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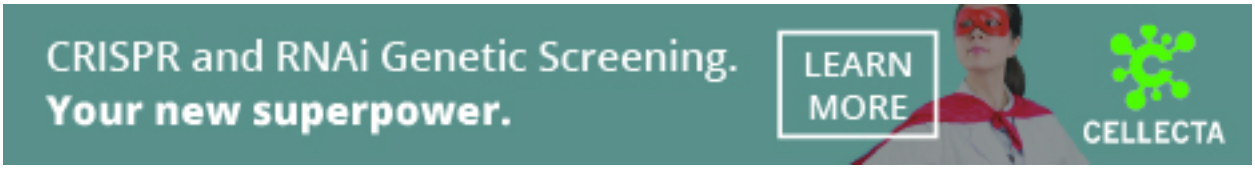

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The Random Amplification of Polymorphic DNA for Fingerprinting Plants

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Molecular markers based upon DNA sequence variation have proved extremely effective tools for distinguishing between closely related genotypes. With the advent of PCR technology, methods have been developed that use amplified DNA sequences as molecular markers. These methods require very little template DNA, which can usually be obtained using a simple minipreparation protocol; the production of marker bands is very fast and far less labor intensive than when using restriction fragment length polymorphism (RFLP) technology. PCR-based techniques have already been used in the analysis of relationships between plants.[1–10] In this paper, we describe methods for producing plant molecular markers using randomly amplified polymorphic DNA (RAPD) technology. In particular, we describe the effect of varying a range of factors during the procedure and define the protocol that we have found most useful.

DNA was extracted from 0.1–0.5 grams of fresh leaf material using a rapid minipreparation method based upon the protocols of Sgai-Maroof et al.[11] and Murray and Thompson.[12] Yields of DNA were measured using the Hoechst dye assay method with a TKO 100 Minifluorometer according to the manufacturer's instructions. For PCR, all reaction mixtures had a total volume of 50 µl. Unless otherwise indicated, the mixture contained 1 unit of *Taq* polymerase (Cetus), 0.2 nmole (= 4 µM) of a single 10-mer oligonucleotide, 200 µM with respect to dATP, dCTP, dTTP, and dGTP, 2.5 mM magnesium chloride, the appropriate dilution of the reaction buffer prepared by the company supplying the polymerase, and not more than 0.1 µg of plant DNA (see below). All reaction mixtures were overlayed with mineral oil before being placed in a Hybaid Thermal Reactor HBTR1. Unless otherwise indicated, all programs had an initial cycle with 94°C for 7 min, the annealing temperature was 36°C (for 1 min), and the extension time was 4 min (at 72°C). The subsequent 35 cycles were the same except that the 94°C denaturation step was applied for only

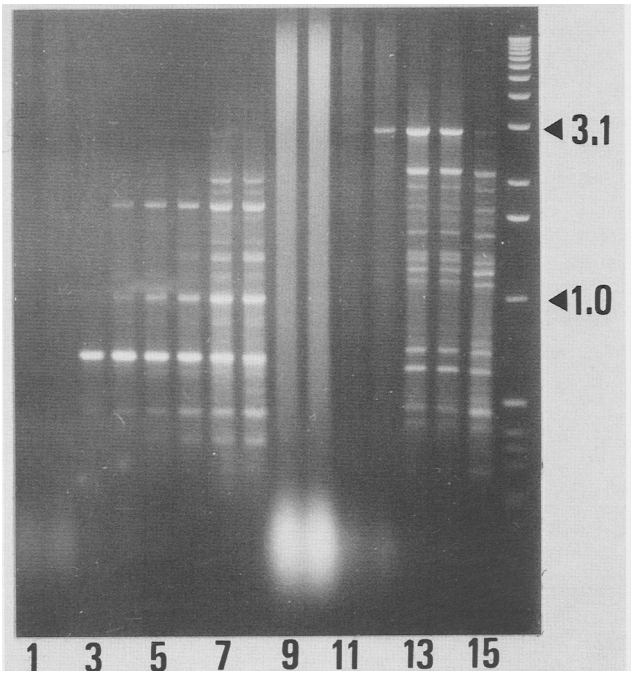

FIGURE 1 Amplification supported by different amounts of template DNA. After the initial cycle, the running program was 94°C for 1 min, 36°C for 1 min, and 72°C for 4 min for 35 cycles. Using banana DNA: (lanes *1* and *2*) 320 ng of DNA; (lanes *3* and *4*) 32 ng of DNA; (lanes *5* and *6*) 13 ng of DNA; (lanes *7* and *8*) 3.2 ng of DNA. Using rice DNA: (lanes *9* and *10*) 4.2 µg of DNA; (lanes *11* and *12*) 420 ng of DNA; (lanes *13* and *14*) 170 ng of DNA; (lane *15*) 42 ng of DNA. Molecular weight markers were run in lane *16* (sizes in kbp).

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1 min. In this paper, as in some others describing RAPD results,[1,8–10] the primers used have been arbitrarily defined 10-mers. Amplifications have been carried out using DNA extracted from sugar beet, banana, *Phaseolus* bean, *Chamaecytisus*, rice, and potato.

