Theor Appl Genet (1991) 82:209–216

THEORETICAL AND APPLIED GENETICS

Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction

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Received January 8, 1991; Accepted January 23, 1991
Communicated by G. Wenzel

Summary. The polymerase chain reaction (PCR) can be used to detect polymorphisms in the length of amplified sequences between the annealing sites of two synthetic DNA primers. When the distance varies between two individuals then the banding pattern generated by the PCR reaction is essentially a genetic polymorphism and can be mapped in the same way as other genetic markers. This procedure has been used in a number of eukaryotes. Here we report the use of PCR to detect genetic polymorphisms in cereals. Known gene sequences can be used to design primers and detect polymorphic PCR products. This is demonstrated with primers to the α-amylase gene family. A second approach is to use semi-random primers to target diverse regions of the genome. For this purpose the consensus sequences at the intron-exon splice junctions were used. The targeting of the intron-exon splice junctions in conjunction with primers of random and defined sequences, such as α-amylase, provides a source of extensive variation in PCR products. These polymorphisms can be mapped as standard genetic markers.

Key words: α-Amylase – Cereal DNA – Genome mapping – Intron splice junction – Polymerase chain reaction

Introduction

Genetic markers are of great value in practical breeding programme and genetic research. Traditionally, markers based on morphological differences between individuals have been used. The subsequent development of isozyme and other biochemical markers represented a significant improvement since they offered greater diversity (Tanksley and Orton 1983). However, markers based upon DNA probes have introduced a new dimension to the development of genetic maps and the mapping of agronomically and physiologically important characters. The major strength of DNA probes, so far exclusively restriction fragment length polymorphisms (RFLP's), is that they have the potential to reveal an almost unlimited number of polymorphisms (Kan and Dozy 1978; Botstein et al. 1980; Wyman and White 1980).

RFLP's depend on the use of probes to identify single or low copy sequences in DNA. They are very powerful and have been used to construct detailed linkage maps of several crop species including tomato, potato and maize (reviewed by Tanksley et al. 1989). However, they are expensive and time consuming and technically difficult to use in some species with large and complex genomes, particularly wheat. This complexity of the wheat and related genomes has delayed the development of such maps for these plants even though recent world-wide efforts are likely to soon remedy this situation.

An alternative method, with the potential to overcome some of the current limitations in cereal genome mapping, is based on the Polymerase Chain Reaction (PCR) (Saiki et al. 1985; Mullis and Faloona 1987). In this case, polymorphisms are sought in the distance between two short target sequences rather than the presence or absence of restriction endonuclease sites as is the case for standard RFLP's (Skolnick and Wallace 1989). Oligonucleotides that anneal to the target sequences are used to prime the polymerase reactions. Careful selection of the primers will allow many polymorphisms to be detected and mapped as standard genetic markers. D'Ovidio et al. (1990) reported that PCR can be used to detect genetic polymorphism in wheat with primer sequences derived from the sequence of a γ-gliadin gene.

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It is necessary for the bases at the 3′ end of each primer to provide a perfect match to the target, whereas extensive mismatching in the remainder of the primer/target can be tolerated (Sommer and Tautz 1989). This gives the option of using sequences with only partial homology to the target sequence to prime the PCR. An extension of this principle has been developed by Williams et al. (1990), who have used entirely random primers to generate polymorphic PCR bands.

PCR has provided an alternative approach to many procedures in molecular biology and is replacing many standard techniques (Erlich 1989). It can be used to amplify specific target sequences for subsequent cloning, and it provides an extremely sensitive method for the detection of specific RNA and DNA sequences. This has led to the use of PCR to reveal variability of simple sequences in eukaryotic genomes (Litt and Luty 1989; Tautz 1989; Weber and May 1989). PCR methods based on families of repeated sequences, such as *Alu*-directed PCR in human genome analysis, provides the prospect of a rapid genome mapping technique (Nelson et al. 1989).

Here we describe the use of PCR to reveal and map polymorphisms in cereals. Two types of primers have been tested: those that target specific, known sequences and those that target intron-exon splice junctions. Both types offer valuable new sources of variability that can be more rapidly and cheaply exploited than RFLP markers.

