For the Record

The RFLP Molecular Marker Closely Linked to the Supernodulation Locus of Soybean Contains Three Inserts

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Received 19 November 1993. Accepted 18 February 1994.

The pA-132 probe, linked to the supernodulation (*nts*) locus of soybean, was generated as a random genomic *Pst*I insert. The insert, however, was not unique, and three *Pst*I fragments were detected in the probe. To clarify whether the three fragments were contiguous, we analyzed a genomic lambda clone as well as the segregation of the three inserts in an F_2 population.

Additional keywords: RFLP, molecular map, molecular genetics, symbiosis, nitrogen fixation.

We are attempting to use marker-based analysis to define the molecular region on the soybean genome that governs autoregulation of nodulation (Gresshoff 1993). A mutational inactivation of the *nts* locus results in abundant nodulation, termed supernodulation (Carroll *et al.* 1985a,b). While a number of supernodulation mutants have been found in soybean and a number of other legumes, there is no information concerning the gene product and the direct biochemical function of the gene or its mutated forms. Using the soybean restriction fragment length polymorphism (RFLP) map generated by the U.S. Department of Agriculture–Agricultural Research Service in collaboration with Iowa State University (Keim *et al.* 1990), an RFLP marker was placed closely to the *nts* locus. F_2 populations from a cross between mutant nts382 and the ancestral soybean *Glycine soja* demonstrated less than 1% recombination (Landau-Ellis *et al.* 1991; Landau-Ellis and Gresshoff 1992). The probe used in the 1991 study was pUTG-132a, a subclone derived from mapped clone pA-132. Preliminary analysis suggested that pA-132 consisted of three *Pst*I inserts instead of the expected single insert. The largest one, pUTG-132a, was used in mapping studies of the Knoxville soybean populations, while the entire insert (as a polymerase chain reaction product) was used in the construction of the USDA-ARS map (Keim *et al.* 1990). The question arose whether the other two *Pst*I inserts in pA-132 were contiguous with pUTG-132a or whether they represented separate fragments, coincidentally cloned into the vector. Two approaches were used to arrive at the answer that they are separate fragments from unlinked regions of the genome: molecular analysis of a 14-kb lambda genomic clone homologous in part with pUTG-132a (Kolchinsky *et al.*, unpublished), and segregation analysis of the polymorphisms generated by the other two fragments.

The plant material used for crossing in this study was diverse in order to detect segregation among the offspring. The *G. max* (cv. Bragg) line nts1007, a supernodulating mutant homozygous for the *nts* locus and allelic to nts382, was used as the female parent. For the male parent, we used the ancestral soybean *G. soja* PI468.397, which exhibited wild-type nodulation. The 57 F_2 progeny from this cross segregated with 15 supernodulating plants and 42 plants with wild-type nodulation. Only the 15 supernodulating plants were used for this RFLP analysis.

The original probe inserts contributing to the Iowa State–USDA-ARS linkage map were generated by digesting genomic soybean DNA (*G. max*) with the *Pst*I restriction endonuclease and cloning the 0.5- to 3-kb fragments into the vector pBS+ (Stratagene Inc., La Jolla, CA). When purifying pA-132 for labeling, we found that digestion of this plasmid clone with *Pst*I resulted in the vector fragment plus three inserts, unlike most of the other clones in this library, which contain only one insert. Subclones were made from these three fragments. The subclone pUTG-132a contains the largest insert (1.8 kb). Clone pUTG-132bc contained two inserts, namely b (1.2 kb) and c (0.7 kb), and pUTG-132c contains only the smallest fragment (0.7 kb).

DNA was isolated from these plasmid clones as previously described (Landau-Ellis and Gresshoff 1992). After restriction of plasmid DNA with *Pst*I (according to specifications of the manufacturer, New England Biolabs), insert fragments were separated by agarose gel electrophoresis, visualized by ethidium bromide, cut out of the gel by scalpel, and purified by electroelution into dialysis tubing. The lambda clone λ UTG-132a was isolated from the soybean cultivar Bragg genomic library in the vector lambda GEMII (Kolchinsky *et al.*, unpublished).

Southern hybridization was performed as described earlier (Landau-Ellis and Gresshoff 1992). The analysis of total DNA genomic blots with probe pA-132 was complicated, with a pattern showing numerous bands. This indicated that the probe detected many genomic regions (i.e., it contained

MPMI Vol. 7, No. 3, 1994, pp. 432-433
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repeated DNA). As such, the probe would have been of lesser utility in a positional cloning approach (Gresshoff 1993). However, during probe preparation it was noted that the pA-132 clone contained three *Pst*I inserts. Isolation of the largest one (pUTG-132a) gave a simple RFLP pattern. A polymorphism between nts382 and *G. soja* gave close linkage in F_2 segregation analysis. Since *nts1007* is allelic to *nts382* (Delves *et al.* 1988), and both alleles map closely to the same RFLP marker (Landau-Ellis and Gresshoff 1992), we analyzed the cosegregation of the three insert clones in phenotypically selected supernodulating plants derived from an nts1007 × *G. soja* (wild-type) cross.

This separate isolation of the three pA-132 fragments before hybridization produced more legible and scorable autoradiographs. When the three individual inserts were labeled with ^{32}P and hybridized onto genomic blots of F_2 DNA from 15 supernodulating segregants of the A3 population (*G. max* nts1007 × *G. soja* PI468.397), we observed independent segregation of the three fragments; in contrast, pUTG-132a is tightly linked to the *nts* locus. The other two markers (b and c) were found to segregate independently of the *nts* locus as well as each other. The probe pA-36, a flanking marker 10 cM from pA-132 on linkage group H, also segregates independently of the pA-132 b and c fragments.

To confirm this genetic evidence, we utilized the 14-kb lambda clone, λUTG-132a, which contains the complete pUTG-132a segment of DNA along with flanking regions from a cv. Bragg genomic library. When the three pA-132 fragments were probed with λUTG-132a, only the 1.8-kb fragment gave positive hybridization, indicating that these three fragments from the plasmid clone pA-132 are not contiguous and must originate from different parts of the genome.

Despite the obvious cloning artifact, the original map position was determined on the basis of just a small region of the RFLP pattern. The probe used in Iowa for map construction was generated by PCR amplification with plasmid-borne external primers. While this approach is fast and useful, it does not permit the detection of multiple inserts. We determined that the three inserts were not contiguous in the soybean genome and that their presence in pA-132 is a cloning artifact by both genetic and molecular means.

ACKNOWLEDGMENTS

We thank Joanna Deckert for the lambda clone. The CSRS Project No. TEN00137 and Tennessee Soybean Promotion Board are thanked for support.

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