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**Running head:** non-redundant FAD7 and FAD8 responses to stress

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# Tissue Distribution and Specific Contribution of *Arabidopsis* FAD7 and FAD8 Plastid Desaturases to the JA and ABA Mediated Cold Stress or Defence Responses

**Running head:** non-redundant FAD7 and FAD8 responses to stress

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**Abbreviations:** ABA, abscisic acid; ACP, acyl-carrier protein; BiFC, bimolecular fluorescence complementation; DA, Dienoic fatty acids; DGDG, digalactosyl-diacylglycerol; ER, endoplasmic reticulum; FAE, fatty acid elongase; FAS, fatty acid synthase; GFP, green fluorescent protein; GUS, glucuronidase; JA, jasmonate; LOX, lipoxygenase; MGDG, monogalactosyl-diacylglycerol; PG, phosphatidyl-glycerol; RT-qPCR, quantitative real-time PCR; TA, trienoic fatty acid; YFP, yellow fluorescent protein.

To overcome the difficulties to analyze membrane desaturases at the protein level, transgenic *Arabidopsis* plants expressing the plastidial *AtFAD7* and *AtFAD8*  $\omega$ -3 desaturases fused to GFP, under the control of their endogenous promoters, were generated and their tissue relative abundance was studied. Gene expression, GUS promoter activity, immunoblot and confocal microscopy analyses indicated that *AtFAD7* is the major  $\omega$ -3 desaturase in leaves when compared to *AtFAD8*. This higher abundance of *AtFAD7* was consistent with its higher promoter activity and could be related with its specificity for the abundant leaf galactolipids. *AtFAD7* was also present in roots but at much lower level than in leaves. Our data suggest that the *AtFAD7* protein in roots might participate in the supply of precursors for JA biosynthesis rather than membrane lipid unsaturation. *AtFAD7* protein levels increased in response to wounding but not to JA, and decreased upon ABA treatment. Conversely, *AtFAD8* protein levels increased upon cold or JA exposure and decreased at high temperatures, but did not respond to ABA or wounding. These results indicated specific and non-redundant roles for the plastidial  $\omega$ -3 desaturases in defence, temperature stress or phytohormone mediated responses and a tight coordination of their activities between biotic and abiotic stress signalling pathways. Our data suggested that transcriptional regulation was crucial for this coordination. Finally, bimolecular fluorescence complementation analysis showed that both *AtFAD7* and *AtFAD8* interact with the *AtFAD6*  $\omega$ -6 desaturase *in vivo*, suggesting that quaternary complexes are involved in trienoic fatty acid production within the plastid membranes.

**Keywords:** *Arabidopsis thaliana*, fatty acid, FAD7, FAD8, ABA, cold, JA, wounding.

## Introduction

Trienoic fatty acids (TAs) are key components of the plant responses against biotic and abiotic stress. As major constituents of plant glycerolipids (Browse et al., 1986), they influence the function of biological membranes by maintaining their appropriate fluidity, playing a key role in plant adaptation to temperature changes (reviewed by Iba, 2002). The relationship between the unsaturation of membrane lipids and temperature acclimation has been established in the past (Nishida and Murata, 1996; Iba, 2002). In addition, certain lipids like phosphatidylglycerol (PG) have been directly linked to chilling sensitivity (Murata et al., 1982). TAs also serve as precursors of plant hormones, like jasmonates, that are directly involved in defence signalling against pathogen attack, participate in the wound response and are also important for plant development and adaptation to environmental stress (Schaller and Stinzi, 2009). Biosynthesis of jasmonates is initiated in the plastid through the action of specific lipases that release TAs from the glycerol backbone making them accessible to lipoxygenases (LOX), that initiate the conversion of these TAs to jasmonates (Wasternack and Hause, 2013).

TAs are synthesized from dienoic fatty acids (DAs) by the activity of  $\omega$ -3 desaturases that are a family of integral membrane enzymes localized in two different cell compartments: FAD3 is specific of the endoplasmic reticulum (ER; Dyer and Mullen, 2001) while FAD7 and FAD8 are plastid specific (Browse et al., 1986; Wallis and Browse, 2002; Román et al., 2015). In *Arabidopsis*,  $\omega$ -3 fatty acid desaturases are encoded by single genes (Yadav et al., 1993; Gibson et al., 1994) while in other plant species like soybean, several isoforms of the microsomal and plastidial  $\omega$ -3 fatty acid desaturases have been reported (Bilyeu et al., 2003; Andreu et al., 2010; Román et al., 2015). Both plastidial  $\omega$ -3 desaturases, FAD7 and FAD8, are highly homologous (more than 90% identity at the protein sequence level), and perform a similar function in the same sub-cellular compartment, the plastid envelope (Ferro et al., 2003; Román et al., 2015). It was initially reported that FAD8 was a cold-specific desaturase (McConn et al., 1994).

However, recent analysis of a collection of loss-of-function mutants from Arabidopsis for the *AtFAD7* and *AtFAD8* genes revealed that *AtFAD8* is also active at control growth temperatures and differences in substrate specificity at polar lipid head group (FAD8 showed preference for PG and sulfolipids) and acyl chain length (FAD7 uses both 16:2 and 18:2 as substrates while FAD8 uses only 18:2 substrates) existed (Román et al., 2015). These data also indicated that *AtFAD8* was active at normal temperatures and could compensate, at least partially, the absence of a functional *AtFAD7* enzyme at 22 °C (Román et al., 2015), implying the presence of a functional *AtFAD8* protein at 22 °C, but experimental evidences of its presence were still lacking. This non-redundant role of both plastidial  $\omega$ -3 desaturases in the biosynthesis of TAs in the plastid (Roman et al., 2015), could be extended to their participation in plant responses to biotic or abiotic stress. Thus, the wound responsive pattern of the *FAD7* gene has been reported in different plant species, consistent with the increasing demand of TAs for JA biosynthesis (Hamada et al., 1996; Reymond et al., 2000; Nishiuchi et al., 1997). However, no data are available of the responsiveness of the *FAD8* gene to JAs or its implication in defence responses. On the other hand, induction of *FAD8* gene expression at low temperatures has also been reported (McConn et al., 1994; Berberich et al., 1998; Román et al., 2015), consistent with a higher  $\omega$ -3 desaturase activity on plastid lipids to maintain plastid membrane fluidity. The use of *myc*-tagged FAD7 and FAD8 proteins expressed in Arabidopsis indicated that protein stability might contribute to the FAD8 specific role at low temperatures (Matsuda et al., 2005). However, there has been no experimental support showing that translation is the key regulatory mechanism controlling the activity of the FAD8 enzyme in response to cold. Nevertheless, we are still far from understanding how the activity of both plastidial  $\omega$ -3 desaturases is specifically regulated in response to these stimuli. Most of our conclusions were obtained through the correlation of *FAD* gene expression levels with changes in the fatty acid content (Gibson et al., 1994; Berberich et al., 1998; Horiguchi et al., 1998; Collados et al., 2006; Martz et al., 2006;

Román et al., 2012; Lagunas et al., 2013; Roman et al., 2015). However, our comprehension of desaturase function at the protein level and more concretely, the relative abundance of FAD7 and FAD8 proteins in the different plant tissues and organs, as well as their variation in response to environmental or developmental stimuli is poorly understood. This analysis of desaturase regulation at the protein level is hampered by their high degree of homology as well as their highly hydrophobic nature, limiting the utilization of specific antibodies or the development of purification protocols. In addition, transgenic lines that expressed the protein under constitutive promoters like 35S (Matsuda et al., 2005; Román et al., 2015), were useful for localization purposes but their protein levels did not represent their actual regulation. This limits our comprehension of the relevance of translation or post-translational mechanisms in the regulation and coordination of plastidial  $\omega$ -3 desaturases to stress or hormone responses. Question arises whether the different response of both plastidial  $\omega$ -3 desaturases is regulated mainly at the transcriptional level, through the action of specific transcription factors, or are post-transcriptional regulatory mechanisms behind some of these specific responses. Furthermore, much of the data are focused in leaves, but very little information, if any, is available about the relative abundance and function of both plastid  $\omega$ -3 desaturases in other plant tissues and organs like roots or flowers, where TAs participate in developmental processes like root elongation or pollen development (Staswick et al., 1992; McConn and Browse, 1996).

