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Protocol

Detection of Minisatellite Sequences in *Phaseolus vulgaris*

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Abstract: An improved procedure for detecting minisatellite sequences in *Phaseolus vulgaris* is described. Both M13 protein III tandem repeat and the 33.15 human mini-satellite sequences revealed polymorphisms with a high number of sharp bands after digestion of genomic DNA with *Hae* III, *Hinf* I, or *Taq* I. Improved resolution of the numerous restriction fragments detected by these probes is accomplished by one or more of the following: varying agarose concentration, using high SDS hybridization buffer, exposure of the autoradiograph without intensifying screens, and transfer of the autoradiograph to electrophoresis paper. Increased stability of the DNA-DNA hybridizations with these heterologous probes is obtained by reducing hybridization temperature. Labeling probes with the polymerase chain reaction can accentuate some restriction fragments depending upon the radiolabeled nucleotide used.

Minisatellite sequences (also known as VNTRs or Variable Number of Tandem Repeats) consist of arrays of tandemly repeated DNA sequences and are present in both animal and plant genomes (*e.g.*, Jeffreys et al., 1985a, 1987; Dallas, 1988; Rogstad et al., 1988; Kuhnlein et al., 1989; Zimmerman et al., 1989; Nybom, 1990; Nybom and Schaal, 1990; Reeve et al., 1990). Because of substantial variability for

Abbreviations: PCR, polymerase chain reaction; BSA, bovine serum albumin; SSC, 150 mM NaCl, 15 mM Na citrate, pH 7.0.

repeat unit number, these sequences can provide highly informative markers (*e.g.*, Jeffreys, 1987; Jeffreys et al., 1987). They have been or could potentially be used in linkage mapping (Donis-Keller et al., 1986; Nakamura et al. 1987), in following introgression in breeding programs (Hillel et al., 1990), in the positive identification of cultivars (Dallas, 1988; Nybom et al., 1989), in forensics (Jeffreys et al. 1985b; Hagelberg et al., 1991), and in population studies (e.g., Balazs et al., 1989; Nybom and Schaal, 1990; Reeve et al., 1990).

Numerous probes (Table I) are available to screen for these sequences in plants (Vassart et al., 1987; Dallas, 1988; Rogstad et al., 1988; Schafer et al., 1988; and Weising et al., 1989). At present, heterologous sequences are the main source of probes available for screening for hypervariable sequences in plants because less is known about the genomes of plants compared to those of animals (specifically humans). The set of minisatellite probes related to 33.6 were constructed and isolated only after extensive sequence information about the myoglobin gene had been obtained in humans (Jeffreys et al., 1985a). In contrast, the discovery that the

Table I. Probes assayed for detection of minisatellite sequences in *Phaseolus vulgaris*

Probe	Source	Characteristics
33.6	Synthetic primer	Monomer of the human minisatellite 33.6: 5'TGGAGGAAGGGCTGGAGGAGGG3'
pHSD42[a]	Synthetic imert	360 bp insert containing the diverged repeat (AGGGGCTGGAGG)N from 33.6 with N = ca. 33; cloned in Bluescript
p33.15[b]	Human nuclear DNA	0.6 kbp *BamHI/KpnI* insert in pBluescript KS-
33.15	Synthetic primer	Monomer of the human minisatellite 33.15
$(GACA)_4$ or $(GATA)_4$	Synthetic primers	
M13 protein III tandem repeat	Bacteriophage M13	781 bp *BsmI/ClaI* fragment of M13; or PCR amplified tandem repeat region of M13; primers (Bellamy et al. 1990): 5'TCCTATTGGGCTTGCTATCC3' and 5'TTTCGGTCATATAGCCCCCTTA3'

[a]Provided by H. Judelson, R. Michelmore; [b]Provided by A. Jeffreys

sequence encoding the tandem repeat of the M13 protein III detected hypervariable repeats in humans and other organisms was accidental (Vassart et al., 1987).

Some of the human minisatellite sequences have limited availability in the cloned form due to the competitive nature of companies concerned with human DNA fingerprinting. However, some sequences can be obtained synthetically as primers, synthetic inserts of clones (Ledwith et al., 1990), or amplified directly from the human genome (Jeffreys et al., 1990), obviating the need for cloned minisatellite sequences (Table I).

