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ORIGINAL PAPER

Seed-specific expression of sesame microsomal oleic acid desaturase is controlled by combinatorial properties between negative *cis*-regulatory elements in the *SeFAD2* promoter and enhancers in the 5′-UTR intron

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Abstract The regulation of genes involved in primary lipid metabolism in plants is much less well understood than that in many other pathways in plant biology. In the investigation reported here, we have characterized transcriptional regulatory mechanisms controlling seed-specific *FAD2* expression in sesame (*Sesamum indicum*). *FAD2* codes for extra-plastidial FAD2 desaturase, which catalyzes the conversion of oleic acid to linoleic acid. Promoter analysis of the sesame *FAD2* gene (*SeFAD2*) using the β-glucuronidase (GUS) reporter system demonstrated that the − 660 to − 180 promoter region functions as a negative *cis*-element in the seed-specific expression of the *SeFAD2* gene. Sesame and *Arabidopsis FAD2* genes harbor one large intron within their 5′-untranslated region. These introns conferred up to 100-fold enhancement of GUS expression in transgenic *Arabidopsis* tissues as compared with intron-less controls. Prerequisite *cis*-elements for the *SeFAD2* intron-mediated enhancement of gene expression and the promoter-like activity of *SeFAD2* intron were identified. *SeFAD2* transcripts were induced by abscisic acid (ABA) in developing sesame seeds, and the − 660 to − 548 and − 179 to − 53 regions in the *SeFAD2* promoter were implicated in ABA-responsive signaling. Theses observations indicate that an intron-mediated regulatory mechanism is involved in controlling not only the seed-specific expression of the *SeFAD2* gene but also the expression of plant *FAD2* genes, which are essential for the synthesis of polyunsaturated fatty acids.

Keywords *Arabidopsis thaliana* · *FAD2* · Intron-mediated enhancement · Microsomal oleic acid desaturase · Seed-specific promoter · *Sesamum indicum*

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The nucleotide sequence of the microsomal oleic acid desaturase (*FAD2*) gene from *Sesamum indicum* reported here has been registered in the GenBank™/EBI Data Bank under accession number AY770501.

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Introduction

Storage compounds such as lipids and proteins accumulate in abundance in seeds during the middle and late phases of embryo development. Some of the genes involved in the biosynthesis and accumulation of storage lipids are exclusively expressed in the developing seed. As such these genes are useful target genes in plant transformation studies aimed at improving the quality of vegetable oils or increasing the production of unusual fatty acids (Cahoon et al. 1992; Kinney 1994, 1996; Millar and Kunst 1997; Liu et al. 2000; Moon et al. 2001; Bao et al. 2002). However, to date relatively

little is known about the regulatory mechanism(s) controlling the seed-specific expression of these genes.

Higher plants express one or more microsomal oleic acid desaturase (FAD2; EC1.3.1.35) isoforms, which catalyze the insertion of a double bond between carbons 12 and 13 of oleic acid at both the *sn*-1 and *sn*-2 positions of phosphatidylcholine (Shanklin and Cahoon 1998). *Arabidopsis* harbors only a single copy of the *FAD2* gene (At3g12120), which is constitutively and abundantly expressed (http://www.plantbiology.masu.edu/lipid/genesurvey/EST_data.htm; Beisson et al. 2003). In contrast to *Arabidopsis*, crops, including soybean (*Glycine max*), cotton (*Gossypium hirsutum*), sesame, corn (*Zea mays*), and canola (*Brassica napus*), express at least one additional *FAD2* gene(s), which is tightly regulated during seed development (Okuley et al. 1994; Heppard et al. 1996; Jin et al. 2001; Pirtle et al. 2001; Kinney et al. 2002). Oleic and linoleic acids constitute the predominant fatty acids in vegetable oils, and their relative proportions in the seeds of oil crops determine the nutritional properties and oxidative stability of edible oils (Vos and Cunnane 2003). The seed-specific FAD2 has been reported to be responsible for the high content of linoleic acid in seed oil (Liu et al. 2000; Jin et al. 2001). Consequently, with the aim of improving the quality of vegetable oils, it is of great importance to increase our understanding of the regulation of seed-specific *FAD2* gene expression.

Introns not only have important functions in the generation of new proteins, via exon shuffling or alternative splicing, but they also have a role in determining the expression level or the spatial and developmental expression of genes (Long et al. 1995; Bolle et al. 1996; Deyholos and Sieburth 2000; Maniatis and Tasic 2002). Intron-mediated enhancement of gene expression (IME) was initially observed in the first intron of the maize *Adh1* gene, and it has subsequently been observed for many plant genes (for review, see Rose and Beliakoff 2000; Clancy and Hannah 2002). In particular, *Arabidopsis* polyubiquitin, transcription elongation factor EF-1α, and rice *β-tubulin* isotype 16 (*Ostub16*) genes are known to harbor an intron within the 5′-untranslated region (UTR) that increases the expression of the reporter gene by 2.5- to 1,000-fold relative to the intronless controls (Curie et al. 1993; Norris et al. 1993; Morello et al. 2002). Because the occurrence of introns within the 5′-UTR of plant *FAD2* genes appears to have been evolutionarily conserved (Okuley et al. 1994; Verwoert et al. 2000; Liu et al. 2001; Pirtle et al. 2001), the question arises as to whether or not the *FAD2* introns are involved in the enhancement of gene expression and/or in the spatial and developmental expression of their respective genes.

Internal (e.g., phytohormones) and external (e.g., light and temperature) signals are involved in the regulation of spatially and temporally expressed genes during seed development. Abscisic acid (ABA) appears to play a key role in storage product deposition, maturation, and desiccation tolerance of developing seeds (Finkelstein et al. 2002). When a single-chain Fv antibody gene capable of immunomodulating ABA was expressed in transgenic tobacco seeds, the embryos developed green cotyledons containing chloroplasts and generated fewer oil bodies and seed storage proteins (Phillips et al. 1997). A similar phenomenon was observed in *Arabidopsis* ABA-deficient mutants (Ooms et al. 1993; Nambara et al. 1994; Parcy et al. 1994). However, ABA signaling in the biosynthesis and accumulation of storage lipids remains somewhat obscure and, to date, ABA has been reported to control the expression of only a select few genes. For example, ABA modulates the levels of oleosin and Δ^{15}-desaturase transcripts and increases the concentrations of very long-chain monounsaturated fatty acids (20:1 and 22:1) in triacylglycerols in *Brassica napus* embryos (Finkelstein and Somerville 1989; Zou et al. 1995). The expression of the Δ^{9}-stearoyl-ACP desaturase gene has also been shown to be transiently modulated by ABA in mature olive (*Olea europaea*) embryos (Haralampidis et al. 1998).

We have isolated the *SeFAD2* genomic clones and the *At*FAD2 promoter region containing the intron with the aim of studying the intron-mediated regulatory mechanism involved in controlling not only the seed-specific expression of the *SeFAD2* gene but also the expression of plant *FAD2* genes. The *cis*-elements in the *SeFAD2* promoter required for seed-specific expression and the effects of the *SeFAD2* and *AtFAD2* introns on gene expression were assessed in transgenic *Arabidopsis* plants using the β-glucuronidase (GUS) reporter system. The sequences of the *SeFAD2* intron, which are essential for the IME and the promoter-like activity of the *SeFAD2* intron, were also identified. An investigation of the factors responsible for the control of *SeFAD2* gene expression during seed development revealed that ABA was clearly involved in the regulation of *SeFAD2* expression. ABA-responsive elements were also analyzed in the *SeFAD2* promoter.

Materials and methods

Plant materials

Sesamum indicum cv. Yangbaeck plants were cultivated from seed in a greenhouse at 26 ± 3°C, and *Arabidopsis thaliana* (ecotype Columbia) plants were

cultivated from seed in a growth chamber, under a 16/8-h [light (24°C)/dark (22°C)] photoperiod. To facilitate the harvesting of the seeds at the predefined developmental stages, we tagged each flower on the day it opened. The developing seeds were then collected by means of dissection from the sesame fruits and *Arabidopsis* siliques.

Isolation of the *SeFAD2* genomic clone and the *AtFAD2* promoter region

Chromosomal DNA was isolated from 10-day-old sesame and *Arabidopsis* seedlings, as described by Dellaporta et al. (1983). A genomic DNA fragment (approx. 3.0 kb) containing the *SeFAD2* coding region and intron within its 5′-UTR was then amplified by PCR using W6-3/SiW6-TR1 primers, as shown in Table 1 (Jin et al. 2001). The 5′-flanking region of the *SeFAD2* gene was subsequently amplified by IPCR (inverse PCR) according to Digeon et al. (1999). A 5-μg aliquot of the genomic DNA was digested with *Nde*I and self-ligated. The first round of PCR amplification was then conducted using W6-1/W6-R6 primers and consisted of five cycles of 94°C for 30 s and 72°C for 4 min; five cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. The second round of PCR was conducted under conditions identical to those of the first PCR round, except that the W6-2 and W6-R7 primers and a 50-fold diluted aliquot of the first PCR mixture were used. Ex *Taq* polymerase (Takara, Japan) with 3′–5′ proofreading activity was used in all PCR experiments.

