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Functional Complementation of a Perilla ω-3 Fatty Acid Desaturase under the Seed-specific SeFAD2 Promoter

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Functional characterization of the fatty acid desaturase genes and seed-specific promoters is prerequisite for altering the unsaturated fatty acid content of oilseeds by genetic manipulation. The ω-6 fatty acid desaturase (FAD2) and ω-3 fatty acid desaturase (FAD3) catalyze extra-plastidial desaturation of oleic acid to linoleic acid and linoleic acid to linolenic acid, respectively. These are major constituents in seed storage oils. Here, we report the complementation of a perilla linoleic acid desaturase (*PrFAD3*) cDNA under the seed-specific sesame *FAD2* (*SeFAD2*) promoter in the *Arabidopsis fad3* mutant. PrFAD3 is functionally active and the *SeFAD2* promoter is applicable for modifying fatty acid composition in developing seeds. Transient expression of the GUS gene under that promoter in the developing seeds and leaves of sesame, soybean, and corn via microprojectile bombardment indicated that the *SeFAD2* promoter likely will be useful for altering the seed phenotypes of dicot and monocot crops.

Keywords: FAD2, FAD3, ω-3 fatty acid desaturase, ω-6 fatty acid desaturase, *Perilla frutescens*, seed-specific promoter

Fatty acids are not only essential components of all cellular membranes in living organisms, but also a major form of chemical energy storage in seeds. Renewable seed oils are especially utilized for food and non-food products, including soups, paints, resins, and biodiesel (Ohlrogge, 1994). In plants, *de novo* fatty acid synthesis occurs in the plastids. Those fatty acids are further reduced *via* the prokaryotic pathway in the plastid and eukaryotic pathway within the endoplasmic reticulum (ER). The ω-6 fatty acid desaturase (FAD2) and ω-3 fatty acid desaturase (FAD3) bound on the ER membrane catalyze the reduction of oleic acid ($18:1^{\Delta 9}$) to linoleic acid ($18:2^{\Delta 9,12}$) and linoleic acid to linolenic acid ($18:3^{\Delta 9,12,15}$), respectively, both of which are major constituents in seed storage oils (Somerville et al., 2000). The properties of those seed oils for industrial application are determined by the degree of saturation of fatty acids that are esterified to triacylglycerol. For example, soybeans that are genetically modified to have oil with an elevated oleic acid content are considered healthier than conventional linoleic acid-rich soybeans due to their high oxidative stability. In contrast, oil from transgenic soybeans containing high levels of linolenic acid, associated with low oxidative stability, can be useful as a drying agent in coating applications, e.g., for paints and inks (Cahoon et al., 2002; Kinney et al., 2002). Therefore, functional characterization of the desaturase genes from a variety of sources is prerequisite for altering the unsaturated fatty acid content of oilseeds by manipulating the expression of their desaturase genes.

Several genes encoding FAD3 have been isolated from *Arabidopsis*, soybean (*Glycine max*), rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*), tung (*Aleurites fordii*), flax (*Linum usitatissimum*), perilla (*Perilla frutescens*), and rice (*Oryza sativa*) (Yadav et al., 1993; Hamada et al., 1994; Kodama et al., 1997; Chung et al., 1999; Bilyeu et al., 2003; Dyer et al., 2004; Vrinten et al., 2005). *Arabidopsis* harbors only a single copy of *FAD3*, which is constitutively expressed (Beisson et al., 2003). In contrast, other plants, including soybean, perilla, and flax, express additional *FAD3* gene(s) that are tightly regulated during seed development (Chung et al., 1999; Bilyeu et al., 2003; Vrinten et al., 2005). The linolenic acid level in *Arabidopsis* seeds is significantly reduced by a mutation of *FAD3* gene (James and Dooner, 1990). Furthermore, a soybean mutant line A5, with low seed linolenic acid, shows a deletion of the seed-specific *FAD3* gene *GmFAD3A* (Bilyeu et al., 2003). Two other seed-specific *FAD3* genes, *LuFAD3A* and *LuFAD3B*, control the amount of linolenic acid in flax seed (Vrinten et al., 2005). Therefore, these seed-specific *FAD3* genes may be closely associated with linolenic acid contents in seed oils.

