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The SebHLH transcription factor mediates trans-activation of the SeFAD2 gene promoter through binding to E- and G-box elements

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Abstract Microsomal oleic acid desaturase (FAD2) catalyzes the first extra-plastidial desaturation in plants, converting oleic acid to linoleic acid, which is a major constituent in all cellular membranes as well as in seed storage oils. Seed-specific FAD2 (SeFAD2) produced 40% of linoleic acids in the total fatty acids of sesame (*Sesamum indicum*) seeds. The expression of *SeFAD2* transcripts was spatially and temporally controlled during seed development. To investigate the regulatory mechanism controlling seed-specific *SeFAD2* expression, we isolated a well-matched sequence homologous to the basic region/helix-loop-helix proteins by yeast one-hybrid screening and named it SebHLH. *SebHLH* transcripts were expressed in developing seeds and roots of sesame. SebHLH:GFP fusion protein localized in the nucleus. Recombinant SebHLH protein bound E-box (CANNTG) and G-box (CACGTG) elements in the region from –179 to –53 of the *SeFAD2* gene promoter, and the external C and G nucleotides in the E- and G-box motifs were essential for SebHLH protein binding. The *SebHLH* gene, under the CaMV35S promoter, and the GUS reporter gene driven by E- and G-box motifs were co-expressed in developing sesame seeds and *Arabidopsis* transgenic leaves. This co-expression demonstrated that SebHLH protein mediates transactivation of the *SeFAD2* gene promoter through binding to E- and G-box elements. E- or G-box elements frequently occur in the 5′-flanking region of genes that are involved in triacylglycerol biosynthesis and that exhibit seed-specific expression in *Arabidopsis* and other plants, suggesting that bHLH transcription factors play a key role in the transcriptional regulation of genes related to storage lipid biosynthesis and accumulation during seed development.

Keywords bHLH transcription factor · FAD2 · Microsomal oleic acid desaturase · SebHLH · Sesame · *Sesamum indicum*

Introduction

In plants, de novo fatty acid synthesis from acetyl-CoA to palmitic acid (16:0), stearic acid (18:0) or oleic acid ($18:1^{\Delta 9}$) occurs in plastids. The fatty acids are further reduced to linoleic acid ($18:2^{\Delta 9,12}$) and linolenic acid ($18:1^{\Delta 9,12,15}$) via a prokaryotic pathway in plastids or a eukaryotic pathway in endoplasmic reticulum (Somerville et al. 2000). The synthesized polyunsaturated fatty acids serve as an essential component in all cellular membranes and in storage oils. Oleic acid desaturase (FAD2) on the endoplasmic reticulum catalyzes the formation 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 a single copy of the *FAD2* gene, but crops including sesame (*Sesamum indicum*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), corn (*Zea mays*), and canola (*Brassica napus*) contain at least two *FAD2* isoforms; one is constitutively expressed

The nucleotide sequence of the SebHLH cDNA from *Sesamum indicum* reported here has been registered in the GenBank™/EBI Data Bank under accession number EF397568.

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and the other 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; Tang et al. 2005). Seed-specific *FAD2* is the key enzyme responsible for the production of linoleic acid in seeds (Liu et al. 2000; Jin et al. 2001). Furthermore, the ratio of the various saturated and unsaturated fatty acids is reported to be one of the critical factors that influence the quality and commercial application of seed oils (Cahoon et al. 2002; Kinney et al. 2002; Tang et al. 2005).

During seed development, storage lipids accumulate in abundance in the middle and late phases of embryogenesis. Coincident with storage lipid accumulation, some genes involved in biosynthesis and accumulation of storage lipids, including seed-specific *FAD2* gene, are spatially and temporally regulated in developing seeds (Cahoon et al. 1992; Slocombe et al. 1992; Kinney 1994, 1996; Suh et al. 1999; Liu et al. 2000; Moon et al. 2001; Rossak et al. 2001; Bao et al. 2002; Tang et al. 2005; Kim et al. 2006). The transcriptional control of these genes through a complex set of promoter DNA or promoter DNA-specific regulatory proteins has been studied. In microspore-derived cell suspension culture of *Brassica napus*, the seed-specific activator ABI3 promotes oleosin gene expression via interaction with the ABA response element at –160 bp of the oleosin gene promoter (Crowe et al. 2000). Promoter regions at –117 and –312 of acyl carrier protein (*Cs-ACP1*) and Δ^4-palmitoyl-acyl carrier protein desaturase (*Cs-4PAD*) genes, respectively, both of which are involved in biosynthesis of petroselinic acid in coriander (*Coriandrum sativum*) endosperms, include *cis*-regulatory elements such as the GCN4-like motif (GTCA), E-box, G-box, AACA motif, and Prolamin-box. These elements are required for endosperm-specific expression of *Cs-ACP1* and *Cs-4PAD* genes (Kim et al. 2005). In sesame, the region from –179 to –53 in the *SeFAD2* promoter, including E-box (CANNTG), G-box (CACGTG), ABRE motif (ACGTGKC), G-box-like element (ACGT), RY repeat element (CATGCA), and Prolamin-box (AAAG), is essential for expression of the *SeFAD2* gene in developing seeds (Kim et al. 2006).

The basic region/helix-loop-helix (bHLH) proteins are a superfamily of transcription factors that have been found in all eukaryotes. The proteins bind as homodimers or heterodimers to specific target sequences, E-box (CANNTG) or G-box (CACGTG) motifs, and function in cell proliferation and cellular differentiation pathways (Littlewood and Evan 1998; Atchley et al. 1999; Ledent and Vervoort 2001). The bHLH domain contains two functionally distinct regions. The basic region consists of approximately 15 amino acids with a high number of basic residues and is involved in DNA binding. The HLH region is comprised of hydrophobic residues that form two α-helices separated by a loop region of variable sequence and length and functions as the dimerization domain (Murre et al. 1989; Ferre-D'Amare et al. 1994; Nair and Burley 2000). Outside of the conserved bHLH domain, these proteins exhibit considerable sequence divergence (Atchley et al. 1999). Since the *R* gene product, Lc, which is involved in the control of anthocyanin synthesis, was reported to possess the bHLH motif in plants (Ludwig et al. 1989), several bHLH transcription factors have been identified including PIF1, PIF3, PIF4, HFR1, SPT, ALC, TT8, GL3, AMS, AtMYC2, ATR2, BEE1, BEE2, BEE3, and ICE1. These proteins regulate a broad range of growth and developmental processes at all phases of the plant life cycle (see review in Toledo-Ortiz et al. 2003). *Arabidopsis* and rice genome sequencing analyses provided genetic information for 147 and 167 bHLH transcription factors, respectively (Toledo-Ortiz et al. 2003; Li et al. 2006). However, approximately 10% of these proteins in *Arabidopsis* have been characterized through mutational or functional analysis (Toledo-Ortiz et al. 2003).

To understand the transcriptional control of the *SeFAD2* gene, which is spatially and temporally regulated during seed development, a trans-acting factor that interacts with E- and G-box elements in the region from –179 to –53 in the *SeFAD2* gene promoter was isolated from developing sesame seeds. The isolated gene, named *SebHLH*, had good sequence homology with the basic region/helix-loop-helix proteins. SebHLH protein was targeted to the nucleus, and the N-terminal region of the protein functioned as a transcriptional activator. Finally, SebHLH protein induced transcriptional activity from E- and G-boxes of the *SeFAD2* promoter in developing sesame seeds and transgenic *Arabidopsis* leaves.

Materials and methods

Plant materials

Sesame (*Sesamum indicum* cv. Yangbaeck) plant was grown in soil in the green house at 26 ± 3°C under a 16-h light/8-h dark regimen. *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia-0) was grown in soil in the *Arabidopsis* room under a photocycle of 16 h of light (24°C) and 8 h of darkness (22°C). To facilitate harvesting of seeds at predefined developmental stages, flowers were tagged on the day of flower opening. The developing seeds were then collected via dissection from sesame fruits and *Arabidopsis* siliques.

