

A PCR-based method to test for the presence or absence of β -conglycinin α' - and α -subunits in soybean

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Abstract The soybean cultivar Yumeminori, which lacks the α' - and α -subunits of β -conglycinin, carries both naturally occurring and induced mutations. While the cause of the natural mutation resulting in the α' -subunit deficiency has been determined, the induced mutation in the *CG-2* gene encoding the α -subunit has not been characterized at the molecular level. In this study, we identified a four base pair insertion in the first exon of *CG-2*, which introduced a premature stop codon. The insertion co-segregated with the lack of α -subunit, indicating that this mutation is the cause of the α -subunit deficiency. A multiplex PCR method of testing for the presence or absence of α' - and α -subunits was developed based on the sequences of mutated and wild-type alleles. This PCR-based test was also capable of detecting the presence of wild-type genes when Yumeminori DNA samples were contaminated with wild-type DNA at levels of 0.2% or greater. Thus, this method will be useful both for marker-assisted selection in soybean breeding programs, and for seed purity tests in food industries.

Keywords Allergen · *Glycine max* · Polymerase chain reaction · Seed purity test

Introduction

β -Conglycinin (7S globulin) and glycinin (11S globulin) are the major components of storage protein in soybean (*Glycine max* [L.] Merr.). β -Conglycinin is low in sulfur-containing amino acids, and thus exhibits poorer nutritional and food processing properties than glycinin (Koshiyama 1968; Saio et al. 1969; Fukushima 1991; Kohyama and Nishinari 1993). Humans are unable to synthesize essential amino acids, including the sulfur-containing amino acid methionine. Tests with animals indicate that soybean protein does not provide adequate methionine to meet human dietary requirements (Young 1991). Furthermore, the α -subunit of β -conglycinin is a major allergenic protein of soybean that is recognized by sera from approximately 25% of soybean-sensitive patients with atopic dermatitis (Ogawa et al. 1995). Since a decrease in the level of β -conglycinin leads to an increased level of glycinin (Ogawa et al. 1989), there is a great deal of interest in the development of soybean lines with reduced amounts of β -conglycinin (Krishnan 2005).

β -Conglycinin consists of three subunits, α' , α and β (Thanh and Shibasaki 1976, 1978; Higgins 1984). In a survey of soybean germplasm collections, Kitamura and Kaizuma (1981) identified two mutants, Keburi and Mo-shi-dou Gong 503, with reduced

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β -conglycinin levels. Keburi lacked the α' -subunit, while Mo-shi-dou Gong 503 had lower levels of the α - and β -subunits. These two cultivars were crossed to produce Kari-kei 434, a line lacking the α' -subunit and having lower levels of the α -subunit. The glycinin/ β -conglycinin ratios of Keburi, Mo-shi-dou Gong 503 and Kari-kei 434 were 1.61, 2.59, and 4.43, respectively, while ratios in normal cultivars range from 0.95 to 1.46 (Kitamura and Kaizuma 1981; Takahashi et al. 2004). Later, a mutant completely lacking both the α' - and α -subunits was selected from the progeny of a Kari-kei 434 plant treated by γ -ray irradiation (Takahashi et al. 1994). The line generated from this mutant is known as Yumeminori, but has also been referred to as T line or Tohoku 124 (Takahashi et al. 1996; Yoshino et al. 2002). The pedigree of Yumeminori is summarized in Fig. 1.

Yumeminori is unique in its high nutritional value and low allergenic risk (Takahashi et al. 1996, 2004; Ogawa et al. 2000). Protein from Yumeminori has an extraordinarily high glycinin/ β -conglycinin ratio of 8.41, and has 30% more sulfur-containing amino acids than protein from normal cultivars. Furthermore, a major soybean allergen, Gly m Bd 30K, could be almost completely removed from the soymilk of Yumeminori without the addition of any reducing agents (Samoto et al. 1996, 1997). To maintain these superior traits, Yumeminori seed must be handled in a manner that keeps it separate from that of other cultivars, and a method of testing seed purity is required. To date, the purity of Yumeminori seed has been tested by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude proteins. However, with this method, test samples are restricted to proteins from mature or developing soybean seeds. Recently, PCR-based detection methods have gained support from food industries because of their high sensitivity and effectiveness, particularly in the detection of genetically modified organisms (GMOs) (reviewed in Gachet et al. 1999). However, these methods depend on the availability of molecular information for genes or traits of interest, and such information has not been available for the β -conglycinin genes of Yumeminori.