Metabolic engineering of complex traits often requires more than one promoter for the introduction of multiple genes. The napin storage protein promoter from *Brassica napus* and the rice glutelin promoter are now used extensively to express introduced genes in dicotyledonous and monocotyledonous transgenic seeds, respectively (Poirier et al., 1999; Rezzonico et al., 2004; Paine et al., 2005). However, because the timing of expression for such promoters, including that of the napin promoter, is not optimal for modifying seed oil content, the promoters associated with storage oil biosynthesis and accumulation have become the focus of increasing attention. Activity of the *FAE1* (fatty acid elongase 1) promoter is superior to that of the napin promoter with regard to the production of hydroxy fatty acids in transgenic *Arabidopsis* seeds (Rossak et al., 2001). Nevertheless, one possible disadvantage of the *FAE1* promoter is that its expression peak occurs at a later time than many of the enzymes involved in seed lipid biosynthesis (Ruuska et al., 2002). The seed-specific *FAD2* gene (*SeFAD2*), encoding

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microsomal ω-6 fatty acid desaturase, has been isolated from sesame (*Sesamum indicum*). Promoter region required for its seed-specific expression has been identified in *Arabidopsis* using the b-glucuronidase (GUS) reporter system (Kim et al., 2006).

In this study, we examined the activity of a perilla (*Perilla frutescens*) linoleic acid desaturase (*PrFAD3*) through complementation of its gene in the *Arabidopsis fad3* mutant. The *SeFAD2* promoter aids in altering fatty acid composition in *Arabidopsis* seed oils. Transient expression of *GUS* gene under the *SeFAD2* promoter indicates that this promoter can be applied to important seed crops, both dicots and monocots, and may provide a useful alternative to currently available seed promoters.

MATERIALS AND METHODS

Plant Materials

Plants of sesame (*Sesamum indicum* cv. Yangbaeck), soybean (*Glycine max* L. Merrill cv. Eunha), and corn (*Zea mays* cv. Chalok1) were cultivated in a greenhouse, and the *Arabidopsis thaliana fad3-2* mutant (Browse et al., 1993) was cultivated in a growth chamber under a photocycle of 16 h of light (24°C) and 8 h of darkness (22°C). To facilitate seed harvests at predefined stages, flowers were tagged on the day they opened. Their developing seeds were collected on ice via dissection for use in microprojectile bombardments.

Binary Vector Construction and *Arabidopsis* Transformation

The *SeFAD2* promoter (from −660 to +140, GenBank Accession No. AF192486; Kim et al., 2006) was amplified by PCR using gene-specific primers containing the *Hin*dIII or *Pst*I restriction enzyme sites (forward: 5′-CCG AAG CTT CAT ATG TGA AAT GTA ATG GAA AAT GCG AC-3′; reverse: 5′-CTG CTG CAG TTG GAA GGA GAA ATC GC-3′). The *PrFAD3* coding region (1 to 1332 bp, GenBank Accession No. AF047039; Chung et al., 1999) was amplified by PCR with gene-specific primers containing the *Pst*I or *Bam*HI restriction enzyme sites (forward 5′-GCC CTG CAG CGG CAC GAG CTC CTC C-3′; reverse: 5′-GCC GGA TCC ACT AAA TCT TTT TGG-3′). The putative *SeFAD2* terminator (from 1244 to 1447, 204 bp) was PCR-amplified using gene-specific primers with the *Bam*HI or *Eco*RI restriction enzyme sites (forward: 5′-CCG GGA TCC AGC CGA ATA ACA TGT GG-3′; reverse: 5′-CTG GAA TTC CAC TAA ACT TGA CAA T-3′). The three DNA fragments of the *SeFAD2* promoter, the *PrFAD3* coding region, and the *SeFAD2* terminator were sequentially ligated into the corresponding cloning sites in the pUC18 cloning vector, and this ligated DNA fragment was then inserted into the *Hin*dIII and *Eco*RI sites in the pMOG413 binary vector (Pen et al., 1993). The expression vector, under the control of the seed-specific *SeFAD2* promoter, was then introduced into *Agrobacterium tumefaciens* strain C58C1 via the freeze-thaw method (An, 1987) and transformed into *Arabidopsis fad3-2* mutants by the floral dip method (Clough and Bent, 1998).

