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Molecular characterization of two high-palmitic-acid mutant loci induced by X-ray irradiation in soybean

Toyoaki Anai*, Tomoki Hoshino, Naoko Imai and Yutaka Takagi

Laboratory of Plant Genetics and Breeding, Faculty of Agriculture, Saga University, Honjyo-machi 1, Saga 840-8502, Japan

Palmitic acid is the most abundant (approx. 11% of total fatty acids) saturated fatty acid in conventional soybean seed oil. Increasing the saturated acid content of soybean oil improves its oxidative stability and plasticity. We have developed three soybean mutants with high palmitic acid content by X-ray irradiation. In this study, we successfully identified the mutated sites of two of these high-palmitic-acid mutants, J10 and M22. PCR-based mutant analysis revealed that J10 has a 206,203-bp-long deletion that includes the *GmKASIIA* gene and 16 other predicted genes, and M22 has a 26-bp-long deletion in the sixth intron of *GmKASIIB*. The small deletion in M22 causes mis-splicing of *GmKASIIB* transcripts, which should result in nonfunctional products. In addition, we designed co-dominant marker sets for these mutant alleles and confirmed the association of genotypes and palmitic acid contents in F_2 seeds of J10 X M22. This information will be useful in breeding programs to develop novel soybean cultivars with improved palmitic acid content. However, in the third mutant, KK7, we found no polymorphism in either *GmKASIIA* or *GmKASIIB*, which suggests that several unknown genes in addition to *GmKASIIA* and *GmKASIIB* may be involved in elevating the palmitic acid content of soybean seed oil.

Key Words: Gm*KASIIA*, Gm*KASIIB*, palmitic acid, *Glycine max*, X-ray irradiation.

Introduction

Soybean oil stability and quality is determined primarily by the relative proportions of saturated versus unsaturated fatty acids (Liu and White 1992, Shen *et al.* 1997). Soybean oil containing high concentrations of polyunsaturated fatty acids have low oxidative stability. In addition, soybean oil with high concentrations of saturated fatty acids can potentially be used to make *trans*-fatty acid-free shortening and margarine (Kok *et al.* 1999). Palmitic acid is the most abundant saturated fatty acid in soybean seed oil, accounting for about 11% of total fatty acids in conventional cultivars (Stoltzfus *et al.* 2000). Many soybean mutants with a high palmitic acid content have been developed by using *N*-methyl-*N*-nitrosourea, ethyl methane sulfonate, or X-irradiation as the mutagen (Erickson *et al.* 1988, Fehr *et al.* 1991, Narvel *et al.* 2000, Rahman *et al.* 1996, Schnebly *et al.* 1994, Stoltzfus *et al.* 2000, Takagi *et al.* 1995). Major alleles in at least five loci have been reported to cause an increase in palmitic acid content (Palmer *et al.* 2004), and the palmitic acid content has been elevated to above 40% of total fatty acids in lines with combinations of these high-palmitic-acid mutant alleles (Stoltzfus *et al.* 2000).

Palmitoyl-acyl carrier protein (16:0-ACP) is synthesized via a fatty acid condensation reaction by *β*-ketoacyl-acyl-carrier protein synthases (βKAS), and then palmitic acid is released from 16:0-ACP by a specific acyl-ACP thioesterase, FatB in plastid. The βKAS family consists of three distinct members, βKASI, βKASII and βKASIII. βKASIII mediates the condensation of acetyl-CoA with malonyl-ACP to form 4:0-ACP, βKASI is responsible for the elongation of 4:0-ACP to 16:0-ACP and βKASII mediates the elongation of 16:0-ACP to 18:0-ACP (Ohlrogge and Browse 1995). In contrast, the structure of mitochondorial βKAS (mtKAS) is closely related to βKASII-type, but the substrate specificity of it is wider (Yasuno *et al.* 2004). The most promising gene for producing high-palmitic-acid mutants encodes βKASII. However, the gene alleles corresponding to the developed high-palmitic-acid soybean mutants have not been characterized at the molecular level except in the Century-derived mutant line C1727. The *GmKASIIA* gene of C1727 contains a single base pair substitution that converts a tryptophan codon into a premature stop codon (Aghoram *et al.* 2006).