In this work, we tried to overcome some of the above-mentioned issues through the obtention of stable transgenic lines of *Arabidopsis thaliana* that expressed both *AtFAD7* and *AtFAD8* proteins fused to a green fluorescent protein (GFP) under the control of their endogenous promoter sequences. This strategy allowed us to distinguish both *AtFAD7* and *AtFAD8* proteins, which are highly identical at the protein sequence level, and analyze their specific regulation. Data obtained with these lines, together with gene expression analysis, indicated that *AtFAD7* is the major  $\omega$ -3 desaturase either in leaves or in non-photosynthetic tissues like

roots, when compared to *AtFAD8*. Relative abundance of both *AtFAD7* and *AtFAD8* proteins was analysed in response to wounding, temperature or hormones like JA and ABA and a specific behaviour of both proteins with a clear specialization among both desaturases was observed. In most cases, a positive correlation between transcript and protein levels could be established, suggesting that transcriptional control is at the basis of their coordination. Finally, bimolecular fluorescence complementation (BiFC) analysis using both FAD7 and FAD8  $\omega$ -3 desaturases with the  $\omega$ -6 desaturase FAD6, responsible of 18:2 biosynthesis in the plastid, indicated that FAD7 and FAD8 interacted with FAD6 *in vivo*, but not among them, at least significantly. These results suggested the existence of supramolecular complexes involved in the production of TAs within plastid membranes.

## Results

### **Generation of transgenic lines expressing *AtFAD7* and *AtFAD8* proteins fused to GFP under the control of their endogenous promoters.**

A strategy based in the use of protein fusions to GFP expressed under the control of the endogenous promoters of both genes was developed to analyse *AtFAD7* and *AtFAD8* at the protein level. First, two sequences of 1682 and 2958 bp upstream of the *AtFAD7* and *AtFAD8* ATG codons, respectively, were chosen to be analysed for their promoter activity. The 1682 bp upstream sequence of the *AtFAD7* (*At3g11170*) gene, located at chromosome 3, contained 853 bp of the non-coding sequence upstream the *AtFAD7* gene and 843 bp of the coding sequence of the *At3g11165* gene, located in the antisense strand, that encodes a protein with unknown function according to TAIR (Supplementary Fig. 1). In the case of the *AtFAD8* gene (*At5g05580*), the 2958 sequence upstream its ATG was located in chromosome 5 and contained



438 bp corresponding to the non-coding upstream sequence from the *AtFAD8* gene and 2520 bp of the coding sequence of the *At5g05570* gene, located in the same strand than *AtFAD8* (Supplementary Fig. 1). *At5g05570* encodes a nuclear transducing protein with methyltransferase activity, according to TAIR. Election of these long sequences was made after analysis with PLACE and PlantCARE tools, which located several putative regulatory elements in these upstream sequences from both promoters that **could be involved in their specific regulation** (Supplementary Fig. 1). Prior to their utilization for protein analysis, these promoter sequences were fused to GUS to check for promoter activity. Several independent T3 lines carrying the 1.7 kb *AtFAD7* promoter fused to GUS (*AtFAD7p1,7kb::GUS*) were analyzed for GUS staining. High GUS activity was detected upon one h staining on leaf tissue including vasculature in either cotyledonal or rosette leaves from 14-d old plants (Fig. 1A and B). GUS staining was also detected in either primary or secondary roots as well as in the root tip, concretely in the root cap (Fig.1C). No GUS staining was detected in leaves or roots from transgenic lines expressing the empty pMDC163 vector (Fig.1L-M). In independent T3 lines harboring the *AtFAD8* gene putative promoter (*AtFAD8p3kb::GUS*), 1 h staining only produced significant GUS staining in cotyledonal leaves (Fig. 1D-F). When the time of staining was increased up to three hours, GUS activity was high in cotyledonal leaves and could also be detected in rosette leaves from 14-d old plants, but with much lower intensity than that of the *AtFAD7p1,7kb::GUS* lines after 1 hour (Fig.1H-I). In the *AtFAD8p3kb::GUS* lines, very low GUS staining was detected in roots, never in the root-tip (Fig. 1K).

In order to confirm these results, those lines which showed a reproducible pattern of GUS staining were selected for the determination of GUS activity. To that end, either leaf or root extracts were obtained from several independent transgenic lines (n=6). As shown in Fig. 1 O, leaf extracts from transgenic lines carrying the *AtFAD7* 1,7 kb promoter sequence showed a glucoronidase activity with values that ranged from 20 to 25 nmol MUG min<sup>-1</sup> mg. protein<sup>-1</sup>

(Fig. 10). These MUG activity values were very similar to those reported previously by Nishiuchi et al., (1995) when they assayed an Arabidopsis 0,7 kb *FAD7* promoter fragment in transgenic tobacco lines. Plants carrying the 2,9kb *AtFAD8* promoter fragment showed reduced levels of GUS activity when compared with those carrying the 1,7 kb *AtFAD7* promoter fragment, with values between 4-5 nmol MUG min<sup>-1</sup> mg. protein<sup>-1</sup> (Fig. 10). These differences in promoter activity might be consistent with the differences in GUS incubation time required for proper GUS staining detected in both promoters. GUS activity was also monitored in root extracts from these transgenic lines. Root extracts from transgenic lines carrying the *AtFAD7* 1,7 kb promoter sequence showed values lower than 2 nmol MUG min<sup>-1</sup> mg. protein<sup>-1</sup>; much lower than that detected in leaves. GUS activity in plants carrying the 2,9kb *AtFAD8* promoter fragment was barely undetectable under our experimental conditions.

Once the activity of both *AtFAD7* and *AtFAD8* gene promoter sequences was analysed, transgenic lines were obtained that expressed the *AtFAD7*-GFP fusion under the control of the 1,7 kb *AtFAD7* promoter (designated as TG7 lines) and lines expressing the *AtFAD8*-GFP fusion under the control of the 2,9 kb *AtFAD8* promoter (designated as TG8 lines). Since the transgenic lines were generated on a wild-type background, containing the endogenous *AtFAD7* and *AtFAD8* genes, we first analysed the expression of both *AtFAD7* and *AtFAD8* genes in these transgenic lines compared to that from the Col0 line. Several lines were checked and those showing no changes with respect to Col0 were discarded for subsequent analysis. Another group of lines showed increased *AtFAD7* or *AtFAD8* mRNA levels with respect to Col0. Thus, TG7 lines showed an average increase in *AtFAD7* transcript levels of 2-3 fold, with respect to Col0 lines (supplementary Fig. 2A). *AtFAD8* gene expression in the TG7 background was comparable to that from Col0 (supplementary Fig. 2A). Similarly, TG8 lines showed an average increase in *AtFAD8* transcript levels of 2 fold, with respect to Col0 lines (supplementary Fig. 2A), without significant changes in *AtFAD7* transcript levels. These results suggested that the

transgene did not significantly affect to the expression of the other  $\omega$ -3 plastidial desaturase counterpart gene. This was confirmed by the analysis of the expression of the reticular  $\omega$ -3 desaturase *AtFAD3* in either Col0, TG7 and TG8 lines, with no changes in *AtFAD3* transcript levels (supplementary Fig. 2A). The phenotype of the selected TG7 and TG8 plants was also analysed. Essentially, no differences in growth, leaf or rosette size or flowering pattern were detected in TG7 or TG8 lines when grown under control culture conditions (supplementary Fig. 2B).

### **Confocal microscopy analysis of *AtFAD7*-GFP and *AtFAD8*-GFP protein distribution in TG7 and TG8 lines.**

Confocal microscopy analysis of transgenic lines expressing *AtFAD7*-GFP or *AtFAD8*-GFP fusions, under the control of their respective endogenous promoters, is shown in Fig. 2. Fourteen day-old TG7 transgenic lines expressing the 1682 bp *FAD7* promoter::*FAD7*-GFP construct, showed a strong GFP signal associated with chloroplasts from either cotyledonal or rosette leaves. In all cases, the GFP fluorescent signal was detected as a typical ring covering the whole plastid surface that fully co-localized with the chlorophyll red auto-fluorescence, indicating that the signal was plastid specific (Figs. 2A-C). It is worth mentioning that the signal was detected abundantly in plastids from both young (Fig. 2B) and mature rosette leaves (Fig. 2C), being less abundant in cotyledonal leaves (Fig. 2A). In young rosette leaves and to a lesser extent in mature rosette leaves, a strong GFP signal was detected also in small chloroplasts (Figs. 2B and 2C). When TG8 transgenic lines carrying the 2958 bp *FAD8* promoter::*FAD8*-GFP construct were analysed, low GFP fluorescent signal was observed in plastids from cotyledonal leaves (Fig. 2D). Low GFP fluorescence signal was detected in plastids from young or mature leaves from TG8 lines (Fig. 2E and F). Transgenic plants carrying the empty vector (pMDC83) did not show any GFP fluorescent signal, detecting only the chlorophyll red auto-

fluorescence signal from plastids (Supplementary Fig. 3). These results altogether strongly suggested that the FAD7 protein was much more abundant than FAD8 in Arabidopsis leaves.

