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Genetic diversity of soybean (*Glycine max* (L.) Merrill) 7S globulin protein subunits

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Abstract

Out of 851 soybean accessions from Vietnam, China and Japan analyzed for 7S β-subunit variants, a new β-reduced subunit line with normal growth was collected from the Mekong Delta, Vietnam. Protein of the 'β-reduced' line is composed of β-reduced and extremely low-β types. (α-null + β-reduced) type and β-null (or extremely low-β) type were screened in the progeny seeds of the Japanese mutant '(α + β) null' line. Therefore, recombination between α- and β-subunits was identified. By comparing the nucleotide sequence of the partial β-subunit gene of Enrei (standard), Mo-shi-dou Gong 503 (α + β low), β-5_{33} seed (β-reduced), β-5_7 seed (extremely low-β), and (α + β)-$null_{16}$((α + β) null) seed, we found that the base 'T' at 166 bp, in Enrei changed to 'G' in Mo-shi-dou Gong 503, β-5_{33} and β-5_7. Using the (α + β)-$null_{16}$ individual as template, a distinct 305 bp β-subunit gene fragment was identified, instead of a 285 bp fragment.

Abbreviations: bp – base pair; kDa – kilo dalton

Introduction

Soybeans are a most important plant protein source for human nutrition and animal feed because of their high protein content and quality. From the viewpoint of soybean breeding, novel sources of genetic variability in seed proteins play an important role in the improvement of soybean protein quality and quantity.

Several spontaneous and induced mutants with an altered composition of soybean 7S storage protein subunits have been obtained. Mutant lines lacking α', α, (α' + α), (α + β), or (α' + α + β) subunits or with reduced levels of α- and β-subunits have already been reported. (Kitamura and Kaizuma 1981; Kitamura et al. 1984; Ladin et al. 1984; Tsukada et al. 1986; Kaizuma et al. 1989; Odanaka and Kaizuma 1989; Takahashi et al. 1994).

The β-subunit of 7S globulin is not a desirable soybean protein subunit in that it contains no methionine residues (Coates et al. 1985) that are considered to be largely responsible for the soybean sulfur amino acid defect. Therefore, by

producing a recombinant 7S globulin subunit with suppressed expression of the β-subunit of soybean 7S globulin, it should be possible to improve the quality of soybean. However, genetic analysis of the β-subunit gene(s) has proven very difficult because quantitative variation of this subunit is associated with changes in environmental conditions (Bray and Beachy 1985; Gayler and Sykes 1985; Holowach et al. 1986;). Furthermore, α- and β-subunits have been found to be tightly linked (Kitamura et al. 1984; Davies et al. 1985; Tsukada et al. 1986; Phan 1996). Although stable α'-null and α-null mutants have been identified (Kitamura and Kaizuma 1981; Takahashi et al. 1994), a β-less variant has not been found. Thus it has not been possible to genetically manipulate the β-subunit.

In the present study, we collected and evaluated 7S protein β-subunit variants of soybean germplasm in Asia. Electrophoretic profiles of total seed protein in 851 soybean accessions were analyzed. A new β-reduced line was obtained. Recombination between α- and β-subunits that occurred at a low frequency was detected. Variants of the $(\alpha + \beta)$ null line were screened. The relationships between protein subunit(s) and partial gene structure were also studied.

Materials and methods

Plant materials

We evaluated 628 soybean accessions collected from Vietnam and 222 soybean accessions collected from China. One line, F5-3 (Insoy), an accession with a reduced level of the β-subunit of 7S globulin was detected from screening over 628 soybean accessions in Vietnam by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) analysis. F5-3 (Insoy) was then crossed with MTD176, a high-yield variety collected in the Mekong Delta Vietnam (Thanh 1997, 2003; Thanh et al. 2004). Derived from the cross between F5-3 (Insoy) and MTD176 (local landrace), we obtained a new 'β-reduced' line.

Odanaka and Kaizuma (1989) and Odanaka (1990) obtained a '$(\alpha + \beta)$ null' line by irradiating the dry seeds of a cultivar 'Wasesuzunari' with γ-ray. This mutant line was originally supplied by Professor Takahata of Iwate University.

SDS-PAGE analysis

All seeds of each generation were screened for variation in subunit composition by SDS-PAGE. Individual seeds were excised with a razor blade to remove approximately 10 mg of the cotyledon from the side opposite of the hilum and crushed in coin envelops with a hammer. Total proteins were extracted from the crushed protein powder with extraction buffer consisting of 0.05 M Tris–HCl, pH 8.0, 0.2 SDS, and 5 M urea. The supernatant was obtained after centrifugation at 14,000 rpm for 15 min. Around 10 μL of extracts were separated by SDS-PAGE using a 11.98% separation gel and a 4.38% stacking gel, and were stained with 0.2% Coomassie brilliant blue R250.

