

Two Plastid Fatty Acid Exporters Contribute to Seed Oil Accumulation in Arabidopsis¹

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Triacylglycerols (TAGs) are the major storage form of seed oil in oilseed plants. They are biosynthesized de novo in seed plastids and then transported into the endoplasmic reticulum. However, the transport mechanism for plastid fatty acids in developing seeds remains unknown. Here, we isolated two novel plastid fatty acid exporters (FATTYACID EXPORT 2 [FAX2] and FAX4, respectively) specifically abundant in seed embryos during the seed-filling stage in *Arabidopsis thaliana*. FAX2 and FAX4 were both localized to the chloroplast membrane. FAX2 and FAX4 loss-of-function mutations caused deficiencies in embryo and cotyledon development. Seeds of *fax2fax4* double mutants exhibited significantly reduced TAG contents but elevated levels of plastid lipid contents compared with those of wild-type plants. By contrast, overexpression of FAX2 or FAX4 enhanced TAG deposition. Seed-feeding experiments showed that the two FAX proteins transported ¹⁴C-plastid fatty acids and ¹³C-oleic acids for TAG biosynthesis during the seed-filling stage. Together, our data demonstrate that FAX2 and FAX4 play critical roles in transporting plastid fatty acids for TAG biosynthesis during seed embryo development. These two transporters may have broad application for increasing oil yield in oilseed crops.

Plant oils are necessary for providing unsaturated fatty acids (FAs) in the diet to promote human health. Triacylglycerols (TAGs) are the majority form of storage lipids in seeds of oilseed plants, including *Arabidopsis thaliana*, *Brassica napus*, and *Canarium album* (Li-Beisson et al., 2013). In these oilseed plants, TAGs are synthesized mainly in the embryo and contribute up to 50% of the seed dry weight. Therefore, the

characterization of TAG biosynthesis in the seeds of oil plants is vital for increasing seed oil yield.

In plants, TAG biosynthesis requires the transport of plastid-synthesized FAs from the plastids to the endoplasmic reticulum (ER). In *Arabidopsis*, ~62% of plastid free FAs are exported to the cytosol through the eukaryotic pathway (Browse et al., 1986; Somerville and Browse, 1991). It is well demonstrated that in *Arabidopsis* leaves, free FAs are exported by FATTY ACID EXPORT1 (FAX1), which is localized at the plastid inner envelope. The exported toxic free FAs are used for vectorial acylation driven by the plastid outer envelope-localized long-chain acyl-CoA synthetase 9 (LACS9). Then, the esterified acyl-CoAs are shuttled from the plastid/cytosol to the plastid envelope-ER contact site by acyl-CoA binding proteins (Leung et al., 2006; Benning, 2009; Xiao et al., 2009; Meng et al., 2014; Xie et al., 2015). The acyl-CoAs are further imported into the ER by ATP-binding cassette transporter 9 (ABCA9), which is localized at the ER membrane of seed cells (Kim et al., 2009, 2013). The ER-localized acyl-CoAs are further incorporated into TAG biosynthesis (Li-Beisson et al., 2013). Overexpression of *ABCA9* enhanced TAG contents in seeds (Kim et al., 2009, 2013). Therefore, regulation of the transcription levels of membrane protein genes involved in TAG

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biosynthesis should be one option to increase seed storage lipid content in oilseed plants.

TAGs are usually synthesized in three organs, the seeds, leaves, and flower buds. To identify the transporters that export free FAs from plastids for TAG synthesis, we focused on FAX proteins in Arabidopsis. Previous studies showed that overexpression of *AtFAX1* increased ER-derived TAGs in flowers and leaves but not in seeds (Li et al., 2015). In Arabidopsis, at least seven genes are predicted to encode FAX transporters, implying that other FAX members may mediate TAG synthesis in seeds. Among these FAX members, FAX2 and FAX4 are predicted to localize at plastids, and their transcripts are abundant in seeds (Li et al., 2015, 2016). We speculated that these FAX proteins may mediate plastid FA export specifically in seeds during the seed oil biosynthesis stage. Here, we characterized the function of FAX2 and FAX4 in seed TAG biosynthesis. Our current study provides an important genetic engineering strategy to improve seed lipid yield for oil plants.

RESULTS

fax2 and *fax4* Mutants Exhibit Abnormal Seed Size Phenotypes

To identify the potential FAX transporters involved in free FA export from plastids during seed

development, we characterized the expression levels of these FAXs, which are predicted to encode proteins with chloroplast-targeting peptides, in developing seeds of Arabidopsis. The phylogenetic tree reveals that these FAX proteins are classified into two major clades, and that *AtFAX1*, *AtFAX2*, and *AtFAX4* belong to the same group (Supplemental Fig. S1, A and D). *AtFAX2* (At2g38550) and *AtFAX4* (At1g33265) are highly expressed during the early stage of seed development (Supplemental Fig. S1B). We grew transfer DNA insertion mutants of *AtFAX2* (*fax2*), *AtFAX4* (*fax4*), and double mutant *fax2fax4* under normal conditions (Supplemental Fig. S1, A and C). Many dry seeds of the mutants were abnormal compared with those of wild-type plants (ecotype Columbia of Arabidopsis [Col-0]; Fig. 1B; Supplemental Fig. S2A). The seeds were classified into four groups: normal, large, shrunken, and small, according to the area of seeds (Fig. 1B; Supplemental Fig. S2B). Only 74% of *fax2fax4*, 83% of *fax2*, and 89% of *fax4* seeds were normal compared with wild-type seeds (Fig. 1C; Supplemental Fig. S2B). Furthermore, *fax2fax4* seeds were more varied in size than wild-type seeds when measured using ImageJ (Supplemental Fig. S2B). The complementation lines of FAX2 and FAX4 (*AtFAX2-comp* and *AtFAX4-comp*, respectively) showed the same size seeds as in wild-type plants (Supplemental Fig. S3, A and B).