In this study, we describe an improved protocol for the detection of minisatellite sequences using the tandem repeat sequence of the gene encoding M13 protein III as a probe in common bean (*Phaseolus vulgaris* L.). The concepts and procedures outlined in this protocol can be used in detecting minisatellite sequences with other probes such as the 33.15 and the 33.6 human minisatellite sequences in *Phaseolus* and other plants. There are, however, a number of technical difficulties in detecting these sequences in plants as compared to animals, attributable to differences ingeneome organization (*e.g.*, Seshandri and Ranjekar, 1980a,b) or size (Bennett and Smith, 1976; Bennett, 1987) or to wide evolutionary divergences between the two kingdoms and among plant taxa.

Solutions Needed

DNA extraction buffer: 50 mM Tris-HCl, pH 9.5, 0.7 M NaCl, 10 mM EDTA, 1% SDS, 5% (w/v) polyvinyl polypyrrolidone, 2% 2-mercaptoethanol.
Chloroform:isoamyl alcohol: in 24:1 (v/v) proportion
TE: 1 mM EDTA, 10 mM Tris-HCl, pH 8.0
TNE (10 x): 100 mM Tris, 10 mM EDTA, 1.0 M NaCl, pH 7.4
Bisbenzimidazole (Hoechst 33258): 10% in H_20 (w/v)
TAE (50 x): 2 M Tris-acetate, 0.05 M EDTA
Alkaline transfer buffer: 0.4 N NaOH, 0.6M NaCl
Primers for PCR radiolabeling of M13: 5'TCCTATTGGGCTTGC-TATCC3' and 5'TTTCGGTCATAGCCCCCTTA3'
PCR buffer (10 x): 15 mM $MgCl_2$, 100 mM Tris pH 9.0, 500 mM KCl, 1% Triton X-100
0.2 mM of each nucleotide: dATP, dTTP, dGTP, dCTP
Hybridization buffer: 5 x SSPE (900 mM NaCl, 58 mM NaH_2PO_4, 5mM EDTA), 7% SDS, 1% bovine serum albumin (Fraction V), and an additional 5 mM EDTA
Post-hybridization buffer: 2 x SSC, 0.1% SDS
Isopropanol

Protocol

DNA extraction[1]

- Weigh 5 g of tissue and grind in mortar and pestle with liquid nitrogen. Add to 10–12 mL of DNA extraction solution.
- Incubate the mixture at 60°C for 1 hour with occasional shaking.
- Cool tubes. Extract aqueous phase two times with an equal volume of chloroform:isoamyl alcohol (24:1)
- Precipitate DNA with 2/3 volume isopropanol alcohol. Either hook out DNA or centrifuge. Wash precipitated DNA with 80% (v/v) ethanol. Dry precipitated DNA and dissolve in TE solution.

DNA quantitation and digestion

- Two µL of DNA stock solution is diluted to 2 mL in 1X TNE buffer containing 0.1 µg/mL bisbenzimidazole (Hoechst 33258)and the concentration of the DNA determined by fluorescence.[2]
- 4 µg of DNA from each accession is digested overnight[3] with 160 units restriction endonuclease (*Hae* III, *Hinf* I, or *Taq* I) in buffer supplied by New England Biolabs and the addition of 0.5 µg of acetylated BSA.

Electrophoresis

- Equal quantities[4] of DNA are loaded onto 1.5% agarose gels. Electrophoresis is run at no greater that 2mV/cm (Kirby, 1990) for at least 16 hours in 1 x TAE buffer until the bromophenol blue dye is within 2 cm of the bottom of the 25 x 21-cm gel tray[5].

Southern transfer

- Capillary transfer of DNA fragments to Zetabind membrane (AMF-CUNO, Meridan, CT, USA) is accomplished in alkaline conditions[6] (0.4 M NaOH, 0.6 M NaCl) in overnight transfer.

