Bull. Fac. Agr., Saga Univ. No. 83 : 31~36 (1998)

Genetic Analysis of Restriction Fragment Length Polymorphism on the Fatty Acid Synthesis in Soybean Mutants and Their Progenies: I. Low linolenic acid mutants with a microsomal ω-3 fatty acid desaturase cDNA as a probe

Yukie YAMASHITA, Takehito KINOSHITA, Shaikh Mizanur RAHMAN, Kazuhiko OKADA*, Toyoaki ANAI and Yutaka TAKAGI
(Laboratory of Plant Breeding)
Received September 7, 1998

Summary

Mutant lines of soybean [*Glycine max* (L.) Merr.], J18, KL-8, M-5 and M24, were selected from X-ray treated Bay cultivar. While the linolenic acid content of Bay was about 8.3% of total fatty acid, the contents of this faffy acid in these mutants were about 4.8%, 6.9%, 4.4% and 5.9%, respectively. The remarkable decreases of linolenic acid content in these mutants were expected to be the result of mutations at the *Fan* (J18 and M-5) and *Fanx* (KL-8 and M24) loci induced by X-ray irradiation. In this study, we analyzed for mutations by restriction fragment length polymorphism (RFLP) analysis using a microsomal ω-3 fatty acid desaturase cDNA as a probe. The probe which hybridized with one band (4.5 kbp) was recognized in Bay and other lines except J18. Thirty-two plants grown from the embryonic part of F_2 seeds made from a cross between J18 and Bay were analyzed with the same method. The F_2 seeds were also analyzed by gas chromatography for their linolenic acid content. The result of this study showed that the segregation ratio for intensities of the 4.5 kbp band fitted the expected 1: 2: 1 ratio (Bay: F1: J18; $\chi^2=0.19$, $P>0.9$) . Moreover, the intensities of this fragment were completely comparable with their linolenic acid contents. These results suggest that the low linolenic acid content in J18 line was caused by some nucleotide modification on the *Fan* locus encoding for an isozyme of microsomal ω-3 fatty acid desaturase.

Key words: *Glycine max*, *Fan* locus, RFLP analysis, oil quality, microsomal ω-3 fatty acid desaturase

Introduction

The soybean oil contains two types of saturated fatty acid and three types of unsaturated fatty acid. The composition of fatty acids in the dietary oil affects its nutritional value and physicochemical characteristics[1]. The unsaturated fatty acids easily convert into their oxidized form. The soybean oil quality can be improved by increasing saturated fatty acid and decreasing unsaturated fatty acid content. The linolenic acid is the most

* MORIMITSU Co., LTD, 9-7 Wakazakura, Fujiki-machi, Tosu, Saga 841.

unstable fatty acid in soybean oil because it has three unsaturated bonds in its molecule at the position of ω-3, -6 and -9. Four soybean mutant lines, J18, KL-8, M-5 and M24 were established by X-ray irradiation that contain about 4.8%, 6.9%, 4.4% and 5.9% linolenic acid, respectively, compared with 8.3% of their original cultivar Bay. Transgressive segregation for linolenic acid content in the F_2 generation suggested that these mutants were derived from the mutations at two loci, *Fan* and *Fanx* [2),3),4)].

The mechanism of fatty acid synthesis was evaluated for biochemical and molecular biological approaches in recent years[5)]. There are two distinct pathways for the biosynthesis of polyunsaturated fatty acids in higher plants. One pathway is located in plastid membranes and the other one is in microsomal membranes. In developing seeds, the microsomal pathway predominates[5)]. The ω-3 fatty acid desaturase is an essential enzyme to produce linolenic acid, which introduces the third unsaturated bond at the ω-3 position of linoleic acid and converts into linolenic acid. The cDNA encoding ω-3 fatty acid desaturase was first isolated from *Arabidopsis thaliana* by map-based cloning[6)] and T-DNA tagging[7)]. Moreover, some putative cDNAs of microsomal and plastid ω-3 fatty acid desaturase were isolated from soybean and other plant species[7)]. However, the genomic structure and gene assignment of this clone are still unknown.

In this study, we tried to determine the molecular basis of soybean mutants harboring *Fan* and *Fanx* loci for low linolenic acid content and to assign this microsomal ω-3 fatty acid desaturase cDNA using hybridization technique.