Genomic DNA Isolation and PCR Analysis

Leaves from individual transgenic *Arabidopsis* plants were ground in liquid nitrogen, and genomic DNA was isolated as described by Edwards et al. (1991). The genomic DNA was amplified by PCR with the *SeFAD2* promoter-specific primer set (SiW6-YF2: 5′-CCG GAA TTC GGG ACA TGC CAC ATT ATG TG-3′; SiW6-YR4: 5′-CCC GAG CTC CCT GTC TGA GCA GGG G-3′) and *Taq* DNA polymerase (iNtRON, Korea). Amplification was conducted for 5 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 50 s; then 5 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 50 s; then 25 cycles of 94°C for 30 s and 72°C for 1 min 20 s.

Total RNA Isolation and RT-PCR Analysis

Total RNAs were isolated from the leaves of independent transgenic *Arabidopsis* plants, using TRIzol reagent (Sigma, USA) according to the manufacturer's instructions. Reverse transcription was carried out by following the manufacturer's protocol (Invitrogen, USA). For *PrFAD3*, PCR was performed using the *PrFAD3* gene-specific primer set (PrFAD3F1: 5′-GCT GGG GAA TTA CTA CAG GGA-3′; PrFAD3R2: 5′-AGA ACT AGA ATC CGA CCA GCA C-3′). The *Arabidopsis* actin2 gene (At3g18780) was amplified with Actin2F1 (5′-CAT CCA AGC TGT TCT CTC CTT GTA C-3′) and Actin2R1 (5′-CAG ACA CTG TAC TTC CTT TCA GGT G-3′) primers to determine the quantity and quality of those cDNAs. PCR reactions were conducted in a final volume of 30 μL that contained 200 ng of cDNA, 1X *i*-Taq buffer (iNtRON, Korea), 2.5 mM of each dNTP, 1 unit of *i*-Taq polymerase (iNtRON, Korea), and 10 pmol of each primer. The amplification program included: 1 cycle of 94°C for 5 min; then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and a final cycle of 72°C for 7 min.

Fatty Acid Analysis

To analyze the fatty acid content in transgenic plants, individual T_2 transgenic seeds and leaves were used. Each sample was transmethylated at 90°C for 1 h in 0.5 mL of toluene and 1 mL of 1 N methanolic HCl. Heptadecanoic acid (17:0) was added to each sample as an internal standard. After transmethylation, 1 mL of aqueous 0.9% NaCl was added, and the fatty acid methyl esters (FAMEs) were recovered by three sequential extractions with 1 mL of hexane and analyzed via gas chromatography on a 30 m×0.32 mm (i.d.) Stabil Wax column (Varian, USA). The oven temperature was ramped from 160°C to 220°C at 2.5°C min^{-1} (Shimadzu, Japan). Fatty acids were identified by comparing their sample retention times and mass spectra with those of standards.