The staining level of soybean β-subunit was classified into three types, normal (almost the same level as the band of β-subunit of Enrei which is the standard of normal type), β-reduced (half the level of normal type) and β-null or extremely low-β (band of β-subunit undetectable, this level needs to be confirmed by further immunological studies).

Immunological method

Proteins separated on the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and incubated with an antibody against the 7S globulin β-subunit. Goat anti-rabbit IgG coupled to horseradish peroxidase (Cappel) was used as a secondary antibody for the detection of the products that had bound with the antibodies (Lingappa et al. 1978).

DNA isolation and PCR analysis

Total genomic DNA was isolated from fresh young soybean leaves of individual soybean plants of the 'β-reduced' line and '$(\alpha + \beta)$ null' line using the modified cetyltriethylammonium bromide (CTAB) method (Murray and Thompson 1980). Enrei was used as a positive control. Polymerase chain reaction (PCR) amplifications were performed with the following β-subunit gene-specific primers: 5′-TAG CCC TAA TCT CAC T-3′; and 5′-GTC CAG TTT CAG TCA A-3′. The expected fragments were amplified as follows: preheated at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1.5 min, 55 °C for 2 min, and 72 °C for 1.5 min, a

final extension was performed at 72 °C for 7 min (Tierney et al. 1987; Phan et al. 1996).

Cloning and sequencing analysis

The resulting DNA fragments for each PCR were purified, cloned into pT7 blue T-vector kit (Novagen). The identity of the cloned insert was confirmed by PCR using the universal T7 and U19 primers. Sequencing was performed by the dideoxy-chain-termination method (Sambrook et al. 1989), using a DNA sequencer (Auto-Sequencer, DSQ 1000L, Shimadzu) in accordance with the manufacturer's instructions. The reactions were repeated at least 3 times to confirm the reproducibility of the results. Sequence comparison was performed with the program BESTFIT from the GCG software package version 7.1 using the default values.

Results

Genetic variations of 7S protein subunits of Vietnamese soybean accessions

We analyzed 628 Vietnamese soybean accessions including 185 local cultivars, 2 wild species accessions and 441 introduced accessions by SDS-PAGE (Table 1). No variations deficient in 7S protein subunits were detected among the local Vietnamese cultivars. We identified only one introduced accession with a reduced level of β-subunit (F5-3) among the introduced accessions. This mutant accession was then crossed with MTD176 (Vietnamese high-yield landrace). We obtained a new 'β-reduced' line (designated as 'β-reduced' line) derived from F5-3 × MTD176 (Thanh 1997, 2003; Thanh et al. 2004). Figure 1a shows the electrophoretic separation of the soybean 7S β-subunit of the 'β-reduced' line on the SDS-gel: lane 1 corresponds to Enrei (standard of normal type). Lanes 2 and 3 correspond to MTD176 and F5-3, respectively. Lane 4 corresponds to the variant individual β-5_{33} seed that has a β-reduced type. Lane 5 corresponds to the variant individual β-5_7 seed characterized by β-null (or extremely low-β). SDS-PAGE revealed some individuals of the line were characterized by a β-reduced type while some were β-null (or extremely low-β) type, and no normal type individual was segregated in this line. The plants of β-subunit variants grew and produced progeny without apparent physiological abnormalities (Figure 5a). Furthermore, two new type of wild soybean relatives (*Glycine* spp.): 'Dau Han The' and 'Dau Hoang' were detected in the Mekong Delta Vietnam (Figure 1b). Compared to MTD176 (normal type control), both 'Dau Han The' and 'Dau Hoang' lack the α'-subunit, 'Dau Han The' had the α-type subunit and neither had the β-type subunit of 7S globulin; 'Dau Hoang' had β-type subunit. The two soybean-related wild species were characterized by creeping stolon, nodule ability, dormancy and pod-borer-resistant feature (Thanh 2003). The introduction of some valuable characteristics of the two soybean-related wild species, such as pod-borer-resistance, into soybeans is being attempted by hybridization breeding.

Table 1. Variations of 7S seed storage protein subunits in soybean accessions collected from Asia.