Yeast one-hybrid screening

Yeast one-hybrid screening was performed according to the manufacturer's instructions (Clontech). The region from

–179 to –53 of the *SeFAD2* gene promoter was amplified by PCR using SiW6-YF4/SiW6-YR4 and inserted into the *Eco*RI/*Sac*I site of the pHIS2 vector (SeYF4:HIS2). Plasmid SeYF4:HIS2 containing the *SeFAD2* promoter region was linearized with *Kpn*I and cotransformed into yeast strain Y187 (Clontech) with developing sesame seed cDNAs (10–30 DAF) in pGADT7-Rec2 vector. Recombinants with positive one-hybrid interaction were selected on SD/-His-Leu-Trp medium supplemented with 45 mM 3-AT (3-amino-1,2,4-triazole). Recombinants containing inserts larger than 500 bp were further selected by colony PCR screening using 5′LD and 3′LD amplimers. The selected plasmids isolated from recombinant yeast cells were re-transformed into *E. coli* XL1-Blue, and nucleotide sequencing was performed. The genetic information of the sequenced clones was obtained from BLASTX and TRANSFAC analyses (http://www.ncbi.nlm.nih.gov; http://www.gene-regulation.com).

The full-length cDNA encoding the SebHLH protein was isolated by 5′-cRACE (*c*ircular first-strand cDNA-mediated *r*apid *a*mplification of *c*DNA *e*nds) as described by Maruyama et al. (1995). The sequence information of all primers used in 5′-cRACE is listed on Table 1. Total RNA isolated from sesame endosperm (10–30 DAF) was reverse-transcribed, self-ligated, and then subjected to PCR. After subcloning the PCR products, the nucleotide sequence was analysed.

Northern blot analysis and RT-PCR

Total RNAs were isolated from various sesame tissues using TRIzol reagent (Sigma, USA) according to the manufacturer's instructions. Approximately 20 μg of total RNA was fractionated via electrophoresis on 1.2% agarose gels with formaldehyde, blotted onto nitrocellulose membranes (Amersham Biosciences), and hybridized at 63°C. Radioactive ^{32}P-dCTP-labeled hybridization probes were generated via random priming (Rediprime II Random Prime Labelling System, Amersham Biosciences) using SebHLH gene-specific DNA fragments (SF4-FF2 and SebHLH-CR primer set, 761 bp of DNA fragments), including the unique 3′-UTR sequence. After hybridization, the membranes were 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 to X-ray film or to a PhosphorImager (Bio-Rad) for 15–32 h.

Reverse transcription was carried out as described by the manufacturer (Invitrogen). PCR was performed using gene-specific SF4-FF2 and SebHLH-ER primers. The sesame elongation factor gene (MC01037C03, Suh et al. 2003) was amplified using the Se-Elon-F and the Se-Elon-R primers to determine the quantity and quality of the cDNAs. PCR was conducted in a final volume of 30 μl containing 500 ng of cDNA, 1× *i*-Taq buffer (iNtRON, Korea), 10 mM of each dNTP, 1 unit of *i*-Taq polymerase (iNtRON, Korea), and 10 pmol of each primer. The following amplification program was used: 1 cycle of 94°C for 5 min (denaturation step), 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min, and then 1 cycle of 72°C for 7 min (elongation step).

Subcellular localization assay

The coding region of the *SebHLH* cDNA was amplified using the SebHLH-GFPS and SebHLH-GFPA primers and subcloned into the pBIN35S-smGFP vector under the control of the CaMV35S promoter (Davis and Vierstra 1998). The resultant plasmid was linearized with *Eco*RI restriction enzyme. As described in Klein et al. (1988), gold particles (1.0 μm, Bio-Rad) were coated with the plasmid DNA mixture (2 μg of the linearized plasmid DNA, 50 μl of 2.5 M $CaCl_2$, and 20 μl of 0.1 M spermidine) and introduced into onion epidermis via a gene gun (Bio-Rad, USA). The tissues were bombarded twice at 1,100 psi with a biolistic helium gun device (Bio-Rad PDS-1000/He). The stopping screen was positioned 3 cm below the rupture disk, and the target tissue was positioned 6 cm below the stopping screen. After bombardment, samples were incubated for 24 h at 25°C in the dark and observed under a confocal laser scanning microscope (OLYMPUS BX51).

Electrophoretic mobility shift assays

The coding region of the *SebHLH* cDNA was amplified using the SebHLH-EF and SebHLH-ER primers, and subcloned between *Eco*RI and *Sal*I sites in pET32b expression vector (Novagen, Germany). After transformation of the resultant pSebHLH:His plasmid into *E. coli* strain BL21(DE3), expression of His-tagged SebHLH protein was induced with 0.2 mM IPTG for 4 h. Cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM Imidazole, pH 8.0) containing 1 mM PMSF and 1 mg/ml lysozyme, and then sonicated. Supernatants obtained by centrifugation were loaded on Ni-NTA resin columns. The column was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl and 30 mM Imidazole, pH 8.0) and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM Imidazole, pH 8.0) as described in the manufacturer's instructions of the His-Bind Buffer Kit (Novagen).

The region from –179 to –53 in the *SeFAD2* gene promoter was amplified by PCR using the SeMF4 and SeMR4 primers, and eluted. To generate double-strand oligonucleotides, single-strand oligonucleotides (SeEbox-F,

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

Reactions	Primer name	Sequence information	Annealing Tm for PCR (°C) [a]	Enzyme site
Yeast one hybrid	SiW6-YF4	5′-CCGGAATTCGGAATGTGCACACTCCATG-3′	66	*Eco*RI
	SiW6-YR4	5′-CCCGAGCTCCCTGTCTGAGGAGGGG-3′	66	*Sac* I
	5′LD Amplimer	5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′	56	
	3′LD Amplimer	5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3′	56	
5′-cRACE	SF4-FF1	5′-GCTAAGGATAACTCCAGTGGTGCAGAAGC-3′	68	
	SF4-FF2	5′-CAGTCACAGCCTTGCTGAACGGGTGAGA-3′	68	
	SF4-FF3	5′-GGCTGGAGTGCAGAATACGGTG -3′	68	
	SF4-FF4	5′-GATGCTCAGGATCACCGCCAAG -3′	68	
	SF4-FR2	5′-CCTCTTCCCATTAGGTGAAGCTCCTAACACCC-3′	68	
	SF4-FR3	5′-GCCAGGAACCAGTTCTTGCAGCAATCTCAT-3′	68	
	SF4-FR4	5′-CCGAAATTCCCACTTTGATCCTGTG -3′	68	
	SF4-FR6	5′-GACTCTGAACAGATTGACATTCCTGC-3′	68	
Northern blot and RT-PCR	SF4-FF2	5′-CAGTCACAGCCTTGCTGAACGGGTGAGA-3′	56	
	SebHLH-CR	5′-CACACAATTCTGTACTAAAGATTTGCACC-3′	56	
	SebHLH-ER	5′-AACGTCGACCAGCCCATTTTTGACAGCCCGC-3′	56	*Sal* I
	SeElon-F	5′-GTGAAGGAAGTGTCTTCATACC-3′	56	
	SeElon-R	5′-GACCGCCTGTCAATCTTGGTC-3′	56	
Subcellular localization	SebHLH-GFPS	5′-TCGTCTAGA*ATG*GGGGATAGAGTA-3′	56	*Xba* I
	SebHLH-GFPA	5′-TCGTCTAGATTGACAGCCCGCTTGG-3′	56	*Xba* I
In vitro expression	SebHLH-EF	5′-GCCGAATTCA*ATG*GGGGATAGAGTATTCCAGC-3′	55	*Eco*RI
	SebHLH-ER	5′-AACGTCGACCAGCCCATTTTTGACAGCCCGC -3′	55	*Sal* I
EMSA	SeMF4	5′-GGAATGTGCACACTCCATGTG-3′	60	
	SeMR4	5′-CCCCCCAACCCCCGCCC-3′	60	
	SeEbox-F	5′-GTGCACACTC*CATGTG*GGCCAATG-3′		
	SeEbox-R	5′-CATTGGCCCACATGGAGTGTGCAC-3′		
	SeGbox-F	5′-GCGGATGA*CACGTG*GCGGGCAACT-3′		
	SeGbox-R	5′-AGTTGCCCGCCACGTGTCATCCGC-3′		
	mSeEbox-F	5′-GTGCACACTC*aATGTc*GGCCAATG-3′		
	mSeEbox-R	5′-CATTGGCCGACATTGAGTGTGCAC-3′		
	mSeGbox-FA	5′-GCGGATGA*CtctaG*GCGGGCAACT-3′		
	mSeGbox-RA	5′-AGTTGCCCGCCTAGAGTCATCCGC-3′		
	mSeGbox-FB	5′-GCGGATGA*tACGTa*GCGGGCAACT-3′		
	mSeGbox-RB	5′-AGTTGCCCGCTACGTATCATCCGC-3′		
Transcriptional activation assay in yeast	SebHLH-NF1	5′-AGCGAATTC*ATG*GGGGATAGAGTATTCCAG-3′	60	*Eco*RI
	SebHLH-NR1	5′-AGCGTCGACTTCTTTAGAAGCTTCTGCACC-3′	60	*Sal* I
	SebHLH-CF1	5′AGCGAATTC*ATG*AATTACATTCATGTGAGAGCC-3′	60	*Eco*RI
	SebHLH-CR1	5′-AACGTCGACCAGCCCATTTTTGACAGCCCGC-3′	60	*Sal* I
Over-expression of in *Arabidopsis*	SebHLH-CF-Xb	5′-TCGTCTAGA*ATG*GGGGATAGAGTA-3′	60	*Xba* I
	SebHLH-CR-Bg	5′-TGAAGATCTCACACAATTCTGTACTAAAGA-3′	60	*Bgl* II