Harada et al. (1989) showed that β -conglycinin is encoded by at least 15 genes, entitled *CG-1–CG-15*, that are clustered in six genomic regions, designated regions A–F. The α' -subunit is encoded only by the *CG-1* gene in region C, and the lack of α' -subunit in Keburi is caused by the complete deletion of this gene (Ladin et al. 1984; Harada et al. 1989; Yoshino et al. 2002). However, the α -subunit is encoded by both the *CG-2* and *CG-3* genes in region A (Harada et al. 1989; Yoshino et al. 2001). Yoshino et al. (2001, 2002) showed by Southern analyses that Keburi lacks the *CG-2* gene and Mo-shi-dou Gong 503 lacks the *CG-3* gene (Fig. 2). Although both of these genes contribute to α -subunit protein levels, the *CG-2* gene is expressed at a much lower level than the *CG-3* gene (Yoshino et al. 2002). Therefore, despite the lack of the *CG-2* gene, Keburi shows a normal amount of α -subunit protein, while the level of this protein is decreased in Mo-shi-

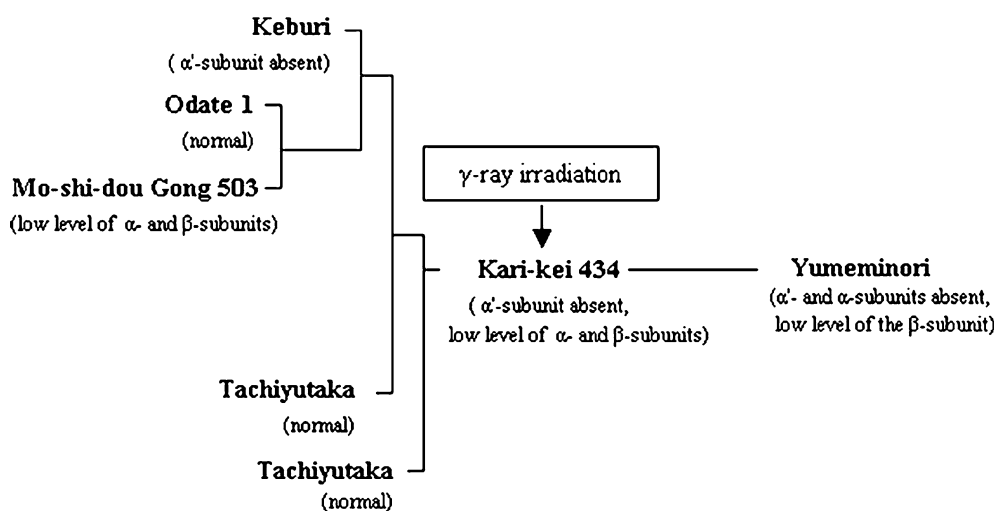


Fig. 1 Pedigree of the soybean cultivar Yumeminori, which lacks the α' - and α -subunits of β -conglycinin. The β -conglycinin subunit profiles are given in parentheses

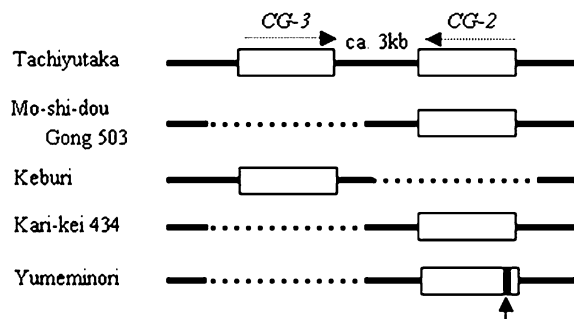
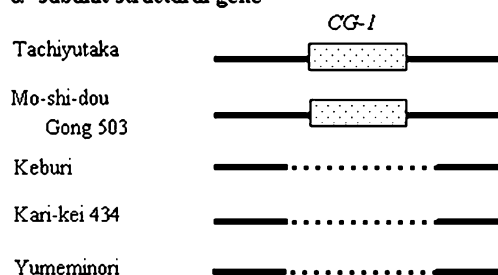
α -subunit structural genes **α' -subunit structural gene**

Fig. 2 Structural organization of the α - and α' -subunit structural genes in Yumeminori and in related lines. Dotted lines indicate a complete deletion of the gene, as revealed by Southern blot analyses (Harada et al. 1989; Yoshino et al. 2001, 2002). An arrow indicates the position of the 4-bp insertion in Yumeminori identified in this study

dou Gong 503. Kari-kei 434 inherited the mutated *CG-1* gene from Keburi and the mutated *CG-3* and wild-type *CG-2* genes from Mo-shi-dou Gong 503 (Yoshino et al. 2002). The complete lack of the α -subunit in Yumeminori is thought to be due to a new radiation-induced mutation in the *CG-2* gene. However, this mutation has not been characterized at the molecular level.

