The Early *enod2* and the Leghemoglobin (*lbc3*) Genes Segregate Independently from Other Known Soybean Symbiotic Genes

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Recombinant inbred lines (RILs) as well as an F_2 segregating population of soybean *Glycine max* facilitated the mapping of two expressed sequence tags involved in early nodulation and subsequent nitrogen fixation in soybean. For the early nodulin gene *enod2*, the parents of RILs, Minsoy and Noir1, showed a polymorphism (5.5 vs 5.9 kb) after *EcoRV* digestion. Restriction fragment length polymorphism (RFLP) patterns of 42 RILs were analyzed with the MAPMAKER program, linking *enod2* to the seed coat color gene, *I*, with a distance of 11.1 cM on linkage group U3 of RIL map. *enod2* and *I* are located close to *Rhg4*, a soybean cyst nematode resistance gene, and a locus for seed coat hardness. The molecular marker pA-110 and seed coat color were used to integrate *enod2* on an F_2 segregating population (72 plants) generated from a cross between cv. Bragg and *G. soja* PI468.397. *enod2* was mapped in the same order as on the RIL map but 18.5 cM from the *I* locus on the TN map. A microsatellite from the 5' region of *enod2b* was mapped in the same position, demonstrating that *enod2b* and not *enod2a* was mapped. An RFLP for *lbc3* (leghemoglobin) segregated independently from *enod2* and the *nts-1* supernodulating locus suggesting that in soybean symbiotically significant loci (including *rj1*, *Rj2*, and *rj6*) are not clustered.

Genetic properties of both bacteria (e.g., *Rhizobium*, *Bradyrhizobium*) and host plants are essential for the development of nitrogen-fixing root nodules. Plant control of nodulation was confirmed by the isolation of symbiotically altered mutants (see Gresshoff 1993; Caetano-Anollés and Gresshoff 1991) and by the demonstration of nodule-specific or nodule-enhanced proteins, namely nodulins, such as leghemoglobin, uricase, or glutamine synthase (Verma and Delauney 1988). Such nodulins are classified into early and late nodulins based on their temporal sequence of induction. Early nodulin genes are expressed during infection and nodule morphogenesis (Gloude-mans and Bisseling 1989; Govers et al. 1986) and may shed light on events related to cell division, hormonal responses, and plant defense mechanisms as well as the signal transduction chain connecting nod-factor recognition and nod-

ule initiation. Late nodulin gene expression is correlated with the onset of nitrogen fixation (Govers et al. 1985, 1987) and reveals information about carbon/nitrogen metabolism and transport, symbiosome membrane transport, functions related to the maintenance of the intracellular symbiosis, and oxygen regulation and transport.

ENOD2, one of the first discovered early nodulins, was initially characterized in soybean (Franssen et al. 1987). The gene product is a hydroxy-proline-rich cell wall protein with a molecular mass of 75 kDa, and has the alternative label of GmN-75 nodulin. It was postulated to be involved in the oxygen barrier inside of legume nodules (Pawlowski and Bisseling 1996; Long 1996), although there is no evidence for ENOD2 function other than the localization of the protein. Indeed, no difference was observed in the level of the *enod2* protein and mRNA of alfalfa nodules under different concentrations of oxygen (Hunt et al. 1995). Two soybean genomic clones, *enod2A* and *enod2B*, of 16 and 25 kb, respectively, were isolated from a soybean genomic library in lambda charon 35 (Franssen et al. 1989). These two clones contained no introns and were identical in their coding domains but differed in the 5' regions. The deduced amino acid sequences revealed two pentapeptide, proline-rich repeats (PRO-PRO-GLU-TYR-GLN and PRO-PRO-HIS-GLU-LYS) that were similar to pentapeptide repeats of a soybean proline-rich protein designated SbPRP1 (Hong et al. 1987). The strong conservation of coding sequence suggests recent gene duplication, genome tetraploidization, or functional restriction.