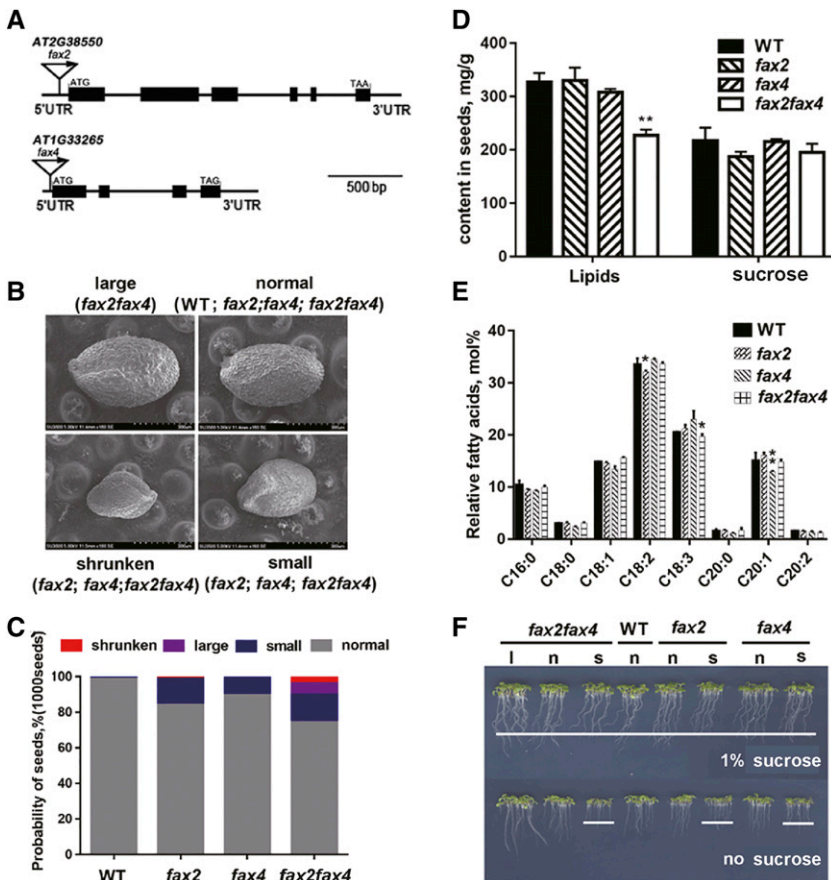


Figure 1. Characterization of *fax2* and *fax4* mutants. A, Schematic representation of the FAX2 and FAX4 genes. Two transfer DNA insertion sites in the 5' untranslated (UTR) regions are indicated by triangles. The mutants are SALK_135883 (*fax2*) and SAIL_671_D03 (*fax4*). B, The four seed classification groups of *fax2fax4* mutants according to seed area. Large seeds are shown only in the *fax2fax4* mutant. Shrunken and small seeds are found in the *fax2*, *fax4*, and *fax2fax4* mutants. WT, wild type. C, Probability of the four classified seed groups in 1,000 seeds of each mutant and the wild type. D, Total lipid and sucrose weight per seed in *fax2*, *fax4*, and *fax2fax4* mutants and the wild type (mean ± SD of n = 3–5 seeds). E, FA composition of TAG in mature seeds of *fax2*, *fax4*, *fax2fax4*, and the wild type (mean ± SD of n = 3–5 seeds). *P < 0.05 and **P < 0.01, Student's t test. F, Abnormal shrunken and small seeds (s) in each mutant line are delayed in seedling growth on one-half strength Murashige and Skoog medium with and without Suc. l, Large seeds; n, normal seeds.

To characterize the potential roles of FAX2 and FAX4, we detected the contents of total lipids and Suc in seeds. The lipid contents in the *fax2fax4* mutant plants were significantly reduced relative to those in the *fax2* and *fax4* mutant and wild-type plants, but the Suc content did not differ (Fig. 1D). The seed FA composition of TAG was comparable in the mutants and wild type (Fig. 1E), which indicates that neither *fax2* nor *fax4* affects the relative probability of FA composition in TAG of seeds. To demonstrate that the genetic seed lipid phenotype of the mutant was caused by FAX2 or FAX4, we also detected the seed TAG and FA composition in the different complementation lines of FAX2 or FAX4. All transgenic lines produced normal seeds and showed similar or even increased TAG content compared with the wild type (Supplemental Fig. S3, C and D). In oilseed plants, early seedling growth usually depends on storage lipids in the absence of Suc (Cernac et al., 2006; Kim et al., 2013). When different abnormal and normal seeds from the mutant and wild-type plants were grown on one-half strength Murashige and Skoog medium with or without Suc, we observed that the growth of small and shrunken seeds in the mutants was defective in the absence of Suc but normal in the medium with Suc (Fig. 1F). These results indicate that the shrunken and small seeds in *fax2*, *fax4*, and *fax2fax4* mutants might be defective in the accumulation or mobilization of storage lipids.

FAX2 and FAX4 Are Localized at the Chloroplast Membrane

To determine whether FAX2 and FAX4 are involved in lipid biosynthesis, we first detected their subcellular localization using transgenic plants expressing FAX2-GFP or FAX4-GFP under the *CaMV 35S* promoter (*Pro-35S::FAX2-GFP* and *Pro-35S::FAX4-GFP*, respectively). Permease in Chloroplasts1 (PIC1), which is a chloroplast inner envelope protein, was used as a positive control (Duy et al., 2007). Green fluorescence signals were detected around the chloroplast in the mesophyll protoplasts isolated (Fig. 2A), which was consistent with the prediction by ARAMEMNON (<http://aramemnon.uni-koeln.de/>) that the FAX2 and FAX4 protein sequences contain chloroplast transit peptides at the N terminus. The results showed that both FAX2 and FAX4 are localized at the chloroplast membrane, similar to PIC1 and FAX1 (Li et al., 2015).

To test the possibility that FAX2 and FAX4 are physical partners at the chloroplast envelope in the seeds, we characterized the interaction between FAX2 and FAX4 using a mating-based split ubiquitin system and bimolecular fluorescence complementation. In the mating-based split ubiquitin system analysis, the coding sequences (CDSs) of both proteins and the sequences without chloroplast transit peptides were inserted into PBT3-N and PPR3-N vectors, respectively. No signal was detected in the synthetic dropout medium without

