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RESEARCH PAPER

Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean

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Abstract

This study analysed the contribution of each omega-3 desaturase to the cold response in soybean. Exposure to cold temperatures (5 °C) did not result in great modifications of the linolenic acid content in leaf membrane lipids. However, an increase in the *GmFAD3A* transcripts was observed both in plant leaves and soybean cells whereas no changes in *GmFAD3B* or *GmFAD3C* expression levels were detected. This increase was reversible and accompanied by the accumulation of an mRNA encoding a truncated form of *GmFAD3A* (*GmFAD3A-T*), which originated from alternative splicing of *GmFAD3A* in response to cold. When the expression of plastidial omega-3 desaturases was analysed, a transient accumulation of *GmFAD7-2* mRNA was detected upon cold exposure in mature soybean trifoliate leaves while *GmFAD7-1* transcripts remained unchanged. No modification of the *GmFAD8-1* and *GmFAD8-2* transcripts was observed. The functionality of *Gm*FAD3A, *Gm*FAD3B, *Gm*FAD3C and *Gm*FAD3A-T was examined by heterologous expression in yeast. No activity was detected with *Gm*FAD3A-T, consistent with the absence of one of the His boxes necessary for desaturase activity. The linolenic acid content of *Sacharomyces cerevisiae* cells overexpressing *Gm*FAD3A or *Gm*FAD3B was higher when the cultures were incubated at cooler temperatures, suggesting that reticular desaturases of the *Gm*FAD3 family, and more specifically *Gm*FAD3A, may play a role in the cold response, even in leaves. The data point to a regulatory mechanism of omega-3 fatty acid desaturases in soybean affecting specific isoforms in both the plastid and the endoplasmic reticulum to maintain appropriate levels of linolenic acid under low temperature conditions.

Key words: desaturase, FAD3, FAD7, FAD8, gene expression, cold, soybean.

Introduction

Temperature is one of the major environmental factors influencing the distribution of plant species, the range of temperatures experienced by plants being extremely variable both at the spatial and temporal scales (Iba, 2002). The adaptability of plants to their temperature environment will depend directly on their capacity for developing mechanisms of temperature adaptation. These mechanisms are rather complex and include the action of temperature stress factors as well as metabolic changes (Tomashow, 1999; Iba, 2002). Membranes are major targets of the temperature acclimation strategies. Biological membranes are organized structures of lipids and embedded proteins that surround cells and organelles, in which essential processes such as photosynthesis, respiration,

or solute transport take place. Thus, membrane lipids provide a dynamic and fluid environment essential for living organisms. Not surprisingly, a strong association has been observed between environmental temperature and the lipid and fatty acid content of plant membranes (Rennie and Tanner, 1989; Nishida and Murata, 1996; Iba, 2002). Certain specific lipids have been directly involved in chilling sensitivity in plants. Thus, a relationship between the levels of palmitic (16:0) and trans-hexadecanoic (t16:1) acids in phosphatidylglycerol (PG) and chilling sensitivity in plants was established (Murata *et al.*, 1982). Furthermore, experiments overexpressing in tobacco the plastidial glycerol-3-phosphate acyl transferase gene (*GPAT*) from a chilling-sensitive (squash) or resistant (*Arabidopsis*) plant indicated that increasing levels of saturated PG were correlated to greater sensitivity to cold temperatures (Murata *et al.*, 1992). Apart from the role of specific lipids, cooler temperatures are often associated with an increase in the production of polyunsaturated fatty acids (PUFAs), mainly β-linolenic acid (18:3) (McConn *et al.*, 1994; Heppard *et al.*, 1996; Horiguchi *et al.*, 2000; Martz *et al.*, 2006; Li *et al.*, 2007; Kargiotidou *et al.*, 2008). These PUFAs are thought to maintain membrane fluidity because of their lower melting temperatures (Nishida and Murata, 1996; Iba, 2002; Gushina and Harwood, 2006). The highest increase has been reported in a non-photosynthetic tissue, wheat root tips, which showed a 25% increase in 18:3 levels when exposed to 10 °C (Horiguchi *et al.*, 2000). However, with the exception of *Arabidopsis*, where significant changes in the 10–15% range have been reported (McConn *et al.*, 1994; Falcone *et al.*, 2004), the extent of the increase in 18:3 levels in leaves has seemed to be rather small in other plant species (Martz *et al.*, 2006; Li *et al.*, 2007; Upchurch and Ramirez, 2011).

Desaturation of fatty acids is performed by a class of enzymes called fatty acid desaturases (FADs). These enzymes are encoded by nuclear genes and differ in their substrate specificity and subcellular localization. The genes encoding plant fatty acid desaturases have been cloned and sequenced from a great variety of plant species. In *Arabidopsis*, three genes encode the omega-3 desaturases responsible for the synthesis of trienoic fatty acids (TAs): one for the endoplasmic reticulum omega-3 desaturase *At*FAD3 and two for the plastidial enzymes *At*FAD7 and *At*FAD8 (Iba *et al.*, 1993; Yadav *et al.*, 1993; Gibson *et al.*, 1994). *AtFAD8* is believed to encode a cold-specific plastidial omega-3 desaturase since its activity has been observed in a *fad3/fad7* double mutant when exposed to low temperatures (Gibson *et al.*, 1994). The way that temperature regulates the expression of the genes encoding omega-3 fatty acid desaturases has been the subject of considerable research during recent years. Thus, in maize leaves, a decrease in *ZmFAD7* mRNA accompanied by an increase in the *ZmFAD8* mRNA was reported in response to low temperatures (Berberich *et al.*, 1998), suggesting direct transcriptional control. More recently, it was demonstrated that the *At*FAD8 protein was destabilized at high temperatures, without changes in mRNA levels, suggesting a post-translational control mechanism regulating *At*FAD8 activity in *Arabidopsis* in which the C-terminus of the mature protein would be involved (Matsuda *et al.*, 2005). Similarly, analysis of wheat root tips subjected to low temperature conditions showed increased enzyme accumulation with higher linolenic acid production without changes in *TaFAD3* mRNA levels (Horiguchi *et al.*, 2000). Changes in the protein half-life could also be involved in control of the activity of the soybean seed-specific *Gm*FAD2–2 isoform (Tang *et al.*, 2005) or rape *Bn*FAD3 enzymes (O'Quin *et al.*, 2010) in response to temperature. Unfortunately, many of the studies that have analysed the regulation of plastidial desaturases in response to cold did not study the regulation of endoplasmic reticulum desaturases under the same experimental conditions (Berberich *et al.*, 1998; Matsuda *et al.*, 2005). Similarly, the regulation of endoplasmic reticulum desaturases and their response to cold has been studied in non-photosynthetic tissues such as roots, but not in leaves (Horiguchi *et al.*, 2000; Dyer *et al.*, 2001; Tang *et al.*, 2005; O'Quin *et al.*, 2010). This has resulted in a lack of information about one of the two organelles where 18:3 is synthesized in plants in a concerted manner. As a result, research is far from having an integrated view of how omega-3 desaturase enzymes respond to temperature changes and in which tissue and through which mechanism this role is actually executed.