Materials and methods

Plant material

Triticum aestivum cv 'Chinese Spring', *Hordeum vulgare* cv 'Betzes', *Secale cereale* cv 'Imperial' and the wheat-barley addition lines described by Islam et al. (1981) were kindly provided by Dr. K. W. Shepherd (Waite Agricultural Research Institute). The barley 1H* addition line (A.K.R.M. Islam and K. W. Shepherd, unpublished) was very generously provided by Dr A.K.R.M. Islam. The nomenclature for the barley chromosomes is based upon the equivalent wheat homoeologous group (traditional barley chromosome numbers are given in parentheses): 1H* (5), 2H (2), 3H (3), 4H (4), 5H (7), 6H (6) and 7H (1). It should be noted that the 1H* addition line is a double monosomic addition containing one copy each of 1H and 6H. The barley varieties were provided by Dr. D. Sparrow.

DNA isolation

Large-scale DNA isolations were made from approximately 5 g fresh leaves. The leaves were ground to a fine powder under liquid nitrogen and then suspended in 100 ml extraction buffer (4% sarkosyl, 0.1 *M* TRIS-HCl, 10 m*M* EDTA, pH 8.0). An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, and the slurry mixed for 90 min at 4 °C. After separation of the phases by centrifugation, the upper aqueous phase was poured off and filtered through fine nylon mesh. The solution was mixed with 10 ml sodium acetate (3 *M*, pH 4.8) and 250 ml ice-cold ethanol. The DNA was scooped out with a spatula, washed twice with 70% ethanol and dissolved in 7 ml TE (5 m*M* TRIS-HCl, 0.2 m*M* EDTA, pH 7.5). The DNA was further purified via CsCl equilibrium centrifugation (Maniatis et al. 1982).

Small-scale DNA isolations used about 200 mg young leaves. These were ground to a powder in 2 ml Eppendorf tubes under liquid nitrogen. The powder was then mixed with 1 ml extraction buffer and, subsequently, with 0.75 ml phenol/chloroform/isoamylalcohol. The whole mixture was shaken for 20–30 s and the aqueous phase recovered after centrifugation. The phenol/chloroform/isoamylalcohol extraction was repeated, and the DNA precipitated by ethanol precipitation.

PCRs and primers

All oligonucleotide primers were synthesized on an Applied Biosystems 381ADNA synthesizer and purified on an OPC cartridge supplied by the manufacturer. The sequences of the primers and the location of the intron-splice junction primers are shown in Fig. 1. The consensus sequence at the junction is also shown for comparison to the primers. The software package DNA Inspector Ile (Textco) was used to analyse published sequences for wheat and barley α-amylase. Primers A1 and A2 (Fig. 1 b) were based on the barley α-Amy 1 sequence (referred to as p141.117, Knox et al. 1987). The polymerase chain reactions were carried out in a 25-μl volume containing 0.2–0.5 μg of genomic DNA template, 0.2 μ*M* of each primer, 200 μ*M* each of dATP, dCTP, dGTP and dTTP, 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 0.01% gelatin and 0.2–0.5 units of Taq polymerase (Perkin Elmer Cetus). The PCR was performed in an Intelligent Heating Block (Hybaid). The standard reaction consisted of 45 cycles each of 1.5 min at 95 °C, 2 min at 55 °C and 2 min at 72 °C. With the intron splice junction primers the first 6 cycles were at 94 °C for 1 min, 40 °C for 1.8 min, 72 °C

