

The development of a co-dominant marker for the β -conglycinin α' subunit in soybeans

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Received: 12 February 2010 / Accepted: 27 August 2010 / Published online: 12 September 2010
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Abstract The development of soybean varieties that lack the β -conglycinin α' subunit is an attractive goal because the β -conglycinin α' subunit exerts a negative influence on nutrition and tofu gelation, and is also a major allergen. We sought to develop a co-dominant DNA marker for the β -conglycinin α' subunit (*Cgy-1*) gene for use in marker-assisted selection (MAS). We identified a deleted sequence responsible for the null allele of *Cgy-1* in a soybean variety lacking the β -conglycinin α' subunit known as 'Keburi'. The deletion spanned 12,998 bp and included *Cgy-1* and its incomplete tandem duplicate on chromosome 10. Based on the *Cgy-1* sequence from both the Williams 82 soybean reference sequence and the Keburi variety, a set of three

allele-specific primers was designed for multiplex PCR assay. These primers enabled allelic discrimination by the sizes of PCR products. This amplified two distinct DNA fragments of 913 and 649 bp in Williams 82 and Keburi, respectively. The practicality of the developed co-dominant marker for *Cgy-1* was also confirmed by amplification in five other soybean varieties including three wild types and two mutants. The heterozygosity of the F₁ plants at the *Cgy-1* locus was ascertained using our novel co-dominant marker. This PCR-based co-dominant marker is capable of detecting the presence or absence of β -conglycinin α' subunit for soybean marker assisted breeding system.

Keywords β -Conglycinin α' subunit · *Cgy-1* · Co-dominant marker · Seed storage protein · Soybean

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Introduction

β -Conglycinin and glycinin are major components of seed storage proteins in soybeans (*Glycine max* L. Merr.), and organize 7S globulin and 11S globulins, respectively, which are divided by their sedimentation coefficients. 7S and 11S globulins account for about 70% of storage proteins, and the amounts of these globulins are inversely related to each other (Hill and Breidenbach 1974).

β -Conglycinin consists of three subunit, α , α' , and β , resulting in the formation of homo- or

hetero-trimers (Thanh and Shibasaki 1978). Compared to glycinin, β -conglycinin has a lower amino acid score in nutritional quality because it contains fewer sulfur-containing amino acids such as methionine (Kaviani and Kharabian 2008). Furthermore, all three subunit of β -conglycinin are potential major allergens in soybean-sensitive patients (Krishnan et al. 2009). During the processing of soy foods, such as tofu, β -conglycinin has a detrimental effect on gel formation. The degrees of influence of each of the β -conglycinin subunit on gelation properties differ. When amounts of glycinin are equal, soybean genotypes lacking the α' subunit show higher gel hardness than all subunit-containing soybean genotypes and α subunit-lacking genotypes. The β -conglycinin α subunit has the least influence on gel hardness (Salleh et al. 2004).

Due to the adverse impacts of β -conglycinin α' subunit on aspects of nutrition, food, and allergies, it is highly desirable to produce soybean varieties that lack this subunit. In addition, since decreased levels of β -conglycinin α' subunit is related to increased levels of glycinin (Krishnan 2005), the removal of β -conglycinin α' subunit is desirable for the improvement of protein quality in soybean seeds.

The characterization of the β -conglycinin α' subunit gene (*Cgy-I*) was accomplished by identifying a *Cgy-I*-null mutant variety, 'Keburi' (Kitamura and Kaizuma 1981), which was found to have a deletion that included *Cgy-I* (Ladin et al. 1984). By crossing with Keburi, the null allele of *Cgy-I* was introduced into elite soybean varieties that had desirable alleles for other traits (Ishikawa et al. 2006). However, distinguishing between homozygous and heterozygous lines is a challenging task, particularly in early stages of selection of plant breeding program.

