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Sequence-tagged-site-facilitated PCR for barley genome mapping

S. Tragoonrung [1], V. Kanazin [1], P.M. Hayes [2], and T.K. Blake [1]

[1] Plant and Soil Science Department, Montana State University, Bozeman, MT 59717, USA
[2] Crop Science Department, Oregon State University, Corvallis, OR 97331, USA

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Summary. Speed, efficiency, and safety considerations have led many genome mapping projects to evaluate polymerase chain reaction (PCR) sequence amplification as an alternative to Southern blot analysis. However, the availability of informative primer sequences can be a limiting factor in PCR-based mapping. An alternative to random amplified polymorphism detection (RAPD) is the sequence-tagged-site (STS) approach. If informative primer sequences could be derived from known sequences, then current maps, which are based on both known function and anonymous clones, might be easily converted to maps utilizing PCR technology. In this paper, four pairs of primer sequences were obtained from published sequences, and four pairs were obtained by sequencing portions of DNA clones from genomic clones derived from a random genomic library used in the North American Barley Genome Mapping Project (NABGMP). These primers were used to screen for polymorphisms in the progeny of a winter × spring and a spring × spring barley cross. Two types of polymorphisms were distinguished using these primer sets: (1) insertion/deletion events that could be read directly from agarose gels, and (2) point mutation events. The latter were identified using polyacrylamide-gel electrophoresis of PCR products following digestion with restriction endonucleases (four-base cutters). To determine whether the PCR-based polymorphisms were allelic to polymorphisms identified by the clones from which the primer sequences derived, chromosomal assignments and (when possible) co-segregation analysis was performed.

Key words: Sequence-tagged-site – Polymerase chain reaction – Polyacrylamide-gel electrophoresis – Four-base cutter

Correspondence to: T.K. Blake

Introduction

Current genetic maps are generally based on restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980). Conventional detection of RFLPs by Southern blot analysis (Southern 1975) is laborious and costly (Beckmann and Soller 1983; Beckmann 1988). The polymerase chain reaction (PCR) (Saiki et al. 1985; Mullis and Faloona 1987) provides a rapid, safe and efficient method for screening large populations. Unlike Southern blot analysis, PCR can directly distinguish between insertion/deletion and point mutation events. Point mutation polymorphisms can be detected by hybridizing PCR products with allele-specific oligonucleotides (Higuchi et al. 1988; Li et al. 1988; Kurt et al. 1991), DNA sequencing of PCR products (Wong et al. 1987), cleavage of PCR products with a restriction endonuclease (Saiki et al. 1985), and denaturing gradient-gel electrophoresis (Myers et al. 1987; Riedel et al. 1990). Insertion/deletion polymorphisms can be analyzed by sizing of PCR products via gel electrophoresis (Higuchi et al. 1988; Shin et al. 1990). A major limitation for PCR analysis is the need for extensive sequence information in order to synthesize the appropriate primers (Williams et al. 1991). Williams et al. (1990) proposed using arbitrary nucleotide sequences as single primers for amplification of random DNA fragments (RAPD).

In the human genome mapping project, an attempt to convert the genetic map to a physical map has been greatly simplified by using sequence-tagged-sites (STS). An STS is a short, unique sequence, amplified by PCR, which identifies a known location on a chromosome (Olson et al. 1989). To-date, all STSs that have been used in mapping projects have been derived from well-characterized DNA probes or sequences (Cole et al. 1991). D'Ovidio et al. (1990) and Weining and Langridge (1991)

showed that PCR can be used to detect genetic polymorphisms in cereals with primer sequences derived from the sequence of a γ-gliadin gene and a α-amylase gene, respectively.

Here, we propose two approaches to obtain STSs for barley genome mapping. One approach is to utilize sequence data from previously published DNA sequences to construct primers. Another is to sequence anonymous DNA clones used in genome mapping projects. Point mutation polymorphisms within amplified fragments were detected on polyacrylamide gels of PCR products digested with four-base cutters. Insertion/deletion events were observed directly by gel electrophoresis.