Increasing evidence indicates that in soybean, the genes encoding omega-3 fatty acid desaturases are grouped in multigene families. Thus, at least three *GmFAD3* genes, designated as *GmFAD3A*, *GmFAD3B*, and *GmFAD3C* seem to contribute to 18:3 synthesis in the endoplasmic reticulum membranes of soybean (Bilyeu *et al.*, 2003; Anai *et al.*, 2005). More recently, the presence of two soybean *GmFAD7* genes were reported, designated as *GmFAD7-1* and *GmFAD7-2,* which would participate in 18:3 production in plastid membranes (Andreu *et al.*, 2010). Finally, two sequences with homology to known *FAD8* genes were detected in the soybean genome (Chi *et al.*, 2011), but their regulation has not been studied yet.

This work studied the effect of low temperatures on the regulation of the expression of omega-3 fatty acid desaturases from soybean in order to analyse the concerted regulation of each gene family in response to temperature. The experiments were performed on mature soybean trifoliate leaves as well as on soybean photosynthetic cell suspension cultures to extend the analysis to a non-tissue differentiating system. The data suggest the existence of regulatory mechanisms of omega-3 fatty acid desaturases affecting specific isoforms in both the plastid and the endoplasmic reticulum to maintain appropriate levels of 18:3 fatty acids under low temperature conditions.

Materials and methods

Plant materials and experimental treatments

Soybean plants (*Glycine max* cv. Volania) were grown hydroponically as described in Andreu *et al.* (2010), in a bioclimatic chamber under a 16/8 light/darkness photoperiod at 24 °C and a relative humidity of 65%. For cold treatment, mature soybean plants were placed at 5 °C under the same photoperiod and humidity conditions. The plants were kept under these conditions for 3 days and trifoliate leaves (>19 days old) were collected at 24, 48, and 72 h of cold exposure. The plants were then placed at normal growth temperature again and samples were collected after 4 days of recovery. Photosynthetic cell suspensions were cultured as described in Collados *et al.* (2006) and the experiments were performed in the same way as those of soybean plants. When indicated, soybean tissues or cells were collected, frozen in liquid nitrogen, and stored at –80 °C until use.

RNA isolation and cDNA synthesis

Total RNA was isolated from 0.5 g of the different soybean tissues using the Trizol Reagent (Invitrogen) and further purified using the RNeasy

Plant Mini Kit (Qiagen), following the manufacturer's instructions. After DNAse I (Roche) treatment to remove contaminating DNA, cDNAs were synthesized from total RNA (4 μg) using M-MLV reverse transcriptase (Promega) and oligo dT primer, according to the manufacturer's instructions.

Expression analysis of omega-3 fatty acid desaturase genes

The expression patterns of the desaturase genes were examined by semi-quantitative reverse-transcription (RT)-PCR assay. The oligonucleotides used as well as the PCR conditions are shown in Supplementary Table S1 (available in *JXB* online). *ACTIN* was used as a housekeeping gene. The amplification reaction was carried out using Platinum *Taq* DNA polymerase (Invitrogen) according to the manufacturer's instructions. The amplified products were resolved by electrophoresis on 1% (w/v) agarose gels. As the primers for amplification of *GmFAD3* genes recognized and amplified both *GmFAD3A* and *GmFAD3B*, a restriction analysis of the amplified fragments was performed using the *Van*91I enzyme (GE Healthcare), which allowed these different *GmFAD3* to be distinguished. The *Van*91I enzyme generates two fragments of 164 and 755 bp from *GmFAD3A* and three fragments of 161, 164, and 594 bp from *GmFAD3B*. The digestion products were resolved by electrophoresis on 1% (w/v) agarose gel. *GmSCOF-1*, encoding a transcriptional factor of the C_2H_2-type zinc finger family that is specifically activated by low temperatures (Kim *et al.*, 2001), was used as an internal control of the cold response under the experimental conditions. Semi-quantification of the relative expression levels was performed through normalization against *ACTIN* from two independent biological experiments. 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). The expression value of the control treatment was given the relative value of 1. The rest of the expression values were compared to the control.

Functional expression of GmFAD3 *genes in yeast*

For the construction of the yeast expression vectors, the corresponding open reading frames of the soybean *GmFAD3A* (AY204710), *GmFAD3B* (AY204711), *GmFAD3C* (AB051215), and *GmFAD3A-T* (the truncated form of *GmFAD3A*) were amplified by PCR using *Pfu* DNA polymerase (Stratagene) and the following primers: 5'-GAGGATCCGCAATGGTTAAAGACACAAAGCCT-3' and 5'-GAACTCGAGACTCAGTCTCGGTGCGAGTG-3' for *GmFAD3A*, *GmFAD3B*, and the truncated form of *GmFAD3A* as well. Clones containing either *GmFAD3A* or *GmFAD3B* were differentiated by restriction enzyme digestion and further sequencing. For amplification of *GmFAD3C*, 5'-GAGGATCCAAATGGTTCAAGCACAG-3' and 5'-GAACTCGAGTTTAGTTGGACTGGGTCC-3' primers were used. All these primers were extended by a *Bam*HI (in the forward primer) and an *Xho*I (in the reverse primer) restriction site (underlined) for directional ligation behind the inducible *GAL1* gene promoter of the yeast expression vector pYES2 (Invitrogen). The resulting PCR product for each specific *Gm*FAD3 isoform was cloned in a pGEM-T-Easy vector, double-digested with *Bam*HI and *Xho*I, and ligated into the digested destination vector. All constructs were checked by sequencing. *Saccharomyces cerevisiae* UTL-7A cells were transformed with plasmids pYES2 (negative control), pYES2-*Gm*FAD3A, pYES2-*Gm*FAD3B, pYES2-*Gm*FAD3C, and pYES2-*Gm*FAD3A-T by the lithium acetate protocol (Gietz and Woods, 1994) and selected on minimal agar plates lacking uracil (Ausubel *et al.*, 1995). Strains containing the plasmids of interest were inoculated into complete minimal drop-out uracil (CM-Ura) liquid medium supplemented with 2% (w/v) raffinose as the exclusive carbon source and cultivated at 30 °C. When the cultures reached an OD_{600nm} of 1 absorption unit (exponential phase), they were back-diluted to 0.4 absorption units with fresh medium, and gene expression was induced by adding 2% (w/v) galactose. At the same time, cultures were supplemented with 0.5 mM linoleic acid (18:2) and 0.1% (w/v) tergitol (type NP-40) and then grown at 10–35 °C until late log-stationary phase. Yeast cells were harvested by centrifugation at 1500 *g* for 5 min at 4 °C and washed with distilled water. A similar strategy was used to obtain the corresponding constructs in the vector pVT102U (Vernet *et al.*, 1987), which carries the constitutive *ADH1* promoter. Strains containing the same constructs in the pVT102U vector were cultivated in a CM-Ura liquid medium supplemented with 2% (w/v) glucose.

