A temporal regulatory mechanism controls the different contribution of endoplasmic reticulum and plastidial ω -3 desaturases to trienoic fatty acid content during leaf development in Soybean (*Glycine max* cv Volania)

Beatriz Lagunas, Ángela Román, Vanesa Andreu¹, Rafael Picorel and Miguel Alfonso*

Department of Plant Nutrition, Estación Experimental Aula Dei (EEAD-CSIC).

Avda. Montañana 1005, 50059 Zaragoza, Spain.

* Corresponding author. Tel: +34 976 71 60 59; fax: +34 976 716045.

E-mail address: alfonso@eead.csic.es (M. Alfonso).

¹Present address: Instituto Aragonés de Ciencias de la Salud (I+CS). Hospital Universitario Miguel Servet. Paseo Isabel la Católica 1-5, 50009 Zaragoza, Spain.

ABSTRACT

We analyzed the molecular mechanism controlling ω -3 fatty acid desaturases during seed germination and leaf development in soybean. During germination, soybean seeds were characterized by a high 18:2 ^{Δ 9,12} level (more than 50%) and reduced 18:3 ^{Δ 9,12,15} content (10%). Interestingly, transcripts from all endoplasmic reticulum (*GmFAD3A* and *GmFAD3B*) and plastidial (*GmFAD7-1/GmFAD7-2* or *GmFAD8-1/GmFAD8-2*) desaturase genes were detected during seed germination. Upon germination, soybean trifoliolate leaf development was accompanied by an increase in linolenic acid (18:3 ^{Δ 9,12,15}). Our data showed that transcripts corresponding to the endoplasmic reticulum ω -3 desaturases *GmFAD3A* and *GmFAD3B* decreased with leaf development. No changes in the expression profile of the plastidial ω -3 desaturases *GmFAD7-1* and *GmFAD7-2* genes were detected. On the contrary, *GmFAD8-2* transcript levels increased while *GmFAD8-1* transcripts decreased during leaf development. Given this expression profile, our data suggested the existence of a temporal regulatory mechanism controlling ω -3 desaturases during leaf development in which the endoplasmic reticulum ω -3 desaturases would be more important in young leaves while plastidial ω -3 desaturases might contribute to 18:3 ^{Δ 9,12,15} production in mature leaves. Photosynthetic cell cultures showed 18:3 ^{Δ 9,12,15} levels similar to those from leaves. No changes in the 18:3 ^{Δ 9,12,15} content or expression of the ω -3 desaturase genes were detected along the cell culture cycle. A comparison of our data with those available in *Arabidopsis* or wheat suggested that the regulatory mechanism controlling the expression and activity of both endoplasmic reticulum and plastidial desaturases during leaf development might differ among plant species.

Keywords: Soybean, *Arabidopsis*, wheat, leaf development, 18:3^{Δ9,12,15}, ω-3 fatty acid desaturase, regulation.

1. Introduction

Fatty acids are the major constituents of membrane lipids. In plants, fatty acid biosynthesis begins in the stroma of plastids (Browse and Somerville, 1991). Fatty acids are then incorporated into the two glycerolipid synthetic pathways that exist in plants. In the prokaryotic pathway, chloroplastic membrane lipids (phosphatidylglycerol, PG; monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG and sulfoquinovosyldiacylglycerol, SL) are synthesized entirely in plastids. In the eukaryotic pathway, phospholipids are synthesized in the endoplasmic reticulum (ER) while MGDG, DGDG and SL are synthesized from phosphatidylcholine (PC) produced in the ER (Browse and Somerville, 1991). The relative amount of glycerolipid synthesis by these two pathways may vary in different tissues and in different plant species. In some plant species like *Arabidopsis*, both pathways contribute almost equally to the synthesis of MGDG, DGDG and SL. These plant species, named 16:3 plants, contain substantial amounts of 16:3 fatty acids esterified in position *sn*-2 of MGDG (Somerville and Browse, 1996). In other plant species such as soybean or wheat, PG is the only product synthesized by the prokaryotic pathway and the rest of the leaf glycerolipids are synthesized through the eukaryotic pathway. These plant species lack the hexatrienoic acid (16:3) and, therefore, contain α-linolenic acid (18:3^{Δ9,12,15}) as the only trienoic fatty acid (Browse and Somerville, 1991). These plants are called 18:3 plants. Trienoic fatty acids are synthesized from dienoic fatty acids by the activity of ω-3 fatty acid desaturases, which are a family of integral membrane enzymes located in two different

cell compartments; FAD3 is localized in the endoplasmic reticulum while FAD7 and FAD8 are localized in plastids (Andreu et al., 2007; Browse et al., 1986a; Wallis and Browse, 2002). Genes encoding these ω -3 fatty acid desaturases have been identified in both model or crop plants (Andreu et al., 2010; Hamada et al., 1996; Horiguchi et al., 1998; Yadav et al., 1993) and in all cases are encoded by nuclear genes. Their number and substrate specificity in *Arabidopsis* have been inferred from the analysis of a comprehensive collection of mutants defective in membrane lipid unsaturation (Wallis and Browse, 2002). In *Arabidopsis*, ω -3 fatty acid desaturases are encoded by single genes (Gibson et al., 1994; Yadav et al., 1993). In other plant species, like soybean or cowpea, several isoforms of the microsomal ω -3 fatty acid desaturase FAD3 have been reported (Anai et al., 2005; Bilyeu et al., 2003; Torres Franklin et al., 2009). Regarding the plastidial desaturases, single *FAD7* and *FAD8* genes were reported in all species analyzed (Hamada et al., 1996; Horiguchi et al., 1998; Nishiuchi et al., 1997; Torres Franklin et al., 2009; Yadav et al., 1993). However, in soybean, two *GmFAD7* and two *GmFAD8* genes have been detected (Andreu et al., 2010; Chi et al., 2011; Román et al., 2012).