Materials and methods

Genetic stocks

Two doubled haploid populations were generated from barley cvs. Dicktoo × Morex (D × M), a winter × spring cross, and from Steptoe × cv. Morex (S × M), a spring × spring cross, by the North American Barley Genome Mapping Project (NABGMP). For the D × M cross, 30 doubled haploid lines were used for segregation analysis. One hundred and fifty doubled haploid lines were used for segregation analysis in the S × M cross. A set of wheat-barley (cv. Chinese Spring-cv. Betzes cultivars) chromosomal addition lines described by Islam et al. (1981) was utilized for chromosomal assignment of polymorphisms.

DNA isolation

Genomic DNA was prepared using the procedure of Ausubel et al. (1987) modified as follows: 10 g fresh weight of young leaf tissue was ground to a fine powder under liquid nitrogen and incubated at 55 °C in 20 ml of extraction buffer (100 mM Tris-HCl pH 8.5, 100 mM EDTA, 250 mM NaCl, 0.5% SDS, and 100 μg/ml proteinase K) for 2 h. The lysate was extracted with 20 ml phenol:chloroform solution (50% phenol, 49% chloroform, 1% isoamyl alcohol), followed by chloroform extraction, and ethanol precipitation. DNA was centrifuged at 5,000 rpm for 15 min, suspended in 2 ml of sterile water, and treated with 20 μg DNase-free RNase for 2 h. DNA was extracted with phenol:chloroform, and chloroform, adjusted to a concentration of 0.3 M sodium acetate, and ethanol precipitated. The purified DNA was dissolved in sterile water and stored at −20 °C until use.

DNA sequencing

Four previously unmapped clones were selected from a *Pst*1 library provided by Dr. Nora Lapitan (Colorado State University). These clones, *Pst*1-319, *Pst*1-327, *Pst*1-337, and *Pst*1-340, were preselected to exclude repeat sequences and organelle sequences. Sequence data was obtained from the terminal 200 bp from each end of each clone and primers were synthesized. Single-stranded DNA (ssDNA) templates were generated using PCR as described by Higuchi and Ochman (1989). PCR primers, 5′ TACGACTCACTATAGGGC 3′ and 5′ CGCCAAGCTATTTAGGTG 3′, were used to amplify the inserted DNA fragments in the pGEM vector. One of the primers was phosphorylated prior to PCR with T4 DNA kinase. After PCR amplification, the double-stranded DNA (dsDNA) was treated with lambda exonuclease, a 5′ to 3′ nuclease which specifically digests only the DNA strand synthesized from the phosphorylated primer leaving the complementary strand (ssDNA). The ssDNA was purified using Prep-A-Gene (BIO-RAD), and sequenced by the technique of Sanger et al. (1977) using Sequenase and following the manufacturer's protocol (USB).

Primer synthesis

Oligonucleotide primers were synthesized by standard phosphoramidite chemistry on an Applied Biosystems 391 DNA synthesizer. After hydrolysis from the solid support and removal of protecting groups in 30% ammonium hydroxide at 55 °C for 15 h, the DNAs were dried under vacuum, and dissolved in 200 μl of sterile water. DNA yield was approximately 800 μg per synthesis.

PCR amplification

PCR amplifications were performed in a 100 μl reaction containing 2.5 units *Taq* polymerase (Perkin Elmer Cetus), 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, and 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.3 μM primers, and 25 ng of genomic DNA templates. The reaction was overlaid with mineral oil and subjected to one cycle of 95 °C for 5 min, followed by 32 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 90 s.