Omega-3 fatty acids (18:3 and 16:3) are less abundant in roots, with an average mole percent of 24.6% and 1.56% of 18:3 and 16:3, respectively, in 15 day-old roots (Beaudoin et al., 2009; Miquel and Browse, 1992). In spite of this lower TA content, the FAD7 protein was detected in roots from TG7 lines. The distribution of the fluorescent GFP signal in root tissues was not homogeneous. Strong GFP fluorescent signal was detected in the root tip (Fig. 3A) and in the cells surrounding the root vasculature (Fig. 3D). As occurred in leaves, more difficult was to detect a specific signal in TG8 lines. Almost no GFP signal, if any, was detected in the root-tip (Fig. 3B) while some GFP signal was detected in the cells surrounding the root vasculature (Fig. 3E). Transgenic lines expressing the empty pMDC83 vector showed only the red fluorescence signal associated with the propidium-iodine staining (Fig. 3C and 3F), indicating that the GFP signal detected in the root, mostly with the FAD7 construct, was specific. Since light has been reported as a regulatory signal for FAD7 gene expression (Nishiuchi et al., 1995; Collados et al., 2006), we analysed whether the presence of the *AtFAD7* protein in the root could be caused by illumination of the root tissue. To that end, TG7 plants were grown vertically in MS plates with the area of root growth covered to avoid illumination. Confocal microscopy analysis of roots from TG7 plants showed a similar pattern of localization in the root when compared to fully illuminated plants (Supplementary Fig. 4), indicating that the presence of the *AtFAD7* protein in the root was not an artefact of illumination.

### **Relative abundance of the *AtFAD7* and *AtFAD8* proteins in leaf and root tissues**

Relative protein abundance was analyzed by western blot, using an anti-GFP antibody, to confirm the confocal microscopy data. Protein extracts were obtained from rosette leaves and

roots from 14 day-old grown TG7 and TG8 transgenic lines. A band of 70-78 kDa molecular weight was detected in protein extracts obtained from rosette leaves from 14 day-old TG7 plants (Fig. 4, left panel). The apparent size of this band (around 70 kDa) was consistent with the molecular weight of the FAD7 protein (50 kDa) and that of GFP (27 kDa). The 70 kDa band representing the FAD7-GFP monomer was highly abundant in extracts from leaves of TG7 plants. A strong signal in the high molecular weight range ( $> 100$  kDa) was also detected repeatedly in the western blot analysis (Fig. 4), which could be originated by protein aggregates, usual in membrane proteins like FAD7. In addition, several faint bands in the low molecular range ( $< 30$  kDa) were also detected (Fig. 4) that may represent degraded GFP protein. In the case of the *AtFAD8* protein, a band with a molecular weight around 70kDa, compatible with that of the FAD8 protein (50 kDa) fused to GFP (27 kDa) was detected (Fig. 4, left panel). This band had a similar molecular weight with respect to that detected in the extracts from TG7 lines, consistent with the almost identical theoretical molecular mass of both proteins. It is worth mentioning that total amount of protein extract was increased from 20 to 35-40  $\mu$ g in the case of the *AtFAD8*-GFP lines to favour its detection in the view of the data from GUS activity and the confocal analysis (Figs. 1 and 2). These results indicated that, although in lower amounts, *AtFAD8* was present in leaf protein extracts at control temperatures. Nevertheless, these data confirmed that the *AtFAD7* protein is the major  $\omega$ -3 desaturase in leaves. The FAD7-GFP protein was also detected in roots from TG7 lines, but at much lower amounts than those detected in leaves (Fig. 4, right panel). No FAD8-GFP protein was detected in root extracts from TG8 lines (Supplementary Fig. 5), indicating that, as occurred in leaves, *AtFAD7* was also the major plastidial  $\omega$ -3 desaturase in root tissues.

To further investigate the role of plastidial  $\omega$ -3 desaturases, particularly FAD7, in roots, several mutants deficient in fatty acid desaturase activity were grown in MS agar plates for 10 days and fatty acid composition of total root lipids was determined. These mutants included a *fad3-1*

mutant, deficient in FAD3 activity (Browse et al., 1993); two T-DNA insertion lines in the *FAD7* and *FAD8* genes, *fad7i* and *fad8i* (Román et al., 2015) and a *fad7-2 fad8-1* double mutant (McConn et al., 1994). Col-0 plants showed a 19,5% of 18:3 fatty acids, with a high content of 18:2 (25,5%) and 16:0 (20%) as major fatty acids in roots (Table 1). This fatty acid composition is consistent with that reported previously for Arabidopsis root total lipids (Li-Beisson et al., 2013). The *fad3-1* mutant showed a dramatic decrease to 3,5% of 18:3 in its root lipids and a concomitant increase of 18:2 levels up to 45% (Table 1). On the contrary, all mutations affecting plastidial  $\omega$ -3 desaturases showed no reduction of 18:3 content in root lipids, with almost similar or even slightly higher levels of 18:3 (Table 1). These results further confirmed that the major desaturase responsible of TA biosynthesis in roots is FAD3. In spite of the fact that the FAD7 plastidial desaturase was detected in roots, it seemed not to be essential for TA synthesis.

### **Relative abundance of leaf *AtFAD7* and *AtFAD8* proteins in response to wounding and hormones**

The effect of wounding and jasmonate supplementation on *AtFAD7* and *AtFAD8* protein levels was studied. First, the expression of both *AtFAD7* and *AtFAD8* genes was monitored in Col-0, TG7 and TG8 plants after wounding or 100  $\mu$ M MeJA treatment for 1 and 2h, to correlate changes in transcript levels with changes in protein levels. LOX2 was used as internal control of the MeJA treatment (Supplementary Fig. 6). qRT-PCR expression analysis showed that *AtFAD7* mRNA increased up to 4 fold upon 1 hour wounding with respect to control-unwounded Col0 plants (Fig. 5A). These high *AtFAD7* mRNA levels were maintained after 2 hours of wounding (Fig. 5A). A similar increase of *AtFAD7* mRNA levels upon wounding was also detected in TG7 or TG8 lines (Fig. 5A). This response of the *AtFAD7* gene seemed to be specific since no changes were detected for the *AtFAD8* mRNA upon wounding in either Col0,

TG7 or TG8 lines. Exposure of Col-0 plants to MeJA resulted in an average 1.7-fold increase of *AtFAD7* mRNA with respect to Col0 untreated plants after 1 h of treatment, but increased up to 3,5 fold after 2h of MeJA exposure (Fig. 5A). TG7 lines also experienced a high increase of *AtFAD7* mRNA upon JA treatment, reaching average values of 5,5 fold with respect to control untreated TG7 plants (Fig. 5). This increase was also observed in TG8 lines, which showed an expression pattern of *AtFAD7* gene upon wounding almost similar to that from Col0 plants (Fig. 5). Differently to what happened upon wounding, JA treatment also modified the expression of the *AtFAD8* gene. Thus, Col0 plants showed an increase of *AtFAD8* transcript levels that reached average 2-fold values after 2 h of treatment (Fig. 5A). Similar increases were also detected in either TG7 or TG8 lines, suggesting that MeJA induced *AtFAD8* gene expression.

Then, we correlated the changes in transcript levels with changes in protein levels. To that end, mature 14 day-old Arabidopsis plants harboring the *AtFAD7*p1,7kb::*FAD7-GFP* and *AtFAD8*p3kb::*FAD8-GFP* constructs were subjected to wound treatment by pressing the leaf with forceps. Leaves were flash frozen at 1 and 2 h after wounding, protein extracts were obtained and analyzed by western blot, using GFP antibodies. *AtFAD7* protein levels increased upon wounding in TG7 plants (Fig. 5B). This increase in *AtFAD7* protein levels could be correlated with changes in *AtFAD7* transcript levels in TG7 plants and either Col-0 plants upon wounding (Fig. 5A). TG8 lines did not show any changes in *AtFAD8* protein levels upon wounding (Fig. 5B), consistent with the gene expression data and indicating that *AtFAD8* was not sensitive to wounding neither at the gene or protein levels. JA supplementation was also investigated. TG7 and TG8 lines were sprayed with a 100  $\mu$ M MeJA solution. Rosette leaves were taken at 1 h and 2 h after MeJA treatment, protein extracts were obtained and western blot was carried out using the anti-GFP antibody. JA supplementation did not result in significant changes of the *AtFAD7* protein levels (Fig. 5B). On the contrary, an increase in *AtFAD8* protein

levels was observed upon 1 h of MeJA supplementation in TG8 lines (Fig. 5B). This increase was consistent with the qPCR expression data, suggesting that *AtFAD8* was JA-sensitive wound-independent.

