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Plant polyunsaturated fatty acids: Biological roles, regulation and biotechnological applications.

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ABSTRACT:

Polyunsaturated fatty acids (PUFAs) are particularly enriched in land plants and are the products of the editing of fatty acids synthesized in the plastids. The desaturases FAD2, FAD3, FAD6, FAD7 and FAD8 enables the synthesis of linoleic and linolenic acids (C18:2 and C18:3 respectively) that are the most abundant PUFAs in land plants. PUFA content is regulated by environmental conditions mainly temperature and stresses associated with oxidative conditions. Genetic regulation of the FAD desaturases is occurring at the transcriptional and post-transcriptional levels allowing fine tuning of PUFAs levels in changing environment but also during seed development to modulate seed oil content. This review aims in describing PUFA synthesis, their biological roles and the different factors involved in their regulation with a specific focus on omega-3 linolenic acid. It will also detail in several crop species the different genetic leverage that were used to modify omega-3 content for plant breeding.

I. INTRODUCTION

The polyunsaturated fatty acids (PUFAs) are fatty acids with multiple double bonds (>1). Plants are mostly harboring the dienoic omega-6 linoleic acid (C18:2) and omega-3 alpha-linolenic acid or ALA (C18:3) PUFAs that are essentially found in membranes (Harwood, 1980). Common aspects of the function of PUFAs in animals and plants include the maintenance of membrane fluidity (Prades et al., 2003) in particular for low temperature adaptation but also for the synthesis of functional and/or signaling derivatives, such as oxylipins.

In plants, PUFAs represent a major part of the fatty acids bound to triglycerides in seed oil and are thus readily available for nutrition or industrial applications (Iii et al., 2009). PUFAs are also referred to essential fatty acids, since most animals with the notable exception of the worm *Caenorhabditis elegans* lack the enzymes enabling the introduction of additional desaturation (desaturases) in omega 6 and 3 positions in fatty acids (Spsychalla et al., 1997). PUFA consumption are often associated with health benefits such as lower risk for heart disease for example. (Clifton & Keogh, 2017). In animals, ALA can serve as a precursor for very long chain PUFAs that are important fatty acids associated with inflammatory responses and critical physiological roles in neural development (Dyall, 2015). The efficiency of conversion of ALA in long chain omega-3 fatty acids is significantly demonstrated for food intake in fish species, making plant sourced food of use in this application (Glencross, 2009). The review will mostly focus on the specificity of omega-3 linolenic acid synthesis and function relative to omega-6 linoleic acid. We will also discuss how different stresses and physiological processes regulate PUFA synthesis in plants, and finally how the understanding of desaturation metabolism can improve crop selection and breeding.

II. SYNTHESIS AND BIOCHEMISTRY OF POLYUNSATURATED FATTY ACIDS IN PLANTS

A. De novo fatty acid synthesis occurs in plastids

Unlike other eukaryotes, plants synthesize fatty acids exclusively within plastids using acetyl-CoA as carbon donor. The simplest pathway to generate acetyl-CoA is from pyruvate produced after photosynthesis and glycolysis, through the action of the plastidial pyruvate dehydrogenase complex (PDHC). The acetyl-CoA is first converted into malonyl-CoA in plastids through the action of the heteromeric Acyl-CoA-Carboxylase, an enzyme composed of 3 nuclear-encoded subunits (BCCP, BC, alpha-CT) and the plastid-encoded beta-CT (Baud et al., 2003). The malonyl moiety is then transferred to the Acyl-Carrier protein (ACP) by the Malonyl-Coa:ACP malonyltransferase (MCMT) to form malonyl-ACP that is finally used to elongate acyl-ACP chains by three Ketoacyl-ACP-Synthases (KAS I, II, III) (Li-Beisson et al., 2013).

The malonyl-ACP (3 carbons chain) is first extended by KAS III using acetyl-CoA (2 carbons) to generate ACP-4:0 releasing 1 molecule of CO₂ in the process. Then, KASI produces ACP-16:0 from ACP-4:0 through 6 sequential condensation reactions using malonyl-ACP as substrate and also releasing free ACP in the process. Finally, KASII synthesizes ACP-18:0 through the last condensation reaction. ACP-18:0 and ACP-C16:0 can then be desaturated by AADs, plastid localized enzymes, to yield ACP-C18:1 or ACP-C16:1. Upon completion of the elongation cycles and desaturation reactions, the fatty acids can be either used for plastid lipid synthesis or exported to the cytosol and ER.

In plastids, the “prokaryotic” synthesis pathway uses acyl-ACP to produce glycerolipids by transferring fatty acid chains to glycerol-3-phosphate (G3P) or to acyl-G3P. In the ER, the “eukaryotic” synthesis pathway uses acyl chains released from ACP by the thioesterases FATA or FATB enzymes and coupled to Coenzyme-A by thioesterification. The acyl-CoA lipid pool localized in the cytoplasm will be used subsequently by most of the ER based lipid reactions.

B. PUFA are synthesized from monounsaturated fatty acids in the endoplasmic reticulum and plastids.

Desaturases add double bonds in a sequential manner on omega-9 oleic acid (C18:1). First, omega-6 desaturases yield dienoic omega-6 fatty acids C18:2 (Falcone et al., 1994; M. Miquel & Browse, 1992). The omega-6 desaturases are coded by two genes in Arabidopsis that have a distinct localization and function. FAD2 is present in the endoplasmic reticulum outer membrane leaflet where it is inserted co-translationally. FAD2 desaturates C18:1 esterified to phosphatidylcholine (PC) (McCartney et al., 2004) to yield C18:2-PC. The omega-6 desaturase FAD6 is localized within the

chloroplast membrane (Ferro et al., 2003) where it regulates plastid lipid composition. FAD6 has a different substrate specificity compared to FAD2 since it desaturates galactolipids and phosphatidylglycerol (PG) C16:1 and C18:1 (Browse et al., 1989).

The omega-6 class fatty acids can then be desaturated by omega-3 desaturases to yield trienoic fatty acids (TFAs). The omega-3 desaturases exist as 3 isoforms in *Arabidopsis*, FAD3, localized in the ER, and FAD7 and FAD8 which are two plastidial desaturases. Like FAD2, FAD3 acts on PC-C18:2 to yield PC-C18:3 and FAD7 and FAD8 like FAD6, acts on galactolipids and PG C16:2 and C18:2 (Román et al., 2015). The sequential desaturation reactions are illustrated in Figure 1.

[Insert Figure 1 here]

The chloroplastic desaturases FAD6, FAD7 and FAD8 are transmembrane enzymes with 4 integral transmembrane helices and possess three histidine box motifs (Fig. 2A and B) (Shanklin et al., 1994) which are conserved and necessary elements among all desaturases identified so far. The histidine boxes stabilize a di-iron center essential for the desaturation reaction and which has been well characterized in the closely related mammalian SCD1 (Bai et al., 2015). Plastidial desaturase substrate specificity is restricted by the acyl chain length (Broadwater et al., 2002) and their activity requires ferredoxin as electron donor (Schmidt & Heinz, 1990). Table 1 summarizes desaturase accession numbers, localization and respective cofactors. The ER localized FAD2 and FAD3 are also transmembrane proteins and possess three histidine boxes but require cytochrome B5 as electron donors (Kumar et al., 2012). The omega-6 and 3 desaturases can act as homodimers or heterodimers associating FAD2/FAD3, FAD6/FAD7 and FAD6/FAD8 complexes while FAD7 and FAD8 were not reported to associate (Lou et al., 2014).