Construction of Transient Expression Vectors and Microprojectile Bombardments

To construct the transient expression vector p*SeFAD2*::GUS, we amplified the 5′-flanking region of *SeFAD2* using two specific primers - *Si*W6F1 (5′-CCG AAG CTT CAT ATG TGA AAT GTA ATG GAA AAT GCG AC-3′) and *Si*W6R1 (5′-CTT GGA TCC TTG GAA GGA GAA ATC GCG TGA AAG CAC-3′) (Kim et al., 2006). The amplified PCR products were

then digested with *Hin*dIII and *Bam*HI and cloned into pBI221 (Clontech, USA) in place of the CaMV35S promoter. The resultant plasmid, p*SeFAD2*::GUS, was isolated with a plasmid midi kit (Qiagen, USA) and linearized with *Hin*dIII enzyme. In accordance with the methods of Klein et al. (1988), 5 mg of gold particles (1.6 μm, BMS) were mixed with 10 μL of linearized plasmid DNA (2 μg μL^{-1}), 500 μL of 2.5 M $CaCl_2$, and 200 ìL of 0.1 M spermidine for 10 shots. After the mixture had been incubated on ice for 30 min, the pellet was washed twice with 70% ethanol and re-suspended in 100% ethanol. Developing seeds and leaves of sesame, soybean, and corn were arranged in the center of a Petri dish containing an MS agar medium (Murashige and Skoog, 1962). The stopping screen was positioned 6 cm below the rupture disk, and the target tissue was positioned 6 cm below that screen. Tissues were then bombarded twice at 1100 psi with a biolistic helium gun device (Bio-Rad PDS-1000/He, USA). The bombarded tissues were incubated for 20 h at 25°C, then subjected to histochemical analysis as described by Jefferson et al. (1987). They were incubated in GUS staining buffer [100 mM sodium phosphate (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM Na_2EDTA, and 0.1% Triton X-100] for 16 h at 37°C. The stained tissues were rinsed with 70% ethanol until pigments such as chlorophyll had been completely cleared, and the images were then photographed under a SZ4045TRPT microscope (OLYMPUS, Japan).

RESULTS AND DISCUSSION

Expression of the PrFAD3 Gene under the Seed-specific FAD2 Promoter in the Arabidopsis fad3-2 Mutant

Seed-specific PrFAD3 cDNA was previously isolated from perilla seeds, which produce 65 to 75% linolenic acids out of their total fatty acids (Chung et al., 1999). The transcriptional regulatory mechanism controlling expression of the seed-specific SeFAD2 gene has been investigated in Arabidopsis (Kim et al., 2006, 2007). There, activity of the SeFAD2 promoter is restricted to the seed developmental stage, i.e., when storage oils are deposited. This observation prompted us to further evaluate the utility of that promoter for possible use in genetic engineering of seed oil composition. The SeFAD2 promoter (from −660 to +141) and terminator were initially fused upstream and downstream, respectively, of the PrFAD3 gene, and the expression cassette was then inserted into the binary vector pMOG413 (Fig. 1A). We tested the resultant construct for its ability to complement the phenotype of the Arabidopsis fad3-2 mutant (Browse et al., 1993).

After transforming that mutant with the binary vector harboring the PrFAD3 cDNA under the control of the SeFAD2 promoter, we evaluated kanamycin-resistant seedlings for integration of the introduced gene into the Arabidopsis chromosomal DNA. Chromosomal DNA was isolated from 2 fad3-2 mutants and 11 transgenic fad3-2 plants, and PCR was conducted with gene-specific primers predicated on the SeFAD2 promoter sequence (Kim et al., 2006). The expected PCR products were observed in all 11 transgenics, but not in the 2 fad3-2 mutants (Fig. 1B).

To investigate expression of the PrFAD3 transcripts under the control of the seed-specific SeFAD2 promoter, we isolated total RNAs from leaf tissues, rather than from seeds, of wild-type, fad3-2 mutant, and independent transgenic lines (Arabidopsis fad3-2 background). This was because it is difficult to isolate total RNAs from Arabidopsis seeds and SeFAD2 transcripts are only weakly expressed in sesame leaf tissues (Kim et al., 2006). Our isolated total RNAs were then subjected to RT-PCR. The Arabidopsis actin2 gene was used to determine the quantity and quality of cDNAs. Following amplification for 25 cycles, no PrFAD3 transcripts were detected in the wild-type, fad3-2 mutant, or any transgenic fad3-2 plants. However, with amplification for 35 cycles, PrFAD3 transcripts were detected in all transgenic plants, but not in the wild-type or the fad3-2 mutant. This result demonstrated that the introduced PrFAD3 gene is expressed in transgenic plants.

