Theor Appl Genet (2001) 103:1266–1272

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Suppression of soybean β-conglycinin genes by a dominant gene, *Scg-1*

Received: 5 January 2001 / Accepted: 20 March 2001

Abstract β-Conglycinin (7S globulin) is a predominant seed storage protein found in soybean [*Glycine max* (L.) Merr.]. A spontaneous mutant lacking the β-conglycinin subunits, α′, α and β, has been identified among Japanese wild soybean genetic resources. Although a multigene family encodes the subunits and is distributed in different linkage groups, a single dominant gene, *Scg-1* (a suppressor of β-conglycinin), controls the mutant trait. This report characterized the genetic and molecular basis of *Scg-1*. The null trait was caused not by either structural defects or changes in the β-conglycinin subunit genes, but by a lack of transcription of the genes, indicating that *Scg-1* suppresses expression of all the structural genes. Linkage analysis revealed that the *Scg-1* locus was located in the same chromosomal region as the α and β subunit genes, which are tightly linked to each other. Furthermore, the methylation of the chromosomal region containing the *Scg-1* and α and β subunit loci was observed, suggesting that the deficiency is associated with the silencing of multicopy genes. *Scg-1* had no obvious effect on the plant growth, so it will be a useful gene source for manipulation of the protein composition in soybean seeds.

Communicated by H.C. Becker

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Keywords *Glycine soja* · *Glycine max* · 7S Globulin · 11S Globulin · Glycinin · Linkage analysis · Dominant null mutant

Introduction

Soybean seeds are one of the most-important vegetable protein sources for foods and industry because of their high protein content (about 40%). The storage proteins in soybean seed consist of two major components, 7S globulin (β-conglycinin) and 11S globulin (glycinin), which together account for about 70% of the total seed protein (Derbyshire et al. 1976). These ssed proteins are encoded by two multigene families. β-Conglycinin is a glycoprotein with a molecular weight of about 150,000, and consists of three primary subunits, α′, α and β, which interact to form a trimeric protein with different subunit compositions (Thanh and Shibazaki 1978). Glycinin is a hexamer with a molecular weight of about 350,000 consisting of five intermediate subunits, and each subunit is composed of an acidic (A) and a basic (B) polypeptide linked by a single disulfide bond (Mori et al. 1981; Staswick et al. 1984). The two globulins differ in amino-acid composition and in their characteristics for food production. Glycinin has a higher content of sulfur-containing amino acids and better gel-forming ability than β-conglycinin (Koshiyama 1968), and its content is inversely proportional to the content of β-conglycinin (Ogawa et al. 1989). Therefore, a reduction of β-conglycinin may provide improvement in the quality of soybean seed protein without a decrease in seed protein content.

Several spontaneous and induced mutants with an altered composition of β-conglycinin have been obtained and intensively studied with respect to their inheritance and the molecular basis of the mutations (Kitamura et al. 1984; Ladin et al. 1984; Tsukada et al. 1986, Kaizuma et al. 1989). Such mutant traits were generally found to be due to recessive genes and were sometimes accompanied by abnormalities. Furthermore, few mutants lacking the

β subunit of β-conglycinin have been obtained, because there are a large number of β subunit genes in the soybean genome (Harada et al. 1989). A single mutant line lacking the α´, α and β subunits of β conglycinin was isolated after γ-ray irradiation treatment (Kitagawa et al. 1991). Because the deficiency controlled by a single recessive gene, *Cgdef*, is accompanied by some abnormalities, such as sterility and lethality, the mutant line is maintained only in heterozygotes (Hayashi et al. 2000). The normal, *Cgdef*, gene product seems to regulate the expression of the β-conglycinin subunit genes at the transcriptional or post-transcriptional level (Hayashi et al. 1998). A new phenotype lacking β-conglycinin was found among Japanese wild soybean (*Glycine soja* Sieb. et Zucc.) genetic resources (Hajika et al. 1996). Genetic analysis of the wild accession showed that the null trait is controlled by a single dominant gene, *Scg-1* (a suppressor of β-conglycinin) (Hajika et al. 1998). The wild soybeans grows normally without any physiological abnormalities, and *Scg-1* was successfully incorporated into soybean cultivars. *Scg-1* affects the accumulation of β-conglycinin, but does not have adverse effects on any other trait (Hajika et al. 1998). Therefore, *Scg-1* may be useful for controlling the composition of soybean seed protein. In this report, we characterized the molecular basis of *Scg-1* by comparison with normal and other globulin-mutant soybean lines.

