RESEARCH PAPER

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

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Abstract

We 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 the GmFAD3A gene 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 GmFAD3A, GmFAD3B, GmFAD3C and GmFAD3A-T was examined by heterologous expression in yeast. No activity was detected with GmFAD3A-T, consistent with the absence of one of the His boxes necessary for desaturase activity. The linolenic acid content of Sacharomyces cerevisiae cells overexpressing GmFAD3A or GmFAD3B was higher when the cultures were incubated at cooler temperatures, suggesting that reticular desaturases of the GmFAD3 family, and more specifically GmFAD3A, may play a role in the cold response, even in leaves. Our 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 (GPAT) gene 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 α-linolenic acid (18:3) (McConn et al., 1994; Heppard et al., 1996; Horiguchi et al., 2000; Li et al., 2007; Martz et al., 2006; 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 sub-cellular 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 \textit{AtFAD3} and two for the plastidial enzymes \textit{AtFAD7} and \textit{AtFAD8} (Yadav et al., 1993; Iba et al., 1993, Gibson et al., 1994). The \textit{AtFAD8} gene is believed to encode a cold-specific plastidial omega-3 desaturase since its activity has been observed in a \textit{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 \textit{ZmFAD7} mRNA accompanied by an increase in the \textit{ZmFAD8} mRNA was reported in response to low temperatures (Berberich et al., 1998), suggesting direct transcriptional control. More recently, it was demonstrated that the \textit{AtFAD8} protein was destabilized at high temperatures, without changes in mRNA levels, suggesting a post-
translational control mechanism regulating \textit{AtFAD8} activity in Arabidopsis in which the C-terminus of the mature protein would be involved (Matsuda \textit{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 \textit{TaFAD3} mRNA levels (Horiguchi \textit{et al}., 2000). Changes in the protein half-life could also be involved in control of the activity of the soybean seed-specific \textit{GmFAD2-2} isoform (Tang \textit{et al}., 2005) or rape \textit{BnFAD3} enzymes (O'Quin \textit{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 \textit{et al}., 1998; Matsuda \textit{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 \textit{et al}., 2000; Dyer \textit{et al}., 2001; Tang \textit{et al}., 2005; O'Quin \textit{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, we are 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, genes encoding omega-3 fatty acid desaturases are grouped in multigene families. Thus, at least three \textit{GmFAD3} genes, designated as \textit{GmFAD3A}, \textit{GmFAD3B} and \textit{GmFAD3C} seem to contribute to 18:3 synthesis in the endoplasmic reticulum membranes of soybean (Bilyeu \textit{et al}., 2003; Anai \textit{et al}., 2005). More recently, we reported the presence of two soybean \textit{GmFAD7} genes, designated as \textit{GmFAD7-1} and \textit{GmFAD7-2}, which would participate in 18:3 production in plastid membranes (Andreu \textit{et al}.)
Finally, two sequences with homology to the known FAD8 genes were detected in the soybean genome (Chi et al., 2011), but their regulation has not been studied yet. In this work we have 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 our analysis to a non-tissue differentiating system. Our 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 16h/8h 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 three 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 RT-PCR assay. The oligonucleotides used as well as the PCR conditions are shown in Supplemental
Table S1. *ACTIN* was used as a housekeeping gene (Table S1). 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 the *GmFAD3* gene recognized and amplified both *GmFAD3A* and *GmFAD3B* genes, we carried out a restriction analysis of the amplified fragments using the *Van*9lI enzyme (GE Healthcare), which allowed us to distinguish between these different *GmFAD3* genes. The *Van*9lI enzyme generates two fragments of 164 and 755 bp in *GmFAD3A* and three fragments of 161, 164, and 594 bp in *GmFAD3B*. The digestion products were resolved by electrophoresis on 1% (w/v) agarose gels. The *GmSCOF-*1 gene, encoding a transcriptional factor of the C2H2-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 in our experimental conditions. Semi-quantification of the relative expression levels was performed through normalization against the house-keeping gene (*ACTIN*) from two independent biological experiments. Densitometric quantification of the RT-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 from *GmFAD3A*) genes 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'-GAGGATCCAAATGGTCAAGCACAG-3’ and 5’-
GAACTCGAGTTTAGTTGACTGGGTCC-3’ primers were used. All these primers were