With a soybean *enod2* cDNA as a probe, homologous *enod2* sequences were isolated from pea (Govers et al. 1987), white clover, bird's foot trefoil, vetch (Moreman et al. 1987), alfalfa (Dickstein et al. 1988), common bean (Sánchez et al. 1988), and *Sesbania rostrata* (Strittmatter et al. 1989). The major difference among mRNAs from different plants was predominantly in the length of the gene. Some of the alfalfa nodulin genes, such as *enod2*, *enod12*, *nod-22*, and *nod-25*, and leghemoglobin and glutamine synthases have been mapped (Kiss et al. 1997). The ineffective nitrogen fixation phenotype in alfalfa (*in6*) was linked to a random amplified polymorphism DNA (RAPD) marker located on linkage group 7. Non-nodulating (*sym14*) and ineffective nitrogen fixation (*sym13*) loci in pea were mapped on chromosome 2 (Kneen et al. 1990).

It is possible that ENOD2 represents a pericycle-specific protein whose expression is increased during early nodulation.

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The protein has similarity to other cell wall proteins, some of which are expressed during lateral root induction or plant defense responses (Lamb et al. 1989). Expression of *enod2* is initiated in cells at the base of the nodule and ultimately in the nodule parenchyma (Allen et al. 1991; Kouchi et al. 1989; van de Wiel et al. 1990). Expression occurred in empty nodules on alfalfa roots induced by exopolysaccharide-deficient (*Exo*⁻) mutant of *Rhizobium meliloti* (Dickstein et al. 1988). Application of auxin transport inhibitors resulted in empty nodule-like structures that expressed *enod2* (Hirsch et al. 1989). Alfalfa variants that formed nodules in the absence of *Rhizobium* (NAR) also expressed *enod2* (Truchet et al. 1989). The protein therefore is expressed independently of bacterial infection and indeed may be reflective of pericycle cell proliferation. *Sesbania rostrata enod2* (*SrEnod2*) was expressed after exogenous application of cytokinins to roots and stems (Dehio and de Bruijn 1992).

Molecular mapping of nodulation-related genes may permit the determination of function, if complete cosegregation with mutant loci is observed. With an increasing data base containing both quantitative trait locus (QTL) and single gene loci associated with agronomic parameters, it is likely that nodulin genes with secondary function are revealed (Mansur et al. 1996).

Most mapping is done in segregating F₂ populations, but these have the disadvantage of being finite (see Landau-Ellis et al. 1991; Keim et al. 1990; Kiss et al. 1997; Weeden et al. 1990; Diers et al. 1992). While ample supplies of DNA can be collected, eventually they run out. Recombinant inbred lines (RILs), through single seed descent, fix homozygosity in genomic regions, resulting in (more or less) stable seed lines, to be shared indefinitely by the scientific community. Such RILs derived from F₇ or F₆ seed are available for soybean (Lark et al. 1993; Mansur et al. 1993a, 1993b, 1996) and *Lotus japonicus* (Jiang and Gresshoff 1997), respectively. RILs also have the advantage that living tissue can be harvested, permitting physical mapping with high-molecular-weight DNA derived from protoplasts (Funke et al. 1993) or the isolation of telomeres, satellites, or related chromosomal regions (Kolchinsky and Gresshoff 1995).

Of course, most parents used to make RILs do not differ in all desired phenotypic properties. Hence a strategy of map-integration can be used, in which molecular markers associated with a mutant locus (i.e., *nts-1*, Landau-Ellis et al. 1991; Kolchinsky et al. 1997) mapped in a specific cross close to a restriction fragment length polymorphism (RFLP) marker (pA381), which in turn was found on the RIL map (Gresshoff and Filatov, *in press*). Thereby, the mutant locus can be transferred to an RIL map by association with flanking markers. We tested this approach here, attempting to confirm conserved marker order and mapping distances for the region around *enod2*. Furthermore, we wanted to evaluate the hypothesis, developed from mapping in pea, that symbiotically important genes are clustered (as on chromosome 1 of pea; Weeden et al. 1990).

RESULTS

RFLPs derived from Southern hybridizations probed with a 600-bp *Pst*I fragment of the *enod2* clone (pENOD2), with digested genomic DNA from Minsoy (M) and Noir1 (N) (Fig.