Lipid extraction and fatty acid analysis

Total lipids were extracted from mature soybean leaves or photosynthetic cell suspensions (0.5 g) with chloroform/methanol (2:1, v/v) as previously described (Bligh and Dyer, 1959). The 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 a SE2330 column (30 m length, 0.25 mm inner diameter, 0.2 μm film thickness), and flame ionization detector (FID).

Total lipid content and fatty acid composition of whole yeast cells were determined using the one-step method of Garcés and Mancha (1993). Methyl esters were analysed by gas-liquid chromatography (GC), using an HP-7890 (Hewlett-Packard, Palo Alto, CA, USA) fitted with a capillary column (30 m length; 0.32 mm inner diameter; 0.2 μm film thickness) of fused silica (Supelco, Bellafonte, PA, USA) and an FID detector. Hydrogen was used as a carrier gas with a linear rate of 1.34 ml min^{-1} and a split ratio of 1/50. The injector and detector temperature was 220 °C, and the oven temperature was 170 °C.

Statistics and data analysis

The results were the mean of three independent experiments, with duplicate determinations of fatty acid composition in each soybean leaf, cell culture, or yeast experiment. Analysis of variance (ANOVA) was applied to compare treatments, and differences between means were tested with Duncan's multiple range test. Statistical analyses were carried out with the program Statgraphics Plus for Windows 2.1, using a level of significance of 0.05.

Results

Effect of cold temperature exposure on the fatty acid composition and omega-3 fatty acid desaturase gene expression in soybean leaves

The fatty acid composition of total lipids extracted from mature leaves of soybean plants grown at control temperature (24 °C), subjected to cold (5 °C) exposure for 24, 48, and 72 h, and then placed under control temperature conditions again for 4 days is shown in Fig. 1. In mature trifoliate leaves from control plants, the major fatty acid species detected in total lipids corresponded to 18:3, which represented around 60% of total fatty acids. These high 18:3 levels are consistent with those reported previously in soybean plants (Andreu *et al.*, 2010). Upon exposure to 5 °C, slight increases in the mean values for 16:0, 18:1, and 18:2 were detected after 24 and 48 h of cold exposure. These increases preceded that for the mean values of 18:3, around 5–8%, which was observed only after 72 h of cold exposure. Once the plants were returned to control temperatures (24 °C), levels of 18:3 as well as the rest of the fatty acids returned to normal values after 4 days of recovery. ANOVA analysis of the results indicated that significant changes were obtained when the 18:3 levels detected after 72 h of cold exposure were compared with those from 24 or 48 h of cold treatment. However, even though the data suggested the existence of a trend in relation to cold exposure, no significant differences between the 18:3 levels after 72 h of cold exposure and

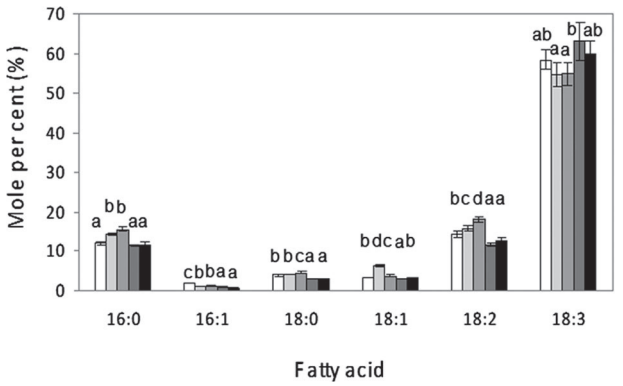

Fig. 1. Effect of exposure to cold temperatures on the fatty acid composition of total lipids from soybean leaves. Total lipids were extracted from plant leaves grown at control temperature (24 °C); after plant exposure for 24, 48, and 72 h to 5 °C; and after recovery for 4 days at control temperature. Data are expressed as molar percentages obtained from the quantitative analysis of peak area chromatogram. White bars indicate fatty acids from control leaves; light-grey bars from 24 h; medium-grey bars from 48 h; dark-grey bars from 72 h of cold treatment; and black bars following 4 days at control temperatures after cold exposure. Data are mean ± SD from three experiments. For the same fatty acid, different letters indicate significant differences among treatments ($P < 0.05$).

those obtained from control plants or from plants that were again placed under normal control temperatures for 96 h were found.

The expression of genes encoding all omega-3 fatty acid desaturases was analysed by semi-quantitative RT-PCR. First, the expression of a cold-sensitive gene was checked as an internal control of the low temperature response under the experimental conditions. To this end, the expression of GmSCOF-1, which encodes a transcription factor of the C_2H_2 family of zinc finger proteins that is strongly activated in soybean upon low temperature exposure (Kim et al., 2001) was monitored. As shown in Fig. 2A, the expression of *GmSCOF-1* was strongly increased upon cold exposure, decreasing once the plants were placed under normal temperatures again. This result indicated that the cold response was induced at the expression level under the experimental conditions. Then the expression of the endoplasmic reticulum omega-3 desaturases was analysed. The PCR primers recognized and amplified both *GmFAD3A* and *GmFAD3B*. A slight increase in the amount of *GmFAD3A+B* transcripts was detected in mature trifoliate leaves upon cold exposure. No significant changes were obtained when the expression of *GmFAD3C* was analysed (Fig. 2A and B). Interestingly, digestion with *Van*91I, which allowed for the differentiation of *GmFAD3*A and *GmFAD3B* transcripts (Andreu *et al.*, 2010), showed an increase in the amount of *GmFAD3A* transcripts upon cold exposure (about 2–3 fold according to the normalized analysis). This increase disappeared after the 4-day recovery of cold-treated plants under control temperature conditions, indicating that the increase in *GmFAD3A* mRNA was a cold-specific response (Fig. 2A and B). It is also worth mentioning that a small band was reproducibly amplified with the *GmFAD3A+B* specific primers (Fig. 2A). This small band (hereafter designated