To examine the expression levels of β -conglycinin α' subunit protein among storage proteins in soybeans, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and antibody tests, such as enzyme-linked immunosorbent assays (ELISA), are commonly used (Mifflin and Shewry 1981). However, these assays are laborious, time-consuming (Gachet et al. 1999), and require seed destruction in order to extract crude proteins. The development of PCR-based DNA markers directly linked to β -conglycinin α' subunit would facilitate the formulation of a simpler method to detect desirable mutants.

Recently, a PCR-based marker for detecting the presence or absence of β -conglycinin α' subunit was

developed for soybeans (Ishikawa et al. 2006). However, this marker is a dominant marker, and is therefore unable to discriminate between homozygotes and heterozygotes. In addition, the absence of a PCR product does not necessarily indicate recessive homozygotes due to the possibilities of false negatives from PCR failure (Winter and Kahl 1995).

In this study, we sought to identify a co-dominant marker for the β -conglycinin α' subunit (*Cgy-I*) gene in soybeans. The deletion site for *Cgy-I* in the Keburi soybean variety was determined, and a set of three allele-specific primers was designed for multiplex PCR analysis. The applicability of the developed co-dominant marker to other soybean genotypes was also evaluated.

Materials and methods

Plant materials

Four soybean genotypes, Williams 82, Daewonkong, Taekwangkong, and Danbaekkong, were used as wild types of the β -conglycinin α' subunit. A mutant lacking the β -conglycinin α' subunit, Keburi, was obtained from the USDA National Plant Germplasm System, and collected twice in 1952 and 1987 as two accessions, PI 200485 and PI 506876, respectively. These samples appeared to be of the same genotype due to identical morphological characteristics (data not shown). To confirm the validity of our newly developed co-dominant marker for *Cgy-I*, two additional mutants collected from China, CS 1226 and CS 1231, were evaluated (Hur et al. 2008). Finally, the Keburi (mutant) and Daewonkong (wild type) genotypes were crossed to produce F₁ progeny.

Extraction of protein and DNA

To extract crude proteins, mature seeds were ground to a fine powder using a mortar and pestle. About 10 mg of ground seed powder was added into 1 ml of extraction solution containing 125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (w/v), 20% glycerol (v/v), 50 μ l of 2-mercaptoethanol, and 0.03 mM bromophenol blue. After the sludge was heated in an incubator at 100°C for 5 min and centrifuged at 12,000 rpm for 20 min, the supernatant was transferred into a new tube. 10 μ l of the extracted protein

was loaded onto an 8% SDS-PAGE gel following the Laemmli method (1970).

Genomic DNA was isolated from young leaves according to the procedure described by Shure et al. (1983). The DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was diluted in Tris–EDTA buffer (pH 8.0) to a concentration of 50 ng/μl and stored at −20°C until use.

Sequence analysis

A complete cDNA sequence for the *G. max* β -conglycinin α' subunit (AB030838.1) was obtained from the NCBI Genbank database (<http://www.ncbi.nlm.nih.gov/Genbank/>). This sequence was compared to soybean reference genome sequences (Glyma1.01, var. Williams 82) from Phytozome (<http://www.phytozome.net/soybean>) using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine its chromosomal location and detect the entire genomic sequence including introns and surrounding sequence.

Cloning and sequencing of the null allele of *Cgy-1* in Keburi

To detect the deletion region of β -conglycinin α' subunit, four sets of primers were designed based on approximately 15 kb of the Williams 82 genomic sequence surrounding the *Cgy-1* gene (Table 1). The putative span size of the deleted region in the Keburi