Material and methods

Plant materials

SDS-PAGE banding patterns and the compositions of β-conglycinin and glycinin in the six soybean cultivars and lines used here are shown in Fig. 1. QT2 is a wild soybean accession containing *Scg-1* (Hajika et al. 1996). Two near-isogenic lines, QY2–5 and QY2–7, were developed by five-times backcrossing with QT2 as the donor parent and Fukuyutaka as the recurrent parent. QY2–5 lacks α´, α and β subunits of β-conglycinin due to the presence of *Scg-1*, and QY2–7 contains the normal components of β-conglycinin due to the presence of *scg-1*. Tohoku 124 is a mutant line that lacks the α´ and α subunits and contains a low level of the β-subunit (Takahashi et al. 1994). A breeding line, EnB1, lacking the five glycinin subunits, was obtained from the Nagano Chushin Agricultural Experiment Station, Japan. Two soybean cultivars, Fukuyutaka and Tachiyutaka, were used as controls. A population of 1,035 individuals in the F_2 generation between QY2–5 and Tohokul124 were used for the linkage analysis of *Scg-1* to each locus of the β-conglycinin subunit genes.

Extraction of seed protein and Western-blot analysis

The detection of globulin subunits was performed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel-electrophoresis) (Laemmli 1970). Proteins were extracted from 3 mg of soybean seed meal by grinding the seeds with 250 µl of 50 mM Tris-HCl buffer solution at pH 8.0, and then letting the extract stand for 60 min at room temperature. The supernatant was obtained after centrifugation at 15,000 g for 10 min. Protein concentration was determined using a BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as a standard. Appropriate quantities of protein were separated by SDS-PAGE using a separation gel containing 12% acrylamide and 0.2% bis-acrylamide, and were stained with 0.2% Coomassie brilliant blue G250.

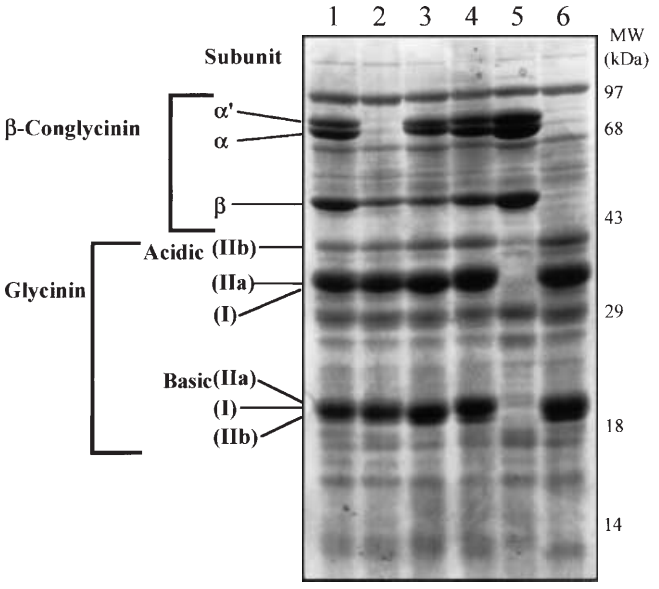

Fig. 1 Genetic variations of soybean seed proteins. *Lane 1* Tachiyutaka, *lane 2* Tohoku 124, *lane 3* Fukuyutaka, *lane 4* QY2–7, *lane 5* EnB1, *lane 6* QY2–5. Seed protein (20 µg) was separated by SDS-PAGE using a separation gel containing 12% acrylamide and 0.2% bis-acrylamide

For the Western-blot analysis, separated proteins on an SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and reacted with polyclonal antibodies against each globulin 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.

Southern-blot analysis

Total plant DNA was isolated from leaves by a modified SDS-potassium method (Draper and Scott 1988). Six micrograms of each total DNA were digested with a restriction enzyme and was fractionated by electrophoresis in a 0.8% agarose gel. After electrophoresis, DNAs were transferred to a nylon membrane (Sambrook et al. 1989). The cDNA clones of each subunit listed in Fig. 2 were used as hybridization probes. Probe labelling, hybridization and detection were used with ECL direct nucleic acid labelling and detection systems (Amersham Pharmacia Biotech.).