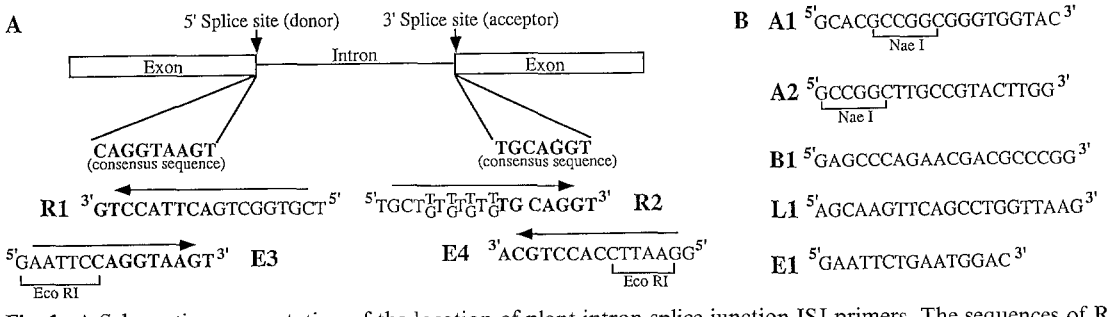

Fig. 1. **A** Schematic representation of the location of plant intron splice junction ISJ primers. The sequences of R1, R2, E3 and E4 are based on the consensus sequences for the exon-intron splice junctions of plants (Brown et al. 1986; Brown 1986; Goodall and Filipowicz 1989). E3 and E4 were synthesized to contain an EcoRI site as indicated. The bases that match the consensus sequence are shown in *bold type*. The orientation of the primers is indicated by the *arrows*. **B** The sequences of the α-amylase primers B1, L1 and E1 primers

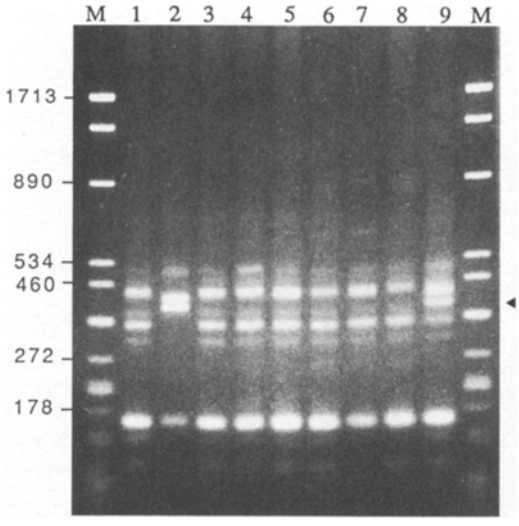

Fig. 2. Amplification of cereal DNA using α-amylase primers A1 and A2. The template DNA was from wheat ('Chinese Spring') in *lane 1*, barley ('Betzes') in *lane 2* and the barley addition lines 1H to 7H, in *lanes 3 to 9*, respectively. DNA size markers are shown in *lanes M*. The barley-specific band at 400 bp in *lanes 2* and *9* is indicated by an *arrow*. The sizes of the marker bands are given on the *left* in base pairs. The PCR products were derived from template DNA isolated by the small scale methods (Materials and methods) and were fractionated on a 3% agarose gel

for 2 min. This was followed by a further 28 cycles of 94 °C for 1 min, 58 °C for 1.5 min and 72 °C for 2 min. The cereal DNA was digested with restriction endonucleases according to the manufacturer's recommendations and used for PCR without further processing. One-fifth of the PCR products were analysed on either 3% agarose gels or 10% polyacrylamide gels and visualized under UV light following ethidium bromide staining. The size marker used for the gels was λdv1 digested with HaeIII (Streeck and Hobom 1975).

Results

PCR with α-amylase primers

The α-amylase genes comprise multigene families in wheat and barley. In Southern hybridizations a large number of bands appear, and these have been correlated to genes on group 5, 6 and 7 chromosomes in wheat and 6H and 7H in barley (Lazarus et al. 1985; Muthukrishnan et al. 1984; Baulcombe et al. 1987). There are various degrees of homology between these gene sequences (Baulcombe et al. 1987; Chandler et al. 1984; Huttly et al. 1988; Knox et al. 1987; Lazarus et al. 1985). The primers were chosen to hybridize with two highly conserved sequences that flank a variable region. In this way several individual α-amylase genes would be targeted simultaneously, and length polymorphisms between the sequences complementary to the primers could be identified. A NaeI site was inserted into each of the primers to allow restriction digestion and cloning of the amplified DNA if required. Figure 2 shows the amplification products generated when the two primers, A1 and A2, were used with DNA from wheat ('Chinese Spring'), barley ('Betzes') and the barley addition line series. The addition lines contain a single pair of barley chromosomes in a wheat background. The predicted length of the reaction product for barley α-amylase (α-Amy 1) is 150 bp. A band of approximately 150 bp is the most prominent band in wheat and was present in barley, wheat and the addition lines. At least two polymorphisms were observed between wheat and barley genomes (compare lanes 1 and 2, Fig. 2). The wheat bands at 350 and 450 bp are replaced by bands at 400 and 440 bp in barley. Using addition lines of barley, the polymorphic bands (cv 'Betzes') band could be mapped to barley chromosome 7H (compare lanes 1 and 9, Fig. 2).