Abscisic acid (ABA) is the major hormone involved in plant responses against abiotic stresses like drought, salt or cold (reviewed in Zhu, 2002). We investigated the effect of ABA on the relative abundance of both *AtFAD7* and *AtFAD8* proteins. Again, expression analysis of both *AtFAD7* and *AtFAD8* genes in Col-0, TG7 and TG8 plants in response to ABA was carried out. *ABI1* was used as internal control of the ABA treatment (Supplementary Fig. 6). Addition of ABA to Col-0 plants resulted in a strong decrease (more than 50% with respect to control values) of the *AtFAD7* mRNA in leaves with respect to Col0 untreated plants (Fig. 6A). A similar decrease was detected in TG7 or TG8 plants (Fig. 6A). This effect of ABA was specific of *AtFAD7* since no significant modifications of *AtFAD8* mRNA levels were observed in leaves upon ABA addition in Col0, TG7 or TG8 plants (Fig. 5A). The effect of ABA was also monitored at the protein level. Both TG7 and TG8 plants grown in MS plates were subjected to ABA treatment (100  $\mu$ M) for 48 h and then western blot analyses using the anti-GFP antibody was performed. As shown in Fig. 6B, ABA treatment resulted in a dramatic decrease of *AtFAD7*-GFP protein in TG7 plants. On the contrary, no changes in *AtFAD8*-GFP protein levels were observed in TG8 lines, indicating that the effect of ABA was specific of *AtFAD7* and seemed to operate both at the transcript and protein levels. Confocal microscopy of TG7 plant lines was performed to contrast the results obtained by western blotting. As shown in Supplementary Fig. 5, TG7 lines treated with 100  $\mu$ M for 48 h showed a dramatic decrease of the GFP fluorescent signal when compared with untreated control plants, indicating that ABA specifically affected *AtFAD7* gene expression in Arabidopsis leaves.



### **Effect of temperature on *AtFAD7* and *AtFAD8* protein levels**

The effect of low (6°C) or high (35 °C) temperatures of *AtFAD7* and *AtFAD8* protein levels was also investigated. When cold temperatures were analysed, two week-old Col0, TG7 and TG8 plants were kept at 6°C for an additional week. Then rosette leaves were frozen for further expression and protein analysis. As shown in Fig. 7A, *AtFAD7* mRNA levels decreased in Col0 plants upon exposure to cold temperatures. This decrease is consistent with previous data in *Arabidopsis* or maize (Román et al., 2015; Berberich et al., 1998). However, no changes in *AtFAD7* transcript levels were detected in TG7 or TG8 lines upon cold temperature exposure (Fig. 7A). On the contrary, *AtFAD8* mRNA showed an increase of 2-fold in Col0, TG7 and TG8 plants upon cold temperature exposure (Fig. 7A), consistent with previous observations in *Arabidopsis* (McConn et al., 1994; Román et al, 2015). Then, total leaf protein extracts from TG7 and TG8 plant lines grown at 22 °C or kept at 6°C for an additional week were obtained and blotted with the anti-GFP antibody. OEE33 was used as internal control. As shown in Fig. 7B, TG7 lines showed no changes in *AtFAD7*-GFP protein levels upon exposure to 6 °C. This result correlated with the absence of changes in *AtFAD7* transcript levels obtained in TG7 and TG8 lines. Conversely, TG8 lines carrying the *AtFAD8p3kb::FAD8-GFP* construct showed a clear increase of the *AtFAD8*-GFP protein levels (Fig. 7B), which again correlated with the changes in *AtFAD8* mRNA in response to cold (McConn et al, 1994; Román et al., 2015). Confocal microscopy analysis was performed to contrast the western blot protein data. TG8 plants carrying the *AtFAD8p3kb::FAD8-GFP* construct showed an increase in GFP fluorescence associated to the *AtFAD8* protein at low temperatures (Supplementary Fig. 6). This increase was specific since no changes were observed in TG7 plants (Supplementary Fig. 7). These results confirmed the western blot analysis and indicated that cold temperatures affected to the *AtFAD8* gene both at the transcriptional and protein levels.

The effect of high temperatures was also studied. To that end, two-week old Col0, TG7 and TG8 plants were exposed to 35°C for 5 days. Then, rosette leaves were frozen and kept for further analysis. Col0 plants showed a decrease of *AtFAD7* mRNA levels upon high temperature exposure, reaching values closer to 50% those of control untreated plants (Fig. 7A). However, this decrease in *AtFAD7* mRNA levels was not observed in TG7 or TG8 plant lines (Fig. 7A). When the expression of *AtFAD8* gene was analyzed, no changes in transcript levels were detected in Col0, TG7 or TG8 plants upon 35°C treatment (Fig. 7A), suggesting that high temperature exposure did not affect to the *AtFAD8* gene at the transcriptional level. Then, changes in protein abundance were also analyzed. *AtFAD7*-GFP protein levels did not show any significant change upon exposure of plants to 35°C for 5 days (Fig. 7B). On the contrary, TG8 lines showed a dramatic decrease of *AtFAD8*-GFP protein levels upon exposure to high temperatures (Fig. 7B). This decrease might be consistent with previous observations in *Arabidopsis* using chimeric myc-FAD8 fusions (Matsuda et al., 2005). These results suggested that *AtFAD8* was highly sensitive to temperature and that high temperatures affected its expression at the post-transcriptional level.

### **Analysis of the *in vivo* interaction of plastidial $\omega$ -3 desaturases with the plastidial $\omega$ -6 desaturase FAD6**

We analyzed whether both plastidial  $\omega$ -3 desaturases FAD7 and FAD8 can interact *in vivo* with their plastidial counterpart, the  $\omega$ -6 desaturase FAD6. To that end, a bimolecular fluorescence complementation assay using the split-YFP system in *Arabidopsis* protoplasts was performed. The different constructs carrying each desaturase (FAD6, FAD7 or FAD8) were co-transformed in protoplasts and the interactions between pairs of proteins were monitored by the detection of the YFP signal. The BiFC analysis showed the existence of interactions between the  $\omega$ -6 FAD6 and the  $\omega$ -3 FAD7 desaturases, obtaining similar results when both proteins carried either the

N-terminus or C-terminus portion of the YFP (Fig. 7C and 7D). When the interaction between FAD6 and FAD8 was tested, the YFP signal was detected with both protein pairs, although the data showed better results when the FAD6 protein carried the N-terminus of the YFP protein and FAD8 the C-terminus of the YFP (Fig. 7 E and F). Since the obtention of the YFP signal is not only dependent of the distance but also the orientation of both proteins, these results might suggest some preference of certain regions from both proteins for the interaction.

The possibility of forming heterodimers between FAD7 and FAD8  $\omega$ -3 desaturases was also tested. Very weak YFP signal was obtained when the FAD7/FAD8 protein pair was assayed (Fig. 7G and 7H), suggesting that, if it occurs; this interaction might be very weak. On the contrary, both  $\omega$ -3 desaturases could form homodimers *in vivo* as deduced from the results obtained when the assay was performed with the FAD7 or FAD8 proteins, respectively (Fig. 7I and 7J).

Protein-protein interaction was also analyzed by co-localization and fluorescence resonance energy transfer (FRET) analysis. To that end, both plastidial  $\omega$ -3 desaturases *AtFAD7* and *AtFAD8* were fused to YFP and expressed under a constitutive 35S promoter. The plastidial  $\omega$ -6 desaturase *AtFAD6* was fused to CFP under the control of a constitutive 35S promoter. Then, transient expression experiments were performed on *N. benthamiana* leaves in which the 35S:FAD7-YFP or 35S:FAD8-YFP were co-transformed with the 35S:FAD6-CFP construct. Confocal microscopy of transiently transformed *N. benthamiana* leaves was performed. Special care was taken to confirm that no YFP signal was detected through the CFP channel and viceversa under our experimental conditions. Blue fluorescent signal from FAD6-CFP protein was detected when transiently transformed leaves were illuminated with the 458 nm CFP laser. This signal was not very abundant but co-localized with the red chlorophyll autofluorescence. When samples were illuminated with the 458 nm argon laser (CFP), FAD7-YFP or FAD8-YFP signals were also detected in the surface of the chloroplast. These signals co-localized with the

red autofluorescence signal from the chlorophyll (Supplementary Fig. 8). It is worth mentioning that intense yellow fluorescent dots were also observed for both proteins (Supplementary Fig. 6). This pattern of localization in discrete *foci* is shared with many plastid proteins, particularly those located in the envelope, expressed under constitutive promoters (Haswell and Meyerowitz, 2006; Breuers et al., 2012). We have already described such a pattern for *AtFAD7*, *AtFAD8* and *Tic40* proteins (Roman et al., 2015). These results suggested that the distance between both proteins was less than 10 Å and therefore compatible with a possible interaction between both proteins.

