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Sequence-based DNA marker for simultaneous genotyping of all three β-conglycinin subunit genes in soybean

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Summary

Soybean (*Glycine max* [L.] Merr.) seed is about 40 % protein, most of which is accounted for by the major storage proteins β-conglycinin (7S globulin) and glycinin (11S globulin). β-conglycinin, which consists of three subunits, α, α', and β, is the main allergen in soybean. Accordingly, elimination of β-conglycinin from seed is one of the goals of soybean breeding programs. Soybean accessions PI 200485 and CS 1150 lack the α'- and α-subunits of β-conglycinin, respectively. In this study, we developed a 3-betacon marker that could simultaneously identify genotypes for the three subunit genes of β-conglycinin. Interestingly, the three subunit genes were amplified with three distinct bands representing varied amplicon sizes. Mutant alleles could be identified in progeny of a three-way cross, and the corresponding α- and α'-subunit proteins were clearly absent in each mutant accession. 3-betacon marker will be highly useful for soybean breeding programs to develop soybean cultivars with decreased β-conglycinin contents.

Introduction

The seeds of soybean (*Glycine max*) consist of 40-50 % protein, the content and profile of which is nutritionally, functionally and economically superior to those of many other crops. The four major storage proteins of soybean are divided into 2S, 7S, 11S and 15S types. Among them, 11S (glycinin) and 7S (β-conglycinin) account for 70 % of the total storage protein (Derbyshire et al., 1976). The quality of soybean seed storage protein has an effect on the biochemical components of tofu and other soy-based foods (Poysa and Woodrow, 2002; Saio et al., 1969).

β-conglycinin (150 kDa) is a glycol protein composed of three subunits, α (72 kDa), α' (76 kDa) and β (52 kDa) (Higgins, 1984; Vu Huu and Shibasaki, 1978). The α-subunit accounts for approximately 25 % of total allergenic protein in soybean (Ogawa et al., 2000). The α'-subunit and β-subunit of β-conglycinin have also been reported to be potential food allergens (Krishnan et al., 2009). Humans cannot synthesize several essential amino acids, including the sulfur-containing amino acid methionine (Fukushima, 1991; Kohyama and Nishinari, 1993). Since β-conglycinin contains less sulfur-containing amino acids than glycinin, it has less value in terms of nutrition. Thus, it was proposed that glycinin content should be increased and β-conglycinin content decreased in soybean breeding to decrease the allergenicity of soybean without detrimental effects on its nutritional value (Krishnan et al., 2009).

In fact, two soybean accessions with increased glycinin have been described. These include CS 1150 (Korea native soybean), in which the β-conglycinin α-subunit is decreased, and PI 200485, which contains decreased levels of the α'-subunit (Hill and Breidenbach, 1974; Honeycutt et al., 1989; Ishikawa et al., 2006; Ogawa et al., 1989). These diminished β-conglycinin accessions can be used in soybean breeding programs to develop elite cultivars for foods such as tofu and soybean gelling hardness with less allergenicity. Although soybean storage proteins can be easily identified by SDS-PAGE, it takes months before seeds can be harvested, and extraction of seed proteins is labor intensive. The use of PCR-based DNA markers could save time and money via the selection of lines with defined protein profiles (Grover and Sharma, 2014).

The sequences of the genes encoding the α'-, α- and β-subunits of β-conglycinin have high similarity (Harada et al., 1989; Li and Zhang, 2011). The gene for the α'-subunit is located on chromosome 10 (GenBank No. M13759), the α-subunit gene is on chromosome 20 (Genbank No. AB051865) and the β-subunit gene is on chromosome 20 (Genbank No. S44893) (Li and Zhang, 2011; Tsubokura et al., 2006). In this study, we attempted to develop DNA markers for identification of the alleles for the α- and α'-subunit of β-conglycinin in the accessions CS 1150 and PI 200485, respectively. We successfully developed a unique marker that could simultaneously authenticate the genotypes of the α- and α'-subunit genes. This marker will be useful for application of PCR-based marker assisted selection (MAS) for breeding of a less-allergenic soybean cultivar.