[Insert Figure 2 here]

C. PUFAs are substrates for oxylipin synthesis.

The PUFAs are molecules sensitive to oxidation either through the action of biological enzymes or by chemical reaction to produce a large variety of lipid derivatives named oxylipins.

Oxylipins are involved in many stress responses ranging from abiotic (freezing) and biotic stresses (pathogens) to mechanical stress (wounding). Among oxylipins, the signaling molecule jasmonic acid (JA) is the most studied (Routaboul et al., 2012). Jasmonate synthesis requires first the release of linolenate by lipases. This step is of critical importance and varies between tissues. For example, DAD1 is a phospholipase A1 required for JA synthesis specifically in stamen filaments of *Arabidopsis* (Ishiguro et al., 2001).

The jasmonate pathway involves first different lipoxygenases (LOX) in plastids that metabolize free trienoic fatty acids. Following their oxidation by LOX, a series of sequential reactions involving the allene oxide synthase (AOS) and the allene oxide cyclase (AOC) in plastids, followed by the 12-OPDA reductase (OPR) in peroxisomes and 3 rounds of beta-oxidation leads eventually to JA

(Schaller & Stintzi, 2009). However, trienoic fatty acids are also implied in pathogen stress tolerance independently of JA since they are essential to plant survival even in *aos* mutants defective in JA (Mène-Saffrané et al., 2009). This result would be worth investigating in other mutants like *lox* to check if C18:3 lipoxygenation products are also involved in biotic stress tolerance.

The non-enzymatic and enzymatic oxylipin generation and subsequent acyl chain breakdown also contributes to the synthesis of malondialdehyde (MDA), a major PUFA by-product that is an oxidation marker involved in stress response as well as carbonyl species that have been well summarized recently (Tsikas, 2017; Yalcinkaya et al., 2019). MDA seems to be involved in large variety of processes. It was involved in post-translational modification of proteins that are regulated by PUFA (Yamauchi et al., 2008). MDA could also be a major sink for reactive oxygen species (ROS) since they are produced by the oxidation of PUFAs (mostly C18:3) and could be re-incorporated into lipids preferentially in trienoic or dienoic galactolipids by a still unknown mechanism (Schmid-Siegert et al., 2016). PUFA oxidative catabolism is complex but recent works suggest that many intermediates of these pathways could be involved in several biological processes related to PUFA regulation and functions.

[Insert Table 1 here]

D. Endoplasmic reticulum and plastid desaturase crosstalks

Genetic analyses of omega-6 and 3 desaturases have demonstrated the existence of a rather complex metabolic network leading to C18:3 accumulation that depends on the tissue and subcellular localization but also the specificity of the enzymes (Table 2).

In seeds, the loss of FAD2 function (*fad2-1* mutant) led as expected to a 10 and 4-fold reduction of C18:2 and C18:3 contents respectively mostly in triacylglycerol. However in leaves, *fad2-1* mutation is characterized by a 70% reduction of C18:2 content (from 14 to 4 mol%) but only a 20% decrease in C18:3 content in total lipids (Lemieux et al., 1990). The loss of function of the plastidial omega-6 desaturase FAD6 has however a more important impact on leaf lipid composition since it resulted in 50% decreased in C18:3 content associated with an important increase in C16:1 and C18:1, but with little effect on C18:2 composition. So, the accumulation of C18:3 is more dependent on plastidial activity of FAD6 than FAD2 in photosynthetic tissue. Interestingly, leaf C18:2 content is not much affected in *fad6* mutant suggesting that ER FAD2 activity could still compensate for the loss of FAD6 activity and produce C18:2 by using plastidial C18:1 as substrate. But FAD2 does not fully compensate for the function of FAD6 as C18:3 content never reached wild type levels in *fad6* mutant (Falcone et al., 1994). The *fad6* mutation did not affect seed and roots C18:3 content in contrast to *fad2-1*, confirming the importance of FAD6 mainly for photosynthetic tissues and the major role of the microsomal desaturation in non-photosynthetic tissues (Browse et al., 1989).

As expected, *fad2 fad6* double mutants have negligible levels of PUFAs in general and C18:3 in particular and are not capable of autotrophic growth, highlighting the importance of PUFAs for photosynthesis (McConn & Browse, 1998). Interestingly, the light harvesting complexes are quantitatively not much altered and the presence of a single functional FAD2 allele is sufficient to recover autotrophy and normal development (McConn & Browse, 1998).

Omega 3 desaturation in seeds and roots depends mostly on FAD3 activity since *fad3* mutation drastically reduces C18:3 levels in those tissues with a two thirds reduction (29 to 10 mol%) in roots and a 10 fold reduction in dry seeds (20 to 2 mol%) compared to leaf tissues (only 3 mol% reduction) (Lemieux et al., 1990). Omega 3 desaturation in plastids is carried out by the 2 desaturases FAD7 and FAD8. The double mutant *fad7 fad8* could therefore only synthesize C18:3 bound to PC thanks to ER FAD3. FAD3 activity is sufficient to synthesize about one third (15 mol%) of C18:3 in total leaf lipids (Román et al., 2015). Interestingly, FAD7 and FAD8 are not completely redundant. FAD7 appears to be more active at high temperature since C18:3 content decreased by 65% in leaves of *fad7* mutants grown between 26 and 32°C and decreased only by 25% in mutants grown between 10 and 23°C (Browse et al., 1986). On the contrary, FAD8 showed increased expression upon cold application (Gibson et al., 1994; McConn et al., 1994).

Finally, genetic studies highlighted the importance of fatty acid exchange between plastids and ER compartments (McConn & Browse, 1998). This is well illustrated by the absence of phenotype of the single mutants *fad6* or *fad2-1* while the double mutant showed chloroplast defects and chlorosis. Interestingly, the double mutant between *fad6* and *tgdl*, a plastid lipid carrier is also extremely chlorotic like *fad2fad6*. While *tgdl-1* mutation caused a strong decrease in C18:3, the double mutations *tgdl-1fad2* did not further reduce C18:3 levels within plastids confirming that an important part of plastidial C18:3 are from ER origin and that TGD1 is one of the main entry gate for trienoic trafficking (Xu et al., 2010).

[Insert Table 2 here]

III. PUFA PHYSIOLOGICAL ROLES

A. PUFA are required for temperature tuning of photosynthesis.

The desaturase mutants showed the importance of PUFAs for plant photosynthesis as detailed above but also the importance of dienoic and trienoic C18 fatty acids for temperature adaptation. The critical requirement for PUFA synthesis for sustaining cold temperature is obvious in *Arabidopsis fad2-2* mutants as they fail to germinate if the siliques and seeds develop at 6°C for more than 10 days. Even seeds of *fad2-2* mutants developed at permissive temperature showed delayed germination at 6°C (M. F. Miquel & Browse, 1994). At low temperatures after 3 weeks of culture at 5°C, the *fad6* mutant exhibited a two-fold reduction of the chloroplast area associated with slow growth phenotype and chlorotic appearance (Hugly & Somerville, 1992). Similarly, *fad2-2* mutant showed chlorotic

phenotype and an impaired growth at 12°C that could not recover even when re-exposed to normal temperatures. The *fad2-2* plants would even die after a long exposure (50 days) to cold (6°C). But a residual FAD2 activity is enough for cold adaptation since the *fad2-1* allele which was shown to be a leaky mutation exhibited a wild type phenotype at cold temperatures (M. Miquel et al., 1993). These results indicated that for omega-6 desaturation, the plastidial and the ER activities are redundant at normal temperatures but are both necessary for the plant to cope with low temperatures.