Table 1 continued

Reactions	Primer name	Sequence information	Annealing Tm for PCR (°C) [a]	Enzyme site
Transcriptional activation assay in plants	SiPF4-TF1	5′-ATGAATTCGGAATGTGCACACTCCATGTGGG-3′	64	*Eco*RI
	SiPF4-TR1	5′-ATAAGCTTCCCCCCAACCCCCGCCCCA-3′	64	*Hin*dIII
	EGbox-TF1	5′ATGAATTCCACACTC*CATGTG*GGCCAA TGAGCGGATGA*CACGTG*GCGGGCAAGCTTCT-3′		*Eco*RI/*Hin*dIII
	EGbox-TR1	5′AGAAGCTTGCCCGC*CACGTG*TCATCCG CTCATTGGCCC*CACATG*GAGTGTGGAATTCAT-3′		*Eco*RI/*Hin*dIII
	mEGbox-TF1	5′ATGAATTCCACACTC*AATGTC*GGCCAATG AGCGGATGA*AACGTT*GCGGGCAAGCTTCT-3′		*Eco*RI/*Hin*dIII
	mEGbox-TR1	5′AGAAGCTTGCCCGC*AACGTT*TCATCCGCT CATTGGCC*GACATT*GAGTGTGGAATTCAT-3′		*Eco*RI/*Hin*dIII

[a] Annealing temperature used for PCR

SeGbox-F, mSeEbox-F, mSeGbox-FA, and mSeGbox-FB) and their complementary oligonucleotides (SeEbox-R, SeGbox-R, mSeEbox-R, mSeGbox-RA, and mSeGbox-RB) were synthesized and annealed at room temperature (25 ± 2°C). DNA probes were end-labeled with γ-^{32}P [dATP] using T4 polynucleotide kinase and purified on a Sephadex G-50 column. Labeled probes were incubated for 30 min at 25°C with 5 μg of purified SebHLH:His protein in binding buffer (10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 1 mM Na_2EDTA, 5 mM DTT, and 5% glycerol) supplemented with 100 ng poly(dI–dC) in the presence or absence of non-radiolabeled competitors. The resulting DNA–protein complexes were electrophoresed on 5% polyacrylamide gels in 0.5× TBE buffer (44.5 mM Tris–HCl, 44.5 mM boric acid, 1 mM EDTA). Gels were dried on Whatman 3 MM paper and visualized by Fuji BAS-2500 phosphorimager.

β-galactosidase assay in yeast

To construct recombinant plasmids, pGBKT7-SebHLH, pGBKT7-SebHLHn, and pGBKT7-SebHLHc, the SebHLH cDNA was amplified using the following primer sets: the SebHLH-NF1 and SebHLH-CR1 primers for pGBKT7-SebHLH, the SebHLH-NF1 and SebHLH-NR1 primers for pGBKT7-SebHLHn, and the SebHLH-CF1 and SebHLH-CR1 primers for pGBKT7-SebHLHc. Each PCR fragment was digested with *Eco*RI and *Sal*I restriction enzymes and cloned into the same restriction sites of the pGBKT7 vector (BD Biosciences Clontech, USA). The resultant constructs were transformed into yeast strain Y190 (*MATα, HIS3, lacZ, trp1, leu2, cyh*^{r}*2*). Transformants were selected on selective medium (SD-Trp) supplemented with 30 mM 3-amino-1,2,4-aminotriazole [3-AT]. For β-galactosidase assays, transformants were cultured on selective medium (SD-Trp-His) for 1 day at 30°C, and filter-lift assays for blue color development were performed for 4 h at 37°C as described in Breeden and Nasmyth (1985).

Transactivation assay in developing sesame seeds and *Arabidopsis* transgenic plants

To develop the pPF4 construct, the region from –179 to –53 of the *SeFAD2* gene promoter was PCR-amplified using the SiPF4-TF1 and SiPF4-TR1 primers. The resulting 143-bp DNA fragments were eluted. For the pEGbox and the pmEGbox constructs, single-strand oligonucleotides (EGbox-TF1 and EGbox-TR1) and mutated single-strand oligonucleotides (mEGbox-TF1 and mEGbox-TR1) were synthesized and annealed to the complementary strand. These three DNA fragments were double-digested with *Eco*RI and *Hin*dIII and transcriptionally fused to 35S minimal promoter of pCAMBIA 1305.1 plasmid containing β-glucuronidase (GUS) reporter gene, followed by the 3′non-coding region of nopaline synthase gene (NOS). To construct the effector plasmid, the coding region of SebHLH cDNA was PCR-amplified using the SebHLH-CF-Xb and SebHLH-CR-Bg primers and inserted between the CaMV35S promoter and the NOS terminator.

For transient assays, 2 μg of reporter plasmid, alone or in combination with the effector plasmid, was introduced into developing sesame seeds (25–35 DAF) by particle bombardment. The tissues were bombarded twice at 1,350 psi with a biolistic helium gun device (Bio-Rad PDS-1000/He). The stopping screen was positioned 3 cm below the rupture disk, and the target tissue was positioned

3 cm below the stopping screen. After bombardment, samples were incubated for 20 h at 26°C in the dark. For β-glucuronidase (GUS) and luciferase (LUC) assays, 100 μg and 50 μg of total proteins were used to measure GUS and LUC activity, respectively. GUS activity was measured by fluorometric assays as described in Jefferson (1987). Luciferase assays were performed using the Luciferase Assay System Kit (Promega) according to the manufacturer's instructions. Protein concentrations were determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

For transactivation assays in transgenic *Arabidopsis* plants, each reporter plasmid contained the hygromycin resistant marker gene, and the effector plasmid contained the kanamycin resistant marker gene. These plasmids were transformed into *Agrobacterium* strain *GV3101* via the freezing-thaw method (An 1987). Using the floral dip method, *Arabidopsis* plants were then transformed with Agrobacterium transformed with each reporter plasmid alone or in combination with Agrobacterium transformed with the effector plasmid (Clough and Bent 1998). Seeds were harvested, sterilized in a mixture of 50% bleach and 0.02% Triton X-100 for 7 min with agitation, and then germinated on selective MS medium containing 50 mg/l kanamycin and/or 30 mg/l hygromycin. The antibiotic-resistant seedlings were transferred to soil, and the T_1 transgenic leaves were used to measure GUS activity.

Results

SebHLH, which interacted with the region from –179 to –53 of the SeFAD2 promoter, was isolated from developing sesame seeds

To isolate novel proteins that bind to *cis*-elements in the fragment from –179 to –53 of the *SeFAD2* promoter, yeast one-hybrid analysis was performed. The -179 to -53 fragment of the *SeFAD2* promoter was PCR-amplified using SiW6-YF4/SiW6-YR4 primers, inserted into the pHIS2 vector (SeYF4:HIS2), and used as bait. Amplified cDNAs from developing sesame seeds (10–30 DAF) were inserted into the pGADT7-Rec2 plasmid that contained the GAL4 activation domain and used as prey. We screened 3×10^6 transformants, and selected 400 colonies on medium lacking Leu, Trp, and His. Plasmids containing inserts larger than 500 bp were further analyzed by nucleotide sequencing. From the BLASTX and TRANSFAC analyses, one clone was identified that encoded a novel protein with the basic helix-loop-helix domain (bHLH) motif. A full-length cDNA clone was obtained by 5′-cRACE and named SebHLH. Nucleotide and deduced amino acid sequences of *SebHLH* cDNA are shown in Fig. 1A.