Materials and methods

Plant materials and gDNA extraction

DNA was extracted from young, fresh leaves from accessions Seokryangput, Sodam, Malli, Nogwon, Taekwang, Jinpum 2, Cheongja, Cheongja 3, Daewon, Karikei 434 (reduced deletion of α'-subunit mutant), CS 1150 (reduced α-subunit mutant), PI 200485 (reduced α'-subunit mutant), PI 567476, Danmi, PI 200508 and PI 181540 using a QIAprep Spin Miniprep Kit (Cat. No. 27104). For practical application of the developed markers, F_1 seeds were harvested from crosses between Daewon and PI 200485 and the F_1 plants were crossed with CS 1150 as three-way cross hybrid. DNA from 90 progeny of (Daewon × PI 200485) × CS 1150 crosses were used to test the markers.

Seed protein extraction and SDS-PAGE

Crude proteins from PI 567476, Jinpum 2, Seokryangput, Malli, PI 200485, CS 1150, Taekwang and Daewon were extracted by grinding the samples into fine powder using a mortar and pestle. Ground seed powder (10 mg) was then treated with 1 ml extraction buffer (125 mM Tris-HCl pH 6.8, 4 % sodium dodecyl sulfate (w/v), 20 % glycerol (v/v), 4 % 2-mercaptoethanol, and 0.03 mM bromophenol blue). Next, the sludge was denatured in an incubator at 95 °C for

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5 min and then centrifuged at 20,000g for 20 min. Following centrifugation, 10 µl supernatant was loaded onto a 12% SDS-PAGE. After electrophoresis, the gel was stained with 0.1% Coomassie Blue R-250 (LAEMMLI, 1970). Total proteins were extracted from the seeds of 90 plants of (Daewon × PI 200485) × CS 1150 crosses, a three-way cross hybrid, for identification of conglycinin subunits and evaluation of the developed marker.

PCR, cloning and marker development

Seven and eight primer sets were designed to develop markers for genes encoding the α- and α'-subunits of β-conglycinin (Tab. 1). Complete gDNA sequences for the genes encoding the three subunits of β-conglycinin were obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/). The sequences were also compared to the soybean reference genome sequence (Glyma 1.01, var. Williams 82) from Phytozome and soybase.org using BLAST to determine their chromosomal locations and detect the entire genomic sequences. GenBank No. M13759 (α'-subunit sequence: total length 3,636 bp) and GenBank No. AB051865 (α-subunit sequence: total length 3,613 bp) were used to design the primer sets using Primer3 (http://frodo.wi.mit.edu). The designed primers were synthesized at BIONEER (Deajeon, South Korea). The primer set used to amplify the genes encoding the three subunits of β-conglycinin was as follows: 3-betacon Forward primer, 5'-GCCAAATCTAGTTCAAGGAAAACC-3' and 3-betacon Reverse primer, 5'-AAGAGCTCCCTATATTTGTGAAC-3'. PCR was then conducted by subjecting the samples to the following conditions: initial denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. Finally, the PCR products were separated and visualized in 6% polyacrylamide gels with silver staining. To confirmation of sequence reproducibility, cloned PCR products were visualized in 1% agarose gels, purified using a Promega wizard SV gel and PCR clean up system (A9282) and cloned using the TOPcloner blunt kit (EZ002). The resulting plasmids were extracted with a QIAprep spin mini prep Plasmid Extraction kit and sequenced (Macrogen, Seoul, Korea).

Results and discussion

SDS-PAGE analysis of β-conglycinin subunits in soybean seeds

To confirm the genotype and protein structures of α- and α'-subunits, using three germplasms and five cultivars, CS 1150, PI 200485, PI 567476, Jinpum2, Seokryangput, Malli, Taekwang and Daewon, respectively. Since target alleles can be selected at earlier development stages in breeding efficiency using PCR based marker (FONTES et al., 1984). SDS-PAGE analysis among these eight genotypes showed that most carried both α- and α'-subunits, with the exception of CS 1150 and PI 200485 (Fig. 1). We therefore used these two cultivars to generate a three-way cross hybrid [(Daewon × PI 200485) × CS 1150] aimed at reducing the number of subunit genes.

Detection of the genes for the α- and α' subunits of β-conglycinin by PCR

Previous reports have suggested that the α'-subunit was located at Gm10: 46,887,129 - 46,890,714 bp, linked to Sat_109 and Sat_231 closely associated with the QTLs of seed glucose content, pod maturity and response to *Sclerotinia sclerotiorum*. The other subunits, α and β, were located at Gm20: 37,579,255 - 37,582,934 bp and 37,372,657 - 37,383,080 bp linked to Sat_307 and Satt671 associated with drought susceptibility, respectively (Supplementary Fig. 1) (soybase.org and phytozome.net/soybean, (KRISHNAN et al., 2009)).