In order to isolate the promoter region of the *AtFAD2* gene, we cloned the intron-less *AtFAD2* promoter and the intron-containing *AtFAD2* promoter using primers *At*W6F1/*At*W6R1 and *At*W6F1/*At*W6R4, respectively. The amplified PCR products were cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced subsequently (Applied Biosystems, Foster City, CA, USA).

cRACE (*c*ircular first-strand cDNA-mediated *r*apid *a*mplication of *c*DNA *e*nds)

In order to identify the transcription initiation site of the *SeFAD2*, cRACE as described by Maruyama et al. (1995) was carried out using total RNA isolated from the developing sesame seeds [15–30 days after flowering (DAF)] with TRIzol reagent (Gibco/BRL, Gaithersburg, MD, USA). The first-strand cDNA was then synthesized using a cDNA synthesis kit (Promega) with the W6-R4 primer, which was phosphorylated by T_4 polynucleotide kinase (Takara, Japan). Following hydrolysis of the template RNA with 0.5 N NaOH at 37°C for 10 min, the cDNA was precipitated and ligated by T_4 RNA ligase (New England Biolabs, Boston, MA, USA). An aliquot of the ligation mixture was then used as a template for the initial PCR amplification with the W6-1 and W6-R7 primers. The PCR product from the first amplification series was then diluted 100-fold with sterile H_2O, and a 2-μl aliquot was utilized as a template for the second nested PCR amplification series using the W6-5 and W6-R9 primers.

An *Arabidopsis* flower cDNA library was utilized to determine the transcription initiation site of the *AtFAD2* gene. The first and second PCRs rounds were conducted as described above. Adaptor primer 1/*At*W6-R3 and Adaptor primer 2/*At*W6-R4 were utilized in the first and second PCR, respectively. After cloning of the PCR products in pGEM-T easy vector, the entire nucleotide sequence was determined.

Construction of binary vectors and transformation of *Arabidopsis*

In order to clone the 5′-deletion series of the *SeFAD2* promoter into the binary vector, pBI101 (Jefferson et al. 1987), the 5′-flanking region of the *SeFAD2* gene, was amplified using the gene-specific primers SiW6F1/SiW6R1, SiW6F2/SiW6R1, SiW6F3/SiW6R1, SiW6F4/SiW6R1, SiW6F5/SiW6R1, SiW6F1/SiW6R3, and SiW6F4/SiW6R3. The amplified PCR products were then cloned in sequence into the *Hin*dIII and *Bam*HI sites of the pBI101 binary vector; the resultant constructs were named as PF1(-intron), PF2(-intron), PF3(-intron), PF4(-intron), PF5(-intron), P2.4(+intron), and P1.9(+intron), respectively.

The deletion series of the *SeFAD2* intron was constructed by generating each of the DNA fragment by PCR using the specific primers (Forward: SiW6-intronF1, SiW6-intronF2, and SiW6-intronF3; Reverse: SiW6-intronR1, SiW6-intronR2, SiW6-intronR3, SiW6-intronR4, and SiW6-intronR5). Following *Bam*HI enzymatic digestion, each of the DNA fragments was inserted between the *SeFAD2* promoter (− 660/+ 141) and the β-glucuronidase (GUS) gene. The four [P2.4(Δ1-intron) to P2.4(Δ4-intron)] constructs were developed by deleting the following nucleotides of the *SeFAD2* intron (nucleotides 149–1,722): nucleotides 792 through to 1,516 [P2.4(Δ1-intron)], 567 through to 1,516 [P2.4(Δ2-intron)], 424 through to 1,516 [P2.4(Δ3-intron)], and 240 through to 1,516 [P2.4(Δ4-intron)]. The full-length *SeFAD2* intron containing the *Bam*HI flanking sequences was inserted into the pBI101 vector and the pBI121 binary vector to

Table 1 Oligonucleotide sequences used in the isolation and analysis of *FAD2* genes from sesame and *Arabidopsis*

Genes	Reactions		Primers	Sequences
SeFAD2 promoter analysis	Genomic clone	Forward	W6-3	5′-CAGTTCTTGCGAAAGAAGGAAA-3′
			SiW6-TF1	5′-AGCCGAATAACATGTGG-3′
		Reverse	SiW6-TR1	5′-CACTAAACTTGACAATTTATTGCAG-3′
	IPCR	1st PCR	W6-1	5′-GACAAAATGGGAGCCGGAGGACGCATGT-3′
			W6-R6	5′-GGGGGCACGTTACCTGAAAACTTGGAAG-3′
		2nd PCR	W6-2	5′-AGCGTGACGAAGTCTTCGTCCCAAAGCC-3′
			W6-R7	5′-CGCGTGAAAGCACTTCTGCGGAAGCGC-3′
	c-RACE	Phosphorylation	W6-R4	5′-GGTAGCAGTATGGGGATGGCAGCAGATGGAAGTA-3′
		1st PCR	W6-1	5′-GACAAAATGGGAGCCGGAGGACGCATGT-3′
			W6-R7	5′-CGCGTGAAAGCACTTCTGCGGAAGCGC-3′
		2nd PCR	W6-5	5′-GAAGAACCCCCTCCAACGGGTGCC-3′
			W6-R9	5′-GCACTTCTGCGGAAGCGCTTTCCTTC-3′
	Promoter assay	Forward	SiW6F1	5′-CCGAAGCTTCATATGTGAAATGTAATGGAAAATGCGAC-3′
			SiW6F2	5′-CCGAAGCTTGGGACATGCCACATTATGTGG-3′
			SiW6F3	5′-CCGAAGCTTGTCCTAACCAGGTTTGAACAACC-3′
			SiW6F4	5′-CCGAAGCTTGGAATGTGCACACTCCATGTG-3′
			SiW6F5	5′-CCGAAGCTTGGGCCCCTCCTCAGACAGG-3′
		Reverse	SiW6R1	5′-CTTGGATCCTTGGAAGGAGAAATCGCGTGAAAGCAC-3′
			SiW6R3	5′-CAAGGATCCGTCAAGCCGCCCCCAATTTAC-3′
	Intron assay	Forward	SiW6-intronF1	5′-CGGGGATCCAACGCCACTCA-3′
			SiW6-intronF2	5′-CGGGGATCCTTTCAGGTAACG-3′
			SiW6-intronF3	5′-CGGAAGCTTGAGAATGTAGGACG-3′
		Reverse	SiW6-intronR1	5′-CCGGGATCCATTTACCTGCAAAAC-3′
			SiW6-intronR2	5′-CCGAAGCTTCAAACCGAAATAACT-3′
			SiW6-intronR3	5′-CCGAAGCTTCTTCCTGCATCC-3′
			SiW6-intronR4	5′-CCGAAGCTTGGGAAAGCCCAC-3′
			SiW6-intronR5	5′-CCGAAGCTTTCTTGAGACACAC-3′
AtFAD2 promoter analysis	RACE	1st PCR	Adaptor primer1	5′-CCATCCTAATACGACTCACTATAGGGC-3′
			AtW6-R3	5′-CGGCATTCTTCCACCTGCACCCATG-3′
		2nd PCR	Adaptor primer2	5′-ACTCACTATAGGGCTCGAGCGGC-3′
			AtW6-R4	5′-CACCCTACGAAGAAGAAGCTCCTCCGC-3′
	Promoter assay	Forward	AtW6F1	5′-CCGAAGCTTGGTGTGTCGTCGTTATACGCGGTAAA-3′
			AtW6F2	5′-CCGAAGCTTCCACCAGTCCACCACAACAC-3′
			AtW6F3	5′-CCGAAGCTTGGTGAAAGATTTGCCATGTGTATTG-3′
			AtW6F4	5′-CCGAAGCTTGAACGTGATATTAAAAAGTTAATGTTCACTG-3′
			AtW6F5	5′-CCGAAGCTTCTCTCTACCTTCGACCCACGG-3′
		Reverse	AtW6R1	5′-CAAGGATCCGGAGATGGAGCTGACGTAGGGGCG-3′
			AtW6R4	5′-CGAGGATCCCTGCAGAAAACCAAAAGCAAAAG-3′

generate the pBI101 + *SeFAD2* and pBI121 + *SeFAD2* introns, respectively.

All of the constructs were introduced into the C58C1 *Agrobacterium tumefaciens* strain via the freezing–thaw method (An 1987). *Arabidopsis* was then transformed by the floral dip method, as described by Clough and Bent (1998). Seeds that had been bulk-harvested from each pot were sterilized in a mixture of 50% bleach and 0.02% Triton X-100 for 7 min with agitation, then germinated on selective medium [4.3 g/l MS (Murashige and Skoog 1962) salts, 1 × B5 vitamins, 1% sucrose, 0.5 g/l MES, pH 5.7, and 0.8% phytagar] containing 100 mg/l carbenicillin and 30 mg/l kanamycin. The kanamycin-resistant seedlings were then transferred to soil to obtain the T_2 generation.

Exogenous application of abscisic acid and Northern blot analysis

Developing sesame (10–15 DAF) and *Arabidopsis* seeds (7–15 DAF) were incubated in MS media supplemented with various concentrations of ABA for 12 or 24 h at 25 ± 2°C and then used in the Northern blot analyses and GUS activity measurements.

Total RNAs were isolated from various tissues of the sesame and ABA-treated sesame seeds using TRIzol reagent (Gibco/BRL) in accordance with the manufacturer's instructions. Approximately 20 μg of total RNA was fractionated by electrophoresis on 1.2% agarose gels with formaldehyde, blotted onto nitrocellulose membranes (Amersham Biosciences, UK), and hybridized at 63°C. The *SeFAD2* gene-specific DNA fragments including the 3′-UTR unique sequence were amplified with SiW6-TF1/SiW6-TR1 and labeled with [^{32}P]-dCTP using the Rediprime II Random Prime labeling system (Amersham Biosciences). The membranes were then washed twice with 2× SSPE and 0.1% SDS for 20 min at room temperature, twice with 0.2× SSPE and 0.1% SDS for 15–30 min at 63°C and finally exposed for 15–32 h to X-ray film or to a PhosphorImager (Bio-Rad, Hercules, CA, USA).