Detection of polymorphisms

PCR products were run on 1% agarose gels to detect insertion/deletion polymorphisms. To detect base substitution polymorphisms, 20 μl of amplified fragments were digested with the four-base cutters *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Rsa*I, and *Taq*I. Two units of the restriction endonucleases (except for *Rsa*I) were directly added to 20 μl of the PCR reaction after amplification without buffer modification. For *Rsa*I, 20 μl of the PCR product was precipitated with three volumes of 95% ethanol and the pellet was washed with 70% ethanol prior to digestion according to the manufacturers' instructions. Digested fragments were electrophoresed in a 7% polyacrylamide gels at 250 V for 100 min.

Results

STS production

Eight PCR primer sets and their sources are shown in Table 1. Four primer sets were designed from published sequences of α-hordothionin, alcohol dehydrogenase 2 (ADH-2), B1-hordein, and the *Bam*HI-*Sst*I fragment of pMSU21. The other four, *Pst*1-319, *Pst*1-327, *Pst*1-337, and *Pst*1-340, were obtained from sequencing short portions of DNA clones from a *Pst*1 genomic library. Figure 1 shows the DNA sequence of short portions at both ends of a genomic clone obtained by the method described. From these sequences, PCR primers were designed to be about 20 bases long, to contain 50% GC and harbor no inverted repeat sequences (Table 1).

PCR amplification and polymorphism detection

Each primer set was used to amplify sequences from total DNA of Dicktoo and Morex barley (Fig. 2).

PCR products amplified by six primer sets did not show size polymorphisms. These products were digested with seven four-base cutters and electrophoresed on 7%

Table 1. Primer sequences, sources, chromosomal locations and allelism analyses

Primer set	Primer sequences	Primer source	Chromosomal location (allelism)
aMSU21	5′GGTCTTTCATGTACCTACC 3′ 5′CGAGCTCCTGTCGAGG 3′	Shin et al. 1990	2[a] (100/100)
B1-Hordein	5′CCACCATGAAGACCTTCCTC 3′ 5′TCGCAGGATCCTGTACAACG 3′	Forde et al. 1985	5 (70/70)
α-Hordothionin	5′CTGGGGTTGGTTCTGG 3′ 5′GGCAGCAACATGGCATTC 3′	Rodriguez-Palenzuela et al. 1988	5 (ND)
Alcohol dehydrogenase 2	5′GGGGAGATATCGACCAAAGT 3′ 5′CACGCCCTCGCCAACGCTCTCCA 3′	Trick et al. 1988	7 Unlinked[b]
*Pst*I-319	5′AGCTGAGCAAGCTTCTTTGG 3′ 5′AACATGCTGGGCAACTCCCA 3′	Genomic library	–
*Pst*I-327	5′GGTACGAACATGGAGGTACT 3′ 5′ATCCAGTTCTTGTGCACCTG 3′	Genomic library	2 (30/30)
*Pst*I-337	5′ATCCAGTTCTTGTGCACCTG 3′ 5′AGCTACGTGGATCACACCAC 3′	Genomic libary	7 (30/30)
*Pst*I-340	5′TAGCATCGGTAATCTCTCGC 3′ 5′CCCTTTATATACACTGCCGA 3′	Genomic library	5 (ND)

[a] Data previously reported (Shin et al. 1990)
[b] Southern blot data communicated by Dr. A. Kleinhofs
–, no polymorphisms were identified either by Southern blot analysis or PCR
ND, data not determined

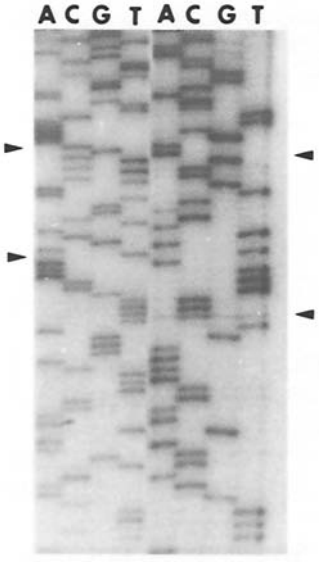

Fig. 1. DNA sequence of the *Pst*I-340 clone. Short portions of both ends (*A and B*) of the clone were used to design PCR primers. The *arrows* indicate the portions of sequence from which primers were designed