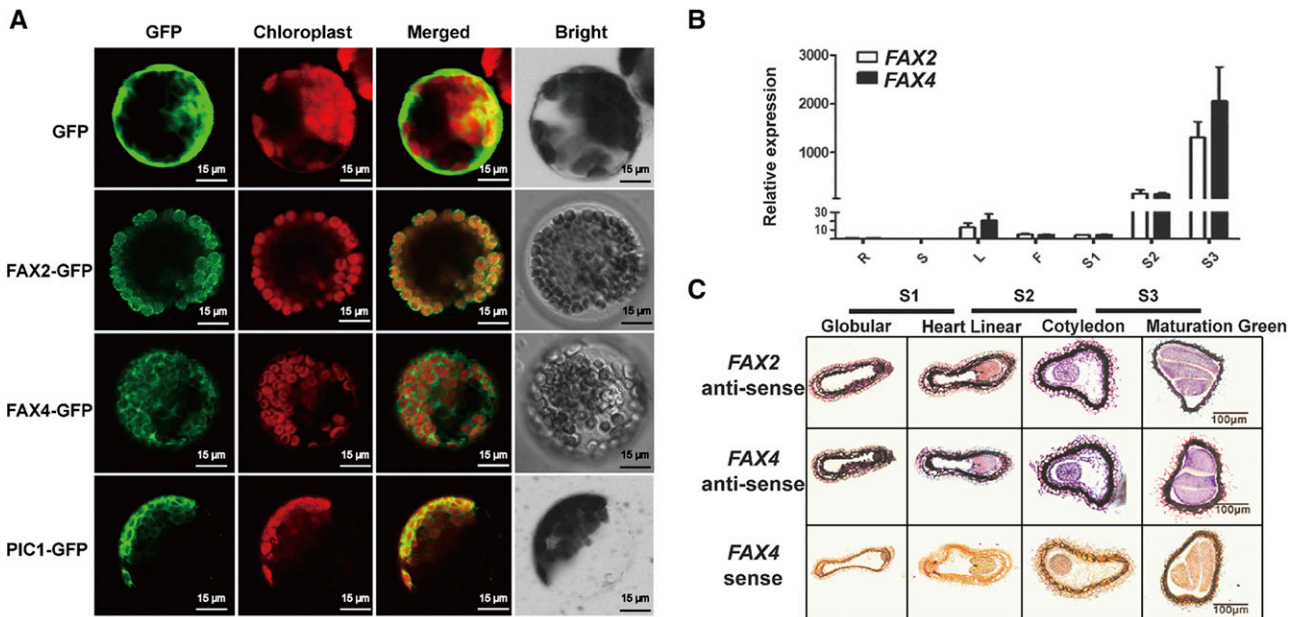


Figure 2. Subcellular localization of FAX2 and FAX4 and their transcript levels in different tissues of Arabidopsis. A, GFP signals and chlorophyll fluorescence signal from the protoplast of the Pro-35s::FAX2 and Pro-35s::FAX4 gDNA-GFP transgenic Arabidopsis. PIC1-GFP was used as a chloroplast inner envelope marker. B, Relative expression of FAX2 and FAX4 transcripts in tissues of Arabidopsis roots (R), stems (S), leaves (L), flowers (F), siliques from 4–6 DAF (S1), siliques from 10–12 DAF (S2), and siliques from 16–18 DAF (S3). Student’s *t* test (mean \pm SD of *n* = 3–4 tissue samples). C, RNA in situ hybridization of FAX2 and FAX4 in siliques at different seed development stages. The siliques of Col-0 were cross-sectioned for hybridization with antisense and sense probes of FAX2 and FAX4.

Trp/Leu/Ade/His when these constructs containing *FAX2* and *FAX4* were incubated together (Supplemental Fig. S4A). Similarly, no interaction signal was observed in the protoplast when these two proteins were cotransformed in the bimolecular fluorescence complementation assay (Supplemental Fig. S4B). These results reveal that *FAX2* and *FAX4* do not physically interact, although both are localized at the chloroplast envelope.

***FAX2* and *FAX4* Are Expressed Specifically in the Embryo of Seeds**

We determined the spatial and temporal expression patterns of *FAX2* and *FAX4* in different tissues of *Arabidopsis* by reverse-transcription quantitative PCR (RT-qPCR). *FAX2* and *FAX4* transcripts accumulated slightly in the root, stem, leaf, flower, and silique from 4 to 6 d after flowering (DAF), whereas high expression levels were detected in the siliques at 10–12 DAF, and even higher levels in the older siliques, such as at 16–18 DAF (Fig. 2B). RNA in situ hybridization further showed that *FAX2* and *FAX4* were preferentially expressed in the embryos of developing seeds (Fig. 2C). RT-qPCR analysis also revealed that amplification of the transcripts of both *FAX2* and *FAX4* was significantly higher in the embryo than at the endosperm (Supplemental Fig. S5). These findings indicate that *FAX2* and *FAX4* may be involved in seed development, especially storage lipid biosynthesis.

***FAX2* and *FAX4* Are Required for Storage Lipid Biosynthesis During Seed-Filling Stages**

Given that both *FAX2* and *FAX4* transcripts mainly accumulate during the seed development process, we investigated the seed morphology of *fax2*, *fax4*, *fax2fax4*, and the wild type at 8, 10, and 14 DAF. Approximately 13% of *fax2*, 13% of *fax4*, and 25% of *fax2fax4* seeds were shrunken and/or smaller at the early stage of seed development (Fig. 3A). We further detected the seed morphology at 10 DAF using scanning electron microscopy (SEM) and found that defective seeds in the *fax2fax4* double mutant were more shrunken and smaller than those in the single mutants (Fig. 3B). The area of the cotyledon and the area of cotyledon cells of the *fax2fax4* mutant were smaller than those of the wild type (Fig. 3C). These results coincided with the spatial and temporal expression of *FAX2* and *FAX4*, supporting our hypothesis that the abnormal seed development in these mutants is caused by knock-out of *FAX2* and/or *FAX4*.

In seed plants, seed formation is an important process involving the coordinated growth of the embryo, endosperm, and seed coat, and embryo maturation (6–20 DAF) determines the size and storage components of seeds (Baud et al., 2003, 2008). We examined the development of the embryo and endosperm in *fax2*, *fax4*, *fax2fax4*, and wild-type plants. The embryo development of the mutants was distinctly delayed compared with that of the wild type. At 3 DAF, the

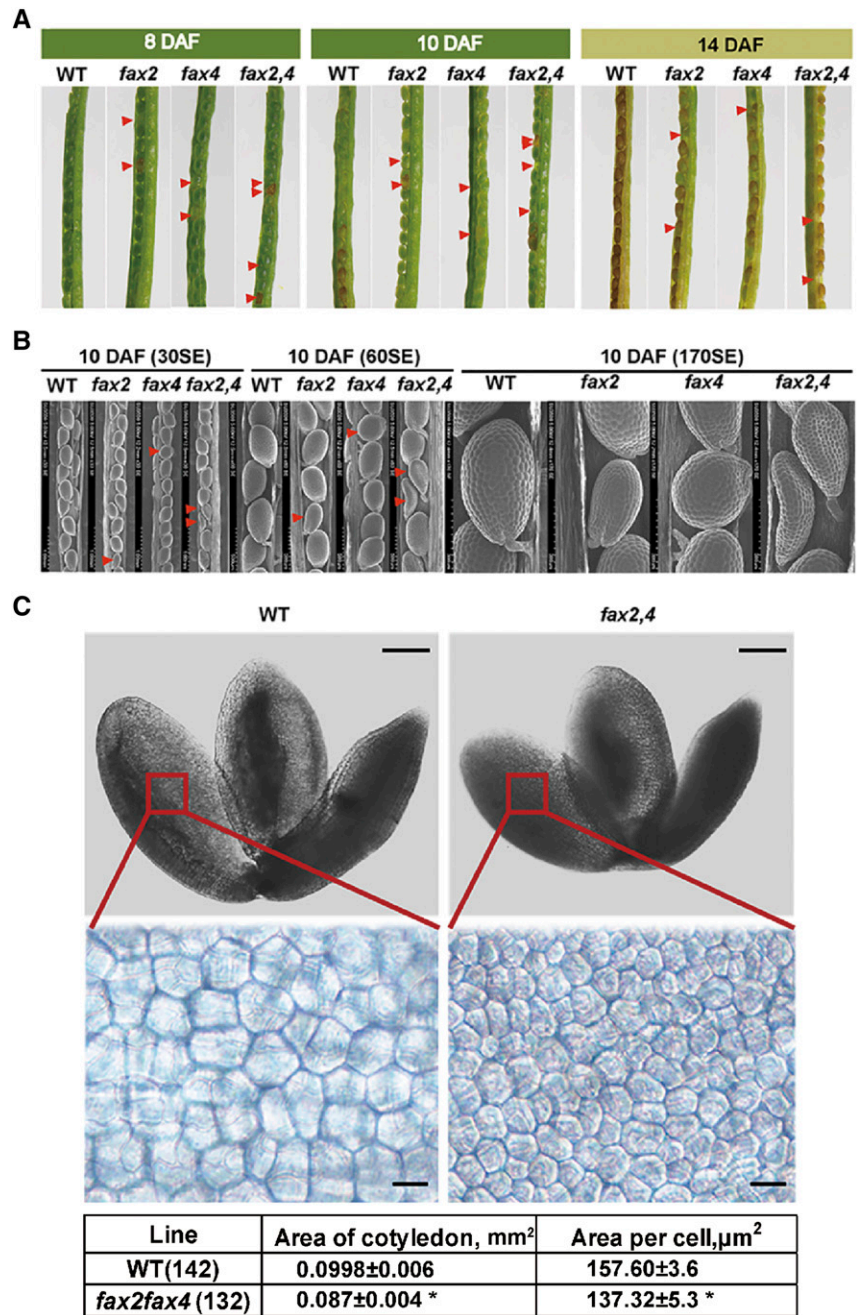
majority of wild-type embryos reached the early heart stage, whereas the mutants reached the globular stage (Fig. 4). At 4 DAF, the embryos and cotyledons of the *fax2fax4* mutant were significantly smaller and more shrunken than those of the wild type. Similar phenotypes were also observed in the *fax2* and *fax4* single mutants. This phenotypic alteration in the mutants was maintained at 6–7 DAF (Fig. 4). These results showed that *FAX2* and *FAX4* are required for the proper development of the embryo and cotyledon, where the majority of storage lipids are synthesized, indicating that *FAX2* and *FAX4* may affect storage lipid biosynthesis during the seed-filling stage.