PCR with primers from plant intron splice junction

The results obtained with the α-amylase primers demonstrate the value of the PCR technique in generating and mapping polymorphisms in cereals. However, for this type of reaction it is necessary to have adequate information about the target sequence to allow synthesis of the primers that are likely to target regions of variable lengths. The need for this information would greatly limited the applicability of PCR as a mapping tool. An alternative strategy was developed based on the consensus sequences for the intron splice junctions (ISJ). Introns have been identified in most plant genes studied (Hawkins 1988), and the junctions to exons are highly conserved sequences. However, since the introns are generally subjected to only weak selective pressure by comparison to exons, they are usually highly variable in sequence and length. These properties would appear to make the ISJ's ideal targets for the identification of polymorphisms in PCR products.

The sequences of the ISJ primers, R1, R2, E3 and E4 (Fig. 1), are based on the consensus sequences of the junctions reported for plants (Brown et al. 1986; Brown 1986; Goodall and Filipowicz 1989). The orientation of these primers at the intron junction is displayed in Fig. 1. The primers used were 15 (E3 and E4) or 18 (R1 and R2) bases in length. The consensus sequence for the splice junctions is 9 bases at the 5′ site and 7 bases at the 3′ site. The additional bases were added at random to extend the length of the primers and provide potential sites for base pairing to the target. For the E3 and E4 primers, an EcoRI site was added to facilitate cloning of the PCR products if required. The results of Sommer and Tautz (1989) suggest that only the three bases at the 3′ end of the primer are critical for efficient PCR; the remainder of the primer serves to stabilize the primer-target duplex as determined by the annealing conditions of the reaction.

