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Pre-germination genotypic screening using PCR amplification of half-seeds

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Abstract. A simple and rapid PCR-based method has been developed for determining the genotype of seeds before germination. Single half-seeds of rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L. em. Thell.) were preincubated, without grinding, in an aqueous extraction buffer. The resulting supernatants were then used in polymerase chain reaction (PCR) with oligonucleotide primers corresponding to rice single-copy sequences or a wheat microsatellite repeat. PCR products of identical size were amplified using either the half-seed extract or DNA isolated from leaf tissue. The remnant half-seeds can be maintained in ordered arrays using microtiter plates allowing the recovery of selected genotypes. Pre-germination genotypic screening of seed populations as described in this report should be useful for a variety of applications in plant breeding and genetics studies.

Key words: PCR – RAPDs – RFLPs – Marker-assisted selection

Introduction

Molecular genetic markers are finding many applications in plant breeding programs and genetic studies. Such applications can require knowledge of the genotypes of many individuals with molecular markers. For example, it is often necessary to identify rare genotypes or recombinants, determine the "fingerprint" of varieties, or assess the varietal purity of seed stocks. However, managing large numbers of seedlings while conducting the genotyping analyses raises many problems. The numbering and ordering of plants is necessary in order to later identify plants with particularly desirable genotypes. To grow and maintain plants in the greenhouse or in the field requires space, time coordination, and other resources. During the genotyping process, plant materials are exposed to pests and environmental stresses that may result in the loss of desirable plants. In order to avoid these problems we have developed a polymerase chain reaction (PCR)-based method that allows the genotyping of seeds before they are germinated.

The polymerase chain reaction has become a basic tool in molecular biology and is employed in a variety of applications including forensic and clinical diagnostic procedures (Saiki et al. 1985; Kazzazian 1989). A particular advantage of PCR is that it requires little biological material (e.g., a single human sperm, blood stains, hair, human tissue) as a starting template and offers a rapid method for the analysis of DNA samples (Li et al. 1988; Higuchi et al. 1988; Higuchi 1989; Mercier et al. 1990; Panaccio and Lew 1991).

The most limiting factor for the use of PCR amplification in analyzing large plant populations is the time and expense of extracting DNA. The extraction of DNA from plant tissue is time consuming, labor intensive, and involves the use of many chemicals (Dellaporta et al. 1983; Bernatzky and Tanksley 1986; Lassner et al. 1989; Doyle and Doyle 1990). There are several recent papers on the rapid extraction of DNA from plant tissues, but almost all of these involve grinding and/or the use of organic solvents and all require tissue from whole plants (Edwards et al. 1991; Langridge et al. 1991; Berthoumieu and Meyer 1991; Guillemaut and Marechal-Drouard 1992).

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We report here the application of PCR to intact, ungerminated seeds. Half of the seed is used for PCR analysis and the remaining ungerminated half-seed can be germinated later after PCR analysis. The ability to select genotypes before germination has implications for both breeding and genetic studies.

Material and methods

Seed and plant material

Dry seeds of indica rice cvs IR24 and 1188 (nearly isogenic for *Xa-21*, *Xanthomonas oryzae* pv. *oryzae* resistance), and an F_2 population derived from a cross between these two lines were supplied by P. Ronald (University of California, Davis, Calif.). Wheat cv 'Papago S' was provided by M. Röder (Cornell University, Ithaca, N.Y.).

Primers

Primers PB7 and PB8 were designed from the sequence of a genomic clone, RAPD248, which detects restriction fragment length polymorphisms (RFLP) linked to *Xa-21* (Ronald et al. 1992). This set of primers has been assayed on leaf-extracted DNA for which the genotypes are already identified by RFLP analysis and similar results obtained for homozygotes and heterozygotes. The PCR products amplified from the *Xanthomonas* resistant and susceptible lines are approximately 900 bp and 700 bp, respectively (Fig. 1; Ronald et al. 1992). Sequences of the primers are as follows: PB7 5′ d[AGACGCG GAAGGGT-GGTT CCCGGA]3′, PB8 5′d[AGACGCGGTAATCGAA-AGATGA AA] 3′.