***fax2fax4* Mutant Seeds Have Reduced TAG Content**

We further determined whether the *fax2fax4* seeds had decreased content of TAGs, which comprise the majority of lipids in seeds, compared with the wild type. The total TAG content in dry seeds of *fax2fax4* mutants was ~30% less than in the wild type (Fig. 5A). Most of the TAG biosynthesis in the *fax2fax4* seeds was reduced compared to that in the wild type. Fold changes were highest for low-abundance TAGs (e.g. 1.6- and 1.8-fold reduction for TAG54:7 and TAG58:6, respectively, in the *fax2fax4* seeds [Fig. 5B]). A massive reduction in highly abundant TAGs was also observed in *fax2fax4* seeds (Fig. 5B). More importantly, the largest reduction, which had the most impact on the total TAG amount, was in the highly abundant species, such as TAG56:5 and TAG56:6, in the *fax2fax4* seeds (Fig. 5B). However, when the relative TAG molecule content (mol %) of these highly regulated TAG species was calculated, almost no difference was detected between the mutant and the wild type (data not shown), indicating that the relative abundance of TAG molecular species was not affected by *FAX2* and *FAX4*.

Glycolipid biosynthesis consists of two pathways: (1) the prokaryotic pathway, occurring in the plastid stroma; and (2) the eukaryotic pathway, where plastid FAs export to the cytosol and synthesized phosphatidylcholine and phosphatidic acid, and are further imported into the plastid (Li-Beisson et al., 2013; Li et al., 2016). The knockout of *AtFAX1* prevented the eukaryotic pathway, while it accelerated the prokaryotic pathway, which increased plastid-derived glycolipid accumulation (Li et al., 2015). In this study, we hypothesized that the plastid-derived glycolipid contents in 10–14 DAF seeds should be increased in the *fax2fax4* mutant line. We determined the plastid-derived 34:x glycolipid contents, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol, and found that more plastid-derived lipids had accumulated in the *fax2fax4* seeds compared to the wild type (Fig. 5C). We further investigated the cytological difference in developing seeds between *fax2fax4* mutants and wild-type plants using transmission electron microscopy. The *fax2fax4* chloroplasts clearly accumulated larger plastoglobule (PG) lipid droplets than

Figure 3. Detection of the abnormal seeds of *fax2* and *fax4* mutants and wild-type (WT) plants at different seed development stages, including 8, 10, and 14 DAF. Red arrowheads indicate defective seeds. B, Detection of abnormal seeds of the mutants and wild type at 10 DAF using SEM. Secondary electron size (SE). C, The *fax2-fax4* double mutant produces abnormal seeds. Shown are mature embryos isolated from mature dry seeds of the wild type and the *fax2fax4* knock-out line after imbibition for 1 h (top) and cotyledon cells from the boxed areas in A (bottom). Scale bars = 100 μm (top) and 10 μm (bottom). The table lists the surface areas of cotyledons and of individual cells of cotyledons (132 from *fax2fax4* and 142 from the wild type) measured from images of the embryos. * $P < 0.05$, Student's *t* test.



did the wild-type chloroplasts (Fig. 5D). Overall, the reduction in TAG contents in the *fax2fax4* seeds supports the idea that FAX2 and FAX4 are involved in chloroplast-localized FA transport for TAG biosynthesis in the embryos of developing seeds of Arabidopsis.

FAX2 and FAX4 Mediate Chloroplast-Localized FA Transport for TAG Biosynthesis

The plastid de novo synthesized FAs are exported mainly as palmitic acid ($\text{C}_{16:0}$), oleic acid ($\text{C}_{18:1}$), and

stearic acid ($\text{C}_{18:0}$), which were derived from acetate. Among these FAs, $\text{C}_{18:1}$ is the major free FA form (Browse et al., 1986). Palmitic acid is the chloroplast-localized secondary high-abundance free FA (Browse et al., 1986). To assess the transport specificity of FAX2 and FAX4 for FAs, which are exported from chloroplasts for TAG biosynthesis in vivo, we separately fed ^{14}C -acetate, ^{14}C -oleic acid, and ^{14}C -palmitic acid ($\text{C}_{16:0}$) to 10-DAF developing seeds isolated from *fax2*, *fax4*, *fax2fax4*, and wild-type silicles and detected the radioactivity of the isolated TAG fraction. Acetate can be converted into acetyl-CoA and malonyl-CoA for further FA biosynthesis in plastids (Kim et al., 2013;

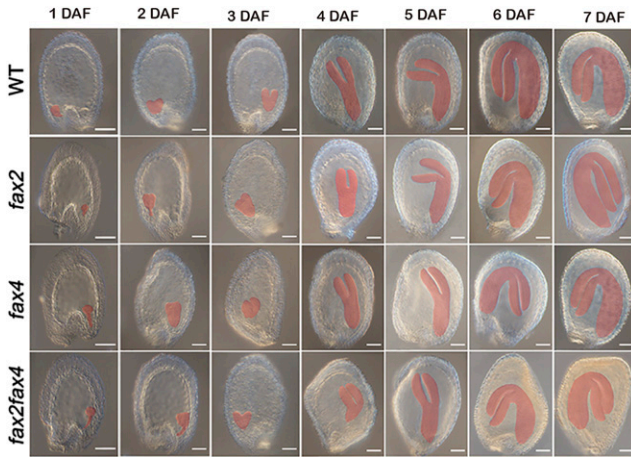


Figure 4. Seed embryo development is delayed to different degrees in *fax2*, *fax4*, and *fax2fax4* mutants. The figure shows whole-mount seeds observed with differential interference contrast optics at different developmental stages of embryogenesis in the wild type (WT) and *fax2*, *fax4*, and *fax2fax4* mutants. Scale bars = 50 μ m.