For trienoic FAs, FAD7 activity is also critical for plastid function since *fad7* mutation affects chloroplast size and also its structure but with minimal physiological impact because this alteration is compensated by a 45% increase in chloroplast number (Mccourt et al., 1987). Both effects are directly related to C18:3 synthesis since these phenotypes are complemented by FAD7 ectopic expression (Iba et al., 1993). To our knowledge, no specific role on chloroplast structure and function had been identified for FAD8 but the modified expression of FAD7 and FAD3 genes in *fad8* mutant as well as the specific FA profile of ER and plastid lipids suggested an alternative function. Interestingly, the overexpression of FAD8 in wild type Arabidopsis did not modify plastid lipid composition but caused a significant increase of C18:3 and a mirrored decrease of C18:2 in ER synthesized glycolipids (Román et al., 2015). These results suggest that ER-plastid PUFA trafficking is important for plastidial homeostasis in particular at low temperature. The different desaturases are required to work in a coordinated manner to tune and adapt photosynthesis to environmental changes. In depth analysis of plastid functions in *fad8* and *fad3* mutants and combination of *fad3 fad7* or *fad3 fad8* mutants are still missing to better understand the interplay between these genes and the precise role of the omega -6 and -3 FA in plastid function but also in non photosynthetic tissues like roots. ER-plastid PUFA trafficking is also important for cold adaptation during seed development and germination. Desaturase mutants suggest that seed germination, particularly at cold temperatures, requires minimal PUFA synthesis from the activity of plastidial desaturases even if their activities have most probably a marginal role during late seed development since chloroplasts are undergoing dedifferentiation (Allorent et al., 2013). For instance, during germination of *fad2-2* mutants, PUFAs can not be provided by storage lipids that are strongly impacted but their synthesis relied mainly on FAD6, FAD7 and FAD8 activities. Plastidial desaturases use the reducing power of ferredoxins which have high cost on photosynthesis and possibly impact seedling viability. Another explanation could be a direct impairment of photosynthesis in the plastids caused by a low-PUFA membrane content (Allorent et al., 2015).

Mirrored effect could be seen with PUFA accumulation and its effect on temperature adaptation. PUFA accumulation is clearly associated with low temperature in seeds of various oilseed crops (Canvin, 2011; Menard et al., 2017, p. 017). Omega-3 desaturases mutants and overexpressors exhibit only subtle phenotypes and are quite difficult to study. The overexpression of FAD8 and to a lesser

extent FAD3, reduced growth of tobacco BY-2 cells at high temperature (M. Zhang et al., 2005), indicating that omega-3 desaturation levels should be tuned down at elevated temperatures to avoid deleterious effects. Nonetheless, trienoic fatty acids are also required for long term thermotolerance, since the inactivation of the three desaturases FAD3, FAD7 and FAD8 led to plant death after 15 days at 33°C. This lethality is not caused by a deficiency in jasmonate or JA response since *aos*, *opr3* and *coil* mutants are thermotolerant (Routaboul et al., 2012).

B. PUFA involvement in abiotic and biotic stress responses.

- **Salt, drought and oxidative stresses**

High salt medium is known to induce oxidative stress delaying *fad2* germination. Interestingly, *fad2* mutants grown in presence of high salt showed 30% increase of MDA (Zhang et al., 2012). Similarly, *fad6* mutant is also sensitive to salt stress and exhibited increased MDA levels (Zhang et al., 2009). The increase in MDA production is relatively counterintuitive since lower levels of C18:2 and C18:3 in *fad2* and *fad6* mutants should have been associated with lower lipid peroxidation (Ayala et al., 2014). As expected, the suppression of all trienoic fatty acids in the *fad3 fad7 fad8* led to reduced steady-state levels of MDA (Mène-Saffrané et al., 2007). The link between trienoic fatty acids and oxidative stress was clearly observed in tomato over-expressing LeFAD3 with an increase of ROS detoxification with the activity of superoxide dismutase. Conversely, the antisense expression of LeFAD3 reduced ROS detoxification and resulted in a net decrease in photosynthetic efficiency (H.-S. Wang et al., 2014). Similarly, the expression in tobacco of antisense *AtFAD7* that most probably impaired the expression of both FAD7 and FAD8, enhanced salt and drought sensitivity (Im et al., 2002).

- **Metals and heavy metals**

Oxidative stress could also be induced by heavy metals leading to the production of lipid hydroxyperoxides and MDA from PUFAs (Skórzyńska-Polit et al., 2009). Nickel accumulation was shown to cause a drastic reduction of PUFAs in favor of saturated fatty acids in roots and shoots of wheat seedlings (Gajewska et al., 2012), and in young leaves of tomato seedlings (Verdoni et al., 2001). In *Arabidopsis*, copper application led to a similar decrease of PUFAs particularly in roots (Yuan et al., 2016). In *Camelina*, the exposure to Cadmium and Copper particularly reduces the accumulation of C18:3 in dry seeds and the overexpression of the Heavy-Metal ATPase CsHMA3 could partially alleviate this defect reducing the associated oxidative stress (Park et al., 2015).

Contrary to heavy metals, limitation of iron bioavailability was shown to induce preferential accumulation of PUFAs in whole plants. Even more importantly, it was shown that the addition of C18:2 or C18:3 can increase the activity of the Ca²⁺ ATPases in isolated wild-type plasma membrane vesicles of *Arabidopsis* roots. The rate of this activity was only significantly reduced in *fad2-2* but

not in *fad3-2* mutants, suggesting that C18:2 is sufficient to maintain membrane dynamics for Ca²⁺-dependent ATPases activity (Oh et al., 2016). Both C18:2 and C18:3 seems to be important for optimal ATPases activities but C18:3 content was more specifically modified by cadmium- or copper-induced stress in camelina.

Interestingly, Boron uptake was shown to be reduced in *fad7-2*, *fad7 fad8* mutants compared to wild type when the plants were exposed to boric acid. However, the chilling sensitive mutant *chs1-1* that exhibits an absence of FAD8 expression (see below) showed an increased Boron uptake (Dordas & Brown, 2000). The link with PUFA content remains to be demonstrated.

- **Biotic stresses**

Linolenic acid is directly involved in biotic stress responses by the fact that it is a direct substrate for jasmonate synthesis. However, several jasmonate independent effects could be associated with C18:3 during pathogen response. C18:3 was found to be important in the tolerance toward *Botrytis cinerea* and the oomycete *Hyaloperonospora* in *Arabidopsis*. Indeed, the triple *fad3 fad7 fad8* mutants showed increased sensitivity toward pathogens compared with *opr3* mutant that is impaired only in JA synthesis suggesting a JA-independent effect of trienoic acid probably based on the alleviation of oxidative stress (Mène-Saffrané et al., 2009).

The subcellular synthesis of C18:3 is also important for pathogen response. The *fad3-2* mutant showed for instance a 45% decrease in crown gall tumor induced by *Agrobacterium tumefaciens* compared to wild type or *fad7 fad8* mutant (Klinkenberg et al., 2014). However, plastidial synthesis of C18:3 is important in *Arabidopsis* to induce Systemic Acquired Resistance (SAR). FAD7 in combination with SUPPRESSOR OF FATTY ACID DEFICIENCY 1 (SFD1 or GLY1) and SFD2 were necessary for the production of a SAR signal in petiole exudate of leaves challenged with an avirulent strain of *Pseudomonas syringae* that promoted subsequent resistance to *Arabidopsis*, tomato and wheat. The SAR signal is independent from JA and is most probably derived from plastid glycerolipid synthesis since the three proteins FAD7, SFD1 and SFD2 are involved in this metabolic pathway and the SAR effect is dependent on the lipid transfer protein DIR1 (Chaturvedi et al., 2008).