Histochemical and fluorometric analyses of GUS

Histochemical and fluorometrical analyses of GUS activity were conducted as described by Jefferson et al. (1987). Developing seeds, silique walls, leaves, caulines, flowers, stems, and roots were hand cut with a sharp razor and incubated immediately 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 then rinsed with 70% ethanol until pigments such as chlorophyll had been completely cleared, and the images were then photographed using a SZ4045TRPT microscope (Olympus, Japan).

Fluorometric GUS activity was evaluated using 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG) as a substrate. Each of the tissues to be tested from the transgenic *Arabidopsis* plants was harvested and homogenized in homogenization buffer (w/v:1/2; 50 mM sodium phosphate, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarcosyl, and 10 mM 2-mercaptoethanol). After the homogenate had been centrifuged for 10 min at 10,000*g*, aliquots (25 μg protein) of the supernatants were incubated for 1 h in homogenization buffer containing 1 mM 4-MUG at 37°C. The reaction was terminated by the addition of 0.2 M Na_2CO_3. Fluorescence was then measured on a fluorescence spectrophotometer (Hitachi F-2000) with 4-methylumbelliferone (4-MU) as the standard. Protein concentrations were measured using bovine serum albumin (BSA) as a standard (Bradford 1976).

Results

Genomic organization of the sesame *FAD2* gene

Previous expression analyses of the *SeFAD2* gene revealed that the *SeFAD2* transcripts are both specifically and temporally controlled in developing seeds (Jin et al. 2001). In order to characterize the mechanisms underlying the seed-specific expression of the *SeFAD2* gene, we isolated a genomic clone encoding SeFAD2 from sesame seeds by PCR and inverse PCR as described in Materials and methods. The nucleotide sequences of the coding regions from three independent clones were 100% identical to those of the *SeFAD2* cDNA. We then used 5′-cRACE to determine the transcriptional initiation site of the *SeFAD2* gene; two clones of the three independent clones exhibited identical transcriptional initiation sites (data not shown). The nucleotide adenine (A) was designated as + 1, as is shown in Fig. 1.

The isolated *SeFAD2* genomic clone harbored a single large intron within the 5′-UTR. This feature is also found in *FAD2* genes sequenced from *Arabidopsis*, cotton, soybean, and rice (Fig. 2) (Okuley et al. 1994; Heppard et al. 1996; Pirtle et al. 2001; http://www.cdna01.dna.affrc.go.jp/cDNA). In the *SeFAD2* gene, a 1,574-bp-long fragment of intron was located 24 bp upstream from the ATG translation initiation codon. This fragment was found to have a 39% T content and 25% A content, which is a

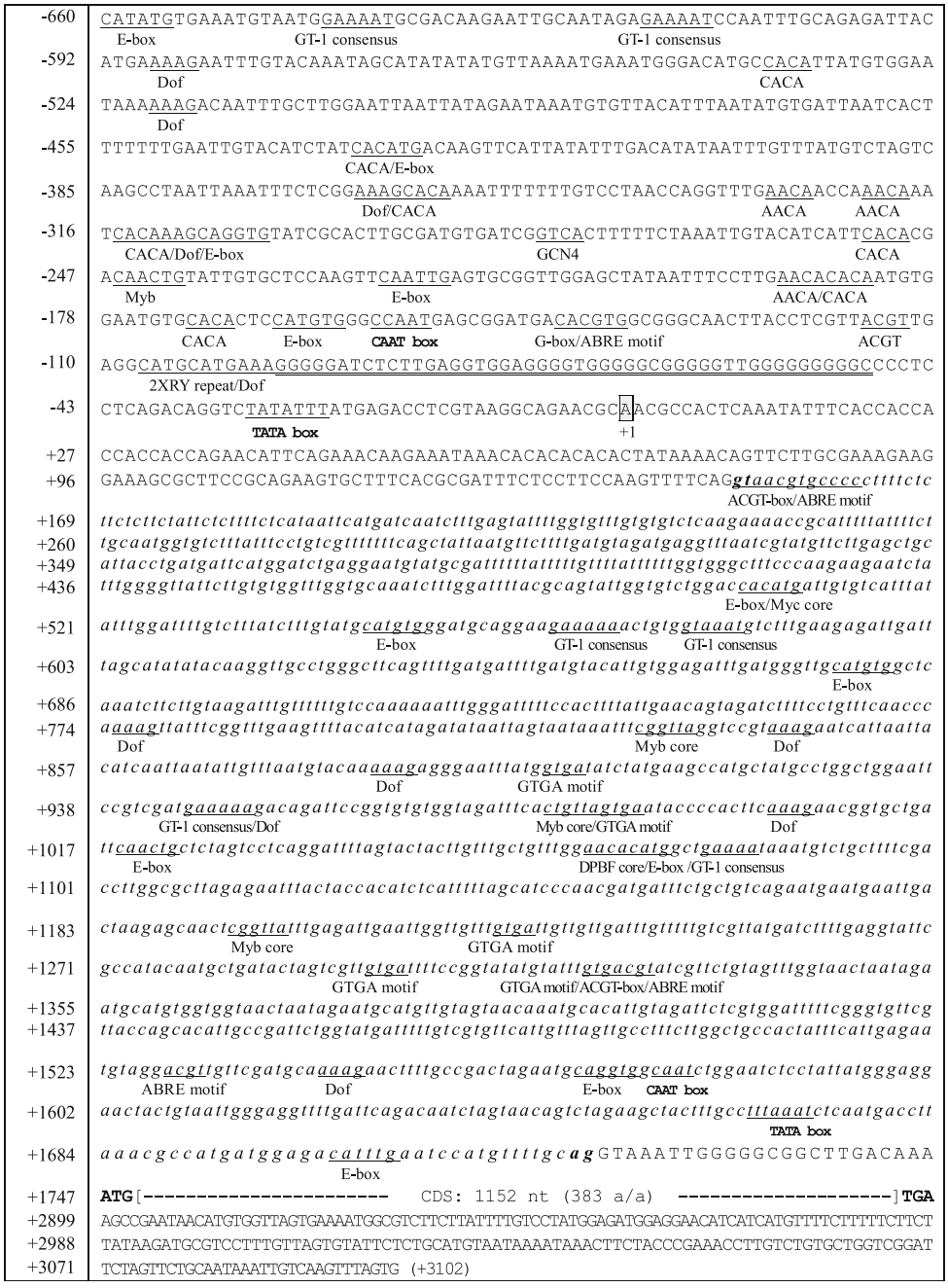

Fig. 1 Nucleotide sequences of the 5′-flanking region and intron of the *SeFAD2* gene. A *black box* represents the transcription initiation site identified by 5′-cRACE and is designated as the + 1 position. The *numbering on the left* refers to the nucleotide sequences. The sequences of the *SeFAD2* intron are shown in *small and italic letters*, including the 5′- and 3′-splice sites (***gt...ag***) at nucleotides 149 and 1,722, respectively. The putative TATA box occurs between − 25 and − 31. Translational initiation (ATG) and termination codon (TGA) are in *bold*, and the coding region of the *SeFAD2* gene (1,152 nucleotides; 383 amino acids) is demarcated by *large brackets*. Several potential *cis*-elements are *underlined* and designated with the names of each of the motifs. G-rich sequences are *double-underlined* between − 49 and − 96

well-known characteristic of higher plant introns. Introns from other plant species studied to date have been found to range in size from 420 to 3,150 bp. In all cases, the intron also contained GT–AG dinucleotides at both ends. Although the sequence identity of their *FAD2* introns was less than 10%, the intron sequences were determined to uniformly harbor a promoter-like feature. The *SeFAD2* intron sequences were then further analyzed for known *cis*-acting elements through a web search of publicly available databases (http://www.dna.affrc.go.jp/htdocs/PLACE and http://www.intra.psb.ugent.be:8080/PlantCARE). Putative TATA box and CAAT box elements were determined to be located at + 1,590 and + 1,575, respectively. Based on this database analysis we were able to identify many seed- or endosperm-specific and ABA-responsive *cis*-elements (ABRE) in the *SeFAD2* intron, including the Dof core (six AAAG motifs), DPBP core (one ACACNNG motif), E-box (seven CANNTG motifs), Myb-core (three cg/tgtta motifs), and ACGT-box (three ACGT/ABRE motifs). In addition, the *SeFAD2* intron also harbored five GTGA and three GT-consensus motifs that are involved in pollen-specific expression and associated with light signaling, respectively (Fig. 1).