Northern-blot analysis

Total RNAs were extracted from immature seeds collected at about 30 days after flowering by the SDS-phenol method (Hall et al. 1978). Denatured RNAs in the buffer (50% formamide, 17.5% formaldehyde, 1% MOPS, 0.5% SDS) were electrophoresed in 0.9% agarose gel (5% formaldehyde, 1% MOPS) and transferred to a nylon membrane. The cDNA clones of each subunit listed in Fig. 2 were provided as hybridization probes. Probe labelling, hybridization and detection were used with ECL direct nucleic acid labelling and detection systems (Amersham Pharmacia Biotech.).

Results

Molecular features of *Scg-1*

Southern-blot analysis (Fig. 2A) was carried out to investigate whether the absence of β-conglycinin subunits in

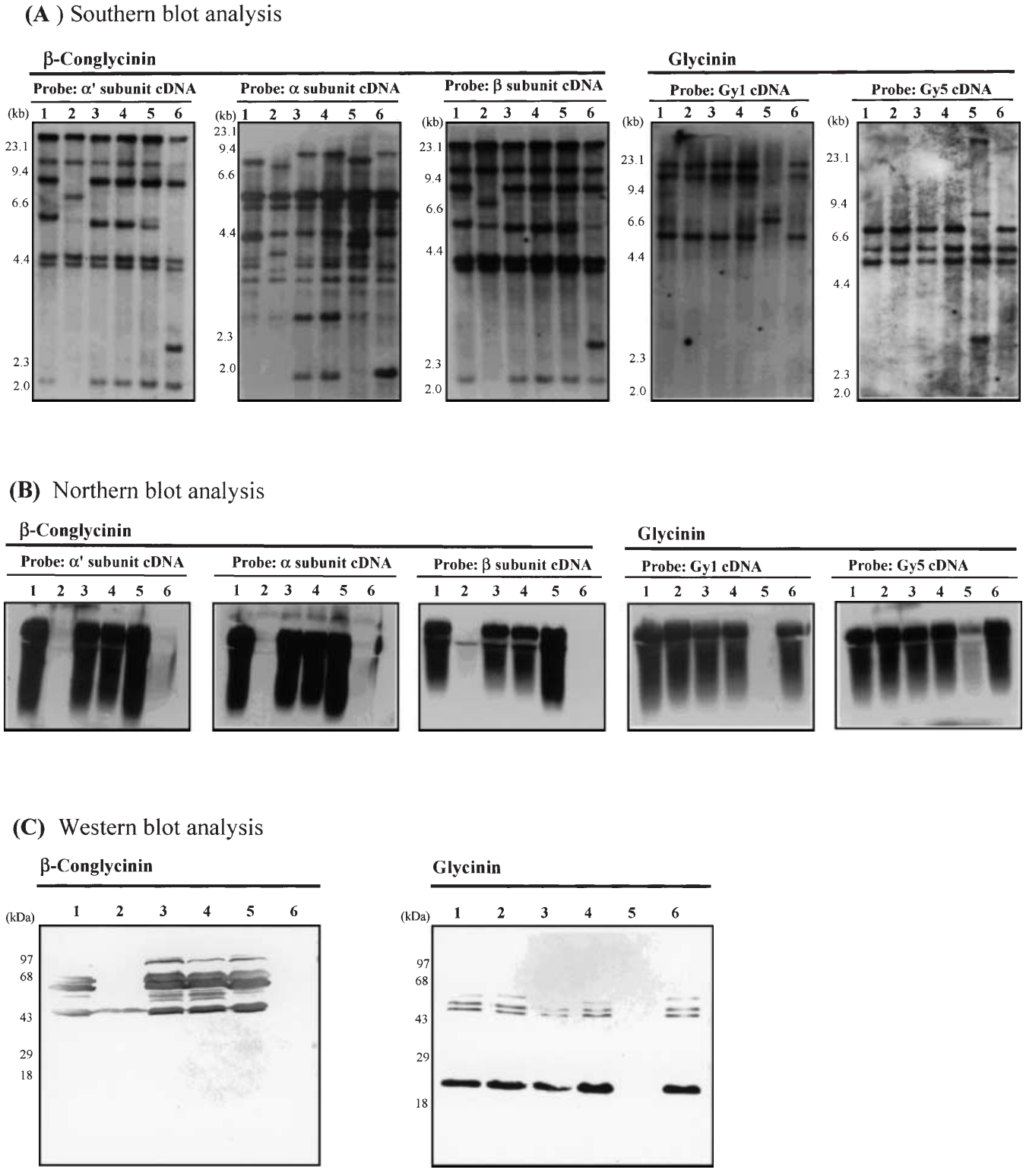

Fig. 2A–C Analysis of genomic DNA and mRNA of β-conglycinin and glycinin globulin in mutant soybeans. (**A**) Southern-blot analysis was performed by hybridization with β-conglycinin cDNA or glycinin cDNA. *Hin*dIII-digested total DNAs were hybridized with the α′ subunit, the β subunit or the Gy5 cDNA probe. *Eco*RV-digested total DNAs were hybridized with the α-subunit or the Gy1 cDNA probe. *Lane 1* Tachiyutaka, *lane 2* Tohoku124, *lane 3* Fukuyutaka, *lane 4* QY2–7, *lane 5* EnB1, *lane 6* QY2–5. (**B**) Northern-blot analysis was performed by the hybridization with β-conglycinin cDNA or the glycinin cDNA probe. The same cultivars and lines were used in the same order as in (**A**). (**C**) Polypeptides were separated by SDS-PAGE, and subjected to immunoblot analysis using polyclonal antibodies against β-conglycinin subunits or glycinin acidic subunits. The same cultivars and lines were used in the same order as in (**A**)

Table 1 Segregation for β-conglycinin subunits in the F_2 seeds derived from crosses between QY2–5 and Tohoku 124. The segregation for *Scg-1* (783 *Scg-1*: 252 *scg-1/scg-1*) fitted a 3:1 ratio (χ^2=0.235, *P*=0.628). The segregation for α subunit (196 presence: 56 absence) fitted a 3:1 ratio (χ^2=1.037, *P*=0.309)

	β-conglycinin subunit			*Scg-1*	No. of seeds		
	α´	α	β				