Previously, we isolated three high-palmitic-acid soybean mutants: J10 (Takagi *et al.* 1995), KK7 (Rahman *et al.* 1996) and M22 (unpublished data), with palmitic acid contents of 18.0%, 15.0% and 16.3%, respectively (Table 1). Reciprocal crosses between J10, KK7 and C1727 have shown that the mutant allele in J10 corresponds to the *fap2* locus in C1727, but that the locus of the mutant allele in KK7 is different from *fap2* (Rahman *et al.* 1999). However, both J10, KK7 and M22, have not yet been characterized molecularly. Moreover, M22 was also isolated by Takagi *et al.* (1995) as

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*Corresponding author (e-mail: anai@cc.saga-u.ac.jp)

Table 1. Fatty acid compositions of three high-palmitic-acid mutants and the original cultivar

Lines	Fatty acid (%, ±SD)				
	Palmitate	Stearate	Oleate	Linoleate	α-Linolenate
J10	18.0 ± 0.3	1.3 ± 0.1	22.4 ± 1.2	50.0 ± 0.7	8.4 ± 0.2
M22	16.3 ± 0.1	0.9 ± 0.2	26.4 ± 0.5	49.4 ± 0.5	7.0 ± 0.1
KK7	15.0 ± 0.1	1.2 ± 0.2	21.9 ± 1.2	54.2 ± 0.7	7.7 ± 0.1
Bay	11.3 ± 0.4	1.2 ± 0.2	31.2 ± 1.4	49.7 ± 1.1	6.6 ± 0.4

Means ± SDs were obtained from three independent experiments.

a high-palmitic-acid mutant from X-ray (200 Gy) irradiated M3 population of Bay using gas chromatography-based screening in 1998 (unpublished data). The characterization of these high-palmitic-acid mutants would be valuable for development of a molecular marker for use in marker-assisted selection for soybean seed oil with improved oxidative stability.

In this study, we analyzed the nucleotide sequences of *GmKASIIA* (Phytozome accession no. Glyma17g05200) and *GmKASIIB* (Phytozome accession no. Glyma13g17290) to determine the mutation sites of J10, KK7 and M22. In J10, we identified a large deletion including the *GmKASIIA* gene and in M22 we identified a small deletion in the sixth intron of the *GmKASIIB* gene. However, we found no sequence polymorphism in the *GmKASIIA* or *GmKASIIB* coding regions between KK7 and the original cultivar Bay.

Materials and Methods

Plant materials

Soybean [*Glycine max* (L.) Merr.] original cultivar Bay and Bay-derived high-palmitic-acid mutants J10, M22 and KK7 were grown in a field at Saga University, Japan, under natural light. Green leaves and developing seeds of these plants were collected and stored at −80°C until DNA and RNA extraction. Dry seeds were also collected for analysis of their fatty acid composition.

Fatty acid analysis

Fatty acid analysis was performed as reported previously (Anai *et al.* 2008). Soybean seed flour (approx. 100 mg) was resuspended in 0.5 M sulfuric acid in methanol containing 2% (v/v) dimethoxypropane and incubated for 1 h at 80°C. Fatty acid methyl esters were extracted with *n*-hexane and separated under isothermal conditions at 180°C in a Yanaco G6800 series gas chromatograph equipped with a 25-m × 0.25-mm Quadrex 23 bonded fused silica capillary column and a flame ionization detector. Each peak was identified by comparison with the retention time of standard fatty acid methyl esters. The experiment was repeated three times.

Southern blot analysis

Genomic DNA was extracted from green leaves by the CTAB extraction method (Murray and Thompson 1980). Genomic DNA (3 μg) was digested with *Hin*dIII and electrophoresed in 1% agarose gel. The separated DNA fragments were transferred to a nylon membrane and detected with DIG-labeled cRNA of a full-length *GmKASIIA* as a probe. Hybridization and detection were carried out as described previously (Anai *et al.* 2005). Hybridizations were carried out under high-stringency conditions using a standard hybridization buffer with 25% formamide at 65°C according to the manufacturer's instructions (DIG Luminescent Detection Kit, Roch). The membranes were washed three times with 2XSSC and 0.2% SDS at room temperature for 10 min and twice with 0.1XSSC and 0.2% SDS at 65°C for 30 min. The antibody treatment and chemiluminescent reaction with CDP-Star (NEW ENGLAND Bio Labs) were carried out according to the manufacturer's instructions.

Determination of mutation sites by PCR

Genomic DNA was extracted from green leaves by the CTAB extraction method (Murray and Thompson 1980). To determine the mutation sites of J10, M22 and KK7, we performed PCR on extracts from J10, M22 and KK7 by using primers that amplified the coding regions of both *GmKASIIA* and *GmKASIIB* (Table 2). To investigate the extent of the deletion in J10, we generated primer sets both upstream (5′ end) and downstream (3′ end) of the *GmKASIIA* gene. The primer sets were designed by using published soybean genome sequences (Glyma1 at Phytozome v6.0; http://www.phytozome.net/soybean.php) (Table 2). All PCR procedures were performed in an iCycler Thermal Cycler (Bio-Rad). Each PCR amplification was performed in a total volume of 10 μl, containing 25 ng DNA, 40 mM tricine-KOH (pH 9.2), 15 mM KOAc (pH 6.0), 3.5 mM $Mg(OAc)_2$, 3.75 μg/ml BSA, 0.005% Tween-20, 0.005% NP-40, 200 μM of each dNTP, 0.5 μM of each primer and 0.05 units/μl *Pfu* DNA polymerase. The PCR conditions were 95°C for 5 min; then 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 to 90 s (depending on the size of the amplicon); final extension at 72°C for 5 min and storage at 4°C. The PCR products were separated by 1.0% agarose gel electrophoresis.