Li-Beisson et al., 2013). With feeding of 14 C-acetate, we found that the 14 C-labeled TAG content was significantly reduced in the *fax2fax4* seeds compared to the wild type (Fig. 6A; Supplemental Fig. S6A). After feeding with 14 C-oleic acid, the amount of radioactivity incorporated into TAG in the *fax2fax4* seeds was ~40% of that in wild-type seeds (Fig. 6A). The amount of labeled TAGs in the *fax2* and *fax4* seeds was also lower than in the wild type (Supplemental Fig. S6B). With the feeding of 14 C-palmitic acid, the amount of radioactivity incorporated into TAGs in *fax2fax4* mutants was

weakly decreased compared with that in wild-type seeds (Fig. 6A).

To further determine the difference in TAG content between *fax2*, *fax4*, *fax2fax4*, and wild-type seeds, we fed 13 C-oleic acid to 10-DAF developing seeds from *fax2*, *fax4*, *fax2fax4*, and wild-type siliques. The total TAGs and amounts of TAG species were detected by a reverse-phase HPLC electrospray ionization tandem mass spectrometry (MS/MS) approach described previously (Lam et al., 2014; Jiang et al., 2017; Li et al., 2019). The 13 C-labeled TAG content was significantly lower in seeds of *fax2*, *fax4*, and *fax2fax4* mutants than in wild-type seeds (Fig. 6B; Supplemental Fig. S6C). These results indicated that both FAX2 and FAX4 are chloroplast FAXs that specifically mediate free FA efflux for acyl-CoA and further supply FA substrates for TAG biosynthesis in the embryos of developing seeds in Arabidopsis.

Overexpression of FAX2 and FAX4 Elevates Seed Oil Content

To investigate whether FAX proteins mediate free FA transport from plastids and improve lipid synthesis during seed development, we generated transgenic Arabidopsis plants overexpressing FAX2 and FAX4, respectively (Fig. 7). The FAX2- or FAX4-overexpressing plants produced seeds that were enlarged compared to wild-type seeds (Fig. 7, A and B; Supplemental Fig. S7). Biochemical analyses revealed that the lipid content per seed, especially C18:2 and C18:3 unsaturated FA compositions, in the FAX2- or FAX4-overexpressing lines was up to ~127% to 148% of that in the wild type (Fig. 7, C and D). Therefore, we conclude that overexpression

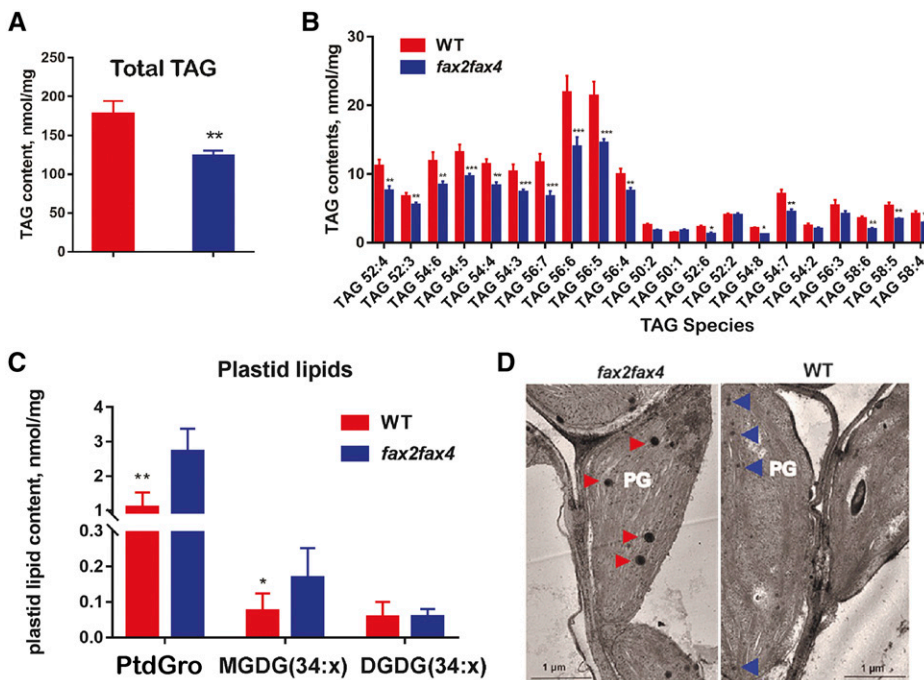
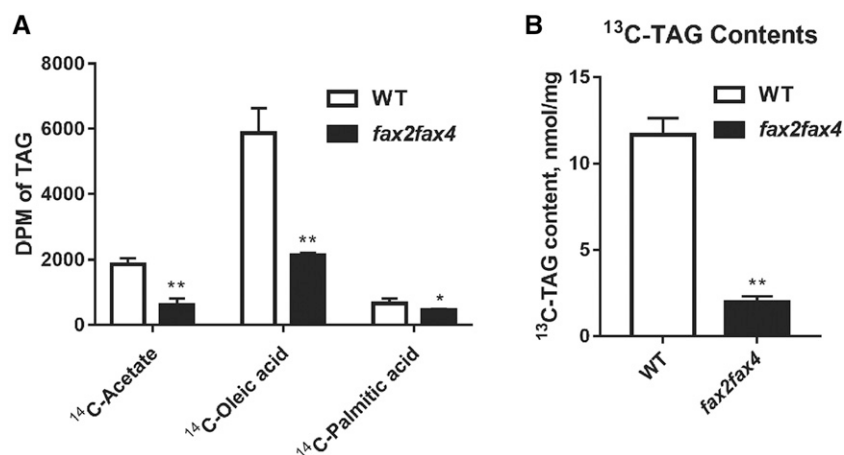


Figure 5. TAG contents in *fax2fax4* and the wild type (WT). A and B, Total TAG contents (A) and TAG species contents (B) in the *fax2fax4* mutant and the wild type. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, Student's *t* test (mean \pm SD of *n* = 4–6 samples). C, The plastid-localized lipid contents in the *fax2fax4* mutant and the wild type. Ptd-Gro, Phosphatidyl-glycerol. **P* < 0.05 and ***P* < 0.01, Student's *t* test (mean \pm SD of *n* = 4–6 samples). D, PG lipid droplets (red arrowheads) were detected in the *fax2fax4* mutant and the wild type by transmission electron microscopy.