P_1 QY2–5	–	–	–	+			
P_2 Tohoku 124	–	–	+	–			
F_1	–	–	–	+			
F_2	–	–	–	+	783		
	–	–	+	–	56	Subtotal	Total
	+	–	+	–	196	252	1,035

QT2 was caused by lack of, or defects in, the structural genes. Hybridization with the glycinin cDNAs produced identical blotting patterns in five types of soybeans, and a different pattern in EnB1. EnB1 is an integrated breeding line with three recessive genes responsible for a triple deficiency of glycinin subunit groups, I, IIa and IIb (Yagasaki et al. 1996). Hybridization with the Gly1 cDNA probe showed only a single band in EnB1 after digestion with *Eco*RV or *Hin*dIII (Fig. 2A and data not shown). Group I subunits consist of three subunits encoded by three tightly linked genes, *Gy1*, *Gy2* and *Gy3*, and the deficiency of group I subunits in EnB1 is due to mutation induced by γ-ray irradiation (Odanaka and Kaizuma 1989). These results suggest that the deficiency of group I subunits may be caused by a deletion in the coding region.

Similarly, some definite differences were observed between the banding patterns of the ordinary type cultivars and the two mutant lines, Tohoku 124 and QY2–5, by hybridization with the β-conglycinin cDNA probes. QY2–5 showed a polymorphism in any combination of the probes and restriction enzymes compared to its sib line QY2–7. For example, QY2–5 lacked a fragment about 5.8 kb, and contained a fragment about 3 kb on the blot hybridized with an α´ subunit probe (Fig. 2A lanes 4 and 6). The two lines, however, shared other comparable bands that hybridized with the probe. Tohoku 124 is an integrated breeding line with two recessive genes for a double deficiency of β-conglycinin subunits, α´ and α, and its genetic background is relatively similar to Tachiyutaka by two-times backcrossing with the cultivar as the recurrent parent (Takahashi et al. 1994). The banding pattern of Tohoku 124 is distinct from that of Tachiyutaka by the presence or absence of some fragments (Fig. 2A lanes 1 and 2). Structural change of the coding or vicinal region of the β-conglycinin gene seemed to be present in the two mutants.