Here we characterized the mutation in the *CG-2* allele carried by Yumeminori and used this sequence information to establish a PCR method capable of simultaneously identifying α' - and α -subunit-deficiencies in soybean samples. This method can be used both for seed purity tests in food industries and for marker-assisted selection of low β -conglycinin lines in soybean breeding programs.

Material and methods

Plant materials

The soybean cultivar Yumeminori and the related lines Kari-kei 434, Tachiyutaka, Keburi, and

Mo-shi-dou Gong 503 were used in these experiments. These cultivars were obtained from the Tohoku National Agriculture Research Center. Yumeminori (formerly T line or Tohoku 124) lacks both the α' - and α -subunits of β -conglycinin (Takahashi et al. 1994, 1996). The pedigree of Yumeminori and the β -conglycinin subunit profiles of Yumeminori and related lines are shown in Fig. 1. The structural organization of the genes encoding the α' - and α -subunits in the ancestors of Yumeminori is summarized in Fig. 2 based on data from Harada et al. (1989) and Yoshino et al. (2001, 2002). As shown in Fig. 1, Kari-kei 434, the irradiated donor line of Yumeminori, is thought to possess the mutated *CG-3* and *CG-1* genes from Mo-shi-dou Gong 503 and Keburi, respectively. The new mutation described in this study occurred in the *CG-2* gene originating from Mo-shi-dou Gong 503.

DNA extraction and sequence determination

Seed was grown in a pot in the greenhouse for 2 weeks, and DNA was extracted from 100 mg of young leaf tissue using the Nucleon PhytoPure Plant DNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. To amplify the complete *CG-2/CG-3* gene, two primers (CG23TF: 5'-GGGTCATCGG-GAACTAGTTCA-3' and CG23TR: 5'-TTCATAG-ATGAAGCTCTAGTCATAGTTAC-3') were designed based on the registered wild-type *CG-2/CG-3* gene sequence (Accession No. AB051865). Each 25- μ l reaction included 50–100 ng DNA, 1.5 mM MgCl₂, 5 pmol of each primer, 0.2 mM dNTP (each), 1 $\times$ Ex *Taq* buffer (Takara, Osaka, Japan), and 1.25 U Ex *Taq* polymerase (Takara, Osaka, Japan). The PCR cycle consisted of an initial 5 min denaturation at 95°C, followed by 32 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 2 min, and 1 cycle of 72°C for 7 min. PCR was conducted using a GeneAmp PCR system 9700 (Applied Biosystems, Foster city, CA, USA). PCR products were cut out of the gel after electrophoresis and purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Purified products were directly sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA).

Sequence comparisons

The online software program CLUSTAL W, which is provided by the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>), was used to compare the previously published wild-type *CG-2/CG-3* gene sequence (Accession No. AB051865) and the *CG-2/CG-3* gene sequences obtained from Keburi, Kari-kei 434, and Yumemino. The *CG-1* (Accession No. AB030838) and *CG-2/CG-3* (Accession No. X17698) cDNA sequences were compared in the same way.

PCR analysis

PCR was conducted to determine genotypes at the *CG-1*, *CG-2* and *CG-3* loci simultaneously. The PCR components and conditions were the same as those given above, with the exception of primer concentrations. Primer sequences, working concentrations, and product sizes are described in Table 1. The primer set for amplifying the first exon of Gly m Bd 30K (Accession No. AB013289) was included in reaction mixes as a positive control. Gly m Bd 30K is a major soybean allergen, and despite extensive screening, no soybean cultivars or lines that lack this protein have been identified. A 5- μ l aliquot of the PCR mixture was analyzed by electrophoresis on a 4% agarose gel in 0.04 M Tris buffer containing 0.04 M glacial acetic acid and 0.001 M EDTA (pH 8.0).

Protein extraction and SDS-PAGE analysis

Crude protein was prepared using 10 mg of seed according to the method of Takahashi et al (1996).

The presence or absence of the α' - and α -subunit was determined by SDS-PAGE according to the method of Laemmli (1970).