Many evidences indicate that fatty acid desaturases are very sensitive to environmental cues such as light (Collados et al., 2006), temperature (Iba, 2002) or development (Horiguchi et al., 1996a; Horiguchi et al., 1998; Horiguchi et al., 1996b). The level of 18:3 usually increases upon leaf development in almost all plant species analyzed like alfalfa (Klopfenstein and Shigley, 1967), barley (Appelqvist et al., 1968), maize (Leech et al., 1973), tobacco (Koiwai et al., 1981), wheat (Horiguchi et al., 1996a; Horiguchi et al., 1998) or *Arabidopsis* (Horiguchi et al., 1996b). This increase of 18:3^{Δ9,12,15} is known to be associated with the biogenesis of plastid membranes, since it is preferentially

found esterified in the plastid galactolipids MGDG and DGDG, (Horiguchi et al., 1998; Leech et al., 1973). However, the molecular basis of this increase of ω -3 desaturase activity is poorly understood.

Most efforts to understand the relative contribution of endoplasmic reticulum and plastidial desaturases to trienoic fatty acid production at the molecular level during leaf development have been focused on monocotyledonous plants. This is because their leaves provide a more simple system than dicotyledonous ones as in the former, cell division occurs at the basal meristem and creates a linear gradient of cell age from basal to apical parts of the leaf (Leech et al., 1973; Mullet, 1988). Thus, in wheat, the spatial distribution patterns of *TaFAD3* and *TaFAD7* mRNAs were different during plant development. *TaFAD3* mRNA accumulated to high levels in proliferating tissues from both shoot and root meristems, but it was not detected during leaf development (Horiguchi et al., 1998). *TaFAD7* mRNA levels remained constant in green leaf tissues throughout development, and no transcript was detected in roots (Horiguchi et al., 1998). These results suggested a different spatial distribution of both fatty acid desaturases to regulate the amount of 18:3^{Δ9,12,15} available for membrane biogenesis or tissue development in monocotyledonous plants. It is worth mentioning that no data concerning the developmental regulation of the *TaFAD8* gene(s) were reported in these works, assuming the cold inducible role of FAD8 in plants (Gibson et al., 1994; McConn et al., 1994). Furthermore, there are no data available about this regulation in dicotyledonous plants, probably because the complexity of leaf development with cell division and elongation taking place simultaneously in various areas of the leaf (Tsukaya, 1995).

In this paper, we studied the accumulation of fatty acids and the expression profiles of ω -3 fatty acid desaturases during seed germination and leaf development in a dicotyledonous plant with high agronomic value like soybean. Our data suggest the existence of a temporal regulatory mechanism controlling the different contribution of endoplasmic reticulum and plastidial ω -3 fatty acid desaturases to the changes in trienoic fatty acids during leaf development. Our data in soybean were compared with those available from *Arabidopsis* or wheat in order to analyze whether differences in embryo development or the contribution of both glycerolipid biosynthetic pathways affected this regulatory mechanism.

2. Results

2.1. Fatty acid composition of germinating soybean seeds

With the exception of flax (*Linum usitatissimum*), that contains high levels of 18:3^{Δ^{9,12,15}} in seeds (Vrinten et al., 2005), most plant species analyzed up to now contain reduced levels of 18:3 (around 10-20 %), with 18:2^{Δ^{9,12}} being the major fatty acid species in soybean (Andreu et al., 2010; Heppard et al., 1996) or *Arabidopsis* (Lemieux et al., 1990). Although the seed fatty acid content has been analyzed in many plant species, data on the fatty acid composition of germinating oilseeds are very scarce. Fig. 1 shows the fatty acid composition of germinating soybean seeds. The fatty acid composition of germinating seeds was very similar to that of developing seeds (Andreu et al., 2010), with high levels of 18:2^{Δ^{9,12}}, which accounted for more than 50% of total fatty acids, and very low levels of 18:3^{Δ^{9,12,15}} (10%). This high 18:2^{Δ^{9,12}} content slightly but significantly decreased after 12 days of seed germination, upon cotyledon greening, without any observable changes in 18:3^{Δ^{9,12,15}} content (Fig.1). Only a slight increase in 18:0 and 16:0 was observed at this stage.

2.2. Expression analysis of ω-3 fatty acid desaturases during soybean seed germination

We analyzed the expression of ω-3 fatty acid desaturase gene families during seed germination by semi-quantitative RT-PCR. The copy number of each ω-3 desaturase gene family, as well as the high degree of homology (70 % including UTR regions on a nucleotide basis) difficult the primer design for appropriate amplicon size and reaction efficiency requirements in accurate q-PCR analysis (Bustin et al., 2009). The results from the RT-PCT analysis are shown in Fig. 2A,B. Upon seed germination, all the ω-3 desaturase genes were expressed. In the case of the endoplasmic reticulum ω-3 fatty

acid desaturases, both RT-PCR and restriction analysis with Van91I indicated that the levels of *GmFAD3A* and *GmFAD3B* transcripts were high after 3, 6 or 9 days of germination, decreasing after 12 days of seed germination (Fig. 2A,B). This is coincident with the greening of cotyledons. In the case of the plastidial fatty acid desaturases, *GmFAD7-1* transcripts decreased during seed germination, while transcript levels of the *GmFAD7-2* gene did not vary significantly under the same experimental conditions (Fig. 2B). Similarly, transcripts from both *GmFAD8* genes presented no significant variation in their expression profile with the exception of a slight but significant decrease of *GmFAD8-1* mRNA after 12 days of seed germination. It is worth mentioning that a lower size band was detected in the RT-PCR expression analysis of the *GmFAD8-1* gene from germinating seeds (Fig. 2A). This band is originated from the splicing of a small intron (70 bp) at the UTR region of the *GmFAD8-1* gene. Nevertheless, the relative amount of this band followed that of the major *GmFAD8-1* transcript. The accumulation of transcripts from *GmFAD3*, *GmFAD7* and *GmFAD8* genes during seed germination did not result in higher levels of ω -3 desaturase activity, according to the levels of 18:3 shown in Fig.1.