Long PCR and nucleotide sequencing

Long PCR was performed with a TaKaRa LA Taq kit (Takara) according to the manufacturer's instruction, using the forward primer −148 and reverse primer +57 (Table 2). PCR was performed in a total volume of 20 μl, containing 50 ng DNA, 2 μL of 10× LA PCR buffer II (Takara),

Table 2. Nucleotide sequence and amplicon size of each primer used in this study

Primers	Forward (5′-3′)	Reverse (5′-3′)	Amplicon size (bp)
For determination of deleted region in J10			
KASIIA-ORF	TTGCGAAGGGTCATATTAGTGCATC	TTAAGAACAAGCCTAACAGGCTGAC	2109
KASIIB-ORF	AATTTGCTTGATGGTGTTAGTGGCATAAGT	GGGTTGATGAACTAATGAACCTTCAG	1376
For examination of J10 deletion upstream (5′ end) of GmKASIIA)			
−84	CAAACGCTACGAGCTAGAGAGGTTG	CCACTTAGGCTTTGTGTTGTCTCCTT	507
−127	TGGACCGATTCAACGACCAAGTCGAAC	TTACACGCGTCTTGCCGCATGCTCAA	400
−142	GGAATGCTGCGACAAAACTCAACAC	GTGTACAACTGGCATGTTTGCGAAGA	792
−148	TACAGTGCATCGAAATGAGCAACAC	AAGATCTGTTTCTCCGCATTGGACT	651
−154	AATGGGACTTTGTGTTTCCAATCCC	GTGTCAGAAAATCTCTTCTCCAACTC	187
−169	CCTTATCACGTGCGTATCCGACACT	ACTTGCCCAATCCAACTCTCAACAC	992
For examination of J10 deletion downstream (3′ end) of GmKASIIA)			
+12	ATTAACAAGTCCAACTATGACTCTT	TATATGAATGCTTTATCATTGCCAAC	749
+43	TTGCTAATTTTCGTCACCATCGCAA	AGCCAACTTAACACATCATGCATCT	720
+53	AATGCGAAGAACACCATAAAGTCCA	ACGATTCTGGAATACACCACTCTCT	502
+57	TAGGCAAGCTATCTAAATCTCGTCA	TATCACTTATGGATTCCGTACCTCT	794
For identification of mis-spliced GmKASIIB *transcripts*			
KASIIB-RT	TCTCGCAAGTCTAATTCAACGTC	GTTTTGACCAAAACAATGCATTAGAGCTT	906
For identification of mis-spliced GmKASIIB *transcripts*			
JM (J10 marker)	CTTTGGAATGCAGTACGTGTCGGAT	CTCAGCGCAAATGTCATTACGGAGA	207,301 (Bay) 1099 (J10)
MM (M22 marker)	TTACTCATTCTCTGTGGCTTGTGTG	GGGTTGATGAACTAATGAACCTTCAG	254 (Bay) 228 (M22)

400 μM of each dNTP, 0.5 μM of each primer and 1 unit of LA *Taq* DNA polymerase (Takara). The PCR conditions were 94°C for 1 min; 30 cycles of 98°C for 10 s and 68°C for 15 min; final extension at 72°C for 10 min and storage at 4°C. The long PCR and RT-PCR (see next section) fragments were cloned into the pGEM vector by using a TA cloning kit (Promega) according to the manufacturer's instructions. Nucleotide sequences were determined with a Big Dye Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems) and automatic sequencer (Model 3100, Applied Biosystems).

RNA extraction and RT-PCR analysis

To identify the mis-spliced products of M22 caused by the internal deletion in an intron, we performed RT-PCR analysis. Total RNA was isolated from leaves and developing seeds of Bay and M22 by using RNAiso Plus (Takara) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg total RNA by using a PrimeScript II 1st strand cDNA synthesis kit (Takara) according to the manufacturer's instructions, and then PCR was performed with the same primer set (KASIIB-ORF) and conditions as when genomic DNA was used for the template.

Genotyping for the presence of the J10 and M22 mutant alleles

Genomic DNA was prepared from dry seed powder using the DNeasy Plant Mini Kit (QIAGEN). To genotype plants for the presence of the J10 mutation, we designed a primer set (JM) flanking the deleted region in the J10 mutant and used it along with the −142 primer set (Table 2) to perform multiplex PCR. The multiplex PCR procedure was performed as described above for standard PCR, except that 0.4 μM of each primer was used. Then the PCR product was separated by 1.0% agarose gel for the J10 genotyping. To genotype plants for the presence of the M22 mutation, we designed a primer set (MM) flanking the region deleted in the M22 mutant (Table 2). PCR was performed as described above for standard PCR. Then the PCR product was separated by 3.0% agarose gel electrophoresis for the M22 genotyping.