Segregation analysis of the α' - and α -subunits

Protein was extracted from the distal portions (approximately 10 mg) of seeds from parental lines and 10 F_3 individuals of a cross between Tohoku 139 (α' - and α -subunits absent) and Tohoku 142 (α' - and α -subunits present). The remaining proximal portions of the seeds were grown in a pot in the greenhouse for 2 weeks, and 100 mg of young leaf tissue was used for DNA extraction. SDS-PAGE and PCR analyses were conducted as described above.

Contamination analysis

Artificially contaminated samples for PCR were prepared by adding genomic DNA of Tachiyutaka to that of Yumemino at levels of 0.1, 0.2, 0.5 and 1.0% (w/w). PCR was conducted with these samples according to the methods outlined above.

Accession number

The genomic sequence of the *CG-2* gene encoding the truncated α -subunit of β -conglycinin in Yumemino is registered as DDBJ/EMBL/GenBank Accession No. AB207802.

Table 1 Primer sets for identifying the presence or absence of α' - and α -subunits of soybean β -conglycinin

Set name	Trait	Gene or allele	Primer name	Sequence (5' → 3')	Working conc. (μ M)	Product size (bp)
A	PC*	Gly m Bd 30K	30KF	AGCTCACTAATCACTATATATACGACATGCT	0.05	554
			30KR	CACAGCCCCCTTGGTACTTTAC	0.05	
	α -subunit absence	Mutated	CG2MF	GAAGAATGTGAAGAAGGTGAAAAAAT	0.2	450
		<i>CG-2</i>	CG23R	GAATGCGACCATATGGTTTTGA	0.2	
	α' -subunit presence	Wild-type	CG1F	GTCACAACAAGTGCCTCCGA	0.2	408
B		<i>CG-1</i>	CG1R	GGTGCTTTTCCTGCTTGTG	0.2	
	PC	Gly m Bd 30K	30KF	AGCTCACTAATCACTATATATACGACATGCT	0.05	554
			30KR	CACAGCCCCCTTGGTACTTTAC	0.05	
	α -subunit presence	Wild-type	CG23F	AAGAAGAATGTGAAGAAGGTGAAAT	0.2	448
		<i>CG-2/CG-3</i>	CG23R	GAATGCGACCATATGGTTTTGA	0.2	

*Positive control

Results

Amplification and sequence analysis of the *CG-2* and *CG-3* genes

Previous studies using Southern analyses have indicated that the *CG-2* and *CG-3* genes, which encode the α -subunit of β -conglycinin, are highly similar (Harada et al. 1989; Yoshino et al. 2001). Most soybean cultivars carry both these genes; however, Keburi has only the *CG-3* gene, while Kari-kei 434 and Yumeminori have only the *CG-2* gene (Fig. 2). In this study, genomic DNA samples from these three lines were used as PCR templates to determine the sequences of the *CG-2* and *CG-3* genes.

A single 2.4-kb product was amplified from all three lines using the CG23TF and CG23TR primers (data not shown). The sequences of the products from Keburi and Kari-kei 434 matched perfectly, indicating that the *CG-2* and *CG-3* genes are identical within the amplified region (Fig. 3). The product amplified from Yumeminori contained an insertion consisting of four adenine bases in the first exon of the *CG-2* gene (Fig. 3). This insertion led to a frameshift mutation, and a premature termination codon was generated at amino acid position 93.

PCR and SDS-PAGE analysis of Yumeminori and related lines

Using the 4-bp polymorphism in the *CG-2* gene of Yumeminori, we designed two allele-specific primers to distinguish mutant and wild-type alleles at this locus. *CG-1* gene-specific primers were designed based on a comparison between the *CG-1* (Accession No. AB030838) and *CG-2/CG-3* (Accession No. X17698) cDNA sequences. The primer sequences used for PCR analyses are shown in Table 1. PCR analysis using primer set “A” detects the presence of the *CG-1* gene and the mutated *CG-2* gene. A 408-bp product is amplified with the CG1F and CG1R primer pair only when the *CG-1* gene is present, and this amplification product indicates the presence of the α' -subunit protein. With the CG2MF and CG23R primer pair, a 450-bp fragment is expected only when the mutated *CG-2* gene is present. With the “B” primer set, the expected size of fragment produced by the CG23F and CG23R primers is 448 bp. This product

indicates the presence of the wild-type *CG-2* and/or *CG-3* genes and thus the presence of the α -subunit protein. The primer pair 30KF and 30KR, which was included in both the A and B primer sets as a positive control, was designed to amplify a 554-bp fragment from the gene encoding Gly m Bd 30K, a major soybean allergenic protein. To date, no soybean germplasm lacking this protein has been reported in the literature. Thus, the presence of the 554-bp product can be used to confirm the success of the PCR reactions.