(PI 200485) genotype was predicted by PCR banding patterns using the primer sets according to physical position. To precisely identify the site of deletion, the Keburi genomic sequence corresponding to the null allele of *Cgy-1* was amplified. Additional forward and reverse primers (*Cloning_F* and *Cloning_R*) were designed based on the flanking sequences of the putative deletion region, which was tailed with 5 extra nucleotides and the restriction sites of *EcoRI* and *SalI* (Table 1). All PCR amplification was performed in a final volume of 50 μl containing 1× PCR buffer (75 mM Tris–HCl (pH 8.5), 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂), 0.2 nM dNTPs, 1.0 μM each of the forward and reverse primers, 1.6 U *Taq* DNA polymerase (Vivagen, Sungnam, Korea), and 50 ng of genomic DNA. The PCR reaction was conducted in a PTC-225 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA) with an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, optimal temperature of 60–64°C for 30 s and 72°C for 30 s, and the final extension step at 72°C for 10 min.

To clone the Keburi genomic sequence for the null allele of *Cgy-1*, the gel-purified PCR product was digested with restriction enzymes of *EcoRI* and *SalI*, and then cloned into the pUC19 vector. The recombinant plasmid was transformed into an *E. coli* strain DH10b followed by the selection of white colonies as true positives. A sequencing reaction was performed using M13 forward and reverse primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied

Table 1 Sequences and chromosomal locations of the primers used in this study

Primer name	Primer sequence (5' → 3')	Position ^a	Purpose
A_F	cgcattgtcttactggtg	46885065	Detection of the deletion region
A_R	agcaacaaataggagactc	46885435	
B_F	cctccatttgccgttatta	46887494	
B_R	tgatgggttgagttggtgaa	46887861	
C_F	ggtgtcgaccaaacaacct	46898394	Cloning and sequencing
C_R	ttgtctgatcacaagcagtgg	46898617	
D_F	ggcacgggcttaaaaagtaa	46899312	
D_R	accgccccctctaaagtaa	46899707	
<i>Cloning_F</i> ^b	tctacgaattccaataacagaagtgactcctggg	46885115	Marker development
<i>Cloning_R</i> ^b	tctacgtcgacgaaccagcatgcgtatcaaag	46899021	
Cgy-1_1F	cgcttggtctccaccctcactttcag	46885385	
Cgy-1_2F	ggtgtcgaccaaacaacct	46898394	
Cgy-1_R	caaccagcatgcgtatcaaag	46899022	

^a The location was represented for the physical position of the 5' end nucleotide of each primer on Chr 10

^b The underlined sequences in the primers *Cloning_F* and *Cloning_R* indicate the restriction sites of *EcoRI* and *SalI*, respectively

Biosystems, Foster City, CA, USA). The reaction product was resolved on an ABI 3730xl DNA sequencer and ABI trace files were analyzed using Seqscape software v2.0 (Applied Biosystems).

Multiplex PCR analysis

We used a multiplex PCR method with three different primers (Chamberlain et al. 1988; Edwards and Gibbs 1994). The three primers were designed based on the genomic sequences of Williams 82 and Keburi, corresponding to each allele of the *Cgy-1* locus (Table 1). To optimize multiplex PCR conditions, various ratios of the primer concentrations (1:0.1:1, 1:0.5:1 and 1:1:1) and different annealing temperatures (60, 62 and 64°C) were tested. The final PCR mixture contained 50 ng of genomic DNA, 0.2 nM dNTPs, 1× reaction buffer, 1× Q Buffer (Elpis Biotech, Daejeon, Korea), 1.6 U *Taq* DNA polymerase (Vivagen), and each primer at an appropriate concentration ratio in a total volume of 10 µl. The multiplex PCR was performed using a PTC-225 Peltier Thermal Cycler (MJ Research Inc.) as follows: an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60, 62 or 64°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 10 min to optimize PCR conditions. PCR products were resolved on a 1.0% agarose gel stained with ethidium bromide.