2.3. Fatty acid composition in soybean developing trifoliolate leaves

Once the cotyledons were fully opened, trifoliolate leaves emerged and began to expand. Figure 3A shows the fatty acid composition from total lipids isolated from soybean trifoliolate leaves from Recently Opened (hereafter RO), 3, 7, 14 and 18 days of leaf development. The fatty acid profile corresponds to a typical 18:3 species; *i.e.* absence of 16:3 and high accumulation of C18 fatty acids. At the leaf developmental stages studied, α -linolenic acid ($18:3^{\Delta 9,12,15}$) was the most abundant polyunsaturated fatty acid,

increasing from the RO stage (around 40 %) to mature trifoliolate leaves (almost 70 %), (Fig. 3A). This increase in 18:3^{Δ9,12,15} was accompanied by a subsequent decrease in 18:2. In fact, RO trifoliolate leaves showed the highest accumulation of 18:2^{Δ9,12} (around 30 %) while mature trifoliolate leaves showed the lowest one (around 10 %). These results indicate an increase of ω-3 fatty acid desaturase activity during leaf development. As occurred in other plant species analyzed up to now (Horiguchi et al., 1998; Leech et al., 1973), lipid analysis by TLC indicated that most part of the 18:3^{Δ9,12,15} that were detected upon soybean leaf development were associated to MGDG or DGDG plastidial lipids (data not shown).

To further correlate our fatty acid data with chloroplast membrane formation, we monitored the total chlorophyll content and the accumulation of D1 protein during soybean trifoliolate leaf development. Total chlorophyll content, on a fresh weight basis, increased with leaf development (Fig. 3B). This increase correlated well with that of 18:3 levels (Fig. 3A). We also monitored the accumulation of photosynthetic proteins. D1 is one of the protein subunits of the Photosystem II reaction center (Mattoo et al., 1984). Its accumulation is directly related with Photosystem II synthesis and, subsequently, thylakoid membrane formation. D1 protein accumulation during leaf development was monitored by western blotting (Fig. 3B). One band corresponding to the D1 monomer (35-36 kDa) was detected. D1 protein levels were already high in RO leaves, consistent with the high turnover of the D1 protein upon illumination (Alfonso et al., 2004; Mattoo et al., 1984). Nevertheless, D1 protein levels increased dramatically during leaf development (Fig. 3B). These data altogether indicate that the increase observed in 18:3 levels during leaf development correlated well with chloroplast membrane biogenesis. It is noteworthy that even at the beginning of leaf development

(RO stage), relevant amounts of D1 protein and chlorophyll, as well as 18:3^{Δ9,12,15} were detected (Fig. 3A,B), indicating that ω-3 desaturase activity, although increased with leaf age, was already operating at the very early stages of leaf development.

2.4. Expression analysis of ω-3 fatty acid desaturases in soybean developing leaves

As a next step in our analysis, we measured transcript levels of both endoplasmic reticulum and plastidial ω-3 desaturase genes during trifoliolate leaf development. The results of the RT-PCR analysis are shown in Fig. 4A,B. Transcripts from the genes encoding the endoplasmic reticulum ω-3 desaturases (*GmFAD3A* and *GmFAD3B*) were higher at RO or 3 days, and then decreased to low levels in mature trifoliolate leaves (Fig. 4A,B). Digestion with *Van91I* enzyme revealed that both *GmFAD3A* and *GmFAD3B* transcripts decreased upon trifoliolate leaf development, remaining *GmFAD3B* mRNA to residual levels in mature soybean trifoliolate leaves (Fig. 4A,B).

The expression profiles of plastidial ω-3 desaturases showed a completely different pattern of transcript accumulation during trifoliolate leaf development. As showed in Fig. 4, expression levels of *GmFAD7-1* and *GmFAD7-2* genes did not change significantly during leaf development. Expression of both *GmFAD8* genes was also detected in all stages of leaf development (Fig. 4A,B) at control temperatures. Interestingly, both *GmFAD8* isoforms showed a different expression pattern: levels of *GmFAD8-1* mRNA decreased while those of *GmFAD8-2* increased during trifoliolate leaf development (Fig. 4A,B).

2.5. Fatty acid composition in soybean photosynthetic cell suspension cultures

Photosynthetic cell suspensions provide a good model system to examine fatty acid synthesis and turnover in plant cells since they have similar amounts of phospholipids or galactolipids to those present in leaf cells (MacCarthy and Stumpf, 1980; Martin et al., 1984). From the point of view of our analysis, photosynthetic cell suspensions behave similarly to young leaf mesophyll cells (Rogers et al., 1987).

Figure 5 shows the fatty acid composition of total lipids isolated from soybean cell suspensions grown under photoautotrophic conditions during a complete culture cycle (21 days). As occurred in plant leaves, polyunsaturated fatty acids represented more than 75 % of total fatty acids, being α -linolenic acid ($18:3^{\Delta 9,12,15}$) the major species detected (50 – 55 %). $18:3^{\Delta 9,12,15}$ levels showed almost no changes all through the culture cycle with only a slight but not significant increase at the early stages of cell culture at the beginning of the exponential phase (Fig. 5). This fatty acid profile was consistent with that reported previously (Martin et al., 1984; Rogers et al., 1987; Collados et al., 2006) and resembled that of 3 to 7 day-old soybean trifoliolate leaves (Fig. 3A).

