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Alteration of the omega-3 fatty acid desaturase gene is associated with reduced linolenic acid in the A5 soybean genotype

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Abstract Reducing the linolenic acid ($18:3^{\omega 3,6,9}$) concentration of soybean [*Glycine max* (L.) Merr.] oil may lessen the need for chemical hydrogenation and enhance flavor stability. Soybean genotypes A5 and A23 have reduced linolenic acid concentration compared with current cultivars. Seed linolenic acid is synthesized primarily by the ω-3 fatty acid desaturase located in the microsomes. The objective of this research was to study whether this enzyme has a role in reducing the fatty acid levels in the soybean genotypes A5 and A23. DNA from A5 and A23 was analyzed by gel-blot hybridization with a cDNA encoding the ω-3 fatty acid desaturase. A5 and lines selected from it have a DNA fragment missing compared to A23 and lines with normal linolenic acid concentration. Seventy $F_{4:5}$ lines from a population segregating for linolenic acid concentration were scored for presence or absence of the fragment. The absence of the fragment was significantly ($P \leqslant 0.0001$) associated with a reduced linolenic acid level and accounted for 67% of the variation for linolenic acid in the population. These results suggest that the reduced linolenic acid concentration in A5 was at least partially the result of a full or partial deletion of a microsomal ω-3 desaturase gene. No DNA polymorphisms were found for the desaturase gene in A23, so no mutations could be studied in this line.

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Introduction

The fatty acid composition of soybean seed oil affects its nutritional value and physical and chemical characteristics (Neff et al. 1992). The fatty acid α-linolenate ($18:3^{\omega 3,6,9}$) is commercially important because it has been identified as an unstable component of soybean oil responsible for undesirable odors and flavors (Liu and White 1992). For this reason, reducing linolenic acid concentration of oil has been a target for genetic selection.

The lines A5 and A23 were selected for reduced linolenic acid levels in a chemical mutagenesis program (Hammond and Fehr 1983; Bubeck et al. 1989). These lines each have at least one major gene which reduces linolenic acid concentration in the seed. The gene in A5 was designated *fan*(A5) (Rennie and Tanner 1991) and it was shown to be nonallelic to the gene in A23 that was designated *fan2*(A23) (Fehr et al. 1992). When A5 and A23 were crossed, transgressive segregants with lower linolenic acid concentration than either parent were identified. These segregants were designated A16 and A17, and they were considered to have the genotype: *fan*(A5)*fan*(A5)*fan2*(A23)*fan2*(A23) (Fehr et al. 1992). Fehr et al. (1992) reported the seed linolenic acid concentration of A16 as $22\,g\,kg^{-1}$ oil, A17 as $24\,g\,kg^{-1}$, A5 as $34\,g\,kg^{-1}$, and A23 as $56\,g\,kg^{-1}$ compared to current cultivars that average approximately $80\,g\,kg^{-1}$.

There are two distinct pathways for the biosynthesis of polyunsaturated fatty acids, one located in the microsomes and the other in the plastid membranes (Browse and Somerville 1991). In leaf tissue, both pathways are important for polyunsaturated fatty acid synthesis, whereas in seeds, the microsomal pathway predominates. The biosynthesis of linolenic acid is

catalyzed by the ω-3 desaturase, which introduces the third double bond into linoleic acid. The genes encoding both the microsomal and plastid forms of this desaturase have been cloned in soybean and other plants (Arondel et al. 1992; Yadav et al. 1993). The objective of the research presented here was to study the role of the microsomal ω-3 desaturase in reducing linolenic acid levels in the A5 and A23 soybean lines.

Materials and methods

Genetic material

The genotypes utilized in this study include soybean lines A5, A23, A16, and A17 and a population of 70 $F_{4:5}$ lines segregating for alleles at the *fan*(A5) and *fan2*(A23) loci. The population was developed from a cross between cv 'Pioneer 9231', which has a normal linolenic acid concentration, and the Iowa State experimental line A89-269077B. A89-269077B was assumed to be homozygous for the *fan*(A5) and *fan2*(23) alleles because it was selected for low linolenic acid concentration in a population descending from A5 and A23. Seed and the fatty acid composition of each line in the population was provided by Pioneer Hi-Bred International. The fatty acid analysis was performed by capillary gas chromatography on five to ten seed samples of each line.