To monitor deletions in the α-subunit gene (Genbank No: AB051865; 3,680 bp), it was retrieved from the NCBI database; the structural organization of the gene is presented in the Fig. 2A. Based on these data, seven primers sets were designed (Tab. 1). Primers alphaseq-1, 2, 3 and 5 are based on the region of the gene encoding the protein targeting sequence, and gave rise to amplicons in all three parental accessions (Fig. 2C). The primers alphaseq-4, 6 and 7 did not produce any amplicons in CS 1150. These results suggest that the lack of α-subunit in CS 1150 arises from a deletion in the middle to 3' end of the corresponding gene.

Similarly, to explore the lack of α'-subunit of β-conglycinin in PI 200485, genomic sequence was retrieved from the NCBI database (Genbank No: M13759) and eight sets of primers were designed

Tab. 1: List of primer sets for identification of genes for the α- and α'-subunits of β-conglycinin.

Gene	Primer name	Forward (5'-3')	Reverse (5'-3')	Product position	Product size (bp)
α-subunit	alphaseq-1	GCAACCATATCAGCATATCA	GGAATTTCACCTTCTTCACA	3-710	708
	alphaseq-2	ACAAGATGAACGTCAATTCC	TCCTTGCAAGTAGGATTGTT	930-1668	739
	alphaseq-3	GTGCCAAATCTAGTTCAAGG	CCTCCTCTTTCTTCTTAGGC	1954-2802	849
	alphaseq-4	AGAAAGGGTCCTTTGTCTTC	CTCGCAAAGAAAGGAAAATA	2814-3521	708
	alphaseq-5	GAAGGTGAAATTCCACGAC	TTCAGAAGAAATGGTTTTCC	697-1991	1295
	alphaseq-6	TATTAATGCCGAGAACAACC	GAAGGACACAAAACAAAAGC	2501-2990	490
	alphaseq-7	CTTCGACAAAGTGTCTAGGA	TTTCAATAAGTTGGGGATCA	3138-3603	466
α'-subunit	primeseq-1	ATCAAAATGGCAAAAACATT	CGGAAGAGAATACGAGTTTG	70-785	716
	primeseq-2	AAACATTCACCAACTCAACC	CAGAACCTTCACTTTCTTGG	707-1554	848
	primeseq-3	ATGATAACACTCGCCATACC	TTCAATGTTTGCTTCTCCTT	2186-2973	788
	primeseq-4	GTTGTGGATATGAACGAGGT	AATAGCCCGATACTTTCCTC	2763-3571	809
	primeseq-5	CATTGCATTTGACTATGTGG	CTTTCTCGCTATTGCAACTT	327-1101	775
	primeseq-6	TCATCAAAAGGAAGAGGAAA	GGTATGGCGAGTGTTATCAT	1418-2205	788
	primeseq-7	TCTAGCACTCAAGCTCAACA	ACCAAAAGCAAAGAAATTCA	2330-3138	809
	primeseq-8	GTTGTGGATATGAACGAGGT	ACACAAAACAAAAGCATCAT	2763-3634	872

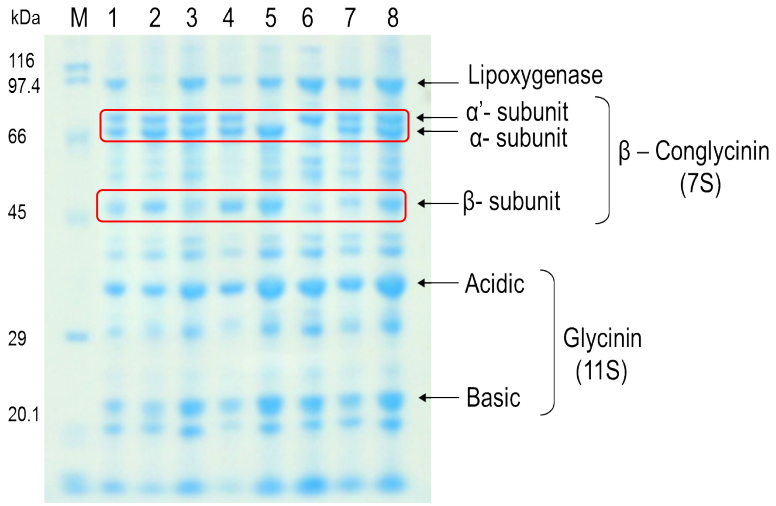

Fig. 1: Comparison of storage proteins among eight soybean genotypes by SDS-PAGE. The α- and α'-subunits of β-conglycinin were absent in CS 1150 and PI 200485, respectively. 1; PI 567476, 2; jinpum 2, 3; Seokryangput, 4; Malli, 5; PI 200485, 6; CS 1150, 7; Taekwang, 8; Daewon, M; protein marker.