When the A primer set was used, only Yumeminori generated a 450-bp product from the mutated *CG-2* gene, while Tachiyutaka and Mo-shi-dou Gong 503 generated a 408-bp product from the *CG-1* gene (Fig. 4A). However, with the B primer set, all samples except that from Yumeminori generated a 448-bp product from the wild-type *CG-2/CG-3* genes. The 554-bp positive control product was observed in all of the samples.

The α' - and α -subunit profiles predicted by PCR were confirmed by SDS-PAGE analysis of seed samples from the plants used for DNA extractions (Fig. 4B).

PCR and SDS-PAGE analysis using segregating individuals

PCR and SDS-PAGE analyses were conducted using 10 F_3 individuals obtained from a cross between Tohoku 139 and Tohoku 142. Tohoku 139 is a sib line of Yumeminori lacking the α' - and α -subunits, while Tohoku 142 represents a wild-type control for the β -conglycinin genes. Genotypes at the *CG-1*, *CG-2* and *CG-3* loci in the segregating individuals were determined by PCR. Two of the ten individuals lacked both the α' - and α -subunits (Fig. 5A, lanes 9, 10). The segregation patterns of the α' - and α -subunits proteins as determined by SDS-PAGE (Fig. 5B) matched perfectly with the PCR results. Furthermore, using the PCR analysis method, we identified three individuals that were heterozygous at the *CG-2* locus (Fig. 5B, lanes 3, 5, 12), whereas heterozygotes could not be distinguished by SDS-PAGE analysis (Fig. 5B). Thus, the PCR method described here is a useful tool for genotyping *CG-1*, *CG-2* and *CG-3* loci encoding α' - and α -subunit proteins.

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1  taagatactATGATGAGAGC  ACGGTTCCCA  TTACTGTTGC  TGGGACTTGT  TTTCCTGGCT
61  TCAGTTTCTG  TCTCATTTGG  CATTGCTTAC  TGGGAAAAAG  AGAACCCCAA  ACACAACAAG
121  TGTCTCCAGA  GTTGCAATAG  CGAGAGAGAC  TCGTACAGGA  ACCAAGCATG  CCACGCTCGT
181  TGCAACCTCC  TTAAGGTGGA  GAAAGAAGAA  TGTGAAGAAG  GTGAAATTCC  ACGACCACGA

                                     I P R P R
Yumeminori  AAAATTCC  ACGACCACGA
                                     K N S T T T

241  CCACGACCAC  AACACCCGGA  GAGGGAACCT  CAGCAACCCG  GTGAGAAGGA  GGAAGACGAA
      P R P Q H P E R E P Q Q P G E K E E D E
      CCACGACCAC  AACACCCGGA  GAGGGAACCT  CAGCAACCCG  GTGA
      T T T T T T P G E G T S A T R
      *