SSR marker analysis

The SSR markers of Satt243, Satt440, Satt445 and Satt188, were used to confirm the genotypes of F₁ hybrids. For SSR marker analysis, the PCR mixture contained 10 ng of template DNA, 0.5 µM of each forward and reverse primer, 0.25 nM dNTPs, 1× reaction buffer (75 mM Tris–HCl (pH 8.5), 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂), and 0.8 U *Taq* DNA polymerase (Vivagen) in 10 µl of total volume. The SSR amplification was conducted with the following cycling conditions: an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 25 s, 46°C for 25 s and 72°C for 25 s, and final extension 72°C for 10 min. The PCR products were resolved with 3.5% NuSieve® GTG® agarose (Lonza Group Ltd., Rockland, ME USA) involving a 1.0% standard agarose gel stained with ethidium bromide.

Results

Determination of the deletion site for *Cgy-1* in Keburi

The *Cgy-1* gene was found to be located on chromosome (Chr) 10 through a BLAST search with its cDNA sequence against the soybean reference sequence. To predict the size of the deletion region in Keburi, four different sets of primers, which covered about 15 kb including *Cgy-1* on Chr 10, were designed and used for PCR amplifications in both the wild type Williams 82 and mutant Keburi genotypes. Two outer primer sets of A and D amplified the PCR products with the expected size in both the wild type and mutant phenotypes (Fig. 1a). However, the inner primer sets of B and C produced PCR amplicons only with the Williams 82 genotype. These results enabled us to predict the putative deletion region in Keburi, which spanned at least 11 kb.

Using specific primers designed from the flanking sequences of the putative deletion region (Table 1), the Keburi genomic sequence for the null allele of *Cgy-1* was amplified for cloning followed by sequencing. The deletion region in Keburi was 12,998 bp in size, located between 46,885,600 and 46,898,597 bp in the long arm of Chr 10 (Fig. 1a). This deletion region contained 10,268 bp of non-genic sequences, which appeared 2,435 bp upstream and 7,833 bp downstream of *Cgy-1*. Within this deleted boundary, the other gene model (Glyma10g39160) was predicted without any expressed sequence tags and showed about 78% identical nucleotides to *Cgy-1* across 1,100 bp, indicating incomplete tandem duplication (Fig. 1a).

Development of a co-dominant marker for *Cgy-1*

A co-dominant marker for the 12,998-bp deleted sequence in Keburi was developed, which enabled allelic discrimination according to the sizes of PCR products. To apply multiplex PCR, a set of three primers were designed for a single PCR reaction; two primers (*Cgy-1_1F* and *Cgy-1_R*) were designed to amplify the genomic region between 5' upstream and 3' downstream of the deletion region, respectively, whereas the other forward primer (*Cgy-1_2F*) was located within the deletion region (Fig. 1b). For wild

