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Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR)

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Summary. Using a novel procedure based on the polymerase chain reaction, we have developed a rapid, efficient, and economical method for identifying plant genotypes. The arbitrarily primed polymerase chain reaction (AP-PCR) generates reproducible fingerprints from any organism, without the need for DNA sequence information. These fingerprints include DNA fragment polymorphisms that can be (1) used for varietal identification and parentage determination, (2) followed in segregating populations produced by crosses, (3) used as markers for the construction of genetic maps, and (4) used to generate dendograms of phylogenetic relationships, especially at the intraspecific level. AP-PCR requires only minute quantities of DNA (10–25 ng per reaction) and therefore can be used in situations in which DNA is limiting. We demonstrate the use of AP-PCR to identify inbred parents of hybrid maize plants in double-blind experiments.

Key words: *Zea mays* – Parentage determination – Varietal identification – Genetic polymorphisms – Genetic markers

Introduction

Methods have been developed over the past two decades that allow the detection of polymorphisms in DNA. DNA polymorphisms can be used as molecular markers and, along with protein markers, are making a significant impact on applied plant breeding. The use of molecular markers in applied plant sciences has resulted in linkage maps for many important crop species (Helentjaris et al. 1986; Tanksley et al. 1987; Landry et al. 1987; McCouch et al. 1988). The most widely used method for detection of DNA polymorphisms is restriction fragment length polymorphisms (RFLPs) (Bishop and Skolnick 1980; Botstein et al. 1980). RFLPs are a product of change(s) in the bases within a restriction enzyme target site, deletion(s) or insertion(s) within a restriction fragment, or rearrangement(s) of DNA. An alternative method for detecting sequence variation in DNA uses denaturing gradient gel electrophoresis (DGGE) (Lerman et al. 1984; Lerman 1986; Riedel et al. 1990). Recently, DGGE has been used to identify polymorphisms in maize (Riedel et al. 1990). Most recently, techniques based on the polymerase chain reaction (PCR) have been used to detect polymorphisms in various plant, animal, and bacterial species (Welsh and McClelland 1990, 1991; Welsh et al. 1991; Williams et al. 1990). Both the arbitrarily-primed polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990) and the random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) can generate fingerprints of DNA products, some of which are polymorphisms that are present in one parent and absent in another. RAPD is a subset of AP-PCR that uses short (9–10 bp) primers. Polymorphisms detected by AP-PCR or RAPD are inherited in a Mendelian manner and can be generated for any species without DNA sequence information (Welsh and McClelland 1990; Williams et al. 1990; Welsh et al. 1991). Polymorphisms are frequent, are useful as genetic markers (Welsh et al. 1991), and can be easily detected by using fluorescent stains or by addition of alpha-[^{32}P] dCTP in the PCR reaction (Welsh and McClelland 1990; Williams et al. 1990). Detection of polymorphisms by using AP-PCR or RAPD technology is faster and less laborious than by using RFLP technology, as long as primers of approximately the same length and GC content are used in a given set of experiments, so that other reaction parameters can be kept constant.

The applications of AP-PCR are similar to those for RFLPs, i.e., the polymorphisms may be used as domi-

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nant genetic markers to determine genetic relationships (such as varietal identification, protection of breeders' rights, and parentage determination), to identify and map loci affecting quantitative traits (Welsh et al. 1991) and to monitor loci during introgression or selection programs. The main advantages of AP-PCR over traditional RFLP technology are increased speed of analysis and dramatic reduction in the amount of DNA required for analysis. For a genome the size of maize, 10–25 ng of genomic DNA is required per AP-PCR reaction. This is approximately 200–1,000 times less DNA than for RFLP analysis. Reduction in the amount of DNA required for a reaction is particularly important in speeding up early screening (in maize, e.g., screening could be done a few days after germination instead of 2–3 weeks thereafter) and in situations in which DNA amounts are limiting, such as extractions of DNA from the megagametophyte of most species of *Pinus*. Large genome size is also not a problem, since PCR-based methods can detect polymorphisms with much greater sensitivity than Southern hybridization (Sobral et al., unpublished results). Using a sequencing gel apparatus, a single person can complete the analysis of approximately 120 individuals with a primer in a single 36-h period, from the beginning of the AP-PCR reaction to the final results; similar results with RFLP analyses would take at least 1 week, and most likely more. Also, since AP-PCR polymorphisms need not be cloned during their detection and mapping, we can circumvent host and vector limitations, opening the possibility that some of the polymorphisms detected may map to genomic regions for which RFLPs may not be easily identifiable (Williams et al. 1990; Welsh et al. 1991). The main disadvantage of AP-PCR in relation to RFLP analyses is that most of the polymorphisms detected are scored as dominant markers (presence versus absence in each of the parents), and therefore heterozygotes cannot be distinguished accurately. RFLP markers generally are codominant (Burr et al. 1983), thereby allowing the heterozygotes to be identified and yielding additional information that may be important for some applications.

