathway’, because of its similarities with the bacterial synthetic pathway, the chloroplastic membrane lipids (phosphatidylglycerol, PG; monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG; and sulphoquinovosyldiacylglycerol, SL) are synthesized entirely in plastids. In the ‘eukaryotic pathway’, phospholipids are synthesized in the endoplasmic reticulum (ER) while MGDG, DGDG, and SL are synthesized from phosphatidyicholine (PC) produced in the ER (Browse and Somerville, 1991). The relative amount of glycerolipid synthesis by these two pathways may vary in different tissues and in different plant species. In some plant species like Arabidopsis or spinach, both pathways contribute almost equally to the synthesis of MGDG, DGDG, and SL. These plant species, named 16:3 plants, contain substantial amounts of 16:3 fatty acids esterified in position sn-2 of MGDG (Somerville and Browse, 1996). In other plant species such as soybean, maize, or pea, PG is the only
product synthesized by the prokaryotic pathway and the rest of the leaf glycerolipids are synthesized through the eukaryotic pathway. These plant species lack the hexatrienoic acid (16:3) and, therefore, contain ω3-linolenic acid (18:3) as the only trienoic fatty acid (Browse and Somerville, 1991). These plants are called 18:3 plants. In both glycerolipid pathways, desaturation of fatty acids is performed by a series of integral membrane enzymes called fatty acid desaturases. The activity of these fatty acid desaturases is critical for the function of biological membranes by maintaining their appropriate fluidity. The number and properties of these enzymes have been inferred from the isolation of a comprehensive collection of Arabidopsis mutants defective in fatty acid unsaturation (Wallis and Browse, 2002). These enzymes are encoded by nuclear genes and differ in their substrate specificity and subcellular localization. Thus, FAD2 and FAD3 are located in the ER while the rest (FAD4, FAD5, FAD6, FAD7, and FAD8) are located in the plastids (Wallis and Browse, 2002). FAD2 is the only soluble desaturase characterized up to now and catalyzes the desaturation of stearic acid (18:0) to 18:1 in the acyl carrier protein (ACP)-bound form (Murphy and Piffanelli, 1998). FAD2 and FAD6 are ω6 desaturases that synthesize the dienoic fatty acid linoleic (18:2) from oleic (18:1) in the ER and plastids, respectively. FAD3, FAD7, and FAD8 are ω3 desaturases that synthesize linolenic (18:3) from linoleic (18:2) in the ER (FAD3) and plastids (FAD7 and FAD8), respectively. The FAD8 gene encodes a plastidial ω3 desaturase that is cold-inducible (Gibson et al., 1994; Berberich et al., 1998). FAD4 and FAD5 produce 16:1 from 16:0 specifically for PG and MGDG, respectively (Murphy and Piffanelli, 1998).

Genes encoding both microsomal and plastidial ω3 desaturases have been isolated and studied either from model or crop plants (Yadav et al., 1993; Hamada et al., 1996; Horiguchi et al., 1998). Three independent microsomal ω3 desaturase genes, named GmFAD3A, GmFAD3B, and GmFAD3C, have been shown to contribute to soybean seed linolenic acid levels (Bilyeu et al., 2003). The presence of seed-specific isoforms of the microsomal FAD2 desaturase has also been reported in many species (Heppard et al., 1996; Pirtle et al., 2001; Mikkilineni and Rocheford, 2003; Hernández et al., 2005). On the contrary, a single FAD7 gene has been reported in all plant species in which the genes encoding plastidial ω3 desaturases have been analysed into detail (Yadav et al., 1993; Hamada et al., 1996; Berberich et al., 1998; Torres-Franklin et al., 2009).

The activity of plant fatty acid desaturases is tightly regulated. Plant desaturases are sensitive to several environmental cues like temperature (Iba, 2002), light (Collados et al., 2006) or other factors like hormones, pathogen attack or wounding (Nishiuichi et al., 1997; Matsuda et al., 2005; Matsuda et al., 2009). Increasing evidence suggests that post-translational mechanisms are involved in the control of desaturase activity (Okuley et al., 1994; Heppard et al., 1996; Horiguchi et al., 2000; Matsuda et al., 2005; Tang et al., 2005; Collados et al., 2006). However, our knowledge of these control mechanisms is still limited. The highly hydrophobic nature of these membrane-bound desaturases has constrained the development of protein purification methods, the obtention of specific antibodies or their enzymatic analysis. As a result, the information concerning protein abundance, distribution, and conformation of desaturases in plants is almost completely unknown.

In this paper, the identification is described of a novel GmFAD7 sequence (GmFAD7-2) different from the GmFAD7 gene previously reported in soybean (Yadav et al., 1993). Genomic sequence analysis, together with searches at the soybean genome database, further confirmed that both genes were located at two different loci within the soybean genome, suggesting that the plastidial ω3 desaturase FAD7 from soybean is encoded by two different genes. Information is provided about the tissue-specific expression of both GmFAD7 paralogous genes. Data are also provided about the tissue-specific distribution of the soybean GmFAD7 polypeptides and its protein levels are correlated with TA production in the different soybean plant tissues. Our protein data suggest the existence of tissue-specific post-translational regulatory mechanisms, based on specific conformations or modifications of the FAD7 enzyme that might be involved in the control of FAD7 ω3 desaturase activity.