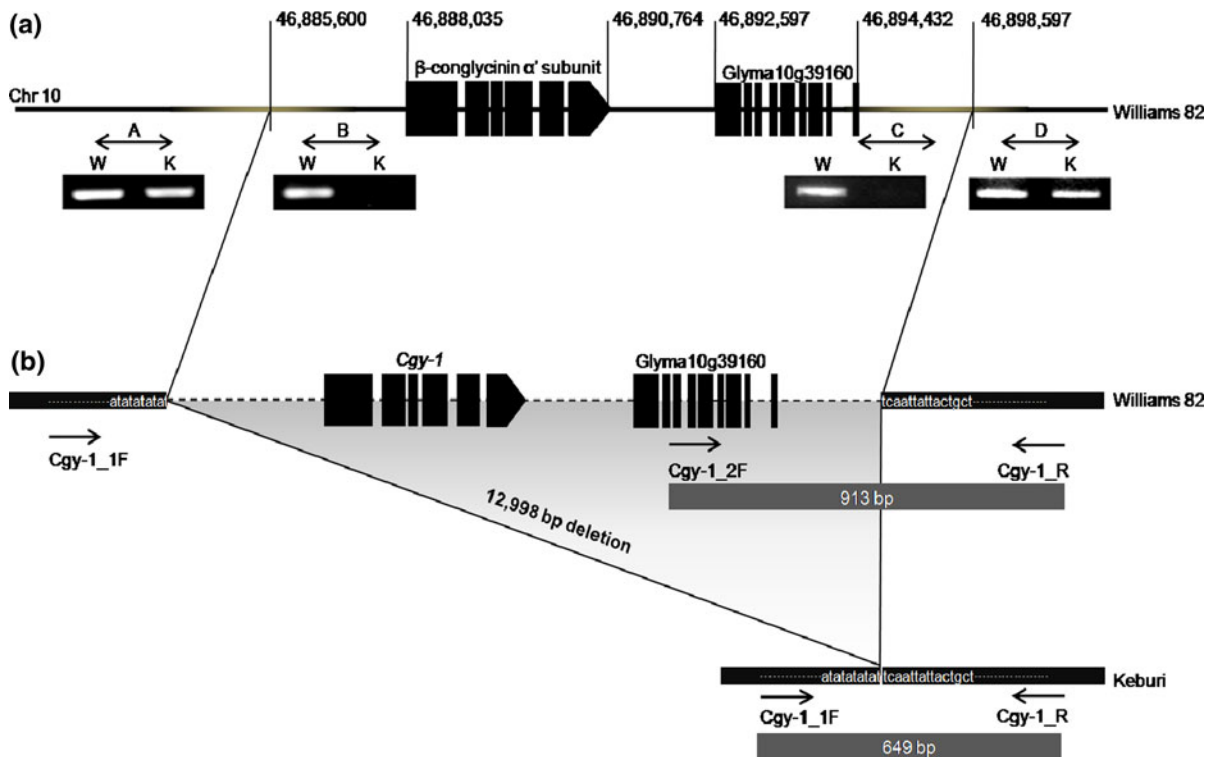


Fig. 1 Sequence comparison of the β -conglycinin α' subunit (*Cgy-1*) between Williams 82 and Keburi varieties, and the development of a co-dominant marker for *Cgy-1*. **a** PCR banding pattern for the prediction of the deletion region in Keburi. The primer sets A and D amplified PCR products in both the wild type and mutants, but primer sets B and C amplified only the wild type. **b** The deletion site in Keburi and the positions of the allele-specific primers for the multiplex PCR. The deletion ranged from 46,885,600 to 46,898,597 bp

types, the primer pair (Cgy-1_2F and Cgy-1_R) was subjected to the amplification of 913-bp fragments. The mutants lacking *Cgy-1* generated smaller PCR products (649-bp) than the wild types, with the combination of Cgy-1_1F and Cgy-1_R.

Since PCR conditions, such as compatibility among the primers within the reaction mixture and

on Chr 10 and covered a total of 12,998-bp including *Cgy-1*, as indicated by the gray triangle. This deletion region contained the incomplete nonfunctional tandem duplicate (Glyma10g39160). Three allele-specific primers were designed to develop a co-dominant marker for *Cgy-1*. Using the primer pair of Cgy-1_2F and Cgy-1_R, the PCR product of 913 bp was obtained in Williams 82, and Keburi represented the amplicon of 649 bp using Cgy-1_1F and Cgy-1_R

annealing temperature, were of great technical importance, it was necessary to optimize multiplex PCR conditions. Three different conditions of annealing temperatures and primer working concentrations were tested to increase the specificity and fidelity of multiplex PCR (Fig. 2). These tests revealed that the annealing temperature had more influence on PCR