Phylogenic analysis

The deduced amino acid sequences were obtained from the Phytozome database. GmKASIIA, GmKASIIB, AtKASI, AtKASII, AtKASIII and AtmtKAS correspond to Glyma17g05200, Glyma13g17290, AT5G46290, AT1G74960, AT1G62640 and AT2G04540, respectively. The phylogenic tree was generated by using the CLUSTALW program (Thompson *et al.* 1994) and the phylogenetic tree diagram was drawn by TreeView X program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

Characterization of the high-palmitic-acid mutant locus in J10

To evaluate the variations of the *GmKASIIA* genomic sequences, we firstly performed a Southern blot analysis with a *GmKASIIA* probe (Fig. 1A) and detected four hybridized

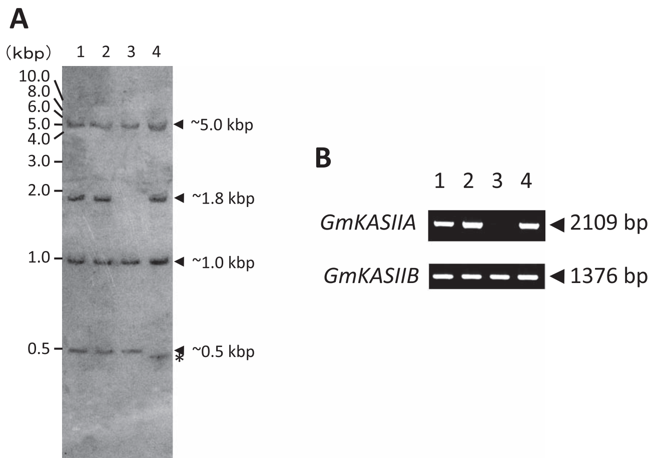

Fig. 1. Southern blot and PCR analysis of *GmKASIIA* and *GmKASIIB* genes in the normal soybean cultivar Bay and three high-palmitic-acid mutants. (A) *Hin*d III-digested DNA fragments (lane 1, Bay; lane 2, KK7; lane 3, J10; lane 4, M22) were hybridized with a *GmKASIIA* cRNA probe. (B) *GmKASIIA* and *GmKASIIB* DNA fragments were amplified with gene-specific primer sets (lane 1, Bay; lane 2, KK7; lane 3, J10; lane 4, M22).

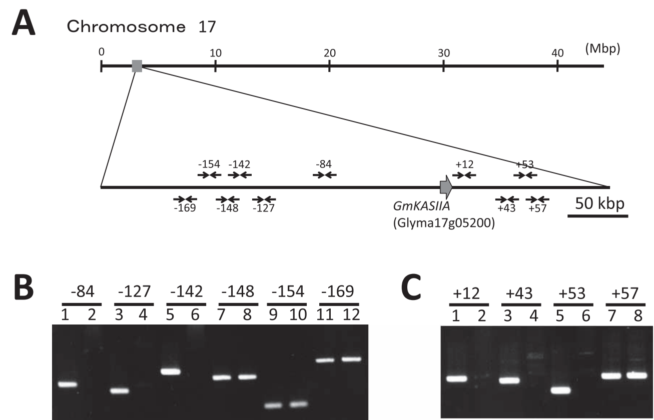

Fig. 2. PCR-based analysis of the deleted region in the J10 mutant. (A) Location of the *GmKASIIA* gene and primer sets on soybean chromosome 17. The large arrow indicates the position of *GmKASIIA*, and the small arrows indicate the primer positions. Amplification result for J10 (even-numbered lanes) and Bay (odd-numbered lanes) with (B) 5′-upstream primer sets and (C) 3′-downstream primer sets.

bands (approx. 5.0, 1.8, 1.0 and 0.5-kbp in length) in the original cultivar Bay Fig. 1A, lane 1), whereas the 1.8-kbp-long band was absent in J10 (Fig. 1A, lane 3). It is difficult to distinguish between *GmKASIIA* and *GmKASIIB* in the Southern blot analysis results because of the high similarity of their nucleotide sequences, but using their genomic sequence data, we predicted that the 1.8-kbp-long band corresponded to *GmKASIIA* and the other three bands to *GmKASIIB*.