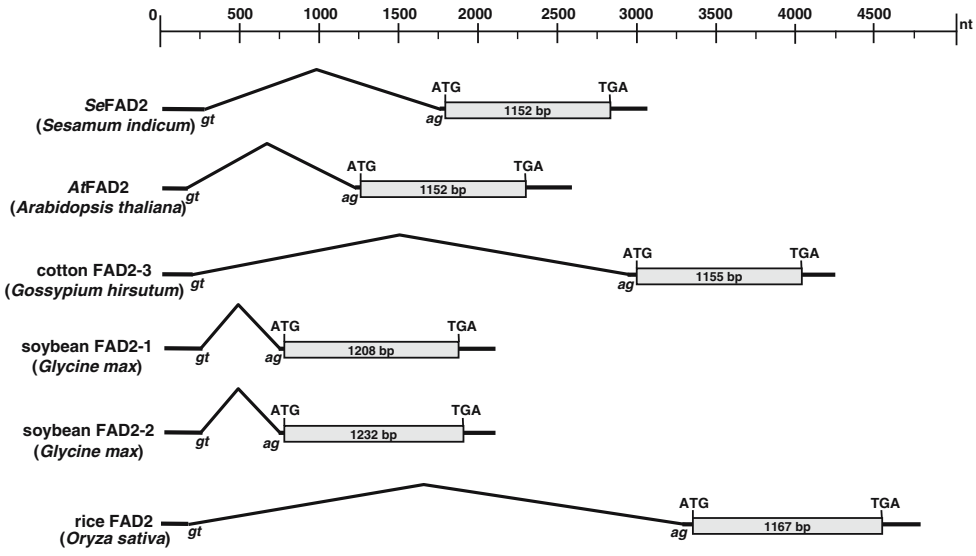

Fig. 2 Comparison of the *FAD2* genomic structures from sesame, *Arabidopsis*, cotton, soybean, and rice

The 5′-flanking region of the *SeFAD2* gene was also evaluated for known *cis*-acting elements. The putative TATA box and CAAT box were located at positions − 25 to − 31 and − 152 and − 156, respectively. Several potential *cis*-elements, including one GCN4 (GTCA) motif, one ACGT motif, three AACA motifs, five E-boxes (CANNTG), five Dof cores (AAAG), 2xRY repeat motif (CATGCATG), one ABRE motif (ACGTGKC), and one G-box, all of which are known to be essential to seed- or endosperm-specific expression, were represented in the 5′-flanking region of the *SeFAD2* gene. In addition, two consensus GT-1 motifs (GRWAAW), which are known to be conserved in the promoters of light-regulated genes, were located at positions − 653 to − 647 and − 616 to − 611, respectively. Seven CACA elements, all of which perform dual positive and negative regulatory functions in the β-phaseolin promoter, were dispersed at various locations between positions − 168 and − 538. Interestingly, several G-rich sequences, which are fairly unusual in plant promoters, were found at positions − 49 to − 96 (Fig. 1). Although the *SeFAD2* promoter and intron shared a number of sequence elements, the overall sequence identity between them was less than 10%, suggesting that they did not arise from tandem duplication.

Deletion of the − 660 to − 180 region of the *SeFAD2* promoter conferred constitutive expression of the *SeFAD2* gene

In order to identify the positive or negative *cis*-elements associated with seed-specific expression in the 5′-flanking region of the *SeFAD2* gene and to determine the effects of the *SeFAD2* intron on gene expression, five 5′-deletion constructs (spanning − 660, − 547, − 346, − 179, and − 52 to + 141 of the 5′-flanking region of *SeFAD2*) and two intron-containing constructs (− 660 to + 1,728 and − 179 to + 1,728) were tested using the GUS reporter gene (Fig. 3a). The resultant binary vectors, as well as the positive [pBI121, with GUS under the cauliflower mosaic virus (CaMV)35S promoter] and negative (pBI101, no promoter) controls were then transformed into *Arabidopsis* plants. For each construct, ten independent transgenic lines (T_2) were examined by histochemical staining and GUS activity measurements. The highest and lowest GUS activities from each of the constructs were excluded in our data analyses.

As is shown in Fig. 4a, histochemical staining revealed that GUS expression under the intron-less 5′-flanking region of the *SeFAD2* gene [PF1(-intron)] was restricted to the mid- and late-developmental phases of the developing seeds. Although the 5′-flanking region of the *SeFAD2* gene had been serially deleted, we noted no changes in the seed-specific patterns of expression. In the case of the PF4(-intron) construct, in which most of the promoter was deleted, GUS expression was detected in the early phases of seed development. When the *SeFAD2* intron sequence was inserted [− 660 to + 1,728, P2.4(+intron)], we observed GUS expression not only in the developing seeds, but also in the silique walls and young seedlings. Surprisingly, the specific combination of the intron with the deletion of the − 660 to − 180 promoter region [− 179 to + 1,728, P1.9(+intron)] resulted in constitutive GUS expression in all of the *Arabidopsis* tissues tested. In the controls, the GUS gene under control of the CaMV35S promoter was found to be constitutively expressed, but no GUS expression was noted in the promoter-less control (pBI101).

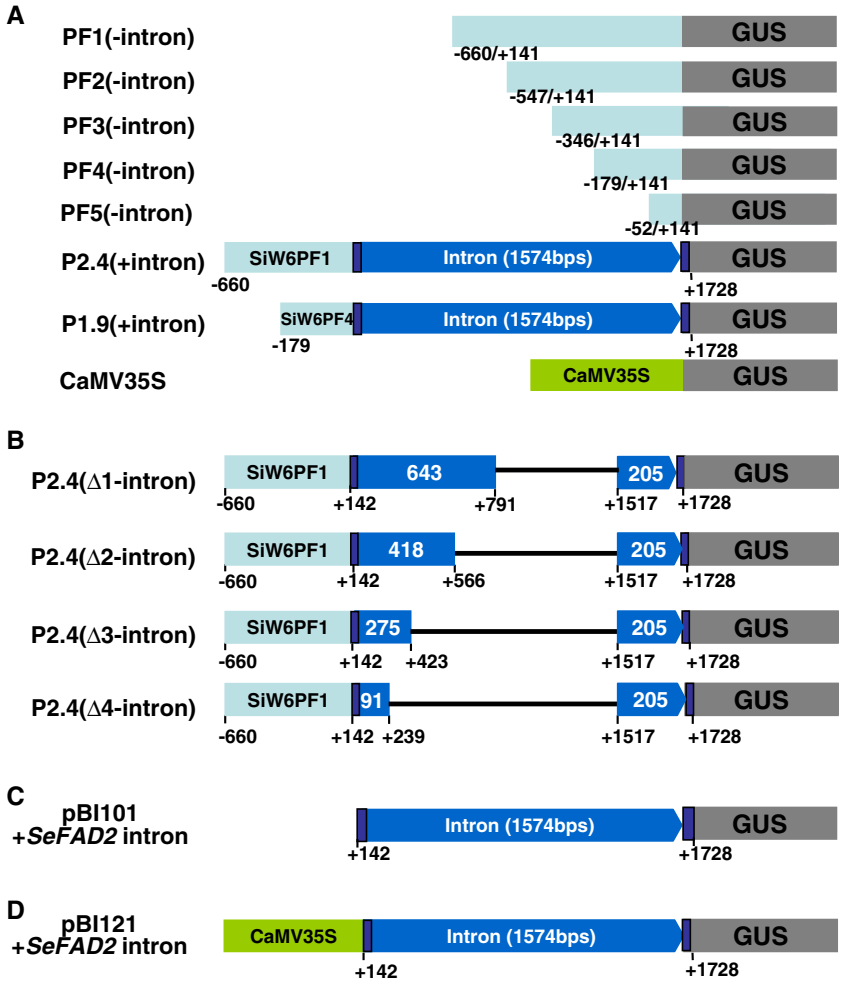

Fig. 3 Schematic diagrams of the *SeFAD2* promoter and GUS fusion constructs with or without intron (**a**), deletion constructs of the *SeFAD2* intron (**b**), the promoter-less *SeFAD2* intron and GUS fusion construct (**c**), and the *SeFAD2* intron and GUS fusion construct under the CaMV35S promoter (**d**)

An analysis of GUS activity revealed that the highest levels of GUS activity in all of the constructs occurred in the mid-developmental phase of seed development (Fig. 4b), which is consistent with the timing of storage lipid accumulation. The PF5(-intron) construct, deleted to − 53 nucleotides in the *SeFAD2* promoter, manifested only minimal promoter activity. However, we noted a greater than 40-fold increase in GUS activity in the PF4(-intron) construct (compared to PF5-intron), which indicated the presence of positively regulatory *cis*-acting element(s) in the − 53 to − 179 region. The upstream region between − 180 and − 547 bp significantly attenuated the promoter activity of PF3(-intron) and PF2(-intron), thereby suggesting the presence of negative *cis*-element(s) within this region. Conversely, we noted an approximately 15-fold increase in the promoter activity of the PF1(-intron), suggesting that some of the element(s) located in the − 548 to − 660 region may also play a role in the up-regulated expression of the *SeFAD2* gene.

A comparison of the GUS activity in the intron-containing P2.4(+intron) and P1.9 (+intron) constructs (Fig. 4c) showed that the GUS activity in the P1.9(+intron) construct was approximately twofold and eightfold higher than that of the P2.4(+intron) construct at all stages of the developing seeds and silique walls. Consistent with the results of the histochemical assay, we measured robust GUS activity in the P1.9(+intron) construct in all of the tissues tested, but detected very little GUS activity in the root, stem, leaf, cauline, and flower of the *Arabidopsis* plants that had been transformed with the P2.4(+intron) construct. On the basis of these results, we concluded that the − 660 (or − 547) to − 180 region of the *SeFAD2* promoter harbors negative *cis*-element(s) for *SeFAD2* gene expression. The seed-specific expression of the *SeFAD2* gene is probably controlled by a gene suppression mechanism that operates through the negative *cis*-elements.