To determine the usefulness of AP-PCR in varietal identification and determination of parentage, we have used six maize inbreds and five hybrids derived from these inbreds to show that determination of parentage can be done by using AP-PCR. Our data not only prove that this is possible, but also suggest that single-gel AP-PCR experiments can be done to identify varieties.

Materials and methods

Inbreds and hybrids used and DNA extractions from maize seedlings

Maize inbred lines used were B73, Mo17, DE811, Oh43, FR16, and H99. These inbreds were maintained as pure stocks and ears were harvested individually. Inbred plants maintained as described were used to produce hybrid seed that was harvested individually. Seeds from these inbreds, as well as from three hybrids, were a kind gift from M. Lee (Department of Agronomy, Iowa State University, Ames) and are being used in an RFLP mapping project. Seeds were germinated in rolls of water-moistened paper towels at 28 °C. DNA extractions from leaves of 5-day-old seedlings were performed according to the method of Doyle and Doyle (1987). For inbred lines and F_1 hybrids, leaves from three seedlings were pooled. The genotypes of the samples were coded prior to DNA extraction.

AP-PCR

Ten-microliter reactions were prepared by using 1 × *Taq* polymerase buffer (Stratagene Cloning Systems, La Jolla/CA) adjusted to 4 m*M* with $MgCl_2$, 0.2 m*M* of each dNTP, 10 μ*M* M13 universal primer (5′-TTATGAAACGACGGCCAGT-3′), 0.25 U *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, CT), and template DNA at various concentrations (see Fig. 1 legend). The high Mg^{2+} concentration was used to enhance the stability of primer/template interactions. The reaction was overlaid with mineral oil and cycled through the following temperature profile: 94 °C for 5 min to denature, 48 °C for 5 min for low stringency annealing of primer, and 72 °C for 5 min for extension for two cycles. This temperature profile was followed by ten high stringency cycles: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. At the end of this reaction, 90 μl of a solution containing 1 × *Taq* polymerase buffer, 0.2 m*M* dNTPs, and 50 μCi alpha-[^{32}P] dCTP were added and the high stringency cycles were continued for an additional 30 cycles. This protocol was designed to allow for high primer concentration during the low stringency cycles (Welsh and McClelland 1990).

Gel electrophoresis and autoradiography

AP-PCR products (2-μl samples) were mixed with 3 μl of formamide dye solution, heated to 72 °C for 20 min, and 3 μl of this was loaded onto a 5% acrylamide, 50% urea gel, prepared in 1 × TBE buffer. Electrophoresis was performed using a Poker-Face sequencing apparatus (Hoefer Scientific Instruments, San Francisco/CA), at 1,500 V, until the tracking dye reached approx. 4/5 the length of the gel (approx. 2.5 h). At this point, the gel was placed on an X-Omat film (Kodak, Rochester/NY) and autoradiography was performed overnight at −70 °C without intensifying screens. It was not necessary to dry the gel before proceeding to autoradiography.

Results and discussion

A double-blind experiment was designed to determine whether or not we could identify inbred parents of hybrid maize plants using AP-PCR. The resulting gel is shown in Fig. 1. We named polymorphic AP-PCR products systematically by giving the name of the primer used followed by the molecular weight of the polymorphic band. The polymorphic bands were also identified by using lowercase letters in Fig. 1.

The inbred parents (groups 1–6 in Fig. 1) of the hybrids (groups 7, 8, and 9 in Fig. 1) can be ascertained by comparing the polymorphisms indicated by dots. For example, the B73 × Mo17 hybrid contains the sum of the polymorphisms that characterize B73 and Mo17, as ex-