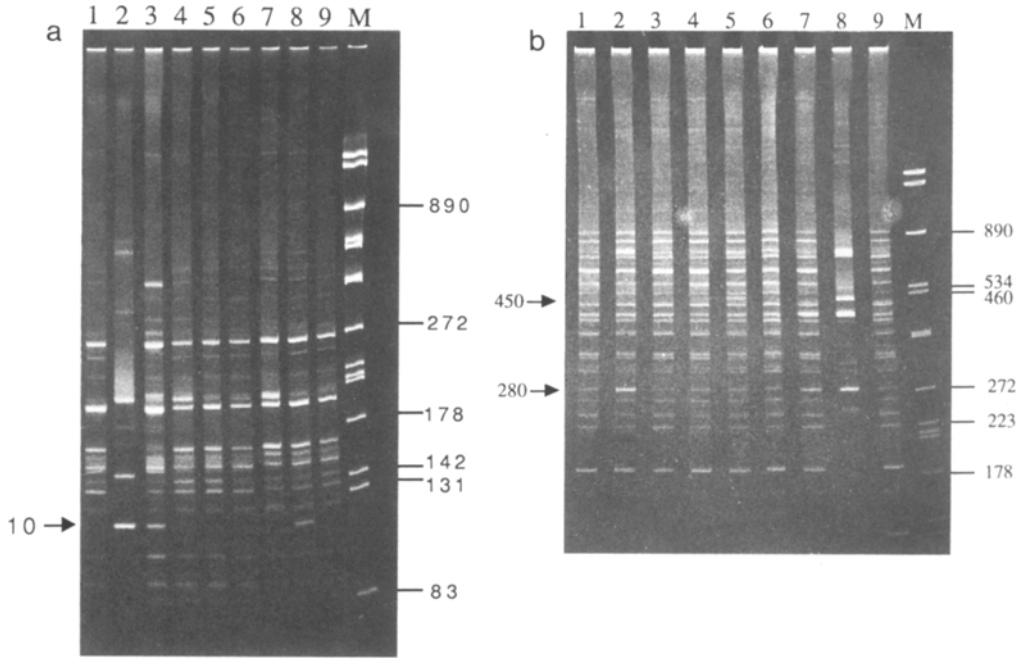

Fig. 3a, b. Amplification of cereal DNA using ISJ primers coupled with random primers. DNA size markers are shown in *lanes M* with the sizes of the markers bands given on the *right* in base pairs. The PCR products were fractionated on a 10% polyacrylamide gel. **a** ISJ primer R1 and random primer B1 were used to amplify cereal DNA. The DNAs were pre-digested with EcoRV prior to amplification. *Lane 1* shows the products obtained with wheat DNA ('Chinese Spring'), *lane 2* barley DNA ('Betzes') and *lanes 3–9* DNA from barley addition lines 1H to 7H, respectively. The barley-specific band in *lanes 2, 3* and *8* at 110 bp is indicated by an *arrow* on the *left*. **b** ISJ primer R2 and random primer L1 were used to amplify cereal DNAs without predigestion. *Lane 9* shows the products obtained with wheat DNA ('Chinese Spring'), *lane 8* barley DNA ('Betzes') and *lanes 7–1* DNA from barley addition lines 7H to 1H, respectively. The barley-specific bands in *lanes 2, 5* and *8* are indicated by *arrows* on the *left*

Primers were produced to generate products from the exon regions (primers R1 and R2) or from the intron region (E3 and E4). The intron-targeted primers used in conjunction with random or specific primers showed more complex and diverse banding patterns than the exon-targeted primers (data not shown).

Two steps were used in the PCR reactions involving the ISJ primers: the annealing temperature during the first 6 cycles was at 40 °C. This was then raised to 58 °C for the final 28 cycles. A low annealing temperature was used for the initial cycles to permit amplification from targets that may only have poor homology to the primers. This was subsequently found to increase the reproducibility in banding patterns generated with ISJ primers and to help avoid the smearing of bands.

The amplification products obtained from cereal DNA with ISJ and random primers are shown in Fig. 2a and b. When ISJ primer R1 (5′ splice site, exon targeting) was used with random primer B1, a faint smear of bands was obtained (data not shown). The PCR products were too complex to allow resolution of single bands and could not be used directly for the identification of polymorphisms. However, if the template DNA was digested with the restriction endonuclease EcoRV prior to the PCR reaction, clear banding patterns were generated. A distinctive pattern of polymorphic bands was observed between wheat and barley (compare lanes 1 and 2 in Fig. 3a). A barley-specific band of 100 bp can be mapped onto 1H* and 6H (lanes 3 and 8 at 110 bp). As 1H* is actually a double monosomic line of the barley chromosomes 1H and 6H (A.K.R.M. Islam and K. W. Shepherd, unpublished), the barley-specific band is probably located on 6H alone rather than on both 1H and 6H. When used alone, the R1 and B1 primers failed to generate any clearly visible bands.

The R2 primer (3′ splice site, exon targeting, Fig. 1) coupled with random primer L1 (L1 is a primer complementary to the imm 434 portion of λgt10 template, Huynh et al. 1985) also revealed extensive polymorphisms between wheat and barley (compare lanes 8 and 9, Fig. 3b). A 450-bp-amplified fragment specific to barley was mapped to barley chromosome 3H using the addition lines (lanes 5 and 8, Fig. 3b). Note also a band at 280 bp present in wheat, barley and the addition lines (Fig. 3b). The intensity of this band is stronger in 1H* and 6H (lanes 7 and 2, respectively, Fig. 3b). The corresponding locus may be situated on 6H in barley as discussed above for the 110-bp band in Fig. 3a.