## Discussion

In this work, we analysed the non-redundant role of the  $\omega$ -3 plastidial desaturases *AtFAD7* and *AtFAD8* in response to stress and hormones, analysing the transcriptional and post-transcriptional components of their different regulation, as well as their relative abundance and protein distribution in the different plant tissues. To that end, stable transgenic lines that expressed both *AtFAD7* and *AtFAD8* proteins fused to GFP under the control of their endogenous promoters were used. Both our expression analysis by qPCR and GUS staining and activity, as well as confocal microscopy and western blot analyses showed that, under normal growth conditions, the *AtFAD7* protein was highly abundant in leaves and was present in lower amounts in roots. The analysis of *AtFAD8*p3kb::*FAD8-GFP* (TG8) lines showed that *AtFAD8* protein was present in leaves from plants grown at 22 °C, but at much lower amounts than *AtFAD7*, indicating that *FAD7* was the major  $\omega$ -3 desaturase in the plastid. This higher abundance of *FAD7*, particularly in leaf tissue, could be consistent with its higher promoter activity and the higher specificity of *FAD7* for galactolipids MGDG or DGDG, which are the major chloroplast lipids with higher TA content. The lower relative abundance of *AtFAD8* could be also explained by its lower promoter activity, as shown in the GUS data. Again, *AtFAD8* has higher specificity for PG or sulfolipids (Román et al., 2015), which are minor components of leaf plastid membranes (around 5%). These results might suggest that transcriptional control is key to determine the differences in relative abundance between both proteins at least in leaves. Our data also showed that the *AtFAD7* gene was expressed and the *AtFAD7* protein accumulated in roots. Root lipids do not contain high levels of TAs, being *FAD3* the major contributor to their synthesis in this tissue (Lemieux et al., 1990). Furthermore, the difficulties to use reduced ferredoxin in root cells as well as the root lipid analysis in the desaturase mutants shown in Table 1 might suggest that the root *AtFAD7* might not be functional or at least not directly involved in the membrane TA synthesis. Whether it could

serve to produce TAs for JA biosynthesis, which has specific roles in root tissues like a negative effector of root length (Staswick et al., 1992), remains to be elucidated.

Our data point to the existence of specific and highly coordinated responses of *AtFAD7* and *AtFAD8* desaturases to stress and hormones. Thus, *AtFAD7*, but not *AtFAD8* protein levels increased in response to wounding, consistent with the gene expression analysis (Fig. 5). Given the position of *AtFAD7* at the basis of the jasmonate biosynthetic pathway to provide precursors (TAs) for its synthesis, this wound-responsive behaviour is not striking. However, no changes in *AtFAD7* protein were detected in response to JA even if an increase in *AtFAD7* mRNA levels was observed (Fig. 5). On the contrary, *AtFAD8* showed a clear JA-dependent response both at the gene expression and protein levels (Fig.5). These results suggest that both plastidial desaturases are coordinated in defence responses in a specific manner, showing differences in their wound and JA-dependent profile. Such differences have been already described for many wound- and JA- responsive genes through different studies, including microarray analysis (Titarenko et al., 1997; Taki et al., 2005; Stotz et al., 2013). It has been proposed that two signalling pathways are activated upon wounding: one of them does not require JA and might be responsible of the activation of the defence response at the wound site (local), while the other one, involves JA perception and biosynthesis to activate the defence response in the rest of the plant (systemic response) (Howe, 2004). *AtFAD7* and *AtFAD8* proteins might differently participate in these two wounding signalling pathways. Furthermore, differences in defence responses against herbivorous insects or necrotrophic pathogens have been reported in which JA-dependent and independent signalling pathways trigger different coordinated responses (Wasternack and Hause, 2013). It is tempting to speculate that the differences in wound and jasmonate response among *AtFAD7* and *AtFAD8* plastidial desaturases could be related with different sensitivity to insects or pathogen attack. More striking was the effect of ABA on *FAD7* protein abundance. Both western blot and confocal microscopy analyses showed a dramatic

decrease of *AtFAD7* protein levels in response to ABA (Fig. 5). This decrease occurred concomitantly with a decrease of *AtFAD7* mRNA levels (Fig. 5). This could be consistent with the presence of two ABA repression sequences, CAACTTG and GAAGTTG (Wang et al., 2011) placed at -278/-267 and -203/-197, respectively, with respect to the ATG in the *AtFAD7* promoter sequence. These sequences are present in most ABA-repressed genes (Wang et al., 2011). Since ABA is the major hormone involved in abiotic stress, this repression of *AtFAD7* gene expression and protein levels by ABA might be related with a higher specialization of FAD7 on biotic and defence responses (as supplier of JA biosynthesis precursors), that could be blocked antagonically by ABA. In that sense, our data also showed an increase of *AtFAD8* mRNA (Román et al., 2015) and protein levels in response to cold. The cold-specific response of *AtFAD8* might be consistent with its specificity for PG, and the specific role of this lipid in maintaining the stability of photosynthetic complexes under stress conditions (Wada and Murata, 2007). Our data also showed that this cold-specific response of *AtFAD8* was not sensitive to ABA neither at the gene nor protein levels (Fig.5). The different effect of cold, wounding, ABA or JA on the expression of *AtFAD7* and *AtFAD8* further demonstrates the non-redundant role of both plastidial  $\omega$ -3 desaturases and the tight coordination of their activity in response to biotic and abiotic stresses. It is worth mentioning that, with the exception of the increase of *AtFAD7* mRNA upon JA supplementation, the increase (or decrease) in FAD7 or FAD8 protein levels, followed the increase (or decrease) of *AtFAD7* and *AtFAD8* transcript levels. These results suggest that transcriptional regulation is crucial for the control of plastidial  $\omega$ -3 FAD7 and FAD8 desaturase activity and it is at the basis of the coordination of the responses of both  $\omega$ -3 desaturases to stress and hormones. Analysis of the promoters from both genes and identification of the different regulatory elements participating in their specific responses might help to clarify this point.

Our bimolecular complementation assay indicated that both plastidial  $\omega$ -3 desaturases FAD7 and FAD8 can form heterodimers *in vivo* with their plastidial counterpart, the  $\omega$ -6 desaturase FAD6, responsible of the biosynthesis of 18:2 (or 16:2) in the plastid. These results are consistent with yeast complementation studies that suggested that the ER  $\omega$ -3 and  $\omega$ -6 desaturases, FAD2 and FAD3 also formed heterodimers that might facilitate metabolic channelling of 18:1 to produce 18:3 *in vivo* (Lou et al., 2014). These results altogether point to the existence of quaternary complexes within the membrane as responsible of the production of polyunsaturated fatty acids. This is not striking if we consider that their orthologs in yeast, the integral membrane desaturase Ole1p, also forms homodimers *in vivo* (Lou and Shanklin, 2010). The soluble stearyl-ACP desaturase, FAB2, the only desaturase for which a tridimensional study is available, also forms homodimers in its functional form *in vivo*. Furthermore, many enzymes involved in the biosynthesis or modification of fatty acids like the fatty acid synthase (FAS) in the plastid or the fatty acid elongase (FAE) are constituted of a complex of specialized membrane proteins, indicating that the production and modification of fatty acids is associated to the formation of membrane quaternary complexes, as seems to be the case of plastidial  $\omega$ -6 and  $\omega$ -3 desaturases. Further studies might be required to investigate how the specific FAD6/FAD7 and FAD6/FAD8 complexes are formed and how they are distributed within the envelope membrane.

## Conclusions

Our strategy based in the obtention of transgenic lines expressing both *AtFAD7* and *AtFAD8* proteins, fused to GFP, under the control of their endogenous promoters, has provided a picture of the tissue specific distribution and relative abundance of both plastidial  $\omega$ -3 desaturases in leaves and non-photosynthetic tissues like roots. Our data have revealed a specific and highly coordinated response of both proteins to defence, temperature and phytohormones, showing a



clear specialization between both  $\omega$ -3 desaturases and a strong coordination between stress and hormone signalling pathways, at the transcriptional level, for the control of their specific activities. Functional dissection of their promoters will help to clarify how this coordination takes place.

## Materials and Methods

### Plant materials

*Arabidopsis thaliana* Col-0 ecotype was used as wild-type. The transgenic lines generated in this work were obtained from Col-0 plants. The fatty acid desaturase mutants *fad3-1*, *fad7i*, *fad8i* and the double mutant *fad7-2 fad8-1* were available from NASC. The *fad7i* and *fad8i* mutants were T-DNA insertion lines characterized in previous works (Román et al., 2015). The EMS *fad3-1* and double *fad7-2 fad8-1* mutants have been characterized previously (McConn et al., 1994). *Arabidopsis* seeds were sterilized and germinated in MS medium or directly in pots. Seeds were vernalized for 3 days at 4 °C and then moved to a growth chamber for 14 days. 14 day-old rosette leaves were frozen in liquid nitrogen and stored at –80 °C until use. Growth conditions were light intensity of 100  $\mu\text{mol. m}^{-2} \cdot \text{s}^{-1}$  in a 16/8 h light/dark photoperiod at 22/18 °C and a relative humidity of 60/65%.