FAD7 was also involved in aphid resistance since antisense inactivation of tomato LeFAD7 reduced aphid infestations in a JA-independent way but with increased SA accumulation. Aphid infestations were also reduced in *Arabidopsis fad7-2* and *fad7-1 fad8* mutants (Avila et al., 2012). It should be noted that the works involving *fad7-1* alleles and pathogen responses have to be analyzed cautiously since it was conducted in the *glabra1* background known to be involved in JA and Gibberellic signaling pathways (Xia et al., 2010).

- **Concluding remark.**

The management of ROS could be a common link between trienoic acid and biotic or abiotic stresses since the trienoic fatty acids were proposed to act as a sink for ROS. The production of MDA could

act as a non-enzymatic reaction to protect cells from oxidative stress (Mène-Saffrané et al., 2009). While free MDA is a marker of oxidative damage, its accumulation after a long time has deleterious effects for the cells, but a transient accumulation could be the sign of oxidative stress adaptation and detoxification (Morales & Munné-Bosch, 2019). A hypothesis could be the incorporation of MDA back into membrane lipids. Application of labelled MDA to plants showed specific carbon flux into C18:3-MGDG as a terminal product, and in a lesser extent in C18:2-MGDG through an unknown metabolic pathway most probably to detoxify this product (Schmid-Siegert et al., 2016).

IV. REGULATION OF OMEGA-6 AND OMEGA-3 DESATURASES

A. Regulation of microsomal desaturases

In Arabidopsis, FAD2 and FAD3 desaturases are responsible for the accumulation of C18:2 and C18:3 in seeds. These fatty acids are constantly accumulated from pollination to full seed maturation without any plateau but C18:3 accumulates at a slower rate and with a delay of 3 days compared to C18:2 (Baud et al., 2002; Rossak et al., 2001). C18:2 and C18:3 accumulation is correlated with enhanced expression of FAD2 and FAD3 that respectively peaked 5 to 11 and 8 and 11 days after flowering and decreased sharply afterward (Ruuska et al., 2002; Schmid et al., 2005). Seed C18:3 content is tightly correlated with FAD3 expression levels. The duplication of the FAD3 locus in two Arabidopsis mutants resulted in an increase of FAD3 expression and C18:3 content in seed oil (O'Neill et al., 2011). The overexpression of Arabidopsis FAD3 with seed specific promoters caused at least a two-fold increase in C18:3 levels compared to wild type plants (Puttick et al., 2009; Yadav et al., 1993). The overexpression of FAD3 using the constitutive 35S promoter increased only by 50% the levels of C18:3 in seeds, stressing the importance of transcriptional regulation of FAD3 expression (Hu et al., 2015). Conversely, the *fad3* SALK-042036 mutant which has a T-DNA insertion between its 5'UTR and promoter which impaired its transcriptional activity but not the enzyme activity showed strong reduction of C18:3 content in seed oil (Claessens, 2012.).

Regulation of FAD3 expression during seed development.

Unbiased genetic analysis of omega 3 desaturation regulation carried out by Genome wide association study (GWAS) demonstrated that C18:3 content is controlled by several factors but most predominantly by the FAD3 locus (Menard et al., 2017). FAD3 promoter defined at least as 1138bp upstream of the FAD3 transcription starting site (TSS) was shown to be sufficient to induce a luciferase activity in shoot apical meristems, flowers and developing siliques in Arabidopsis, as well as providing response to auxin, cytokinins and abscisic acid especially in roots (Matsuda et al., 2001). Several known transcription factor binding sites were found in this region and in particular two G-boxes and one CCAAT-box (respectively 174, 400 and 191bp upstream of FAD3 TSS). This promoter region was also able to be transactivated by several transcription factor complexes including

LEC1, LEC-1-Like (L1L), NFYC2 and one of the following bZIP family transcription factors: bZIP67 or AREB3 or EEL or ABI5. The transcription factor bZIP67 is able to bind the G-box1 (-174) *in vitro* and is required with the G-boxes for the transactivation by the complex L1L/NFYC2. However, only a limited effect has been reported on seed lipid composition in the *bzip67* mutant suggesting that other transcription factors are important for FAD3 expression in the seed (Mendes et al., 2013). EEL, AREB3 and bZIP67 are homologous transcription factors coexpressed with FAD3 in embryos that could be also direct regulators (Bensmihen et al., 2005). Furthermore, it has been shown that bZIP12 could directly bind FAD3 promoters in Camelina (Na et al., 2019). Data also suggest that the histone acetyltransferase GCN5 could promote FAD3 expression in a direct manner within the 0-200bp region upstream of the TSS, suggesting a potential transcriptional regulatory hub near FAD3 TATA Box (Hu et al., 2015, p. 5). The fact that *gcn5* mutation has a larger effect on seed lipid profile and in particular on C18:3/C18:2 ratio compared to individual *bzip* mutants like *bzip67*, indicates the existence of redundant or additive transcriptional effects. The histone methyltransferase CLF28 that is involved in the PRC2 complex appears also to be important for FAD3 expression. The *clf28* mutants showed a 2.5-fold increased FAD3 expression in 5-week-old plant siliques followed by a significant decrease in mature green isolated embryos. In consequence the *clf28* mutation resulted in a reduced C18:2 and C18:3 content but an increase in C20:1. The effect of the *clf28* mutation could be linked to small RNA that would then deregulate other master regulators of seed development like FUS3, ABI3, LEC1 and LEC2 (Liu et al., 2016).

The transcription factors LEC1 and L1L, NFYB factors alone cannot trans-activate FAD3 expression but are nonetheless necessary to fully activate FAD3. Transcriptomic data during Arabidopsis silique development showed that FAD3 expression is reduced in *lec-1* mutants compared to WT (Yamamoto et al., 2014). The inducible overexpression of LEC1 in Arabidopsis increased FAD3 expression and the C18:3 content. This effect is conditional to the presence of functional ABI3 and FUS3 activity confirming the importance of these three master regulators during seed development (Mu et al., 2008). BnLEC1 and BnL1L overexpression in *Brassica napus* led also to significant increase in FAD3 expression and C18:3 content during seed development (Tan et al., 2011). These data suggest that LEC1 and potentially LEC1-Like are sufficient for inducing FAD3 expression even in ectopic tissues. Therefore, these two transcription factors could be considered as a critical integrator of developmental cues required for the correct expression of FAD3 during seed development.

Other transcription factors from the MYB and/or BhLh family have also been shown to have an effect on FAD3 expression. TT2 and TT8 for instance are potentially integrating inhibitory signals since *tt2* and *tt8* mutants showed an increased expression of FAD3. These mutations resulted in modified seed fatty acid composition in specific arabidopsis accessions but with decreased C18:3 accumulation, which is counter-intuitive. The effect on FAD3 expression appeared to be indirect in the case of TT8

since the expression of LEC-1 ABI1 FUS3 LEC2 genes (LAFL) was also modified in *tt8*. However, TT2 did not appear to directly impair master regulator expressions and could therefore be a downstream regulator in the FAD3 transcriptional regulatory network (Chen et al., 2012, 2014). MYB76 is also a candidate for PUFA regulation since FAD2 and FAD3 expressions were decreased as well as PUFA levels in *myb76* mutant (Duan et al., 2017).