The *SebHLH* cDNA consists of an open reading frame encoding 400 amino acids. Amino acid sequence homology searches against the NCBI public database revealed that SebHLH cDNA had 60% sequence homology with *Arabidopsis* bHLH transcription factor (At1g10120), whose biological function was not identified. The amino acid sequence of the N-terminal region (residues 1–227) of SebHLH protein contains a transcription activation domain featuring specific patterns of hydrophobic and aromatic amino acids and acidic amino acid-rich sequences. The C-terminal region (residues 228–400) of the SebHLH protein contains the bHLH motif (residues 241–261) that is highly conserved in the eukaryote bHLH transcription factor family. The histidine (H5), glutamate (E9), and arginine (R12) amino acid residues are also conserved in the bHLH motif of the SebHLH protein (Fig. 1B). A putative nuclear localization signal sequence (KRKRK) identified by the PSORT program (http://www.psort.org) and the putative calcium-binding domain (residues 314–334) are shown in Fig. 1A.

SebHLH transcripts were expressed in sesame developing seeds and roots

To investigate the expression of *SebHLH* transcripts, total RNAs were isolated from sesame roots, stems, leaves, flowers, seed coats, seeds, and seedlings. Northern blot analysis was then performed using the C-terminal domain of the *SebHLH* cDNA as a probe. However, no signal was detected (data not shown), suggesting that the *SebHLH* transcript expression level is very low.

To determine the expression level of *SebHLH* transcripts, RT-PCR was carried out. The sesame elongation factor gene was used to determine the quantity and quality of cDNAs. Following 35 cycles of amplification, *SebHLH* transcripts were detected in developing seeds and roots, and *SebHLH* expression in stems, leaves, and flowers was much lower than in developing seeds and roots. No *SebHLH* expression was detected in seed coats and seedlings (Fig. 2).

The SebHLH:smGFP fusion protein was localized in the nucleus

To investigate the subcellular localization of SebHLH protein, full-length *SebHLH* cDNA without a translation termination codon was translationally fused with the C-terminus of the smGFP gene and transformed into onion epidermal cells by particle bombardment. After incubating in the dark for 24 h, the bombarded tissues were visualized under a confocal laser scanning microscope. As shown in Fig. 3, the SebHLH:smGFP fusion protein was localized to the nucleus of onion epidermal cells whereas smGFP was found in the cytoplasm. Therefore, this result suggests that SebHLH protein might act as a transcription factor in the nucleus.

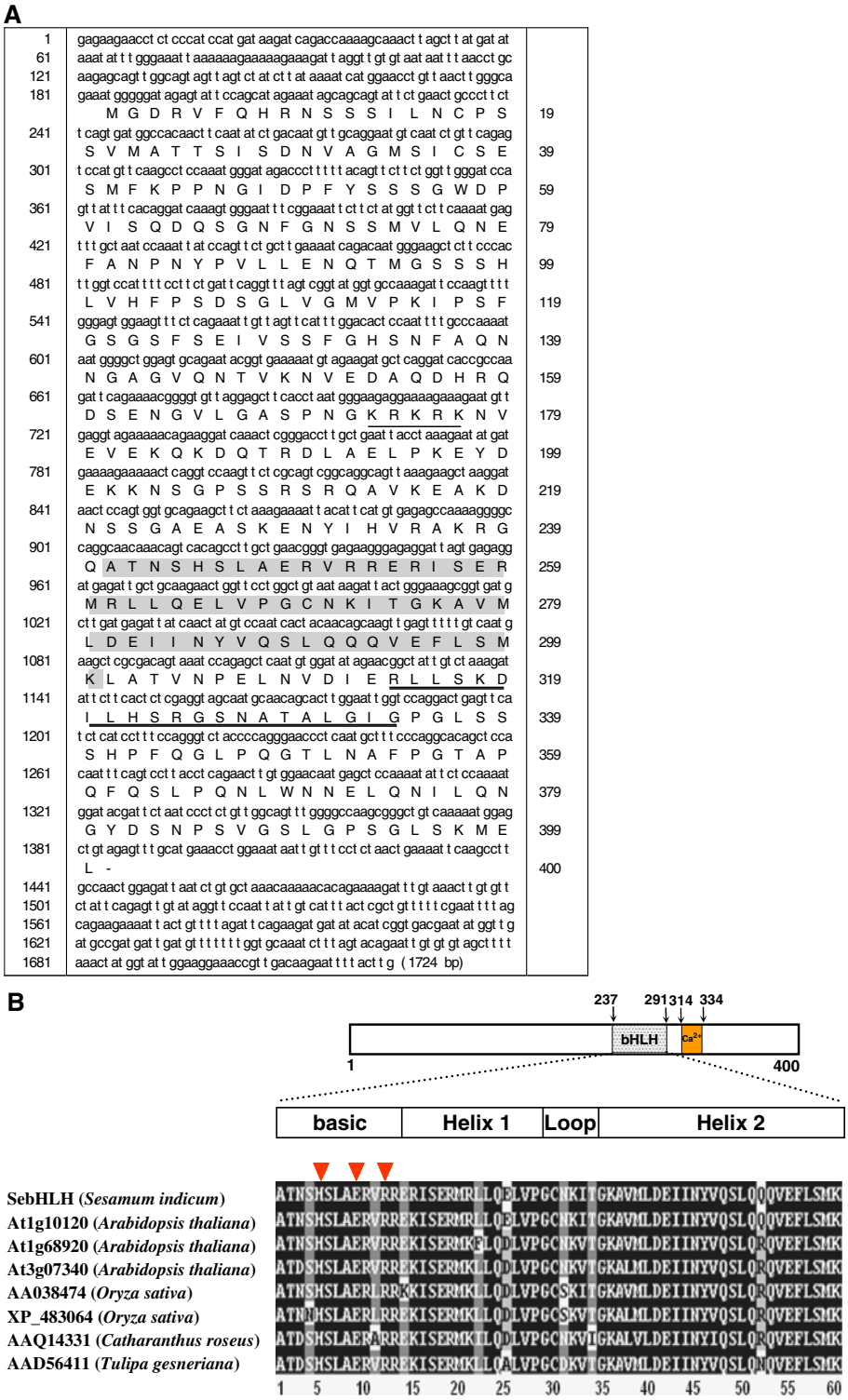

Fig. 1 Nucleotide and deduced amino acid sequences of the *SebHLH* cDNA (**A**) and alignment of deduced amino acid sequences of bHLH domains from sesame and other plants (**B**). A putative NLS and calcium binding sequences are single and double-underlined, respectively. The conserved 60 amino acid residues corresponding to the bHLH motif are shown in the shaded region. The conserved H5, E9 and R12 residues are shown in inverted red-triangles

Recombinant SebHLH protein binds to E- and G-box elements in the region from -179 to -53 of the SeFAD2 promoter

To investigate which *cis*-elements interacted with SebHLH protein, the coding region of *SeFAD2* cDNA was amplified by PCR and ligated into the pET-32b expression vector. His-tagged SebHLH proteins were purified from soluble *E. coli* extracts and incubated with the –179 to –53 fragment of the *SeFAD2* promoter (Fig. 4A), which had been end-labeled with γ-^{32}P[dATP]. Binding of recombinant SebHLH protein to the probe was determined by electrophoretic mobility shift assay (EMSA).