Fatty Acid Composition in Transgenic Arabidopsis fad3-2 Lines Expressing PrFAD3 gene

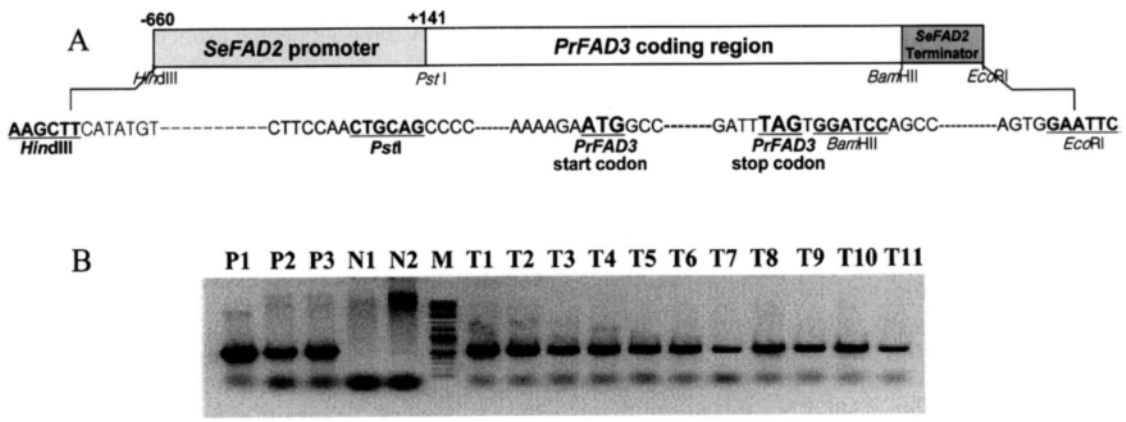

Figure 1. A, Schematic diagram of binary vector harboring *PrFAD3* gene under seed-specific *SeFAD2* promoter and **B**, genomic DNA PCR analysis of *PrFAD3* gene in transgenic plants *fad3-2* mutant background) expressing that gene. P1 to P3, Plasmid DNA carrying *PrFAD3* gene; N1 and N2, *fad3-2* mutants; M, DNA size marker (NEB, UK); 1 to 11, Individual transgenic plants. Chromosomal DNA was isolated from *fad3-2* mutants and transgenic *fad3-2* plants and subjected to PCR using *SeFAD2* promoter-specific primers.

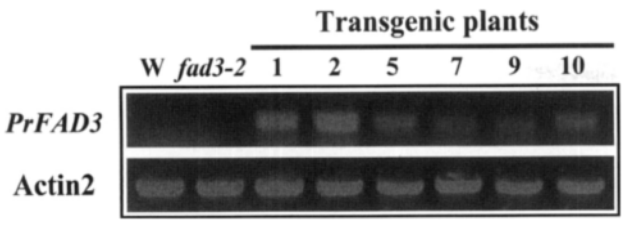

Figure 2. Expression of *PrFAD3* transcripts in transgenic plants (*fad3-2* mutant background) expressing that gene. Total RNAs were isolated from leaves of wild-type (W), *fad3-2* mutant (*fad3-2*), and transgenic plants expressing *PrFAD3* gene (1, 2, 5, 7, 9, and 10) and amplified by RT-PCR using gene-specific primers. *Arabidopsis* actin2 (At3g18780) gene served as internal control.