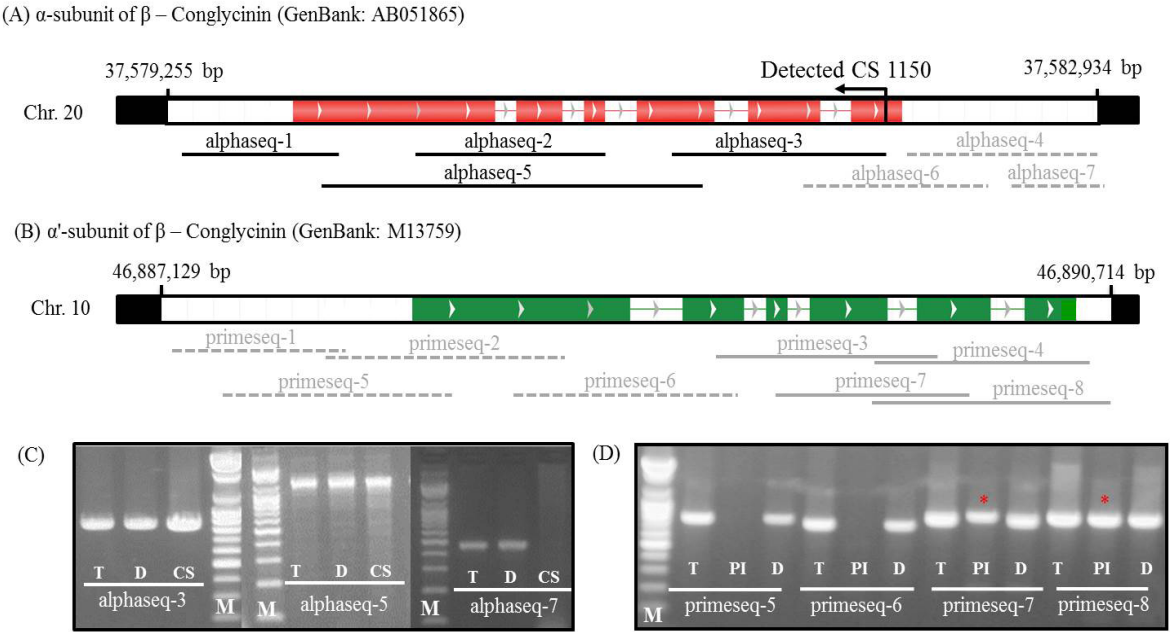

Fig. 2: Schematic of the genes of the α- and α'-subunits of β-conglycinin. These sequences were used to design primer sets (Tab. 1) to identify two mutant loci. PCR analysis confirmed that the 3' end of the α-subunits gene is not present in CS 1150 **(A)** and that the entire α'-subunit gene is absent in PI 200485 **(B)**. Lines below genes denote regions amplified by primer sets used for marker development. Black indicates found in PCR analysis, dotted gray not detected. The gray line indicates similarity of the α'-subunit to the α-subunit. **(C)** Products from the gene for the α-subunit of β-conglycinin were compared in agarose gels. Alphaseq-3 and -5 were detected in CS 1150, but alphaseq-7 was not. **(D)** Products from the gene for the α'-subunit of β-conglycinin were identified in agarose gels. Products with primeseq-5 and -6 were not detected in PI 200485, whereas primeseq-7 and -8 amplicons were detected. Based on sequence analysis, asterisks denote products derived from the gene for the α-subunit. T; Taekwang, PI; PI 200485, D; Daewon, CS; CS 1150, M; 100 bp marker.

(Tab. 1; Fig. 2B). Four set of primers (primeseq-1, primeseq-2, primeseq-5 and primeseq-6) did not produce any amplicons in PI 200485, indicating that the 5' region of the gene might be deleted in PI 200485 (Fig. 2D). The remaining four sets of primers showed amplification.