Figure 6. Reduced TAG biosynthesis in developing seeds of the *fax2fax4* mutant. A, Incorporation of radioactivity from ^{14}C -acetate (50 mCi/mmol), ^{14}C -oleic acid (50 mCi/mmol), and ^{14}C -palmitic acid (50 mCi/mmol) into TAG was detected using developing seeds at 10 DAF. DPM, Disintegrations per minute. B, Total TAG content of 10-DAF developing seeds fed with ^{13}C -oleic acid for 18 h. At least three replicates were averaged, and the SD is shown. * $P < 0.05$ and ** $P < 0.01$, Student's *t* test (mean \pm SD of $n = 3\text{--}5$).



of *FAX2* and *FAX4* is capable of increasing the seed lipid yield in oilseed plants.

DISCUSSION

TAG biosynthesis in seeds is critical to improving the quality and quantity of storage lipids in oilseed plants. FA synthesis provides substrates for TAG biosynthesis during seed filling. However, in contrast to other eukaryotes, *de novo* FA synthesis in plants occurs in the plastid (Li-Beisson et al., 2013). In previous studies, genetic engineering strategies have been reported to increase seed oil content by overexpressing transporter, enzyme, and transcription factor genes specifically involved in TAG biosynthesis (Roesler et al., 1997; Thelen and Ohlrogge, 2002; Masaki et al., 2005; Baud et al., 2007; Maeo et al., 2009; Baud et al., 2010). Although *FAX1* has been demonstrated to mediate plastid FA export for vegetative TAG biosynthesis in flowers and leaves of *Arabidopsis*, the mechanism of plastid FA export for oil accumulation in seeds remains unknown. Here, we showed that two plastid envelope-localized proteins, *FAX2* and *FAX4*, are required for FA transport from plastids to the ER for TAG biosynthesis during seed filling.

We propose that *FAX2* and *FAX4* function as two separate FA transporters specifically for TAG biosynthesis in the embryo of seed development, mainly based on the following evidence: (1) both *FAX2* and *FAX4* are localized at the plastid envelope (Fig. 2A), as we predicted; (2) transcripts of both *FAX2* and *FAX4* are specifically expressed in the seed embryos during the seed-filling stage (Fig. 2, B and C), during which TAGs are rapidly synthesized; (3) the abnormal phenotypes of seed embryos of *fax2*, *fax4*, and *fax2fax4* are correlated with the defects in *FAX2* and/or *FAX4* (Figs. 3 and 4); (4) TAG contents are reduced by 30% in *fax2fax4* double mutant seeds compared with wild-type seeds (Fig. 5); (5) the plastid-localized lipid droplets in *fax2fax4* double mutants are distinctly increased relative to the number in the wild type (Fig. 5, C and D); and (6) TAG synthesis is reduced in *fax2*, *fax4*, and *fax2fax4*

seeds, as demonstrated by feeding experiments in growing seeds with radioactively labeled ^{14}C -acetate, ^{14}C -oleic acid, radioactively labeled ^{14}C -palmitic acid, or stable-isotope-labeled ^{13}C -oleic acid (Fig. 6, A and B; Supplemental Fig. S6). Oleic and palmitic acid, which are known as the most abundant FAs, were synthesized in plastids and then exported to the cytosol and further to the ER for TAG synthesis. Therefore, palmitic and oleic acid were used in ^{14}C -labeling experiments. Although ^{14}C -linoleic acid was also used in the radioactive labeling experiment, no difference was detected between mutant and wild-type seeds (Supplemental Fig. S6). We hypothesized that plastid-derived linoleic acid would account for very low abundance and in the experiment, we detected a very weak radioactive signal derived from ^{14}C -linoleic acid. ^{13}C labeling is not a very sensitive method comparing with ^{14}C labeling, and therefore, only oleic acid was used in the ^{13}C labeling experiment. In a previous study, it was demonstrated that *FAX1* is involved in TAG biosynthesis in vegetative tissues of *Arabidopsis*, but plants generally produce TAGs in developing embryos (Penfield et al., 2006). Our findings support a role for *FAX2* and *FAX4* in seed TAG biosynthesis. We hypothesized a working model for *FAX2* and *FAX4* during seed embryo TAG biosynthesis. When both *FAX2* and *FAX4* were knocked out, plastid FA export of the *fax2fax4* seed embryo was affected, resulting in inefficient TAG biosynthesis.

Although we demonstrated that *FAX2* and *FAX4* mediate FA export for TAG accumulation in seed embryos, the growth, development, and seed quantity of the mutants are not obviously different from those in the wild type (Fig. 1). However, the TAG content of *fax2fax4* seeds was decreased by $\sim 30\%$ relative to that of wild-type seeds (Fig. 5A). Many TAG molecular species were also significantly decreased in *fax2fax4*-seeds. When content (mol %) of relative TAG molecules was calculated, however, very little difference was detected between the *fax2fax4* mutant and the wild type (Supplemental Fig. S8). These findings indicated that the relative abundance of TAG molecular species was stable in both the *fax2fax4* mutant and the wild type.

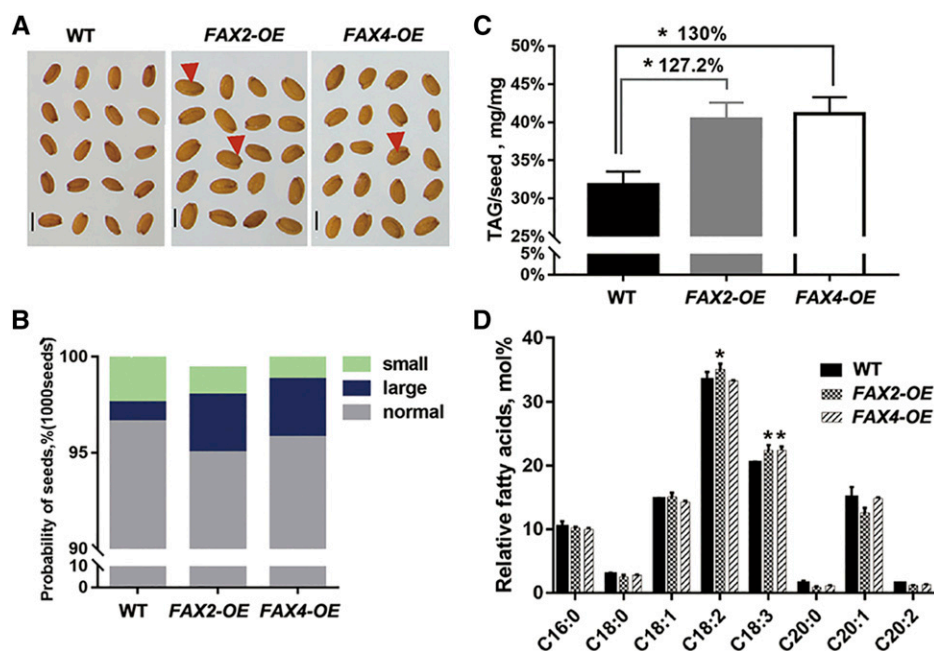


Figure 7. FAX2- and FAX4-overexpressing plants show enlarged seeds with elevated lipid content. A, Enlarged seeds of transgenic plants overexpressing FAX2 or FAX4 (FAX2-OE and FAX4-OE, respectively) as compared to those of the wild type (WT). Scale bar = 200 μ m. B, Probability of the four classified seed groups in 1,000 seeds of the two overexpressing plants and the wild type. C, TAG content in seeds of overexpressing plants. D, FA composition of overexpressing and wild-type seeds. * $P < 0.05$, Student's t test (mean \pm sd of $n = 6$ seeds).