To confirm the deletion in the *GmKASIIA* gene in J10, we performed PCR with both *GmKASIIA*- and *GmKASIIB*-specific primer sets (Table 2: KASIIA-ORF and KASIIB-ORF). We detected no band when we amplified the coding region of *GmKASIIA* in J10, whereas we detected a band when we amplified the coding region of *GmKASIIB* (Fig. 1B, lane 3). This result clearly indicated that a deletion occurred in the coding region of *GmKASIIA* in J10 and that the 1.8-kbp-long band (Fig. 1A) corresponded to a *GmKASIIA*-derived fragment. To investigate the size of the deletion in J10, we performed PCR using primer sets generated both upstream and downstream of the *GmKASIIA* gene (Table 2 and Fig. 2A) based on published soybean genome sequences (Schmutz *et al.* 2010). When we used primer sets that amplified regions from up to about 142 kbp from the 5′ end of the *GmKASIIA* gene in the original cultivar Bay, we detected no amplified product in J10 (−142 in Fig. 2B). In contrast, we successfully obtained a PCR product with a primer set that amplified the region about 148-kbp from the 5′ end of *GmKASIIA* in J10 (−148 in Fig. 2B). Similarly, we detected no amplified product with a primer set that amplified the region from 53-kbp from the 3′ end of *GmKASIIA* in J10 (+53 in Fig. 2C), whereas we detected a PCR product when we amplified the region about 57-kbp from the 3′ end (+57 in Fig. 2C). These results indicate that a nucleotide sequence at least 200-kbp long including *GmKASIIA* is deleted in J10.

To examine the deletion of J10 in detail, we performed long PCR. When we used the forward primer from set −148 and the reverse primer from set +57 (Table 2 and Fig. 2A) in a long PCR with J10 DNA as the template, an approximately 8-kbp-long PCR product was amplified (data not shown). From the nucleotide sequence of this 8-kbp-long PCR product, we determined that a region corresponding to 206,203-bp in the original cultivar Bay had been deleted, and this deleted region in J10 contained 17 annotated genes in the Phytozome database (Glyma17g05030–Glyma17g05250), including *GmKASIIA* (Table 3).

Characterization of the high-palmitic-acid mutant locus in M22

In the Southern blot of M22, a hybridized band slightly shifted to the lower molecular mass side could be observed (Fig. 1A, lane 4, indicated by an asterisk). Using the genomic sequence data, we predicted that this band was derived from *GmKASIIB*. However, we could not detect any difference between M22 and Bay in size in the both PCR products of *GmKASIIA* and *GmKASIIB* (Fig. 1B, lane 4) when the gene-specific primer sets KASIIA-ORF and KASIIB-ORF (Table 2) were used for the amplification.

To clarify the M22 mutation in detail, we compared the nucleotide sequences of *GmKASIIA* and *GmKASIIB* derived from M22 with that of the original cultivar Bay. We identified a 26-nucleotide deletion at positions 2741 to 2766 in *GmKASIIB*, but observed no change in *GmKASIIA* (data not shown). This result was consistent with the band shift observed in the Southern blot results for M22 (Fig. 1A). In addition, since the 26-nucleotide deletion in M22 occurred in the sixth intron of *GmKASIIB*, this mutation could be

Table 3. Comparison of predicted genes located in the deleted region of chromosome 17 in the J10 mutant between soybean and *Arabidopsis*. Gene accession numbers of soybean and *Arabidopsis* are those in the Phytozome (http://www.phytozome.net/) and TAIR (http://www.arabidopsis.org/) databases, respectively

	Paralogs in *Glycine max*		Ortholog in *Arabidopsis*	Predicted gene product/putative function
	Chromosome 17	Chromosome 13		
1	Glyma17g05030	Glyma13g17450	At4g33250	EIF3K (translation initiation factor)
2	Glyma17g05040	Glyma13g17440	At3g43210	Kinecin-like protein (microtubule motor)
3	Glyma17g05080	Glyma13g17410	At2g03140	CAAX amino terminal protease family protein
4	Glyma17g05100	Glyma13g17390	BAB09012	Pectin methylesterase-like protein
5	Glyma17g05120	Glyma13g17380	At3g43120	Auxin-responsive protein-related
6	Glyma17g05130	Glyma13g17370	At4g21090	Adrenodoxin-like ferredoxin 1
7	Glyma17g05140	Glyma13g17360	At1g19240	Unknown protein
8	Glyma17g05150	Glyma13g17350	At3g26060	PRXQ (antioxidant/peroxiredoxine)
9	Glyma17g05160	Glyma13g17340	At1g19250	FMO1 (flavin-dependent monoxygenase 1)
10	Glyma17g05170	Glyma13g17330	At3g43110	Unknown protein
11	Glyma17g05180	–	At5g20740	Invertase / pectin methylesterase inhibitor family protein
12	Glyma17g05190	Glyma13g17300	At2g34350	Noduline-like protein
13	Glyma17g05200	Glyma13g17290		KASII
14	Glyma17g05220	Glyma13g17270	At1g19220	ARF19 (auxin response factor 19)
15	Glyma17g05230	Glyma13g17260	–	GroES chaperonin
16	Glyma17g05240	Glyma13g17250	At5g20720	CPN20 (chaperonin 20: calmodulin binding)
17	Glyma17g05250	Glyma13g17240	At5g20710	BGAL7 (β-galactosidase 7)

expected to cause mis-splicing of its transcript.