Interestingly, the overexpression of the transcription factor AmDREB2C from *Ammopiptanthus mongolicus* in *Arabidopsis* increased FAD3 expression and C18:3 levels during seed development. However, the overexpression of other DREB transcription factors resulted in downregulation of FAD3 and FAD8 indicating complex regulatory pattern of the DREB family of transcription factors (Yin et al., 2018).

Regulation of FAD3 expression in response to stresses.

Cold stress appears to have a positive effect on FAD3, since FAD3 expression can be transiently induced during cold stress in birch (Martz et al., 2006), oilseed rape (Tasseva et al., 2004), *Arabidopsis* (Kilian et al., 2007), tomato roots (Yu et al., 2009) and in wheat (Golizadeh & Kumleh, 2019). Those reports mostly point toward a rapid and transient upregulation of FAD3 in vegetative tissues. Conversely, heat stress downregulates FAD3 expression in FAD3 expression roots during a 3 hours exposure to 38°C and subsequent recovery (Kilian et al., 2007). LeFAD3 expression in tomato roots is also markedly reduced upon exposure to high temperature 40°C (Yu et al., 2009).

FAD3 can be transiently induced in cowpea by drought (Torres-Franklin et al., 2009) and transcriptomic analysis showed that hypoxia induces FAD3 as well in *Arabidopsis* (Klinkenberg et al., 2014). Other transcriptomic reports showed that FAD3 can be downregulated during drought in *Arabidopsis* (de Simone et al., 2017; Perera et al., 2008).

The application of DMTU, a ROS scavenger molecule, in guard cell suspension cultures caused a two-fold increase in *FAD3* expression, an effect that could be blocked by the addition of ABA with DMTU. These results, if confirmed, could be a first step toward a mechanistic link between C18:3, FAD3 expression and ROS-mediated stresses discussed above (Böhmer & Schroeder, 2011).

Very few reports described an effect of soil metals on FAD3 expression which is surprising considering their effect on PUFA levels (see above). FAD3 expression was nonetheless reported to be induced with FAD2 and CBR1 in seedlings after application of the iron chelant ferrozine (Oh et al., 2016).

Similarly, to metals, FAD3 expression was modified by biotic stresses in crown gall infection where its expression correlated with tumor development (Klinkenberg et al., 2014).

FAD3 post-transcriptional regulation.

While transcriptional regulation of FAD3 was clearly associated with tissue specific expression and with several abiotic and biotic stresses, post-transcriptional regulation of FAD3 was mostly associated with temperature control. The *Brassica napus* FAD3 protein could be degraded faster at 30°C than at 20°C by the ER associated degradation pathway while tung tree FAD3 desaturase was more stable (O'Quin et al., 2010). It has later been confirmed that the N-termini of both FAD3 proteins could induce proteasome mediated degradation, potentially coupling transcription with protein degradation (Khuu et al., 2011). Surprisingly, BnFAD3 expressed in yeast showed lower enzymatic activity at cold temperatures suggesting the existence of plant specific factors stabilizing FAD3 at low temperature (Dyer et al., 2001). In wheat roots, accumulation of C18:3 in the cold was linked to higher levels of FAD3 protein compared to RNA (Horiguchi et al., 2001). Wheat 5' untranslated region FAD3 was found to slightly increase the translation efficiency of *Arabidopsis* FAD3 during cold stress (Wang & Xu, 2010) but that effect was not sufficient to explain the accumulation of FAD3 protein in cold-treated wheat.

Another mechanism enhancing desaturase activity is metabolite channeling. FAD2 and FAD3 are able to interact and to form stable heterodimer that enable sequential desaturation reactions from C18:1 to C18:3 with limited release of C18:2 (Lou et al., 2014). The positive effect of this regulation on C18:3 production could be further modulated by ALA10, a phospholipid flippase that also physically interacts with both microsomal desaturases enhancing the availability of the C18:1 or C18:2 substrates from the plastid lipids (Botella et al., 2016).

Finally, FAD3 activity is also involved in a post-translational regulatory loop associating membrane fluidity and stress response since *fad3* mutation triggers the proteolytic cleavage of NTL6 (Seo et al., 2010), a transcription factor involved in drought response. Processed NTL6 moves in the nucleus where it regulates ABA and ER stress related genes (Yang et al., 2014). The NTL6 proteolytic cleavage could be directly inhibited by C18:3 application or activated by cold exposure. Cold activation was enhanced in *fad3* background but not in the triple *fad3 fad7 fad8* mutation suggesting that only ER synthesis of C18:3 are involved in this process (Seo et al., 2010). Similarly, the addition of C18:3 can also induce the nuclear localization of the transcription RAP2.12 linked to hypoxia tolerance (Zhou et al., 2019).

B. Regulation of plastidial PUFA desaturases

The *Arabidopsis* FAD7 promoter expressed in tobacco showed that the -362/-166 region containing two putative boxes II could induce GUS expression after light exposition in leaves (Nishiuchi et al., 1995). Similar induction was also found in *Arabidopsis* cotyledon (Soria-García et al., 2019). Furthermore, the FAD7 promoter is also responsive to wounding in leaves, stems and to a lesser extent in roots of tobacco transgenic plants, and that effect could be reduced by the application of JA

synthesis inhibitors or salicylic acid (Nishiuchi et al., 1997). The wounding response was shown to be dependent on JA-biosynthesis genes and SCF^{CO11}-mediated signalling and was also enhanced by exogenous Me-JA applications. FAD7 promoter is able to integrate both JA but also JA unrelated signaling since wounding response can also be induced in *aos* mutants (Matsuda et al., 2009). FAD7 promoter wounding response was confirmed in Arabidopsis transgenic plants and compared with FAD8 promoter (Soria-García et al., 2019). FAD8 promoter induced steady state GUS reporter expression at lower levels than FAD7 in cotyledons but did not respond to wounding. FAD8 promoter activity in root tips was also undetectable. Interestingly, GFP translational fusion showed that FAD8 proteins are more accumulated than FAD7 after JA application suggesting that JA regulation of FAD8 promoter is not identical as for FAD7 promoter (Soria-García et al., 2019). Arabidopsis FAD7 is expressed constitutively in leaves while FAD8 is essentially cold inducible. The correlations between the relative amount of FAD7 and FAD8 protein and transcript levels suggests that these genes are essentially regulated at the transcriptional level (Soria-García et al., 2019). Table 3 summarises the different studies describing transcriptional regulation of the plastidial desaturases.

[Insert Table 3 here]

Altogether these studies confirmed the involvement of transcriptional regulation of plastid desaturase expression by abiotic stresses to fine tune PUFA accumulation in vegetative tissues. However, the transcriptional factors involved in plastidial desaturases regulation are still not known, with the exception of a potential role of AmDREB2C which upregulated FAD7 expression in Arabidopsis. However the existence of an Arabidopsis ortholog is not known (Yin et al., 2018). It can be noted that several chilling-sensitive *chs* mutants did show altered expression of omega-3 desaturases upon cold application. In particular, the cold inducible desaturase FAD8, was not induced in *chs1*, *chs2* and *chs3* mutants potentially contributing to the lethality phenotype (Provar et al., 2003).

Post-translational regulation was also observed in plastidial PUFA desaturases. Similar to FAD2/FAD3, FAD6 was found to interact *in vivo* with FAD7 or FAD8 resulting in trienoic fatty acids channeling (Lou et al., 2014; Soria-García et al., 2019). In addition, temperature dependent control of plastidial desaturase activity occurs also at the post-transcriptional level since the C-terminal of Arabidopsis *FAD8* was shown to reduce protein stability at 27°C which in combination with reduced FAD8 transcriptional activity led to the decrease of trienoic FAs at high temperatures (Matsuda et al., 2005).