extended by a BamHI (in the forward primer) and an XhoI (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 GmFAD3
isoform was cloned in a pGEM-T-Easy vector, double-digested with BamHI and XhoI, and
ligated into the digested destination vector. All constructs were checked by sequencing.
Saccharomyces cerevisiae strain UTL-7A cells were transformed with plasmids pYES2
(negative control), pYES2-GmFAD3A, pYES2-GmFAD3B, pYES2-GmFAD3C and pYES2-
GmFAD3A-T by the lithium acetate protocol (Gietz et al., 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 30ºC. When the cultures
reached an OD$_{600}$nm 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 10-35ºC until late log-stationary phase.
Yeast cells were harvested by centrifugation at 1,500 x g for 5 min at 4ºC, 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 experiment in the case of the FA determinations from soybean leaf, cell culture experiments or yeast FA determinations. 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 (Fig. 1). These increases preceded that for the mean values of 18:3, around 5-8%, which was observed only after 72 h of cold exposure (Fig. 1). 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 (Fig. 1). 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 those obtained from control plants or from plants that were again placed under normal control temperatures for 96 h were found.
We decided to analyse the expression of genes encoding all omega-3 fatty acid desaturases by semi-quantitative RT-PCR. First, we checked the expression of a cold-sensitive gene as an internal control of the low temperature response in our experimental conditions. To this end we monitored the expression of the *GmSCOF-1* gene that encodes a transcription factor of the C$_2$H$_2$ family of zinc finger proteins that is strongly activated in soybean upon low temperature exposure (Kim *et al.*, 2001). As shown in Fig. 2A, the expression of the *GmSCOF-1* gene 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 in our experimental conditions. Then the expression of the endoplasmic reticulum omega-3 desaturases was analysed. Our PCR primers recognized and amplified both *GmFAD3A* and *GmFAD3B* genes. 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 the *GmFAD3C* gene was analysed (Figs. 2A and 2B). Interestingly, digestion with Van91I, which allowed for the differentiation of *GmFAD3A* 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 (Figs. 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 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 the *GmFAD7*
*I* gene was not altered in response to cold exposure (Figs 2A and B). However, the expression of the *GmFAD7-2* gene showed a transient increase upon exposure to cold temperatures for 24 h (Figs. 2A and 2B), 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 from the *GmFAD7-2* gene. Transcripts of both *GmFAD8* genes were present in high amounts in soybean leaves at control temperature (Figs. 2A and 2B). In fact, both *GmFAD8* transcripts were detected in total RNA extracted from roots, leaves, stems, flowers and mature seeds, indicating that both *GmFAD8* genes were expressed in all soybean tissues analysed even at control temperatures (Supplemental Fig. S1). Interestingly, no modification of the transcript levels from *GmFAD8-1* or *GmFAD8-2* genes was observed upon cold temperature exposure (Figs. 2A and 2B). A lower size band was detected in the expression analysis of the *GmFAD8-1* gene 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; Lagunas and Alfonso, unpublished results). In an attempt to simplify our experimental system, we decided
to compare the results obtained in plant leaves with similar experiments performed on soybean photosynthetic cell suspension cultures. This would allow us 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 three weeks of culture (early-stationary phase) and then exposed to 5ºC 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 (Lagunas and Alfonso, unpublished results). 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 to 35ºC 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 (24ºC) restored the fatty acid composition to standard values (Fig. 3).
We also monitored the expression of genes encoding omega-3 desaturases in response to cold exposure in soybean photosynthetic cell suspensions. The results are shown in Fig. 4. Expression of the GmSCOF-1 gene 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 our photosynthetic suspension cultures. The expression of the GmFAD3 genes was then examined. No expression of the GmFAD3C gene was detected in cell suspensions (data not shown) even at control temperature, so we focused our analysis on the GmFAD3A and GmFAD3B genes. Exposure of photosynthetic cell suspensions to 5°C produced an increase in the GmFAD3A + GmFAD3B transcripts (Figs. 4A and 4B). Digestion with Van91I, 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 (Figs. 4A and 4B). 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 our photosynthetic cell suspension...
Analysis of splice variants originating from the omega-3 desaturase genes in soybean.