The *SeFAD2* intron is involved in the enhancement of gene expression

The deletion experiments clearly showed that a construct harboring the full-length intron sequence

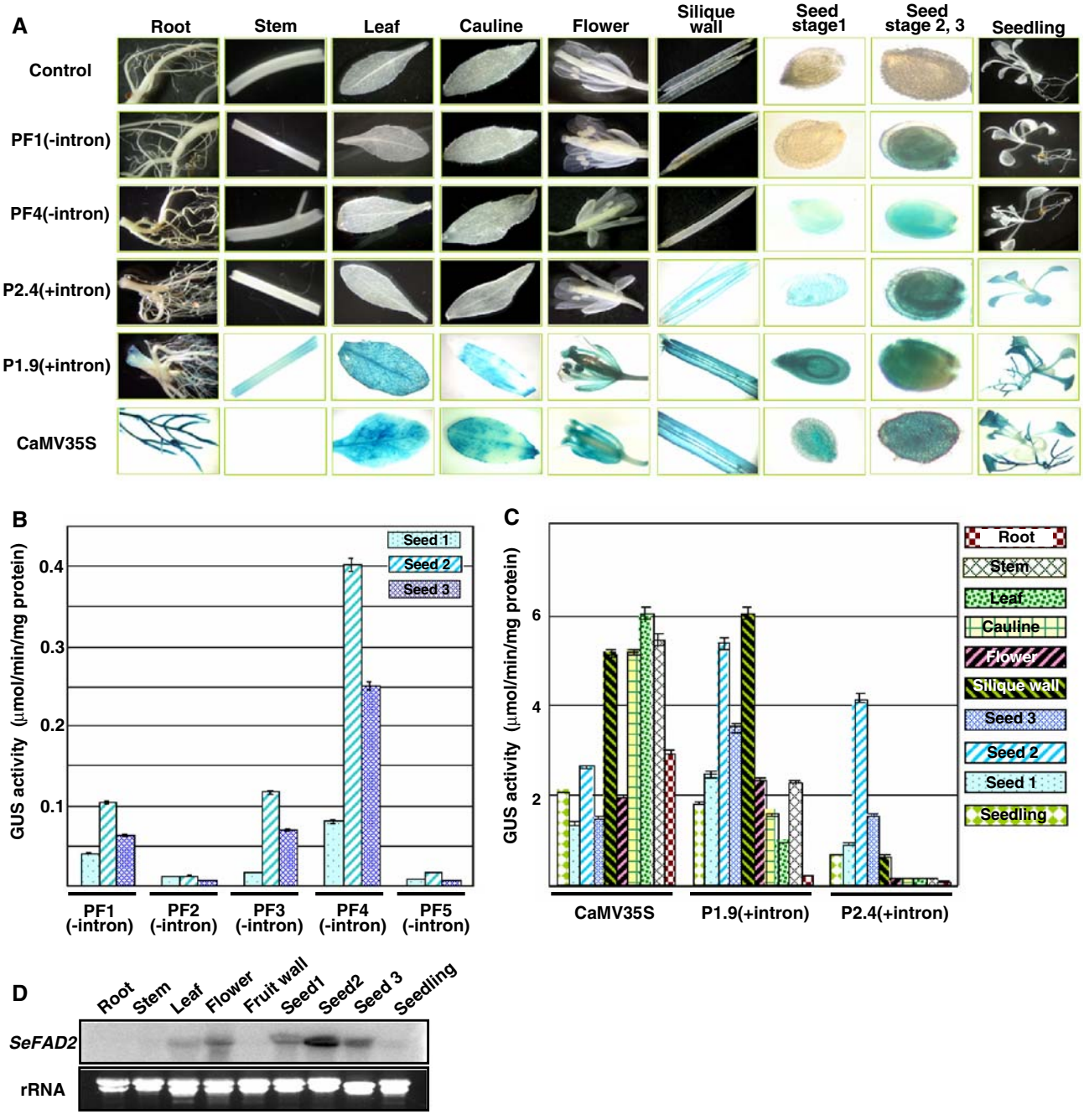

Fig. 4 The *SeFAD2* promoter and intron analyses in transgenic *Arabidopsis* and expression of the *SeFAD2* mRNA in sesame. Histochemical (**a**) and quantitative (**b**, **c**) of GUS expression in transgenic *Arabidopsis*. In histochemical staining and GUS activity measurements, ten independent transgenic lines (T_2) were examined for each construct. The highest and lowest GUS activities from each of the constructs were excluded in our data analyses. The values are average ± standard error. Seed stage 1: *Arabidopsis* developing seeds between 5 and 9 DAF; seed stage 2: *Arabidopsis* developing seeds between 10 and 15 DAF; seed stage 3: *Arabidopsis* developing seeds between 16 and 20 DAF. **d** Northern blot analysis of the *SeFAD2* mRNA in various tissues of sesame. Seed stage 1: sesame developing seeds between 7 and 15 DAF; seed stage 2: sesame developing seeds between 16 and 30 DAF; seed stage 3: sesame developing seeds between 30 and 45 DAF. Ethidium bromide staining of the gel (rRNA, ribosomal RNA) prior to transfer indicates RNA loading

[P2.4(+intron)] increased GUS activity by approximately 25- to 40-fold relative to the intron-less construct [PF1(-intron)] (Fig. 4). When the effect of the construct harboring the in [P1.9(+intron)] sequence was compared to the intron-less construct [PF4(-intron)], GUS activity in the former was approximately 15-fold higher in the developing seeds and more than 100-fold higher in other tissues. Although the magnitude

of the observed gene expression enhancement appeared to be dependent on tissue type, it would appear that the *SeFAD2* intron was clearly involved in IME (Fig. 4b, c).

When *SeFAD2* expression between the heterologous *Arabidopsis* system and the sesame native system was compared, the most abundant expression of the *SeFAD2* transcript was observed in developing sesame seeds. The strongest GUS activity was also detected in the developing seeds of *Arabidopsis*. Therefore, the timing and tissue specificity for the expression of *SeFAD2* transcript in sesame were quite similar with those of GUS expression under the *SeFAD2* promoter in *Arabidopsis*. However, low levels of *SeFAD2* mRNA were detected in flowers and leaves, but not in the fruit walls of the sesame plant (Fig. 4d).

The *Arabidopsis FAD2* intron is also involved in intron-mediated enhancement of gene expression

Plant *FAD2* genes manifest in two distinct isoforms, which are either constitutively or seed-specifically expressed (Okuley et al. 1994; Verwoert et al. 2000; Pirtle et al. 2001). Interestingly, the presence of a single large intron was highly conserved in both isoforms (Fig. 2). As shown in Fig. 4, the intron in the seed-specific *SeFAD2* gene was involved not only in the enhancement of gene expression but also in the change of tissue specificity and of seed-specific expression into constitutive expression. In order to characterize the effects of an intron in the *AtFAD2* gene on the enhancement of gene expression and to determine whether or not the *AtFAD2* intron influences the constitutive expression of the *AtFAD2* gene, we amplified the 5′-flanking regions of the *AtFAD2* gene, both with and without the intron, using *At*W6F1/*At*W6R1 and *At*W6F1/*At*W6R4 primers, respectively.

In the isolated 5′-flanking region, the putative TATA box was determined to be located between positions − 290 and − 286. The *AtFAD2* promoter exhibited several potential *cis*-elements associated with seed- or endosperm-specific elements, including nine Dof cores (AAAG), four E-boxes (CANNTG), two G-boxes (CACGTG), as well as several light-regulated elements, including three GATA motifs (GATA) and five GT-1 consensus motifs (GRWAAW). Although the *AtFAD2* and *SeFAD2* promoters exhibited less than 40% sequence homology, they harbored similar endosperm- and seed-specific *cis*-elements. In the case of the intron sequences, the *SeFAD2* intron sequence harbored a host of seed- or endosperm-specific *cis*-elements. Several stress-related elements [SEBF (silencing element binding factor); YTGTCWC, ERE (elicitor responsive elements); TTGACC, T-box; TTWTWTTWTT, and salicylic acid (SA)-inducible elements (GT-1 consensus); GRWAAW] were localized to the sequences of the *AtFAD2* introns. The transcriptional initiation site of the *AtFAD2* gene was subsequently determined to be located 147 bp upstream from its translational initiation site, as described above (Fig. 5).

We designed two constructs, *AtFAD2*-PF1 and *AtFAD2*-P2.2; the former consisted of approximately 2.2 kb of the 5′-flanking region plus intron of the *AtFAD2* gene fused to the GUS reporter gene, and the latter of the intron-less fragments fused to the GUS reporter gene. These were subsequently transformed into *Arabidopsis* plants (Fig. 6a). The GUS gene under the 5′-flanking region of the *AtFAD2* gene was observed to be expressed in all *Arabidopsis* tissues, regardless of the presence or absence of the *AtFAD2* intron sequence (Fig. 6b). When GUS activity was evaluated in *Arabidopsis* transformed with the *AtFAD2*-P2.2(+intron) and *AtFAD2*-PF1(-intron) constructs, the overall GUS activity in the various tissues was found to be approximately two- to threefold higher in the intron-containing construct (*AtFAD2*-P2.2) than in the intron-less construct (*AtFAD2*-PF1), although the magnitude of the increases associated with the *AtFAD2* intron differed depending on the tissue type (Fig. 6c). The highest levels of GUS activity under the *AtFAD2* promoter were observed in *Arabidopsis* stem tissues. In addition, when compared to the CaMV35S promoter, the expression of GUS under the *AtFAD2* promoter including the intron was over fivefold higher in the developing seed, flower, stem, and root tissues, but was more or less the same in the cauline and leaf tissues. These results show that the *AtFAD2* intron was capable of increasing gene expression but that it was not involved in the constitutive expression of the *AtFAD2* gene, thereby suggesting that the *FAD2* introns from a variety of plant sources are likely to be involved in the enhancement of gene expression.