Region	Source	No. of accessions	7S		
			α'	α	β
Vietnam	Local landraces	185	0	0	0
	Introduced	441	0	0	1
	Wild type	2	2	1	1
China	HeiLongJiang province	42	0	0	0
	JiLin province	68	2	2	0
	LiaoNing province	61	0	0	11
	ZheJiang province	11	0	1	1
	Inner-Mongolia	40	0	2	2
Total		850	4	6	16

Genetic variation of 7S protein subunits of Chinese soybean accessions

It was analyzed 222 soybean landraces collected from HeiLongJiang province (42 lines), JiLin province (68 lines), LiaoNing province (61 lines), northeast in China; ZheJiang province (11 lines), southern in China and Inner-Mongolia (40 lines) (Table 1). Among the soybean accessions from China, quantitative variations of 7S protein subunits were observed. Especially, 14 β-reduced variations were identified from the LiaoNing province, ZheJiang province and Inner-Mongolia, respectively (Table 1 and Figure 1c). The same

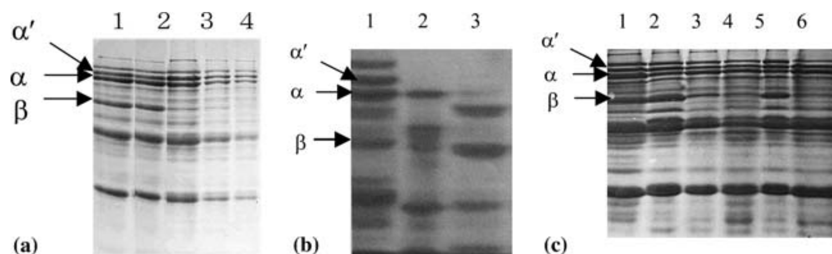

Figure 1. SDS-PAGE banding patterns of collected soybean accessions. (a) SDS-PAGE patterns of the 'β-reduced' line. 1. Enrei (standard of normal type), 2. MTD176, 3. F5-3, 4. β-5_{33} (β-reduced type) and 5. β-5_7 (extremely low-β type). (b) SDS-PAGE patterns of two soybean related wild species. 1. MTD176 (*Glycine max*), 2. Dau Han The (*Glycine* spp.) and 3. Dau Hoang (*Glycine* spp.). (c) SDS-PAGE patterns of β-reduced variations collected from China. 1. Enrei, 2 and 5. Normal types, 3, 4 and 6. β-reduced types: Kaiyu 10 (LiaoNing province), Hongmao 957 (ZheJiang province) and Neimeng-G (Inner-Mongolia).

quantitative variations of 7S globulin β-subunit were not observed in the varieties of HeiLongJiang province and JiLin province. No qualitative variations of 7S globulin β-subunit were detected among the 222 Chinese soybean accessions. We obtained α-subunit variants from the JiLin province, ZheJiang province and Inner-Mongolia, and α'-subunit variants only from the JiLin province (Table 1).

Genetic diversity of the segregation progenies from Japanese mutant '(α + β) null' line

A mutant soybean line lacking both α- and β-subunits of 7S globulin showed lethal chlorosis at the seedling stage soon after germination and stopped growing at about 2 weeks after germination. All of them died about 6 weeks after germination. Therefore, the mutant line could be maintained only in a heterozygous state.

The progeny seeds of the '(α + β) null' line were screened for electrophoretic variation of the soybean protein subunits. Two hundred and eighty three seeds obtained in 2002 were analyzed by the SDS-PAGE method. Segregation between the α- and β-subunits was observed (Figures 2 and 3). In addition to the (α + β) null-type and normal-type (Figure 2a, Lanes 2 and 3), we detected the new phenotypes (α-null + β-reduced) type (Figure 2a, lane 4) and β-reduced type (Figure 2a, lane 5).

More diversity segregations were detected in 2003. Among the progeny seeds derived from normal-type, α-null + β-reduced and β-reduced type were obtained again (1 seed and 61 seeds, respectively) (Figure 3). In addition, 3 seeds

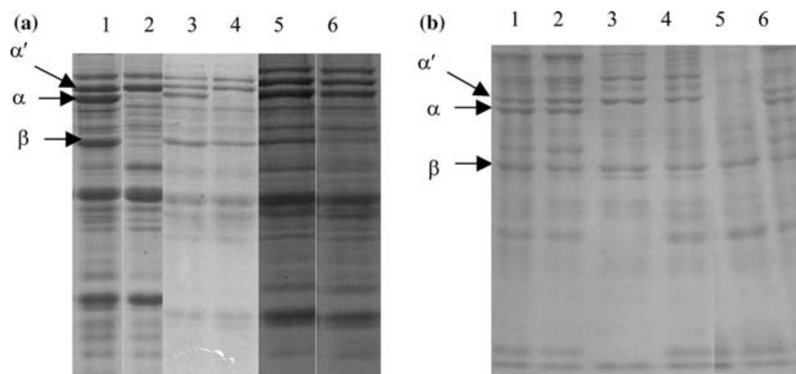

Figure 2. Variations of subunit composition in the progeny seeds derived from the '(α + β) null' line (2001–2003). (a) SDS-PAGE banding patterns of the progeny seeds derived from Normal-type. 1. Enrei (standard of normal type), 2. (α + β) null-type, 3. Normal type, 4. α-null + β-reduced type, 5. β-reduced type and 6. β-null type (or extremely low-β). (b) SDS-PAGE banding patterns of the progeny seeds derived from 'α-null + β-reduced' line. 1. Enrei, 2. Normal type, 3. (α + 11S group I + 11S $A_4A_5B_3$) null type, 4. α-null + β-reduced type, 5. (α' + α) null-type and 6. Enrei.