2.6. Expression analysis of ω -3 fatty acid desaturases in soybean photosynthetic cell suspension cultures

We analyzed the expression levels of the genes encoding the ω -3 fatty acid desaturases in soybean photosynthetic cell suspension cultures throughout the culture cycle. The results are shown in Fig. 6A,B. Transcripts from both *GmFAD3* gene isoforms were detected at all stages of the cell culture cycle (Fig. 6A). Van91I restriction analysis

suggested that the *GmFAD3B* isoform was more expressed than *GmFAD3A* in these photosynthetic cultures (Fig. 6A), but no significant changes in their expression profile were detected along the cell culture cycle. The plastidial ω -3 desaturases *GmFAD7* and *GmFAD8* gene families were also expressed (Fig. 6A,B). However, no significant changes were observed in the expression of any of these genes during the cell culture cycle (Fig. 6B). It is important to note that the presence of *GmFAD3*, *GmFAD7* and *GmFAD8* transcripts in photosynthetic cultured cells resembled the expression profile of young developing trifoliolate leaves (Fig 4A,B).

2.7. eFP Browser analysis of the expression of the ω -3 desaturases from Arabidopsis during leaf development

At this point, question merged whether this regulatory mechanism observed in soybean could be found completely or partially in other plant species. Data from wheat (monocotyledonous and 18:3 plant) were described in Horiguchi et al. (1998). Data from *Arabidopsis* (dicotyledonous 16:3 plant) were available at the eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) obtained during the microarray analysis during plant development (Schmid et al., 2005). *Arabidopsis* ω -3 desaturase gene accession numbers were: *AtFAD3* (At2g29980), *AtFAD7* (At3g11170) and *AtFAD8* (At5g05580). Sequences are available in TAIR <http://www.arabidopsis.org/>. It is interesting to point out that, in *Arabidopsis*, this analysis is complicated by the fact that the array chip used (ATH1) did not distinguish between the *AtFAD7* and *AtFAD8* genes. In general, it is assumed that the contribution of the *AtFAD8* gene at control temperatures is negligible (Gibson et al., 1994). However, a higher contribution of this gene even at control temperatures in *Arabidopsis*, cannot be discarded. Accordingly, we

will refer as *AtFAD7+AtFAD8* expression to designate the expression of both plastidial ω -3 desaturases from *Arabidopsis*. These results are shown in Figure 7. *AtFAD3* and (*AtFAD7+AtFAD8*) gene expression was detected either in young or mature rosette leaves (Fig. 7). The expression of these genes did not show any leaf developmental pattern in contrast to what occurred in soybean. It is noteworthy that absolute expression values of *AtFAD7+AtFAD8* genes were always higher than *AtFAD3* all through leaf development, ranging from 227 to 834 from minimum to maximum in the case of the *AtFAD3* gene and 854 to 1474 from minimum to maximum in the case of the *AtFAD7+AtFAD8* gene expression (Fig. 7B).

3. Discussion

The aim of this work was to analyze the changes in fatty acid content and their correlation with the expression profiles of the genes encoding both the endoplasmic reticulum and plastidial ω -3 desaturases during seed germination and leaf development in soybean. Soybean seeds are characterized by high levels of linoleic acid (18:2 ^{Δ 9,12}) and low levels of 18:3 ^{Δ 9,12,15} (Heppard et al., 1996, Lemieux et al., 1990, Andreu et al., 2010). In contrast with developing oilseeds, the events occurring during seed germination at the fatty acid content and desaturase gene expression levels have not been studied into detail. Our data show that in germinating seeds, high transcript level accumulation of both endoplasmic reticulum (*GmFAD3*) and plastidial (*GmFAD7* and *GmFAD8*) gene families occurs. The presence of these high abundant transcripts was concomitant with the reduced 18:3 ^{Δ 9,12,15} levels detected in soybean seeds (around 10 %), that did not vary during the initial stages of seed germination and shoot differentiation. These results might suggest the existence of a tight control of ω -3 fatty acid desaturase activity in soybean seeds and that this control should operate at the post-transcriptional level. Data obtained from the *Arabidopsis fad3* mutant pointed to endoplasmic reticulum ω -3 desaturase activity as the major responsible of 18:3 ^{Δ 9,12,15} synthesis in seeds (Lemieux et al., 1990). Whether it is the endoplasmic reticulum ω -3 desaturase activity the major responsible of the reduced 18:3 ^{Δ 9,12,15} production during soybean seed germination remains to be experimentally elucidated. However, given the high transcript levels of *GmFAD7* and *GmFAD8* genes (particularly *GmFAD7-2* and *GmFAD8-2*) we cannot preclude a role of plastidial *GmFAD7* and *GmFAD8* ω -3 desaturase activities during seed germination.

Once after germination, linolenic acid levels increased during soybean leaf development. Although this increase has been well documented in many plant species (Klopfenstein and Shigley, 1967; Appelqvist et al., 1968; Leech et al., 1973; Koiwai et al., 1981; Horiguchi et al., 1996a; Horiguchi et al., 1996b), very few studies have covered this question at the molecular level and, to our knowledge, only in a monocotyledonous plant like wheat (Horiguchi et al., 1998). The main objective of this work was to cover this lack of information. In soybean, a temporal regulation pattern of the *GmFAD3* and *GmFAD8* genes was detected. *GmFAD3A* and *GmFAD3B* transcript accumulation decreased with leaf development suggesting a more relevant contribution for the endoplasmic reticulum enzymes in young leaves. A high expression of both *GmFAD3A* and *GmFAD3B* genes was also detected in photosynthetic cultured cells. These photosynthetic cells behave both physiologically and developmentally similar to young mesophyll leaf cells (Rogers et al., 1987). This similar expression pattern in photosynthetic cells and young leaves might further strengthen our hypothesis of the role of endoplasmic reticulum ω -3 desaturases in young proliferating soybean leaf tissue. Interestingly, in spite of the decrease in *GmFAD3* mRNA levels with leaf age, 18:3^{A9,12,15} levels increased during leaf development. Based in the expression data it should be concluded that this increase in 18:3^{A9,12,15} with leaf development resulted mainly from the plastidial ω -3 desaturase activity. Our data showed that *GmFAD8-2* mRNA levels increased during this process, without significant changes in the transcript levels of *GmFAD7-1* or *GmFAD7-2*. The absence of changes at the *FAD7* gene expression pattern in response to leaf development seemed to be also the case in wheat (Horiguchi et al., 1998) or Arabidopsis (Fig. 6). This observation strongly suggests that the main mechanism controlling *FAD7* gene expression and activity in plants during leaf development is based on post-transcriptional regulatory mechanisms. The presence