The 791–1,517 region of the *SeFAD2* intron is essential for the IME of gene expression, and the *SeFAD2* intron exhibits promoter-like activity

In order to determine the length of the intron and of its internal sequences that influence the degree of stimulation of gene expression, four internal deletion series of the *SeFAD2* intron —P2.4(Δ1-intron), Δ792–1,516; P2.4(Δ2-intron), Δ567–1,516; P2.4(Δ3-intron), Δ424–1,516; and P2.4(Δ4-intron), Δ240–1,516—were constructed (Fig. 3b) and transformed into *Arabidopsis* plants. Histochemical analyses of the internal deletion constructs revealed that GUS expression was detectable exclusively in the developing seeds (data not

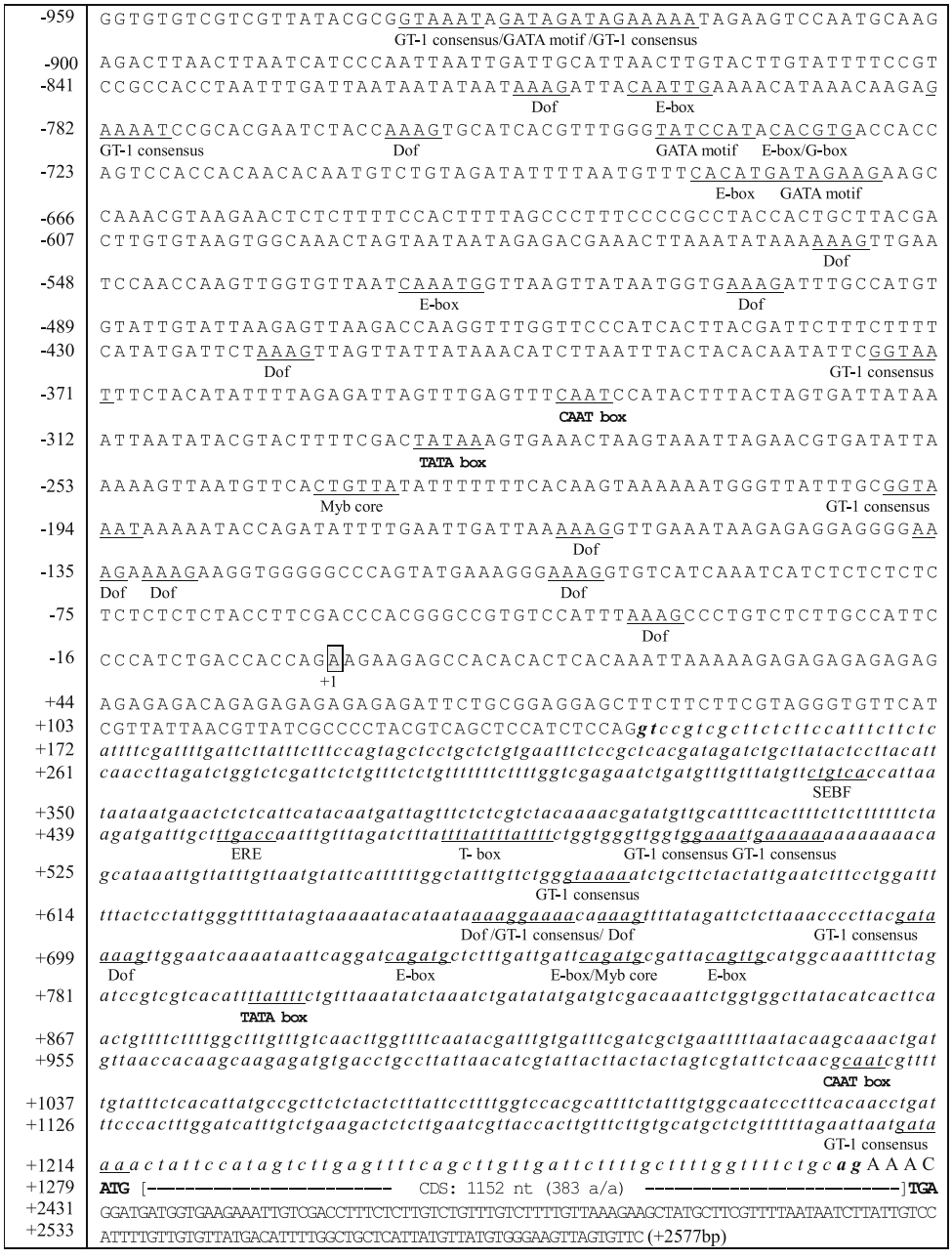

Fig. 5 Nucleotide sequences of the 5′-flanking region and intron of the *AtFAD2* gene. A *black box* represents the transcription initiation site identified by 5′-cRACE and is designated as the + 1 position. The *numbering on the left* refers to the nucleotide sequences. The sequences of the *AtFAD2* intron are shown in *small and italic letters*, including the 5′- and 3′ splice sites (***gt...ag***) at nucleotides 144 and 1,274, respectively. The putative TATA box occurs between − 286 and − 290. Translational initiation (ATG) and termination codon (TGA) are in *bold*, and the coding region of the *SeFAD2* gene (1,152 nucleotides; 383 amino acids) is demarcated by *large brackets*. Several potential *cis*-elements are *underlined* and designated with the names of each of the motifs

shown). Deletion of the region from 792 to 1,516 [P2.4(Δ1-intron)] in the intron sequence (1,574 bp) resulted in an approximately 30-fold attenuation of GUS activity relative to that of the P2.4(+intron) construct that contained the full-length intron. An additional deletion of 567–791 in the P2.4(Δ1-intron) construct resulted in an approximately threefold increase in GUS activity relative to that of the P2.4(Δ1-intron) construct, but the GUS activity of the P2.4(Δ2-intron) construct remained approximately tenfold lower than that of the P2.4(+intron) construct. When the intron sequence was subjected to further deletion, from 566 to 424 [P2.4(Δ3-intron); Δ424–1,516] or from 423 to 240 [P2.4(Δ4-intron); Δ240–1,516], GUS activity decreased gradually, and the intron segment in P2.4(Δ4-intron) ultimately lost its ability to enhance GUS expression (Fig. 7a). These results show that either the full-length *SeFAD2* intron or the specific nucleotide sequences between 792 and 1,516 of the *SeFAD2* intron is essential for the observed enhancements in gene expression.

As described above, sequence analyses of the *SeFAD2* intron indicated that the *SeFAD2* intron sequence manifests a promoter-like feature (Fig. 1). In support of this conclusion based on sequence analysis, we observed that GUS expression in the promoter-less binary (pBI101)

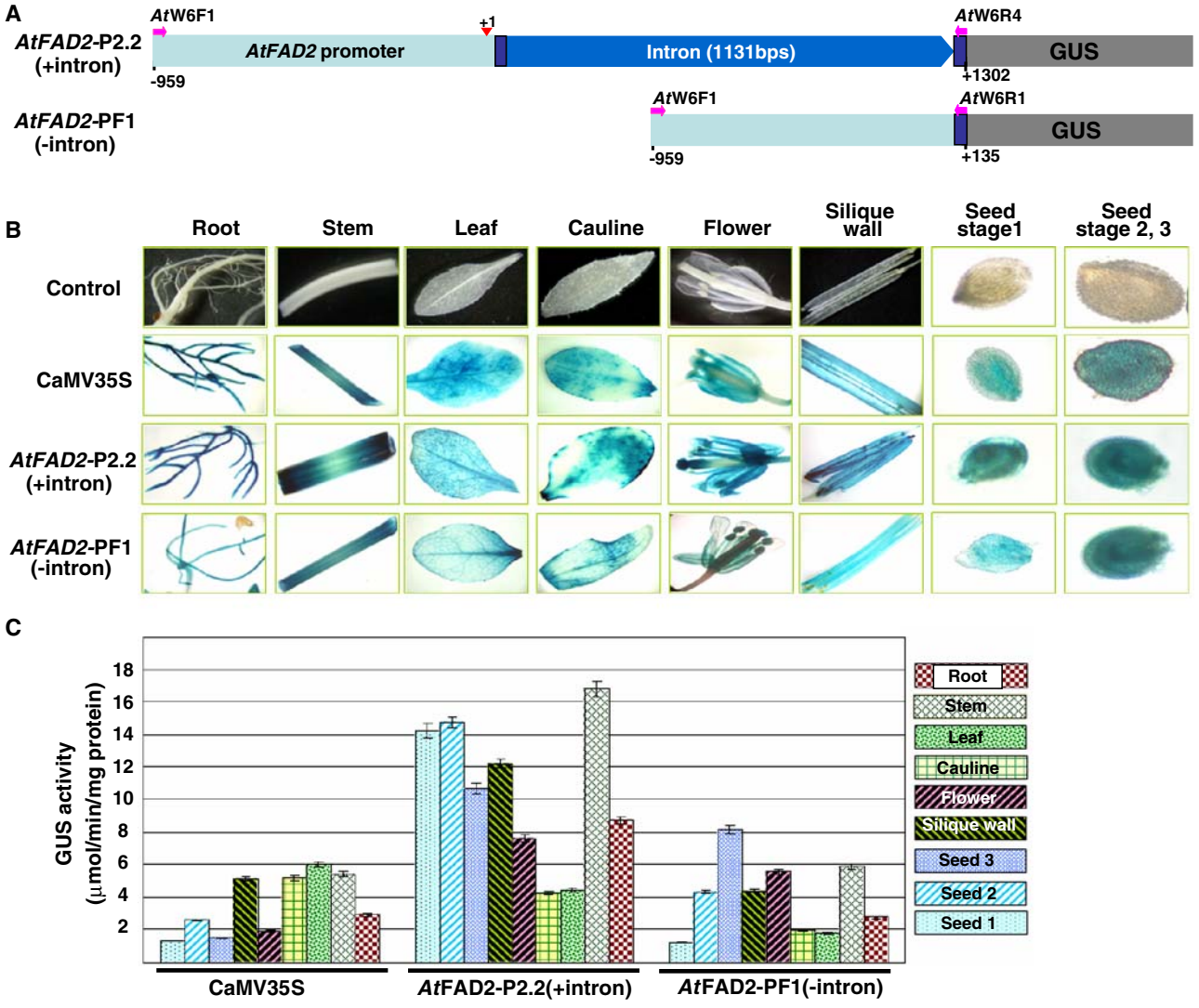

Fig. 6 The *AtFAD2* promoter and intron analyses in transgenic *Arabidopsis*. **a** Schematic diagrams of the *AtFAD2* promoter and GUS fusion constructs with or without intron. Histochemical (**b**) and quantitative (**c**) analyses of GUS expression under the *AtFAD2* promoter with or without the intron in transgenic *Arabidopsis* plants. In histochemical staining and GUS activity measurements, ten independent transgenic lines (T_2) were examined for each construct. The highest and lowest GUS activities from each of the constructs were excluded in our data analyses. The values are average ± standard error. Seed stage 1: *Arabidopsis* developing seeds between 5 and 9 DAF; seed stage 2: *Arabidopsis* developing seeds between 10 and 15 DAF; seed stage 3: *Arabidopsis* developing seeds between 16 and 20 DAF

plus *SeFAD2* intron construct (Fig. 3c) was similar to that associated with the PF1(-intron) construct. These results have led us to conclude that the *SeFAD2* intron has intrinsically a promoter-like activity for controlling GUS expression in transgenic plants (Fig. 7b).