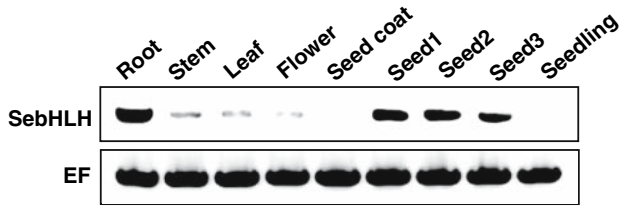

Fig. 2 Expression of *SebHLH* transcript. RT-PCR analysis of *SebHLH* transcript in various sesame tissues. Total RNAs were isolated from various sesame tissues, converted into cDNA, and amplified by RT-PCR using gene-specific primers. The sesame elongation factor gene was used as an internal control

As shown in Fig. 4B, one specific DNA–protein complex (indicated by arrow) was observed. A 250-fold or 500-fold molar excess of unlabeled probe eliminated the strongly shifted complex. Based on previous reports that bHLH transcription factors bind to E- or G-box elements, we determined whether E-box and/or G-box elements in this region of the *SeFAD2* promoter were involved in specific binding between recombinant SebHLH protein and the region from -179 to -53 of the *SeFAD2* promoter. Double-stranded DNA fragments containing the E-box motif (5′-GTGCACACTCCATGTGGGCCAATG-3′;

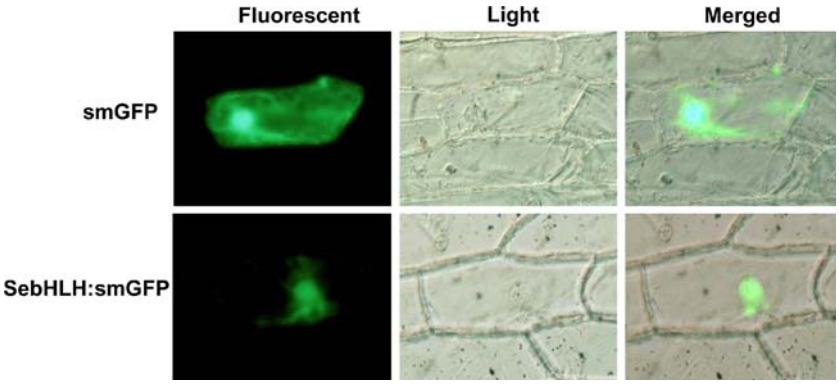

Fig. 3 Subcelluar localization of the *SebHLH* gene. The SebHLH:GFP fusion construct was introduced into onion epidermal cells by particle bombardment and visualized under a confocal laser scanning microscope (OLYMPUS BX51)

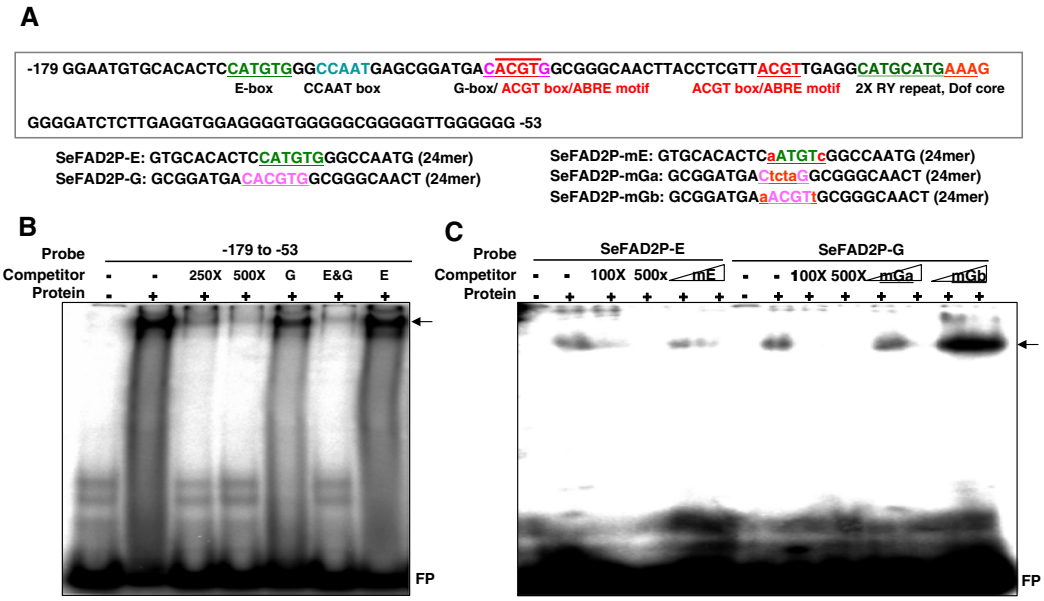

Fig. 4 EMSA of SebHLH protein. (**A**) Nucleotide sequences of the region from –179 to –53 in the *SeFAD2* promoter and DNA fragments containing E- or G-box motifs and mutated E- or G-box motifs. (**B**) Interaction between SebHLH protein and ^{32}P-labeled *SeFAD2* promoter (–179 to –53). (**C**) In vitro interaction between SebHLH protein and ^{32}P-labeled SeFAD2P-E and SeFAD2P-G DNA fragments. The SeFAD2P-mE, SeFAD2P-mGa, and SeFAD2P-mGb were generated by site-directed mutagenesis and used as competitors. 100× and 500× indicate 10 pmol and 50 pmol molar excess of double-stranded oligonucleotides, respectively. An arrow indicates protein-DNA complexes. FP: Free probe

24mer) or G-box motif (5′-GCGGATGACACGTGG CGGGCAACT-3′; 24mer) in this region of the *SeFAD2* promoter were generated, named SeFAD2P-E and SeFAD2P-G, respectively, and used as competitors. A 500-fold molar excess of each unlabeled competitor was not enough to eliminate the strongly shifted complex. However, the strong DNA/protein complex was completely eliminated when 500-fold molar excess of both SeFAD2P-E and SeFAD2P-G were used as competitors.

To further determine whether the E-box and G-box sequences are essential for specific binding between recombinant SebHLH protein and the region from –179 to –53 of the SeFAD2 promoter, one mutated E-box (SeFAD2P-mE, 5′-GTGCACACTCaATGTcGGCCAATG-3′) and two mutated G-boxes (SeFAD2P-mGa, 5′-GCGGATGACtctaGGCGGGCAACT-3′ and SeFAD2P-mGb, 5′-GCGGATGAaACGTtGCGGGCAACT-3′) were generated. In EMSA of recombinant SebHLH protein and end-labeled SeFAD2P-E fragments, a 500-fold molar excess of unlabeled SeFAD2P-E fragments completely eliminated the protein/DNA complex, but a 500-fold molar excess of unlabeled SeFAD2P-mE fragments did not eliminate the protein/DNA complex. Similar results were observed in EMSA of recombinant SebHLH protein and end-labeled SeFAD2P-G fragments when SeFAD2P-mGb fragments were used as a competitor. When a 500-fold molar excess of SeFAD2P-mGa fragments were used as a competitor, the DNA/protein complex was partially eliminated (Fig. 4C).

These observations revealed that SebHLH recombinant proteins bind to E-box (CANNTG) and G-box (CACGTG) elements in the –179 to –53 region of the *SeFAD2* gene promoter and that the external C and G sequences in these motifs are essential for SebHLH protein binding.

The N-terminal region of SebHLH protein contains transcriptional activation domain

To determine whether SebHLH protein acts as a transcriptional activator, and where its activation domain is if it acts as a transcriptional activator, the full-length (1–400 amino acids) protein and the N-terminal (1–229 amino acids) and C-terminal (230–400 amino acids) regions of the SebHLH protein were PCR-amplified and translationally fused with the GAL4 DNA-binding domain in the pGBKT7 plasmid.

Three resultant plasmids, pGBKT7-SebHLH, pGBKT7-SebHLHn, and pGBKT7-SebHLHc, were introduced into yeast Y190 strain harboring the GAL1 promoter and *HIS3* and *lacZ* reporter genes. Transformants were then selected on SD (-His/-Trp) medium supplemented with 30 mM 3-AT. Transformants with pGBKT7-SebHLH or pGBKT7-SebHLHn grew faster than those with pGBKT7-SebHLHc on selection medium. Filter-lift assays revealed β-galactosidase activity in transformants with pGBKT7-SebHLH and pGBKT7-SebHLHn, but not in transformants with pGBKT7-SebHLHc and pGBKT7 (Fig. 5). This result revealed that SebHLH protein functions as a transcriptional activator and that the N-terminal region of SebHLH protein contains the transcriptional activation domain.