polyacrylamide gels. Restriction site polymorphisms were detected in PCR products amplified from the α-hordothionin, alcohol dehydrogenase 2, *Pst*1-327, *Pst*1-337, and *Pst*1-340 primer stes (Fig. 3, Table 2). No polymorphism was detected with the *Pst*1-319 primers. Segregation based on PCR amplification from primers *Pst*1-327 and *Pst*1-337 agreed perfectly with that determined by Southern blot analysis of doubled haploid lines from the D × M cross (Table 1). The product of ADH-2 primer amplification, however, did not show allelism with any of five RFLP polymorphisms identified through the use of the ADH-2 clone in a Southern blot analysis of a series of doubled haploid lines (personal communication, A. Kleinhofs, NABGMP, 1991).

Two primer sets differentiated between the two cultivars based on agarose-gel electrophoresis. Amplified DNA fragments from pMSU21 primers identified an insertion/deletion polymorphism. A 670 bp fragment is amplified from Dicktoo DNA, and a 450 bp fragment is amplified from Morex DNA, when these primers are used. Parental and progeny banding patterns are shown in Fig. 4A. This polymorphism was previously determined to be allelic to that identified by Southern blot analysis with the probe pMSU21 (Shin et al. 1990). B1-hordein primers amplified two fragments in Dicktoo (approximately 960 and 860 bp) and one fragment in Morex (approximately 860 bp). The PCR and protein polymorphisms (determined by SDS-PAGE) demonstrated perfect cosegregation (Table 1). Parental and progeny banding patterns for the analysis based on the *Pst*-337 primers (a restriction site polymorphism) are shown in Fig. 4B.

Chromosomal location

Chromosomal locations of STSs were identified by linkage analysis with previously mapped markers in doubled

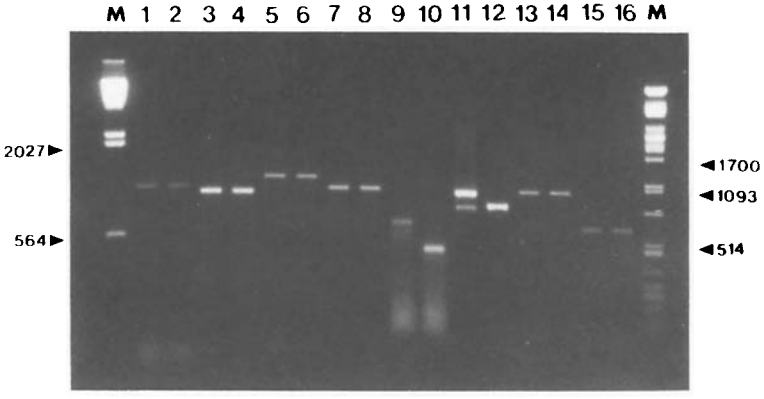

Fig. 2. Amplification of barley DNA from Dicktoo and Morex with eight primer sets on a 1% agarose gel. DNA templates in the odd lanes are Dicktoo, in the even lanes are Morex. Amplified products with *Pst*I-319 in *lanes 1 and 2*, *Pst*I-327 *lanes 3 and 4*, *Pst*I-337 *lanes 5 and 6*, *Pst*I-340 *lanes 7 and 8*, pMSU21 *lanes 9 and 10*, B1-hordein *lanes 11 and 12*, α-hordothionin *lanes 13 and 14*, alcohol dehydrogenase 2 *lanes 15 and 16*. DNA size markers are shown in lane *M*. Sizes of markers bands are shown in base pairs