To compare the fatty acid composition among wild-type, *fad3-2* mutant, and all transgenic plants (*fad3-2* mutant background), fatty acids were isolated from their dry T_2 seeds and leaves and analyzed *via* gas chromatography. Whereas the wild-type *Arabidopsis* seeds contained approximately 40 mol% oleic acid (18:1) and linoleic acid (18:2), and 20 mol% linolenic acid (18:3), the *Arabidopsis fad3-2* mutant had less than 2.5 mol% 18:3 content, with the 18:1 plus 18:2 levels being concomitantly increased to 63 mol% of the total fatty acid content (Fig. 3A). After transformation with the *PrFAD3* gene, the fatty acid composition in all six of the independent transgenic lines was completely restored to that of the wild-type. Moreover a similar result was observed in fatty acid analysis of leaf tissues (Fig. 3B). For example, leaves from of the *fad3-2* mutant contained an increased 16:3 and 18:2 contents and a reduced 18:3 content compared with those of wild-type plants. Therefore, we can conclude that this gene is functionally active and we suggest that seed-specific FAD3 is responsible for the production of linolenic acids in perilla seeds.

Activity of plant FAD3 genes has also been examined in low-linolenic acid plants or yeast. When flax FAD3A gene (Lu-FAD3A), under the flax 2S storage protein conlinin promoter was expressed in solin-type flax (which contains less than 2% linolenic acid of the total seed fatty acids), the mutant phenotype was partially restored, suggesting that Lu-FAD3A and Lu-FAD3B are the major genes responsible for desaturation of linoleic acid in that crop (Vrinten et al.,