DNA gel-blot analysis

Total genomic DNA from young soybean leaves was isolated as described by Anderson et al. (1992). Restriction digests, electrophoresis on agarose gels, Southern blots, hybridizations, and autoradiography was according to Bernatzky and Tanksley (1986) with minor modifications. Ten micrograms of DNA from the parents of the population was digested with *Eco*RI, *Hin*dIII, *Taq*I, *Eco*RV and *Dra*I. To screen for genetic polymorphisms, we analyzed the DNA by means of gel-blot hybridization to a cDNA coding for the microsomal ω-3 desaturase from soybean. This cDNA is a 0.93-kb *Hha*I fragment containing the open reading frame of the gene (Yadav et al. 1993). DNA from each line in the population was digested with *Eco*RI and *Dra*I and analyzed by hybridization with the desaturase cDNA.

Polymerase chain reaction (PCR) analysis

On the basis of the cDNA sequence of the microsomal ω-3 desaturase, two 30-mer oligonucleotides were designed to PCR-amplify a region of the gene. The sense primer starts at base pair 567 of the soybean cDNA (Yadav el al. 1993) and has the sequence CCC CGG AAA GGA AGG CTC TCA CTT CAA TCC. The antisense primer starts at base pair 817 and has the sequence GCC ACC TCT TAA ATA ACT CCA TTC CTT GCC. Amplification was carried out using standard reaction conditions in a thermal cycler (Perkin Elmer Cetus 480) programmed for 94°C/4 min and then 30 cycles of 94°C/1 min, 60°C/1 min, and 72°C/2 min. The amplification products were electrophoresed in 1.6% agarose gels.

Data analysis

Analysis of variance was used to test for a significant association in the population between the segregation of a DNA polymorphism and the linolenic acid concentration of the lines. Data were analyzed using the general linear model procedures of SAS (SAS 1989).

Results and discussion

DNA of soybean genotypes was hybridized with the cDNA for the microsomal ω-3 desaturase. The hybridization revealed that A5, A16, A17, and A89-269077B were missing DNA fragments when compared with A23 and 'Pioneer 9231' (Fig. 1). These lines were further tested by PCR using primers specific for the microsomal ω-3 desaturase gene. One DNA fragment was amplified in genotypes A5, A16, A17, and A89-269077B, and two fragments were amplified in A23 and 'Pioneer 9231' (Fig. 2).

Lines from the cross 'Pioneer 9231' by A89-269077B were tested for linolenic acid concentration and were hybridized with the desaturase gene to score for presence or absence of the DNA fragments. The missing fragments cosegregated and fit expectations for Mendelian segregation (Table 1). The absence of the fragments was significantly ($P \leqslant 0.0001$) associated with reduced linolenic acid levels. The R^2 value for the ANOVA was 0.67, which means that segregation for the fragments accounted for 67% of the variation for linolenic acid in the population (Fig. 3). The average linolenic acid concentration of lines without the DNA fragments was 43 g kg^{-1} oil, and the average for lines with the fragments was 77 g kg^{-1} oil. The lines from the population were also tested with PCR analysis. The segregation of the lines in the PCR and Southern hybridization analyses were identical with the same lines missing fragments in both analyses.

These results suggest that at least part of the reduction of linolenic acid in A5 was caused by a full or partial deletion of a microsomal ω-3 desaturase gene. The hybridization patterns indicate this because for all

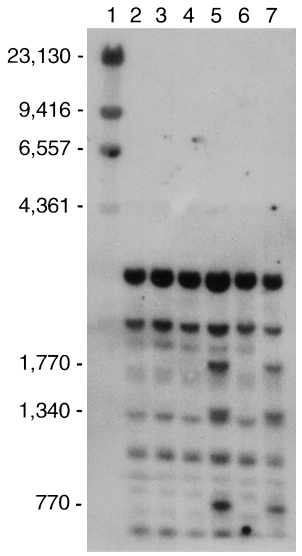

Fig. 1 Autoradiograph of soybean genotypes digested with *Dra*I and hybridized with the cDNA encoding the microsomal ω-3 desaturase. *Lanes 1–7* contain *Hin*dIII cut λ (Gibco BRL, Gaithersburg, Md.) (*1*), A5 (*2*), A16 (*3*), A17 (*4*), A23 (*5*), A89-269077B (*6*), and 'Pioneer 9231' (*7*). Molecular weights are given in base pairs for the *Hin*dIII-cut λ and polymorphic fragments