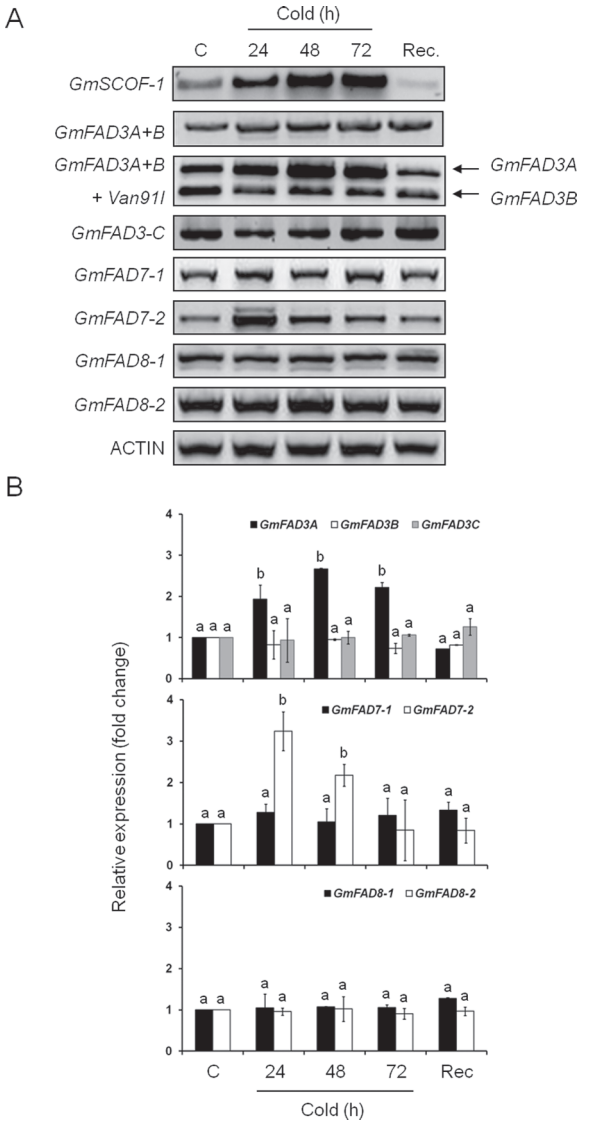

Fig. 2. (A) Omega-3 fatty acid desaturase gene expression in mature leaves from soybean plants kept at control temperature (24 °C); 5 °C exposure for 24, 48, and 72 h; and after recovery for 4 days at control temperature. *GmSCOF-1* was used as an internal control for cold-inducible expression. *ACTIN* was used as a housekeeping gene in all experiments. (B) Normalization of gene expression results against *ACTIN*. Data are mean ± SD from two experiments. For the same time point, different letters indicate significant differences among treatments ($P < 0.05$).

as *GmFAD3A-T*) seemed to accumulate specifically in response to cold exposure, since it disappeared once plants returned to the control temperature.

With respect to the plastid omega-3 fatty acid desaturases, the expression of *GmFAD7-1* was not altered in response to cold exposure (Fig. 2A and B). However, the expression of *GmFAD7-2* showed a transient increase upon exposure to cold temperatures for 24 h (Fig. 2A and B) and then a progressive decrease with time of cold exposure (48 and 72 h), returning to levels similar to control leaves after 4 days of re-exposure to the control temperature. An upper size band was also detected at 24 h of cold treatment (Fig. 2A). Sequence analysis revealed that this band

was a PCR artefact originating from the unspecific annealing of the reverse primer downstream of *GmFAD7-2*. Both *GmFAD8* transcripts were present in high amounts in soybean leaves at control temperature (Fig. 2A and B). In fact, both *GmFAD8* transcripts were detected in total RNA extracted from roots, leaves, stems, flowers, and mature seeds, indicating that both *GmFAD8-1* or *GmFAD8-2* were expressed in all soybean tissues analysed even at control temperatures (Supplementary Fig. S1). Interestingly, no modification of the transcript levels from *GmFAD8-1* or *GmFAD8-2* was observed upon cold temperature exposure (Fig. 2A and B). A lower size band was detected in the expression analysis of *GmFAD8-1* in mature trifoliate leaves (Fig. 2A). However, this band was detected both under control and cold temperatures, indicating that its accumulation was not temperature specific.

Effect of cold temperature exposure on the fatty acid composition and omega-3 fatty acid desaturase gene expression in soybean photosynthetic cell cultures

At this point, the effect of cold temperatures on young (1–3 days) developing trifoliate leaves was analysed. However, due to the length of the experiment (7 days) it was extremely difficult to differentiate the changes in 18:3 produced by the cold response from those originating during leaf maturation in all plant species analysed (Horiguchi *et al.*, 1996; data not shown). In an attempt to simplify the current experimental system, the results obtained in plant leaves were compared with similar experiments performed on soybean photosynthetic cell suspension cultures, to separate the effect of developmental and/or tissue differentiation from that of cold response. Such photosynthetic cell suspensions provide a good model system since they behave similarly to young leaf mesophyll cells (Rogers *et al.*, 1987). Furthermore, these cultures have been previously used as a model system to examine fatty acid synthesis and turnover in plant cells, finding similar amounts of phospholipids or galactolipids to those present in leaf cells (MacCarthy and Stumpf, 1980; Martin *et al.*, 1984). The fatty acid composition of photosynthetic cell suspensions after 3 weeks of culture (early-stationary phase) and then exposed to 5 °C is shown in Fig. 3. The fatty acid composition of control cells was similar to that reported previously (Rogers *et al.*, 1987; Collados *et al.*, 2006) and to that obtained in young soybean trifoliate leaves (data not shown). Fatty acid 18:3 constitutes the most abundant fatty acid species, representing around 50% of the total (Fig. 3). Upon exposure to cold temperatures, the fatty acid composition did not change dramatically. A slight but reproducible increase in 18:1 was observed at 72 h of cold exposure (Fig. 3). This increase was accompanied by a slight decrease in 18:3 levels (i.e., less than 5%). These results are in agreement with those obtained in similar photosynthetic cell suspensions in which desaturase activity was monitored in a range of temperatures from 15–35 °C using radioactive ^{14}C- labelled fatty acids (MacCarthy and Stumpf, 1980). It is also noteworthy that the increase in 18:1 observed after 72 h of cold treatment was similar to that observed in plants during the first 24 h of low temperature exposure (Fig. 1). Finally, as occurred with plants cultivated in a growth chamber, re-exposure to the control temperature (24 °C) restored the fatty acid composition to standard values (Fig. 3).