1A) and their RILs (Fig. 2), were detected. In most cases, the two or more copies of *enod2* were consistent with the ancient tetraploidy nature of soybean (Polzin et al. 1994; Singh and Hymowitz 1988) and the fact that two related but distinct genomic clones were available. However, a few faint bands were detectable stemming presumably from similarity of *enod2* to other cell-wall-protein genes. Four RFLPs were detected from restriction with *Eco*RI, *Eco*RV, *Taq*I, and *Bgl*II. In the case of *Eco*RV, the sizes of polymorphic bands were 5.9 and 5.5 kb for Minsoy and Noir1, respectively. A monomorphic band was observed at 7.5 kb. Identical RFLP patterns for *enod2* (5.5 vs 5.9 kb) and *lbc3* (3.0 vs 2.6 kb) in Minsoy and *G. soja* PI468.397 may indicate a common origin in this region; likewise, Noir1 and Bragg also share common patterns (Fig. 1A, B, and D).

Although several RFLPs were detected with other enzymes, none of them were clearly codominant. An RFLP with codominant pattern is valuable to distinguish heterozygous segregants. Inheritance of the *enod2* RFLP was studied with a chi square test to examine whether the observed RFLP banding patterns in RILs were consistent with an expected 1:1 Mendelian ratio (Table 1). No significant differences between expected and observed values indicated that this RFLP was inherited as a single locus.

We used the MAPMAKER program to analyze the data for *enod2* segregation. *enod2* was mapped to linkage group U3 (Mansur et al. 1996) (Fig. 3A). Flanking are two molecular markers of K401b and BLT24 at 7.4 and 7.3 cM distance, respectively. In addition, *enod2* is linked to the “*I*” gene encoding seed coat color at 11.1 cM distance (LOD score of 6.34). The “*I*” gene was postulated to encode chalcone synthase genes (*CHS*) or modulators of their activity (Wang et al. 1994). This central gene of isoflavone/flavone biosynthesis (Estabrook and Sengupta-Gopalan 1991) is important for plant pigmentation, plant disease response (phytoalexins), and nod-gene inducer biosynthesis (Kosslak et al. 1987; Sutherland et al. 1990). Further analysis revealed strong interactions between the *enod2* region and a region on linkage group U16 close to the molecular marker pA401 and explained quantitative variation for agronomically important traits such as yield and pod filling period (Gordon Lark, *personal communication*). The *enod2* gene was located in a region close to the *Rhg4* gene, a soybean cyst nematode resistance gene (Weisemann et al. 1992; Yazdi-Samadi et al. 1996; Webb et al. 1995; Concibido et al. 1994; Matson and Williams 1965) and a QTL for seed coat hardness (Keim et al. 1990).

The flanking RFLP pA-110 and seed coat color were used to integrate *enod2* onto an F₂ segregating population map (Shoemaker and Olson 1993). The results of Southern blots probed with the *enod2* clone for Bragg and *G. soja* PI468.397 (Fig. 1C) and F₂ individuals showed an inheritance pattern consistent with the expected 1:2:1 Mendelian ratio (Table 1). Since F₂ seed coat color phenotypes were distinguishable only after seed formation, F₃ seed coat color was scored as yellow (Bragg pattern), gray-brown (heterozygote pattern), and black (soja pattern) with a ratio of 16:57:18, respectively. F₂ segregants probed with the pA-110 clone revealed a polymorphism of 6 vs 12 kb (Bragg vs PI468.397) after *Xba*I restriction and segregation of 15:41:17 (Bragg, heterozygote, and soja patterns, respectively). Significant differences for expected vs observed ratio were found neither for seed coat

color nor for pA-110 (Table 1). Cosegregation of *enod2*, pA-110, and seed coat color of 72 F₂ individuals resulted in the integration of *enod2* on the Iowa and TN *G. max* × *G. soja* partial map in the same order as on the RIL map, but 18.5 cM from the *I* locus (Fig. 3A). The numerical differences are

within the expected range of error caused by different parents and sample sizes.