Labeling tandem repeat region of M13 protein III[7]

- Using the random-primer method, the replicative form of M13 is first isolated according to Sambrook et al. (1989) and then digested with *ClaI* and *BsmI*. The desired 781-bp *ClaI/BsmI* fragment is recovered after electrophoresis in low-melting point agarose. Labeling is performed according to Feinberg and Vogelstein (1983, 1984).
- Using PCR labeling (Schowalter and Sommer 1989; Bellamy et al. 1990), M13 is labeled in a 50-µL reaction volume using the following conditions: 5 ng of heat-denatured template is added to 10 µM of each primer (Table I), which is 2 µL of a 50 ng/uL of the oligonucleotide mixture, 5 µL of *Taq* DNA polymerase buffer, 5 µL of 0.2 mM mixture of each non-labeled nucleotide (dATP, dTTP, dGTP), and 50 µCi (5 µL) of labeled nucleotide ($\alpha[^{32}P]$-dCTP, $\alpha[^{32}P]$-dATP or $\alpha[^{32}P]$-dTTP; specific activity = 800 Ci/mmole). The DNA is cycled through 30 cycles of amplification using the conditions

from Bellamy et al. (1990): 1 min at 94°C for denaturation, 2.5 min at 55°C for annealing, and 5 min at 72°C for elongation.

Hybridization

- The amplified tandem repeat-region is hybridized to Zetabind filters at 52°C [8] for 16 to 24 hours in 0.25 mL hybridization buffer[9] per cm^2.

Post-hybridization wash and detection

- Low-stringency washes are performed in 2 x SSC, 0.1% SDS at room temperature for 1 hour with at least 3 changes of excess washing buffer[10].
- A preliminary survey of the ^{32}P signal on the Southern blot can be made with an end-window probe of a Geiger counter. Between 300 to 600 counts per min should be observed to obtain clear banding patterns on the autoradiograph after four days without intensifying screens. Exposures without intensifying screens produce sharper bands and more of the higher molecular weight bands are detectable. Multiple exposures are made with and without the intensifying screen to insure analysis of all restriction fragments.

Notes

1. DNA was extracted according to the procedure of Saghai-Maroof et al. (1984), as modified by Gepts and Clegg (1989).
2. The fluorometer detects the quantity of DNA in the presence of the stain bis-benzimidazole (Hoechst 33258) without interference from RNA (Labarca and Paigen, 1980).
3. Shorter digestion times can be used, in which case complete digestion is determined by comparison with overnight digestions. Inclusion of bacteriophage lambda DNA as an internal digestion control should be avoided because both the 33.15 human minisatellite and the tandem repeat sequence of the M13 protein III cross-hybridize with the lambda *Hind* III 23,130-bp fragment at low stringency.
4. Equal quantities of DNA need to be loaded in each lane of the agarose gel because banding patterns are difficult to compare and interpret if unequal quantities are present between lanes due to background problems and the large number of bands with varying image intensities. For the most precise DNA quantification, one should use the most sensitive range of the fluorometer.
5. Caution should be used in interpreting the sizes of restriction fragments on the 1.5% agarose gels run at 2mV/cm because previous studies have found band inversion under these conditions (Slater et al., 1989).
6. Alkaline transfer (Reed and Mann, 1985) of DNA fragments to a charged nylon membrane is recommended when using 1.5% agarose in order to insure complete denaturation and transfer of restriction fragments. In the higher percentage agarose gels the diffusion rates of the denaturation and neutralization solutions are significantly reduced, which makes other methods such as transfer in 10 x SSC more time consuming and tedious.

7. Probes need to be labeled to a high specific activity (i.e., 0.5 x 10^9 to 5 x 10^9 dpm/μg). For random-primer labeling, relatively high quantities of the desired restriction fragment or insert need to be isolated in low-melting-point agarose. The restriction digest of the plasmid should contain at least 5 to 10 μg of DNA. The fragment is labeled using a[^{32}P]-dCTP of specific activity > 3,000 Ci/mmol. For PCR labeling, the specific activity of a[^{32}P]-dCTP is ~800 Ci/mmol.
8. In general, minisatellite sequences need to be hybridized at lower temperature in plants compared to humans. A general rule for determining the hybridization temperature of minisatellite probes and oligonucleotides is to calculate the T_d (Miyada and Wallace, 1987) of an oligonucleotide monomer in the tandem repeat of the particular minisatellite in question. The equation for T_d (the temperature at which one half of an oligonucleotide duplex becomes associated in 1 M NaCl) is as follows: $T_d = 4°C \times (G + C) + 2°C \times (A + T)$. The best hybridization temperature is then determined empirically and may vary from 0 to 20°C below the T_d value.
9. The hybridization buffer is a modified form of a high SDS buffer that is commonly used for hybridization of minisatellite sequences (Westneat et al., 1988).
10. Stable hybrids between the M13 probe and the *Phaseolus* genomic DNA are maintained in 42–60°C washes. However, some of the bands disappear in the higher stringency washes.