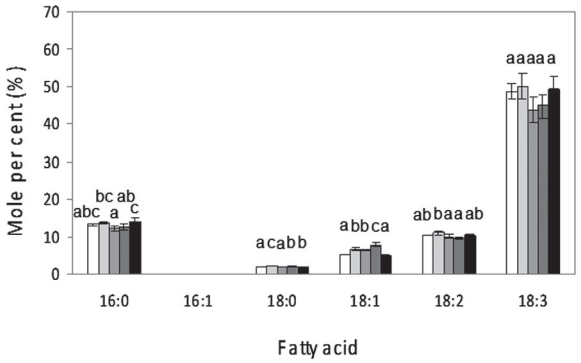

Fig. 3. Effect of exposure to cold temperatures on fatty acid composition of total lipids from soybean photosynthetic cell suspensions. Total lipids were extracted from cell suspensions kept at control temperatures (24 °C); 5 °C exposure for 24, 48, and 72 h; and after recovery for 4 days at control temperature. Fatty acids were determined by gas chromatography (GC). White bars indicate fatty acids from control leaves; light-grey bars from 24 h; medium-grey bars from 48 h; dark-grey bars from 72 h of cold treatment; and black bars after 4 days at control temperatures after cold exposure. Data are mean ± SD from three experiments. For the same fatty acid, different letters indicate significant differences among treatments ($P < 0.05$).

The expression of genes encoding omega-3 desaturases in response to cold in soybean photosynthetic cell suspensions was also monitored. The results are shown in Fig. 4. Expression of *GmSCOF-1* was induced upon cold exposure, decreasing after replacement of the cell cultures in normal growth conditions. These results suggested that, as occurred in mature trifoliate leaves, the cold-induced response at the gene expression level was also activated in the photosynthetic suspension cultures. The expression of the *GmFAD3* genes was then examined. No expression of *GmFAD3C* was detected in cell suspensions (data not shown) even at control temperature, so the analysis was focused on *GmFAD3A* and *GmFAD3B*. Exposure of photosynthetic cell suspensions to 5 °C produced an increase in the *GmFAD3A* + *GmFAD3B* transcripts (Fig. 4A and B). Digestion with *Van*91I, which allowed the *GmFAD3A* and *GmFAD3B* transcripts to be distinguished, showed a similar situation to what happened in mature leaves. A specific increase in the *GmFAD3A* transcript was detected in cell suspensions upon cold treatment, reverting when the cells were returned to control temperatures (Fig. 4A and B). This increase was accompanied by the accumulation of a smaller transcript that amplified with the *GmFAD3A* and *B* specific primers. The smaller transcript was not detected at control temperatures and disappeared upon recovery (Fig. 4A). This behaviour corresponded well to that detected in mature trifoliate leaves under similar temperature conditions (Fig. 2A). It is also worth mentioning that its accumulation seemed to be retarded in photosynthetic cell suspensions when compared with trifoliate leaves (Figs. 2A and 4A).

Transcripts from the plastidial omega-3 fatty acid desaturases (*GmFAD7-1*, *GmFAD7-2*, *GmFAD8-1*, and *GmFAD8-2*) did not show any noticeable changes upon cold exposure or recovery at control temperatures (Figs. 4A and 4B) in the photosynthetic cell suspension cultures.

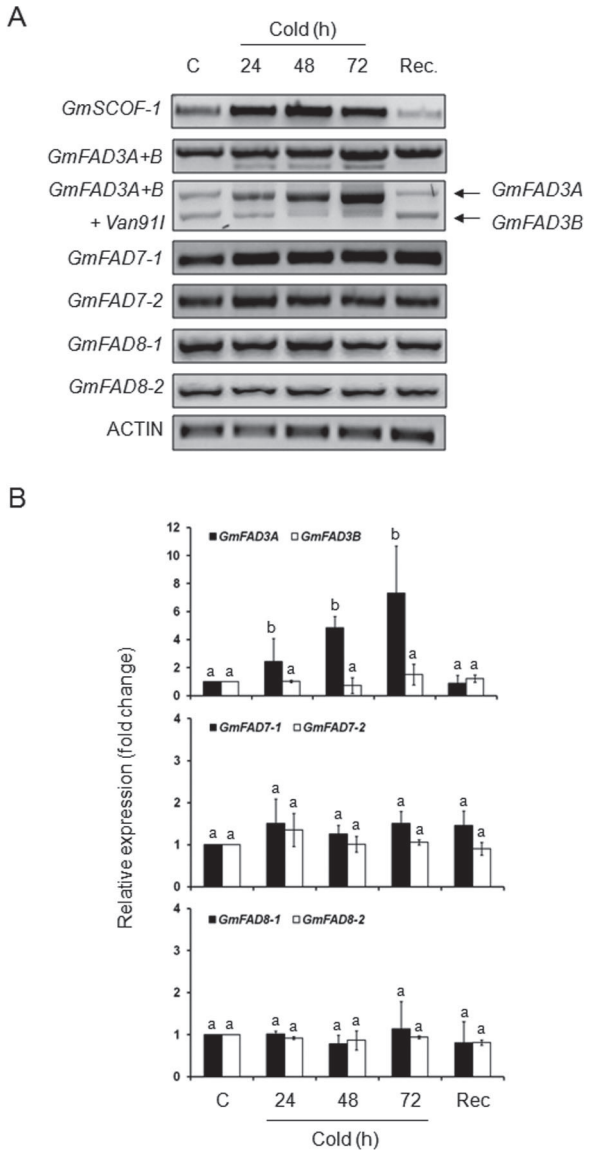

Fig. 4. (A) Omega-3 fatty acid desaturase gene expression in soybean photosynthetic cultured cells kept at control temperature (24 °C); 5 °C exposure for 24, 48, and 72 h; and after recovery for 4 days at control temperatures. *GmSCOF-1* was used as an internal control for cold-induced expression. *ACTIN* was used as a housekeeping gene in all experiments. (B) Normalization of gene expression results against *ACTIN*. Data are mean ± SD from two experiments. For the same time point, different letters indicate significant differences among treatments ($P < 0.05$).