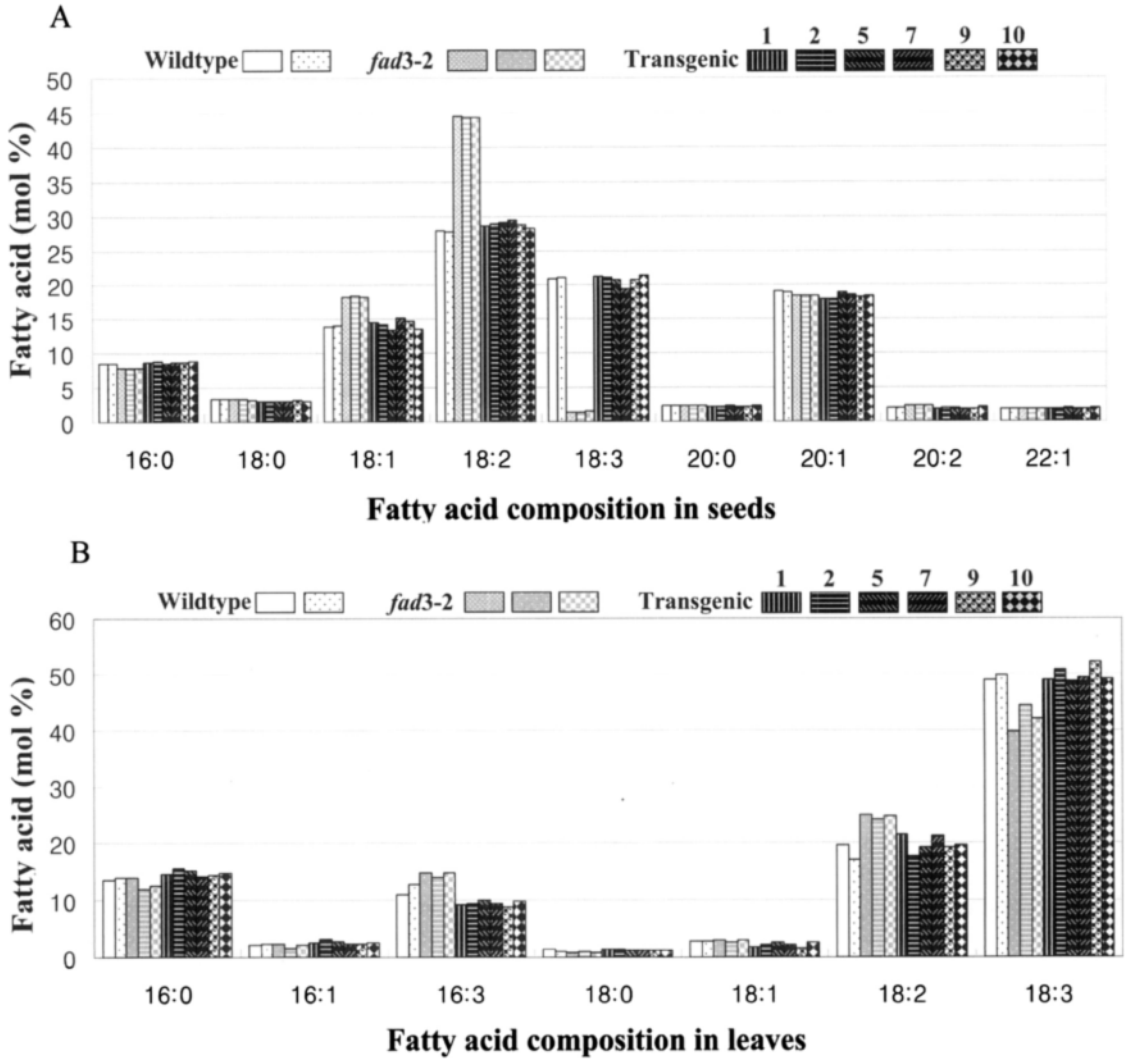

Figure 3. Complementation of *Arabidopsis fad3-2* mutant by expression of *PrFAD3* gene. Fatty acids were extracted from dry seeds (**A**) and leaves (**B**) of wild-type (W), *fad3-2* mutant (*fad3-2*), and transgenic plants (*fad3-2* mutant background) expressing *PrFAD3* gene (1, 2, 5, 7, 9, and 10), then analyzed by GC, and identified by comparing retention times and mass spectra with those of standards.

