Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat
by Xueyan Shan

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Sciences
Montana State University

Abstract:
Conversion of amplified fragment length polymorphisms (AFLPs) to sequence-specific PCR primers would be useful for many genetic linkage applications. We examined 21 wheat nullitetrasomic stocks and five wheat-barley addition lines using twelve and fourteen AFLP EcdBJJMsel primer combinations, respectively. On average, 36.8% of the scored AFLP fragments in wheat nullitetrasomic stocks and 22.3% in wheat-barley addition lines could be mapped to specific chromosomes, providing approximately 461 chromosome specific AFLP markers in wheat nullitetrasomic stocks and 174 in wheat-barley addition lines. Ten AFLP fragments specific to barley chromosomes and sixteen AFLP fragments specific to wheat 3BS and 4BS chromosome arms were isolated from the polyacrylamide gels, reamplified, cloned and sequenced. Primer sets were designed from these sequences. Amplification of wheat and barley genomic DNA using the barley-derived primers revealed that three primer sets amplified DNA from the expected chromosome, five amplified fragments from all barley chromosomes but not from wheat, one amplified a similar sized fragment from multiple barley chromosomes and from wheat, and one gave no amplification. Amplification of wheat genomic DNA using the wheat-derived primer sets revealed that three primer sets amplified a fragment from the expected chromosome, eleven primer sets amplified a similar-sized fragment from multiple chromosomes, and two gave no amplification. We also examined 21 wheat nullitetrasomic stocks using seven methylation sensitive PstHMseI primer combinations. 21.3% of the scored hypomethylated AFLP fragments in wheat nullitetrasomic stocks could be mapped to specific chromosomes. Out of four pairs of sequence-specific primers designed from the cloned wheat chromosome-specific PstI/MseI AFLP fragments, one primer pair amplified a fragment marking the expected chromosome. From these experiments we postulate that conversion of AFLPs to sequence-specific PCR markers in wheat is a promising, feasible, yet not efficient method so far..

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APPROVAL

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Xueyan Shan

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Conversion of amplified fragment length polymorphisms (AFLPs) to sequence-specific PCR primers would be useful for many genetic linkage applications. We examined 21 wheat nullitetrasomic stocks and five wheat-barley addition lines using twelve and fourteen AFLP *Eco*RI/*Mse*I primer combinations, respectively. On average, 36.8% of the scored AFLP fragments in wheat nullitetrasomic stocks and 22.3% in wheat-barley addition lines could be mapped to specific chromosomes, providing approximately 461 chromosome specific AFLP markers in wheat nullitetrasomic stocks and 174 in wheat-barley addition lines. Ten AFLP fragments specific to barley chromosomes and sixteen AFLP fragments specific to wheat 3BS and 4BS chromosome arms were isolated from the polyacrylamide gels, reamplified, cloned and sequenced. Primer sets were designed from these sequences. Amplification of wheat and barley genomic DNA using the barley-derived primers revealed that three primer sets amplified DNA from the expected chromosome, five amplified fragments from all barley chromosomes but not from wheat, one amplified a similar sized fragment from multiple barley chromosomes and from wheat, and one gave no amplification. Amplification of wheat genomic DNA using the wheat-derived primer sets revealed that three primer sets amplified a fragment from the expected chromosome, eleven primer sets amplified a similar-sized fragment from multiple chromosomes, and two gave no amplification. We also examined 21 wheat nullitetrasomic stocks using seven methylation sensitive *Pst*I/*Mse*I primer combinations. 21.3% of the scored hypomethylated AFLP fragments in wheat nullitetrasomic stocks could be mapped to specific chromosomes. Out of four pairs of sequence-specific primers designed from the cloned wheat chromosome-specific *Pst*I/*Mse*I AFLP fragments, one primer pair amplified a fragment marking the expected chromosome. From these experiments we postulate that conversion of AFLPs to sequence-specific PCR markers in wheat is a promising, feasible, yet not efficient method so far.

CHAPTER 1

INTRODUCTION

The improvement of agricultural productivity has been largely accelerated by the genetic improvement of agricultural crops. For example, in order to accommodate changes in agricultural markets, or in biotic and abiotic environments, crop varieties have been developed by introgression of exotic germplasm with elite agronomic traits and introduction of foreign genes conferring stress-tolerance or disease-resistance. These genetic applications require rapid and detailed genetic analysis of the corresponding crop species and this has been achieved by the use of DNA markers. DNA markers play a fundamental role in genetic analyses such as construction of genetic maps, identification of genes for valuable traits from indigenous and exotic germplasms, and interpretation of evolutionary relationships among crop species and their wild relatives (Paterson et al. 1991). DNA markers make it possible to conduct marker-assisted selection which helps to expedite the process of modern crop improvement .

A number of different types of DNA markers have been developed over recent years

(Burow et al. 1997). The first successful and widely used DNA marker was restriction fragment length polymorphism (RFLP) (Botstein et al. 1980). This technique is a hybridization-based DNA marker system and it is a reliable technique in the development of dense genetic maps. The limitation of this method is that it is laborious and not easy to employ on large populations. New generations of DNA marker systems, such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anolles et al.1991), are based on the polymerase chain reaction (PCR). These methods are designed to simultaneously detect a set of random genomic DNA fragments by using arbitrarily selected PCR primers. They provide abundant polymorphisms but have the major disadvantage that they are very sensitive to the reaction conditions and may not be reproducible (Kleinhofs et al. 1993). This limits their applications. Sequence-tagged-site (STS) method (Olson et al. 1989) is also a PCR-based technique. Instead of using arbitrarily selected PCR primers, STS-PCR primers are designed from mapped DNA sequences such as RFLP clones. This technique is more reliable and useful in applications on large populations. The limitation of this technique is that it requires prior knowledge of DNA sequences and it depends on the limited resources of DNA clones which could be used to develop STS markers.