To evaluate the mis-spliced transcripts of *GmKASIIB* caused by the partial deletion of intronic segments in M22, we performed RT-PCR analysis and compared the products between M22 and Bay. By nucleotide sequencing of cloned RT-PCR products, we obtained a normally spliced transcript and two different mis-spliced transcript sequences in both leaves and developing seeds tissues of M22. We estimated from the frequency of each sequence that the amount of abnormal transcripts were approximately 80% of total *GmKASIIB* transcripts in both leaves and developing seeds of M22 (data not shown). However, any abnormal transcripts of *GmKASIIB* could not be observed in Bay. One of these mis-spliced transcripts lost the nucleotide sequence at a position of 770- to 823-base from the initiation codon of mRNA (Fig. 3), and results in a 18-amino acids shorter polypeptide than normal one. The 59-base-long mutated intron sequence was inserted in between the sixth- and seventh-exons of another mis-spliced transcript (Fig. 3). This abnormal transcript encodes a frame-shifted polypeptide with unusual amino acid sequence. Carlsson *et al.* (2002) have reported that the *fab1* mutant of *Arabidopsis* carrying a single amino acid substitution (Leu337Phe) is partially deficient in activity of βKASII enzyme activity. They also indicated that the Leu337Phe is quite important to keep the correct structure of βKASII enzyme. Since the equivalent amino acid residue in *fab1* mutant is Leu285 of GmKASIIB that is located in the seventh exon, just after the mis-spliced site in M22, the mutant products would be judged to be mostly inactive. This result strongly suggests that the high-palmitic-acid trait of M22 was caused by the reduction of the normal *GmKASIIB* transcript level.

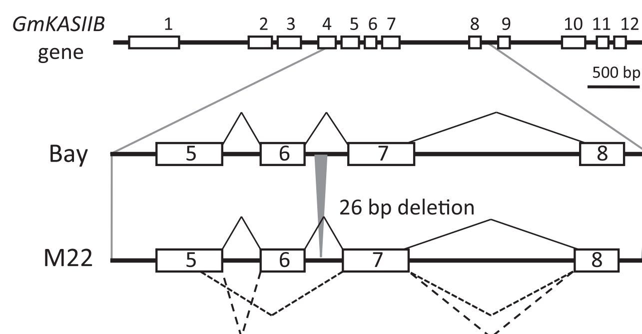

Fig. 3. Gene structures and splicing products from a normal Bay and a mutant M22 *GmKASIIB* gene. The 26-bp-long deleted region is indicated by the gray triangle. The normally spliced transcript is shown by solid lines and two different mis-spliced transcripts are shown by short- and long-dashed lines.

Nucleotide sequence of GmKASIIA and GmKASIIB in KK7

In contrast to the case of J10 and M22, we have detected no difference between KK7 and Bay in the Southern blot analysis (Fig. 1A, lanes 1, 2). Also, there was no difference between KK7 and Bay in size in the both PCR products of *GmKASIIA* and *GmKASIIB* (Fig. 1B, lanes 1, 2). Furthermore, we could not find any difference between KK7 and Bay in the nucleotide sequence in the ORFs of *GmKASIIA* and *GmKASIIB* (data not shown). This result may suggest that an unknown gene beside *GmKASIIA* and *GmKASIIB* controls the high-palmitic-acid trait of KK7.

Development of molecular markers for the high-palmitic-acid mutant loci in J10 and M22

To make it possible to utilize the high-palmitic-acid mutant alleles of J10 and M22 for improving soybean seed oil

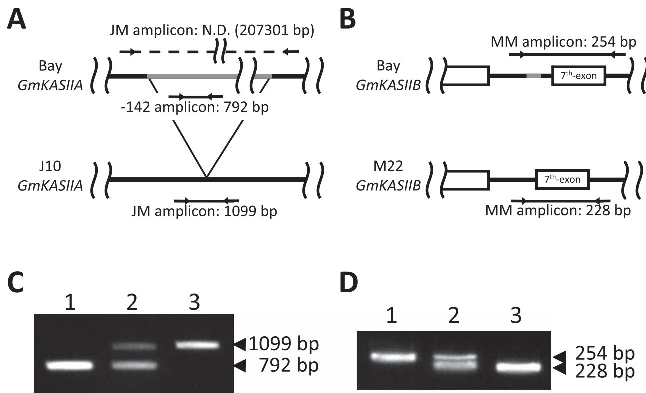

Fig. 4. Development of PCR-based markers for the J10 and M22 mutations. (A) Locations of two primer sets for detecting the mutation in J10. N.D. means not detected. (B) Location of a primer set for detecting the mutation in M22. (C) Detection of the J10 mutation: Bay (lane 1), heterozygous (lane 2) and J10 (lane 3) DNA templates. (D) Detection of the M22 mutation: Bay (lane 1), heterozygous (lane 2) and M22 (lane 3) DNA templates.