The primer set for wheat microsatellite WHGT2 left and right were provided by M. Röder, and amplify three products of about 245, 210, and 130 bp. WHGT2 has the following sequence: left 5′ d[CTGCAAGCCTGTGATCAACT] 3′, right 5′ d[CAT-TCTCAAATGATCGAACA] 3′.

Half-seed treatment

Seeds were cut in half, and the endosperm ends were placed into 200 µl of PCR buffer containing 10 m*M* TRIS-HCl pH 8.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20 and 6–24 µl/1000 µl of 10 mg per milliliter proteinase K (12–48 µg/ 200 µl reaction) in 1.5-ml microcentrifuge tubes. The other halves of the seeds were maintained dry in 96-well plates at room temperature. The tubes with half-seeds were incubated in a water bath at 50 °C for 1h, centrifuged briefly (1 min, 12 000 *g*), and then boiled at 100 °C in a heat block for 5 min to inactivate the proteinase K.

An alternate extraction buffer employed the use of Chelex-100 (Bio-Rad), a chelate resin (Walsh et al. 1991). Half-seeds were immersed in 200 µl 5% Chelex-100 in double-distilled water and boiled for 10 min in a heat block at 100 °C. The tubes were then centrifuged at 12 000 *g* for 5 min and the supernatant used for PCR amplification.

PCR conditions

Ten to 25 microliters of the half-seed extracts were used per 100 µl of PCR reaction (10 m*M* TRIS-HCl pH 8.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 µM each of dNTP, 100 nM of each primer, and 0.2–0.5 µl of 5 units/µl of *Taq* Polymerase). PCR reactions were performed in a Perkin Elmer Cetus Thermocycler model 480 using the following profile: 94 °C, 1 min; 45–55 °C, 1–2 min; and 72 °C, 2 min for 35 cycles. PCR products were resolved by electrophoresis (1.2 V/cm) for 18 h in a gel composed of 1% Agarose or 1% Agarose/1% NuSieve Agarose GTG or 1% Agarose/2% NuSieve Agarose GTG. The running buffer was either 0.5 × or 1 × NEB [1 *M* TRIS (Sigma 7–9), 10 m*M* Na_2EDTA and 125 m*M* NaOAc, pH 8.1]. Photographs were taken after ethidium bromide staining and destaining for 10 minutes each.

Remnant half-seeds were tested for their ability to germinate at 1-week intervals for 8 weeks. A total of 640 IR24 rice seeds were divided into four treatments: full seed and half-seed with and without fungicide (Captan). Seeds of each treatment were stored at 25 °C in a dry place and at weekly intervals 20 seeds from each treatment were germinated in 9-cm petri plates held at 30 °C in a dark incubator. Germination rates were calculated by counting germinated seeds after 7 days.

Result and discussion

PCR using extracts from rice half-seeds

Proteinase K treatment

Half-seeds of two nearly isogenic lines (NILs), differing for alleles at the *Xa-21* locus, were used as substrates for PCR amplification using primers specific to *Xa-21*-linked markers (Ronald et al. 1992). Purified DNA isolated from leaf tissue from these same NILs was amplified with the same primers as a control. PCR using half-seeds or purified DNA resulted in amplified products of the expected molecular weights for both NILs (Fig. 1). To further test PCR on seed extracts and to examine amplification from a heterozygous locus, individual F_2 seeds derived from a cross between the two NILs were randomly selected and analyzed. The *Xa-21* primers produced the expected banding patterns for each of the three genotypic classes (Fig. 1).