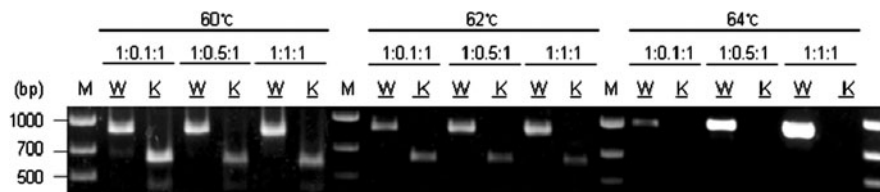


Fig. 2 Optimization of the annealing temperature and primer concentration in multiplex PCR. Three annealing temperatures of 60, 62, and 64°C were tested. The ratio of primer

concentration was represented by Cgy-1_1F, Cgy-1_2F, and Cgy-1_R. W Williams 82, K Keburi, M marker

specificity than the ratios of primer concentrations. Regardless of the ratios of primer concentrations, an annealing temperature of 60°C appeared to generate non-specific fragments with low molecular weight besides the bands of target. No PCR products were detected in the mutants at an annealing temperature of 64°C. Therefore, 62°C was identified as the optimal annealing temperature, since clear amplified specific bands without any non-specific bands were observed. Although there was little difference in PCR products among the ratios of primer concentrations, a reduced concentration of Cgy-1_2F was used, according to the suggestion by Edwards and Gibbs (1994). The concentrations of Cgy-1_1F, Cgy-1_2F and Cgy-1_R were determined to be 1, 0.5, and 1 μ M, respectively (Fig. 2). Using the determined PCR conditions, two specific DNA fragments in the size of 913 and 649 bp corresponding to each allele of the *Cgy-1* locus in wild type and mutant, respectively, were successfully obtained.

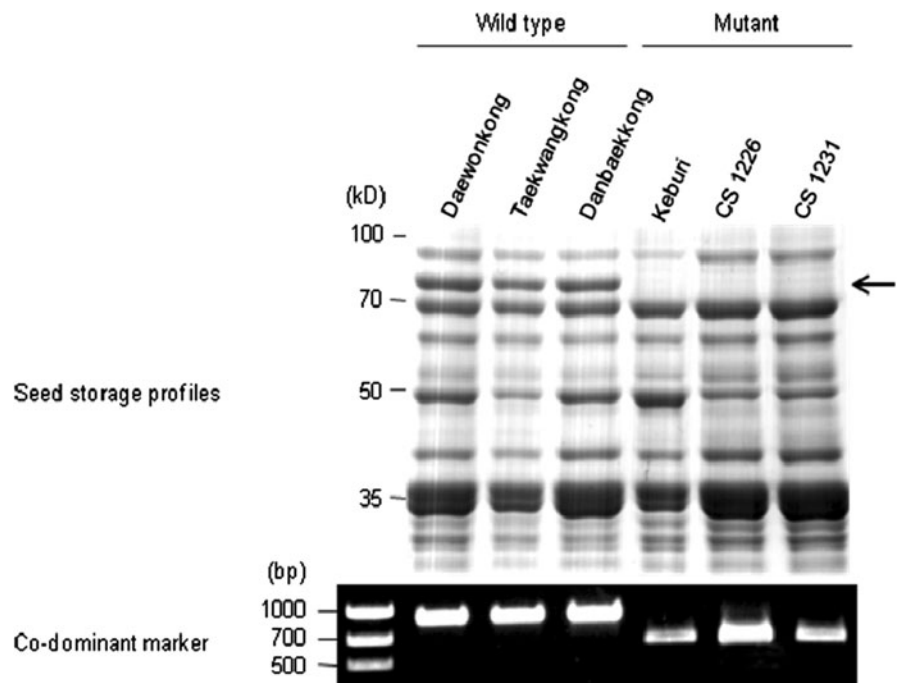
Evaluation of co-dominant marker for *Cgy-1*