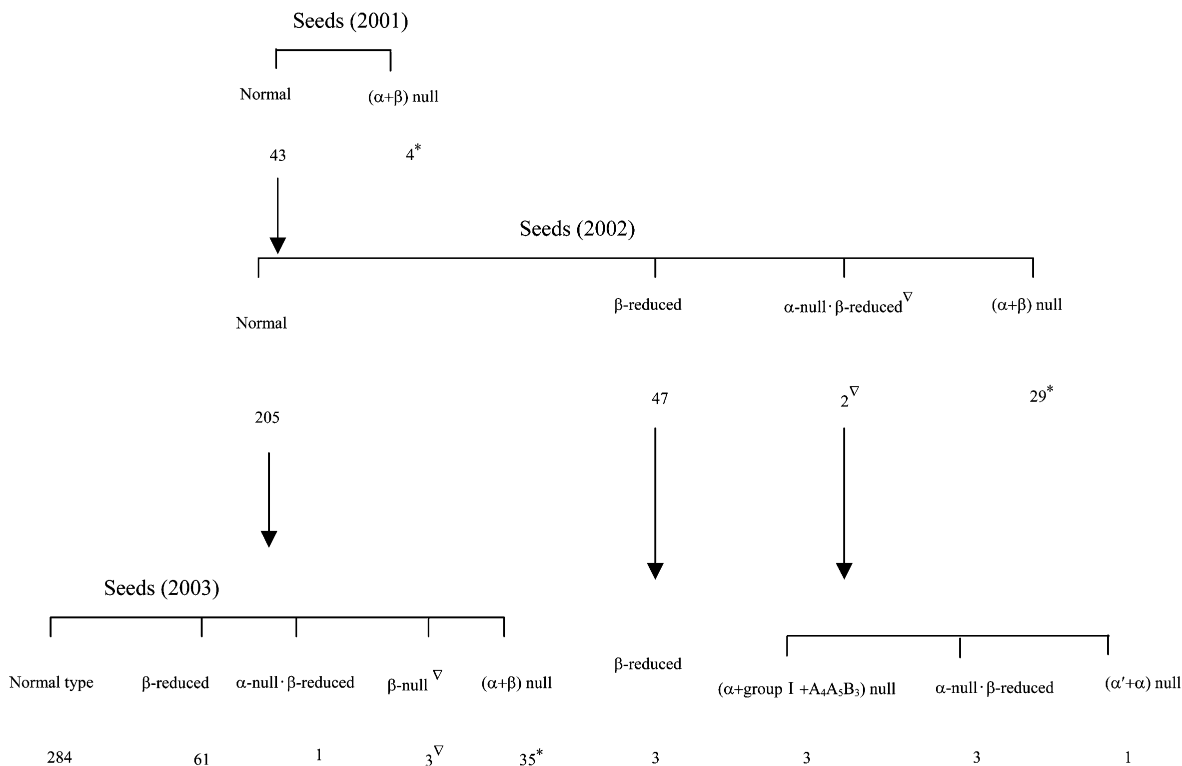

Figure 3. Pedigree of the '(α + β) null' line (2001–2003). Numbers represent the seed numbers. *: (α + β) null and the lethal chlorosis traits are inherited together. ∇: Two recombination types, α-null + β-reduced and β-null (or extremely low-β) types were detected and the recombination values are 0.71% (2/283) (2002) and 1.04% (4/384) (2003), respectively.

characterized by β-null (or extremely low-β) were also detected (Figure 2a, lane 6). So far, two recombination types, i.e., α-null + β-reduced and β-null (or extremely low-β) were detected. The recombination values are 0.71% (2/283) in 2002 and 1.04% (4/384) in 2003, respectively (Figure 3). Both recombinants are mutant types derived from normal-type seeds obtained in the previous year. No variation was detected from the progeny seeds of β-reduced individuals. However, three subunit types of α'-, α- and 11S-subunits were observed in the seeds derived from α-null + β-reduced individuals (Figure 2b and 3): One type lacking α-, 11S group I and 11S $A_4A_5B_3$-subunits (Figure 2b, lane 3); one type characterized by α-null + β-reduced (Figure 2b, lane 4) and one type lacking α'- and α-subunits (Figure 2b, lane 5).