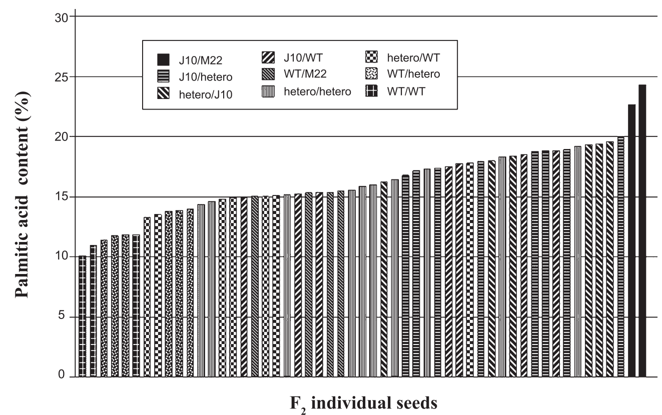

Fig. 5. Comparison of palmitic acid content and marker segregation on F_2 seed individuals obtained from the cross between J10 and M22. The palmitic acid contents are arranged in the order of increasing palmitic acid content. The patterns of individual bars indicate nine genotypes as illustrated in the figure.

quality, we designed a PCR-based molecular marker to distinguish each allele. To detect the J10 mutation, we designed a new primer set, JM (Table 2 and Fig. 4A), corresponding to the two flanking regions of the deleted nucleotide sequence in J10. When we performed multiplex PCR with the JM and −142 primer sets, a 792-bp-long PCR product was amplified when Bay genomic DNA was used, whereas a 1099-bp-long product was amplified with J10 genomic DNA as the template (Fig. 4A). In addition, to detect the M22 mutation, we designed a new primer set, MM (Table 2 and Fig. 4B), corresponding to the two flanking regions of the deleted nucleotide sequence in M22. When we performed PCR with the MM primer set, a 254-bp-long product was amplified from Bay genomic DNA and a 228-bp-long product from M22 genomic DNA (Fig. 4B). Furthermore, we confirmed that both of these primer sets functioned as co-dominant markers (Fig. 4C, 4D).

Segregation analysis of genotypes and high-palmitic-acid trait in F_2 seeds of J10 X M22

To evaluate the efficacy of the newly developed markers, we tested these markers on 53 F_2 seed individuals obtained from the cross between J10 and M22 (Fig. 5). We ground each seed into a fine powder and then divided it for DNA extraction and fatty acid analysis. The genotypes of this F_2 population were segregated into 9 types, and the range of palmitic acid contents in the same population showed continuous values ranging from 10.2% to 24.3% (Fig. 5). The genotypes of this F_2 population were clearly segregated into nine genotypes and the segregation ratio of these genotypes satisfactorily fitted a 1:2:2:1:4:1:2:2:1 ratio ($\chi^2 = 6.84$, $p > 0.55$). In contrast, the range of palmitic acid contents in the same population showed continuous values ranging from 10.2% to 24.3%, but the phenotype of each seed was closely correlated with its genotype. Moreover, the palmitic acid contents of two double homozygous mutant lines (J10/M22 in Fig. 5) were 22.7% and 24.3%, respectively (Fig. 6). It suggests that the molecular markers for two mutant alleles (*GmKASIIA* of J10 and *GmKASIIB* of M22) are well linked with the high-palmitic-acid trait.

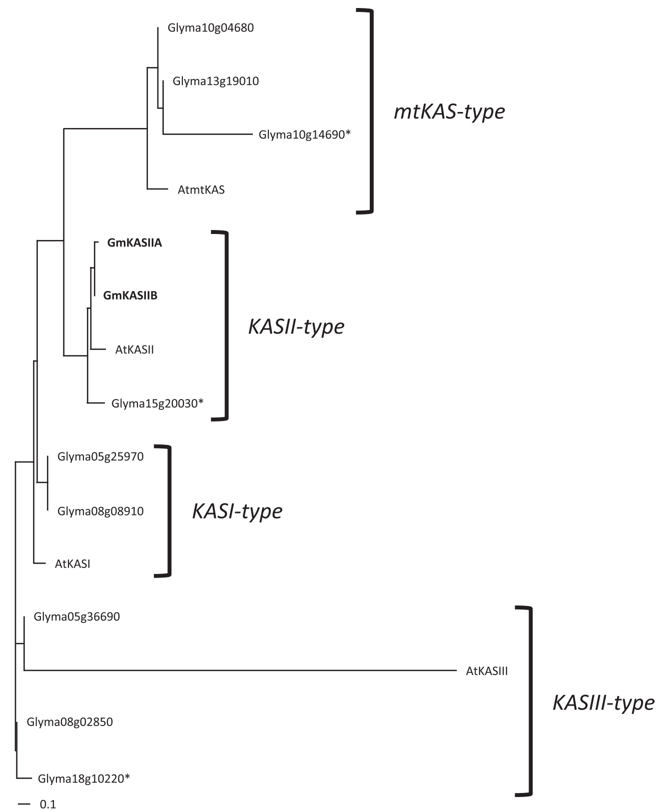

Fig. 6. Phylogenic tree of βKAS family proteins in soybean. The AtKASI, AtKASII, AtKASIII and AtmtKAS were used as outgroups. The reliability of each branch was tested by bootstrap analysis with 1,000 replications. The asterisks indicated only partial amino acid sequences were registered in Glyma1 at Phytozome v.6.0.