V. ISSUES AND STRATEGIES FOR BREEDING HIGH OMEGA-3 CROPS

A. High omega 3 oilseed crops

High omega-3 oilseed crops are bred mainly for the high nutritional value of linolenate for food and feed uses but also for its physical properties (fluidity and oxidation) that is of interest for the lubricant, solvent and deperlant industry (Iii et al., 2009).

The most popular omega 3 crop is linseed or Flax (*Linum usitatissimum*) for its high linolenate content and generally high yields. For instance, M5791 is a recent flax cultivar containing the highest fraction of linolenate in seeds, reaching almost 70 mol% in field conditions. Higher expression of FAD2A, FAD3A and FAD3B microsomal desaturases in this cultivar was proposed to explain the high omega-3 content in seed oil (Thambugala & Cloutier, 2014). Further breeding enabled Agriculture and Agri Food Canada (AAFC) to generate plants with ALA seed oil contents greater than 70 mol% of total FAs (Kenaschuk, 2010). Conversely, the very low linolenic acid flax cultivar (1.3 mol%) Solin, showed stop mutations in both FAD3A and FAD3B homeologs confirming that the microsomal FAD3 are the main source of seed linolenate in this crop (Vrinten et al., 2005).

Chia (*Salvia hispanica*) is an oilseed crop from South America that belongs to the Lamiaceae family. This crop is characterized by a high linolenate content in seed oil (up to 69%) most probably because its two FAD3 homeologs are particularly highly expressed during early seed development (Xue et al., 2018). Chia shows seed yields that can reach up to 2500 kg/ha in Chile (Baginsky et al., 2016) but with a high variability. Chia was introduced in Europe with seed yield peaking around 1200kg/Ha (Grimes et al., 2018). Another interesting feature of Chia is that its major triglyceride is trilinolenin where all three FA positions are filled by C18:3 (Ixtaina et al., 2011). Chia is therefore able to transfer efficiently C18:3 acyl moieties to sn1 or 2 positions of TAG with specific acyltransferases that remain to be characterized.

Perilla (*Perilla frutescens*) is another omega-3 rich oilseed crop also belonging to the lamiaceae family and originating from South East Asia. Perilla seed has a high oil content (more than 40%) in which ALA can represent more than 60% of total fatty acids (Lee et al., 2016). Recent varieties can reach grain yield over 2200 kg/ha in China (Qi et al., 2019). Perilla FAD3 expression was found to be 192-fold higher in developing seeds than in other tissues, and correlated with the highest 18:3 contents of perilla seed fatty acids confirming the importance of the expression of this desaturase during seed development (Lee et al., 2016).

Finally, camelina (*Camelina sativa*) albeit with lower linolenate seed content (30%) turned out to be a valuable omega-3 crop. As a Brassicaceae, this ancient European crop is close to the model organism *Arabidopsis thaliana* and the widely cultivated *Brassica napus*, which has a long history of breeding. *Camelina sativa* is also very easy to transform by flower dip and its genome is sequenced in many accessions and cultivars (Kagale et al., 2014; Li et al., 2021; Luo et al., 2019). Camelina is a polyploid species with three closely related sub-genomes coding three FAD3 homeologs (Kang et al., 2011).

B. Crop improvement through gene engineering of omega-3 desaturases.

1. Seed oil tailoring

Oilseed crop breeding focused primarily in modifying seed oil profile and yield. The discovery of desaturase genes immediately provided tools to change monounsaturated vs polyunsaturated profiles in seed oils. Mutations in FAD2 genes resulted in plants producing seed oil essentially devoided of polyunsaturated fatty acids as described above. The FAD2 gene was found in many plant species but its loss of function seemed to have varied effects. While *Arabidopsis fad2* mutants showed mainly reduced tolerance to several stresses (J. Zhang et al., 2012), camelina *fad2* mutants showed stunted growth and altered development (Lee et al., 2021; Morineau et al., 2017). The different engineering strategies involving FAD2 has been covered recently in detail (Dar et al., 2017).

FAD3 engineering was more specifically used to modify omega-3 content in seed oil. For instance, overexpression of FAD3 using a constitutive promoter enables the accumulation of omega-3 fatty acids in rice seeds that normally accumulate almost no ALA but mainly C18:1 and C18:2. ALA reached about 30% of total fatty acids (Anai et al., 2003). The overexpression of FAD3 with seed specific promoter caused a dramatic increase in C18:3 content in whole seeds of plants accumulating C18:1 and C18:2 causing severe depletion of these fatty acids, suggesting a significant pull effect. Furthermore, the association of microalgae elongases with desaturases, including FAD2 and FAD3 in order to cause a metabolic push effect in *Camelina sativa* and *Brassica napus* allowed the production of very long chain polyunsaturated fatty acids: EPA and DHA (Petrie et al., 2020; Usher et al., 2017). Similarly, soybean could be engineered to produce stearidonic acid that can efficiently increase EPA accumulation in blood cells (Harris et al., 2008). These fatty acids have significant health benefits and can be immediately metabolized by mammals (Schuchardt & Hahn, 2013). Specific desaturases were also used to improve ALA content in crops. The identification of a bifunctional desaturase from the three fungi *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* that can act on both C18:1 to produce C18:2 and on C18:2 to produce C18:3 led to the possibility of generating soybean producing high linolenic plants that could produce 71 mol% of total fatty acid of ALA in dry seeds, while decreasing oleic acid (Damude et al., 2006). Two clusters of amino acids were shown to be responsible for the combined delta 12 and 15 activities, but the latter activity was clearly dependent on the former in *F. moniliforme* (Cai et al., 2018). Furthermore, a recent field trial of T1 soybean plants overexpressing an isoform of FAD3 under the control of a seed specific promoter improved ALA content in seed along with plant growth and pod number as well as grain yield (Yeom et al., 2020). Even if more field trials in different environmental conditions are still needed, these results suggest that seed specific modification of PUFA desaturase activity seem to affect plant fitness.

Stress tolerance

It is now admitted that the overexpression of omega-3 desaturases genes improves cold resistance and tolerance, while loss-of-function mutations increases heat tolerance. For example, the overexpression of LeFAD3 improved chilling tolerance in tomato (Yu et al., 2009) but also improve salt stress tolerance in seedlings (H.-S. Wang et al., 2014). LeFAD7 overexpression led to an increased photosynthetic activity in tomatoes exposed to 4°C for 6 hours providing a possible explanation for cold tolerance (X. Y. Liu et al., 2013). The combined expression of FAD3 and FAD7 further enhanced plant cold tolerance and doubles the content in omega-3 FAs in ripe tomatoes compared to their single overexpression and as a consequence led to an important accumulation of cis-3-hexenal at the expense of hexanal, produced by the lipoxygenase activity and endoperoxydation of C18:3 (Domínguez et al., 2010). Conversely, the depletion of FAD3 expression enhanced heat tolerance (H.-S. Wang et al., 2010) while FAD7 loss of function in tomato improved photosynthetic activity (Wickramanayake et al., 2020).