301  GATGAGCAAC  CACGTCCAAT  CCCATTCCCA  CGCCCAACAAC  CTCGTCAAGA  AGAAGAGCAC
361  GAGCAGAGAG  AGGAACAGGA  ATGGCCTCGC  AAGGAGGAAA  AACGCGGAGA  AAAGGGAAGT
421  GAAGAGGAAG  ATGAGGATGA  GGATGAGGAA  CAAGATGAAC  GTCAATTCCC  ATTCCACGCG
481  CCACCTCATC  AGAAGGAAGA  GCGAAAGCAA  GAGGAAGATG  AGGATGAGGA  GCAGCAGCGA
541  GAGAGCGAAG  AAAAGTGAAGA  TTCTGAGTTA  CGAAGACATA  AGAATAAGAA  CCCTTTTCTC
601  TTCGGCTCTA  ACAGGTTCTG  AACTCTCTTC  AAAAAACCAAT  ATGGTCGCAT  TCGCGTCCTC
661  CAGAGGTTCA  ACCAACGCTC  CCCACAACCT  CAGAATCTCC  GAGACTACCG  CATTTTGGAG
721  TTCAACTCCA  AACCCAACAC  CCTCCTTCTC  CCCAACCATG  CTGACGCTGA  TTACCTCATC
781  GTTATCCTTA  ACGgtgagta  ttatttactt  aataaacate  atcaattgct  tctgtctctt
841  gtaaaattat  gatgatgaat  ttgttctglt  aattacaggg  ACTGCCATTG  TTTCCTTGGT
901  GAACAACGAC  GACAGAGACT  CCTACAGACT  TCAATCTGGT  GATGCCCTGA  GAGTCCCTCT
961  AGGAACCACA  TACTATGTGG  TCAACCCTGA  CAACAACGAA  AATCTCAGAT  TAATAACACT
1021  CGCCATACCC  GTTAACAAGC  CTGGTAGATT  TGAGgtactg  ccccttcttt  ataataaatt
1081  aattatttctt  claatllgtt  lccaaatttg  aagatcaate  ttcccttttt  tcccclltag
1141  AGTTTCTTCC  TATCTAGCAC  TGAAGCTCAA  CAATCCTACT  TGCAAGGATT  CAGCAGGAAC
1201  ATTTTtagagg  CCTCCTACGA  Tgtaagaaac  caaacacacca  tctaatlttc  attaccattt
1261  agtagtttat  ccaactgtca  cactacactg  gctaattaaa  tattatcggt  atcccttatat
1321  atttlltcc  glgctllgatt  ttacagACCA  AATTGAGGA  GATAAACAA  GTTCTGTTTA
1381  GTAGAGAGGA  AGGGCAGCAG  CAAGGGGAGC  AGAGGCTGCA  AGAGAGCGTG  ATTGTGGAAA
1441  TCTCGAAGGA  ACAGATTCCG  GCACTGAGCA  AACGTGCCAA  ATCTAGTTCA  AGGAAAACCA
1501  TTTCTTCTGA  AGATAAACCT  TTTAACTTGA  GAAGCCGCGA  CCCATCTAC  TCCAACAAGC
1561  TTGGCAAGTT  CTTTGAGATC  ACCCCAGAGA  AAAACCCCA  GCTTCGGAC  TTGGATATCT
1621  TCCTCAGTAT  TGTGGATATG  AACGAGgtaa  gcagaaaaac  catgtagaca  aactgaacag
1681  gacaattlag  ttctactatt  cactctcttc  ttagttagag  aagcctatta  ttgagacttt
1741  aaataatgat  ttactttttt  ctttlltca  aaatataggG  AGCTCTTCTT  CTACCACACT
1801  TCAATTCAAA  GGCGATAGTG  ATACTGGTAA  TTAATGAAGG  AGATGCAAA  ATTGAACCTG
1861  TTGGCCTAAA  AGAACAAACA  CAGGAGCAGC  AACAGGAAGA  GCAACCTTTG  GAAGTGCGGA
1921  AATATAGAGC  CGAATTGTCT  GAAACAAGTA  TATTTGTAAT  CCCAGCAGGT  TATCCAGTTG
1981  TGGTCAACGC  TACCTCAAA  CTGAATTTCT  TTGCTATTGG  TATTAATGCC  GAGAAACAAC
2041  AGAGCAACTT  CCTCGCAGgt  atataattta  tatatcaca  tcaaatgaa  tattgttgtt
2101  acttttttgt  ttatttgcatt  gtgaaacata  gccgcctgag  ttatttcaaa  cctaaacttg
2161  gtgattgaaa  acttgaagGT  TCGCAAGACA  ATGTGATAAG  CCAGATACCT  AGTCAAGTGC
2221  AGGAGCTTGC  ATTCCCTGGG  TCTGCAACA  CTGTTGAGAA  GCTATTAAG  AACCAAGAG
2281  AATCCTACTT  TGTGGATGCT  CAGCCTAAGA  AGAAAGAGGA  GGGGAATAAG  GGAAGAAAGG
2341  GTCCTTTGTC  TTCAATTTTG  AGGGCTTTTT  ACTGAataag  tatgtactaa  aatgtatgct
2401  gtaatatgctc  atagttagcg  aggaaagt

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Fig. 3 Nucleotide sequence of the α -subunit genes in Kari-kei 434 and Keburi, and position of a 4-bp insertion (underlined in bold) in Yumeminori. The 4-bp insertion changes the reading frame, creating a premature termination codon in the first exon (bold box). Upper and lower case indicate exons and introns of

the α -subunit gene, respectively. Boxes indicate start and stop codons in Kari-kei 434 and Keburi. In comparison to the previously published α -subunit sequence (Accession No. AB051865), a single base substitution (from C to A) was observed in the last intron (underlined)

Detection of α' - and α -subunit contamination by PCR

To investigate the sensitivity of detection of α' - and α -subunit contamination, PCR analysis was conducted with samples in which varying amounts of

genomic DNA of Tachiyutaka, a wild-type line, was mixed with DNA of Yumeminori. The presence of the wild-type *CG-1* and *CG-2/CG-3* genes could be detected when Yumeminori samples were contaminated with wild-type DNA levels of 0.2% or greater (Fig. 6).