of such regulatory mechanisms acting on FAD7 has already been postulated in *Arabidopsis* or soybean photosynthetic suspension cultures acting either at mRNA stability or at the protein level. (Collados et al., 2006; Matsuda et al., 2005). More intriguing is the behavior of the *GmFAD8* genes that showed a specific and opposite pattern of expression during soybean leaf development. The role of the FAD8 protein is still subject of debate. Although believed to be a cold-induced ω -3 desaturase, in the absence of specific biochemical data, the presence of *GmFAD8-1* and *GmFAD8-2* transcripts in developing leaves (or seeds) at control temperatures as well as their specific pattern of expression make it difficult to preclude a specific contribution of *GmFAD8* (particularly *GmFAD8-2*) to 18:3^{Δ9,12,15} biosynthesis during leaf development. However, in the absence of functional or biochemical data of these enzymes, it is very difficult to determine the contribution of each specific desaturase to the 18:3^{Δ9,12,15} content detected during leaf development.

We also compared our data from soybean with those available at the eFP browser obtained from microarray analysis of *Arabidopsis* development (Schmid et al., 2005) and those from Horiguchi et al. (1998) in wheat. The idea was to evaluate common and different regulatory mechanisms participating in the control of fatty acid unsaturation during leaf development. The temporal regulatory pattern observed during soybean leaf development was not observed in *Arabidopsis*. According to the eFP browser data, transcripts from both endoplasmic reticulum (*AtFAD3*) and plastidial (*AtFAD7* and *AtFAD8*) ω -3 desaturases were detected in *Arabidopsis* leaves without significant changes during leaf development. Furthermore, the absolute expression values of the genes encoding the plastidial enzymes (*AtFAD7+AtFAD8*) were higher than those from *AtFAD3*, independently of leaf developmental stage. This observation could be in

agreement with the fact that, in *Arabidopsis*, a 16:3 plant, the plastid is more important for glycerolipid biosynthesis than in 18:3 plants like soybean (Browse et al., 1986b). In that sense, a knock-out mutation in the *fad7* gene substantially reduced the accumulation of 18:3^{Δ9,12,15} and 16:3^{Δ7,10,13} fatty acids during leaf development in all membrane lipids, particularly in the major plastid lipids MGDG and DGDG, demonstrating the essential role of *AtFAD7* in the trienoic fatty acid biosynthesis in *Arabidopsis* and the high importance of the plastid in this process (Horiguchi et al., 1996b).

In wheat (an 18:3 monocotyledonous plant), a spatial regulatory mechanism separates the expression of the endoplasmic reticulum *TaFAD3* gene in non-photosynthetic tissues (like root or in highly proliferating meristems) while *TaFAD7* mRNA was detected in high amounts in leaves without any significant changes with leaf age (Horiguchi et al., 1998). Whether this expression pattern is related to the different leaf developmental models between monocotyledonous and dicotyledonous plants would require a more extensive analysis in other plant species. However, from the point of view of the glycerolipid synthesis pathways, the expression pattern of the *TaFAD3* and *TaFAD7* genes suggest that in wheat, all 18:3^{Δ9,12,15} detected in plastidial and extra-plastidial lipids in leaves might be originated from the plastidial ω-3 desaturase activity while in non-photosynthetic tissues, like root or highly proliferating meristems, it would be the endoplasmic reticulum FAD3 activity the major responsible for 18:3^{Δ9,12,15} production.

4. Conclusions

Our results indicate the existence of a tight control of the ω -3 desaturase activity during seed germination and leaf development in soybean. The reduced 18:3 ^{Δ 9,12,15} content in the presence of *GmFAD3*, *GmFAD7* and *GmFAD8* transcripts suggest that during seed germination the ω -3 desaturase activity is controlled at the posttranscriptional level. A concerted temporal regulatory mechanism seemed to control the different contribution of the endoplasmic reticulum and plastidial ω -3 desaturases to 18:3 ^{Δ 9,12,15} biosynthesis during leaf development in soybean. Our data suggest that the contribution of endoplasmic reticulum ω -3 desaturases would be more important in young leaves while plastidial ω -3 fatty acid desaturases would be more important in mature leaves. Furthermore, the comparative analyses with other plant species like Arabidopsis or wheat suggests that the regulation of endoplasmic reticulum and plastidial ω -3 fatty acid desaturases and their contribution to the 18:3 ^{Δ 9,12,15} levels in membrane lipids during leaf development might differ among plant species. These differences could be related to the different contribution of the plastid and the endoplasmic reticulum to glycerolipid biosynthesis as well as differences in their embryo developmental pattern. This information might be relevant to understand how plants exchange lipids and fatty acids among membranes during leaf or plant development.