To check the hypothesis of clustering of nodulation and nitrogen fixation genes, one soybean leghemoglobin gene, *lbc3*, was hybridized to *TaqI* restricted genomic blot of Minsoy,

Fig. 1. Autoradiograph of Southern hybridizations for *enod2*, pA-110, and *lbc3* probes. **A**, Minsoy (M) and Noir1 (N) probed with ENOD2 cDNA clone (pENOD2). Two copies of *enod2* loci were detected in most cases. Restriction fragment length polymorphism (RFLP) from *EcoRV* restriction (5.9 vs 5.5 kb) indicated by arrows, was used for screening recombinant inbred lines (RILs). **B**, Bragg (B) and *Glycine soja* (S) probed with pENOD2. RFLP from *EcoRV* restriction (5.9 vs 5.5 kb) and monomorphic band (7.5 kb), indicated by arrows, was used for screening F₂ individuals. **C**, Bragg and *G. soja* probed with the flanking marker pA-110. RFLP from *XbaI* restriction (12.0 vs 6.0 kb) and monomorphic band (8.5 kb) was used for screening the F₂ population. **D**, Autoradiograph of *TaqI* restricted DNA from Minsoy, Noir1, Bragg, *G. soja*, and RILs probed with the leghemoglobin (*lbc3*) probe (pAR10). Two copies of *lbc3* were detected. RFLP for *TaqI* restriction (3.0 vs 2.6 kb) was used for screening RILs.

Noir1, and 226 RILs (Fig. 1D). An RFLP for *lbc3* was mapped at the end of linkage group U31 (Fig. 3B) and segregated independently from the *enod2* locus.

The *enod2* RFLP and the microsatellite marker cosegregated in the RIL population, and the F₂ population (Fig. 4), indicating that the *enod2* RFLP that we mapped was the

enod2B copy. The size of single-sequence-repeat, polymorphic bands was 184 vs 189 bp in Minsoy vs Noir1 and 165 vs 264 bp in Bragg vs *G. soja*, respectively. We note with interest the 99-bp size difference in Bragg and *G. soja*, suggesting perhaps that *enod2b* is transcriptionally inactive and that the monomorphic fragment (7.5 kb) contained the functional *enod2A* copy.

DISCUSSION

We mapped the *enod2B* gene on the linkage group U3 of Utah map in a region of soybean genome that seems to contain a number of genes encoding monogenic or polygenic traits (QTL) that may be associated with cell wall proteins, e.g., seed hardness (Keim et al. 1990), pod filling period, and soybean cyst nematode resistance genes (Weisemann et al. 1992; Webb et al. 1995). Furthermore, *enod2* was linked to the *I* gene, which encodes seed coat color and affects *CHS* gene expression (Wang et al. 1994).

Seed hardness in soybean is a QTL that is encoded by three genes affecting germination rate, viability, and quality of stored seed (Kilen and Hartwig 1978; Rolston 1978; Potts et al. 1978). This trait has been genetically analyzed with 72 RFLP markers (Keim et al. 1990). Five independent RFLPs were associated with seed hardness trait. However, a major portion of variation (32%) was explained by the genomic region containing the *I* locus.

The *I* gene seems to be involved in the distribution of anthocyanin pigments (reviewed in Palmer and Kilen 1978). When a dominant *I* allele is present, production of anthocyanin is inhibited in the epidermal layer of the developing seed coat, resulting in yellow seed color. In yellow-coated seed (*I*), activity of *CHS* was seven- to 10-fold less than in the pigmented (*i/i*) seed coats. *CHS* mRNA was barely detectable in seed coats carrying the *I* allele. Association of *CHS* and *I* locus was further confirmed with detection of multiple restriction site polymorphisms in genomic DNA blots of the *CHS* gene family in near-isogenic lines of *I* locus (Wang et al. 1994).

Although the order of the *enod2* map is the same in all the maps, the differences in distance might be due to different parents, environmental effects during recombination, or experimental error. Sequence analysis of the *enod2b* genomic clone of cv. Wayne revealed a microsatellite (AT) of 17 repeats between nucleotide 866 and nucleotide 900 (about 2 kb upstream from the ATG start site for *enod2*). This single sequence repeat was detected by polymerase chain reaction (PCR) primers and detected a polymorphism between Minsoy and Noir1. Mansur et al. (1996) mapped this *enod2*-associated single sequence repeat to 9.3 cM from *I*, confirming our RFLP-based data.

The second copy of the *enod2* gene (represented by the 7.5 kb monomorphic *EcoRV* fragment) seems to be highly conserved in *G. max* cultivars (Minsoy, Noir1, and Bragg) and wild-type *G. soja* PI468.397. We postulate that it maps in a homologous region of soybean. It would be of value to test whether both loci are actively involved in expression of ENOD2.