Materials and methods

Plant materials and experimental treatments

Soybean plants (Glycine max cv. Volania) were grown hydroponically as described by Andreu et al. (2007). Roots, stems, flowers, mature leaves, and developing seeds were collected at the times indicated, quickly frozen in liquid nitrogen, and stored at –80 °C until use. When indicated, two other soybean cultivars, Safrana and Corsoy (the latter available as photosynthetic cell suspensions) were also used. Photosynthetic cell suspensions were cultured as described in Collados et al. (2006). For wound experiments, mature leaves (18-d-old) from plants were used. Incisions were made with a razor blade across the main vein at intervals of approximately 3 mm. The edges of the leaf were left undamaged. Two other soybean cultivars, Safrana and Corsoy (the latter available as photosynthetic cell suspensions) were also used. Photosynthetic cell suspensions were cultured as described in Collados et al. (2006). For wound experiments, mature leaves (18-d-old) from plants were used. Incisions were made with a razor blade across the main vein at intervals of approximately 3 mm. The edges of the leaf were left undamaged. Wounded leaves were harvested after 30 min or 4 h of wound treatment. The jasmonate effect was also tested. To that end, a methyl-jasmonate solution (50 μM) was applied to the leaf with a paintbrush. Treated leaves were harvested after 30 min or 4 h of jasmonate treatment.

RNA isolation and cDNA synthesis

Total RNA was isolated from 0.5 g of the different soybean tissues (except for roots where 1 g was used) using the Trizol Reagent (Invitrogen) and further purified using the RNeasy Plant MINI Kit (Qiagen) following the manufacturer’s instructions. cDNAs were synthesized from total RNA (4 μg) using M-MLV Reverse transcriptase, (Promega) according to the manufacturer’s instructions.

Database searching, identification, and sequence analyses and manipulation

The DFCI (Dana Faber Cancer Institute) database was used for BLAST searches of soybean sequences with homology to the previously annotated GmFAD7 gene sequence (GenBank accession no. L22965). Protein alignment was performed using the ClustalW Multiple alignment (BioEdit 7.0.9.0 software). Phylogenetic trees
were generated using the PHYML software (Guindon and Gascuel, 2003; ww.atgc-montpellier.fr/PHYML) with bootstraps 500. Compute pl/Mw (ExPaSy Server; http://www.expasy.ch/tools/pi_tool.html) was used for the theoretical pl (isoelectric point) and MW (molecular weight) calculation. The presence of a chloroplast transit peptide together with the prediction of subcellular localization was performed with ChloroP, PSORT, and TargetP analysis tools. The complete genomic sequence from soybean has recently been made available to the public at www.phytozome.net/soybean.php, (Schmutz et al., 2010).

RACE and RT-PCR expression analysis of the desaturase genes

For amplification of unknown 5’ and 3’ ends of the different GmFAD7 sequences, 5’- and 3’-RACE were performed according to the protocol described in the GeneRacer Kit (Invitrogen), using total RNA (4 μg) from 18-d-old mature leaves. The annealing temperature of the first-strand synthesis reaction was 50 °C. A series of gene-specific primers (GSP), summarized in Supplementary Table S1 available at JXB online, were used for these experiments. The full-length sequences obtained from the RACE experiments were confirmed by RT-PCR using cDNA isolated from 18-d-old leaves as a template and GSP primers. All PCR reactions were performed with the Platinum Taq High Fidelity polymerase from Invitrogen. In all cases, the PCR products obtained were cloned into the pGEM T-Easy vector (Promega) and sequenced (CNIO services, Madrid, Spain). The sequencing results obtained from the analysis of the clone population confirmed that single sequences were amplified with each pair of specific primers. The results were further confirmed by PCR amplification of genomic sequence fragments corresponding to both GmFAD7-1 and GmFAD7-2 genes from the Volania cultivar. Genomic DNA was isolated from soybean young leaves as described in Alfonso et al. (1996).

The expression patterns of the desaturase genes were examined by semi-quantitative RT-PCR assay. The oligonucleotides used were as follows (see Supplementary Table S1 at JXB online): GmFAD7-1 sense primer, GSP9; GmFAD7-1 antisense primer, GSP8; GmFAD7-2 sense primer, GSP7; GmFAD7-2 antisense primer, GSP4. Specific primers amplifying the GmFAD8 and GmFAD3 genes, as well as ACTIN as the housekeeping gene, were also used (see Supplementary Table S1 at JXB online). The amplification reaction was carried out using Platinum Taq High Fidelity DNA polymerase (Invitrogen) according to manufacturer’s instructions. The annealing temperatures used for amplification of the GmFAD7-1 and GmFAD7-2 were 56 °C for GmFAD7-1 and GmFAD7-2, respectively. The amplified products were resolved by electrophoresis on 1% agarose gels. Semi-quantification of the relative gene expression levels was performed through normalization against the housekeeping gene (ACTIN). Expression levels in roots or in 4 mm seeds were taken as reference values for relative expression calculations. Densitometric quantification of the PCR bands under non-saturating conditions was performed using an image densitometer (Gel Doc XR, Bio-Rad) and the image analysis software Quantity One (Bio-Rad).

Restriction analysis of the GmFAD3 isoforms

As the gene-specific primers for amplification of the GmFAD3 gene recognized and amplified both GmFAD3A and GmFAD3B genes, a restriction analysis was carried out of the amplified fragments using the Van9111 enzyme (GE Healthcare, UK), that allowed us to distinguish between these different GmFAD3 genes. The Van9111 enzyme cuts to GmFAD3A in position 164 generating two fragments of 164 and 755 base pairs, respectively, while it cuts to GmFAD3B in positions 164 and 758 generating three fragments with 161, 164, and 594 base pairs, respectively. The digestion products were resolved by electrophoresis on 1% agarose gels.