5. Experimental

5.1 Plant materials and growth conditions

Soybean plants (*Glycine max* cv. Volania) were germinated in a mixture of water humidified perlite/vermiculite (50 % v/v). Seeds were vernalized for 48 h at 4 °C in darkness and then moved to a growth chamber and left for 48 h in darkness (wrapped in aluminum foil). Then, the foil was removed to illuminate the seeds, and seed germination started. Samples were taken at 3, 6, 9 and 12 days of seed germination. Growth conditions were a 16/8 h light/darkness photoperiod at 24 °C and a relative humidity of 65 %. Shoots were transferred to hydroponic culture as described in Andreu et al. (2010). For leaf development experiments, developing trifoliate leaves were tagged and maintained in hydroponic culture. Leaves corresponding to RO (recently opened), 3, 7, 14 and 18 days of development were collected for subsequent analysis. All samples were collected, rapidly frozen in liquid nitrogen and stored at -80 °C until use.

Photosynthetic cell suspension cultures from soybean (*Glycine max* cv. Corsoy) were grown as previously described in Collados et al. (2006). Cell suspensions were grown in KN⁰ medium (without sucrose supplement) at 24 °C in a rotary shaker at 130 rpm in a 5 % CO₂ atmosphere under continuous light (35 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Culture samples were taken at 0, 7, 14 and 21 days, corresponding to the different stages of a cell growth cycle, frozen in liquid nitrogen and stored at -80 °C until use.

5.2. RNA isolation and cDNA synthesis

Total RNA was isolated from 0.5 g of tissue using the Trizol Reagent (Invitrogen) and further purified using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. A DNase treatment with recombinant DNase from Roche was performed. cDNA synthesis was performed using oligodT and M-MLV reverse transcriptase from Promega according to the manufacturer's instructions.

5.3. Expression analysis of ω -3 fatty acid desaturases

The expression levels of the genes encoding the ω -3 fatty acid desaturases (*GmFAD3A*, *GmFAD3B*, *GmFAD7-1*, *GmFAD7-2*, *GmFAD8-1* and *GmFAD8-2*) were examined by a semi-quantitative RT-PCR assay. Soybean ω -3 desaturase gene accession numbers were: *GmFAD3A* (Glyma14g37350), *GmFAD3B* (Glyma02g39230), *GmFAD7-1* (Glyma18g43210), *GmFAD7-2* (Glyma07g18350), *GmFAD8-1* (Glyma01g29630) and *GmFAD8-2* (Glyma03g07570). Sequences are available in <http://www.phytozome.net/v8.0>. The specific oligonucleotides used for amplification of each gene as well as the PCR conditions were described in Román et al. (2012). Additional primers were used for amplification of a second reference gene, EF1 α _F: 5' CTATGCCCCTGTCCTCGAC 3' and EF1 α _R: 5' GG TAGGATCCTTCTTCTCAACGT 3'. Amplification reactions were carried out using BioTaq DNA polymerase (Bioline) according to manufacturer's instructions. Amplified products were resolved by electrophoresis on 1 % (w/v) agarose gels. Densitometric quantification of the PCR bands was performed using an image densitometer (Gel Doc XR, Bio-Rad) and the image analysis software Quantity One (Bio-Rad). Semi quantification of the relative gene expression levels was performed through normalization against the reference genes (*ACTIN* and *EF1 α*). For

quantification of the *GmFAD3A* and *GmFAD3B* transcripts, a restriction analysis of the *GmFAD3AB* amplification product was performed using Van91I as previously described in Andreu et al. (2010).

5.4. Lipid analysis

Total lipids were extracted from different soybean tissues as described in Bligh and Dyer, (1959). Lipids were transesterified with potassium hydroxide in methanol. The fatty acid methyl esters were analyzed and quantified using a gas chromatograph (HP model 5890 series 2 plus) equipped with SE2330 column (30 x 0.25 mm inner diameter, 0.2 μ m df) and a flame ionization detector (FID). Hydrogen was used as a carrier gas with a linear rate of 1.34 ml.min⁻¹. Injector and detector temperature was 220°C and the oven temperature was 170°C.

5.5. Chlorophyll extraction

Total chlorophyll was extracted with 80 % (v/v) aqueous acetone following the Arnon method (Arnon, 1949).

5.6. Western blot analysis

Total protein was extracted from leaves at different stages of development using the procedures described in Andreu et al. (2010), and quantified using the BIO-RAD protein assay reagent. Five micrograms of total protein were loaded per lane. Western blot procedures were performed as previously described in Andreu et al. (2007) using a

commercial antibody (AS05-084 from Agrisera, Sweden) against the Photosystem II reaction centre D1 subunit (product of the chloroplastic *psbA* gene).

5.8. Statistics and data analysis

Data from fatty acid analysis were obtained from two independent biological experiments with two technical repeats for experiment. Data from the expression analysis of ω -3 fatty acid desaturase genes were obtained from three independent biological experiments. Values in the graphics represent means and standard deviations. Differences between sampling points were analyzed by one-way ANOVA, followed by posthoc multiple comparisons of means using the Duncan test ($P < 0.05$). The statistical program SPSS 19 was used for this purpose.