ISJ primers can be used in conjunction with specific primers based on the sequence information from defined

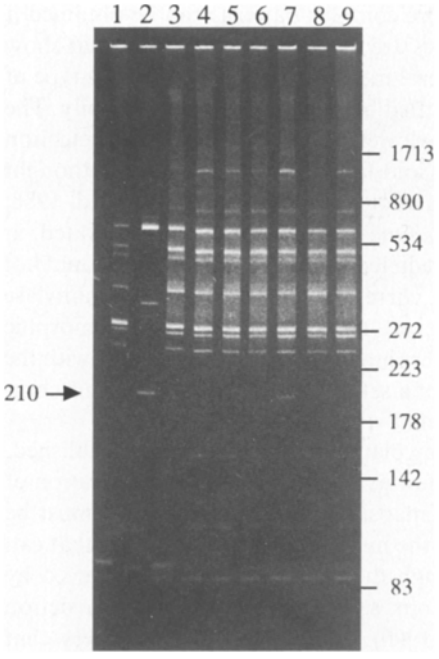

Fig. 4. Amplification of cereal DNA using the α-amylase primer, A2, in conjunction with the ISJ primer, R1. *Lane 1* shows the products obtained with wheat DNA ('Chinese Spring'), *lane 2* barley DNA ('Betzes') and *lanes 3–9* DNA from barley addition lines 1H to 7H, respectively. The positions of DNA size markers is indicated on the *right* in base pairs. The barley-specific band at 210 bp in *lanes 2* and *7* is indicated by an *arrow* on the *left*. The PCR products were fractionated on 10% polyacrylamide gels

cereal genes. Figure 4 shows the results of amplification of cereal genomic DNA with the α-amylase A1 and R1 (5′ splice site) primers. A clear polymorphic band can be localized to barley chromosome 5H (at lane 7).

Identification of barley varieties

The ISJ primers are valuable in detecting and mapping polymorphisms between wheat and barley. However, for these polymorphisms to be useful in the development of genetic maps and in their application, it is important that they also detect polymorphisms between varieties. Figure 5 shows an example of the products obtained when the R1 primer (5' splice site) is used in conjunction with the random primer E1 to amplify bands from 21 commercial varieties and breeding lines of barley. Several polymorphic bands can be seen in this example. The most reliable bands are those seen in the size range below 1000 bp. The larger size region also shows extensive polymorphism, but these tend to be less reproducible.

The banding patterns shown in Fig. 5 show at least nine polymorphisms between varieties. These allow classification of the 21 varieties into 15 classes. Unique banding patterns are shown by 12 varieties (lanes 3, 4, 6, 7, 8, 12, 13, 14, 15, 16, 17 and 19). Other primer combinations allow the differentiation of the remaining varieties from one another.