In pea, *sym* cluster 1 on chromosome 1 consists of glutamine synthetase, leghemoglobin genes, *sym-2* (strain specificity), *sym-5* (low nodule number), *sym-18* (strain specificity)

Fig. 2. Segregation of restriction fragment length polymorphism (RFLP) for *enod2*. **A**, *EcoRV* restricted DNA of Minsoy, Noir1, and recombinant inbred lines (RILs) was probed with pENOD2 clone. Top row labels the RFLP patterns (M or N type); numbers are the same as original ones obtained from the University of Utah. **B**, *EcoRV* restriction of the parents of two populations (RIL and F₂) and some of the RILs and F₂ individuals. Those F₂ individuals with either 5.9- or 5.5-kb bands were scored as B and S type patterns, respectively. Heterozygotes possessed all three bands and were scored as H.

Table 1. Five independent chi square tests^a

Character	RFLP patterns	Observed values	Expected values	Mendelian ratios	Calculated chi squares	Table chi squares
<i>enod2</i> RIL population	M	25	20.5	1	1.560	3.840 $\alpha = 5\%$ NS
	N	16	20.5	1		
<i>enod2</i> F ₂ population	B	26	22	1	3.217	5.991 $\alpha = 5\%$ NS
	H	47	44	2		
	S	15	22	1		
F ₂ seed coat color	B (yellow)	16	22.75	1	5.902	5.991 $\alpha = 5\%$ NS
	V (variegated)	57	45.5	2		
	S (black)	18	22.75	1		
F ₂ pA-110	B	15	18.25	1	1.220	5.991 $\alpha = 5\%$ NS
	H	41	36.5	2		
	S	17	18.25	1		
<i>lbc3</i> RIL population	M	121	113	1	0.994	3.840 $\alpha = 5\%$ NS
	N	105	113	1		

^a Tests were for the inheritance of restriction fragment length polymorphism (RFLP) banding patterns of *enod2* gene in the recombinant inbred line (RIL) and the F₂ populations, for seed coat color and pA-110 RFLP in the F₂ population, and the *lbc3* gene in the RIL population of soybean. M, Min-soy; N, Noir1; B, Bragg; H, heterozygote; S, *G. soja*; and V, variegated color (gray-brown). NS, non-significant differences.

and late flowering), and *sym-19* (non-nodulating mutant). In soybean, *Rj2*, controlling ineffective nodulation, was integrated from the classical genetic map onto the USDA/Iowa map (Polzin et al. 1994) in a cluster of disease resistance QTLs (Kanazin et al. 1996). However, an RFLP for one of the leghemoglobin genes (*lbc3*) segregated independently from the *enod2B* locus, and the *nts-1* supernodulation locus mapped on linkage group H of the USDA/Iowa map (Landau-Ellis et al. 1991) (Table 2). Two loci for P³⁴ protein kinase (*cdc2*) were mapped onto separate linkage groups (U20 and U25) (Taranenko 1995), as was the region of the supernodulation locus *nts-1* (U23- close to pA381) (Gresshoff and Filatov, *in press*). Moreover, *nod139* (*rj6*) and *nod49* (*rj1*) (non-nodulating loci) as well as *nod49* and *nts-1* segregated independently (Table 2), suggesting that in soybean symbiotically significant loci are not clustered.

MATERIALS AND METHODS

Parent plants of the Utah soybean (*Glycine max* (L.) Merr.) RIL population, Minsoy and Noir1 cultivars, and RIL plants (at F_{7.5}) were grown in an environmentally controlled greenhouse. Unifoliolates and young trifoliolates (0.5 g of fresh weight) were harvested and immediately placed in liquid nitrogen. Average DNA yield was 100 µg per g of fresh weight measured by DNA fluorometry (Model TKO 100, Hoefer Sci-

entific Instruments, San Francisco, CA). After DNA isolation (Dellaporta et al. 1983), samples from parents were digested with seven restriction enzymes (*Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, and *Taq*I), with buffers recommended by the manufacturer. Restricted genomic DNA (5 µg per lane) was electrophoresed on 0.9% agarose gels and then blotted onto Zeta Probe Nylon membranes by vacuum blotting (Sambrook et al. 1989).

enod2 cDNA cloned in pUC18 (confirmed by partial DNA sequencing), provided by Ton Bisseling (Wageningen, Netherlands), was isolated (Sambrook et al. 1989) and restricted with *Pst*I. Southern blot hybridization was carried out with ³²P-labeled (Random Primer Labeling Kit, Boehringer Mannheim, Mannheim, Germany) *enod2* probe and parental DNA blots. After detection of polymorphisms in the parents, Southern hybridization was carried out for 42 RILs.