Total RNAs were extracted from developing seeds and subjected to Northern-blot analysis (Fig. 2B). Transcripts of glynicinin subunit genes were detected in five soybean lines, but not in EnB1, in which no Gy1 mRNA was detected and only a small amount of Gy5 mRNA was detected. Signals for the three β-conglycinin subunit gene transcripts were observed in four lines, but not in QY2–5 or Tohoku 124. Tohoku 124 seeds accumulated no α´ or α subunit mRNAs, but did accumulate a low level of β subunit mRNAs. Tohoku 124 is a mutant breeding line that lacks α´ and α subunits and contains a low level of β subunit (Figs. 1 and 2C) (Takahashi et al. 1994). Since soybean contains at least six clustered β subunit genes (Harada et al. 1989), the above result suggests that one or more of the β subunit genes in Tohoku 124 retain its transcription activity. In contrast, none of the β-conglycinin subunit genes were transcribed in QY2–5, which resulted in the absence of the α´, α and β subunits (Fig. 2C). Moreover, these polypeptides were not observed in the developing seeds of QY2–5 (data not shown). *Scg-1* seems to regulate β-conglycinin gene expression transcriptionally or post-transcriptionally (e.g. at the level of mRNA stability).

Linkage analysis of *Scg-1* and the β-conglycinin subunit genes

The F_2 population obtained from a cross between QY2–5 and Tohoku 124 was used to determine linkage relationships between *Scg-1* and the β-conglycinin subunits. A total of 1,035 F_2 seeds were analyzed by SDS-PAGE to determine the composition of the β-conglycinin subunits. Although Tohoku 124 contains only the β subunit and QY2–5 lacks all subunits, segregation of the α´ and β subunits was observed (Table 1 and Fig. 3). Among the F_2 seeds, 783 lacked all the β-conglycinin subunits due to the presence of *Scg-1*. The segregation of *Scg-1* fitted the expected ratio if *Scg-1* was a single dominant gene (χ^2=0.235, *P*=0.628). Among the 252 F_2 seeds in which the genotype was judged as *scg-1/scg-1*, 196 F_2 seeds contained the α´ and β subunits, and 56 F_2 seeds contained only the β subunit. The segregation for the α´ subunit fitted a 3:1 ratio (χ^2=1.037, *P*=0.309). these results suggest that QY2–5 contains a normal α´ subunit gene, and that the α´ subunit gene is inherited independently from *Scg-1* and other subunit genes. However, no F_2 seeds containing the α subunit appeared. These observation and the tight linkage between the α and β subunit genes (Tsukada et al. 1986; Harada et al. 1989) suggest that *Scg-1* is located near the α and β subunit genes, although other possiblities, such as a lack or defect of the α subunit gene in QY2–5, cannot be excluded.

Ninety eight plants randomly selected from the F_2 population were subjected to Southern-blot analysis in order to characterize the relationship between the *Scg-1* and the β-conglycinin subunit genes. When the *Hin*dIII-digested DNAs were hybridized with an α subunit cDNA probe, tight linkage between the α´ subunit and two bands, named B1 and B2, was observed in the F_2

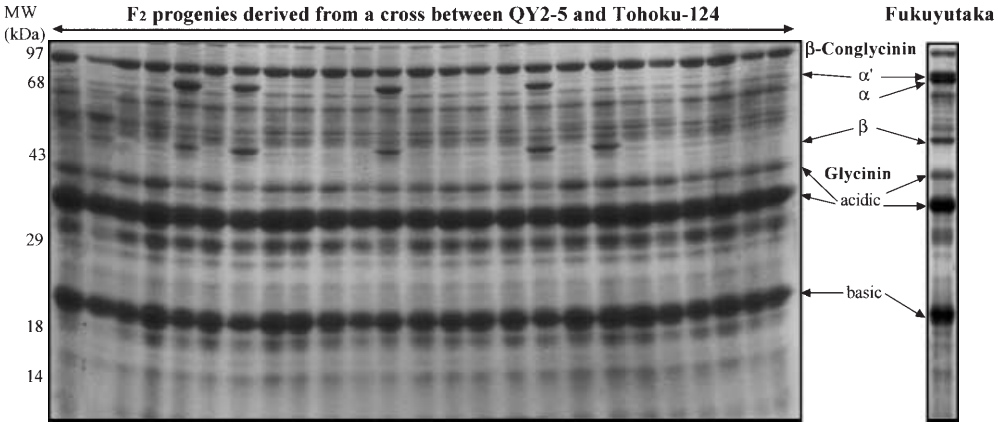

Fig. 3 Segregation patterns of β-conglycinin subunits in the F_2 progeny derived from crosses between QY2–5 and Tohoku 124. Seed protein (20 μg) was separated by SDS-PAGE on a 12% acrylamide separation gel. The *left lane* shows a banding pattern of Fukuyutaka with normal globulin components