Lipid analysis

Total lipids were extracted from different soybean tissues with chloroform:methanol (2:1, v/v) as described by Bligh and Dyer (1959). Lipids were transesterified with potassium hydroxide in methanol. The resultant fatty-acid methyl esters were analysed and quantified using a gas chromatograph (HP model 5890 series 2 plus) equipped with SE3230 column (30×0.25 mm inner diameter, 0.2 μm df) and flame ionization detector (FID).

Obtention of protein extracts from soybean plant tissues and immunoblotting analysis

Protein extracts were obtained from roots, stems, leaves, and seeds. To that end, 0.5 g of tissue (except for roots where 2 g were used) were ground in a mortar to a fine powder with liquid nitrogen. The powder was then resuspended in buffer A (0.1 M TRIS-HCl, pH 7.5, 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM MgCl₂, 14 mM β-mercaptoethanol (β-MeOH), and homogenized with a Teflon homogenizer for 2 min. The sample was then centrifuged to remove unbroken material and debris. The protein content of the different fractions was estimated using the Bio-Rad protein assay reagent (Bio-Rad). All solutions contained Pefabloc (100 μg ml⁻¹, Fluka), antipain (1 μg ml⁻¹, Sigma-Aldrich) and leupeptin (1 μg ml⁻¹, Sigma-Aldrich) as protease inhibitors.

When indicated, the distribution of the FAD7 protein in chloroplasts isolated from soybean leaves or from other plant species (Arabidopsis thaliana, Lotus japonicus, Phaseolus vulgaris, Medicago sativa) was also studied. Chloroplasts were isolated as described by Andreu et al. (2007). Finally, the role of thiol groups in the distribution of the different conformations of the GmFAD7 proteins in seeds was analysed using specific thiol reagents. To that end, seed extracts (15 μg protein) were treated with sample buffer in the absence or presence of 170 mM β-mercaptoethanol or 20 mM dithiothreitol (DTT) as thiol group reducing agents for 90 min at room temperature and then analysed by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For diamide treatment (i.e. thiol group oxidizing conditions), an aliquot of seed extracts was incubated in buffer A containing 15 mM diamide and kept for 15 min at room temperature. These samples were subsequently treated with sample buffer in the absence or presence of β-mercaptoethanol or DTT as described above to re-reduce oxidized thiol groups. To examine the effect of metals in the seed FAD7 protein conformation, an aliquot of the extract was incubated with buffer A (without β-mercaptoethanol) containing 100 mM metal-free EDTA for 15 min at room temperature. Alternatively, seed extracts were treated with buffer A containing 1 mM Fe²⁺ in the presence of 5 mM ascorbate for 15 min at room temperature. In this case the incubation buffer did not contain EDTA. Then, samples were denatured in sample buffer in the absence or presence of reducing agents depending on the experiments and loaded onto SDS-PAGE gels.

The electrophoretic separation of total proteins and immunoblotting analysis were performed essentially as described by Andreu et al. (2007) in gels (12% acrylamide) containing 1% SDS and 4 M urea. 15 μg of total protein were loaded per lane. For correct visualization of both GmFAD7 bands it was necessary to extend the migration of the gels, allowing the smaller molecular weight (MW) marker (19.4 kDa) to leave the gel and the second one (28.8 kDa) to reach the bottom of the gel.

Results

Identification and sequence analysis of a novel plastidial ω3 desaturase gene, GmFAD7-2, in soybean

A single soybean sequence with homology to the known AtFAD7 gene was isolated by screening of a cDNA library
from developing seeds (Yadav et al., 1993). However, when the DFCI soybean gene index was used for BLAST searches of soybean sequences with homology to the GmFAD7 gene sequence obtained in Yadav et al. (1993), it provided several homologous tentative consensus (TC) sequences (identified as TC204932, TC204936, and BE610928) with high homology to the FAD7 gene. At least two separate genes could be predicted from the overlap analysis of the TC entries, supporting the existence of several GmFAD7 genes in the soybean genome. To confirm these findings further, several 5' and 3' RACE strategies, together with specific PCRs using GSPs (see Supplementary Table SI at JXB online) were designed to obtain the full-length cDNA of this additional FAD7 gene from soybean. According to this analysis, two full-length cDNA sequences have been identified that encoded proteins with high identity to the previously annotated GmFAD7 sequence. A protein sequence alignment is shown in Fig. 1. The first full-length cDNA sequence was predicted to encode a protein of 453 amino acids with very high identity to the previously annotated GmFAD7 sequence (98%). This protein had a theoretical molecular mass of 51.2 kDa and a pI of 8.45. All these parameters were very similar to those obtained for the previously annotated GmFAD7 sequence (51.3 kDa; pI of 8.77). The second full-length cDNA was predicted to encode a protein of 453 amino acids also with high homology to the previously annotated GmFAD7 sequence (92.3%). This protein had a theoretical molecular mass of 51.2 kDa and a pI of 7.80. At this point, these two novel sequences were named as GmFAD7-1b because of its very high degree of identity to the previously annotated GmFAD7 sequence (which was designated as GmFAD7-1a) and GmFAD7-2. These new sequences were deposited in Genbank with accession nos GQ144962 and EU621390, respectively.