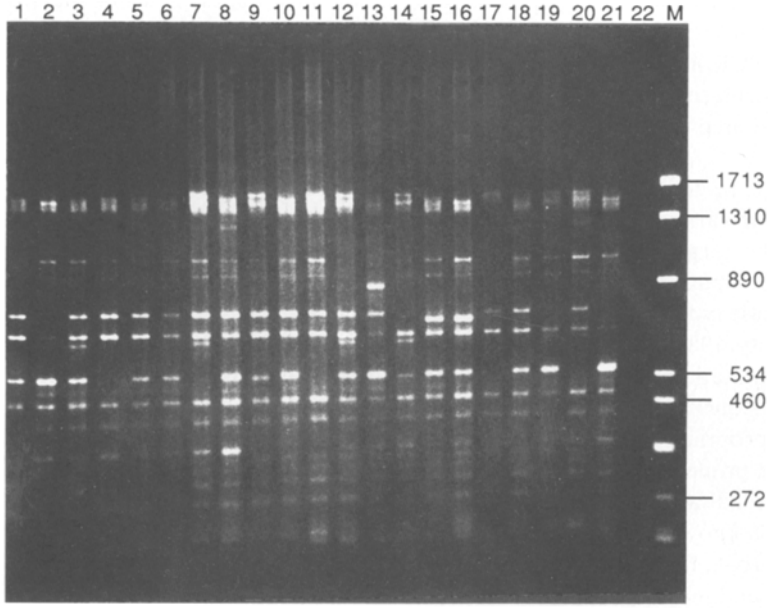

Fig. 5. Identification of barley varieties using an ISJ primer coupled with a random primers. DNA was amplified from a series of commercial barley varieties and some breeding lines using ISJ primer R1 and ramdom primer E1. *Lane 1*, 'Clipper'; *lane 2*, 'Galleon'; *lane 3*, 'Schooner'; *lane 4*, WI 2692; *lane 5*, WI 2736; *lane 6*, WI 2737; *lane 7*, 'O'Conner'; *lane 8*, 'Stirling'; *lane 9*, 'Onslow'; *lane 10*, 'Moondyne'; *lane 11*, 'Forrest'; *lane 12*, 'Windich'; *lane 13*, 'Yagan; *lane 14*, 'Malebo'; *lanes 15*, and *16*, 'Franklin'; *lane 17*, 'Weeah'; *lane 18*, 'Ingrid'; *lane 19*, 'Orge Martin'; *lane 20*, CYMMT 42002 and *lane 21*, CM 72. *Lane 22* shows a control where no genomic DNA was added to the reaction. DNA size marker (*lane M*) is shown on right with the sizes of the bands given in base pairs. The PCR products were fractionated on a 3% agarose gel

Efficiency of PCR primers in generating polymorphic bands

A comparison was made between the ISJ-based primer system and random primers alone in generating PCR products that could be clearly classified as bands and in revealing polymorphic bands between wheat, rye and barley and between barley varieties. When the ISJ primers were used in pairs (R1 + R2 or E3 + E4) to target either introns or exons, a smear of bands was obtained and no useful bands for mapping could be generated under the reaction conditions described above. Conversely, most random primers of 15–25 bases in length failed to give any bands. A total of 20 combinations of random primers were tested and only 5 combinations yielded bands. Similar results were obtained if the random primers were used singly to prime the PCR. The ISJ primers were tested in 40 different combinations with random primers. Of the 40 combinations 38 yielded clear banding patterns and 10 provided polymorphic bands that could be assigned to chromosomes.

Discussion

Genetic maps are valuable tools in crop breeding and genetic studies, but they depend upon the easy identification and availability of large numbers of markers distributed evenly over the chromosomes of the organism. The great strength of RFLP mapping is that it offers an almost unlimited number of markers that can be mapped over the entire genome and has no detrimental phenotypic effect. However, the methods involved in detecting RFLP's are technically difficult, particularly for complex organisms such as wheat, and they are expensive to assay. The PCR offers an alternative method with simple and cheap assay protocols when compared to RFLP analysis. The major cost associated with the PCR is in the synthesis of the primers. The problem with the PCR lies in the identification of large numbers of polymorphisms that can be mapped to specific chromosomes and used to generate genetic maps.

Polymorphisms identified by the PCR are in the length of the region amplified. One is looking for variation in the length of sequences separating the target regions with which the primers will anneal. In mammalian systems it has been demonstrated that these types of polymorphisms are abundant (Litt and Luty 1989; Tautz 1989; Weber and May 1989). A major limitation for the PCR approach is the need for extensive sequence information in order to synthesize the appropriate primers. With good sequence information, the primers can be synthesized to sit on each side of regions that are likely to be of variable length. In this way specific, known sequences can be amplified out of the genomic DNA; for a simple target such as an individual gene one band may be generated. More complex patterns can be obtained if multi-gene families are targeted using primers that show homology to several members of the family. This type of marker is exemplified by the α-amylase gene family. The primers A1 and A2 were selected to allow the detection of multiple genes and to flank an area that was thought to be variable (Baulcombe et al. 1987; Huttly et al. 1988; Knox et al. 1987). One of the major bands generated, at 150 bp, was as predicted. The remaining bands could not be unequivocally correlated to the published α-amylase sequences. However, the localization of polymorphic bands to barley chromosome 7H is in agreement with the known location of a set of α-amylase genes on this chromosome (Knox et al. 1987).

Although many plant sequences have been published, there are far too few available to allow the generation of extensive genetic maps. Alternative procedures must be sought to extend the number of polymorphisms that can be detected through the PCR. This can be achieved by the use of random sequences to prime the reaction (Williams et al. 1990) or by developing primers that target a range of genes. For the second system to function one must identify sequences that occur frequently throughout the genome but are likely to be evenly dispersed. It is a further advantage if the target sequences occur rarely, if at all, in heterochromatic regions since these areas are unlikely to contain genes of agronomic importance to which linkage would subsequently be sought. The criteria outlined are best met by the conserved regions of genes. Several options exist; for example, the translation start signals, the poly-A addition sites, promoter and enhancer regions and the intron-exon splice junctions. In the study reported here, we have used consensus sequence information for plant intron splice junctions (ISJ) (Brown 1986).