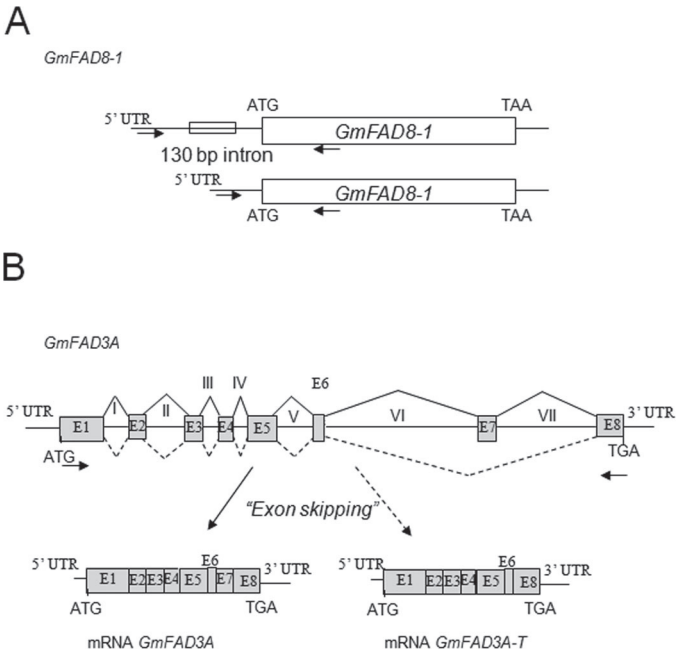

Fig. 5. Schematic diagram showing the proposed alternative splicing mechanisms observed during the expression analysis of *GmFAD8-1* (A) and *GmFAD3A* (B). Boxes represent exons while introns are represented by lines and numbered in Roman numerals. The positions of the ATG and stop codons as well as the primers used for amplification are also shown.

Analysis of splice variants originating from the omega-3 desaturase genes in soybean

The additional transcript bands detected during the course of the expression analysis of the genes encoding omega-3 desaturases in response to low temperatures were also studied. To analyse their molecular origin in detail, these bands were excised from the agarose gels, purified, cloned in a pGEM-T-Easy vector, and sequenced. In the case of *GmFAD8-1*, analysis of the lower size band revealed that the only difference between the two bands was the processing of a small intron (130 bp) present in the 5'-untranscribed region, 5 bp upstream of the ATG of the *Gm*FAD8-1 protein (Fig. 5A). The splicing of this intron might eliminate a canonical Shine-Dalgarno sequence located 7 bp upstream of the ATG, suggesting that it could be related with control of the translation of the *GmFAD8-1* mRNA. This splicing mechanism seemed to operate independently of temperature since the intron was detected both in control and cold-exposed samples.

The nature of the small band accumulating during cold exposure upon RT-PCR analysis of the *GmFAD3A+B* transcripts was also analysed. This small band (*GmFAD3A-T*) corresponded to a truncated form of the *GmFAD3A* transcript that presented a deletion of 138 nt eliminating 47 amino acid residues with respect to the mature *GmFAD3A* protein (Fig. 5B). The study compared the deduced sequences obtained from the analysis of the RT-PCR-amplified bands with the genomic sequence of *GmFAD3A* obtained from the soybean database. This analysis showed that the *GmFAD3A-T* transcript originated from an alternative splicing of *GmFAD3A* that eliminated exon 7, joining exon 6 with exon 8 (Fig. 5B) in a typical exon-skipping mechanism. These results indicate that cold induced an alternative splicing of *GmFAD3A*, producing a putative truncated form of the *Gm*FAD3A protein. Two features were interesting in this *GmFAD3A-T* transcript. First, the deletion eliminated one of the three His boxes necessary for the enzymatic desaturase activity (Shanklin *et al.*, 1994). Second, the C-terminus of the *Gm*FAD3A-T form was identical to the mature *Gm*FAD3A protein (Fig. 6), suggesting that all of the sequences necessary for membrane anchoring and insertion were present in *Gm*FAD3A-T.

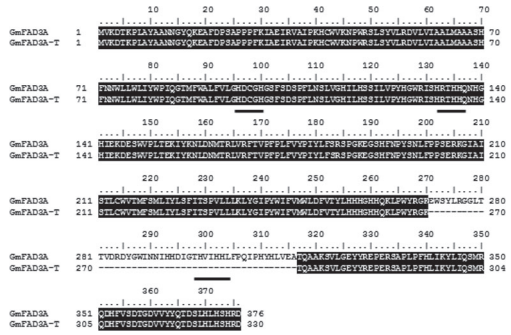

Fig. 6. Protein sequence alignment of *Gm*FAD3A and *Gm*FAD3A-T. Black boxes indicate residues that are strictly identical, and dashes show the region that was eliminated in the truncated form after alternative splicing. The His boxes characteristic of the desaturase active site are underlined.

Effect of temperature on the linolenic acid content of S. cerevisiae *cells overexpressing the soybean* GmFAD3 *genes*

Yeast has been proven as a suitable heterologous expression system for studying the functionality of endoplasmic reticulum desaturases such as FAD3 (Dyer *et al.*, 2001). In the presence of the appropriate substrates (18:2), the FAD3 enzymes expressed in yeast can obtain reducing power and electrons for the omega-3 desaturase activity (Dyer *et al.*, 2001). Unfortunately, these studies are not suited for the analysis of plastid desaturases as they require electron transport chains from the chloroplast (Shanklin *et al.*, 1994). The current study decided to take advantage of the yeast system to further analyse the functionality of *Gm*FAD3A, *Gm*FAD3B, *Gm*FAD3C, and *Gm*FAD3A-T as a function of temperature. To this end, these four isoforms of *Gm*FAD3 were expressed in *S. cerevisiae* under the galactose-inducible yeast promoter of the pYES2 vector. The fatty acid analysis of transformed yeast cells revealed a high quantity of linoleic acid (18:2; Table 1 and Supplementary Fig. S2), which was not present in the wild-type yeast (data not shown), showing a correct uptake of the supplemented substrate. Table 1 shows the fatty acid compositions of yeast cells transformed with *GmFAD3A*, *GmFAD3B, GmFAD3C*, and *GmFAD3A-T* using the pYES2 vector. The fatty acid analysis of the *GmFAD3A*- and *GmFAD3B*-transformed yeast cells showed the presence of linolenic acid (18:3) that was present neither in wild-type yeast nor in cells transformed with the empty vector. The percentage of 18:3 obtained with the inducible pYES2 vector at normal yeast growth temperature (30 °C) was 3.8, 6.2, and 0.9% for *Gm*FAD3A, *Gm*FAD3B, and *Gm*FAD3C, respectively (Table 1). The percentage of 18:3 obtained was consistent with similar data from the literature (Dyer *et al.*, 2001; O'Quin *et al.*, 2010). These results indicate that the expression of the three genes is functional, as they code for isoforms capable of desaturating exogenous substrate to produce the corresponding 18:3. Under the experimental conditions, the 18:3 content of yeast cells overexpressing *Gm*FAD3B was slightly higher than that obtained for *Gm*FAD3A at yeast growth temperature (Table 1). By contrast, in the case of *Gm*FAD3C, it was always significantly lower than that obtained in yeast transformed with *Gm*FAD3A or *Gm*FAD3B under the same experimental conditions (Table 1). No production of 18:3 was detected in yeast cells transformed with *GmFAD3A-T* (Table 1), even though the 18:2 levels were similar to those from yeast cells transformed with *GmFAD3A, GmFAD3B*, or *GmFAD3C*, indicating that the absence of 18:3 was not due to the low availability of 18:2 as a substrate. Similar results were obtained with the pVT102-U vector, which carries a constitutive promoter (Vernet *et al.*, 1987), except that lower 18:3 percentages were routinely obtained (data not shown).