The differences found between the GmFAD7-2 gene sequence and that from GmFAD7-1b (32 residues, 15 of which were conservative) or GmFAD7-1a (34 residues, 13 conservative) were consistent with the hypothesis that GmFAD7-2 represented a novel GmFAD7 gene in soybean. However, the high degree of identity found between our GmFAD7-1b sequence and that isolated by Yadav et al. (1993; GmFAD7-1a) suggested that these two sequences could represent the same gene with polymorphic changes attributable to the different cultivar origin of the soybean plants used for the analysis rather than to different GmFAD7 genes. All the sequence data shown above were obtained from soybean plants of cultivar Volania.

![Fig. 1. Protein sequence alignment of GmFAD7-1b, GmFAD7-2, and GmFAD7-1a genes encoding plastidial ω3 fatty acid desaturases from soybean. The GmFAD7-1a sequence was obtained from Yadav et al. (1993) (GenBank accession number L22965). Black boxes indicate residues that are strictly identical and grey boxes indicate conservative changes between each desaturase sequence. The location of the synthetic peptide used to obtain the FAD7 antibody is shown as a black box. The three histidine boxes characteristic of the desaturase active site are underlined.](image-url)
hypothesis was further confirmed when the same RACE and RT-PCR analysis was performed on the other two cultivars from soybean, Corsoy and Safrana, available in our laboratory (see Supplementary Fig. S1 at JXB online). At this point it was decided not to make any more differences between GmFAD7-1a and GmFAD7-1b and simply to designate both sequences as GmFAD7-1. It is also worth mentioning that when a similar analysis was performed with the GmFAD7-2 sequence, only three amino acid substitutions (one of them conservative) were detected (data not shown). This result suggests that during evolution, genetic variability was focused on the GmFAD7-1 gene rather than on the GmFAD7-2 gene.

As shown in the protein sequence alignment (Fig. 1) both GmFAD7-1 and GmFAD7-2 contained a N-terminal domain with the characteristic features of a plastid targeting peptide including a high content of hydroxylated residues (Ser, Thr), a low content of acidic residues and the conserved N-terminal Met-Ala dipeptide. In fact, when targeting prediction tools like ChloroP, pSORT or TargetP were used for subcellular localization prediction of the different GmFAD7 sequences, all predicted a chloroplast transit peptide and chloroplast localization. Another expected feature of this novel desaturase gene was the presence of the three conserved histidine clusters or H-box (Fig. 1), which have been shown to be essential for desaturase activity, acting as potential ligands for non-haem iron atoms (Shanklin and Cahoon, 1998). Also, the existence of four putative membrane spanning domains was predicted through the use of the bioinformatics tool ProTHMM and was consistent with the predicted folding model proposed for membrane desaturases (Dyer et al., 2001; Tang et al., 2005).

Genomic characterization of the GmFAD7 genes

The complete sequence of the soybean genome has recently been available at www.phytozome.net. Since our GmFAD7-1 and GmFAD7-2 sequences were also available at that time, it was decided to query the soybean phytozome database to contrast and complete the information concerning both desaturase genes. In parallel, partial genomic fragments corresponding to the GmFAD7-1 and GmFAD7-2 genes were amplified by PCR with gene-specific primers. The results from the in silico analysis of the soybean phytozome database were consistent with our experimental data. The soybean phytozome database showed only two different targets localized to chromosome 7 and chromosome 18, encoding for proteins with high homology to FAD7. The sequence at chromosome 7, identified by phytozome as Glyma07g18350.1, matched perfectly with the GmFAD7-2 sequence (see Supplementary Fig. S2 at JXB online). Similarly, the sequence located at chromosome 18, identified by phytozome as Glyma18g43210.1, also matched with the GmFAD7-1 sequence (see Supplementary Fig. S2 at JXB online). It is worth mentioning that the protein sequence provided by phytozome from translation of Glyma18g43210.1 presented a 135 amino acids deletion from the C-terminus of the protein when compared with our GmFAD7-1 sequence from cv. Volania, Corsoy, or that reported by Yadav et al. (1993; L22965). However, a careful analysis of the exon 5 sequence from Glyma18g43210.1 showed an annotation error from phytozome in the assignment of the end of exon 5 and the beginning of intron V. When this was properly corrected, the three exons (6–8) could easily be identified obtaining a normal full-length GmFAD7-1 protein. These results confirmed the presence of two GmFAD7 genes located at different loci within the soybean genome.

Phylogenetic relationships of GmFAD7s

To study the phylogeny of GmFAD7 proteins, a phylogenetic tree including the GmFAD7-1 and GmFAD7-2 sequences described in this work, the two GmFAD7 sequences annotated in the soybean phytozome database as well as most of the FAD7 and FAD8 protein sequences available in public databases, was performed. The phylogenetic tree is shown in Fig. 2 and the multiple protein alignment is shown in Supplementary Fig.S2 at JXB online. With the exception of the Chlamydomonas FAD7 protein, which was clustered apart, the phylogenetic analysis revealed two distinct clusters within the plant plastidial ω3 desaturase family essentially consistent with the conventional species tree, separating monocotyledonae from dicotyledonae (Fig. 2).