In tobacco, FAD3 and FAD8 overexpression improved drought tolerance and recovery (M. Zhang et al., 2005) while antisense expression had the opposite effect (Im et al., 2002). A more recent work showed that the overexpression of the cryophyte *Chorispora bungeana* *CbFAD3* in tobacco improved resistance to drought, salt and cold stresses (Shi et al., 2018). The expression of *CbFAD3* reduced electrolyte leakage and MDA formation while increasing photosynthetic yield in these different stress conditions (Shi et al., 2018). More recently, it has been shown in cotton that the overexpression of a *Brassica napus* FAD3 also accelerated seed germination in sub-optimal condition while slightly improving photosynthesis and fiber resistance. (Gao et al., 2020)

In conclusion, omega 3 desaturases appear to represent an interesting leverage to modify agronomic traits including seed oil quality as well as resistance to various stresses. However, the pleiotropic nature of PUFAs in general and omega 3 in particular limits their biotechnological applications. Editing tissue-specific isoforms or the use of tissue-specific promoters could circumvent these limitations by restricting PUFA modification to seed development for instance.

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Tables :**Table 1:** *Arabidopsis* Omega-6 and Omega-3 desaturase characteristics.

| | Substrate | Product | Cofactor | Localisation | Uniprot accession |
|------|--|--|------------|-----------------------|-------------------|
| FAD2 | PC-C18:1 | PC-C18:2 | Cyt-B5 | ER, external leaflet. | P46313 |
| FAD3 | PC-C18:2 | PC-C18:3 | Cyt-B5 | ER, external leaflet. | P48623 |
| FAD6 | MGDG-C18:1 DGDG-C18:1 SQDG-C18:1 PG-C18:1 MGDG-C16:1 DGDG-C16:1 SQDG-C16:1 PG-C16:1 | MGDG-C18:2 DGDG-C18:2 SQDG-C18:2 PG-C18:2 MGDG-C16:2 DGDG-C16:2 SQDG-C16:2 PG-C16:2 | Ferredoxin | Plastid | P46312 |
| FAD7 | MGDG-C18:2 DGDG-C18:2 SQDG-C18:2 PG-C18:2 MGDG-C16:2 DGDG-C16:2 SQDG-C16:2 PG-C16:2 | MGDG-C18:3 DGDG-C18:3 SQDG-C18:3 PG-C18:3 MGDG-C16:3 DGDG-C16:3 SQDG-C16:3 PG-C16:3 | Ferredoxin | Plastid | P46310 |
| FAD8 | MGDG-C18:2 DGDG-C18:2 SQDG-C18:2 PG-C18:2 MGDG-C16:2 DGDG-C16:2 | MGDG-C18:3 DGDG-C18:3 SQDG-C18:3 PG-C18:3 MGDG-C16:3 DGDG-C16:3 | Ferredoxin | Plastid | P48622 |

| | | | | | |
|--|------------------------|------------------------|--|--|--|
| | SQDG-C16:2 PG-C16:2 | SQDG-C16:3 PG-C16:3 | | | |
|--|------------------------|------------------------|--|--|--|

Table 2: Fatty acid content in desaturases mutants compared to wild type expressed as log₂-fold change from published work (reference). Change of fatty acid content in desaturase mutants compared to wild type are highlighted in red (accumulation) or in blue (reduction). Empty table box indicates absence of available data; nd, not detectable.

| Tissue | Mutant | C16:0 | C16:1 | C16:3 | C18:0 | C18:1 | C18:2 | C18:3 | Reference |
|------------------|---------------|-------|-------|-------|-------|-------|-------|-------|--------------|
| <i>leaves</i> | <i>fad2-1</i> | 0,03 | -0,26 | 0,21 | | 3,18 | -1,86 | -0,34 | Lemieux 1990 |
| | <i>fad6</i> | 0,23 | 1,46 | nd | 0,15 | 1,55 | 0,22 | -0,10 | Falcone 1994 |
| | <i>fad3-1</i> | -0,40 | 0,37 | 0,19 | | -1,06 | 0,43 | -0,11 | Lemieux 1990 |
| | <i>fad7</i> | 0,14 | 1,27 | -2,42 | 0,11 | 1,31 | 1,21 | -1,39 | Browse 1986 |
| | <i>fad8-1</i> | | | | | | | | McConn 1994 |
| <i>roots</i> | <i>fad2-1</i> | -0,78 | -0,48 | | -0,91 | 3,04 | -2,23 | -1,16 | Lemieux 1990 |
| | <i>fad6</i> | -0,01 | 0,58 | | 0,00 | 0,17 | -0,14 | -0,01 | Browse 1989 |
| | <i>fad3-1</i> | -0,52 | -0,36 | | -0,48 | 0,82 | 0,88 | -1,47 | Lemieux 1990 |
| | <i>fad7</i> | -0,04 | -0,39 | | 0,08 | -0,05 | 0,02 | 0,05 | Browse 1986 |
| | <i>fad8</i> | | | | | | | | McConn 1994 |
| <i>dry seeds</i> | <i>fad2-1</i> | -0,22 | | | 0,78 | 1,82 | -3,32 | -1,86 | Lemieux 1990 |
| | <i>fad6</i> | 0,15 | | | 0,00 | -0,15 | -0,02 | -0,58 | Browse 1989 |

| | | | | | | | | | |
|--|---------------|-------|-------|--|-------|------|------|-------|-----------------|
| | <i>fad3-1</i> | -0,38 | | | 0,06 | 0,58 | 0,49 | -3,14 | Lemieux 1990 |
| | <i>fad7</i> | 0,00 | -0,32 | | -0,09 | 0,02 | 0,01 | -0,02 | Browse 1986 |
| | <i>fad8</i> | | | | | | | | McConn 1994 |

Table 3. Reports of differential regulation of FAD7 and FAD8 in response to stress related signals.

| Organism | Gene | Stress | Regulation | References |
|-------------|--------|---------|------------|--------------------------------|
| Olive tree | FAD7 | Cold | Up | (Hashempour et al., 2019) |
| Soybean | FAD7-1 | Cold | Up | (Román et al., 2012) |
| Arabidopsis | FAD7 | Cold | Down | (Román et al., 2015) |
| Birch | FAD7 | Cold | Down | (Martz et al., 2006) |
| Maize | FAD7 | Cold | Up | (Berberich et al., 1998) |
| Arabidopsis | FAD8 | Cold | Up | (Román et al., 2015) |
| Birch | FAD8 | Cold | Up | (Martz et al., 2006) |
| Rice | FAD8 | Cold | Up | (J. Wang et al., 2006) |
| Cowpea | FAD8 | Drought | Down | (Torres-Franklin et al., 2009) |

Figures legends

Figure 1: Simplified biosynthesis pathway of polyunsaturated fatty acids in the plastids and the endoplasmic reticulum. C4:0 elongation is carried out by the FAS complex and fatty acids are extended by KAS consuming malonyl-ACP. The desaturation reactions of the desaturases are carried out on specific substrates in organelles and on lipid classes described in Table 1.

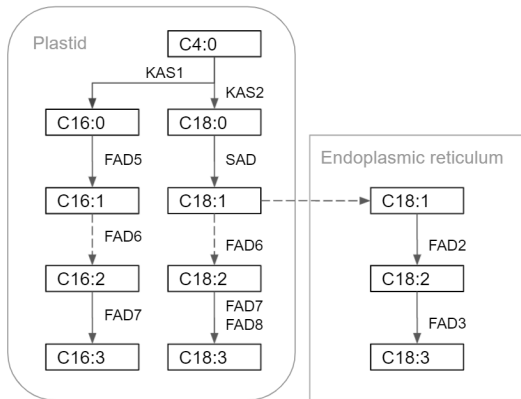


Figure 2: Protein sequence and structure analysis of Arabidopsis FAD2, FAD3, FAD6, FAD7 and FAD8.