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References

- Alfonso, M., Collados, R., Yruela, I., Picorel, R., 2004. Photoinhibition and recovery in a herbicide-resistant mutant from *Glycine max* (L.) Merr. cell cultures deficient in fatty acid unsaturation. *Planta* 219, 428-439.
- Anai, T., Yamada, T., Kinoshita, T., Rahman, S. M., Takagi, Y., 2005. Identification of corresponding genes for three low-alpha-linolenic acid mutants and elucidation of their contribution to fatty acid biosynthesis in soybean seed. *Plant Sci.* 168, 1615-1623.
- Andreu, V., Collados, R., Testillano, P., Risueño, M., Picorel, R., Alfonso, M., 2007. In situ molecular identification of the plastid omega-3 fatty acid desaturase FAD7 from soybean: evidence of thylakoid membrane localization. *Plant Physiol.* 145, 1336-1344.
- Andreu, V., Lagunas, B., Collados, R., Picorel, R., Alfonso, M., 2010. The *GmFAD7* gene family from soybean: identification of novel genes and tissue-specific conformations of the FAD7 enzyme involved in desaturase activity. *J. Exp. Bot.* 61, 3371-3384.
- Appelqvist, L. A., von Wettstein, D., Boynton, J. E., Stumpf, P. K., 1968. Lipid biosynthesis in relation to chloroplast development in barley. *J. Lipid Res.* 9, 425-436.
- Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1-15.
- Bilyeu, K. D., Palavalli, L., Sleper, D. A., Beuselinck, P. R., 2003. Three microsomal omega-3 fatty-acid desaturase genes contribute to soybean linolenic acid levels. *Crop Sci.* 43, 1833-1838.
- Bligh, E. G., Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* 37, 911-917.
- Browse, J., McCourt, P., Somerville, C., 1986a. A mutant of *Arabidopsis* deficient in C(18:3) and C(16:3) leaf lipids. *Plant Physiol.* 81, 859-864.
- Browse, J., Somerville, C., 1991. Glycerolipid synthesis: Biochemistry and regulation. *Annu. Rev. Plant. Phys.* 42, 467-506.
- Browse, J., Warwick, N., Somerville, C. R., Slack, C. R., 1986b. Fluxes through the prokaryotic and eukaryotic pathways of lipid syntehsis in the 16:3 plant *Arabidopsis thaliana*. *Biochem. J.* 235, 25-31.
- Bustin, S., Benes, V., Garson, J., Hellemans, J., Huggett, J., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55, 611-622.
- Collados, R., Andreu, V., Picorel, R., Alfonso, M., 2006. A light-sensitive mechanism differently regulates transcription and transcript stability of omega3 fatty-acid desaturases (*FAD3*, *FAD7* and *FAD8*) in soybean photosynthetic cell suspensions. *FEBS lett.* 580, 4934-4940.
- Chi, X., Yang, Q., Lu, Y., Wang, J., Zhang, Q., 2011. Genome-Wide Analysis of Fatty Acid Desaturases in Soybean (*Glycine max*). *Plant Mol. Biol. Rep.* 29, 769-783.
- Gibson, S., Arondel, V., Iba, K., Somerville, C., 1994. Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol.* 106, 1615-1621.

- Hamada, T., Nishiuchi, T., Kodama, H., Nishimura, M., Iba, K., 1996. cDNA cloning of a wounding-inducible gene encoding a plastid omega-3 fatty acid desaturase from tobacco. *Plant Cell Physiol.* 37, 606-611.
- Heppard, E. P., Kinney, A. J., Stecca, K. L., Miao, G. H., 1996. Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. *Plant Physiol.* 110, 311-319.
- Horiguchi, G., Iwakawa, H., Kodama, H., Kawakami, N., Nishimura, M., 1996a. Expression of a gene for plastid omega-3 fatty acid desaturase and changes in lipid and fatty acid compositions in light- and dark-grown wheat leaves. *Physiol. Plant.* 96, 275-283.
- Horiguchi, G., Kawakami, N., Kusumi, K., Kodama, H., Iba, K., 1998. Developmental regulation of genes for microsome and plastid omega-3 fatty acid desaturases in wheat (*Triticum aestivum* L.). *Plant Cell Physiol.* 39, 540-544.
- Horiguchi, G., Kodama, H., Nishimura, M., Iba, K., 1996b. Role of omega-3 fatty acid desaturases in the regulation of the level of trienoic fatty acids during leaf cell maturation. *Planta* 199, 439-442.
- Iba, K., 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant. Biol.* 53, 225-245.
- Klopfenstein, W. E., Shigley, J. W., 1967. Changes in fatty acid composition of sulfolipid and phospholipids during maturation of alfalfa. *J. Lipid Res.* 8, 350-351.
- Koiwai, A., Matsuzaki, T., Suzuki, F., Kawashima, N., 1981. Changes in total and polar lipids and their fatty acid composition in Tobacco leaves during growth and senescence. *Plant Cell Physiol.* 22, 1059-1065.
- Leech, R. M., Rumsby, M. G., Thomson, W. W., 1973. Plastid differentiation, acyl lipid, and fatty acid changes in developing green maize leaves. *Plant Physiol.* 52, 240-245.
- Lemieux, B., Miquel, M., Somerville, C., Browse, J., 1990. Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. *Theoretical and Applied Genetics* 80, 234-240.
- MacCarthy, J. J., Stumpf, P. K., 1980. Effect of different temperatures on fatty acid synthesis and polyunsaturation in cell suspension cultures. *Planta* 147, 389-395.
- Martin, B. A., Horn, M. E., Widholm, J. M., Rinne, R. W., 1984. Synthesis, composition and location of glycerolipids in photoautotrophic soybean cell cultures. *Biochim. Biophys. Acta.* 796, 146-154.
- Matsuda, O., Sakamoto, H., Hashimoto, T., Iba, K., 2005. A temperature-sensitive mechanism that regulates post-translational stability of a plastidial omega-3 fatty acid desaturase (FAD8) in *Arabidopsis* leaf tissues. *J. Biol. Chem.* 280, 3597-3604.
- Mattoo, A. K., Hoffmanfalk, H., Edelman, M., Hoffman Falk, H., Marder, J. B., 1984. Regulation of protein metabolism: Coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. *Proc. Natl. Acad. Sci. USA* 81, 1380-1384.
- McConn, M., Hugly, S., Browse, J., Somerville, C., 1994. A mutation at the FAD8 locus of *Arabidopsis* identifies a second chloroplast omega-3 desaturase. *Plant Physiol.* 106, 1609-1614.
- Mullet, J. E., 1988. Chloroplast development and gene expression. *Annu. Rev. Plant. Phys.* 39, 475-502.
- Nishiuchi, T., Hamada, T., Kodama, H., Iba, K., 1997. Wounding changes the spatial expression pattern of the *Arabidopsis* plastid omega-3 fatty acid desaturase gene (FAD7) through different signal transduction pathways. *Plant Cell* 9, 1701-1712.