Materials and Methods

Plant materials

The soybean lines used in this study were J18, KL-8, M-5 and M24 mutants, and their original cultivar Bay. The F_1 and F_2 seeds of the cross J18 with Bay, and of parental seeds were harvested in the field at Saga University in 1997. The half of dry seeds were cut off and subjected to analyze the fatty acid composition, and the remaining part of dry seeds with embryo were planted in the field for DNA preparation.

Fatty acid analysis

Fatty acid composition was determined by gas chromatography, as described earlier[8)].

Probe preparation

A cDNA of microsomal ω-3 fatty acid desaturase was prepared from total RNA of developed soybean (Bay cultivar) seeds by RT-PCR method as described earlier[9)] except for PCR reactions. Two primer sequences were 5′-caatggttaagacacaaagcc-3′ and 5′-gcgagtggaggagcagagaatcagtc-3′, which designed to amplify the coding region of microsomal ω-3 fatty acid desaturase gene[7)]. The conditions of PCR reaction were followed, for 94°C/2 min and then 30 cycles of 94°C/1 min, 55°C/1 min, and 72°C/2 min. The amplified cDNA fragment (approximate 1.1 kbp) was cloned into *Eco*RV site of pBluescript II KS$^-$ vector by T-A cloning method[10)]. After checking this clone by nucleotide sequencing, ω-3

cDNA fragment was labeled with DIG-11-dUTP by DIG-High Prime Kit (BOEHRINGER MANNHEIM).

Southern-blot analysis

Total DNA was extracted from young green leaves by CTAB method[11] and quantified with ethidium bromide staining after 1% agarose gel electrophoresis using uncut lambda DNA as a standard. Two μg of DNA was completely digested with *Eco*RI restriction endonuclease. The digested DNA was separated on 0.8% agarose gel, followed by transfer to nylon membrane (Hybond-N$^+$, Amersham) with alkaline solution (0.5 M NaOH and 1.5 M NaCl). The membrane was hybridized with DIG-labeled probe, subsequently washed under high-stringent conditions (0.1 X SSC and 0.1% SDS at 65°C) and detected as described in manufacture's protocol (DIG Luminescent Detection Kit, BOE-

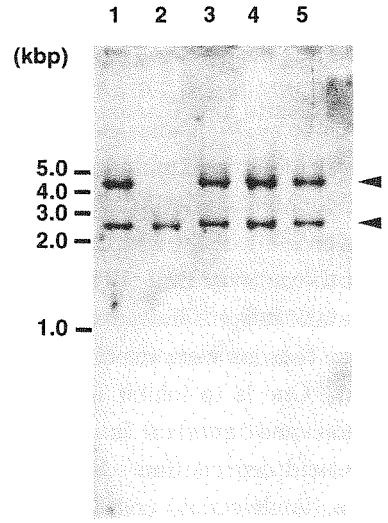

Fig. 1 Hybridization patterns of soybean genotypes with a microsomal ω-3 fatty acid desaturase probe. Total DNA was digested with *Eco*RI and molecular weights were given in kbp for the 1kbp DNA ladder (NEW ENGLAND Biolabs). Two μg of Bay (lane 1), J18 (lane 2), KL-8 (lane 3), M-5 (lane 4) and M24 (lane 5) were subjected for southern-blot analysis. Two bands (2.3 and 4.5 kbp) hybridized with the probe indicate with arrow heads.