We focused our attention on the additional transcript bands detected during the course of the expression analysis of the genes encoding omega-3 fatty acid desaturases in response to low temperatures. 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-UTR region, 5 bp upstream of the ATG of the GmFAD8-1 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). We compared the deducted sequences obtained from the analysis of the RT-PCR-amplified bands with the genomic sequence of the GmFAD3A gene obtained from the soybean database. This analysis showed that the GmFAD3A-T transcript originated from an alternative splicing of the GmFAD3A gene 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
the GmFAD3A gene, producing a putative truncated form of the GmFAD3A 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 GmFAD3A-T form was identical to the mature GmFAD3A protein (Fig. 6), suggesting that all of the sequences necessary for membrane anchoring and insertion were present in GmFAD3A-T.

Effect of temperature on the linolenic acid content of S. cerevisiae cells overexpressing 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). We decided to take advantage of the yeast system to further analyse the functionality of GmFAD3A, GmFAD3B, GmFAD3C and GmFAD3A-T as a function of temperature. To this end, we expressed these four isoforms of GmFAD3 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 Supplemental 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 composition 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 (30ºC) was 3.8, 6.2, and 0.9% for GmFAD3A, GmFAD3B and GmFAD3C, 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. In our experimental conditions the 18:3 content of yeast cells overexpressing GmFAD3B was slightly higher than that obtained for GmFAD3A at yeast growth temperature (Table 1). By contrast, in the case of GmFAD3C, it was always significantly lower than that obtained in yeast transformed with GmFAD3A or GmFAD3B 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).

We next studied the effect of growth temperature on the 18:3 content of S. cerevisiae cells overexpressing GmFAD3A, GmFAD3B and GmFAD3C 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 et
Yeast cells transformed with *GmFAD3A* or *GmFAD3B* 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 higher in *GmFAD3B*- than in *GmFAD3A*-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 *GmFAD3A* and *GmFAD3B*, the 18:3 content increased (4.1%) upon exposure of yeast cells transformed with pYES2-*GmFAD3C* to 15°C. This result indicated that the 18:3 content of *S. cerevisiae* cells overexpressing *GmFAD3C* was also higher at lower temperatures, although this percentage was again lower than that detected for *GmFAD3A* and *GmFAD3B*. Finally, it is noteworthy that when similar experiments were performed with the truncated *GmFAD3A-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 (see Table 1).
DISCUSSIO

In this work we 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 8ºC 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 20/16ºC 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, our data showed specific changes at the level of the expression of genes encoding omega-3 desaturases in response to cold in soybean. Our 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, we detected a rapid transient activation of the GmFAD7-2 gene 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, we had reported previously that the \textit{GmFAD7-1} isoform seemed to be more sensitive to the wound response than \textit{GmFAD7-2} (Andreu \textit{et al.}, 2010). These results together might suggest a certain degree of specialization among \textit{GmFAD7} isoforms, with a specific role for the \textit{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 \textit{FAD8} induction. Thus, Gibson \textit{et al.}, (1994) identified the \textit{FAD8} locus in a \textit{fad3/fad7} double mutant from Arabidopsis that was capable of producing TAs only at cold temperatures. Low temperatures seem to induce \textit{FAD8} mRNA in Arabidopsis (Gibson \textit{et al.}, 1994), maize (Berberich \textit{et al.}, 1998), rice (Wang \textit{et al.}, 2006) and birch (Martz \textit{et al.}, 2008).