### Generation of *AtFAD7*p1,7kb::*FAD7-GFP* (TG7) and *AtFAD8*p3kb::*FAD8-GFP* (TG8)

#### Arabidopsis lines

*Arabidopsis* genomic DNA was isolated by CTAB method (Doyle and Doyle, 1990). Fragments of -1682 and 2958 bp corresponding to the *AtFAD7* and *AtFAD8* putative promoter sequences, respectively, were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo) and cloned in a pENTR-D-TOPO vector. *AtFAD7* and *AtFAD8* promoter fragments, flanked by the appropriate *attL* sites, were subcloned in a pMDC163 plasmid (Curtiss and Grossnicklaus, 2003) through Gateway technology using LRclonaseII. *Agrobacterium* mediated transformation (GV3101 strain) of *Arabidopsis* plants was performed by floral dip (Clough and Bent, 1998). Positive transformants were selected for hygromycin resistance and genotyped by Phire® Plant direct PCR kit (Thermo). Homozygote T3 lines were fully segregated and

multiple independent transgenic events were used for the analysis of GUS activity. To create the promoter::protein-GFP constructs, the two -1682 and 2958 bp fragments corresponding to the *AtFAD7* and *AtFAD8* putative promoter sequences, respectively, were amplified by PCR using Phusion High-Fidelity DNA Polymerase lines and specific primers (Supplementary table 1) that contained *KpnI* and *XmaI* restriction sites to allow their cloning in the pUC57 L4-*KpnI*\_XmaI-R1 vector. This introduced two *attL4* and *attR1* sequences flanking both promoters to facilitate their directional multisite gateway cloning into the entry vector. In parallel, pENTER D-TOPO vectors carrying the coding sequences from both *AtFAD7* and *AtFAD8* genes without their respective STOP codons were used (Román et al., 2015). A third plasmid, pEN-R2F-L3, (Plant Systems Biology, Ghent University, Belgium; Karimi et al., 2007) contained a GFP coding sequence flanked by two *attR2* and *attL3* sequences. Multisite gateway cloning was performed using the plasmid pH7m34GW (Plant Systems Biology, Ghent University, Belgium; Karimi et al., 2005) as destination vector and the enzyme LR Clonase II®. Positive transformants were selected in streptomycin/spectinomycin selective media and further checked by *KpnI*, *XmaI* digestion and sequencing of the positive selected clones. Positive *Arabidopsis* transformants were selected for hygromycin resistance and were genotyped by Phire® Plant Direct PCR Kit (Thermo). Homozygote lines T3 were segregated. Lines producing 100% resistant plantlets were selected (T3 homozygous lines, single insertion locus) and used for further analysis. Plants carrying the *AtFAD7*p1,7kb::*FAD7-GFP* were designated as TG7 lines while those carrying the *AtFAD8*p3kb::*FAD8-GFP* were designated as TG8 lines. In all the experiments, data from three independent transgenic events were used for the analysis.

### **Histochemical and fluorimetric GUS assays**

GUS staining protocol was adapted from Jefferson et al., (1987). Samples were vacuum infiltrated for 15-20 min with GUS staining buffer (50 mM sodium phosphate pH=7.2, 10 mM

EDTA, 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100) containing 1 mg/ml 5-bromo-4-chloro-3-indolylglucuronide (Thermo). GUS staining was performed at 37 °C in darkness for 1-3 hours. Chlorophyll was removed after 15 min 3:1 (v/v) EtOH:acetic acid incubation and subsequent 70% (v/v) EtOH overnight incubation. Samples were visualized in a Leica M165 FC stereomicroscope. Results shown are representative of 3-6 individual plants of at least six transformation events. GUS fluorometric protocol was adapted from Vitha et al. (1993). Samples were homogenised in GUS extraction buffer (50 mM sodium phosphate pH=7.2, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) N-lauroylsarcosine sodium salt, and 0.7 µl/ml β-mercaptoethanol), and centrifuged at 14,000x g for 5 min. Aliquots of the supernatants (100 µg protein) were assayed fluorometrically using 4-methyl-umbelliferyl-β-D-glucuronide as substrate. Fluorescence of aliquots was measured using a Synergy™ HT plate reader (BioTek) at 365 nm excitation wavelength and 455 nm emission wavelength. Results shown are representative of at least three biological samples of at six independent transformation events.

### **Experimental treatments**

For wounding treatments, 2 week-old Col0, TG7 and TG8 plants were wounded by pressing the leaf with forceps and kept in the growth chamber for 1 or 2 h. Wounded tissues were rapidly stored at -80 °C. For low-temperature treatments, 2 week-old Col0, TG7 and TG8 plants were transferred to a 6-8 °C bioclimatic chamber for an additional week, before being analyzed or stored at -80 °C. For high temperature experiments, 2 week-old Col0, TG7 and TG8 plants were transferred to a growth chamber at 35°C for an additional week, before being analyzed or stored at -80 °C. In both cases, some plants were kept at 20-22 °C as control treatment. For ABA treatments, after one week of growth in MS plates, Col0, TG7 and TG8 plants were transferred to MS plates containing 100 µM (+/-)-abscisic acid (ABA, Sigma) for 48 h before being

analyzed or stored at -80 °C. For MeJA treatment, two-week old Col0, TG7 and TG8 plants were sprayed with 100 µM methyl jasmonate (MeJA, Sigma) for 1 or 2 hours, before being analyzed or stored at -80 °C. ABA was dissolved in methanol and MeJA in water. Methanol and water were used for mock treatments in the hormone experiments. Analysis of variance (ANOVA) was applied to compare treatments. Statistical analyses was carried out with the program Statgraphics Plus for Windows 2.1, using a level of significance of 0.05.

### **Quantitative PCR analysis**

Total RNA was extracted from 0.5 g of rosette leaves and 0.1 g of roots with Trizol (Life Technologies) according to manufacturer's instructions. First-strand cDNA was synthesized from 3 µg of DNAase-treated RNA with M-MLV reverse transcriptase (Promega) and oligo dT. Quantitative PCR (qRT-PCR) was performed using a 7500 Real Time PCR System (Applied Biosystems), SYBR Green Master Mix (Applied Biosystems), and specific primers (Supplementary Table 1). The Ct values were calculated relative to EF1α reference gene (*At5g60390*) using  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). Data were obtained from the analysis of at least three biological samples with three independent technical repeats for each sample.

### **Bimolecular Fluorescence Complementation (BiFC) Analysis**

Three fragments of 1341, 1308 and 1347 bp, respectively, corresponding to the coding sequences of *AtFAD7*, *AtFAD8* and *AtFAD6* genes (without their corresponding STOP codons) were amplified by RT-PCR using Phusion® High Fidelity polymerase. Primers used for amplification (Supplementary Table 1) contained two BamHI and XmaI restriction sites to facilitate their cloning in the corresponding vectors. pUC-SPYNE and PUC-SPYCE vectors

(Walter et al., 2004), were used for the analysis. These vectors expressed the two target proteins fused to yellow fluorescent protein (YFP) in either N-terminal (pUC-SPYNE) or C-terminal (pUC-SPYCE) sites under the control of a 35S promoter. Protoplasts from *Arabidopsis* were obtained using the sandwich method (Wu et al., 2009) that uses Scotch® tape to remove the cuticle. Once the cuticle was removed with the tape, ten to fifteen leaves were incubated in a digestion mixture containing both Cellulase and Macerozyme (20 mM MES, pH: 5,7; 0.4 M Mannitol; 20 mM KCl; 10 mM CaCl<sub>2</sub>; 1.5% (w/v) Cellulase and 0.4% (w/v) Macerozyme 0.4% (w/v). Leaves were incubated in this mixture in darkness, at room temperature for no more than 1 h without agitation. Then, the mixture was diluted in washing buffer (20 mM MES, pH: 5,7; 125 mM CaCl<sub>2</sub>; 5 mM KCl) and filtered through a 0,2 µm filter to eliminate debris and undigested material. Protoplasts were precipitated at 100 x g at 4 °C for 5 min and carefully resuspended in a 15 mM MES, pH: 5,7; 0.4 M MgCl<sub>2</sub> solution. Integrity of the isolated protoplasts was analyzed in a Leica M165 FC stereomicroscope (Leica Microsystems).