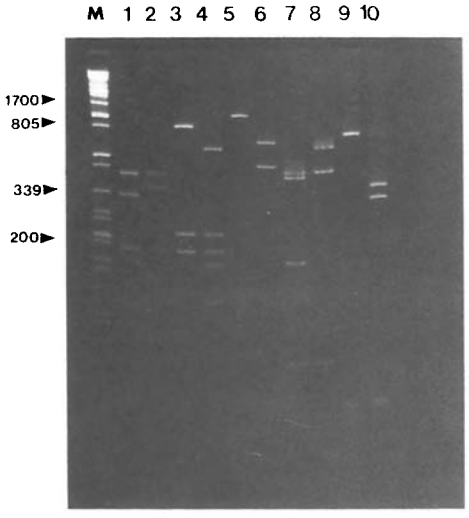

Fig. 3. Digestion of PCR products with restriction endonucleases with four-base recognition sequences were electrophoresed on a 7% polyacrylamide gel. *Hin*fI digestion of amplified products with *Pst*I-327 primers are in *lanes 1* (Dicktoo) *and 2* (Morex), digestion of products amplified with *Pst*I-337 primers are in *lanes 3* (Dicktoo) *and 4* (Morex). *Hae*III digestion of products amplified with *Pst*I-340 primers are in *lanes 5* (Dicktoo) *and 6* (Morex). *Taq*I digestion of products amplified with α-hordothionin primers are in *lanes 7* (Dicktoo) *and 8* (Morex). *Rsa*I digestion of products amplified with ADH 2 primers are in *lanes 9* (Dicktoo) *and 10* (Morex). DNA size markers are shown in lane *M* with the size of the marker bands given in base pairs

haploid progenies and by PCR amplification of wheat-barley addition lines (Table 1 and Fig. 5). The recombination analyses were performed using MAPMAKER (Lander and Botstein 1989). The *Pst*1-327 and α-hordothionin amplification products were evaluated in both the S × M (population size, 150) and D × M (population size, 30) crosses. They were located on chromosome 2 and 5 respectively in both crosses. Identical chromosomes were identified using recombination analysis and PCR amplification of DNA from the wheat-barley addition lines. The polymorphism identified by the ADH-2 based primers mapped to barley chromosome 7 by both recombination analysis and PCR amplification of wheat-barley chromosome addition lines.

Efficiency of PCR analysis

Seven restriction endonucleases with 4 bp restriction sites were utilized with the six PCR products from the barley cultivars Morex and Dicktoo. A total of 79 restriction sites were surveyed, and a total of 16 were absent in one of the two genotypes (Table 2). This provides a conservative estimate of sequence divergence between these two genotypes of 16/316 bases, about 5%. Since the clones were not preselected on the basis of polymorphisms, we have no reason to expect any bias in this estimate of base pair differentiation between homologous single-copy sequences of Dicktoo and Morex barley.

Discussion

Three of the four randomly selected single-copy clones from which we obtained primer sequences identified allelic variation between Morex and Dicktoo barley. Further, it appears as though about one base in 20 varies between Morex and Dicktoo. Approximately 37% of the randomly isolated single-copy genomic clones tested against Morex and Dicktoo identified polymorphisms when six 6-base restriction endonculeases were utilized. When individual restriction sites were evaluated, we actually observed a frequency of one site in five lost by mutation (39 mutations/1188 bases evaluated), or 3.3% (data not shown). These frequencies (5% vs. 3.3%) seem fairly similar and suggest that polymorphisms between winter and spring barley cultivars are frequent enough to permit effective mapping using either RFLP or PCR-based approaches.