There are four major advantages in using the ISJ sequences to generate primers. First, the core of the junctions are highly conserved. In our experiments the primers were synthesized to match for the 3 bases at the 3′ end; this is within the highly conserved core of the consensus sequence (see Fig. 1). Since there is a degree of degeneracy in the consensus sequence only a sub-set of splice junctions will actually be targeted. Mismatches in the remainder of the primer-target pairing increase the number of targets and the complexity of the amplification products. The second advantage of the ISJ primers is that introns are present in most plant genes and one can, therefore, target an extremely diverse range of genes. The third and major advantage is that through the ISJ primers one avoids targeting heterochromatic regions and is likely to get a good chromosomal distribution of polymorphisms. These aspects are of particular importance in cereal mapping since repeat sequence regions are abundant, ranging from about 84% in barley to over 90% of the rye genome (Flavell et al. 1974). The fourth

advantage is that the cost of PCR is reduced through the use of the ISJ primers since only one addition primer is required for each reaction and the ISJ primer can be produced in large-scale synthesis.

The PCR can be based on primers derived from sequences that are defined, semi-random, such as the ISJ, or purely random. If both primers are random, the probability of finding the target sequence for both primers in close vicinity to each other is low. Random sequences of about 20 bases in length succeeded in generating clear bands in only 25% of the cases tested. These primers were substantially longer than the 9 or 10 base primers described by Williams et al. (1990) and it is, therefore, not possible to make a direct comparison between the two types of PCR. For the long random primers the likelihood of success in the PCR is increased through the use of the ISJ primers in conjunction with either random or defined primers. The examples shown here demonstrate the use of both defined and random primers with the ISJ primers.

Pre-digestion of the template DNA with a restriction endonuclease is required in some cases when the ISJ primers are used alone or in conjunction with other primers (e.g. Fig. 3a). The major effect of pre-digestion is the reduction in complexity of bands to a point where individual bands can be resolved. The type of enzyme used appears to have little effect on the actual banding pattern although some variation has been seen (data not presented). There are two possible explanations for this effect. First, the digestion may prevent the amplification of certain regions by cleaving between some target sequences. This would lead to a reduction in band complexity and occasional variation between the patterns generated by templates digested with different endonucleases. Second, the pre-digestion may allow improved denaturation and transcription of some of the template DNA fragments as a result of the reduced length and better access for the Taq polymerase to the DNA.

Two types of PCR can be used to detect and map polymorphisms in wheat and barley. The first uses specific primers that can target known genes or gene families. This method is rapid, cheap and does not require pre-digestion of the template with restriction endonucleases. However, sequence information is needed. PCR based on specific primers will be a valuable technique for screening with one or few markers in a breeding programme. For example, if an RFLP marker were identified that is closely linked to a gene of interest, the RFLP probe could be sequenced and PCR primers synthesized. The locus could then be monitored through the PCR rather than the more difficult RFLP. This procedure would be justified if large numbers of plants are to be screened.

A second type of PCR uses the ISJ primers. These allow the generation of a great diversity of markers without any additional sequence information. The ISJ-based markers can be used for generating maps of the cereal genomes. Although pre-digestion of the template may be required in some cases, these markers are still cheaper and faster to assay than RFLP's. They are technically less demanding to use than RFLP's and, as with standard PCR, do not require radioactivity to detect.

Acknowledgements. We wish to thank Drs. K. Shepherd, A.K.R.M. Islam and D. Sparrow for the genetic material, Dr. P. Rogowsky for helpful discussion and critical reading of the manuscript, Dr. M. Schwall and A. Karakousis for the random primers. We are also grateful to Dr. K. Trainer (Flinders Medical Centre) for his help in the PCR studies of α-amylase. S. W. is a recipient of University of Adelaide postgraduate scholarship. This work was supported by the Wheat Research Council.

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