Other putative transporters might possess functions similar to those of FAX2 and FAX4 in TAG biosynthesis in seeds. Furthermore, ABCA9 has been described as a FA transporter, as demonstrated by a 14 C-acetate, 14 C-oleic acid seed-feeding experiment in *abca9* mutants (Kim et al., 2013). We hypothesize that putative ABCA9, FAX2, and FAX4 homologs may perform the same functions for FA export at the plastid membrane. The exported FAs are used not only for TAG synthesis but also for synthesis of surface lipids, which constitute 1% of seed storage lipids, and membrane lipids, which account for 5% of seed storage lipids (Ohlrogge and Browse, 1995; Li et al., 2006; Molina et al., 2006; Beisson et al., 2007). In addition, half of the exported FAs are returned for plastid-intrinsic lipid assembly by the eukaryotic pathway (Browse et al., 1986). Therefore, FAX2 and FAX4 contribute to part of plastid FA export for TAG biosynthesis in seed embryos.

Recently, we also identified two homologous genes of *Arabidopsis* FAX1, *CrFAX1* and *CrFAX2*, in the green alga *Chlamydomonas reinhardtii*. The overexpression of both *CrFAXs* increased TAG content by 38% (Li et al., 2019). We found that embryo cells from FAX4 overexpression lines are larger and have higher TAG levels than the corresponding wild-type seeds (Fig. 7). However, FAX4 overexpression did not change the number of seeds per silique. Interestingly, there was no difference in protein and starch contents between FAX4-overexpressing and wild-type seeds. Therefore, our findings suggest that FAX2 and FAX4 are important for their potential to specifically increase seed oil production without reducing storage protein and starch in oilseed crops.

In summary, we conclude that FAX2 and FAX4 are plastid-localized FA exporters for TAG biosynthesis in seed embryos. This discovery provides an important

strategy to boost seed oil yield without reducing storage protein and starch content in seeds and without reducing shoot biomass. These two transporters would be very valuable in improving oil production and increasing carbon and energy storage in crop plants.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) was used as the wild type. The mutants used in the current study, including *fax2* (SALK_135883) and *fax4* (SAIL_671_D03), were obtained from ABRC (Ohio State University). The double mutant *fax2fax4* was screened from the F3 generation following artificial pollination between *fax2* and *fax4*. Seeds of wild-type and mutant plants were surface sterilized and plated on one-half strength Murashige and Skoog medium with or without 1% (w/v) Suc. To generate complementation lines and overexpressing lines of FAX2 and FAX4 under the *CaMV* 35S promoter, the coding sequences of FAX2 and FAX4 were subcloned into the pCambia1301M vector, a binary expression vector (Xu et al., 2019). The constructs were transformed into *Agrobacterium tumefaciens* GC3101, which were then transfected into *fax2* or *fax4* mutants (FAX2-comp and FAX4-comp, respectively) and Col-0 (FAX2-OE and FAX4-OE, respectively) plants (Li et al., 2015). The plates were cold-treated at 4°C in the dark for 2 d and then grown under sterile conditions with a 16 h/8 h day/night cycle at ~20°C to 22°C for 2 weeks. For further analysis, the plants were transferred to soil and grown in an incubator with a 16 h/8 h day/night cycle at ~20°C to 22°C.

Phylogenetic Analysis

A phylogenetic tree was constructed using the neighbor-joining method, and a bootstrap test was performed using 1,000 iterations in MEGA 6.0 according to a previous study (Li et al., 2015).

Seed Lipid and Suc Analysis

For FA composition and oil content measurement, total lipids were extracted using a method described previously (Kim et al., 2013; Lu et al., 2018, 2019). Dried seeds (3–5 mg/sample) were added to methanol with 5% (v/v) H_2SO_4 and 0.01% (v/v) butylated hydroxyl toluene and incubated for 90 min in an

85°C water bath for transmethylation. FA methyl esters (FAMES) were extracted with hexane. FAMES were quantified using gas chromatography supplied with a hydrogen flame ionization detector and a SUPELCOWAX capillary column with a helium carrier at 20 mL/min. The oven temperature was maintained at 170°C for 1 min and then increased in steps to 210°C, raising the temperature by 3°C/min. FAMES from TAGs were identified by comparing their retention times with known standards. Heptadecanoic acid (C_{17:0}) was used as the internal standard to quantify the amounts of individual FAs. FA composition was calculated as mole percent. Total Suc quantification was performed according to previous methods (Kim et al., 2013).

Subcellular Localization Analysis

Primers containing enzyme cleavage sites were designed to amplify the coding sequences (CDSs) of candidate genes (Supplemental Table S1). Then, the CDSs of *FAX2*, *FAX4*, and *PIC1* were inserted into the pAN580 vector, in which *CaMV35S* was the promoter and GFP was the reporter gene. We examined the subcellular location of genes by PEG/Ca²⁺-mediated protoplast transformation. We cultured Arabidopsis seedlings in nutrient soil. When the seedlings had grown for 20 d, young leaves were selected for experimentation. Cellulase R10 and Macerozyme R10 were applied to separate the mesophyll cell protoplast in the dark at 23°C, and 10–20 µg of plasmids, protoplast, and PEG/Ca²⁺ solution were cocultured for transformation. The protoplast after transformation was incubated overnight. Then, the green fluorescence signal and chlorophyll fluorescence were observed by laser scanning confocal microscopy (Leica, SP8), and the chloroplast in each cell was used as the control.

RT-qPCR Analysis

Total RNA was extracted, and complementary DNA (cDNA) was synthesized as described previously (Li et al., 2019). Diluted cDNA (2 µL) was used for RT-qPCR analysis according to the manufacturer's instructions on an ABI Prism 7900 Sequence Detection system. Gene-specific primers are listed in Supplemental Table S1. The housekeeping gene *ACTIN* was used as the internal reference. At least three biological replicates were performed for each gene.

RNA In Situ Hybridization

For probe preparation, the cDNAs of *FAX2* or *FAX4* were amplified for *FAX2* and *FAX4* detection, respectively. The two probes were labeled according to the DIG RNA Labeling Kit (Roche). Section pretreatment, hybridization, and immunological detection were performed as previously described (Xu et al., 2019).