However, the size of the primeseq-7 amplicon differed between PI 200485 and the controls. When we explored this further, sequencing results revealed that both the primeseq-7 and the primeseq-8 products of PI 200485 were identical to the α-subunit gene, not to the α'-subunit gene. Thus, the products in the primeseq-7 and primeseq-8 bands likely arose from both α- and α'-subunit gene in the controls. By contrast, consistent with PI 200485 lacking the α'-subunit, only sequence for the α-subunit was detected in that genotype. In PI 200485, the deleted region of the gene for the α'-subunit of β-conglycinin is known to be 12,998 bp, located on chromosome 10 (46,885,660 to 46,898,597) (Kim et al., 2011). The deleted region includes non-genic sequence (10,268 bp) 2,435 bp upstream and 7,833 bp downstream of the α'-subunit gene. The lack of bands corresponding to sequences encoding the α'-subunit of β-conglycinin provide further evidence of this deletion.

Sequence comparison and marker development for β-conglycinin subunits

PCR-based markers are important tools for marker-assisted selection (MAS) in the breeding programs for various crop species (THRO et al., 2004). Our results with the primer sets described above confirmed that the genes for the α- and α'-subunit had similar sequences, as evidenced by co-amplification. The sequences of genes for all three α-, α'- and β-subunits were retrieved from Genbank (AB051865, M13759 and S44893, respectively) and aligned (TSUBOKURA et al., 2006). The genes for the α- and α'-subunits showed 87% similarity, while the α- and α'-subunit genes showed 76% and 75% similarity to the β-subunit gene, respectively, indicating that the genes for the α- and α'-subunits were more similar to each other than the gene for the β-subunit. Additionally, we compared all three subunit gene sequences to identify common sequences. Highly conserved sequences appeared from the 5' end to the middle regions of the genes; 13 regions had at least 15 bp in common among all three subunits in genes (Tab. 2).

We used this information to develop DNA markers (3-betacon) for simultaneous amplification of the three respective genes. A primer set was designed for the longest common sequence regions, regions 3 and 5 (Tab. 2). Importantly the conserved regions 3 and 5 were present in genes for all three subunits, but overlapped with the deleted regions in PI 200485 and CS 1150. The designed primers were expected to amplify a part of each subunit gene in all respective cultivars. Due to the deletions in the parental PI 200485 and CS 1150 α- and α'-subunit genes, respectively compared to the β-subunit gene, PCR using the progeny of the three-way cross amplified products of 305, 288 and 279 bp corresponding to the β-, α- and α'-subunit genes, respectively (Fig. 3A).

(POYSA and WOODROW, 2002) checked the effects of glycinin and β-conglycinin subunit on tofu quality using 28 mutant genotypes. In the past, to identify mutant genotypes with unusual storage proteins, each protein needed to be isolated and analyzed. Here, we used the 3-betacon marker designed for region 3 and region 5 to compare 16 other accessions to CS 1150 and PI 200485, which lack the α-subunit and α'-subunit, respectively, as shown above (Fig. 3B). As expec-ted, CS 1150 showed only two bands corresponding to the genes for the α'-subunit and β-subunit, and no band corresponding to the α-subunit gene. Karikei 434 (ISHIKAWA et al., 2006) and PI 200485 each had two bands corresponding to the α- and β-subunit genes, but no band for the α'-subunit gene. The other 13 accession had bands for genes corresponding to all three subunits of β-conglycinin.

Confirmation of DNA marker for three subunits of β-conglycinin

To evaluate the usefulness of our DNA marker, we tested whether the presence/absence of DNA bands was associated with the presence/absence of the corresponding proteins. Hence, we evaluated its the field-level application for potential selection of ideal genotypes with two combined mutant alleles.

As described above, hybrid F_1 plants between Daewon and PI 200485 were crossed with CS 1150 to select individuals lacking the α- and α'-subunits (Fig. 4A). DNA was extracted from the leaves of 94 individual F_2 plants and tested using our 3-betacon marker (Fig. 4B, Supplementary Fig. 2). F_2 individuals showing bands indicating presence or absence of the α- and α'-subunits could be clearly distinguished. To determine the presence or absence of the corresponding storage proteins in F_3 seeds, seed proteins were separated by SDS-PAGE. These results confirmed that all individuals predicted based on the DNA marker results to have α- and α'-subunits did, and the individuals missing either the α- or α'-subunits showed clear segregation (Fig. 4C).