Results

In our program, we have been interested in identifying genotypic differences among individuals within populations. Our past experience with biochemical or molecular markers in *P. vulgaris* [*e.g.*, phaseolin seed protein (Gepts et al., 1986; Koenig et al., 1990), allozymes (Koenig and Gepts 1989; Singh et al., 1991), RFLPs of nuclear DNA (Nodari et al., in press), and RFLPs of cpDNA (Llaca and Gepts, 1991)] indicated that these markers were in most cases not variable enough for our purpose. Instead, minisatellite markers could potentially provide the tool needed for intra-population studies based on results in other organisms (*e.g.*, Jeffreys, 1987; Dallas, 1988; Nybom et al., 1989). Hence, in a first step, we examined the potential of various mini- or micro-satellite sequences to detect polymorphisms within *P. vulgaris*. In a second step, we compared the effect of various labeling procedures on the banding patterns revealed by mini-satellite sequences.

Relative usefulness of various probes. All the probes listed in Table I hybridized to genomic *P. vulgaris*; however, only the M13 protein III tandem repeat (Figs. 1 and 2) and 33.15 sequences (not shown) displayed polymorphisms involving a large number of sharp bands. The oligonucleotide $(GACA)_4$ hybridized to distinct bands but revealed little

polymorphism. Sequences related to the 33.6 human minisatellite (33.6 primer and pHSD42⁻) hybridized to a dispersed smear (not shown). These sequences might detect useful polymorphisms in other taxa. Thirdly, we examined the use of electrophoresis paper to increase the contrast among bands by decreasing background signal.

Influence of the labeling method. Significant differences in banding pattern and band intensity could be observed in blots hybridized with the gene encoding M13 protein III labeled with the random-primer method versus PCR depending on the radiolabeled nucleotide used (Figs. 1 and 2). In both reactions, the entire hybridizing region of the M13 protein III tandem repeat is labeled (Van Wezenbeek et al., 1980; Vassart et al., 1987). When PCR was used to label the probe with a[^{32}P]-dCTP, differences in banding pattern were observed compared to the banding pattern generated after random primer labeling with the same radionucleotide (compare Figs. 2, left and middle). However, banding patterns generated after PCR labeling with a[^{32}P]-dATP or a[^{32}P]-dTTP were similar to the banding pattern obtained after random primer labeling with a[^{32}P]-dCTP (compare Fig. 2, left vs. right and Fig. 1, left vs. right).

Transfer of images to electrophoresis paper. To obtain images of the Southern hybridization with much less of the background smear than appears on the autoradiograph (Figs. 1 and 2), the image on the autoradiograph is duplicated with electrophoresis duplicating paper (EDP Kodak 182-7831). The autoradiograph is placed on the duplicating paper which is subsequently exposed to light for 5 to 40 seconds. The paper is developed with Dektol and fixed with Kodak rapid fixer.

Discussion

Several key aspects to be considered when attempting to develop fingerprints with mini-satellite sequences.

The first aspect is the probe-enzyme combination, which should reveal a banding pattern with sharp bands and reduced background hybridization. In our hands, the tandem repeat sequence of M13 protein III , the 33.15 human minisatellite sequence, and the $(GACA)_4$ sequence revealed such a banding pattern, after digestion with either *Hae*III, *Hin*f I, or *Taq* I. In addition, the first two sequences were able to detect polymorphisms among closely related genotypes, making them useful tools for population genetic studies (Stockton, Sonnante, and Gepts,