A: Alignment of *Arabidopsis* FAD2, FAD3, FAD6, FAD7 and FAD8 protein sequences using clustal omega and accession numbers described in Table 1. Integral transmembrane regions are highlighted in yellow and histidine boxes highlighted in red. **B:** Schematic representation of FAD desaturase structure. Grey box represents membrane lipid bilayers, Fe indicates the stabilized di-iron center, yellow bars represent transmembrane domain.

A

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SP|P46313|FAD2 -----
SP|P48623|FAD3 -----
SP|P46312|FAD6 MASRIADSLFAFTG-----PQQCLPRVPKLAASSARVSPGVYAVKPI 42
SP|P46310|FAD7 MA----NLVSECGIRPLPRIYTTPRSNFLSNNN---KFRPSLSSSSYKT-----SSSPL 48
SP|P48622|FAD8 MA----SSVLECGFRPLPRFYPKHHTTSFASNPKPTFKFNPLL-----KPP 42

SP|P46313|FAD2 -----MGAG-GRMPVPTSSKKSETDITKR-----VP 25
SP|P48623|FAD3 -----MVMAMDQRTNVNGDPGAGDRKKEERF-----DP 28
SP|P46312|FAD6 DLLLKGRTSRRCVAPVKRRIGCI---KAVAAPVAPPSADSAEDREQLAESYGFQIGE 99
SP|P46310|FAD7 SFGLNSRDG-----FTRNWALNVSTP-LTTPIFEESPLEEDNKQRF-----DP 90
SP|P48622|FAD8 SSSLNSRYG-----FYSKTRNWALNVATP-LTTLQ---SPSEEDTERF-----DP 83

SP|P46313|FAD2 CEKPPFVSGDLKKAIPPHCFKRSIPRSFYSYLISDII-IASCFYVATNYFSLLPQPLSYL 84
SP|P48623|FAD3 SAQPPFKIGDIRAAIPKHCWVKSPLRSMSYVVRDII-AVAALAAIAAV-----YVDSWF 80
SP|P46312|FAD6 DLPENVTLKDIMDTLPEKVEFIDDLKALKSVLISVTSYTLGL-----FMIAKSPWY 150
SP|P46310|FAD7 GAPPPFNLAIRAAIPKHCWVKNPWKLSYVVRDVA-IVFALAAGAA-----YLNNNWI 142
SP|P48622|FAD8 GAPPPFNLAIRAAIPKHCWVKNPWMSMSYVVRDVA-IVFGLAAVAA-----YFNNWL 135
      ..*: :*: . : . : : : . : : : . : : :
SP|P46313|FAD2 AWPLYWACQGCVLTDGIWVIAHECGHAFSDYQWLDLDTVGLIFHSFLLVPYFSWKYSHRRH 144
SP|P48623|FAD3 LWPLYWAAQGTLFWAI FVLGHDCGHGSFSDIPLNSVVGHLHHSF ILVPYHGWRISHRTH 140
SP|P46312|FAD6 LLPLAWAWTGTAITGFFVIGHDCAHKSF SKNKLVEDIVGT LAF LPLVYPYEPWRFKHDRH 210
SP|P46310|FAD7 VWPLYWLAQGTMFWALFVLGHDCGHGSFSDNDPKLSNVVGHLLHSSILVPYHGWRISHRTH 202
SP|P48622|FAD8 LWPLYWFAQGTMFWALFVLGHDCGHGSFSDNDPRLNSVAGHLHSSILVPYHGWRISHRTH 195
      ** * * : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
SP|P46313|FAD2 HSNTGSLERDEVFVPKQSAIKWYGKYLNNPLGRIMMLT-VQFVLGWPLYLAFNVSGRPY 203
SP|P48623|FAD3 HQNHGHVENDES WVLP ERVYKKLPH---ST-RMLRYTVPLMLAYPLYLCYRSPGKE- 194
SP|P46312|FAD6 HAKTNMLVHDTAQVPPPEEFESSPVMRKA---IIFGYGPIRPWLSIAHW-----257
SP|P46310|FAD7 HQNHGHVENDES WHPMSEKIYNTLDK---PT-RFFRFTLPLVMLAYPFYLWARS PGKK- 256
SP|P48622|FAD8 HQNHGHVENDES WHPLESIYKNLEK---TT-QMFRFTLPPMLAYPFYLWNRSPGKQ- 249
      * : : . : * : * : * : : : : : * : * :
SP|P46313|FAD2 DGFACHFFNPAP IYNDRLERLQIYLS DAGILA-VCFGLYRYAAAQGMASMICLYGVPLLIIV 262
SP|P48623|FAD3 ---GSHFNPYSSLFAPSERKLIATSTTCWSI-MFVSLIALSFVFGPLAVLKVYGVPIYIF 250
SP|P46312|FAD6 ---VNWHFNLKFRASEVNRVKISLACVFAFMVAVGWPLIYKVGILGWKFWLMPWLGY 313
SP|P46310|FAD7 ---GSHYHPDSDLFLPKERKDVLTSTACWTA-MAALLVCLNFTIGPIQMLKLYGIPYWIN 312
SP|P48622|FAD8 ---GSHYHPDSDLFLPKERKDVLTSTACWTA-MAALLVCLNFVMGPIQMLKLYGIPYWIF 305
      : : : * : * : : : : * : : : * :
SP|P46313|FAD2 NAFVLVITYLQHT--HPSLPHYDSS EWDWLRGALA-TVDRDYG-ILNKVFHNITDTHVAH 318
SP|P48623|FAD3 VMWLDVAVTYLHHHGHD EKL PWYRGKEWSYLRGGLT-TIDRDYG-IFNNIHDI-GTHVIH 307
SP|P46312|FAD6 HFWMSTFTMVHHT--APHIPFKPADEWNAQAQLNGTVHCDYPSWIEILCHDI-NVHIPH 370
SP|P46310|FAD7 VMWLDVAVTYLHHHGHD EKL PWYRGKEWSYLRGGLT-TLDRDYG-LINNIHDI-GTHVIH 369
SP|P48622|FAD8 VMWLDVAVTYLHHHGHD EKL PWYRGKEWSYLRGGLT-TLDRDYG-WINNIHDI-GTHVIH 362
      : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
SP|P46313|FAD2 HLFSTMPHYNAMEATKAIKPI LGDYYQFDGTPWYVAMY-----REAKECIYVEPDREGD 372
SP|P48623|FAD3 HLFQPIPHYLV DATKAAKHLVGRYREPKTSGAIP IHLVESLVA SIKKDHYV-----SD 362
SP|P46312|FAD6 HISPRIPSYNLRAAHESI QENWGKYTNLATWNWRLMKTIMTVCHVYDKEENYIPFDRLAP 430
SP|P46310|FAD7 HLFQPIPHYLV EATEAAKPV LGKYREPKSGPLPLHLEILAKSIKEDHYV-----SD 424
SP|P48622|FAD8 HLFQPIPHYLV EATEAAKPV LGKYREPKNSGPLPLHLLGSLIKSMKQDHFV-----SD 417
      * : : * * : * : : * * : : : * : : :
SP|P46313|FAD2 K--KGVYWYNNKL-----383
SP|P48623|FAD3 T--GDIVFYETDPDLYVYASDKSKIN 386
SP|P46312|FAD6 EESQPITFLK KAMPNYTA-----448
SP|P46310|FAD7 E--GEVYVYKADPNLYGEVKVRAD--446
SP|P48622|FAD8 T--GDVYVYEA DPKLNQRT-----435

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B