In this line, the new novel β-reduced type, α-null + β-reduced type, β-null (or extremely low-β) type, (α + 11S group I + 11S $A_4A_5B_3$) null-type and (α' + α) null-type variants or segregants have not been reported before (Odanaka and Kaizuma 1989; Odanaka 1990; Phan et al. 1996).

Immunological analysis of β-subunit variants

Immunological analysis was performed to confirm the electrophoretic variants of β-subunit that were detected by using SDS-PAGE analysis (Figure 4). Visible bands of immunological cross-reactions were detected in both β-5_{33} (β-reduced type) and β-5_7 (β-null or extremely low-β type) individuals of the 'β-reduced' line (Figure 4, lanes 1 and 2), but not in (α + β)-$null_{16}$ ((α + β) null-type) individuals of the '(α + β)' null line (Figure 4, lane 3). These results indicated that the β-5_7 individual of the 'β-reduced' line detected by SDS-PAGE still contains β-subunit. However, the β-null character of (α + β)-$null_{16}$ individual derived from (α + β) null line was confirmed by immunological analysis.

Distinct PCR band pattern of the (α + β) null mutant

DNA obtained from Enrei (standard of normal type), Mo-shi-dou Gong 503 (standard of α + β

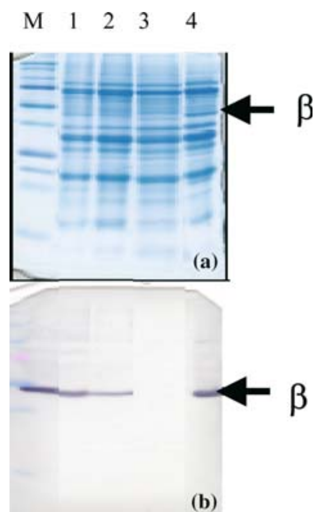

Figure 4. Western immunoblot analysis of the mutants of the 'β-reduced' line and '(α + β) null' line. (a) SDS-PAGE banding patterns (b) Western blot analysis. M: Marker, 1. β-5_{33} (β-reduced type), 2. β-5_7 (extremely low-β type), 3. (α + β)-$null_{16}$ ((α + β) null-type) and 4. Jack (Normal-type control).

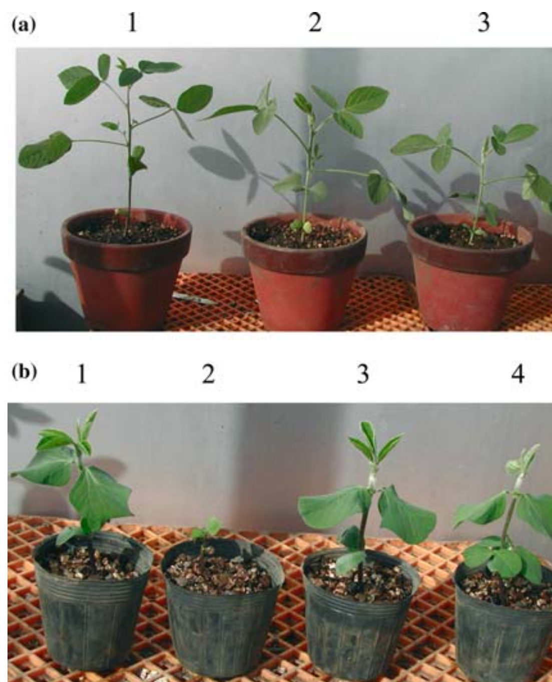

Figure 5. Plants of the 'β-reduced' line and the '(α + β) null' line. (a) Plants of the 'β-reduced' line 1. Enrei (standard of normal type), 2. β-5_{33} (β-reduced type), 3. β-5_7 (extremely low-β type).(b) Plant of the '(α + β) null' line. 1. Enrei, 2. (α + β)-$null_{16}$ ((α + β) null type), 3. (α + β) $null_{71}$ (α-null + β-reduced type) and 4. (α + β)-$null_9$ (β-reduced type).

low type), β-5_{33} (β-reduced type), β-5_7 (extremely low-β type) seeds of 'β-null' line, (α + β)-$null_{71}$ (α-null + β-reduced type), (α + β)-$null_9$ (β-reduced type), and (α + β)-$null_{16}$ ((α + β) null-type) seeds of '(α + β) null' line were used as templates. PCR amplifications were carried out. Since three major subunit genes (α', α and β) of soybean 7S globulin have very high homology (91%) in their nucleotide sequences (Schuler et al. 1982; Harada et al. 1989), we chose specific primers for a partial coding region of β-subunit gene to confirm the uniqueness of amplified sequences. The oligonucleotides used as primers for the amplification of β-subunit gene were synthesized based upon the previously reported sequence of the genomic clone pGmg91 that encode the β-subunit of β-conglycinin in soybean (Tierney et al. 1987).