Discussion

We identified the mutations corresponding to two of three high-palmitic-acid mutant lines obtained by X-ray irradiation of a soybean population. One line, J10, has a large (206,203 bp) deletion that includes the *GmKASIIA* gene. There are a total of 17 annotated genes in this region (Table 3), but we did not observe notable growth defects under field conditions, presumably because other, redundant genes partly complement the functions of the deleted genes. In fact, 16 of the 17 genes (all except Glyma17g05180) were present in the corresponding region around *GmKASIIB* on chromosome 13 (Table 3). Glyma17g05180, which is likely to encode an invertase/pectin methylesterase inhibitor protein, is not present on chromosome 13, but members of this gene family are redundantly distributed on other chromosomes of soybean. Aghoram *et al.* (2006) have also identified a base substitution in *GmKASIIA* in C1727, obtained from an ethyl methane sulfonate-treated population. In M22, we identified a small (26-bp) deletion in the sixth intron of *GmKASIIB*. RT-PCR analysis showed that this deletion sequence resulted in two different mis-spliced transcripts, one in which a part of the fifth exon and the sixth exon of *GmKASIIB* are missing, and the other in which the sixth intron is not spliced out of the gene transcript (Fig. 3). This result suggests that a small deletion in an intronic segment can occasionally produce a null or knockdown mutation. Carlsson *et al.* (2002) have reported that in *Arabidopsis* the high-palmitic-acid *fab1* mutant has a mutated *βKASII* gene causing a Leu337Phe substitution. In this study, we identified two mis-splicing transcripts of the mutated *GmKASIIB* gene in M22, and these mutated sites were located in near the substituted amino acid residue of *Arabidopsis fab1* mutant. Since these mis-splicing transcripts are changed more drastically than the transcript in the *Arabidopsis fab1* mutant, their products cannot have any KASII activities in M22. These nonfunctional transcripts are more abundant than normal transcripts in M22, the level of normal transcript in M22 was estimated to be decreased to about 20% or less of that in Bay.

On the other hand, we consider it to be quite reasonable that we obtained both J10 and M22, both of which show deletion-type mutations, in an X-ray irradiated population. The trigger that generated these deletions was the induction of double-strand breaks (DSBs) by ionizing radiation. Naito *et al.* (2005) suggested that large deletions are generated by nonhomologous end joining (NHEJ) between nonadjacent DNA fragments resulting from two DSBs, whereas small deletions are contrastingly generated from one DSB by NHEJ.

We have not detected any change at nucleotide sequence of the ORF in either *GmKASIIA* or *GmKASIIB* in KK7. Palmer *et al.* (2004) reported that at least five loci are involved in elevating palmitic acid content. We tried to identify the other high-palmitic-acid gene candidates from soybean genome sequence data based on their structural homology, but it was quite difficult to determine them. Although genetic studies (Stoltzfus *et al.* 2000) indicate that more than three unidentified high-palmitic-acid genes should be present in the soybean genome, in this study only one other gene (Glyma15g20030) was categorized into the same clade as *GmKASIIA* and *GmKASIIB* (Fig. 6). Since the amino acid sequence of Glyma15g20030 obtained from Glyma1 database was partial and there was no information about the expression yet, it may be necessary to take into consideration this gene as a candidate gene which is involved in the high-palmitic traits. This discrepancy may suggest that the high-palmitic-acid genes encode not only βKASII but also or enzymes belonging to other phylogenic clades or other enzymes (e.g., acyl-ACP thioesterase). Therefore, further investigations into other possible mechanisms of the high-palmitic-acid trait are needed in the future.

Here we developed PCR markers to enable genotyping of two different high-palmitic-acid mutant alleles of *GmKASIIA* and *GmKASIIB*. Furthermore, the integration of these two mutant alleles induced the significant elevation of palmitic-acid level (above 20%) than that of each mutant allele. Aghoram *et al.* (2006) previously developed a cleaved amplified polymorphic sequence marker for a high-palmitic-acid allele of *GmKASIIA* in C1727. Despite the importance of soybean oil with high palmitic acid, no cultivars for a high-palmitic-acid genotype are yet commercially available (Fehr 2007). Therefore, our high-palmitic-acid mutants and their molecular markers will be quite helpful in the development of novel soybean cultivars with improved palmitic acid content through marker-assisted selection.

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