Once isolated, protoplasts were transformed following the PEG/Ca method described by Yoo et al. (2007) with several modifications. The PEG percentage was adjusted to 30% to avoid protoplast lysis. For transformation, 100 µl of protoplasts and 100 µl of protoplast transformation solution (0.2 M mannitol; 100 mM CaCl<sub>2</sub>; 30% (w/v) PEG4000) were mixed and 20 ng of DNA from each of the plasmids containing each of the protein pairs to be analyzed were added and incubated at room temperature for 20 min. The transformation reaction was stopped by adding 400 µL of washing buffer and the protoplasts were precipitated by centrifugation at 100 x g at 4 °C for 5 min. Protoplasts were carefully resuspended in 2 mL of a 4 mM MES pH: 5.7; 0.5 M KCl solution and kept under illumination at 22-24 °C for 12-24 h before visualization of fluorescence. Fluorescence was detected with a Leica DM2500 fluorescence microscope using a 40x immersion objective. YFP fluorescence emission was detected with the YFP filter (excitation 500/20 nm; emission 535/30 nm). Chlorophyll auto-

fluorescence was detected with the N2.1 filter (excitation 515/560 nm; emission 590 nm).

### **Confocal microscopy analysis**

Fresh leaf imaging was carried out on a Leica TCS SP2 confocal microscope (CIBA-IACS, Zaragoza). Images were acquired using a 40x or 60x oil immersion objectives as described in Román et al., (2015). For root analysis, samples were incubated with 15  $\mu$ M propidium iodide for 10 min in the dark and rinsed twice in water to stain the cell wall. Propidium iodide fluorescence was detected exciting with an Argon laser at 488 nm and emission was detected in the 600-680 nm range. Imaging of GFP emission was performed by sequential scanning. GFP was excited with a 488 line of an argon laser and the emission collected through a 505-560 nm emission filter. Autofluorescence of chlorophyll was excited with the 633 nm line of an argon laser, and the emission was collected through a 651-717 nm emission filter. Images were taken with 6-8 line averaging. Images were analyzed and merged using Leica AF and Image J software. In all cases, special care was taken to avoid overlapping of fluorescence signals.

For co-localization purposes, Fluorescence Resonance Energy Transfer (FRET) analysis was carried out. To that end, two constructs expressing both FAD7-YFP and FAD8-YFP proteins under the control of a constitutive 35S promoter were generated using pEarly101 as destination vector. A third 35S:FAD6-CFP construct was also generated using pEarly102 destination vector. Once available, these constructs were transformed individually in *Agrobacterium tumefaciens* (GV3101 strain). For co-localization and FRET analysis, *N. benthamiana* leaves from 5-week old plants, grown at 22 °C, 16 h light, 8 h dark photo period were infiltrated with a combination of *A. tumefaciens* cultures (OD<sub>600nm</sub> 0.5), harbouring either FAD7-YFP and FAD6-CFP or FAD8-YFP and FAD6-CFP. All constructs were co-infiltrated with the p19

plasmid to avoid silencing (Roman et al., 2015). Plants were kept in the growth chamber for 3-5 days before analysis. Imaging of YFP and CFP emissions were performed by sequential scanning. CFP was excited with a 458 nm line of an argon laser and the emission collected through a 518-580 nm emission filter for YFP and 465-571 nm for CFP. Autofluorescence of chlorophyll was excited with the 633 nm line of an argon laser, and the emission was collected through a 651-717 nm emission filter. Images were taken with 6-8 line averaging. Images were analyzed and merged using Leica AF and Image J software.

### **Immunoblot analysis**

Protein extracts were obtained from 0.5 g of Arabidopsis rosette leaves or 0.1 g of roots homogenized in a mortar with liquid nitrogen. The powder was dissolved in buffer A (0.1 M Tris-HCl, pH: 7.5; 20% (w/v) glycerol; 1 mM EDTA; 10 mM MgCl<sub>2</sub>; 14 mM  $\beta$ -mercaptoethanol; 100  $\mu$ g/ml Pefabloc (Fluka); 1  $\mu$ g/ml antipain (Sigma-Aldrich) and 1  $\mu$ g/ml leupeptin, (Sigma-Aldrich) and filtered with Miracloth paper (Calbiochem). The protein content of the different fractions was estimated using the BioRad protein assay reagent (BioRad). Except when specifically mentioned, total protein of 20  $\mu$ g was loaded per lane. Western blot procedures were performed as described in Román et al., (2015) using a GFP antibody (ab290, Abcam). Anti-OEE33 (Alfonso et al., 2004), that recognizes the 33 kDa oxygen evolving extrinsic protein from Photosystem II, was used as an internal loading control.

### **Root lipid analysis**

Total lipids were obtained from 0.1 g of roots from plants of the different lines grown vertically in MS plates for two weeks, extracted as described (Bligh and Dyer, 1959) and analysed by gas chromatography (GC), (Román et al., 2015). Data from fatty acid analysis were obtained from



two independent biological experiments with two technical repeats per experiment. Analysis of variance (ANOVA) was applied to compare treatments with the program Statgraphics Plus for Windows 2.1, using a level of significance of 0.05.

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## FIGURE LEGENDS

**Figure 1.** Characterization of the *AtFAD7* and *AtFAD8* gene promoter sequences used in this work. GUS histochemical staining of transgenic lines carrying the 1682 bp sequence corresponding to the *AtFAD7* gene promoter after 1 h GUS-staining in (A) cotyledonal leaves; (B) rosette leaves; (C) roots. GUS histochemical staining of transgenic lines carrying the 2958 bp sequence corresponding to the *AtFAD8* gene promoter after 1 h (D-F) or 3 h (H-K) GUS-staining in (D, H) cotyledonal leaves; (E, I) rosette leaves; (F, K) roots. (L-N) plants carrying the pMDC163 empty vector. 14-d old rosette leaves and roots were used for the analysis. (O) GUS activity (nmol of 4-methylumbelliferone, MU) produced per minute per milligram of protein using 4-methylumbelliferyl  $\beta$ -D-glucuronide as substrate. Each bar represents the activity of an individual transgenic event. Data represent means of at least three biological replicates.

**Figure 2.** Confocal microscopy analysis of the relative abundance of both *AtFAD7* and *AtFAD8* proteins in leaves from transgenic lines carrying the *AtFAD7*p1,7kb:FAD7-GFP and *AtFAD8*p3kb:FAD8-GFP constructs. Analysis was performed in two week-old rosette leaves. Merged images (chlorophyll autofluorescence in red and GFP in green) of plants carrying the *AtFAD7*p1,7kb:FAD7-GFP from: (A) cotyledonal leaves; (B) young rosette leaves; (C) mature rosette leaves. Plants carrying the *AtFAD8*p3kb:FAD8-GFP from: (D) cotyledonal leaves; (E) young rosette leaves; (F) mature rosette leaves. Bars correspond to 10  $\mu$ m.

**Figure 3.** Confocal microscopy analysis of the relative abundance of both *AtFAD7* and *AtFAD8* proteins in roots from transgenic lines carrying the *AtFAD7*p1,7kb:FAD7-GFP and



*AtFAD8p3kb:FAD8-GFP* constructs. Analysis was performed in roots from two week-old plants. Merged images of plants carrying the *AtFAD7p1,7kb:FAD7-GFP* from: (A) root tip; (D) root. Plants carrying the *AtFAD8p3kb:FAD8-GFP* from: (B) root tip; (E) root. Plants carrying the empty vector (C) root tip; (F) root. Red fluorescent signal was obtained by propidium iodide staining. Bars correspond to 25  $\mu\text{m}$ .

**Figure 4.** Western blot analysis of the relative abundance of *AtFAD7* and *AtFAD8* proteins in transgenic lines carrying the *AtFAD7p1,7kb:FAD7-GFP* and *AtFAD8p3kb:FAD8-GFP* (TG8) constructs. Protein extracts from two week old rosette leaves from transgenic lines carrying the *AtFAD7p1,7kb:FAD7-GFP* (TG7) and the *AtFAD8p3kb:FAD8-GFP* constructs (TG8), (left panel). 20  $\mu\text{g}$  of total protein were loaded per lane for the *AtFAD7p1,7kb:FAD7-GFP* plants. For detection of the *AtFAD8-GFP* protein, 35-40  $\mu\text{g}$  of total protein were loaded per lane. AntiOEE33 was used as internal control. Protein extracts from roots and leaves of transgenic lines carrying the *AtFAD7p1,7kb:FAD7-GFP* construct (right panel). 20  $\mu\text{g}$  of total protein were loaded per lane. Anti-GFP was used as primary antibody. Total protein stained by Coomassie is shown as loading control.