In order to determine whether the *SeFAD2* intron also functions as an enhancer, the full-length of *SeFAD2* intron, flanked by 7 bp (5′) and 6 bp (3′) of the 5′-UTR sequence, was inserted between the CaMV35S promoter and the GUS gene (Fig. 3d) and then transformed into *Arabidopsis* plants. GUS expression was detected only in the developing seeds despite the fact that CaMV35S is a constitutive promoter. In addition, the insertion of the *SeFAD2* intron behind the CaMV35S promoter resulted in an approximately 20-fold reduction of GUS activity, as compared with the intron-less CaMV35S promoter (pBI121) (compare Fig. 7b with Fig. 4c). This result shows that the *SeFAD2* intron has reduced rather than enhanced expression controlled by the CaMV35S promoter.

Exogenous abscisic acid application increased *SeFAD2* gene expression

In order to assess any possible hormonal control on expression of the *SeFAD2* gene during seed

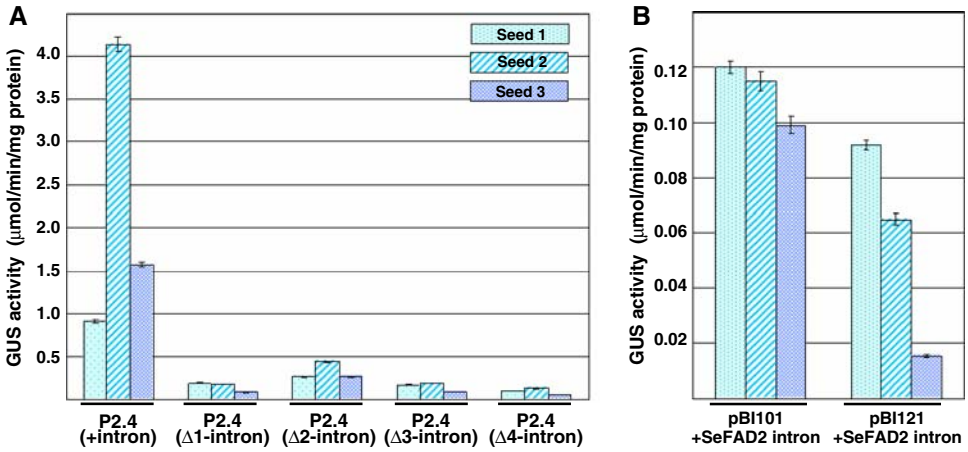

Fig. 7 The *SeFAD2* intron analysis in transgenic *Arabidopsis*. Quantitative GUS activity of deletion constructs of the *SeFAD2* intron (**a**), the promoter-less *SeFAD2* intron and GUS fusion constructs (**b**), and the *SeFAD2* intron and GUS fusion construct under the CaMV35S promoter in transgenic *Arabidopsis* (**b**). In histochemical staining and GUS activity measurements, ten independent transgenic lines (T_2) were examined for each construct. The highest and lowest GUS activities from each of the constructs were excluded in our data analyses. The values are average ± standard error. Seed stage 1: *Arabidopsis* developing seeds between 5 and 9 DAF; seed stage 2: *Arabidopsis* developing seeds between 10 and 15 DAF; seed stage 3: *Arabidopsis* developing seeds between 16 and 20 DAF

development, developing sesame seeds (10–15 DAF) were incubated with various ABA concentrations. As shown in Fig. 8a, Northern blot analyses revealed that exogenous ABA application resulted in an approximately twofold increase in the expression of the *SeFAD2* transcripts in the developing sesame seeds.

To further determine the locations of the ABA-responsive element(s) in the *SeFAD2* promoter, and whether or not the *SeFAD2* intron is involved in ABA response, we analyzed the P2.4(+intron), PF1(-intron), four 5′-deletion constructs of the *SeFAD2* promoter [PF2(-intron), − 547 to + 141; PF3(-intron), − 346 to + 141; PF4(-intron), − 179 to + 141; PF5(-intron), − 52 to + 141], as well as a CaMV35S promoter construct. Three independent T_2 transgenic lines, all manifesting an average amount of GUS activity, were selected from

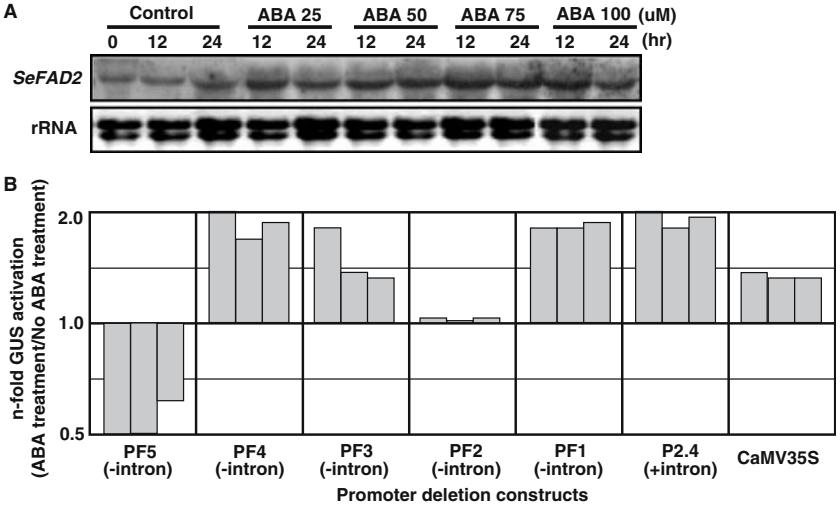

Fig. 8 Expression of the *SeFAD2* mRNA in developing sesame seeds (**a**) and quantitative GUS activity in developing *Arabidopsis* seeds (**b**) after application of ABA. **a** Developing sesame seeds (10–15 DAF) were incubated with 0, 25, 50, 75, 100 μM ABA for 12 and 24 h, then subjected to Northern analysis of the *SeFAD2* gene. Ethidium bromide staining of ribosomal RNAs (rRNA) indicates similar RNA loading in all lanes. **b** Each column represents one individual transgenic line. Three transgenic lines with average GUS activity from each construct were used. T_3 immature seeds (7–15 DAF) were incubated in MS media supplemented with or without 50 μM ABA for 12 h. The relative GUS activity was calculated by the ratio of GUS activity from the ABA-treated seeds to the GUS activity from the nontreated seeds

each of the constructs and then T_3 immature seeds (7–15 DAF) were incubated for 12 h in MS media supplemented either with or without 50 μM ABA. Figure 8b shows the relative amount of GUS activity, as defined by the ratio of GUS activity in ABA-treated seeds to GUS activity in the nontreated seeds. Similar levels of induced GUS activity were observed in the P2.4(+intron) and PF1(-intron) following exogenous ABA application, thereby indicating that the *SeFAD2* intron sequence is not required for the ABA response. An approximately twofold induction of GUS activity observed in the PF1(-intron) was consistent with the results of Northern blot analysis for the *SeFAD2* mRNA in the sesame developing seeds treated with ABA. The results of this experiment enabled us to delineate two regions that harbored ABA-response elements. Deletion of − 660 to − 548 of the *SeFAD2* promoter abrogated the twofold induction of GUS activity associated with the previous administration of ABA. Furthermore, the deletion of the − 179 to − 53 region resulted in an approximately fourfold down-regulation in GUS activity. Therefore, the ABA elements responsible for the response were determined to exist in the − 660 to − 548 and − 179 to − 53 regions of the *SeFAD2* promoter (Fig. 8b).

Discussion

We have identified the transcriptional regulatory mechanisms through which seed-specific *SeFAD2* expression is controlled by combinatorial properties between negative *cis*-regulatory elements in the *SeFAD2* promoter and enhancers in the 5′-UTR intron. The intron-mediated regulatory mechanism was found to be essential in controlling expression of the plant *FAD2* genes, which play a pivotal role in the synthesis of polyunsaturated fatty acids. For the development of new crop cultivars through the application of plant biotechnological methodology, one critical factor is the availability of suitable promoters that are able to provide strong expression of genes within a specific tissue (in this case, the seeds). We have provided preliminary evidence that the seed-specific *SeFAD2* promoter is probably applicable to the modification of seed phenotypes of transgenic plants.