Tissue-specific expression of the ω3 desaturase genes

The tissue-specific distribution of the mRNA from both GmFAD7 desaturase genes in soybean plants was examined. To that end, total RNA was isolated from leaves, stems, roots, and flowers. The transcript level was examined by semi-quantitative RT-PCR using gene-specific primers for each ω3 desaturase gene and the constitutively expressed ACTIN gene was used as an internal control. The expression profile of the ω3 desaturase genes in soybean plants is shown in Fig. 3A and the normalization of the expression results against the constitutive ACTIN control is shown in Fig. 3B. Both GmFAD7-1 and GmFAD7-2 mRNAs were detected in all the vegetative tissues analysed, being more abundant in mature leaves when compared with the rest of the tissues. The tissue expression pattern of the GmFAD7-1
and GmFAD7-2 genes was compared with that of the cold-inducible plastid ω3 desaturase GmFAD8 gene. As shown in Fig. 3A and B, the GmFAD8 mRNA seemed to be constitutively expressed in all vegetative tissues examined at optimal growth temperatures. At this point, it was found interesting to compare the expression of the plastidial ω3 desaturase genes with that from the reticular ω3 desaturase GmFAD3 gene in the same tissues and conditions. Three independent microsomal ω3 fatty acid desaturase genes (GmFAD3A, GmFAD3B, and GmFAD3C) have been identified in soybean plants (Bilyeu et al., 2003). Our analysis was focused on the GmFAD3A and GmFAD3B genes since the GmFAD3C gene seemed to be expressed at significant levels only in flower buds (Bilyeu et al., 2003). In our analysis, the gene-specific primers used for amplification of GmFAD3 recognized and amplified both GmFAD3A and GmFAD3B genes. Thus, a strategy was designed to distinguish them by analysis with the Van91I restriction enzyme (see Materials and methods). The mRNA originated from both GmFAD3A and GmFAD3B genes was present in roots and flowers and to a lesser extent in stems (Fig. 3A, B). By contrast, very small amounts of GmFAD3 mRNA were detected in mature leaves (Fig. 3A, B), indicating that the reticular ω3 desaturase GmFAD3 genes may have little, if any, role in lipid desaturation in this tissue. When the restriction analysis with Van91I was made, it was observed that both GmFAD3A and GmFAD3B mRNAs were present in roots.
at very similar amounts while in stems or flowers the GmFAD3B mRNA was the major species detected (Fig. 3A, right panel).

It was also investigated whether the expression of the GmFAD7-1 and GmFAD7-2 genes was developmentally regulated in seeds. To that end, total RNA was isolated from developing seeds of <4, 4, 5–6, 7–9, and 9–11 mm size. Expression analysis was performed as described above. The results are shown in Fig. 4A and B. Both GmFAD7-1 and GmFAD7-2 transcripts were present in developing seeds (Fig. 4A, B) and their mRNA levels were not significantly modified as a result of seed development. Similar results were obtained when the expression of the GmFAD8 gene was analysed (Fig. 4).

In developing seeds, a small increase of the total GmFAD3 mRNA was observed, specially between the smallest size (<4 mm) when compared with the biggest ones (9–11 mm; Fig. 4A). When the restriction analysis of the GmFAD3 PCR product was carried out in developing seeds to distinguish between the GmFAD3A and GmFAD3B isoforms, it was observed that both GmFAD3A and GmFAD3B mRNA increased, this increase being much more important in the case of GmFAD3A mRNA (Fig. 4A, right panel). This result suggests that GmFAD3A is more abundant than GmFAD3B in mature seeds.

**Fatty acid composition of the different soybean plant tissues**

The relationship between the accumulation of the transcripts of the GmFAD7-1 and GmFAD7-2 genes with the distribution of trienoic fatty acids among the different soybean tissues was investigated. To that end, the fatty acid composition of the different plant tissues used in the expression analysis (roots, stems, mature leaves, flowers, and 9–11 mm seeds) were analysed. The fatty acid composition of soybean seeds or leaves has been analysed in the past by many other groups. However, other tissues like stems or roots have not been analysed in detail. The results are shown in Fig. 5. The highest amount of trienoic fatty acids (i.e. linolenic acid, 18:3) was observed in leaves. In fact, linolenic acid (18:3) represented more than 60% of total fatty acids, being the major fatty acid species detected. These data were consistent with those obtained in other 18:3 plants and similar to the 18:3 levels found in photosynthetic cell cultures from soybean (Collados et al., 2006). In roots,
The 18:3 levels reached 30 mole per cent (Fig. 5), which means a reduction of 50% with respect to the TA levels detected in leaves. This reduction was accompanied by an increase from 15% to 30% of dienoic fatty acids (i.e. linoleic acid, 18:2) with respect to leaves (Fig. 5). Similarly, linoleic acid (18:2) was the most abundant fatty acid in seeds (38 mole per cent) while linolenic acid (18:3) represented a 20% of total fatty acids (Fig. 5). In fact, 18:2 levels in seeds were the highest with respect to all the other tissues (see also Heppard et al., 1996).

Fig. 4. (A) Expression of ω3 fatty acid desaturase genes in developing seeds. Total RNA was extracted from developing seeds of <4, 4, 5-6, 7-9, or 9-11 mm size. RT-PCR reactions were performed with gene-specific primers. All PCRs were done under non-saturating conditions. ACTIN was used as the housekeeping gene in all experiments. Right panels show the digestion of GmFAD3 PCR product with the Van91I restriction enzyme. The 755 bp fragment corresponded to the GmFAD3A gene, the 594 bp fragment to the GmFAD3B gene, and the 164 bp fragment was common to both genes. Marker sizes (M) are shown in base pairs. (B) Normalization of gene expression results against the housekeeping gene (actin).
Analysis of the GmFAD7 protein levels