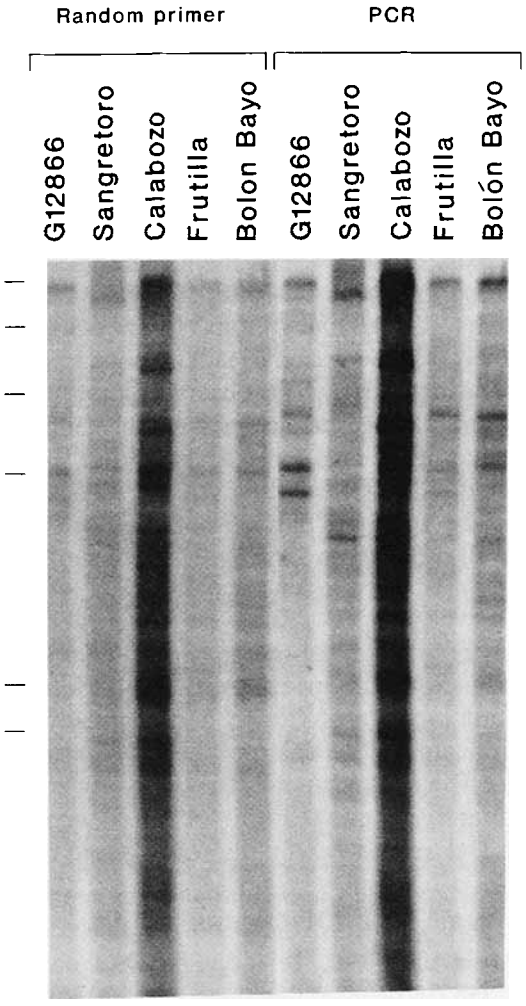

Fig. 1. Southern hybridization of total genomic DNA from *Phaseolus vulgaris* with M13 labeled either by the random primer-method or PCR. Total genomic DNA was digested with *Taq*I and electrophoresed on an agarose gel. After Southern transfer, the blot was hybridized first with the M13 protein III tandem repeat region labeled by the random primer method with $\alpha[^{32}P]$-dCTP (left) and, following probe removal, with the M13 protein III tandem repeat region labeled by PCR with $\alpha[^{32}P]$-dATP (right). Size markers are the upper 6 fragments of λDNA digested with *Hin*dIII, ranging between 23,130 bp (top) and 2,027 bp (bottom). Photos were taken directly from the autoradiogram on X-ray film.

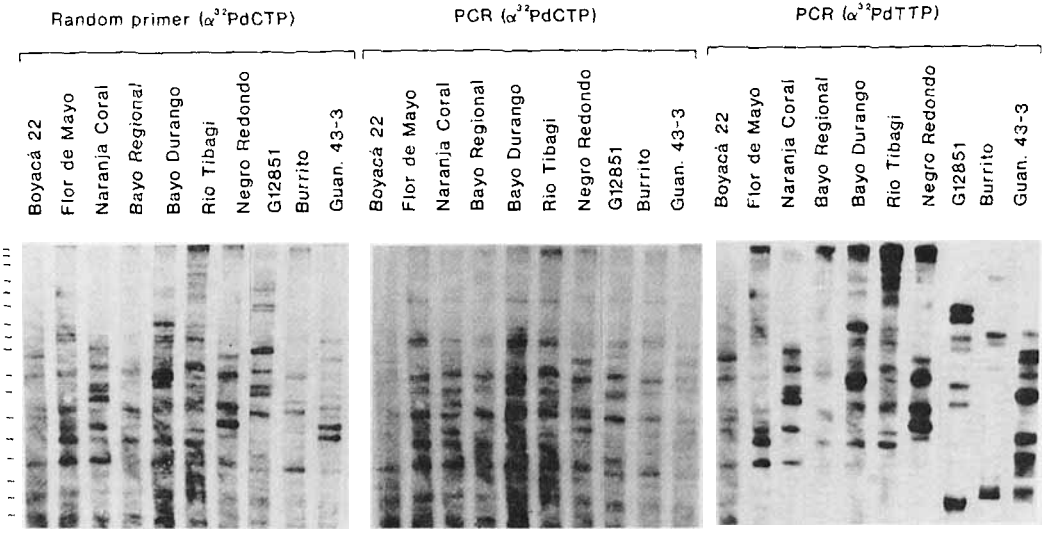

Fig. 2. Southern hybridization of total genomic DNA from *Phaseolus vulgaris* with M13. Total genomic DNA was digested with *Taq* I and electrophoresed on an agarose gel. After Southern transfer, the blot was hybridized first with the M13 protein III tandem repeat region labeled by the random primer method with α[^{32}P]-dCTP (left). Following probe removal, the same blot was probed with the M13 protein III tandem repeat region labeled by PCR with α[^{32}P]-dCTP (middle) or α[^{32}P]-dTTP (right). Size markers are the upper 17 bands of the λ DNA Analysis Marker System of BRL (Cat. No. 4401SA), ranging between 22,621 bp (top) and 2,433 bp (bottom). Photos were taken from an image on electrophoresis paper after duplication from the autoradiogram.