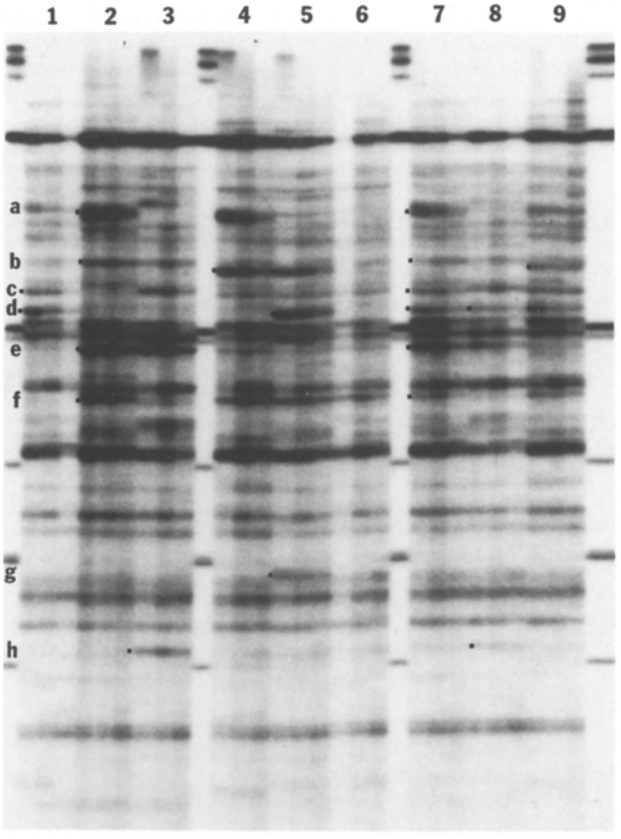

Fig. 1. AP-PCR fingerprints of maize DNA, arranged in groups of three. The first, second, and third lanes of each group correspond to 64, 16, and 4 ng of genomic DNA per reaction. Groups 1–6 are inbred lines of maize: group 1, B73; group 2, Mo17; group 3, DE811; group 4, Oh43; group 5, FR16; group 6, H99. Groups 7, 8, and 9 are F_1 progeny, as follows: group 7, B73 × Mo17; group 8, B73 × DE811; group 9, B73 × Oh43. The parents of the F_1 group can be determined by comparing the polymorphisms indicated by dots. Size markers are the 1-kb ladder from Gibco/BRL. AP-PCR and electrophoresis conditions are described in the text. The lowercase on the *left* identify the polymorphisms (see text)

pected. The polymorphisms that characterize B73 are M13-565 (lowercase c) and M13-505 (d), and the polymorphisms that characterize Mo17 are M13-736 (a), M13-623 (b), M13-492 (e), and M13-441 (f). The B73 × Mo17 hybrid contains all of these polymorphisms. Other polymorphisms are as follows: DE811, M13-302; Oh43, M13-576; FR16, M13-595, M13-530, and M13-332.

Soller and Beckmann (1983) determined that 15 polymorphisms gives a combined probability of exclusion (CPE) of 0.95 if the female parent is scored and the combined allelic frequencies are 0.5. If the female parent is not scored, then 22 polymorphisms are required for a CPE of 0.95. In our case with maize, strain identification will usually involve a situation where the likelihood is that a given hybrid came from a series of known inbreds. In this case, the plausibility of origin from a given inbred can be taken as the ratio of specific inbred probability to the sum of the probabilities of all inbreds (Soller and Beckmann 1983).

Our data show that one primer, such as the M13 primer, can produce as many as four (Mo17) and as few as one (DE811 and Oh43) diagnostic polymorphisms in a single experiment. The M13 primer revealed fewer polymorphisms than did other primers, at least in rice, sugar cane, and pinyon pine (B.W.S. Sobral, unpublished results). Therefore, it is possible to obtain more polymorphisms per primer by adjusting the sequence of the primer. Using the current experimental setup, we can analyze 60 lanes of data in a single, 1-day, one-gel experiment. To achieve larger numbers of polymorphisms required for a high level of certainty in parentage determinations, three or four different primers could be used, thereby generating a large number of polymorphisms. If the primers were approximately of the same length and GC content, it would not be necessary to adjust the AP-PCR conditions, as other experiments have shown (Welsh and McClelland 1990; Welsh et al. 1991). We have found that the conditions reported here work well with most primers of similar GC content and length (Welsh et al. 1991 and data not shown). Other refinements in AP-PCR, such as the use of pair-wise primer combinations, are being investigated. Preliminary data suggest that such modifications may further reduce the cost and time inputs of parentage determinations, as well as of other applications of AP-PCR. Although it was not the purpose of this investigation to do AP-PCR-based mapping in maize, results using other genomic DNAs, such as from mouse recombinant inbreds (Welsh et al. 1991), rice (Welsh and McClelland 1990), soybeans (Williams et al. 1990), and sugar cane (Sobral et al., unpublished results), show that AP-PCR polymorphisms are inherited from generation to generation, thereby making them extremely useful for genetic mapping. The dominant nature of the markers is offset by the large number of polymorphisms generated by a single marker using AP-PCR. As long as it is unnecessary to identify heterozygotes, or if backcrosses can be easily made, we feel that AP-PCR-detected polymorphisms can be used to generate linkage maps much more quickly than RFLP-based procedures.

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