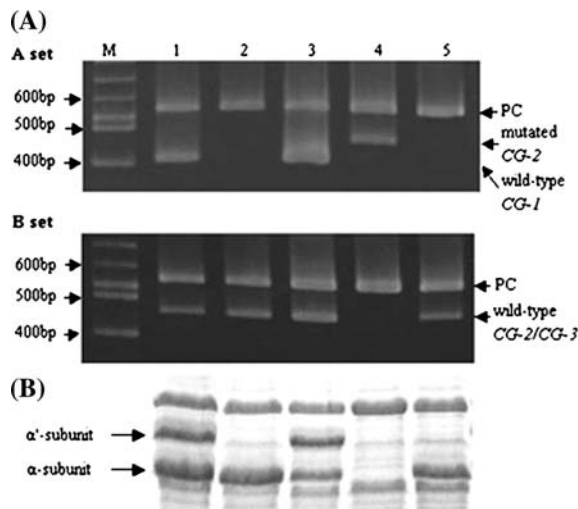


Fig. 4 Analysis of the α - and α' -subunits in Yumeminori and related lines by PCR (A) and SDS-PAGE (B). Lane 1, Tachiyutaka; lane 2, Keburi; lane 3, Mo-shi-dou Gong 503; lane 4, Yumeminori; lane 5, Kari-kei 434; M, Size marker

Discussion

Amplification products with identical sequences were generated with primers CG23TF and CG23TR from Keburi, which carries only the *CG-3* gene, and Kari-kei 434, which carries only the *CG-2* gene, indicating

that the regions between the start and stop codons of the *CG-2* and *CG-3* genes are not distinguishable (Fig. 3). Thus, DNA markers capable of distinguishing between wild-type *CG-2* and *CG-3* genes cannot be generated from this region. However, for our purposes is not necessary to discriminate between wild-type *CG-2* and *CG-3* genes, because Yumeminori lacks the coding region of the *CG-3* gene (Fig. 2) and carries a *CG-2* gene containing a 4-bp insertion (Fig. 3). This information is sufficient to identify the mutated *CG-2* and *CG-3* genes harbored in Yumeminori.

Segregation analysis indicated that the presence or absence of the 4-bp insertion was consistent with the presence or absence of the α -subunit protein (Fig. 5), indicating that the lack of the α -subunit protein likely results from this insertion. A large number of radiation-induced mutations in plants have been analyzed at the sequence level; these mutations include deletions, translocations, and transposable element-related insertions (Shirley et al. 1992; Bruggenmann et al. 1996; Yano et al. 2000; Shikazono et al. 2001, 2003; Nakazaki et al. 2003; Naito et al. 2005). However, there are few reports of insertional mutations consisting of several nucleotides, as was observed in this study. Ionizing radiation causes DNA damage including base damage and single- and