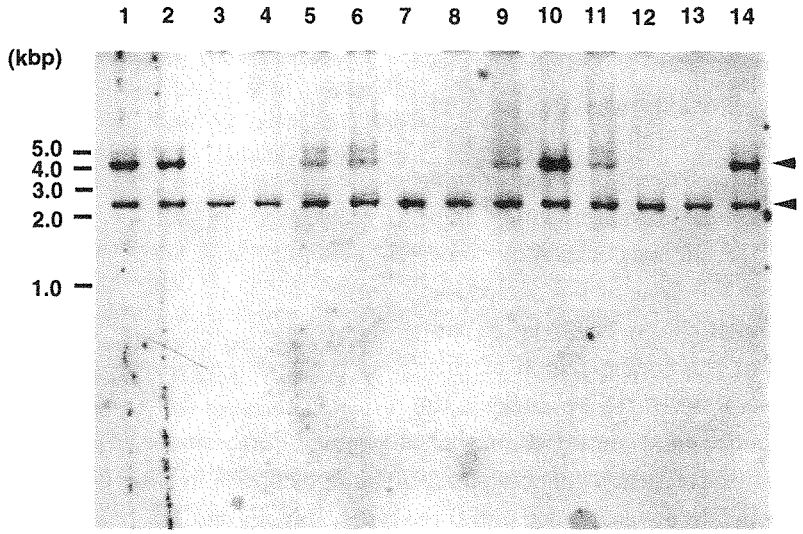

Fig. 2 Hybridization patterns of Bay, J18, their F_1 and F_2 progenies with same probe. Bay (lanes 1 and 2), J18 (lanes 3 and 4), F_1 (lanes 5 and 6) and F_2 plants (lanes 7 to 14) were analyzed with same as Figure 1. Each F_2 plant was completely comparable with No.1 to 8 plant of Table 1.

HRINGER MANNHEIM) .

Results and Discussion

Low linolenic acid mutants of soybean, J18, KL-8, M-5 and M24 were selected from the progeny of X-ray irradiated population of the cultivar Bay. In these mutant lines, mutations were independently occurred. Two reasons were expected for their mutation. One is to inhibit linolenic acid production and another is to promote linolenic acid degradation. However, the precursor, linoleic acid contents of these lines were slightly increased than that of Bay[2),3),4)]. Based on this result, we presumed that these lines might have some defection on the conversion step of linoleic acid to linolenic acid. Microsomal ω-3 fatty acid desaturase is an essential enzyme to convert linoleic acid to linolenic acid in microsome, and therefore, putative candidate was isolated from soybean cDNA library[7)].

The line J18 lacked 4.5 kbp band, whereas approximately 2.3 and 4.5 kbp bands were observed in Bay and other lines (Fig. 1). The differential RFLP pattern of this microsomal ω-3 desaturase gene could suggest that the low linolenic acid phenotype of J18 line was caused by some modification on this gene at the nucleotide level. The modification could be a partially deleted at the coding region of this gene. Because, it seems to be difficult to occur two or more point mutations on the same gene. Furthermore, it is well-known that X-ray irradiation frequently induces small or large deletions in their genome.

Table 1 Distribution of linolenic acid content in F_2 seeds and intensity of 4.5 kbp band hybridized with a microsomal ω-3 fatty acid desaturase probe in F_2 plants of cross between Bay and J18.

Number of individual	Linolenic acid content in F_2 seed	Band intensity in F_2 plant
1	4.4	−
2	4.3	−
3	6.8	+
4	9.1	++
5	6.5	+
6	4.6	−
7	4.5	−
8	9.5	++
9	5.2	−
10	6.9	+
11	6.7	+
12	5.9	+
13	9.3	++
14	6.3	+
15	6.2	+
16	8.9	++
17	8.6	++
18	6.1	+
19	6.3	+
20	6.7	+
21	7.0	+
22	6.2	+
23	4.9	−
24	4.7	−
25	4.5	−
26	6.4	+
27	6.4	+
28	7.1	+
29	6.6	+
30	9.0	++
31	9.1	++
32	6.5	+

The F_2 population was divided into three types, Bay, F_1 and J18, based on the intensity of 4.5 kbp band (lanes 7-14, Fig. 2). The content of linolenic acid in F_2 seeds was also devided into three groups. Segregation for the intensity of band and linolenic acid contents were found to be completely associated and fitted the expected 1 : 2 : 1 ratio (Table 1). These results support that this RFLP pattern was completely linked with the low linolenic

acid content of J18.

On the other hand, no difference of RFLP could be detected between other mutants and Bay in this study. This results supported that the mutation for KL-8 and M24 occurred at different loci, *Fanx*, and suggested that the modification at *fan* gene in M-5 could be lesser than that of J18. In this study, the other genes encoding the microsomal ω-3 fatty acid desaturase isozyme could not be detected, because the hybridization and wash conditions were highly strict. However, some bands that were putative candidates of the microsomal ω-3 fatty acid desaturase isozyme gene were detected under the low-stringency conditions (unpublished data). It was already reported that the mutations for KL-8 and M24 occurred at *Fanx* locus[3),4)]. From this evidence, at least two genes of microsomal ω-3 fatty acid desaturase probably exist in the same soybean genome.

In this study, we demonstrate that *Fan* locus is structural for microsomal ω-3 fatty acid desaturase. Molecular cloning of new isozyme gene(s) from soybean is needed to understand the molecular basis of *fanx* gene in KL-8 and M24, and more detail structural analysis may lead to a better understanding about mutations of *fan* gene in J18 and M-5.