To evaluate the co-dominant marker for *Cgy-1*, five other soybean genotypes including three normal cultivars and two mutants were used (Fig. 3). Three

normal cultivars, Daewonkong, Taekwangkong, and Danbaekkong, represented the 913 bp amplicons in the same size as the wild type Williams 82 genotype. Two mutants, CS 1226 and CS1231, lacking the β -conglycinin α' subunit of 76 KD (Beachy et al. 1981), produced 649 bp DNA fragments, as seen in the Keburi genotype (Fig. 3). These mutants, collected from China, were further investigated to ascertain their differences from Keburi. CS 1226 and CS 1231 showed different morphological characteristics from Keburi with regards to leaf shape, growth habit, and flower color. The Keburi genotype had broad leaves and purple flowers, while CS 1226 and CS 1231 genotypes exhibited narrow leaves and white flowers. Keburi and CS 1231 showed determinate habit, but CS 1226 was indeterminate (data not shown). Even though CS 1226 and CS 1231 had different genetic backgrounds from Keburi, the specific primers based on the deleted sequence in Keburi were shown to have direct applicability to these mutants that lacked the β -conglycinin α' subunit.

The practicality of the co-dominant marker for *Cgy-1* to heterozygotes was also assessed using F₁ progeny from the cross of Keburi and Daewonkong. An SSR marker, Satt440, that exhibited parental polymorphism, was used to check for the heterozygosity of the

Fig. 3 Evaluation of a co-dominant marker for five other genotypes. Three wild types, Daewonkong, Taekwangkong, and Danbaekkong, and two mutants, CS 1226, and CS 1231, were tested. Co-segregation of the marker with the presence or absence of the β -conglycinin α' subunit was confirmed using SDS-PAGE analysis. The arrow indicates the β -conglycinin α' subunit (76 kDa)



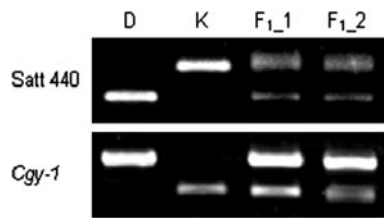


Fig. 4 Genotyping of F_1 individuals heterozygous to Satt440 and *Cgy-1*. The F_1 individuals were derived from the cross between Daewonkong and Keburi. *D* Daewonkong, *K* Keburi, F_1 F_1 individual

F_1 plants (Fig. 4). Using the marker for the β -conglycinin α' subunit, it was seen that the F_1 plants represented two distinct DNA bands which were inherited from each of the parental genotypes (Fig. 4). These results revealed that the co-dominant marker for the *Cgy-1* gene allowed us to select heterozygous individuals into which the null allele of *Cgy-1* had introgressed.

Discussion

β -conglycinin is a major seed storage protein that accumulates during seed development. This protein provides a source of nitrogen and a carbon skeleton for developing soybean seedlings after germination (Wilson et al. 1986; Clarke and Wiseman 2000). There have been a few reports on the molecular expression and function of each of the three β -conglycinin subunit in soybeans (Meinke et al. 1981). None of the subunit was found to synthesize new peptides during seedling development that were likely involved in the photosynthetic activity of cotyledons. Recently, global transcript profiling in the soybean cotyledon using microarray analysis revealed that the β -conglycinin α' subunit could play an important function during seedling development (Gonzalez and Vodkin 2007). The transcripts for the three subunit α , α' , and β showed rapid decrease until cotyledons started emerging from the ground. Afterwards, the α' subunit was only over-expressed during the green photosynthetic stage of cotyledons. The high transcriptional activity of α' subunit indicated that this subunit is likely to be an actual type, contrary to α and β subunit, and involved in the photosynthetic stage of soybean cotyledons. Approximately 1,000 accessions from different origins for

the mutants for each of three β -conglycinin subunits were screened. Some mutants for the α and β subunit were found, but mutants lacking the α' subunit could not be detected by SDS-PAGE analysis (data not shown). Several mutants exhibiting the absence of α and low level of β subunit have been found in soybeans. However, Keburi was reported to be a single natural mutant for the absence of the β -conglycinin α' subunit, screened among 3,000 natural accessions (Kitamura and Kaizuma 1981; Takahashi et al. 1994). This rare mutation in the *Cgy-1* gene might also support the fact that this subunit is more important in seedling development than other subunit, even though significant morphological alterations have not been observed in Keburi during seedling development.