Overall, our study revealed that α- and α'-subunit genes can be used generate gene markers in soybean varieties. 3-betacon marker described here will be useful for MAS to develop soybean lacking the α- and α'-subunits of β-conglycinin in seeds to reduce the allergenicity of soy protein.

Acknowledgments

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Tab. 2: Conserved sequences among the three-subunits of β-conglycinin based on a minimum of 15 conserved bp. Underline denotes primers selected to amplify the genes for the three-subunits of β-conglycinin.

Region	α- of position	α'- of position	β- of position	Consensus sequence	Segment Length
1	1374-	2013-	1517-	CCTTGGTGAACAACGACGAC	20
2	1831-	2488-	1990-	AATTCGAGGAGATAAACA	18
3	1955-	2612-	2099-	TGCCAAATCTAGTTCAAGGAAAACCATTTC	30
4	2079-	2733-	2223-	CAGCTTCGGGACTTGGAT	18
5	2242-	2879-	2396-	TTTGTTCACAAATATAGGGAGCTCTT	26
6	2269-	2906-	2423-	TTCTACCACACTTCAATTCAAAGGC	25
7	2325-	2962-	2479-	GCAAACATTGAACTTGTTGGC	21
8	2349-	2986-	2503-	AAAGAACAACAACAG	15
9	2383-	3020-	2537-	AACCTTTGGAAGTGC	15
10	2463-	3100-	2617-	GTCAACGCTACCTCA	15
11	2511-	3148-	2665-	GAGAACAACCAGAGGAACTTCCT	23
12	2694-	3343-	2827-	CAAGTGCAGGAGCTTGC	17
13	2808-	3457-	2941-	AAGGGAAGAAAGGGTCCTTT	20