With the specific primers for the β-subunit, except for (α + β)-$null_{16}$ seed, the presence of an amplified product about 290 bp in size was observed (Figure 6, lanes 1–6). However, the PCR product of (α + β)-$null_{16}$ seed, presented a unique electrophoretic banding pattern beside other materials used in this study. Three bands appeared in (α + β)-$null_{16}$ (Figure 6, lane 7), while only one band in other materials.

Comparison of partial amplified β-subunit gene fragment sequences of different mutants

At the first step in elucidating the real structure we cloned the mutant gene responsible for the null trait. Each of the PCR-amplified DNA fragments of the β-subunit genes of β-subunit variants derived from different strains were cloned and sequenced. The amplified fragment of the β-subunit gene is expected to be 285 bp, starting near the TATA boxes (Figure 7) through most of the first exon of the β-subunit gene (Tierney et al. 1987).

Figure 7 shows the alignment of the amplified β-subunit gene fragments of the β-subunit variants derived from different strains and the corresponding β-subunit gene sequence of genomic clone pGmg91 (Tierney et al. 1987). The comparison reveals that Mo-shi-dou Gong 503, β-5_{33}, β-5_7 and standard Enrei have nearly identical nucleotide sequences. Only one point mutation, T to G that located at 166 bp was identified. The base 'T' in the β-subunit gene sequence of Gmg91 (Tierney

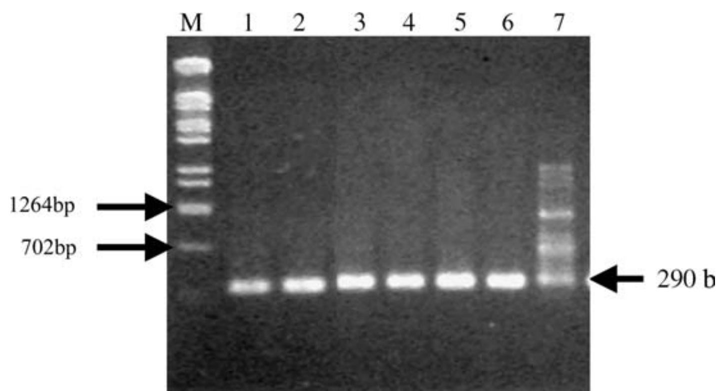

Figure 6. PCR products using primers specific for a partial coding sequence of the β-subunit gene. M: DNA size marker (BstPI digest), 1. Enrei (standard of normal type), 2. Mo-shi-dou Gong 503 (standard of $\alpha + \beta$ low type), 3. β-5_{33} (β-reduced type), 4. β-5_7 (extremely low-β type), 5. ($\alpha + \beta$)-null_{71} (α-null + β-reduced type), 6. ($\alpha + \beta$)-null_9 (β-reduced type) and 7. ($\alpha + \beta$)-null_{16} (($\alpha + \beta$) null-type).

et al. 1987) and Enrei was altered to 'G' in that of Mo-shi-dou Gong 503, β-5_{33} and β-5_7 individuals.

Figure 8 shows the partial amplified β-subunit gene fragments between Enrei and the mutants of the '($\alpha + \beta$) null' line. Identical amplified sequences were detected in the amplified fragments of Enrei, ($\alpha + \beta$)-null_{71} and ($\alpha + \beta$)-null_9. However, a unique sequence was identified in ($\alpha + \beta$)-null_{16} (shown by letters on a gray background in Figure 8). The amplified β-subunit gene sequence of ($\alpha + \beta$)-null_{16} strongly deviates from the standard sequence. A low degree of nucleotide homology, 53.1% (shown with asterisks in Figure 8), exists between the ($\alpha + \beta$)-null_{16} mutant and the standard Enrei sequence. Instead of 285 bp, a 305 bp amplified product was obtained. Furthermore, a 5′-primer sequence replacing the 3′-primer amplified the opposite DNA strands (shown underlined twice in Figure 8).