Previous work from our laboratory (Collados et al., 2006; Andreu et al., 2007) described the obtention and properties of a specific antibody raised against the GmFAD7 protein. It was reported that the GmFAD7 antiserum reacted with a single polypeptide band of approximately 39 kDa (Collados et al., 2006; Andreu et al., 2007). However, when longer electrophoretic protein separation was performed (see the Materials and methods for details), the presence of two bands in the range of 39–42 kDa that reacted with the antiserum (Fig. 6A, lane 1) were reproducibly observed in chloroplasts from mature soybean leaves. Note that the epitope target sequence designed from the GmFAD7-1 sequence was also highly conserved in the GmFAD7-2 sequence (13 out of 17 residues were identical and 2 were conservative substitutions between both sequences; Fig. 1), indicating that the GmFAD7-2 protein was also recognizable by our antibody. It is also worth mentioning that the apparent molecular mass (39–42 kDa) of the GmFAD7 proteins was different from their theoretical mass as deduced from their amino acid sequence. These differences between the apparent and theoretical molecular mass are typical of membrane proteins like FAD7 and are related to alterations in detergent binding in SDS-PAGE (Rath et al., 2009). At this point a western blot analysis was performed in different plant species that included some legumes like Lotus japonicus, Medicago sativa, Phaseolus vulgaris (common bean), Vigna unguiculata (cowpea) or less related plant species like Arabidopsis thaliana. With the exception of P. vulgaris, for which no genetic information was available, searches at the DFCI database or published data (Yadav et al., 1993; Torres-Franklin et al., 2009) revealed that the plastid ω3 desaturase FAD7 was encoded by a single gene in the rest of the plant species selected. As shown in Fig. 6A (lanes 2–6), the GmFAD7 antibody showed a reaction in chloroplasts isolated from all plant species analysed, with the exception of A. thaliana (lane 3). This is not surprising since the epitope target sequence is not conserved in the AtFAD7 gene. With the exception of P. vulgaris, in which a doublet band similar to soybean (same subfamily Phaseoleae) was detected, the rest of the plant species analysed (Medicago, Lotus or cowpea) showed a single reacting band (Fig. 6A).

The distribution pattern of the GmFAD7 protein was analysed in the different soybean plant tissues. To our knowledge, such protein distribution has not been determined for a plant membrane-bound desaturase, reticular or plastidial, in any plant species. To that end, extracts from roots, stems, mature leaves, and developing seeds were analysed. The results are shown in Fig. 6B. In crude extracts...
from leaves, the characteristic doublet bands at 39–42 kDa were obtained (lane 3). In isolated chloroplasts, the signal from both bands was much more intense (lane 4) which is consistent with the chloroplastic localization of the FAD7 protein (Andreu et al., 2007). In roots, the GmFAD7 antiserum reacted with two bands of 47 kDa and 60 kDa, respectively, which varied in intensity depending on the preparations (lane 1). Other bands of higher MW (>90 kDa) could also be observed upon higher exposure of the blots. Surprisingly, no GmFAD7 protein was detected in stems (lane 2) despite the presence of GmFAD7 mRNA, suggesting that, in this tissue, the regulation of GmFAD7 seems to be at the post-transcriptional level.

Finally, the GmFAD7 protein distribution was studied in developing seeds. As shown in Fig. 6C, a band of approximately 80 kDa accumulated during seed development. A low intensity 39 kDa band that fits well with the lower band of the leaf doublet was also detected in the later stages of seed development (Fig. 6C, lane 3). This band profile was obtained in the presence of β-MeOH in the sample buffer. The effect of in vitro changes of the redox conditions from thiol groups on the distribution of the GmFAD7 proteins from 9–11 mm seed extracts was analysed further. The results are shown in Fig. 7. Seed extracts resuspended in sample buffer containing thiol reducing agents like β-MeOH or DTT showed a similar band pattern to that described above, i.e. detection of the 80 kDa band as well as the lower 39 kDa band (Fig. 7A, lanes 1 and 2). Furthermore, when 9–11 mm seed extracts were incubated in sample buffer without thiol reducing agents, the 80 kDa and 39 kDa bands were detected again, but a strong band of approximately 42 kDa was also detected (Fig. 7A, lane 3). This 42 kDa band is coincident with the upper band of the leaf doublet (Fig. 6A, B). Similar results were observed in the presence of diamide, a thiol-oxidizing reagent (Fig. 7A, lane 4). In both cases, the appearance of the 42 kDa GmFAD7 protein band seemed to be accompanied by a decrease in the 80 kDa protein complex. Furthermore, when seed extracts pretreated with diamide were subsequently treated with thiol reducing agents like β-MeOH or DTT, the 42 kDa protein band disappeared again and the major GmFAD7 protein conformation detected corresponded to the 80 kDa protein band (Fig. 7A, lanes 5 and 6), further confirming that the redox conditions from thiol groups affected the distribution and conformation of the GmFAD7 proteins in seed extracts. This effect seemed to be seed-specific since the presence or absence of β-MeOH or DTT in isolated chloroplasts did not have any effect on the protein distribution profile (data not shown). Note that the 39 kDa lower GmFAD7 protein band was detected with very low intensity but in similar amounts under all experimental conditions. These results suggested that the detection of this band was not sensitive to thiol redox conditions. Treatment of seed extracts with denaturing or chaotropic agents or high ionic strength in the incubation buffer resulted in no modification of the protein band pattern described above (data not shown), suggesting that the interactions (probably hydrophobic) that maintained GmFAD7 protein complexes in soybean seed extracts were very strong.