**Figure 5.** Response of *AtFAD7* and *AtFAD8* to wounding and jasmonate. (A) qRT-PCR expression analysis of *AtFAD7* and *AtFAD8* genes in response to wounding (upper panel) or MeJA (lower panel) in Col0, TG7 and TG8 plant lines. Data represent means of at least three biological replicates. Asterisk indicates that the differences significant over than 2-fold or less than 0,5-fold when compared with the control. (B) Western blot analysis of the relative abundance of *AtFAD7* protein in TG7 and TG8 transgenic lines upon wounding (left panel) or 100  $\mu\text{M}$  MeJA supplementation (right panel). 20  $\mu\text{g}$  of total protein were loaded per lane for

the *AtFAD7*p1,7kb:FAD7-GFP plants. For detection of the *AtFAD8*-GFP protein, 35-40 µg of total protein were loaded per lane. Anti-GFP was used as primary antibody. AntiOEE33 was used as internal control.

**Figure 6.** Effect of ABA on *AtFAD7* and *AtFAD8* gene expression and protein levels. (A) qRT-PCR expression analysis of *AtFAD7* and *AtFAD8* genes in response to 100 µM ABA treatment in Col0, TG7 and TG8 plant lines. Data represent means of at least three biological replicates. Asterisk indicates that the differences significant over than 2-fold or less than 0,5-fold when compared with the control. (B) Western blot analysis of the relative abundance of the *AtFAD7* and *AtFAD8* protein in TG7 and TG8 lines in response to 100 µM ABA treatment. For detection of the *AtFAD7*-GFP protein, 15-20 µg of total protein were loaded per lane. For the *AtFAD8*-GFP protein, 35 µg of total protein were loaded per lane. AntiOEE33 was used as internal control.

**Figure 7.** Effect of cold or heat on *AtFAD7* and *AtFAD8* gene expression and protein levels. (A) qRT-PCR expression analysis of *AtFAD7* and *AtFAD8* genes in response to cold (6°C), (upper panel) or heat (35°C), (lower panel) in Col0, TG7 and TG8 plant lines. Data represent means of at least three biological replicates. Asterisk indicates that the differences significant over than 2-fold or less than 0,5-fold when compared with the control. (B) Western blot analysis of the relative abundance of the *AtFAD7* and *AtFAD8* protein in TG7 and TG8 lines at 22, 6 °C and 35 °C. For detection of the *AtFAD7*-GFP protein, 15-20 µg of total protein were loaded per lane. For the *AtFAD8*-GFP protein, 35 µg of total protein were loaded per lane. AntiOEE33 was used as internal control.

**Figure 8.** Bimolecular fluorescence complementation analysis of the *in vivo* interaction of *AtFAD7* and *AtFAD8* proteins with the plastidial  $\omega$ -6 desaturase *AtFAD6*. All the red chlorophyll autofluorescence, yellow YFP and merged channels are shown for each pair. (A) protoplasts were transformed with a pEarley101 construct carrying the *AtFAD8* protein fused to YFP used as a positive control; (B) an empty pUC-SPYNE vector was used as negative control; (C) nYFP-FAD7/FAD6-cYFP pair; (D) nYFP-FAD6/FAD7-cYFP pair; (E) nYFP-FAD8/FAD6-cYFP pair; (F) nYFP-FAD6/FAD8-cYFP pair; (G) nYFP-FAD7/FAD8-cYFP pair; (H) nYFP-FAD8/FAD7-cYFP pair; (I) nYFP-FAD7/FAD7-cYFP pair; (J) nYFP-FAD8/FAD8-cYFP pair. Bars correspond to 100  $\mu$ m.

**Supplementary Figure 1.** Schematic diagram of the constructs used for the analysis of the relative abundance of *AtFAD7* and *AtFAD8* proteins. Location of both *AtFAD7* and *AtFAD8* genes in chromosomes 3 and 5, respectively, is indicated. Position of genes upstream both *AtFAD7* and *AtFAD8* is also indicated. A diagram of the localization and distribution of putative *cis*-acting regulatory sequences found in both promoters using PLANTCARE and PLACE tools is also shown.

**Supplementary Figure 2.** (A) Determination of the *AtFAD7*, *AtFAD8* and *AtFAD3* gene expression ratio in TG7 and TG8 lines with respect to Col0. Data represent means of at least three biological replicates. (B) Growth phenotype of TG7 and TG8 plant lines compared to that from Col0. Plants were grown for 4 weeks in a climatic chamber.

**Supplementary Figure 3.** Confocal microscopy analysis from leaves of transgenic lines carrying the empty vector pEN-R2F-L3. Analysis was performed either in 10 day cotyledonal leaves as well as from two week-old rosette leaves. Bars correspond to 10  $\mu\text{m}$ .

**Supplementary Figure 4.** Confocal microscopy analysis of the *AtFAD7* protein roots from transgenic lines carrying the *AtFAD7*p1,7kb:FAD7-GFP construct grown fully illuminated or with the root growth zone protected from light. Analysis was performed in roots from 7 d-old plants in which the root growth area of the MS plates was covered to avoid illumination (A and B) or kept under illumination (C and D). Merged images of plants carrying the *AtFAD7*p1,7kb:FAD7-GFP from (A and C) root tip; (B and D) root. Red fluorescent signal was obtained by propidium iodide staining. Bars correspond to 25  $\mu\text{m}$ .

**Supplementary Figure 5.** Western blot analysis of the relative abundance of the *AtFAD8* protein in transgenic lines carrying the *AtFAD8*p3kb:FAD8-GFP constructs in leaf and roots. Protein extracts from leaves and roots from two week old transgenic lines grown in MS plates, carrying the *AtFAD8*p3kb:FAD8-GFP construct. To favour its detection in roots, 40-50  $\mu\text{g}$  of total protein were loaded per lane. Anti-GFP was used as primary antibody. Coomassie staining of total proteins from both extracts is shown as loading control.

**Supplementary Figure 6.** RT-PCR of *LOX2* and *ABII* genes as internal controls of the MeJA and ABA treatments applied in this work. ACTIN was used as housekeeping gene.

**Supplementary Figure 7.** (A) Confocal microscopy analysis of the relative abundance of both *AtFAD7* and *AtFAD8* proteins in TG7 and TG8 transgenic lines in response to cold treatment.

Plants were maintained at 22 °C or kept at 6 °C for one week before the analysis. Bars correspond to 10 µm. (B) Confocal microscopy analysis of the relative abundance of the *AtFAD7* protein in TG7 transgenic lines in response to 100 µM ABA treatment. Bars correspond to 10 µm.

**Supplementary Figure 8. Co-localization/FRET analysis of FAD7-YFP and FAD8-YFP fusion proteins with FAD6-CFP by transient expression in *N. benthamiana* leaves.** Upper panel FAD7-FAD6 interaction; lower panel FAD8-FAD6 interaction. (A and E) autofluorescence of the chlorophyll from plastids, (B and F) FAD6-CFP, (C and G) FAD7-YFP and FAD8-YFP signals, respectively; (D and H) merged image from A,B , C and F,G,H, respectively. Scale bar is 10 µm in all pictures.

**Table 1.** Fatty acid composition of total lipids from roots of Col-0 and different fatty acid desaturase mutant lines.

	Col-0	<i>fad3-1</i>	<i>fad7i</i>	<i>fad8i</i>	<i>fad7-2/fad8-1</i>
C14:0	0,58 ± 0.11	0,54 ± 0.1	0,95 ± 0.4	0,52 ± 0.1	0,52 ± 0.2
C16:0	23,85 ± 0.21	23,84 ± 0.4	29,53 ± 0.45	23,72 ± 0.55	24,37 ± 0.42
C16:1	0,98 ± 0.25	0,72 ± 0.17	0,76 ± 0.07	0,7 ± 0.14	0,52 ± 0.1
C18:0	4,21 ± 0.18	4,67 ± 0.16	7,94 ± 0.23	3,42 ± 0.1	4,69 ± 0.31
C18:1	4,76 ± 0.29	5,82 ± 0.32	4,68 ± 0.27	4,64 ± 0.14	4,39 ± 0.2
C18:2	30,28 ± 0.54	53,35 ± 0.46	28,08 ± 0.24	30,13 ± 0.4	31,18 ± 0.59
C18:3	23,06 ± 0.56	4,16 ± 0.01	21,01 ± 0.36	28,78 ± 0.56	26,04 ± 0.79
C20:0	0,96 ± 0.1	0,45 ± 0.05	0,67 ± 0.01	0,73 ± 0.14	0,79 ± 0.14
C20:1	0,22 ± 0.14	0,07 ± 0.02	0	0,32 ± 0.14	0,36 ± 0.1
C22:0	6,59 ± 0.18	2,84 ± 0.17	2,99 ± 0.14	3,32 ± 0.24	3,56 ± 0.31
C22:1	0,43 ± 0.17	0,41 ± 0.06	0,31 ± 0.14	0,46 ± 0.17	0,28 ± 0.17
C24:0	3,74 ± 0.15	2,78 ± 0.24	3 ± 0.26	3,16 ± 0.1	3,22 ± 0.6

Data represent means from two biological replicates.