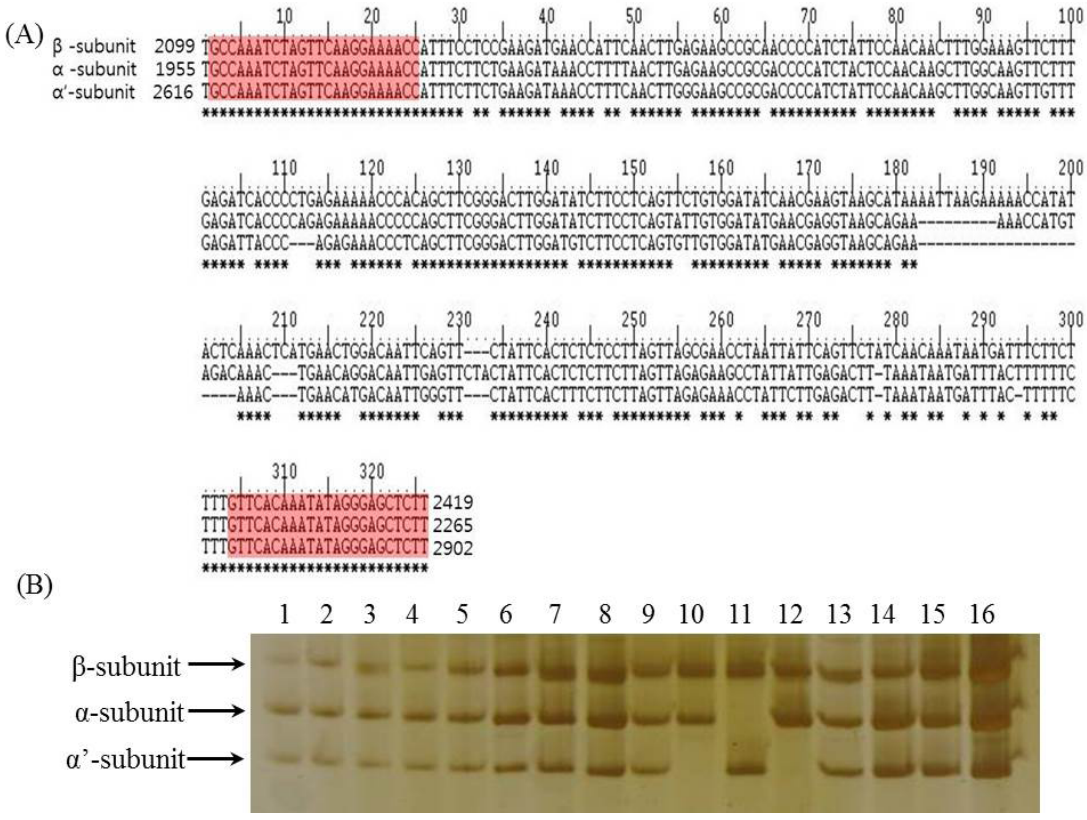

Fig. 3: Sequences and gel for genes encoding the α, α' and β-subunits of β-conglycinin. **(A)** The 30-bp deletion in the α'-subunit and 10-bp deletion in the α-subunit were compared with the β-subunit to develop the molecular marker based on primers complementary to the regions in red (Regions 3 and 5 in Tab. 2). **(B)** PCR marker analysis of the genes for the three subunits of β-conglycinin in a variety of soybean cultivars and germplasm. 1; Seokryangput, 2; Sodam, 3; Malli, 4; Nogwon, 5; Taekwang, 6; Jinpum 2, 7; Cheongja, 8; Cheongja 3, 9; Daewon, 10; Karikei 434 (lack α'-subunit), 11; CS 1150 (lack α-subunit), 12; PI 200485 (lack α'-subunit), 13; PI 567476, 14; Danmi, 15; PI 200508, 16; PI 181540.

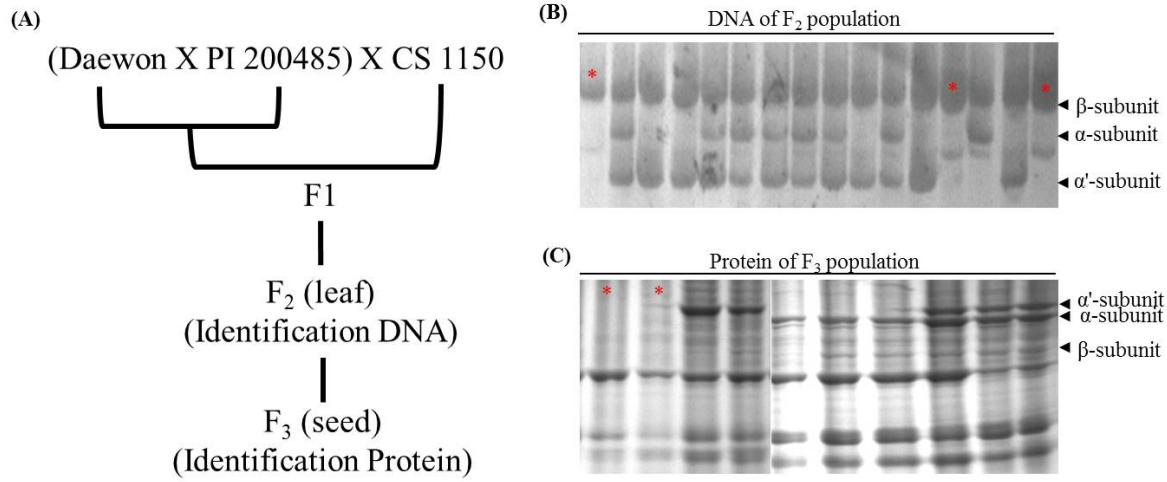

Fig. 4: Confirmation of the consistency of markers using acrylamide gels and SDS-PAGE of protein from three-way cross population of plants lacking the α- and α'-subunits. (**A**) Scheme for testing 3-betacon markers and protein expression using three-way cross hybrids. **(B)** F_2 plants between Daewon and PI 200485 crossed with CS 1150 [(Daewon × PI 200485) × CS 1150] were used for selection of individuals that lack the α- and α'-subunits based on the 3-betacon marker (from regions 3 and 5 in Tab. 2). **(C)** To confirm that the marker selection corresponds to the presence/absence of the proteins, the storage proteins from F_3 seeds were also separated on SDS-PAGE. Red asterisk indicates lack of α- and α'-subunits.