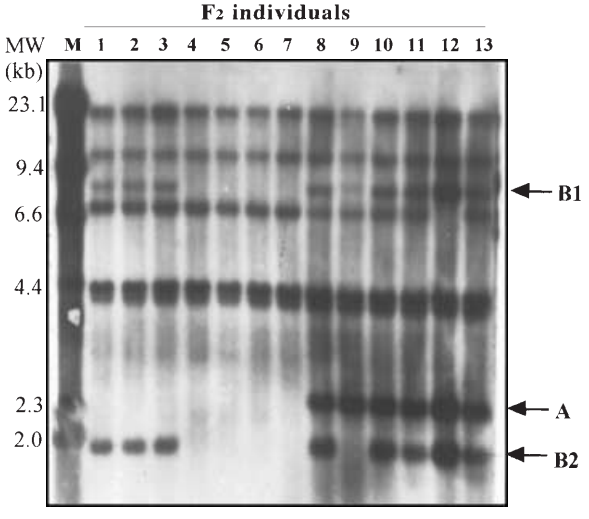

Fig. 4 Southern-blot analysis of the F_2 progeny derived from crosses between QY2–5 and Tohoku 124. *Hin*dIII-digested total DNAs were hybridized with an α subunit cDNA probe. *Lanes 1 to 3* F_2 plants containing *scg-1*/*scg-1*, α′ and β subunits, *lanes 4 to 7* F_2 plants containing *scg-1*/*scg-1* and β subunit, *lanes 8 to 13*. F_2 plants containing *Scg-1*. A band, *A*, was linked to the *Scg-1* locus, and two bands, *B1 and B2*, might be linked to the α′ subunit locus

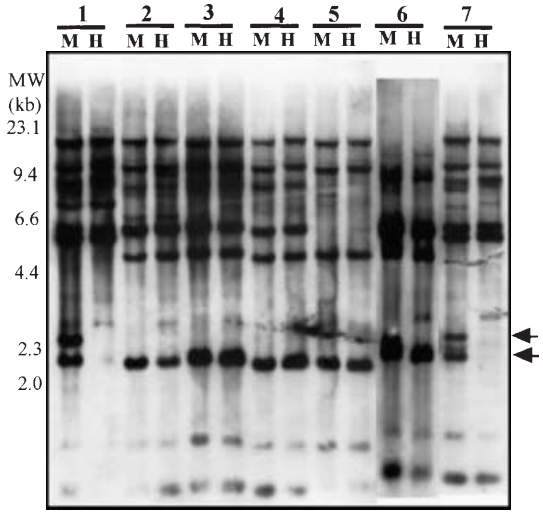

Fig. 5 Southern-blot analysis of mutant soybean lines. *Lane 1* QY2–5, *lane 2* QY2–7, *lane 3* Fukuyutaka, *lane 4* Tohoku 124, *lane 5* Tachiyutaka, *lane 6* EnB1, *lane 7* QT-2. The DNA was digested with *Msp*I (*M*) or *Hpa*II (*H*), and hybridized with a *Bam*HI–*Xho*I fragment of α-subunit cDNA. *Arrows* indicate different bands between the samples digested with *Msp*I or *Hpa*II

plants containing α′ and/or β subunits (Fig. 4). The lack of the α′ subunit in Tohoku 124 results from the deletion of the coding sequence of the α′ subunit gene (Ladin et al. 1984). The absence of bands B1 and B2 may imply the deletion of the α′ subunit gene in the F_2 plants containing no α′ subunit. The F_2 plants lacking β conglycinin showed similar banding patterns to these of the F_2 plants containing α′ and/or β subunits, except for the presence of a band, named A, suggesting that band A is tightly linked to *Scg-1* and is derived from the chromosomal region in which the α and β subunit genes are located in QY2–5.