Next was studied the effect of growth temperature on the 18:3 content of *S. cerevisiae* cells overexpressing *Gm*FAD3A, *Gm*FAD3B, and *Gm*FAD3C isoforms. The results are shown in Fig. 7 and Table 1. The growth temperature modified the 18:3 content in transformed yeast cells. This accumulation of fatty acyl lipid is possibly due to the low levels of β-oxidation displayed by *S. cerevisiae* cells in the presence of an appropriate carbon source (Veenhuis and Goodman, 1990). Yeast cells transformed with *Gm*FAD3A or *Gm*FAD3B showed the highest amount of linolenic acid at lower temperatures (10–15 °C), with percentages ranging between 25–30%, while at higher temperatures (30–35 °C) the percentage decreased to 4–6% (Table 1 and Fig. 7). Although the percentage of 18:3 production was slightly

Table 1. Fatty acid composition of *S. cerevesiae* cells overexpressing soybean *Gm*FAD3 proteins and grown at two different temperatures

		Fatty acid composition (mol%)						
Plasmid	**Temperature (ºC)**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**Conversion 18:2 to 18:3 (%)**
pYES2	30	20.6±1.1	4.1±0.0	6.4±0.3	2.3±0.2	66.3±1.7	0.0±0.0	0.0±0.0
	15	19.5±1.4	5.2±2.8	7.3±0.1	3.3±2.1	64.7±6.3	0.0±0.0	0.0±0.0
pYES2-*Gm*FAD3A	30	19.8±1.0	5.2±0.	7.3±0.4	3.1±0.2	60.7±1.8	3.8±0.3	5.9±0.5
	15	19.5±0.2	4.8±0.2	8.2±0.3	2.9±0.1	39.9±1.7	24.8±1.8	38.8±2.7
pYES2-*Gm*FAD3A-T	30	18.6±1.1	4.2±1.5	7.3±1.0	2.4±1.1	67.2±3.3	0.0±0.0	0.0±0.0
	15	17.4±1.8	4.1±0.6	7.2±0.2	2.4±0.6	68.9±3.0	0.0±0.0	0.0±0.0
pYES2-*Gm*FAD3B	30	20.2±1.0	5.2±1.2	7.4±0.2	3.0±0.9	58.0±1.9	6.2±0.9	9.7±1.5
	15	19.4±1.2	5.4±0.4	7.7±0.2	3.6±0.5	34.3±4.9	29.6±2.7	46.4±5.9
pYES2-*Gm*FAD3C	30	18.6±0.4	6.2±1.1	7.7±1.6	5.0±1.1	61.6±3.9	0.9±0.3	1.4±0.3
	15	16.4±1.2	4.7±0.7	7.1±0.3	3.8±0.5	64.0±2.5	4.1±0.7	6.0±1.2

16:0, Palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. Data are mean ± SD from three independent experiments.

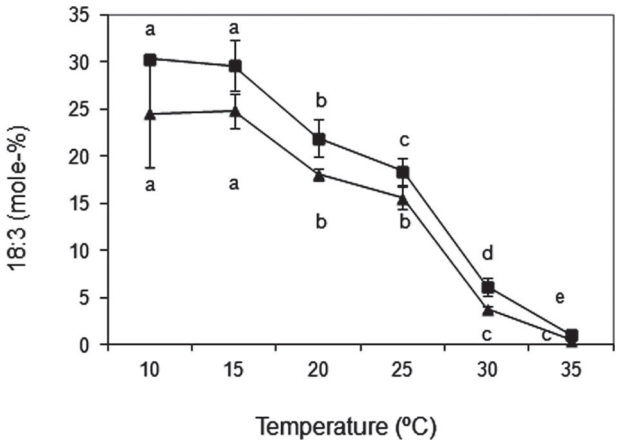

Fig. 7. Production of linolenic acid in *S. cerevisiae* cells overexpressing soybean *GmFAD3A* and *GmFAD3B* and grown at different temperatures. Yeast cultures harbouring the pYES-*Gm*FAD3A (closed triangles) and pYES-*Gm*FAD3B (closed squares) vectors were grown at the temperatures indicated. After reaching the stationary phase, yeast cells were harvested and the fatty acid composition was determined in whole cells using GC/FID. Data are mean ± SD from three experiments. For each gene construct, different letters indicate significant differences among treatments ($P < 0.05$).

higher in *Gm*FAD3B- than in *Gm*FAD3A-transformed yeast cells (Fig. 7 and Table 1), the differences were not statistically significant, indicating that overall the ratio of 18:3 conversion in both types of transformed cells was similar independently of the temperature (Table 1). As occurred with *Gm*FAD3A and *Gm*FAD3B, the 18:3 content increased (4.1%) upon exposure of yeast cells transformed with pYES2-*Gm*FAD3C to 15 °C. This result indicated that the 18:3 content of *S. cerevisiae* cells overexpressing *Gm*FAD3C was also higher at lower temperatures, although this percentage was again lower than that detected for *Gm*FAD3A and *Gm*FAD3B. Finally, it is noteworthy that when similar experiments were performed with the truncated *Gm*FAD3A-T form, no 18:3 was detected irrespective of the temperature, further suggesting that this truncated *GmFAD3A-T* mRNA, if translated, would give rise to an inactive omega-3 desaturase enzyme (Table 1).

Discussion

This work analysed the behaviour of all the endoplasmic reticulum and plastidial omega-3 desaturases in soybean at the level of both fatty acid content and gene expression in order to determine how low temperature exposure affected the concerted contribution of each omega-3 desaturase to the synthesis of 18:3 in response to cold. Exposure of soybean plants to cold did not result in significant modifications of 18:3 in leaf membrane lipids. This result is consistent with previous observations in other plant species. Thus, in birch Martz *et al.* (2006) reported a 3% increase in 18:3 levels in galactolipids in response to cold. In soybean, Li *et al.* (2007) reported a 7% increase in 18:3 levels in total lipids from plants exposed to 8 °C for a week. More recently, Upchurch and Ramirez (2011) reported a 4% increase in 18:3 from total leaf lipids isolated from soybean plants exposed to a 20/16 °C day/night temperature for 72 h. In fact, with the exception of *Arabidopsis*, where significant changes in the 10–15% range were reported (McConn *et al.*, 1994; Falcone *et al.*, 2004), the extent of 18:3 changes in response to cold seems to be rather small in other plant species, suggesting that the effect of cold temperatures on 18:3 is relatively slight and could be limited to specific plant species, tissues, or growth processes (Iba, 2002). The higher 18:3 content present in soybeans (65–70%) when compared with *Arabidopsis* (40%) may account for these differences.