The addition of proteinase K to the extraction buffer is essential to template preparation prior to PCR. When proteinase K was excluded, considerably lower yields of the PCR product were observed. How-

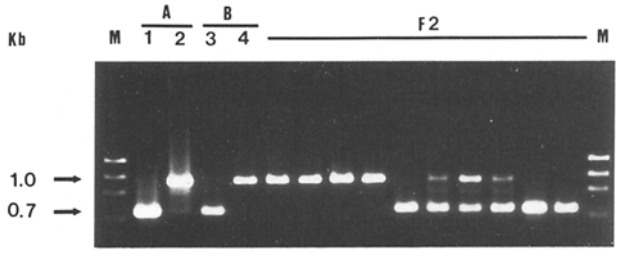

Fig. 1. PCR amplification of DNA from rice lines IR24 and 1188 and an F_2 population derived from a cross between these two lines. Primers PB7 and PB8 were used in the reactions. *A* Template DNA isolated from leaf tissue, *B* template DNA isolated from half-seed, *1*, *3* IR24, *2*, *4* 1188, F_2 template DNA from half-seeds of F_2 population, *M* molecular weight markers (*Hae*III-digested Φ × 174)

ever, for rice, proteinase K concentrations higher than 12 µg/200 µl reaction had no added beneficial effect (data not shown). Extraction buffer containing proteinase K can be stored at −20 °C for at least several months without deleterious effects. The amount of DNA obtained from half-seeds when compared with purified leaf DNA, permit PCR amplifications equivalent to 100 ng of purified DNA. We have encountered no failures with the amplification from more than 100 seeds of rice extracted DNA using primers specific for *Xa-21*.

Chelex-100 treatment

Chelex-100 chelates heavy metal ions, which are thought to play a role in DNA decomposition at high temperatures in solutions of low ionic strength (Singer-Sam et al. 1989; Walsh et al. 1991). This resin was tested as an alternate extraction buffer in half-seed sample PCR analysis and was found to give results comparable to those obtained with the proteinase K treatment (data not presented). The Chelex-100 treatment requires a shorter treatment time than the proteinase K method (10 min versus 65 min).

PCR amplification is from endosperm DNA

Rice, like most other grasses, has seeds that contain several tissue layers. The pericarp, testa, and aleurone are thin, compact layers lying between the hull and the grain. They are derived from the maternal ovary wall, and DNA from these tissues reflects the genotype of the maternal plants, not that of the seed embryo (Grist 1975). Since the objective of half-seed PCR is to determine the genotype of the embryo, DNA from maternal tissues represents a potential source of ambiguity. However, PCR analysis of F_2 half-seeds indicates that the visible amplified products are not derived from the DNA of maternal tissues. If DNA from maternal tissues had been amplified, all F_2 seed samples would reveal only the maternal allele. This was not the case,

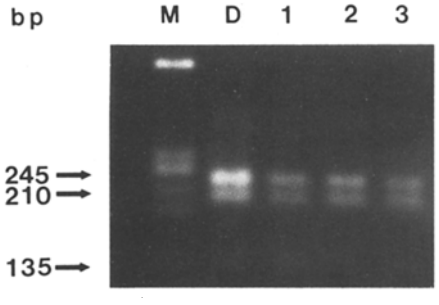

Fig. 2. PCR amplification using primer set WHGT2 on DNA from wheat cv 'Papago S'. *D* Template DNA isolated from leaf tissue, *1–3* template DNA isolated from three half-seeds, *M* molecular weight markers (*Hae*III-digested Φ × 174)

and all three of the expected F_2 genotypes were observed (Fig. 1). We therefore conclude that most, if not all, of the amplification is from endosperm-derived DNA, a conclusion that is consistent with the fact that endosperm represents the bulk of the tissue contained in the rice half-seeds used for PCR analysis.

Half-seed PCR of other plant species

The half-seed PCR protocol was also tested on wheat and found to work well (Fig. 2). However, PCR amplification was greater with the Chelex-100 extraction method than with the proteinase K method. With the proteinase K treatment, optimum amplification was obtained at a proteinase K concentration greater than that used for rice (48 µg/200 µl reaction).