Parents of the F₂ population were *G. max* cv. Bragg and *G. soja* PI468.397. These were previously used to map the supernodulation gene *nts-1* (Landau-Ellis et al. 1991). DNA samples from parents were digested with six restriction nucleases, namely *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Taq*I, and *Xba*I. Parental crosses were made and verified as described by Landau-Ellis et al. (1991) with morphological and RFLP markers. After detection of polymorphisms in parents, Southern hybridization was carried out for 88 F₂ plants. Inheritance of RFLP banding patterns of *enod2* in the RILs and the F₂ population was determined with two independent chi square tests.

The MAPMAKER program (Lander et al. 1987) was used to map *enod2* and *lbc3* in the RIL and the F₂ populations. In the case of the F₂ population, cosegregation of *enod2*, pA-110 molecular marker, and seed coat color (*I*) of 72 plants was studied.

The *lbc3* gene was cloned in pAR10 as a 3.5-kb *Bam*HI fragment carrying a 2-kb 5' region of the *lbc3* gene (provided by Jens Stougaard, Aarhus, Denmark). After detection of RFLP with *Taq*I restriction of Minsoy and Noir1, 226 RILs were screened for RFLP patterns.

Cosegregation of RFLP marker and microsatellite markers in parental materials, RIL, and F₂ populations was studied to determine which *enod2* copy we mapped. The microsatellite represents 17 repeats of AT from nucleotide 866 through nucleotide 900 in the 5' upstream region of the genomic *enod2B* clone of cv. Wayne. PCR conditions for amplification of the microsatellite marker were as follows: 6 min at 95°C and 35 times of 30 s at 95°C, 1 min at 47°C, and 2 min at 72°C in a Twin Block thermocycler (Ericomp, San Diego, CA). The PCR reaction mix contained 50 ng of DNA template, 1 mM

Fig. 4. Mapping of the *enod2* by an AT microsatellite. **A**, Autoradiogram of polyacrylamide gel for the *enod2b* microsatellite in F₂, F₁, parents, and recombinant inbred lines (RILs). **B**, Bragg; S, *Glycine soja*; M, Minsoy; N, Noir1; H, heterozygote; E, expected; and O, observed banding patterns. No amplification product was observed for RIL # 168. **B**, A silver-stained 10% polyacrylamide gel run in a Mini-proteanII gel electrophoresis apparatus (BioRad, Hercules, CA). Additional band at 232 bp may be a heteroduplex or mismatched product.

Table 2. Linkage and genetic data for soybean symbiosis and symbiosis-related genes

Gene symbol	Function	Linkage group
<i>cdc2-M</i>	P34 protein kinase	U20
<i>cdc2-N</i>	P34 protein kinase	U25
pA381-pPV (<i>nts-1</i> region)	Supernodulation mutant	U23
<i>enod2</i>	Cell wall protein	U3
<i>lbc3</i>	O2 carrier protein	U31
<i>Rj2</i>	Ineffective nodulation	Iowa Linkage Group J
<i>rj1</i>	Non-nodulation	Chromosome 3
<i>rj6</i>	Non-nodulation	Segregates independently from <i>rj1</i> and <i>nts-1</i>

Mg²⁺, 0.15 µM of each 3' and 5' primers, 100 µM of each dNTP, 0.1 µl of 3000 Ci/mmol α-³²P dATP, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1% Triton X-100), and 1 unit *Taq* DNA polymerase (Perkin Elmer, Roche Molecular System, Branchburg, NJ). Each sample (3.5 µl) was added to 3.5 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) before loading in a 6% acrylamide sequencing gel containing 8 M urea. The polymorphism was detected by autoradiography as well as silver staining. All photos of autoradiographs were taken by the IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA).

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