Despite the small changes in 18:3 levels, the data showed specific changes at the level of the expression of genes encoding omega-3 desaturases in response to cold in soybean. The data suggest the existence of changes in the regulatory mechanism of omega-3 fatty acid desaturases affecting specific isoforms in both cell compartments to maintain appropriate levels of 18:3 under low temperature conditions. Thus, with respect to the plastidial desaturases, this study detected a rapid transient activation of *GmFAD7-2* in response to cold that was only present in mature trifoliate leaves but not in photosynthetic cell suspensions. This increase preceded the small changes observed in the 18:3 content. Interestingly, it has been previously reported that the *GmFAD7-1* isoform seemed to be more sensitive to the wound response than *GmFAD7-2* (Andreu *et al.*, 2010). These results together might suggest a certain degree of specialization among *GmFAD7* isoforms, with a specific role for the *GmFAD7-2* isoform in the cold response. It has generally been inferred from the results obtained in *Arabidopsis* that the increase in 18:3 observed as a response to low temperatures was due to *FAD8* induction. Thus, Gibson *et al.* (1994) identified the *FAD8* locus in a *fad3/fad7* double mutant from *Arabidopsis* that was capable of producing TAs only at cold temperatures. Low temperatures seem to induce *FAD8* mRNA in *Arabidopsis* (Gibson *et al.*, 1994), maize (Berberich *et al.*, 1998), rice (Wang *et al.*, 2006), and birch (Martz *et al.*, 2006). The current data in soybean showed high levels of both *GmFAD8* transcripts even at control temperatures (i.e., unlike *Arabidopsis*, maize, rice, or birch) with no apparent changes upon cold exposure. This result suggests that, if there is a specific effect of cold temperatures on the *GmFAD8* genes, it is not at the transcriptional level. In this sense, a post-translational regulatory mechanism acting on the stability of the *At*FAD8 protein in response to temperature has been described in *Arabidopsis* (Matsuda *et al.*, 2005). Given the results presented in this paper, and in the absence of data on specific protein or enzyme activity, the existence of additional control points controlling the amount and activity of *Gm*FAD8 proteins in response to cold remains to be elucidated.

The current data show that in soybean leaves, the expression of endoplasmic reticulum omega-3 desaturases is also tightly regulated in response to cold temperatures. An increase in *GmFAD3A* transcripts was detected both in mature leaves and cell suspensions. These results were consistent with previous observations for *BnFAD3* (Tasseva *et al.*, 2004). The higher activity levels (Fig. 7 and Table 1) observed for *Gm*FAD3A and *Gm*FAD3B compared to *Gm*FAD3C in transformed yeast indicate that these two genes/isoforms might contribute to the 18:3 content to a greater extent than *Gm*FAD3C. As occurred with the plastidial *GmFAD7-2*, the selection towards the *Gm*FAD3A isoform in response to cold might suggest a more specific role for this *Gm*FAD3 isoform in these conditions. However, the yeast expression experiments showed no significant differences in activity at a low temperature between the

*Gm*FAD3A and B isoforms, suggesting that this exchange was not related with higher activity of *Gm*FAD3A at a low temperature. It cannot be ruled out that in its natural environment this could be the case. However, other factors differentially regulating the expression of *GmFAD3A* in response to cold might account for the different behaviour of these two *GmFAD3* isoforms. Another important point that can be inferred from the data on transformed yeast expression is the high *Gm*FAD3 omega-3 desaturase activity detected in yeast at low temperatures (Fig. 7 and Table 1). These values are consistent with those previously reported in other plant species (Dyer *et al.*, 2001; O'Quin *et al.*, 2010), and are also consistent with the highest percentage of 18:3 reported in wheat root tips exposed to cold (Horiguchi *et al.*, 2000). Endoplasmic reticulum enzymes have been shown to be the major contributors to root linolenic acid levels (Yadav *et al.*, 1993). All these results suggest that the role of FAD3 and the endoplasmic reticulum membranes in the cold response cannot be precluded, even in leaves.

Finally, another interesting question emerging from these data is the involvement of alternative splicing mechanisms in the regulation of the expression of omega-3 desaturases in soybean. One of the spliced variants seemed to be cold specific (*GmFAD3A*), while that derived from *GmFAD8-1* was not related with temperature. The existence of an intron in the 5'-untranscribed region strongly suggests a role in the translation of the *Gm*FAD8-1 protein that could be more closely related with the relative abundance of *Gm*FAD8 isoforms. By contrast, this study detected an alternative spliced form of *GmFAD3A* that gave rise to a putative truncated form of the *GmFAD3*A protein that was specifically accumulated upon cold exposure of soybean plants. It is worth noting that a similar truncated form of *AtFAD3* was also found in the databases (accession NM179808), suggesting that this alternative splicing is not a unique feature of soybean. Although the specific role of these spliced variants is still far from being understood, the list of the alternatively spliced genes associated with abiotic stress responses is rapidly expanding (Reddy, 2007). The putative truncated form of *Gm*FAD3A proved to be inactive in yeast, consistent with the loss of one of the three His boxes that have been reported to be essential for the desaturation activity (Shanklin *et al.*, 1994). It is tempting to speculate on other functions more closely related with gene regulation, as for example acting as regulatory RNAs in order to control gene expression by different means.

In conclusion, these data show that in soybean there is a cold-specific response by omega-3 desaturases at least at the transcriptional level, involving both endoplasmic reticulum (*GmFAD3A*) and plastidial (*GmFAD7-2*) omega-3 desaturases, in order to maintain appropriate 18:3 levels in membrane lipids. Given this coordinated expression of these omega-3 desaturase genes and their different subcellular localization, the data highlight the relevance of the mechanisms of lipid exchange between membranes in these acclimation responses in plants.

Supplementary material

Supplementary data are available at *JXB* online.Supplementary Table S1. Gene specific primers used in this studySupplementary Fig. S1. Tissue-specific expression of *Gm*FAD8-1 and *Gm*FAD8-2 genes in soybean plantsSupplementary Fig. S2. GC-FID chromatograms showing the fatty acid profile obtained in yeast transformants containing the different constructions corresponding to each of the *Gm*FAD3 isoforms

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