Iron is a cofactor necessary for the desaturase reaction (Somerville et al., 2000). Metalloproteins are dependent on their cofactors for their correct folding and functioning. Therefore, the effect of iron on the distribution of the GmFAD7 protein conformations in seed extracts was examined. The results are shown in Fig. 7B. The effect of metals was first analysed in the absence of reductants like β-MeOH, when all GmFAD7 conformations are present. Under such conditions, sequestration of metals by 100 mM EDTA reduced considerably the amount of the 42 kDa GmFAD7 protein fraction compared with control samples (Fig. 7B, lanes 1 and 2). Again little or no effects were observed in the amount of the 39 kDa GmFAD7 protein band. In the presence of an excess of iron (Fe²⁺), the relative distribution of GmFAD7 protein conformations was similar to that from control extracts (Fig. 7B, lane 3). The same experiments were performed in the presence of β-MeOH. As expected, control seeds showed the 80 kDa and the low intensity 39 kDa GmFAD7 protein bands (Fig. 7B, lane 5). Addition of EDTA did not change this band distribution profile (Fig. 7B, lane 6). However, when seed extracts were incubated with excess of iron (Fe²⁺), even in the presence of thiol reductants, the 42 kDa GmFAD7 protein band increased dramatically (Fig. 7B, lane 7).
Similar results were obtained when Fe$^{3+}$ instead of Fe$^{2+}$ was supplied to seed extracts (data not shown). Our results indicated that iron promoted the 42 kDa GmFAD7 protein band accumulation.

**Stress-induced response of GmFAD7-1 and GmFAD7-2 genes**

In an attempt to identify specific roles for each GmFAD7 genes, we studied whether any of both GmFAD7 isoforms was specifically affected under some stress situations known to stimulate o3 desaturase activity, like cold or wounding (Iba, 2002; Nishiuchi et al., 1997). No changes in the expression level were observed when soybean plants were subjected to cold treatment (data not shown). The expression of both GmFAD7 genes upon wounding was also analysed. To that end, mature leaves from soybean plants were wounded and leaf tissue was taken after 30 min or 4 h of wound treatment. As shown in Fig. 8A left panel and B, GmFAD7-1 mRNA levels rapidly accumulated after wounding with a 2.5–3-fold increase at 30 min after treatment. These mRNA levels remained high (more than 2-fold) 4 h after wounding (Fig. 8A, left panel). The GmFAD7-2 mRNA levels showed a small increase upon wounding when compared with GmFAD7-1 (Fig. 8A, left panel, B).

Wounding activates the transcription of a wide number of genes than JA by stimulating both JA-dependent and independent wound signalling mechanisms (Devoto et al., 2005). It was determined whether this wound-responsive behaviour of both GmFAD7 genes was JA-dependent and which was the expression profile of both genes in response to JA treatment. As shown in Fig. 8A, right panel, when mature leaves from soybean plants were treated with MeJA no noticeable changes in the mRNA levels of GmFAD7-1 or GmFAD7-2 genes were observed, suggesting that the wound-response observed of both GmFAD7 genes was JA-independent.

**Discussion**

In this paper, the identification of a novel GmFAD7 sequence (GmFAD7-2) from soybean, different from the GmFAD7-1 gene previously reported (Yadav et al., 1993) is described. Both genomic and in silico analyses of the soybean genome database confirmed that both GmFAD7 genes were located at two different loci, suggesting that the soybean o3 plastidal desaturase FAD7 is encoded by two different paralogous genes. Genes encoding for microsomal desaturases (FAD2 and FAD3) are well known to be present in several copies in the genomes of many plant species, giving rise to distinct protein isoforms (Heppard et al., 1996; Pirtle et al., 2001; Bilyeu et al., 2003; Mikkilineni and Rocheford, 2003; Hernández et al., 2005). However, to our knowledge this is the first report in which the existence of different paralogous genes encoding for plastidial desaturases is described. The presence of these two GmFAD7 paralogous genes is not striking since soybean is a tetraploid for which there is molecular evidence of genome duplications that occurred at approximately 59 and 13 million years ago (Shoemaker et al., 1986; Schmutz et al., 2010). Recent analysis of the extent to which duplicated genes have been retained in the soybean genome revealed that of the 46,430 high-confidence genes analysed, 31,264 exist as paralogues and 15,166 have reverted to singletons (Schmutz et al., 2010). In fact, the number of genes involved in plastid fatty acid synthesis was 63% higher in soybean compared to Arabidopsis.

In many cases, duplicate genes are conserved during evolution because they provide a new or advantageous specialized function. With the exception of the data obtained upon wounding, showing that the GmFAD7-1 mRNA increased to a higher extent than GmFAD7-2, no great differences were observed at the expression level between both GmFAD7 genes. Our data showed that both GmFAD7 paralogous genes were expressed in all soybean tissues analysed, indicating the absence of tissue-specific expression patterns. It is worth mentioning that, in contrast to our results in soybean, in other plant species like Arabidopsis (Yadav et al., 1993; Nishiuchi et al., 1997), tobacco (Hamada et al., 1996) or wheat (Horiguchi et al., 1998) no FAD7 mRNA was detected in roots. However, tissue-specific differences emerged from the analysis when the expression of both GmFAD7 genes was compared to the reticular o3 desaturase GmFAD3 genes. Thus, in leaves,
both GmFAD7 genes were expressed at very high levels while the GmFAD3A and GmFAD3B mRNAs were present at very low amounts. This result is consistent with the expected role of FAD7 in the plastids and with previous observations in wheat (Horiguchi et al., 1998). By contrast, in Arabidopsis AtFAD3 mRNA levels in leaves were similar to AtFAD7 (Yadav et al., 1993). These results suggest that, in soybean, there is a control mechanism that seems to operate only in mature leaves up-regulating the expression of the plastid ω3 desaturase FAD7 and down-regulating the reticular ω3 desaturase FAD3. This leaf-specific control mechanism could be part of a wider mechanism controlling the fluxes between the endoplasmic reticulum and the chloroplast for co-ordinated trienoic fatty acid synthesis in 18:3 plants, like soybean.