Five of the six PCR-based evaluation systems in which allelism was determined for Southern blot-based polymorphisms provided expected linkages to previously

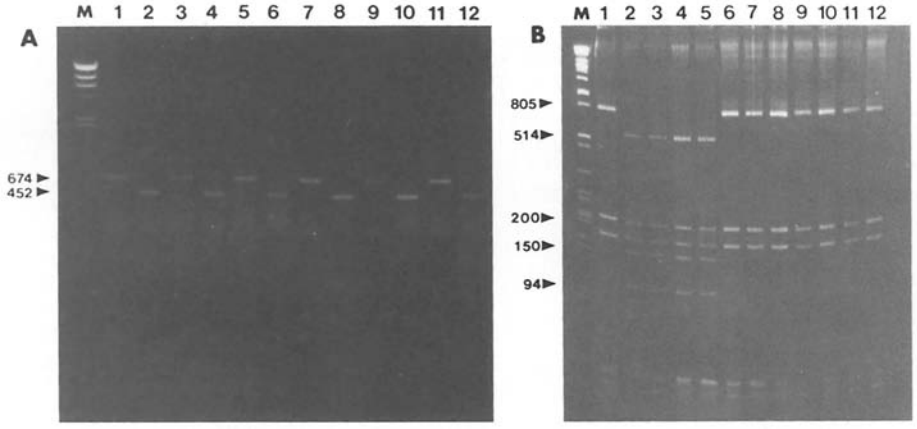

Fig. 4 A, B. Segregation of D × M doubled haploid lines. DNA size markers are shown in lane *M* with the size of markers bands given in base pairs. The template DNA was from Dicktoo in *lane 1*, Morex in *lane 2* and doubled haploids in *lanes 3–12*. **A** Products amplified with pMSU21 primers showed insertion/deletion polymorphisms on a 1% agarose gel. Sizes of the two allelic fragments are given in base pairs. **B** *Hin*fI digestion of products amplified with *Pst*I-337 primers were electrophoresed on a 7% polyacrylamide gel

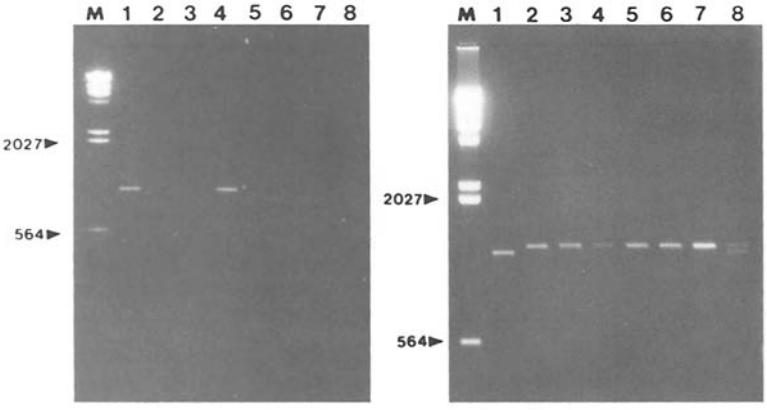

Fig. 5 A, B. Amplification of wheat-barley chromosome addition lines. Betzes in *lane 1*, Chinese spring wheat in *lane 2*, barley chromosome 1, 2, 3, 4, 6, 7 addition lines are in *lanes 3–8*, respectively. DNA size markers are shown in lane *M* with a given size of marker bands in base pairs. **A** Amplification with *Pst*I-327 primers electrophoresed on a 1% agarose gel. **B** Amplification with *Pst*I-337 primers electrophoresed on a 1% agarose gel

Table 2. Restriction site variation in PCR products

Primer sets	DNA source	PCR amplified fragment sizes	Restriction sites						
			*Alu*I	*Hae*III	*Hha*I	*Hin*fI	*Msp*I	*Rsa*I	*Taq*I
*Pst*I-319	Dicktoo	1,100 bp	4	3	1	1	1	1	3
	Morex	1,100 bp	4	3	1	1	1	1	3
*Pst*I-327	Dicktoo	1,000 bp	1	2	1	3	1	ND	1
	Morex	1,000 bp	2	3	1	2	0	ND	1
*Pst*I-337	Dicktoo	1,200 bp	2	3	1	2	3	ND	4
	Morex	1,200 bp	4	3	3	4	4	ND	5
*Pst*I-340	Dicktoo	1,100 bp	5	0	0	5	1	0	1
	Morex	1,100 bp	5	1	0	5	1	0	1
α-hordothionin	Dicktoo	1,050 bp	1	2	1	1	0	ND	3
	Morex	1,050 bp	1	2	1	1	0	ND	2
Alcohol dehydrogenase 2	Dicktoo	630 bp	ND	3	2	0	2	0	1
	Morex	630 bp	ND	3	3	0	2	1	1