To produce a protocol for RAPD that will efficiently yield reproducible results, a number of decisions must be made concerning a range of experimental parameters. To optimize the method used, we have carried out experiments in which we have varied a number of factors.

1. We have found no advantage in using more than 35 cycles during the amplification program. Presumably, inactivation of the polymerase by repeated incubation at 94°C prevents the accumulation of amplification products in later cycles. However, where amplification products are apparent, we have not detected any changes in banding patterns due to variation in cycle number.
2. We have carried out experiments in which the extension time in the reaction has been varied. It is clear that a different banding pattern can be observed by varying this factor with a longer extension time allowing (e.g., 4 min) the accumulation of larger amplification products.
3. The effects of different annealing temperatures on amplification were also examined. An increase in the annealing temperature can lead to the loss of some bands but not others; eventually all bands disappear. Once again, different banding patterns can be obtained using the same genotype and primer if a single reaction parameter is altered.
4. Control of the amount of template DNA in the reaction is critical for obtaining reproducible results. In Figure 1, rice and banana DNA have been used. For rice DNA, the optimum concentration was 170 ng per reaction. With banana DNA, no bands were obtained using 320 ng, but amplification of differing numbers of bands occurred as the DNA concentration was decreased, with the most extensive pattern emerging at 3.2 ng per reaction. However, amplification of banana DNA was not very reproducible because an unacceptable proportion of reactions produced no amplification products. In view of this, an altered DNA extraction procedure, designed specifically for banana tissue[13] was employed. The amplification success rate was greatly improved and DNA concentrations from 7 ng to 40 ng yielded the same banding patterns (not shown).
5. Differences in primer concentration can also lead to changes in the pattern of amplified bands. The appearance of some bands is favored by a low primer concentration (e.g, 1.6 μM), whereas other bands appear only at high primer concentrations (e.g., 19 μM).
6. The level of magnesium has a profound effect upon the banding patterns produced. In our experiments, no bands were observed in the absence of magnesium, and the number of bands increased as the concentration was increased to 4 mM.

If the parameters described above are controlled, then consistent banding patterns for DNA samples from individual genotypes are obtained. For example, a single marker band could be used to distinguish between two varieties of *P. vulgaris* (Edmund and Tendergreen), and this marker was shown to segregate in a Mendelian fashion (Fig. 2). A band present among the amplification products of Tendergreen, but not Edmund, behaves as a dominant marker. It is present in three-quarters of the F_2 progeny.

However, it is clear from the results of the experiments described, that a specific combination of plant genotype and primer can yield different RAPD banding patterns if a number of experimental parameters are altered. Although one would expect these to be controlled in a laboratory carrying out RAPD analyses routinely, variation in any of them between laboratories will lead to different results. So is there an optimum protocol? We think that there is not. A very large range of combinations of the parameters listed will yield amplification products. As long as the same set of conditions is always used, then the pattern of amplification products will always be the same. In

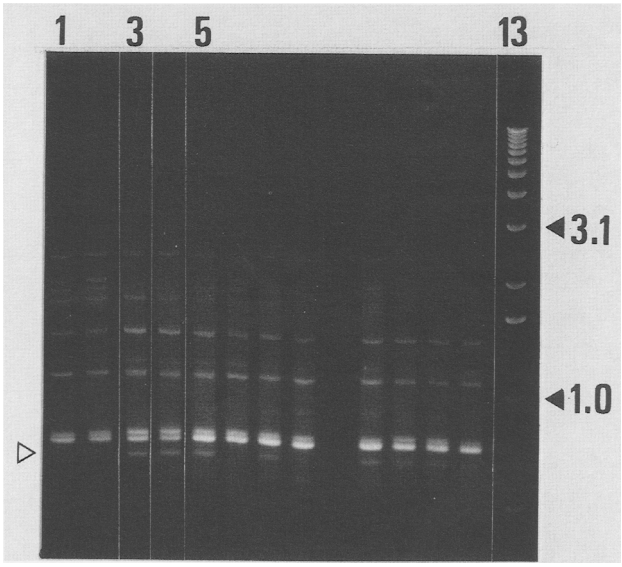

FIGURE 2 Amplification of *Phaseolus* DNA. After the initial cycle, the running program was 94°C for 1 min, 36°C for 1 min, and 72°C for 4 min for 35 cycles. The genotypes used were Edmund (lanes *1* and *2*) and Tendergreen (lanes *3* and *4*), followed by eight F_2 progeny of a cross between these two genotypes; in all, 39 F_2 progeny were used to confirm a 3:1 segregation of the band extra (marked by open triangle) from Tendergreen. Molecular weight markers were run in lane *13* (sizes in kbp).

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our RAPD work, we have defined a set of conditions (see initial description) and we stick rigidly to them in all except one respect. We have discovered that the optimal amount of DNA, extracted by the same procedure, in the reaction mix is different for different species. It may be that this is due to varying concentrations of inhibitory compounds in our DNA preparations from different species. The extraction technique we have used is quick but yields a fairly crude DNA pellet, which is usually discolored in the case of beet and banana. The particular problems with the success rate of amplification of banana DNA sequences have been dramatically improved by using a more intensive DNA purification protocol, and the levels of this purer banana DNA that support amplification are similar to the levels of beet and rice DNA routinely used.

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