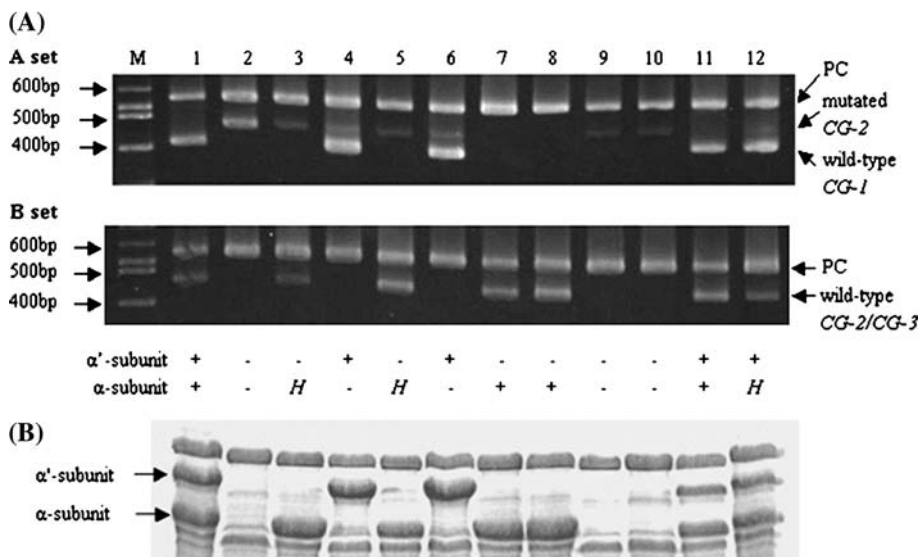


Fig. 5 Analysis of the α - and α' -subunits in segregating individuals by PCR (A) and SDS-PAGE (B). Lane 1, Tohoku 142 (α' - and α -subunits presence); lane 2, Tohoku 139 (α' - and α -subunits absence); lanes 3–12, F_3 individuals derived from

the cross between Tohoku 142 and Tohoku 139. Genotype determination: –, null allele; +, wild-type allele; H, heterozygote

double-strand breaks and insertional mutations of one or more base pairs can occur during the repair of the breaks (reviewed in Britt 1999; Gorbunova and Levy 1999; Lieber et al. 2003). In addition, it has been reported that misalignment at a continuous sequence of the same nucleobase during polymerization may induce minus or plus frameshift mutations (Kunkel 1990). In this study, the insertion of four additional adenine bases in a string of three adenine bases was probably induced by misalignment during the repair of γ -ray irradiation induced damage in the *CG-2* gene.

We showed that contamination of a cultivar carrying the mutated *CG-1* and *CG-2* genes with a wild-type cultivar could be detected at levels of 0.2% weight percentage of genomic DNA (Fig. 6). There have been a number of reports describing multiplex PCR methods for detecting genetically modified organisms (GMOs), particularly in maize and soybean (Tao et al. 2001; James et al. 2003; Germini et al. 2004). The detection sensitivities of these methods were high enough to clear the threshold levels for labeling of foods containing GMOs (0.9% in the European Union). New techniques, such as real-time PCR and peptide nucleic acid array (PNA), show even higher quantification accuracies and detection sensitivities (Germini et al. 2005; Huang and Pan 2005). Rapid and efficient methods to extract DNA from various foodstuffs have been established, and we feel that the PCR method outlined in this study can be used to assay wild-type contamination of cultivars lacking the α' - and α -subunits.

PCR-based markers are important tools for marker-assisted selection (MAS) in the breeding

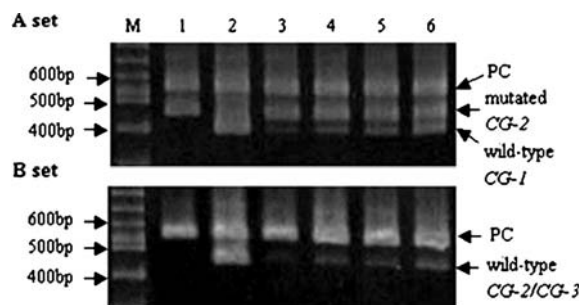


Fig. 6 PCR detection of α - and α' -subunit contamination in genomic DNA of Yumeminori. Lane 1, Yumeminori; lane 2, Tachiyutaka; lanes 3–6, genomic DNA of samples from Yumeminori spiked with DNA of Tachiyutaka at levels of 0.1, 0.2, 0.5 and 1.0% (w/w), respectively

programs of various crop species (Thro et al. 2004). In soybean, markers for disease resistance genes have been used to select elite lines (Klos et al. 2000; Mienie et al. 2002; Jeong and Saghai Maroof 2004). In this study, the lack of α' - and α -subunits of β -conglycinin could be detected simultaneously using a PCR-based method (Figs. 4, 5).

Recently, β -conglycinin has been shown to have positive effects on lipid metabolism, thereby suppressing serum triglyceride levels (Fukui et al. 2004; Moriyama et al. 2004). Also, specific β -conglycinin subunit compositions may affect the gelling properties of soybean protein (Salleh et al. 2004). Nevertheless, since low β -conglycinin lines have hypoallergenic properties (Ogawa et al. 2000) and novel processing characteristics (Takahashi et al. 2004), it is advantageous to have lines with both normal and low levels of β -conglycinin available. Therefore, low β -conglycinin lines are being developed in Japanese breeding programs, and Yumeminori and its sib lines have been used as donor parents for this trait (Takahashi, personal communication). The PCR-based DNA markers described here will be useful for MAS of lines lacking the α' - and α -subunits.

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