SEM and Image Processing

Seeds of the wild type and *fax2fax4* mutant were collected at three developmental stages: 8, 10, and 14 DAF. The seeds in the same siliques were detected using a stereoscopic microscope (SteREO Discovery V20, Zeiss) and a SEM (SU3500, Hitachi).

Cytological Analysis of Developing Seeds

Green embryos were dissected from wild-type and *fax2fax4* seeds and fixed in freshly prepared FAA (10% [v/v] formalin, 5% [v/v] acetic acid, and 45% [v/v] ethanol) at 4°C for 2 h. The embryos were dehydrated in 30%, 50%, 75%, 95%, and 100% (v/v) ethanol for 30 min each at 4°C. After dehydration, methyl salicylate was used for transparency treatment. ImageJ software (<https://imagej.nih.gov/ij/>) was used to quantify the cotyledon area as well as the average size of epidermal cells in the central region of the cotyledons.

Differential Interference Contrast Analysis of Cotyledon Cells

Seeds of wild-type and *fax2fax4* mutant were collected at various developmental stages and fixed in formaldehyde-acetic acid overnight at 4°C. The samples were then stepwise dehydrated with different concentrations of ethanol, and methyl salicylate was used to render them transparent. The development of cotyledon cells in the decolorized seeds was observed by differential interference contrast optics of the Olympus BX53.

¹³C Labeling and Measurement of Plastid Lipids and TAG Contents in Seeds

When the siliques of Arabidopsis plants had grown for 12–16 d, we peeled the green siliques under a microscope, and all of the intact seeds from five siliques were collected as the sample. The weights of all the samples were measured first. ¹³C-oleic acid (Absin Biotechnology) was dissolved in methyl alcohol at a concentration of 1 mg/mL. The intact seeds were dipped into reagent (500 µL) containing ¹³C-oleic acid for 18 h. And then samples were collected and rinsed three times with reagent without isotope oleic acid to wash off the isotope on the surface of seeds. Butylated hydroxyl toluene and isopropanol were applied at 75°C for 20 min to deactivate lipid-hydrolyzing enzymes. Chloroform, methyl alcohol, and 300 mM ammonium acetate at a volume ratio of 30:41.5:3.5 were used to extract the single phase containing FA, and 1 M KCl was essential in the experiment. The Termovap sample concentrator was used to dry all the samples before storage at –80°C. Phospholipids and galactolipids were analyzed by a shotgun lipidomics method described by a Triple Quad 4000 liquid chromatography (LC) MS/MS (Welti et al., 2002). TAGs were quantitatively analyzed by a modified version of the reverse-phase HPLC electrospray ionization MS/MS approach, as described previously (Lam et al., 2014; Lu et al., 2018). LC-multiple reaction monitoring (MRM) scans of TAG species were used to determine the species of TAG (Lam et al., 2014; Lu et al., 2018). Briefly, separation of the aforementioned lipid extracts was carried out on a Phenomenex Kinetex 2.6 µ-C18 column using an isocratic mobile phase of chloroform:methanol:0.1 M ammonium acetate (100:100:4). Neutral-loss MS/MS scans of FAs were used to determine the levels of TAGs. TAGs were quantified against the relative amounts of TAG-14:0/14:0/14:0-d5, TAG-15:0/15:0/15:0-d29, and TAG-18:0/18:0/18:0-d5 internal standards (CDN Isotopes). MGDGs and DGDGs were separated using Phenomenex Kinetex 2.6 µ-C18 column (internal diameter 4.6 × 100 mm) using an isocratic mobile phase of chloroform:methanol:2% 50 mM sodium acetate (49:49:2) at a flow rate of 160 µL/min for 25 min, as previously described (Lu et al., 2018). Individual MGDG and DGDG molecular species were quantitated by referencing to MGDG-16:0/18:0 and DGDG-18:0/18:0 internal standards (Matreya). ¹³C-labeled TAGs were detected by a method described previously (Jiang et al., 2017). Lipid standards were purchased from Avanti Polar Lipids, and the LIPID Metabolites and the Pathways Strategy.

¹⁴C-Labeling Assay Monitoring the Incorporation of Precursors into TAG

Fifty Arabidopsis seeds from the wild type and *fax2*, *fax4*, and *fax2fax4* mutants at 10 DAF were collected and transferred to 200 µL of 20 mM MES buffer (pH 5.8) as previously described (Kim et al., 2013). Then, 0.5 µCi of ¹⁴C-acetate (50 mCi/mmol), 0.5 µCi of ¹⁴C-oleic acid (50 mCi/mmol), and 0.5 µCi of ¹⁴C-palmitic acid (50 mCi/mmol) were added separately to the solution. The labeled seeds were incubated for 18 h in the dark with rotation at 100 rpm. Next, the seeds were washed with ice-cold water three times and homogenized in 50 µL of chloroform:methanol:formic acid (10:10:1 [v:v:v]). The organic and aqueous phases were then divided by adding 12.5 µL of solution containing 1 M KCl and 0.2 M H₃PO₄. After centrifugation at 16,000g for 5 min, the total lipids in the lower layer were separated and added to a silica thin-layer chromatography plate. The TAGs were separated after a 1–2 h incubation of the thin-layer chromatography plate in hexane:diethylether:acetic acid (80:30:1 [v:v:v]) buffer in a glass incubator. After dyeing with iodine pellets, the TAG signal was shown on the silica plate and then scraped from the plate and mixed with the scintillation mixture. Radioactivity was measured by scintillation counting. At least six replicates were performed for each FA fed.

Statistical Analysis

Significant values were determined using Student's *t* test with **P* < 0.05 and ***P* < 0.01. All data were statistically analyzed using SPSS 18.0 and GraphPad Prism 5 with at least three biological replicates.

Data Availability

The authors declare that all data supporting the findings of this study are available within the manuscript and its supplementary files or available from the corresponding author on request.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: At2g38550 (*AtFAX2*) and At1g33265 (*AtFAX4*).

Supplemental Data

The following supplemental information is available.

Supplemental Figure S1. Sequence alignment and transcript levels of *FAX1*, *FAX2*, and *FAX4* in seeds.

Supplemental Figure S2. Seed size distribution of the four classified seed groups in *fax2fax4* mutants.

Supplemental Figure S3. The seeds of *FAX2* and *FAX4* complementation lines show no difference in seed lipids and seed size compared with the wild type.

Supplemental Figure S4. Physical interaction between *AtFAX2* and *AtFAX4*.

Supplemental Figure S5. Transcript levels of *FAX1*, *FAX2*, and *FAX4* in seeds and siliques during seed-filling stages.

Supplemental Figure S6. TAG biosynthesis is reduced in developing seeds of *fax2*, *fax4*, and *fax2fax4* mutants.

Supplemental Figure S7. Seed size distribution of the *FAX2* and *FAX4* complementation and overexpression lines.

Supplemental Figure S8. Content (mol %) of relative TAG molecules in wild-type and *fax2fax4* mutant seeds.

Supplemental Table S1. Primers used in this study.

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