Seeds of several other plant species, including both monocotyledonous and dicotyledonous species (rice, wheat, maize, tomato, and soybean), were also tested. Not all of the species were amenable to half-seed PCR assay. Seed anatomy appears to be an important factor. For example the spiral shape of the tomato embryo makes it difficult to cut the seed without also damaging the embryo. The most desirable seed structure is one in which the embryo is restricted to one end of the seed so that the seed can be dissected without harm to the embryo. Seed size is also important. Seeds of *Brassica* species and some *Solanaceous* species, for example, are very small and difficult to cut. Maize and bean seeds contain large amounts of starch, which reduces the recovery of DNA after boiling. More work is needed to optimize the protocol for these species.

Germination of half-seed after PCR analysis

Cutting seeds in half may reduce viability or make the seeds more susceptible to pathogen infection. In addition, since the endosperm is the sole source of nutrition during the initial growth of seedlings, the remnant half of the endosperm might not be sufficient for germination. To examine the effect of removing one-half of the seed, time-course germination tests were conducted. The germination rates of half-seeds were generally high, though not as high as those of uncut seeds (Fig. 3). Moreover, the seedlings derived from half-seeds were vigorous and survived transfer to soil. We observed that there is a tendency for the half-seeds to become infected by fungal pathogens. Treatment with a fungicide (Captan) appeared to reduce fungal contamination to some extent. In addition, we noticed that half-seeds will germinate faster if the hulls are removed.

Application of half-seed PCR analysis to breeding and genetics

Half-seed assays have been used to evaluate fatty acid content in oil seed crop breeding (Tiwari et al. 1988;

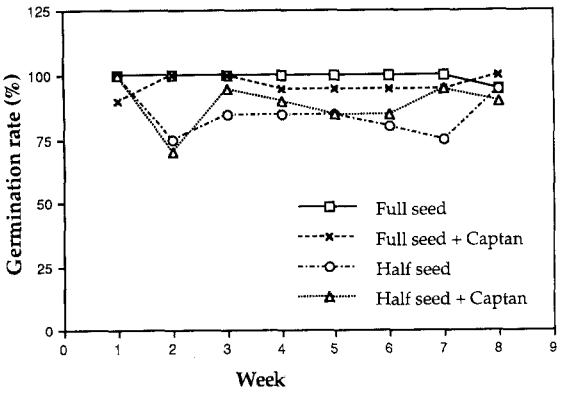

Fig. 3. Germination rate of rice half-seeds with and without a fungicide (Captan) treatment compared to those of full seeds

Conte et al. 1989). They have also been used for analyzing isozyme or protein polymorphisms (Hash and Blake 1981). However, the traits that can be analyzed in these assays are limited to seed characteristics and certain enzymes. In contrast PCR primers can be generated for virtually any DNA sequence in the genome. Half-seed PCR analysis thus open the possibility of developing rapid, pre-germination screens for virtually any locus in the genome, including those linked to major genes of agronomic importance and QTLs.

Each PCR reaction requires 10–25 µl of extract from a total of 200 µl. Thus, it should be possible to obtain between 8 to 20 reactions per half-seed. Currently, thermocyclers can process approximately 200 samples per day, and the automation of PCR techniques is likely to greatly extend this capacity in the near future. Methods of direct detection of PCR products without gel electrophoresis may be useful in examining even larger numbers of PCR reactions (Higuchi et al. 1992).

The half-seed PCR amplification method requires only standard PCR equipment (e.g., a thermocycler) and can be done routinely by trained technicians. Seeds to be subjected to PCR-based selection could be sent to a centralized laboratory for analysis and the result received back in a few days along with the half-seeds of the population. The breeder could then germinate only the selected genotypes.

Finally, half-seed PCR analysis should be useful in the identification of rare recombinant plants important in fine structure mapping. Geneticists, when analyzing specific loci, often need to identify cross-over events in very small genetic intervals (e.g., less than 1 cM), which may require the screening of a very large numbers of segregating plants (Messeguer et al. 1991). The half-seed assay method should allow a researcher to select rare recombinant plants more expeditiously and without the handling of plant tissue.

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