Seed-specific and temporal regulation of the *SeFAD2* gene expression

The data presented here describe a novel mechanism by which the seed-specific expression of the *SeFAD2* gene is controlled through *cis*-acting negative regulatory elements. As is shown in Fig. 4b, c, the − 547 to − 180 region of the *SeFAD2* promoter harbors negative *cis*-elements responsible for the repression of gene expression. The deletion of the − 660 to − 180 region within the intron-containing *SeFAD2* promoter resulted in a switch from seed-specific expression to constitutive gene expression in all of the *Arabidopsis* tissues tested. This control mechanism for seed-specific expression is quite different from the well-characterized positive regulation mechanism, which operates through interactions between *cis*-elements in the 5′-flanking regions of seed storage protein genes and seed-specific *trans*-acting factors (Bustos et al. 1991; Chandrasekharan et al. 2003). For example, the interaction between the prolamin box (P-box, AAAG, or CTTT) and endosperm-specific transcription factors, such as zinc-finger protein and the basic leucine zipper (bZIP) transcriptional activator, performs a positive function in the coordinate activation of zein gene expression during the development of the endosperm (Vicente-Carbajosa et al. 1997). Although the negative regulation mechanism for seed-specific expression is currently fairly obscure, it appears likely that *SeFAD2* expression is repressed in all tissues, including the developing seeds. In particular, the *SeFAD2* transcripts were strictly repressed in the endogenous sesame roots, stems, fruit walls, and seedling tissues, although the magnitude of this repression varied according to tissue type (Fig. 4). It remains to be determined which types of *cis*-elements and *trans*-acting factors are responsible for the repression mechanism of the seed-specific expression of the *SeFAD2* gene.

Another interesting aspect of the *SeFAD2* promoter is that the − 179 to − 53 region of the *SeFAD2* promoter was found to harbor positive *cis*-elements for *SeFAD2* gene expression in developing seeds (Fig. 4). Eight potential *cis*-elements—the $(CA)_2$ element, E-box, CCAAT box, G-box, ABRE motif, G-box-like element, RY repeat element, and P-box—all of which have been implicated in the regulation of gene expression in developing seeds, are represented in this region (Fig. 1). A − 179-bp fragment of the *SeFAD2* promoter is quite similar to a − 150-bp section of the 2S seed storage protein (*nap*A) promoter in *Brassica napus*, and also to a − 295-bp section of the promoter for the β-phaseolin storage protein in *Phaseolus vulgaris*. Mutations in the ABRE/E-box-like elements (GCCACgTGTCc), (CA)n elements (CAAACAC), RY (CATGCA), or G-boxes (CACGTG) of the *nap*A promoter result in a profound reduction of the *nap*A promoter activity in seeds (Ellerstrom et al. 1996; Stalberg et al. 1996; Ezcurra et al. 2000). Only 2.6% of − 295*phas* promoter activity could be detected after induced mutations of the G-box. The CCAAT box, the E-box, and the RY elements in the − 295*phas*

promoter were also determined to be positively involved in the high expression levels observed in embryos (Chandrasekharan et al. 2003). The similarity of *cis*-regulatory elements such as the GCN4-like motif (GTCA), G-box-like element (ACGT) or G-box (CACGCC), AACA motif, and Prolamin-box (AAAG) has also been reported to be located in the seed-specific promoters of acyl carrier proteins and Δ^4-palmitoyl-acyl carrier protein desaturase genes, which in turn are involved in the biosynthesis of petroselinic acid in *Coriandrum sativum* (Kim et al. 2005). Therefore, future research should focus on defining which *cis*-elements in a − 179 bp section of the *SeFAD2* promoter are associated with the quantitative regulation of *SeFAD2* gene expression during seed development.

In addition to the seed-specific expression of the *SeFAD2* gene, which occurs via combinatorial properties between the negative *cis*-regulatory elements in the *SeFAD2* promoter and the IME of *SeFAD2*, the expression of the *SeFAD2* gene is temporally controlled during seed development. *SeFAD2* mRNA levels are quite low in the early stages of embryogenesis, achieve maximum levels in the seeds during mid- and late embryogenesis, and then fall to low levels as the seeds mature (Fig. 4d). The ABA phytohormone is one of the signals that control the expression of the *SeFAD2* gene during seed development, and the ABA-response elements are located in the − 660 to − 548 and − 179 to − 53 regions of the *SeFAD2* promoter (Fig. 8). These two regions contain a series of ABA-responsive elements, including the E-box, G-box, ABRE motif, ACGT motif, and RY repeat motif, all of which have also been previously identified in the promoters of maize and wheat *Em* (embryo) genes, the rice *Osem* (a rice homolog to the wheat *Em* gene), the *Lea* (late embryo abundant) genes from monocots and dicots, and the barley *HVA1* (group 3 *Lea* protein) genes (Guiltinan et al. 1990; Izawa et al. 1993; Shen and Ho 1997). The ABA-regulated expression of the *Em*, *Osem*, and *HVA1* genes has been reported to be induced by a transcriptional activator, VP1 (Hattori et al. 1995; Hill et al. 1996; Shen et al. 1996). Therefore, further investigations are clearly warranted into the identification of these possible ABA-response elements and their interacting transcription factors, both which are required for the regulation of *SeFAD2* gene expression.

SeFAD2 and *AtFAD2* intron-mediated enhancement of gene expression

We determined that both *SeFAD2* and *AtFAD2* introns are capable of stimulating GUS gene expression in transgenic *Arabidopsis* plants, although the magnitude of IME varied considerably in different tissues (Figs. 4, 6). In addition, the IME associated with *SeFAD2* was sufficient to effect a switch from a seed-specific expression pattern to a constitutive expression pattern in all of the *Arabidopsis* tissues tested (Fig. 4). This constitutive expression pattern was observed to be strictly repressed via the negative *cis*-elements (− 660 or − 547 to − 180) in the vegetative tissues, such that the *SeFAD2* mRNAs were expressed most abundantly in the reproductive tissues, particularly in the developing seeds. Moreover, the internal deletion (+ 791 to + 1,517) of the *SeFAD2* intron resulted in a dramatic reduction of GUS expression to levels similar to those in the intron-less construct (Fig. 7a). When the *SeFAD2* intron was inserted downstream of the CaMV35S promoter, we were unable to detect the IME associated with the *SeFAD2* intron (Fig. 7b). Collectively, our findings strongly support the notion that the IME mechanism of *SeFAD2* is attributable primarily to specific interactions with one or several *cis*-acting elements within the − 179 to − 53 region of the *SeFAD2* promoter and in the *SeFAD2* intron (+ 791 to + 1,517), rather than to the length of the intron or to some posttranscriptional effect. Similar *SeFAD2* IME mechanisms were observed in the first introns of the maize *Adh1* and *Sh1* genes as well as in the *Arabidopsis AtEF-1α* intron (Maas et al. 1991; Curie et al. 1993; Luehrsen and Walbot 1994). The *Arabidopsis* T-DNA mutant with an insertion in the 5′-UTR of the *AtFAD2* gene (*fad*2-5) showed greatly reduced levels of *AtFAD2* mRNA in the shoots and roots (Okuley et al. 1994), thereby supporting our result of the IME being associated with the *AtFAD2* intron.

The regulatory sequence elements positively and negatively affecting IME have also been studied in the sequences of a variety of plant introns (Deyholos and Sieburth 2000). Among the best characterized of these are the U-rich motif in the maize *Sh1* intron1 and the *Arabidopsis PAT*1 intron1, which is ostensibly operant with regard to intron recognition (Clancy and Hannah 2002; Rose 2002). Based on sequence analysis of the + 791 to + 1,517 region of the *SeFAD2* intron, this region contains approximately 38% of the U-rich sequence (Fig. 1). Future studies should focus on whether the U-rich sequence in the *SeFAD2* intron is essential for the IME associated with the *SeFAD2* gene.

Interestingly, the *SeFAD2* intron was found to increase GUS activity in developing seeds, even though no promoter sequence was inserted upstream of the GUS gene (Fig. 7b). Because this result was observed in many independent transformants, it is unlikely that this observation resulted from the influence of upstream *Arabidopsis* chromosomal genomic sequences that were

active when the promoter-less intron construct became integrated into *Arabidopsis*. Our data are similar to previous findings, namely that the maize ubiquitin first exon and intron-fragment can promote GUS expression in *Tritordeum* inflorescences and wheat scutella (Salgueiro et al. 2000). Also, an intron within the 5′-UTR of the rice beta-tubulin gene (*Ostub*16) has been shown to autonomously promote transcription both in vivo and in vitro, although the level of expression was not induced strongly (Morello et al. 2002). Both introns were determined to harbor putative TATA and CAAT boxes and consensus sequence targets for known transcription factors. As is shown in Figs. 4 and 7, the GUS activity associated with the *SeFAD2* intron was as high as the GUS activity associated with the *SeFAD2* promoter (− 179 to + 141). When the sequence of the *SeFAD2* intron was evaluated for known *cis*-acting elements, the *SeFAD2* intron was also determined to harbor promoter-like structures, including putative TATA and CAAT boxes, as well as a host of potential *cis*-elements (Fig. 1). Therefore, these elements may play some role in the ability of the *SeFAD2* intron to drive GUS expression, acting as regulatory sequence elements. The seed-specific GUS expression produced by the *SeFAD2* intron in transgenic *Arabidopsis* plants is probably influenced by many of the seed- or endosperm-specific and ABA-responsive *cis*-elements in the *SeFAD2* intron.

It would be of interest to determine whether or not the *SeFAD2* and/or *AtFAD2* genes are transcribed at the two different initiation sites, one of which is in the promoter and the other in the intron. Our study was too limited to answer this question: only three colonies were sequenced in each 5′-cRACE, and tissues other than developing seeds of sesame and flowers of *Arabidopsis* were not examined. When 570 *Arabidopsis FAD2* expressed sequence tags (ESTs) from publicly available databases (http://www.arabidopsis.org, http://www.mips.gsf.de, http://www.plantbiology.masu.edu/lipid/genesurvey/EST_data.htm) were analyzed for the presence of *AtFAD2* mRNA, which is initiated from the intron site, no ESTs were observed.

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