The recent development of a GmFAD7 antibody in our laboratory has provided us with an invaluable tool to study the distribution of the different GmFAD7 isoforms in soybean plant tissues and to correlate their accumulation with the production of TAs. With the exception of stems, in which no protein was detected, in the rest of the tissues analysed several bands reactive with the GmFAD7 antibody were observed. Overall, the results obtained in leaves, roots or developing seeds at the protein level suggest the existence of tissue-specific post-translational regulatory mechanisms affecting the distribution and conformation of the different GmFAD7 family members that could be correlated with the FAD7 ω3 desaturase activity in each specific tissue. Thus, the higher amounts of 18:3 are found in leaves, where both GmFAD7-1 and GmFAD7-2 genes are highly expressed. This fact as well as the absence of GmFAD3 mRNA suggests that 18:3 accumulation in leaves corresponded to the highest level of FAD7 activity. Under such conditions, the characteristic doublet band of the GmFAD7 protein in leaves was detected. With the results presented in this paper, the presence cannot be precluded of some kind of post-translational modification affecting any or both GmFAD7 isoforms differently, resulting in changes in their mobility and, thereby, originating the double bands in leaves. On the other hand, the single FAD7 protein band obtained in legume species like cowpea or Medicago, where only one FAD7 gene is present in their genomes, suggests that the doublet bands observed in soybean could be related to the presence of the additional GmFAD7 gene. Moreover, the differences in size of the chloroplast transit peptide as predicted by ChloroP (longer in the case of GmFAD7-1) might well account for the differences in size observed in our western blot analysis.

Roots and developing seeds are characterized by a strong reduction of TA levels when compared with mature leaf tissue (Fig. 6). Microsomal enzymes have been shown to be the major contributors to seed or root linolenic acid levels (Lemieux et al., 1990; Yadav et al., 1993; Heppard et al., 1996). This necessarily implies that FAD7 activity must be tightly down-regulated in non-photosynthetic tissues. In roots, our data suggest the presence of some kind of post-translational modification or interaction with another protein of small size as part of the mechanism controlling FAD7 activity in this tissue. In soybean developing seeds, our protein data point to the formation of GmFAD7 protein complexes. Our data cannot preclude the possibility of an interaction between GmFAD7 with another protein of similar size. On the other hand, the 80 kDa band could be compatible with a GmFAD7 dimer. Whether this dimer is a homodimer of any of the seed GmFAD7 isoymes or represents a heterodimer from both GmFAD7 products remains to be elucidated. Since in this tissue FAD7 activity is strongly reduced, it can be suggested that the formation of these GmFAD7 protein complexes is at the basis of the post-translational down-regulatory mechanism controlling FAD7 ω3 desaturase activity in soybean developing seeds.

At least two factors, iron availability and redox conditions affecting thiol groups seemed to alter the distribution of the different conformations of the FAD7 enzymes in vitro. The involvement of iron is not striking since this metal is a cofactor of the enzyme active site (Somerville et al., 2000). Metaloproteins are dependent on their cofactors for their correct folding and functioning (Wilson et al., 2004). Our data with thiol-specific reagents suggest that thiol groups from cysteine residues are also involved in the control of the GmFAD7 protein conformation in seeds. More precisely, thiol-oxydizing conditions favoured the increase in the putative monomer fraction, pointing towards the existence of a disulphide bridge, probably intramolecular, involved in monomer formation. In that sense, two Cys residues (Cys118 and Cys174) are conserved among all plastid ω3 desaturases. Cys174 is in fact located within one of the His boxes that are essential for desaturase activity. In the absence of structural data for membrane-bound desaturases, it is tempting to speculate that Cys174 together with other conserved Cys residues of the protein (like Cys118) may participate in an intramolecular disulphide bridge that might contribute to the proper folding and/or stabilization of the di-iron active centre of the desaturase.

Questions arise about the biological significance of the GmFAD7 protein conformations found in seeds. During seed development and maturation, lipid biosynthetic pathways are shifted towards storage lipid biosynthesis and tryacylglycerol (TAG) production. TAG lipids are enriched in linoleic (18:2) fatty acids. Under such conditions, ω3 desaturase activity must be tightly regulated. These seed-specific GmFAD7 protein complexes may serve as a reserve pool to initiate FAD7 activity upon germination and plant differentiation, when leaf tissue is forming and membrane lipids are needed.

In conclusion, our data indicate that the synthesis of TAs in soybean by plastidial ω3 desaturases is under the control of rather complex mechanisms in which two FAD7 ω3 desaturase genes are present and tissue-specific post-translational regulatory mechanisms control the enzyme activity, distribution, and conformation. The tissue-specific nature of these control mechanisms most probably reflects specific tissue requirements for concerted synthesis of 18:3 fatty acids.
Supplementary data

Supplementary data are available at JXB online.

Supplementary Table S1. List of the gene specific primers used in this study.

Supplementary Fig. S1. Protein sequence alignment of the GmFAD7-1 gene from different soybean cultivars.

Supplementary Fig. S2. Multiple protein sequence alignment of the plastid FAD7 and FAD8 ω3 desaturases from different plant species.

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