unpubl. results). It is not clear why certain sequences revealed fingerprints whereas others could not. In a general sense this may depend on the genome organization of *P. vulgaris*. Common bean has a longer interspersion pattern than wheat, rye, soybean, and tobacco, and of most animal genomes (Gurley et al., 1979; Seshadri and Ranjekar, 1980b). In contrast the lengths of the interspersion in common bean is less than that in pearl millet (Wimpee and Rawson, 1979). These genomic organization differences combined with differences in repeat sequence composition and methylation/mutation patterns may be responsible for differential restriction endonuclease restriction patterns seen between the plant and animal kingdoms and among plant taxa. We are currently mapping the polymorphic bands on the genome map of the common bean (T. Stockton, R. Nodari, and P. Gepts, unpubl. results). We have also cloned a *P. vulgaris* sequence with homology to M13. In Southern hybridizations, this clone reveals a banding pattern that is similar to that revealed by M13; we are currently determining its sequence (T. Stockton and P. Gepts, unpubl. results).

The second aspect is the size distribution of polymorphic bands. In our experience, such bands can reliably be identified over a molecular weight range from 1 to 10 kb. To obtain a good separation of all bands, it may be necessary to run agarose gels at different concentrations: *e.g.*, gels at 1.5 % agarose may separate primarily bands in the 2- to 6-kb range, whereas 0.8% agarose gels may separate bands in the 6- to 10-kb range.

The third aspect is probe labeling. The two labeling methods—random primers and polymerase chain reaction—yielded probes revealing different but reproducible banding patterns. Differences were also observed according to the radiolabeled nucleotide used in the reaction. Such differences may be due to differential incorporation of the different radionucleotides into the probe during the labeling reaction or to the different base composition of the various repetitive sequences hybridizing to the minisatellite probes. These effects might have been accentuated due to the high G/C content (60%) of the tandem repeat of the M13 protein III gene.

The fourth aspect involves the hybridization conditions and particularly the hybridization temperature. In *Phaseolus*, highly unstable DNA hybridizations were observed at standard stringencies of hybridization (i.e., 5 x SSC and 50% formamide at 42°C or 5 x SSPE at 65°C) using the M13 protein III tandem repeat and the human minisatellite sequences. Similar instabilities requiring washes of extremely low stringency have been observed in grape (*Vitis vinifera*; T. Bandman and C. Meredith, personal communication). For this reason, hybridization temperatures were calculated using T_d and, subsequently, the actual hybridization

temperature to these heterologous probes was determined empirically using T_d as a reference point. Depending on the probe, this actual temperature ranged anywhere between 0 and 20°C below T_d. For example, the T_d for the monomers of the M13 tandem repeat ranged between 48 and 52°C; the empirically determined temperature was approximately 52°C (T_d - 0). On the other hand, the oligonucleotide $(GACA)_4$ was hybridized at 37°C (T_d - 11) and the human minisatellite 33.6 primers and pHSD42- were hybridized at 54°C (T_d - 20). Although hybridization temperature had to be lowered to detect sequences hybridizing to M13 protein III tandem repeat in *Phaseolus*, Zimmerman et al. (1989) were to able obtain more stable hybrids of M13 protein III tandem repeat region with various plant taxa under standard hybridization stringency (50% formamide, 6 x SSC, 42°C). However, the probe they used differed from that used in *Phaseolus* and other studies (Rogstad et al., 1988; Nybom and Rogstad, 1990) in that it consisted of a 309-bp *Hae*III fragment that included the 117-bp tandem repeat element, which is essentially two-thirds of the sequence previously implicated in the detection of hypervariable RFLPs (Vassart et al., 1987). In contrast, the sequence used to probe *Phaseolus* and other taxa (present results and Rogstad et al., 1988) is a 781-bp *Cla*I/*Bsm*I fragment containing both regions implicated in human minisatellite detection (Vassart et al., 1987). An explanation for differing stabilities of these hybridizations could be that there may be more heteroduplex formation with the 781-bp fragment containing two separated regions of tandem repeats and that a smaller proportion of the total 781-bp probe hybridizes to the genomic DNA on the Southern blot. Another explanation for the varying hybridization temperatures between studies is the wide evolutionary divergence among the plant taxa examined. Although Zimmerman et al. (1989) included several leguminous taxa in their study, fewer copies of sequences cross-hybridizing to their M13 probe were present in the legume genomes as compared to the grass taxa genomes (*Zea mays* and *Lolium perenne*) using the same hybridization conditions. The similarity of the probe to the sequences in these various taxa is unknown and could have been partially responsible for differences in copy number among taxa.

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