Discussion

In recent years several mutants of 7S globulin subunits have been obtained (Kitamura and Kaizuma 1981; Odanaka and Kaizuma 1989; Ogawa et al. 1989; Takahashi et al. 1994; Hajika et al. 1996). The molecular basis of these mutations has also been intensively studied (Beachy et al. 1985; Chen et al. 1986, 1988; Bray et al. 1987; Naito et al. 1988; Allen et al. 1989; Lessard et al. 1991; Teraishi et al. 2001). As a stable β-null variation has not been found, free recombination among 7S globulin subunits has not been successful and the mechanism underlying seed-specific regulation is still unclear. In the previous studies, α- and β-subunit genes proved to be tightly linked (Kitamura et al. 1984; Davies et al. 1985; Tsukada et al. 1986). However, in our study, α-null + β-reduced type and β-null (or extremely low-β) type in the progeny seeds of the '($\alpha + \beta$) null' line in 2002 and 2003, respectively, were detected (Figures 2 and 3). Recombination between α- and β-subunits of the '($\alpha + \beta$) null' line was identified for the first time. Furthermore, we identified β-subunit variations, i.e., β-reduced, extremely low-β and β-null, among the progeny of the Vietnamese hybrid line, Chinese cultivated species and the progeny of Japanese '($\alpha + \beta$) null' line. The mutants lacking both α- and β- subunits (($\alpha + \beta$) null-type) were associated with lethal chlorosis at the young seedling stage (Figures 5b and 2). All other mutants characterized by various protein subunits compositions germinated and grew normally without apparent abnormalities (Figure 5). These results suggest that recombination among 7S globulin subunits is possible. Using these 7S-related specific variants, we are trying to elucidate the mechanism for the control of the expression of ($\alpha + \beta$) null deficiency and the relationship of soybean 7S globulin subunit genes.

Some alleles affect the viability of individuals that carry them. In most cases the homozygous recessive individual does not survive, but the heterozygotes may have a normal life span. In some instances, a homozygote may appear in reduced numbers. In