SebHLH protein activates transcription from E- and G-box elements of the SeFAD2 gene promoter

The functional relevance of the interaction observed in vitro between SebHLH protein and E- and G-box elements of the *SeFAD2* gene promoter were determined by transient expression assays in co-bombarded developing sesame seeds and by transgenic approaches with effector and reporter constructs. Figure 6A depicts the scheme of reporter and effector constructs used in transactivation

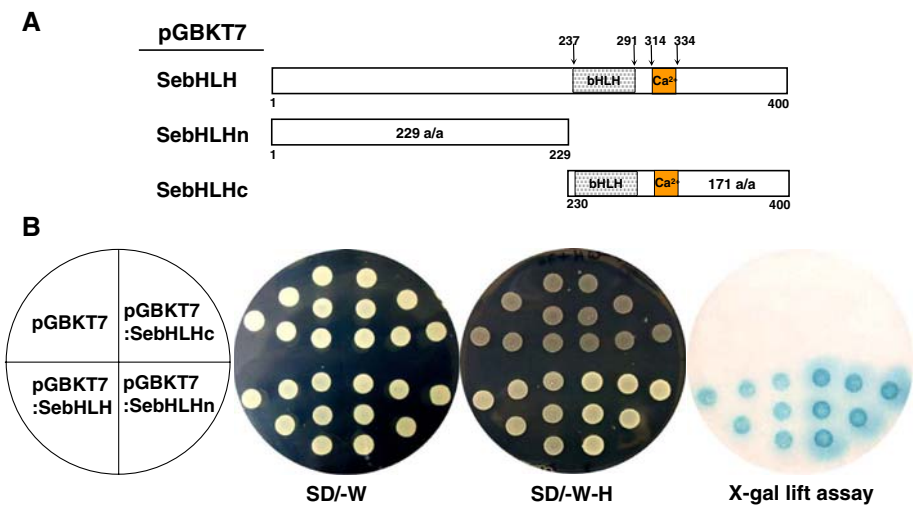

Fig. 5 Transcriptional activity assay of SebHLH protein in yeast. (**A**) Schematic diagram of full-length and deletion constructs of SebHLH protein in pGBKT7 vector. (**B**) Growth of yeast cells in medium SD/-W (left) and SD/-W-H (middle) containing 30 mM 3-AT. The X-gal lift was carried out using SD/-W plate (right)

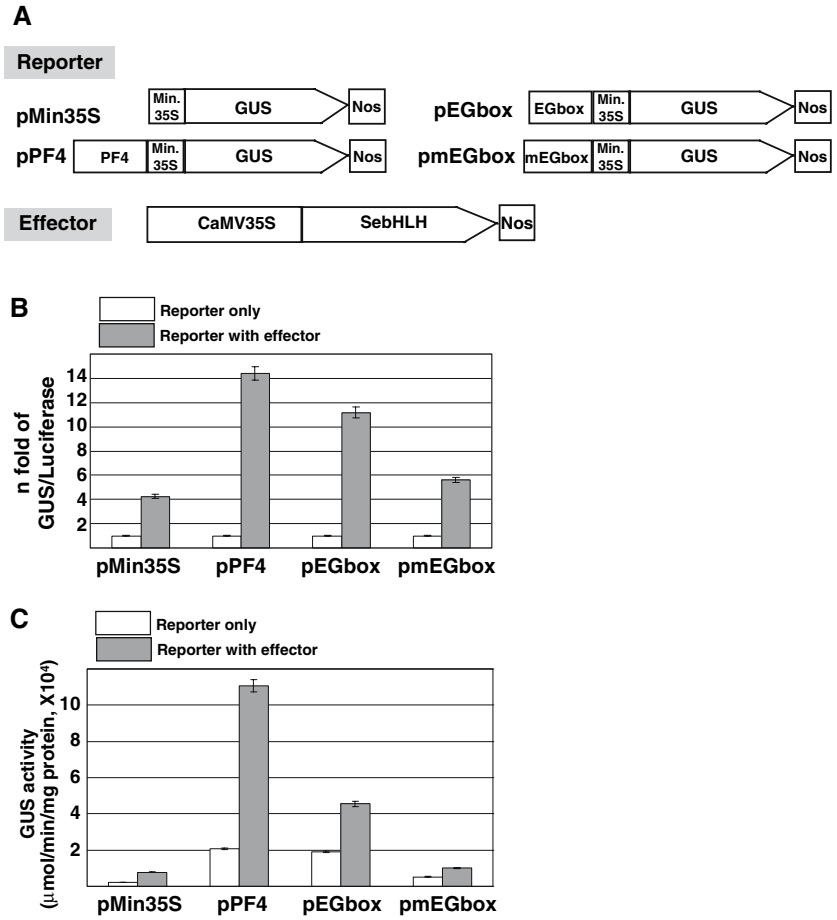

Fig. 6 Transactivation assay in developing sesame seeds and transgenic *Arabidopsis* plants. (**A**) Scheme of reporter and effector constructs. The pMin35S contains the GUS reporter gene between the minimal CaMV35S promoter and the NOS terminator in the pCAMBIA 1305.1 vector. The pPF4, pEGbox, and pmEGbox were constructed by insertion of the region from –179 to –53, DNA fragments containing the E- and G-box motifs, and DNA fragments containing mutated E- and G-box motifs upstream of the minimal CaMV35S promoter in the pMin35S. Effector construct contains the *SebHLH* cDNA between the CaMV35S promoter and the NOS terminator in pMBP1 vector. (**B**) Transactivation assay in developing sesame seeds. Developing sesame seeds (25–35 DAF) were bombarded with each reporter construct alone or in combination with the effector construct and incubated. GUS activity was measured by fluorometric assays as described by Jefferson et al. (1987). Luciferase expression was used to normalize GUS activity. (**C**) Transactivation assay in transgenic *Arabidopsis* plants. *Arabidopsis* was transformed with each reporter construct alone or in combination with the effector construct, and T_1 transgenic leaves were used to measure GUS activity

studies of the *SeFAD2* promoter. The region from -179 to -53 of the *SeFAD2* promoter was PCR-amplified, and 41-bp DNA fragments (EG-box) containing E- and G-box elements, as well as 41-bp DNA fragments (mEG-box) containing mutated E- and G-box elements, were generated by annealing the synthesized single-strand oligonucleotides. The DNA fragments were transcriptionally fused to the 35S minimal promoter of pCAMBIA 1305.1 plasmid containing the β-glucuronidase (GUS) reporter gene, followed by the 3′ non-coding region of nopaline synthase gene (NOS). The resultant plasmids were named pPF4, pEGbox, and pmEGbox. An effector plasmid containing the SebHLH cDNA between the CaMV35S promoter and the NOS terminator was also constructed. Developing sesame seeds (25–35 DAF) were transiently transformed by particle bombardment with the pPF4, pEGbox, or pmEGbox reporter, alone or in combination with the effector construct. A construct with the luciferase gene between the CaMV35S promoter and the NOS terminator was transiently cotransformed to monitor transformation efficiency.

As shown in Fig. 6B, co-transfection of pPF4 and effector or pEGbox and effector resulted in 11- to 12-fold increase in GUS activity over that directed by pPF4 or pEGbox alone. In addition, mutation of the E- and G-boxes decreased trans-activation by the effector approximately 2-fold. We further confirmed the interaction between SebHLH protein and E- and G-box elements of the *SeFAD2* promoter in planta. *Arabidopsis* plants were transformed with each reporter construct alone or in combination with the effector construct. T_1 transgenic leaves of each

transformant were used to measure GUS activity. When pPF4 reporter and effector were co-expressed, a 5-fold increase in GUS activity level was observed compared to the activity measured when the pPF4 reporter was expressed without an effector. Similarly, co-expression of pEGbox and effector resulted in more than 2-fold increase in GUS activity over that directed by pEGbox alone. The effector did not transactivate the reporter construct pmEGbox with mutations in the E- and G-box-binding site. Taken together, these results indicate that SebHLH protein mediates trans-activation of the *SeFAD2* gene promoter through binding to E- and G-box elements.

Discussion

Seed-specific FAD2 functions in the committed step of polyunsaturated fatty acid synthesis during triacylglycerol accumulation. *SeFAD2* mRNA levels are quite low in early embryogenesis, achieve maximum levels in seeds during mid- and late embryogenesis, and fall to low levels as seeds mature (Kim et al. 2006). In this study, we investigated the transcriptional control mechanism for expression of the *SeFAD2* gene during seed development. We determined that the SebHLH trans-acting factor activates transcriptional activity from E- and G- boxes in the *SeFAD2* promoter. This result strongly implicates the bHLH protein as an important trans-acting factor in combinatorial regulation of genes that are involved in the biosynthesis and accumulation of storage lipids in developing seeds.