Involvement of methylation in the deficiency of β-conglycinin

Gene silencing of transgenes and endogenous muticopy sequences is a common feature in plants and is frequently associated with DNA methylation (Selker 1999). In order to explore the relationship between *Scg-1* and methylation, we compared the hybridization patterns of the genomic DNA of each line after cleavage with *Msp*I or *Hpa*II. *Msp*I and *Hpa*II cleave at the sequence CCGG, but they have different sensitivities to cytosine methylation. *Msp*I is blocked from digesting ^{m}CCGG, whereas *Hpa*II is blocked from digesting ^{m}CCGG and C^{m}CGG. Total DNAs from seven soybean cultivars and lines were treated with either *Msp*I or *Hpa*II, and subjected to Southern-blot analysis with an α′ subunit cDNA probe. QT2 and QY2–5, which are *Scg-1* mutants, displayed different banding patterns depending on whether the samples were digested with *Msp*I or *Hpa*II, while other soybean cultivars and mutant lines showed the same patterns (Fig. 5). The two bands of around 2.3 kb were not detected in the *Hpa*II-digested samples (Fig. 5 lanes 1 and 7), indicating that β-conglycinin genes and/or their

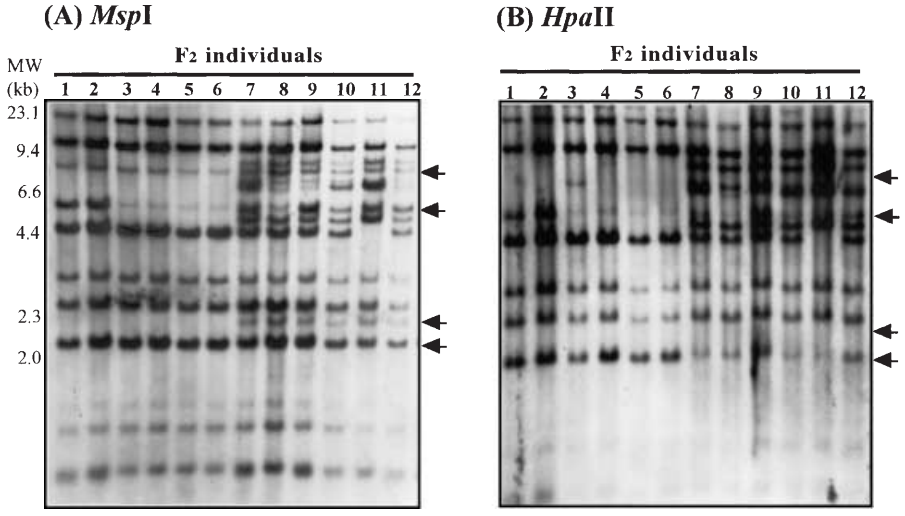

Fig. 6A, B Comparison of hybridization patterns in the F_2 progeny derived from crosses between QY2–5 and Tohoku 124. The DNA was digested with *Msp*I (**A**) or *Hpa*II (**B**), and hybridized with a *Bam*HI–*Xho*I fragment of α-subunit cDNA. DNA samples from a given F_2 plant were applied in the same-numbered lanes in figures (**A**) and (**B**). *Lanes 1 to 6* F_2 plants containing *scg-1*/*scg-1*, *lanes 7 to 12* F_2 plants containing *Scg-1*. *Arrows* indicate different bands between the samples digested with *Msp*I or *Hpa*II

homologous gene regions are highly methylated. The digests diagnostic for DNA cytosine methylation were analyzed in the F_2 population obtained from a cross between QY2–5 and Tohoku 124 (Fig. 6), and the same phenomenon was also observed in the F_2 populations: The chromosomal region, hybridized with the β-conglycinin gene probes, was highly methylated in the F_2 plants lacking β-conglycinin. The inactivation of β-conglycinin genes in *Scg-1* mutants might be related to the DNA methylation, although we can not exclude the possibility that tight linkage between *Scg-1* and a highly methylated chromosomal region is a coincidence.