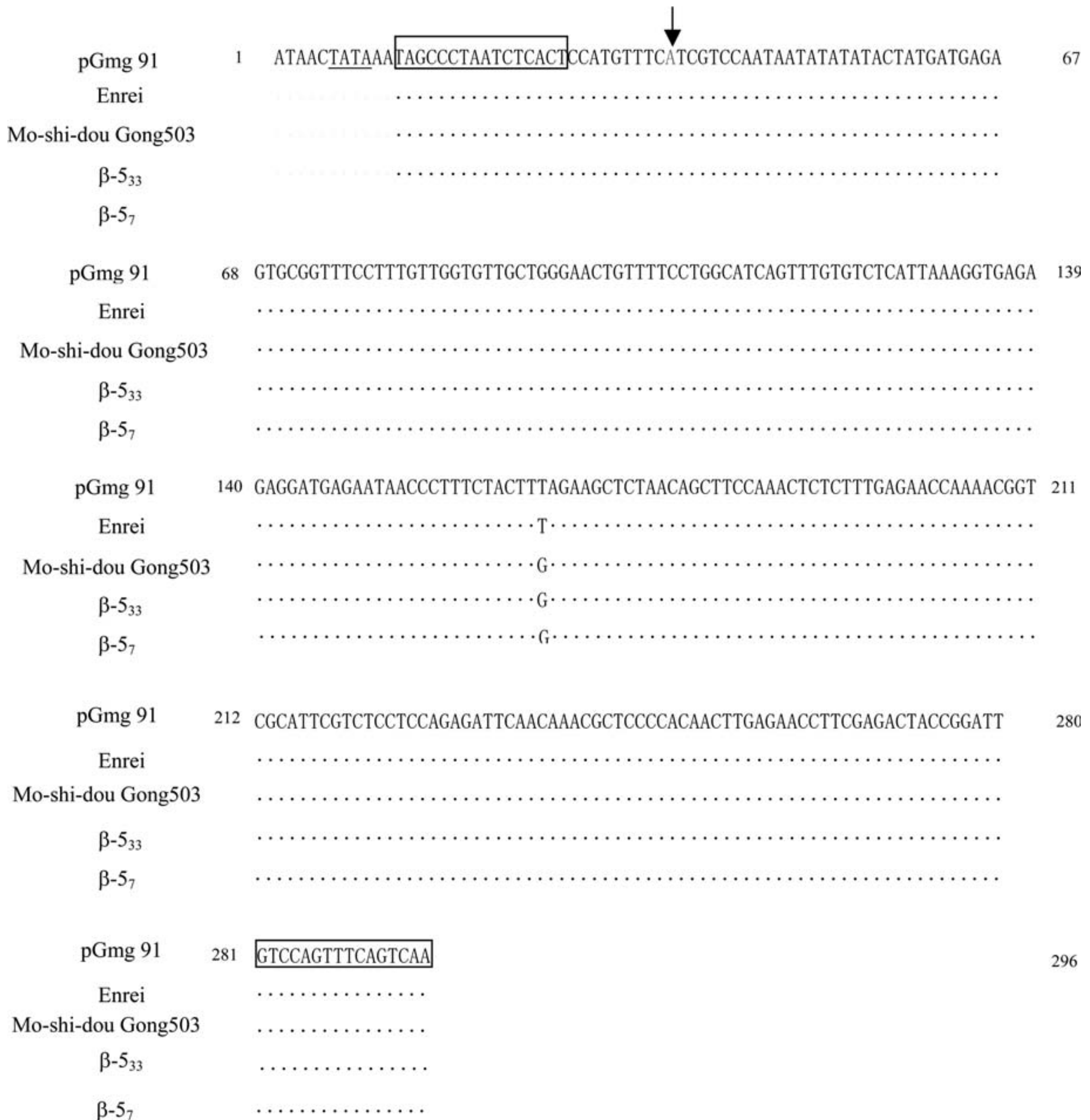

Figure 7. Sequence comparison between Enrei (standard of normal type) and β-reduced type mutants. Nucleotides identical to those of Gmg91 are shown by dots and only the nucleotide that differs among the five β-subunit gene sequences are shown. TATA box is underlined. Oligonucleotides used as primers for PCR amplification are marked by box. An arrow indicates the probable transcription start site (Slightom et al. 1983). Numbers indicate the position of nucleotides (Tierney et al. 1987).

the case of the '($\alpha + \beta$) null' line, the mutant trait of ($\alpha + \beta$) deficiency accompanying lethal chlorosis was considered to be caused by a single gene mutation (Odanaka and Kaizuma 1989; Odanaka 1990; Phan et al. 1996), and homozygous recessive individuals with ($\alpha + \beta$) deficiency showed lethal chlorosis at the seedling stage. Instead of the segregation of 1:2:1 for a single gene trait, a distorted segregation ratio of 3 normal:4 intermediate:1 ($\alpha + \beta$) null has been reported (Phan et al. 1996). In our study, the segregation ratio of normal type:($\alpha + \beta$) null type was 10:1 (43/4) in 2001, 7:1

Figure 8. Sequence comparison between Enrei (standard of normal type) and the mutants of '($\alpha + \beta$) null' line. Oligonucleotides used as primers for PCR amplification are marked by box. Letters on a gray background show the distinct 305 bp β-subunit gene sequence amplified from ($\alpha + \beta$)-$null_{16}$ mutant. Asterisks indicate nucleotides in Enrei identical to those in ($\alpha + \beta$)-$null_{16}$ (homology is 53.1%). The inverted 5′-primer segment identified in the ($\alpha + \beta$)-$null_{16}$ are underlined twice. Arrows indicate the direction of PCR amplification.

(205:29) in 2002 and 8:1 (284/35) in 2003, respectively (Figure 3). These results are similar ($p > 0.05$) to the previously reported segregation ratio 7:1 ((3 + 4):1) (Phan et al. 1996). Reduced numbers of homozygous recessive individuals were screened in 2001 (10:1) and 2003 (8:1). These results seem attributable to the lethal chlorosis trait of ($\alpha + \beta$)-deficient individuals. Further studies such as cross combination among different variants of '($\alpha + \beta$) null' line with a larger number of independent mutants will help elucidate the inheritance of the variations of the '($\alpha + \beta$) null' line.

Using the DNA template of the ($\alpha + \beta$) null mutant, with the same specific primers for β-subunit genes and the same amplification condition, we detected PCR amplified products (Figure 6). This is quite different from the report by Phan et al. (1996) where no PCR product was detected by the PCR using the DNA template of ($\alpha + \beta$) null mutant, and therefore they inferred that the α and β null mutant is related to the deletion of a chromosome segment with the genes encoding α- and β-subunits of soybean 7S globulin (Phan 1996). However, PCR products of the ($\alpha + \beta$)-

$null_{16}$ mutant gave three bands in the present study (Figure 6, lane 7) suggesting that the absence of α- and β-subunits is not caused by the structural defect of both α- and β-subunit genes as reported by Phan et al. (1996).

One base change of T to G between standard Enrei and β-less variants was detected (Figure 7). However, similar point variation was also identified in other mutant lines, 'Karike 434' (α′ null-type) and 'Tohoku 124' (α′ + α null-type) (data not shown). These results suggested that the base change has no effect on the β-reduced mutant. Further studies such as site-directed mutagenesis and run-on transcription studies are necessary to determine whether the absence of β-subunit is associated with alteration of the single base change.

On the other hand, using the DNA template of (α + β)-$null_{16}$ mutant individuals, a unique 305 bp amplified PCR product sequence that strongly deviated from the standard sequence (Figure 8) was screened. We are performing further studies such as PCR southern blot hybridization analysis, RFLP analysis, cloning and sequencing of the 702 bp, 1264 bp PCR products (Figure 6) and the whole β-subunit gene sequence of the (α + β) null mutant to determine whether (α + β)-null accompanied with lethal chlorosis traits is attributed to the deviated gene sequence of the (α + β) null mutant.

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