Our data in soybean showed high levels of both \textit{GmFAD8} gene 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 \textit{GmFAD8} genes it is not at the transcriptional level. In this sense, a post-translational regulatory mechanism acting on the stability of the \textit{AtFAD8} protein in response to temperature has been described in Arabidopsis (Matsuda \textit{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 \textit{GmFAD8} proteins in response to cold remains to be elucidated.

Our 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 \textit{GmFAD3A} transcripts was detected both in mature leaves and cell suspensions. These results were consistent with previous observations for the \textit{BnFAD3} gene (Tasseva \textit{et al.}, 2004). The higher activity levels (Fig. 7 and Table 1) observed for \textit{GmFAD3A} and \textit{GmFAD3B} compared
to GmFAD3C in transformed yeast indicate that these two genes/isoforms might contribute to
the 18:3 content to a greater extent than GmFAD3C. As occurred with the plastidial GmFAD7-2
gene, the selection towards the GmFAD3A isoform in response to cold might suggest a more
specific role for this GmFAD3 isoform in these conditions. However, the yeast expression
experiments showed no significant differences in activity at a low temperature between the
GmFAD3A and B isoforms, suggesting that this exchange was not related with higher activity
of GmFAD3A 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 the
GmFAD3A gene in response to cold might account for the different behaviour of these two
GmFAD3 gene isoforms. Another important point that can be inferred from the data on
transformed yeast expression is the high GmFAD3 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 our 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’-UTR strongly suggests a role in the translation of the GmFAD8-1 protein that could be
more closely related with the relative abundance of GmFAD8 isoforms. By contrast, we
detected an alternative spliced form of the \textit{GmFAD3A} gene giving rise to a putative truncated form of the \textit{GmFAD3A} protein that was specifically accumulated upon cold exposure of soybean plants. It is worth noting that a similar truncated form of the \textit{AtFAD3} gene was also found in the databases (accession number NM-179808), suggesting that this alternative splicing is not a unique feature of soybean. Although we are still far from understanding the specific role of these spliced variants, the list of the alternatively spliced genes associated with abiotic stress responses is rapidly expanding (Reddy, 2007). The putative truncated form of \textit{GmFAD3A} 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 \textit{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, our 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 (\textit{GmFAD3A}) and plastidial (\textit{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 sub-cellular localization, our data highlight the relevance of the mechanisms of lipid exchange between membranes in these acclimation responses in plants.
Acknowledgements

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FIGURE LEGENDS

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 (24ºC); after plant exposure for 24, 48, and 72 h to 5ºC; 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 (mean ± S.D.) were obtained from three different experiments. For the same fatty acid, different letters indicate significant differences among treatments at P< 0.05.

Fig. 2 (A) Omega-3 fatty acid desaturase gene expression in mature leaves from soybean plants kept at control temperature (24ºC); 5ºC exposure for 24, 48, and 72 h; and after recovery for 4 days at control temperature. The GmSCOF-1 gene 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 the housekeeping gene (ACTIN). Data were obtained from two (mean ± S.D.) different experiments. For the same time point, different letters indicate significant differences among treatments at P< 0.05.

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 (24ºC); 5ºC 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 (mean ± S.D.) were obtained from three different experiments. For the same fatty acid, different letters indicate significant differences among treatments at P< 0.05.

Fig. 4 (A) Omega-3 fatty acid desaturase gene expression in soybean photosynthetic cultured cells kept at control temperature (24ºC); 5ºC exposure for 24, 48, and 72 h; and after recovery for 4 days at control temperatures. The GmSCOF-1 gene 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 the housekeeping gene (ACTIN). Data (mean ± S.D.) were obtained from two different experiments. For the same time point, different letters indicate significant differences among treatments at P< 0.05.