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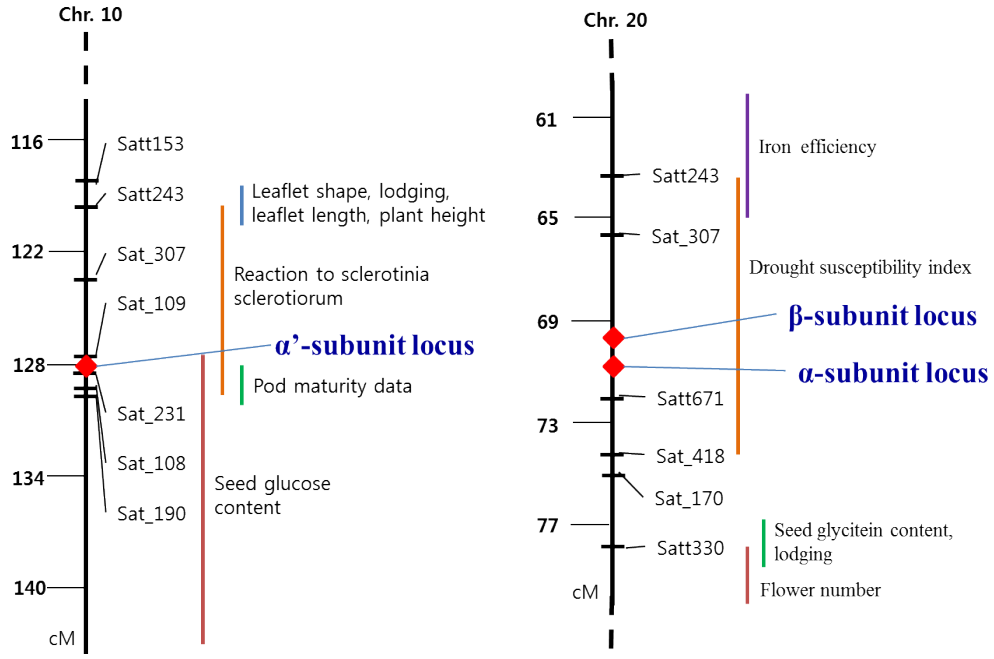
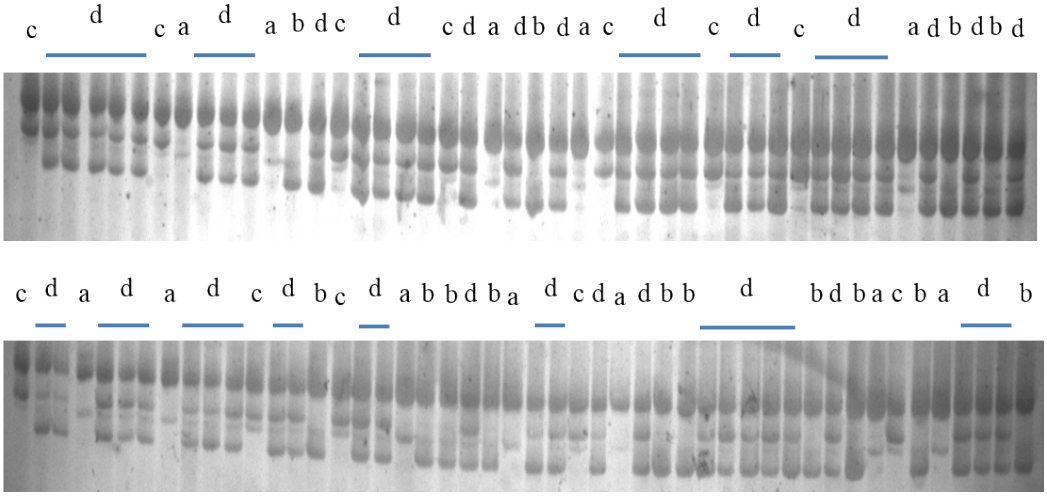


Supplementary Fig. S1. Schematic of genetic and physical loci for the genes encoding the three subunits of β-conglycinin. The α'-subunit gene was determined to be between Sat_109 and Sat_231 on Gm10, while the α-subunit and β-subunit genes were between Sat_307 and Satt671 on Gm20. Colored bars show QTL regions.



Lacking α- and α'-subunit	Lacking α-subunit	Lacking α'-subunit	Normal	Total
12	14	12	56	94

Supplementary Fig. S2. F_2 plants between Daewon and PI 200485 crossed with CS 1150 [(Daewon × PI 200485) × CS 1150] for selection of individuals lacking α- and α'-subunits using the 3-betacon marker (from regions 3 and 5 in Tab. 2). a; α and α' null, b; α null, c; α' null, d; Control.