The lack of the β -conglycinin α' subunit in Keburi was shown to be caused by a large deletion that included *Cgy-1* (Ladin et al. 1984). Relative to the wild type, however, the nucleotide sequence of the deletion in Keburi was not previously identified. The precise deletion site of *Cgy-1* in Keburi was determined in this study. The genomic region ranging from 46,885,600 to 46,898,597 bp on Chr 10 is deleted in Keburi, spanning approximately 13 kb (Fig. 1). Around the deletion site, AT-repeats and nine inserted nucleotides were found in Keburi, which appear to cause failures in direct sequencing and PCR walking in the corresponding region. Therefore, specific primers tailed with restriction sites for the cloning of Keburi were designed, and the nucleotide sequence for the null allele of *Cgy-1* in Keburi was successfully determined.

Multiplex PCR is a variant of PCR that enables the simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. This technique is commonly used in deletion screening (Chamberlain et al. 1988; Edwards and Gibbs 1994) and was applied to develop the DNA marker for the β -conglycinin α' subunit in this study. By comparing the sequences for both alleles of the *Cgy-1* locus in the Williams 82 and Keburi genotypes, three allele-specific primers were designed including two forward primers and one reverse primer (Table 1). The forward primer *Cgy-1*_1F, upstream of the deletion region, did not amplify the PCR product with the reverse primer *Cgy-1*_R in the wild type due to the long distance between the regions. The other forward primer *Cgy-1*_2F was

annealed only in the wild type, resulting in the PCR amplification of the target region. Even though the three primers had different GC-contents, which led to various annealing temperatures and self-secondary structures following the optimization of the annealing temperature, clear PCR products in different sizes distinguishing dominant and null alleles of the *Cgy-1* were obtained (Fig. 2). The developed co-dominant marker for *Cgy-1* enabled marker assisted selection (MAS) against germplasm or breeding individuals for the presence or absence of the β -conglycinin α' subunit. The reliability of this marker was confirmed in five other soybean genotypes including three wild types and two mutants (Fig. 3). In addition, heterozygosity of the F_1 plants to the *Cgy-1* locus was ascertained using the co-dominant marker (Fig. 4).

More interestingly, primers specific to the null allele of *Cgy-1* from Keburi produced DNA fragments in the same size in two other mutants, CS 1226 and CS 1231. This result indicated the same region was deleted in all of these mutants. Keburi is a Japanese variety, while CS 1226 and CS 1231 originate from China. Although the parentages of CS 1226 and CS 1231 are not known, it is suggested that these mutants are descendants of Keburi. It is also possible that the corresponding region is a mutation hotspot in *Cgy-1*. The imperfect nonfunctional tandem duplication of *Cgy-1*, 1.8 kb downstream of the original *Cgy-1*, was also identified. Between both flanking sequences of this repeat gene cluster, unequal crossing over due to homologous recombination may be responsible for the reciprocal recombination product of the large deletion.

The conventional phenotypic assay for the presence or absence of the β -conglycinin α' subunit requires seed destruction followed by protein extraction. This makes it difficult to select early generation lines for use in breeding programs. The DNA marker for *Cgy-1*, presented here, is allele-specific and directly linked to the presence or absence of the β -conglycinin α' subunit. Furthermore, only a single PCR reaction is required for the selection of β -conglycinin α' subunit mutants. Thus, this marker will be highly useful for mutant screening and MAS for the development of soybean cultivars that lack the β -conglycinin α' subunit.

Acknowledgments This work was supported by a grant (No. CG3121) from the Crop Functional Genomics Center of the

21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, South Korea. We would like to thank the National Instrumentation Center for Environmental Management at Seoul National University in South Korea. The genomic sequence for the null allele of the *Cgy-1* locus from Keburi was obtained from the NCBI Genbank database (GU723691).

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