Discussion

Many mutants for seed protein components have been reported in various crops. However, most mutant traits are controlled by recessive genes and dominant mutants are rarely observed. A rice mutant which contains a low amount of glutelin and a high amount of prolamine compensating for the reduction of glutelin was obtained by induced mutation (Iida et al. 1993). The mutant seed lacks the largest polypeptide of the glutelin subunits, and a single dominant gene governed these characters. However, the mechanism underlying the deficiency has not been clarified. The β-conglycinin null trait of the soybean mutant used in this study is also controlled by a single dominant gene, *Scg-1*, and can be successfully incorporated into soybean cultivars without causing any physiological abnormalities (Hajika et al. 1998). β-Conglycinin is composed of α′, α, and β subunits encoded by a multigene family containing about 20 genes (Ladin et al. 1984), and the α subunit and β subunit genes are inherited independently from the α′ subunit gene (Tsukada et al. 1986). In addition, the results of Southern- and Northern-blot analyses demonstrated clearly that the deficiency is caused not by a deletion or defect of the structural genes encoding the β-conglycinin subunits, but by a lack of the transcription of the genes. Another mutant line lacking β-conglycinin was obtained by γ-ray irradiation, and the null mutation was controlled by a single recessive gene, *cgdef* (Kitagawa et al. 1991; Hayashi et al. 2000). The *cgdef* mutant is also characterized by a lack of the transcripts for the β-conglycinin subunit genes (Hayashi et al. 1998). The *cgdef* mutant phenotype appears to result from changes in regulatory genes that cause pleiotropic effects such as sterility and lethality, so that plants homozygous for the *cgdef* locus have never been obtained. The mechanism of the deficiency in the *Scg-1* mutant may be different from that in the *cgdef* mutant.

The segregation analysis of the F_2 plants derived from a cross between QY2–5 and Tohoku 124 suggested that the *Scg-1* locus resides in the vicinity of the α and β subunit gene loci. Linkage analysis of β-conglycinin variants and molecular analysis of β-conglycinin gene organization has indicated that the α subunit genes, the β subunit genes, and homologous genes are tandemly organized and interspersed among each other (Davies et al. 1985; Harada et al. 1989). There are at least ten β-conglycinin genes and homologous sequences in this region. Furthermore, the methylation of the chromosomal region containing the *Scg-1* and α and β subunit loci was observed by comparison of hybridization patterns of genomic DNA cleaved with *Msp*I or *Hpa*II. These findings suggested that homology dependent gene silencing occurs in the *Scg-1* mutant. Gene-silencing phenomena have been observed in the transgenic plants with repeated transgenes, and have also been found in repeated endogenous genes (Bender and Fink 1995; Ronchi et al. 1995). Recent findings suggest that certain repeats, such as inverted repeats, are likely to be powerful inducers of silencing and methylation (Luff et al. 1999). Since inverted repeats are produced during natural chromosomal rearrangements, the complex region containing β-conglycinin genes might contain β-conglycinin-homologous inverted repeats. It will be necessary to clone *Scg-1* or analyze the chromosomal region containing the β-conglycinin genes in order to elucidate the mechanism that leads to the null trait.

β-Conglycinin and glycinin are composed of three and five major subunits, respectively, and constitute ap-

proximately 70% of soybean seed proteins (Derbyshire et al. 1976). Several mutants that affect the accumulation of glycinin subunits have been detected in soybean germplasms or have been obtained by induced mutation. Null mutations of group I, IIa, or IIb glycinin subunits make it possible to control glycinin composition (Kitamura and Kaizuma 1981; Kaizuma et al. 1989; Yagasaki et al. 1996). Moreover, since *Scg-1* controls the presence of β-conglycinin, it may be possible to develop a soybean line lacking both β-conglycinin and glycinin. Soybean seeds possess great ability to produce and store proteins in the seeds. Breeding of a soybean line lacking these major storage proteins could lead to new applications in addition to improvement of the nutritional and functional quality of the seed. For example, instead of the accumulation of the two major seed proteins, a large amount of foreign products such as pharmaceutical preparations could be generated in soybean seeds. We are now attempting to select a line lacking the major β-conglycinin and glycinin subunits and to evaluate them.

Acknowledgments We thank Dr. S. Utsumi for providing the cDNA probes of the β-conglycinin and glycinin subunits, Dr. K. Yagasaki for providing the EnB1 line and the antibodies against glynicinin, and Dr. S. Naito for providing the antibodies against β-conglycinin. We also thank Dr. A. Kaga for his technical suggestions. This work was in part supported by the Cooperative System for Supporting Priority Research providid by the Japan Science and Technology Corporation.

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