- Rogers, S. M., Ogren, W. L., Widholm, J. M., 1987. Photosynthetic characteristics of a photoautotrophic cell suspension culture of soybean. *Plant Physiol.* 84, 1451-1456.
- Román, A., Andreu, V., Luisa Hernández, M., Lagunas, B., Picorel, R., Hernandez, M. L., Martínez-Rivas, J. M., Alfonso, M., 2012. Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean. *J. Exp. Bot.* 63, 4973-4982.
- Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., 2005. A gene expression map of *Arabidopsis thaliana* development. *Nat. Genet.* 37, 501-506.
- Somerville, C., Browse, J., 1996. Dissecting desaturation: plants prove advantageous. *Trends in Cell Biol.* 6, 148-153.
- Torres Franklin, M.-L., Repellin, A., Huynh, V.-B., d'Arcy Lameta, A., Zuily Fodil, Y., 2009. Omega-3 fatty acid desaturase (*FAD3*, *FAD7*, *FAD8*) gene expression and linolenic acid content in cowpea leaves submitted to drought and after rehydration. *Environ. Exp. Bot.* 65, 162-169.
- Tsukaya, H., 1995. Developmental genetics of leaf morphogenesis in dicotyledonous plants. *J. Plant Res.* 108, 407-416.
- Vrinten, P., Hu, Z. Y., Munchinsky, M. A., Rowland, G., Qiu, X., 2005. Two *FAD3* desaturase genes control the level of linolenic acid in flax seed. *Plant Physiol.* 139, 79-87.
- Wallis, J., Browse, J., 2002. Mutants of *Arabidopsis* reveal many roles for membrane lipids. *Prog. Lipid Res.* 41, 254-278.
- Yadav, N. S., Wierzbicki, A., Aegerter, M., Perezgrau, L., Caster, C. S., Pérez-Grau, L., Kinney, A. J., Hitz, W. D., Booth, J. R., Schweiger, B., Stecca, K. L., 1993. Cloning of higher plant omega-3 fatty acid desaturases. *Plant Physiol.* 103, 467-476.

Figure legends

Fig. 1. Fatty acid composition analysis of germinating soybean seeds. Data are expressed as molar percentages from the quantitative analysis of peak areas obtained by GC analysis. Data are mean $\pm$ S.D. from two independent experiments. For the same fatty acid, different letters indicate significant differences among sample points ($P < 0.05$).

Fig. 2. Expression of ω -3 desaturase genes during soybean seed germination. (A) Semi-quantitative RT-PCR of ω -3 desaturase genes during soybean seed germination. *ACTIN* and *EFL α* were used as reference genes in all experiments. (B) Relative expression values after normalization against reference genes. Data are mean $\pm$ S.D. from three independent experiments. For each gene different letters indicate significant differences among sample points ($P < 0.05$). Absence of letters indicates no significant differences among sample points.

Fig. 3. Changes in fatty acid and chlorophyll composition during soybean leaf development. (A) Fatty acid composition analysis of developing trifoliate leaves. Data are expressed as molar percentages from the quantitative analysis of peak areas obtained by GC analysis. Data are mean $\pm$ S.D. from two independent experiments. For the same fatty acid, different letters indicate significant differences among sample points ($P < 0.05$). (B) Total chlorophyll accumulation (on a fresh weight basis) and Western blot analysis of the Photosystem II reaction centre D1 subunit during trifoliate leaf

development. Chlorophyll data are mean $\pm$ S.D. from two independent experiments. For western blot analysis, five micrograms of total leaf protein were loaded per lane.

Fig.4. Expression of ω -3 desaturase genes during soybean leaf development. (A) Semi-quantitative RT-PCR of ω -3 desaturase genes during soybean trifoliolate leaf development. *ACTIN* and *EFL α* were used as reference genes in all experiments. (B) Relative expression values after normalization against reference genes. Data are mean $\pm$ S.D. from three independent experiments. For each gene different letters indicate significant differences among sample points ($P < 0.05$). Absence of letters indicates no significant differences among sample points.

Fig. 5. Fatty acid composition analysis of soybean photosynthetic suspension cells during a complete cell culture cycle. Data are expressed as molar percentages from the quantitative analysis of peak areas obtained by GC analysis. Data are mean $\pm$ S.D. from two independent experiments. For the same fatty acid, different letters indicate significant differences among sample points ($P < 0.05$). Absence of letters indicates no significant differences among sample points.

Fig. 6. Expression of ω -3 desaturase genes during a soybean photosynthetic cell suspension culture cycle. (A) Semi-quantitative RT-PCR of ω -3 desaturase genes during a soybean photosynthetic cell suspension culture cycle. *ACTIN* and *EFL α* were used as reference genes in all experiments. (B) Relative expression values after normalization

against reference genes. For each gene different letters indicate significant differences among sample points ($P < 0.05$). Absence of letters indicates no significant differences among sample points.

Fig. 7. ω -3 desaturase gene expression in *Arabidopsis* leaves. (A) Relative ω -3 desaturase gene expression during *Arabidopsis* leaf development. Absolute normalized values were obtained from eFP Browser. Microarray ATH1 chip data do not allow to differentiate between *AtFAD7* and *AtFAD8*. (B) eFP browser images from both *AtFAD3* and *AtFAD7+AtFAD8* genes. Absolute value scale is different for both genes.

Fig. 1

Fig.2, Lagunas et al.

A

B

Fig. 3, Lagunas et al.

Fig.4, Lagunas et al.

A

B

Fig.5, Lagunas et al.

A **B**

Fig.7, Lagunas et al.