References

1. Neff, W.E., Selke, E., Mounts, T.L., Rinsch, W., Frankel, E.N. and Zietoun, M.A.M. 1992. Effect of triacylglycerol composition and structures on oxidative stability of oils from selected soybean germplasm. *J. Am. Oil Chem. Soc.* **69**: 111-118.
2. Rahman, S.M., Takagi, Y. and Kumamaru, T. 1996. Low linolenate sources at the *Fan* locus in soybean lines M-5 and IL-8. *Breed. Sci.* **46**: 155-158.
3. Rahman, S.M. and Takagi, Y. 1997. Inheritance of reduced linolenic acid content in soybean oil. *Theor. Appl. Genet.* **94**, 299-302.
4. Rahman, S.M., Kinoshita, T., Anai, T., Arima, S. and Takagi, Y. 1998. Genetic relationships of soybean mutants for different linolenic acid contents. *Crop Sci.* **38**: 702-706.
5. Browse, J. and Somerville, C. 1991. Glycerolipid synthesis: biochemistry and regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 467-506.
6. Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M. and Somerville, C.R. 1992. Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science* **258**: 1353-1355.
7. Yadav, N.S., Weirzbicki, A., Aegerter, M., Caster, C.S., Perez-Grau, L., Kinney, A.j., Hitz, W.D., Booth, J.R.Jr., Schweiger, B., Stecca, K.L., Allen, S.M., Blackwell, M., Reiter, R.S., Carlson, T.J., Russell, S.H., Feldmann, K.A., Pierce, J. and Browse, J. 1993. Cloning of higher plant ω-3 fatty acid desaturases. *Plant Physiol.* **103**, 467-476.
8. Takagi, Y., Hossain, A.B.M.M., Yanagita, T. and Kusaba, S. 1989. High linolenic acid mutant in soybean induced by X-ray irradiation. *Jpn. J. Breed.* **39**: 403-409.
9. Anai, T., Miyata, M., Kosemura, S., Yamamura, S., Tsuge, T., Matsui, M., Uchida, H. and Hasegawa, K. 1997. Comparison of *abp*1 primary sequences from monocotyledonous and dicotyledonous species. *J. Plant Physiol.* **151**: 446-449.
10. Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19**: 1154.
11. Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.

ダイズ脂肪酸生合成突然変異体とその子孫を用いた制限酵素断片長多型の遺伝分析
Ⅰ．小胞体型 ω-3脂肪酸不飽和化酵素 cDNA をプローブとした低リノレン酸突然変異体の解析

山下　幸恵・木下　剛仁・S.M.ラーマン・岡田　和彦*・
穴井　豊昭・高木　胖
（生物工学講座・*森光商店(株)）

平成10年9月7日　受理

摘　　要

ダイズ［*Glycine max*（L.）Merr.］低リノレン酸系統 J 18，KL8，M5及び M24，は，X 線照射された品種 Bay より分離された突然変異体である．親品種である Bay ではリノレン酸含量が総脂肪酸の約8.3%であったのに対して，これらの突然変異体では各々約4.8%，6.9%，4.4%及び5.9%であった．この著しいリノレン酸含量の減少は，X 線照射時に *Fan* 及び *Fanx* 遺伝子座において引き起こされた突然変異によるものであろうと考えられた．そこで，本研究において，我々は小胞体型 ω-3脂肪酸不飽和化酵素の cDNA をプローブとした RFLP 解析を行い，これらの突然変異について検出を試みた．その結果，J18系統においてのみ，他の系統には認められる１本のバンドが欠失することを見出した．さらに，J18及び Bay を交配して得られた32系統の F_2集団についても同様の解析を行った．また，これらの種子のリノレン酸含量についてもガスクロマトグラフィーを用いて解析を行ったところ，これらのバンドの濃度は明確に１：２：１の比に分離し（Bay：F_1：J18型；$\chi^2=0.19$，$P>0.90$），更に，これらのバンドの濃度とリノレン酸含量との間には完全な相関が認められた．以上の結果より，J18における低リノレン酸含量は小胞体型 ω－3脂肪酸不飽和化酵素遺伝子のアイソザイムの一つをコードする *Fan* 遺伝子座での何等かの塩基配列の変化に起因する事が明らかとなった．