X-ray crystallography analyses of Max, E47, MyoD, Pho4, and USF bHLH transcription factors have shown that the interaction between two identical or similar monomers, each with two helical segments separated by a loop, leads to the formation of a four helix bundle of homo- or heterodimers. The invariant glutamate (Glu) in the basic region of each partner binds to conserved CA bases in each palindromic half of the CAN/NTG (E-box) motif. The arginine (Arg) confers specificity for CACGTG versus CAGCTG E-boxes by directly contacting the central G of the G-box. Histidine (His) has asymmetrical contact and also interacts with the G residue complementary to the first C in the G-box (Ellenberger et al. 1994; Ferre-D Amare et al. 1994; Ma et al. 1994; Shimizu et al. 1997; Fuji et al. 2000). SebHLH protein has the conserved His (H5), Glu (E9), and Arg (R12) residues that constitute the G-box (CACGTG) recognition motif in its basic region (Fig. 1). As shown in Fig. 4, SebHLH protein bound E-box (CATGTG) and G-box (CACGTG) elements in the region from –179 to –53 in the *SeFAD2* gene promoter. The external C and G sequences in the E- and G-box motifs were essential for the interaction between SebHLH protein and E- and G-box motifs, but mutation of the internal ACGT core sequence of the G-box motif did not influence their interaction. This result suggests that the external C and G nucleotides in the palindromic halves (CAN/NTG) of the E- and G-box motifs are the most critical sequences for SebHLH protein binding to E- and G-box motifs. Moreover, the flanking nucleotides outside of the hexanucleotide core have been shown to play a role in binding specificity (Littlewood and Evan 1998; Atchley et al. 1999; Massari and Murre 2000), and there is evidence that a loop residue in the protein functions in DNA binding through elements that lie outside of the core recognition sequence (Nair and Burley 2000). The flanking nucleotide sequences of the E- and G-box motifs in the region from –179 to –53 in the *SeFAD2* gene promoter that are involved in the interaction with SebHLH protein remain to be investigated.

The N-terminal region (amino acids 1 to 229) of the SebHLH protein, which shares moderate sequence conservation with other plant bHLH proteins, contains a putative nuclear localization signal (NLS), the KRKRK sequence, at amino acids 163–167 (Fig. 1). Subcellular localization assays revealed that SebHLH protein was targeted to the nucleus, suggesting that the KRKRK sequence probably functions in nuclear targeting (Fig. 3). Typically, the NLS is the sequence Pro-Lys-Lys-Lys-Arg-Lys (PKKKRK) (Jans 1995). The N-terminal domain of the R gene product Lc, which is involved in the control of anthocyanin synthesis in maize, was identified as the sequence DRRAAPARPA from residues 100 to 109 (Ludwig et al. 1989). However, this NLS is not conserved among other maize B, *Antirrhinum* DEL, and bean PG1 bHLH transcription factors (Radicella et al. 1991; Goodrich et al. 1992; Kawagoe and Murai 1996).

Transcriptional activators are composed of distinct domains including separable DNA-binding and activation domains. The activation domains have been classified into acidic-type activation domains, glutamine-rich domains, and proline-rich domains, according to the type of amino acid enrichment in this domain (Triezenberg 1995). In acidic-type activation domains, specific patterns of hydrophobic and aromatic amino acids were reported to be equally, or even more, critical than the acidic amino acids (Regier et al. 1993; Drysdale et al. 1995). Similar hydrophobic and aromatic amino acid patterns and acidic amino acid-rich sequences were scattered in the N-terminal region of SebHLH protein (Fig. 1). The N-terminal region (amino acids 1–229) of SebHLH protein functioned as a transcription activation domain in yeast (Fig. 5). When SebHLH protein under the CaMV35S promoter was co-expressed with GUS reporter gene driven by E- and G-box motifs in developing sesame seeds and transgenic *Arabidopsis* leaves, GUS activity increased approximately 11-fold and 2-fold, respectively (Fig. 6B,

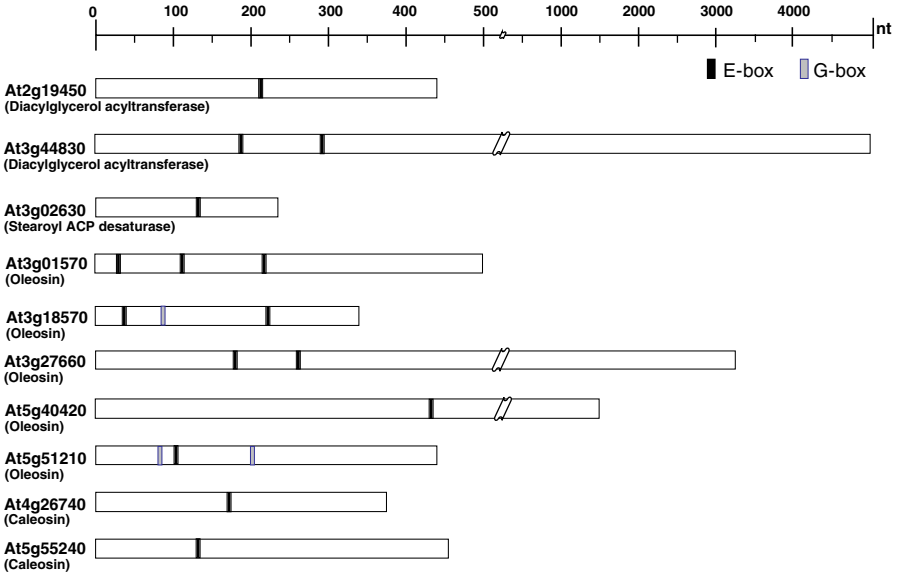

Fig. 7 Occurrence of E- or G-box elements in the 5′-flanking region of some genes that are involved in triacylglycerol biosynthesis and show seed-specific expression in *Arabidopsis*

C). The acidic-type activation domain was observed in the N-termini of several bHLH transcription factors, including *Arabidopsis* MYC (AtMYC2), MYB (AtMYB2), and ICE1 proteins (Urao et al. 1993; Abe et al. 1997; Chinnusamy et al. 2003). The AtMYC2 and AtMYB2 transcription factors specifically bound the MYC and MYB recognition sites, respectively, in the 67-bp regions of the *rd22* and *AtADH1* genes and these sites function as *cis*-acting elements in drought and ABA-induced expression of these genes. Both AtMYC2 and AtMYB2 activated the transcription of the GUS gene fused to the 67-bp region of the *rd22* promoter in *Arabidopsis* leaf protoplasts (Abe et al. 1997, 2003). ICE1 bound specifically to MYC recognition sequences (CANNTG) in the *CBF3* promoter and activated the transcription of *CBF* genes in the cold (Chinnusamy et al. 2003).

In *Arabidopsis*, the AtbHLH protein family consists of at least 147 members. Based on the amino acid sequence of the bHLH domain in the AtbHLH proteins, 109 and 11 proteins are predicted to be E-box binders and non-E-box binders, respectively. Eighty-nine of 109 E-box binders are categorized as G-box binders (Toledo-Oritz et al. 2003). Based on the conservation of amino acid sequence in the bHLH domain and of DNA-binding capacity, SebHLH protein is considered a typical G-box binder. The *SebHLH* gene was preferentially expressed in non-photosynthetic tissues, seeds and roots (Fig. 2). According to previous reports, the E- and G-box elements in the *SeFAD2*, *Cs-ACP1*, and *Cs-4PAD* promoters were essential *cis*-elements for seed-specific expression (Kim et al. 2005, 2006). *Arabidopsis* fatty acid elongase gene (*FAE1*), which catalyzes very-long-chain fatty acid biosynthesis, was specifically expressed in embryonic tissues. Two E-box elements are found at –105 to –100 (CACATG) and at –358 to –353 (CACATG) in the FAE1 promoter (Rossak et al. 2001). Interestingly, the E- and G-box elements are concentrated in the region from –250 to –50 in the promoters of acyl-CoA-diacylglycerol acyltransferase (At2g19450), phosphatidylcholine:diacylglycerol acyltransferase (At3g44830), several oil-body oleosins (At3g01570, At3g18570, At3g27660, At5g40420, and At5g51210) and two caleosins (At4g26740 and At5g55240) genes, which are involved in triacylglycerol synthesis (http://www.plantbiology.msu.edu/lipid/genesurvey/EST_data.htm; Beisson et al. 2003; http://www.arabidopsis.org) (Fig. 7). Based on the *Arabidopsis* microarray analysis database, GENEVESTIGATOR (https://www.genevestigator.ethz.ch/), these genes were specifically expressed in developing *Arabidopsis* seeds. In conclusion, SebHLH transcription factor binds to E- or G-box elements in the *SeFAD2* gene promoter and contains domains for DNA binding and transcriptional activation. Through their interaction with E- and G-box *cis*-elements, the bHLH transcription factors, including SebHLH protein, probably play a key role in transcriptional regulation of genes involved in storage lipid biosynthesis and accumulation during seed development.