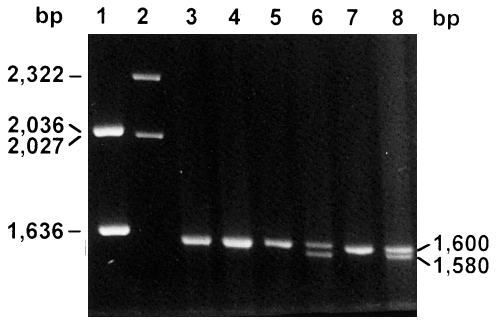

Fig. 2 Results from PCR amplification of a region of the microsomal ω-3 desaturase. *Lanes 1–8* contain 1-kb ladder (Gibco BRL) (*1*), *Hin*dIII-cut λ (Gibco BRL) (*2*), A5 (*3*), A16 (*4*), A17 (*5*), A23 (*6*) A89-269077B (*7*), and 'Pioneer 9231' (*8*)

Table 1 Segregation of the microsomal ω-3 desaturase fragments in the soybean population

Class	Observed	Expected[a]
Fragments present	31	36
Fragments absent	33	28
$\chi^2 = 1.6$ not significant		

[a] Expected segregation of 9/16 present and 7/16 absent for a dominant marker among F_4 derived lines

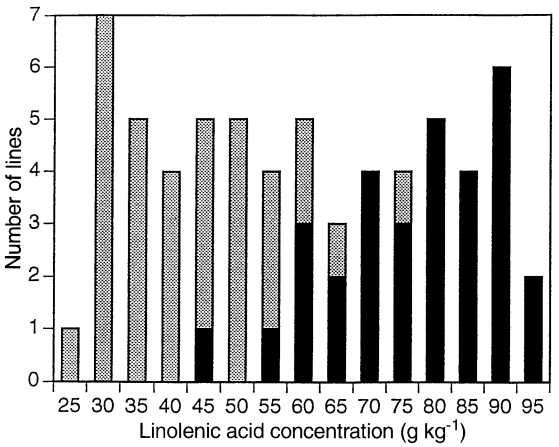

Fig. 3 Distribution of $F_{4:5}$ lines from the cross between 'Pioneer 9231' and A89-269077B. The *gray portion* of the *bars* represents the number of lines missing a copy of the microsomal ω-3 desaturase gene and the *black portion* represents the number of lines with the desaturase gene

enzymes used to digest the soybean DNA, one or more DNA fragments were deleted in A5 and no changes in DNA fragment size were observed. Had regions flanking the gene been deleted or had the gene been mutated in other ways, such as through base substitutions or rearrangements, changes in DNA fragment sizes would have been observed. A16, A17, and A89-269077B were also missing the same DNA fragments as A5. Thus, they likely received the mutation from A5, which was a parent (Fehr et al. 1992) or ancestor of these lines. The experimental line from which A5 was developed was not available for this study. Therefore, the possibility that the deletion was present prior to the ethyl methanesulfonate (EMS) treatment cannot be completely ruled out.

A5 was developed by treating seeds with EMS. Ethyl methanesulfonate typically causes base substitution mutations by acting as an alkylating agent (Hartl 1994). Therefore, the results showing EMS causing a deletion of the microsomal ω-3 desaturase gene were unexpected. However, there are other reports of EMS causing gene deletions (Wood and Moses 1989).

A DNA fragment missing in A5 was polymorphic between the *G. max* and *G. soja* parents of the population used to develop the USDA-ARS soybean map (Keim et al. 1990). The two parents had a different size fragment, and this polymorphism was used to map the microsomal ω-3 desaturase gene to linkage group B2 (Byrum et al. 1995), which is consistent with the placement of the *Fan* locus to the same location in another population (Brummer et al. 1995). This further indicates that the *Fan* locus is the microsomal ω-3 desaturase gene that was altered in A5.

Both the hybridization and PCR analysis suggests there is at least one more copy of the microsomal ω-3 desaturase gene present in A5 (Figs. 1 and 2), which could explain why there remains 34 g linolenic acid kg^{-1} oil in seeds of this line. If only one copy of the microsomal ω-3 desaturase gene was present in soybean, and the gene was at least partially deleted, a functional enzyme would not be encoded and there would be little, if any linolenic acid in the seed. It is possible that the *fan2*(A23) locus could be a second copy of the desaturase gene that was mutated in A23. However, hybridizations of the microsomal ω-3 desaturase gene did not reveal any DNA polymorphisms in A23. Therefore, a molecular analysis of the *fan2*(A23) locus could not be done.

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