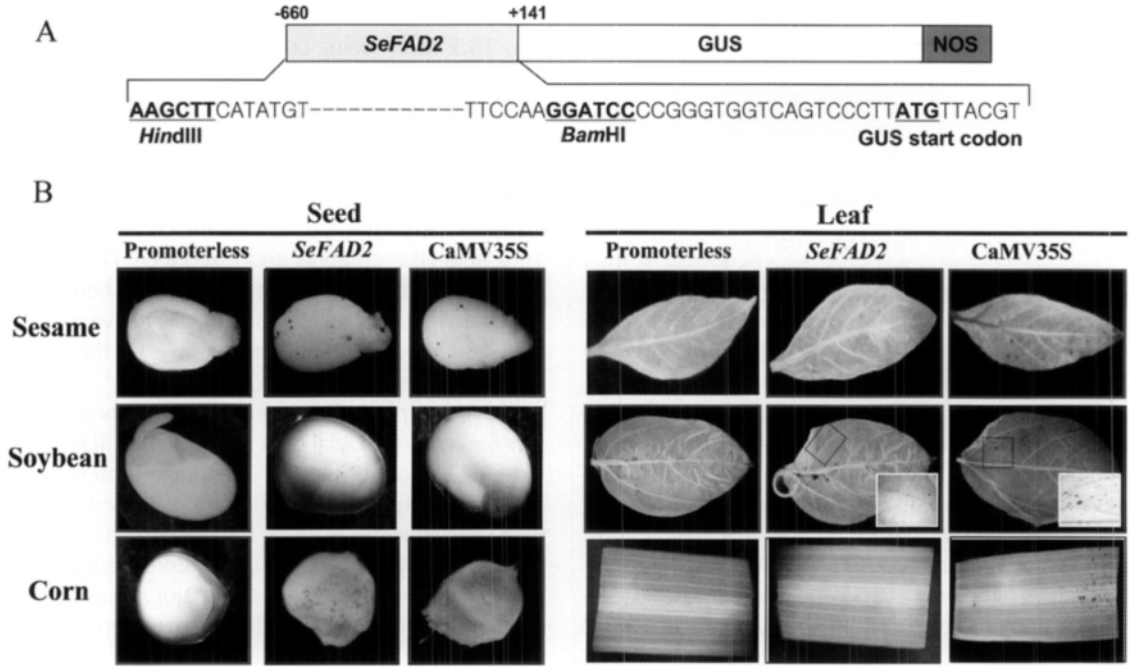

Figure 4. Transient expression of GUS gene under seed-specific *SeFAD2* promoter in developing seeds and leaves of sesame, soybean, and corn. **A**, Schematic diagram oftransient expression vector harboring *GUS* gene under seed-specific *SeFAD2* promoter. **B**, Histochemical analysis of *GUS* expression in developing seeds and leaves of sesame, soybean, and corn. Inner boxes in soybean leaves were magnified 10-fold. Negative control (promoterless; pBI101) and positive control (pBI221, CaMV35S::GUS).

2005). Moreover, the substrate specificity and regioselectivity of Brassica napus FAD3 have been investigated in a heterologous yeast system (Reed et al., 2000). Although occasionally the activity of an enzyme expressed in yeast does not exactly mirror its activity in plants (Heilmann et al., 2004), expression of desaturase genes from a variety of sources in low-linolenic acid plants or yeast is useful to the assessment of enzyme activity.

Transient GUS Gene Expression in Developing Seeds and Leaves of Sesame, Soybean, and Corn

Based on the rescue of fatty acid composition by the expression of PrFAD3 gene under the SeFAD2 promoter, we investigated whether that promoter can be utilized in important seed crops. Here, we replaced the CaMV35S promoter with the seed-specific SeFAD2 promoter (from –660 to +141) in the pBI221 transient expression vector, which harbors the GUS reporter gene (Fig. 4A). This construct was then introduced into the developing seeds and leaves of sesame, soybean, and corn via microprojectile bombardment. Those bombarded tissues were then stained histochemically after 24 h. The GUS gene, under the control of the SeFAD2 promoter, was transiently expressed in developing sesame, soybean, and corn seeds, but not in the sesame and corn leaves. A very low level of GUS expression was found in the soybean leaves. We detected no signals in the developing seeds and leaves of sesame, soybean, and corn that had been bombarded with the promoter-less control DNA-coated particles. GUS expression, under the control of the CaMV35S promoter, was detected in both the developing seeds and leaves of all three species (Fig. 4B).

This seed-specific *SeFAD2* promoter was able to complement the *PrFAD3* cDNA in *Arabidopsis fad3-2* mutant seeds (Fig. 3). In addition, the GUS reporter gene under the *SeFAD2* promoter was transiently expressed in the seeds of both dicots and monocots upon particle bombardment. Our results are supported by other reports that some promoters of seed storage protein genes, such as zein (corn), napin (rape), and α-globulin (cotton), appear to have similar mechanisms for transcriptional regulation despite the evolutionary distance between monocots and dicots (Schernthaner et al., 1988; Sunilkumar et al., 2002). Seed storage proteins and oils accumulate in developing seeds at high levels, but their timing is differentially controlled during that growth process. The accumulation of seed storage proteins also is earlier than that of storage oils (Ruuska et al., 2002). Although the seed-specific *SeFAD2* promoter is leaky in leaf tissues, it is strictly regulated during the biosynthesis of those oils.

One of the more practical applications of plant oil biotechnology involves the manipulation of seed metabolism in order to generate industrially applicable vegetable oils. When using such tools to develop new crop cultivars, one critical factor is the availability of suitable promoters that can induce strong expression of genes within a specific tissue (in this case, the seeds) as well as of genes with functional activity. In conclusion, both complementation of PrFAD3 in the Arabidopsis fad3 mutant and transient expression of GUS under the SeFAD2 promoter indicate that the PrFAD3 gene is functionally active and that the SeFAD2 promoter is valuable to the modification of seed-phenotypes from dicot and monocot crops.

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