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References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9:1859–1868

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

An G (1987) Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol 153:292–305

Atchley WR, Terhalle W, Dress A (1999) Positional dependence, cliques, and predictive motifs in the bHLH protein domain. J Mol Evol 48:501–516

Bao X, Katz S, Pollard M, Ohlrogge JB (2002) Carboxylic fatty acids in plants: biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of *Sterculia foetida*. Proc Natl Acad Sci USA 99:7172–7177

Beisson F, Koo AJ, Ruuska S, Schwender J, Pollard M, Thelen JJ, Paddock T, Salas JJ, Savage L, Milcamps A, Mhaske VB, Cho Y, Ohlrogge JB (2003) Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol 132:681–697

Bradford MM (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Breeden L, Nasmyth K (1985) Regulation of the yeast HO gene. Cold Spring Harb Symp Quant Biol 50:643–650

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. Proc Natl Acad Sci USA 89:11184–11188

Cahoon EB, Ripp KG, Hall SE, McGonigle B (2002) Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. Plant Physiol 128:615–624

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Crowe AJ, Abenes M, Plant A, Moloney MM (2000) The seed-specific transactivator, ABI3, induces oleosin gene expression. Plant Sci 151:171–181

Davis SJ, Vierstra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol 36:521–528

Drysdale CM, Dueñas E, Jackson BM, Reusser U, Braus GH, Hinnebusch AG (1995) The transcriptional activator GCN4 contains multiple activation domains that are critically dependent on hydrophobic amino acids. Mol Cell Biol 15:1220–1233

Ellenberger T, Fass D, Arnaud M, Harrison SC (1994) Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop helix dimer. Genes Dev 8:970–980

Ferre-D'Amare AR, Pognonec P, Roeder RG, Burley SK (1994) Structure and function of the b/HLH/Z domain of USF. EMBO J 13:180–189

Fuji Y, Shimizu T, Toda T, Yaganida M, Hakoshima T (2000) Structural basis for the diversity of DNA recognition by bZip transcription factors. Nat Struct Biol 7:889–893

Goodrich J, Carpenter R, Coen ES (1992) A common gene regulates pigmentation pattern in diverse plant species. Cell 68:955–964

Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. Plant Physiol 110:311–319

Jans DA (1995) The regulation of protein transport to the nucleus by phosphorylation. Biochem J 311:705–716

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jin UH, Lee JW, Chung YS, Lee JH, Yi YB, Kim YK, Hyung NI, Pyee JH, Chung CH (2001) Characterization and temporal expression of a ω-6 fatty acid desaturase cDNA from sesame (*Sesamum indicum* L.) seeds. Plant Sci 161:935–941

Kawagoe Y, Murai N (1996) A novel basic region/helix-loop-helix protein binds to a G-box motif CACGTG of the bean storage protein b-*phaseolin* gene. Plant Sci 116:47–57

Kim MJ, Shin JS, Kim JK, Suh MC (2005) Genomic structures and characterization of the 5′-flanking regions of acyl carrier protein and Δ^4-palmitoyl-ACP desaturase genes from *Coriandrum sativum*. Biochim Biophys Acta 1730:235–244

Kim MJ, Kim HJ, Shin JS, Chung CH, Ohlrogge JB, Suh MC (2006) 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. Mol Genet Genomics 276:351–368

Kinney AJ (1994) Genetic modification of the storage lipids of plants. Curr Opin Biotechnol 5:144–151

Kinney AJ (1996) Development of genetically engineered soybeans for food applications. J Food Lipids 3:273–292

Kinney AJ, Cahoon EB, Hitz WD (2002) Manipulating desaturase activities in transgenic crop plants. Biochem Soc Trans 30:1099–1103

Klein TM, Fromm M, Weissinger A, Tomas D, Schaaf S, Sletten K, Sanford JC (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc Natl Acad Sci USA 85:4305–4309

Ledent V, Vervoort M (2001) The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis. Genome Res 11:754–770

Li X, Duan X, Jiang H, Sun Y, Tang Y, Yuan Z, Guo J, Liang W, Chen L, Yin J, Ma H, Wang J, Zhang D (2006) Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and *Arabidopsis*. Plant Physiol 141:1167–1184

Littlewood TD, Evan GI (1998) Basic helix-loop-helix transcription factors. Oxford University Press, Oxford

Liu Q, Singh S, Green A (2000) Genetic modification of cotton seed oil using inverted-repeat gene-silencing techniques. Biochem Soc Trans 28:927–929

Ludwig SR, Habera LF, Dellaporta SL, Wessler SR (1989) Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc homology region. Proc Natl Acad Sci USA 86:7092–7096

Ma PCM, Rould MA, Weintraub H, Pabo CO (1994) Crystal structure MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell 77:451–450

Maruyama I, Rakow TL, Maruyama HI (1995) cRACE: a simple method for identification of the 5′-end of mRNAs. Nucleic Acids Res 23:3796–3797

Massari ME, Murre C (2000) Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol Cell Biol 20:429–440

Moon H, Smith MA, Kunst L (2001) A condensing enzyme from the seeds of *Lesquerella fendleri* that specifically elongates hydroxy fatty acids. Plant Physiol 127:1635–1643

Murre C, McCaw PS, Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56:777–783

Nair SK, Burley SK (2000) Functional genomics: recognizing DNA in the library. Nature 404:715–718

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J (1994) *Arabidopsis* FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6:147–158

Pirtle IL, Kongcharoensuntorn W, Nampaisansuk M, Knesek JD, Chapman KD, Pirtle RM (2001) Molecular cloning and functional expression of the gene for a cotton Δ-12 fatty acid desaturase (FAD2). Biochim Biophys Acta 1522:122–129

Radicella JP, Turks D, Chandler VL (1991) Cloning and nucleotide sequence of cDNA encoding B-Peru, a regulatory protein of the anthocyanin pathway in maize. Plant Mol Biol 17:127–130

Regier JL, Shen F, Triezenberg SJ (1993) Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. Proc Natl Acad Sci USA 90:883–887

Rossak M, Smith M, Kunst L (2001) Expression of the FAE1 gene and FAE1 promoter activity in developing seeds of *Arabidopsis thaliana*. Plant Mol Biol 46:717–725

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol 49:611–641

Shimizu T, Toumoto A, Ihara K, Shimizu M, Kyogoku Y, Ogawa N, Oshima Y, Hakoshima T (1997) Crystal structure of PHO4 bHLH domain-DNA complex: flanking base recognition. EMBO J 16:4689–4697

Slocombe SP, Cummins I, Paul Jarvis R, Murphy DJ (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol Biol 20:151–155

Somerville C, Browse J, Jaworski JG, Ohlrogge J (2000) Lipids. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry & molecular biology of plants. American Society of Plant Physiologists, Rockville, pp 456–527

Suh MC, Schultz DJ, Ohlrogge JB (1999) Isoforms of acyl carrier protein involved in seed-specific fatty acid synthesis. Plant J 17:679–688

Suh MC, Kim MJ, Hur C-G, Bae JM, Park YI, Kang C-W, Ohlrogge JB (2003) Comparative analysis of expressed sequence tags from *Sesamum indicum* and *Arabidopsis thaliana* developing seeds. Plant Mol Biol 52:1107–1123

Tang GQ, Novitzky WP, Carol Griffin H, Huber SC, Dewey RE (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. Plant J 44:433–446

Toledo-Ortiz G, Huq E, Quail PH (2003) The Arabidopsis basic/helix-loop-helix transcription factor family. Plant Cell 15:1749–1770

Triezenberg SJ (1995) Structure and function of transcriptional activation domains. Curr Opin Genetics Dev 5:190–196

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539