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

**Fig. 6.** Protein sequence alignment of *GmFAD3A* and *GmFAD3A-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.

**Fig. 7.** Production of linolenic acid in *S. cerevisiae* cells overexpressing soybean *GmFAD3A* and *GmFAD3B* genes and grown at different temperatures. Yeast cultures harbouring the pYES-*GmFAD3A* (closed triangles) and pYES-*GmFAD3B* (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 (mean ± S.D.) were obtained from three different experiments. For each gene construct, different letters indicate significant differences among treatments at P< 0.05.
Table I. Fatty Acid composition of *S. cerevesiae* cells overexpressing soybean *GmFAD3* proteins and grown at two different temperatures.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Temp. (°C)</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>%conversion 18:2 to 18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES2</td>
<td>30</td>
<td>20.6 ± 1.1</td>
<td>4.1 ± 0.0</td>
<td>6.4 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>66.3 ± 1.7</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>19.5 ± 1.4</td>
<td>5.2 ± 2.8</td>
<td>7.3 ± 0.1</td>
<td>3.3 ± 2.1</td>
<td>64.7 ± 6.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>pYES2-</td>
<td>30</td>
<td>19.8 ± 1.0</td>
<td>5.2 ± 0.0</td>
<td>7.3 ± 0.4</td>
<td>3.1 ± 0.2</td>
<td>60.7 ± 1.8</td>
<td>3.8 ± 0.3</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>GmFAD3A</td>
<td>15</td>
<td>19.5 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>8.2 ± 0.3</td>
<td>2.9 ± 0.1</td>
<td>39.9 ± 1.7</td>
<td>24.8 ± 1.8</td>
<td>38.8 ± 2.7</td>
</tr>
<tr>
<td>pYES2-</td>
<td>30</td>
<td>18.6 ± 1.1</td>
<td>4.2 ± 1.5</td>
<td>7.3 ± 1.0</td>
<td>2.4 ± 1.1</td>
<td>67.2 ± 3.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>GmFAD3A-T</td>
<td>15</td>
<td>17.4 ± 1.8</td>
<td>4.1 ± 0.6</td>
<td>7.2 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>68.9 ± 3.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>pYES2-</td>
<td>30</td>
<td>20.2 ± 1.0</td>
<td>5.2 ± 1.2</td>
<td>7.4 ± 0.2</td>
<td>3.0 ± 0.9</td>
<td>58.0 ± 1.9</td>
<td>6.2 ± 0.9</td>
<td>9.7 ± 1.5</td>
</tr>
<tr>
<td>GmFAD3B</td>
<td>15</td>
<td>19.4 ± 1.2</td>
<td>5.4 ± 0.4</td>
<td>7.7 ± 0.2</td>
<td>3.6 ± 0.5</td>
<td>34.3 ± 4.9</td>
<td>29.6 ± 2.7</td>
<td>46.4 ± 5.9</td>
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<tr>
<td>pYES2-</td>
<td>30</td>
<td>18.6 ± 0.4</td>
<td>6.2 ± 1.1</td>
<td>7.7 ± 1.6</td>
<td>5.0 ± 1.1</td>
<td>61.6 ± 3.9</td>
<td>0.9 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>GmFAD3C</td>
<td>15</td>
<td>16.4 ± 1.2</td>
<td>4.7 ± 0.7</td>
<td>7.1 ± 0.3</td>
<td>3.8 ± 0.5</td>
<td>64.0 ± 2.5</td>
<td>4.1 ± 0.7</td>
<td>6.0 ± 1.2</td>
</tr>
</tbody>
</table>

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 (mean ± S.D.) were obtained from three independent experiments.
Figure 1 Román et al, (2012)
Figure 2 Román et al, (2012)
Figure 3 Román et al, (2012)
Figure 4 Román et al, (2012)
Figure 5 Román et al., (2012)
Figure 6. Román et al, (2012)
Figure 7. Román et al, (2012)