ND, data not determined

mapped markers. The sixth, ADH-2, provided a clear polymorphism but no linkage to polymorphisms previously identified by Southern blot analysis. We have not yet sequenced the PCR products produced using the ADH-2 products, and so are unable to determine whether these products are truly homologous to ADH-2, or whether mere chance homology exists for primer sequences which flank an unrelated sequence. On one hand, it is clear that the ADH-2 primers used in this study provide a useful genetic marker. On the other, it is likely that the locus amplified by these primers has no relationship to alcohol dehydrogenase. Further study could help explain this anomaly.

The MSU21 primers direct the amplification of products which differ in size between Morex and Dicktoo. This size polymorphism was previously noted and mapped using an F_2 population (Shin et al. 1990). The PCR polymorphism identified using these primers and the RFLP polymorphism using the probe which was sequenced to derive the primer sequences showed perfect cosegregation in both F_2 and doubled haploid populations. While "allele dropout" can be a real problem in systems such as HLA-DQ alpha genotyping of humans (Saiki et al. 1986), the MSU21 primer set appears to direct amplification of both characterized alleles with similar efficiency.

This study demonstrates an alternative method to Southern blot analysis for genome mapping. However, the need for primer sequences can be a limiting factor in PCR-based genome mapping. To overcome this problem, the RAPD approach was introduced (Williams et al. 1990). D'Ovidio et al. (1990) and Weining and Langridge (1991) derived PCR primers from published DNA sequences. We extended the STS idea by evaluating four randomly selected DNA clones from a genomic library. PCR products of three of these four clones identified variation between Morex and Dicktoo. PCR-based sequencing methods (Gyllensten and Erlich 1988; Innis et al. 1988; Higuchi and Ochman 1989; Kusukawa et al. 1990) make the production of STS from selected DNA clones practicable.

The production of medium density genetic maps using RFLPs has proven to be feasible in many crop species. Using these maps for efficient QTL manipulation demands the use of a technology simpler than Southern blot analysis. In this experiment we found that seven of the eight marker sequences tested could be easily manipulated to permit evaluation of segregation by PCR. As significant QTL loci are identified in crops, converting mapped RFLP markers which flank agronomically important loci to PCR-based detection systems will provide a user-friendly technology to the ultimate users of genetic maps, the plant breeders.

Although variation outside the STS region can not be detected as in Southern blot analysis, it has been demonstrated that there exists sufficient variation within amplified fragments in mammalian systems (Li et al. 1988; Litt and Luty 1989; Tautz 1989; Weber and May 1989). In plant systems, our study supports a similar conclusion. In contrast to Skolnick and Wallace (1988), this study demonstrates that PCR polymorphisms among the barley genotypes tested are mainly due to the absence or presence of restriction endonuclease sites rather than the fragment length differences.

Although relative costs might be debated, the ease of PCR analysis, the clarity of restriction pattern of PCR products, and the ease of distribution of primer sequence data (as opposed to the maintenance of clones) all argue in favor of PCR as a tool in genetic analysis. Clearly, the chromosomal location of a polymorphism must be well-characterized and stable over crosses if it is to be generally useful. All seven polymorphisms evaluated in this study appear stable and appear to map to single loci. One of the primer sets (ADH-2) amplified a product from an unexpected locus. The clone from which our sequence data derived clearly hybridized to sequences at several dispersed loci. We would suggest that markers which hybridize to dispersed, multigene families may be unsuitable candidates for PCR-based genetic analysis.

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