Each type of DNA markers has its advantages and disadvantages depending on different applications. Multiplex PCR-based DNA fingerprinting techniques such as RAPD and DAF can easily reveal large number of polymorphisms, but they also reveal large number of non-specific DNA fragments at the same time. To be used for purpose such as screening genomic or cDNA libraries or tracking valuable traits in large

populations, these RAPD or DAF markers first have to be converted into other types of DNA markers, such as RFLP or STS-PCR markers, which confer higher specificity. The value to such a conversion is that RFLP markers are more reliable and STS-PCR markers are less expensive and can be more easily employed using large populations. Therefore, conversion between different types of markers are sometimes necessary in many genetic applications. Conversion of RFLP, RAPD and microsatellite markers into their simplified consensus PCR-based markers, such as STS markers, has been reported for several crops (D'ovidio et al. 1992; Storlie et al. 1993; Bradshaw et al. 1994; Chen et al. 1994; Sowokin et al. 1994; Chee et al. 1995; Hittalmani et al. 1995; Salentijn et al. 1995; Blake et al.1996; Brady et al. 1996; Talbert et al. 1996; Bryan et al. 1997; Cheung et al. 1997). Many of these simplified PCR-based markers amplified homologous sequences which were highly informative as indicated by the original marker types. The efficiencies of conversion between different marker types varied from case to case.

Amplified fragment length polymorphism (AFLP) is a newly developed DNA fingerprinting technique (Vos et al. 1995) that permits analysis of a subset of restriction fragments from a complete digest of genomic DNA. AFLP analysis entails digestion of genomic DNA with restriction enzymes, followed by amplification of a subset of the restriction fragments using PCR. PCR products are resolved on denaturing polyacrylamide gels, providing an efficient tool for revealing polymorphisms. The high efficiency, reproducibility and reliability of AFLP has been supported by a number of recent publications. Abundant AFLP polymorphisms have been found in many plant species, confirming its use in plant genetic studies. AFLP has been used to assess genetic

diversity in wheat (*Triticum aestivum* L.) (Barret and Kidwell 1998; Barrett et al. 1998; Burkhamer et al. 1998), barley (*Hordeum vulgare* L.) (Ellis et al. 1997; Schut et al. 1997), maize (*Zea mays* L.) (Ajmone Marsan et al. 1998), lettuce (*Lactuca sp.* L.) (Hill et al. 1996), sunflower (*Helianthus annuus* L.) (Hongtrakul et al. 1997), pea (*Pisum sp.* L.) (Lu et al. 1996), soybean (*Glycine max* L.) (VanToai et al. 1997), potato ( ) (Milbourne et al. 1997), *Manihot* (Roa et al. 1997), and *Eucalyptus urophylle* (Gaiotto et al. 1997). AFLP has been used to construct high density genetic maps of barley (*Hordeum vulgare* L.) (Becker et al. 1995; Qi and Lindhout 1997; Castiglioni et al. 1998), rice (*Oryza sativa* L.) (Mackill et al.1996; Maheswaran et al. 1997), soybean (*Glycine max* L.) (Keim et al. 1997), melon (*Cucumis melo* L.) (Wang et al. 1997) and potato (Rouppe van der Voort et al. 1997). AFLP analysis has been used in quantitative trait analysis (Nandi et al. 1997; Roa et al. 1997; Pakniyat et al. 1997; Powell et al. 1997), and in the enrichment of DNA markers near a locus of interest (Ballvora et al. 1995; Meksem et al. 1995; Thomas et al. 1995; Cnops et al. 1996; Rouppe van der Voort et al. 1997; Kaloshian et al. 1998; Lu et al. 1998; Simons et al. 1998). A comparison of AFLP with RAPD and sequence-tagged microsatellites (SSR) markers (Jones et al. 1997) showed that AFLPs were relatively reproducible.

AFLP detects restriction fragments of genomic DNA and resembles in RFLP technique at this point. PCR amplification, instead of Southern hybridization, is used in AFLP technique, allowing high numbers of restriction fragments to be analyzed at the same time. Therefore AFLP is able to combine the reliability of RFLP with the advantage of the PCR technique. However, just like other comprehensive DNA fingerprinting techniques, AFLP

can also reveal large number of non-polymorphic DNA fragments while it provides abundant polymorphisms. Conversion of AFLPs to more specific DNA markers such as RFLPs and sequence-specific PCR-based markers would be useful for many genetic applications. Despite the reported use of the AFLP technique in various genetic analyses, little information is available regarding cloning of AFLP fragments for conversion to other marker types. In the few cases in which AFLP marker conversion has been attempted (Meksem et al. 1995; Cho et al. 1996; Qu et al. 1998), only a few of the corresponding RFLP markers or sequence-specific PCR markers retained the specificity indicated by the original AFLP markers. The efficiency and difficulties associated with conversion of AFLPs are unknown.

In the experiments described in this dissertation, we attempted to address issues concerning AFLP cloning and conversion of AFLPs to sequence-specific markers. Several hundred sequence-tagged-site (STS) PCR markers have been developed from different marker types (Talbert et al. 1994, 1995; Blake et al. 1996; Erpelding et al. 1996) for use in genetic analysis and marker-assisted selection in wheat and barley. However, regions of chromosomes that are not marked by available primer sets still exist. Conversion of AFLPs to sequence-specific PCR primers would allow further saturation of the wheat and barley genetic maps. The goal of this study was to determine the feasibility and the efficiency of cloning and conversion chromosome-specific AFLPs to sequence-specific PCR-based markers in wheat and barley.

CHAPTER 2

IDENTIFICATION OF CHROMOSOME-SPECIFIC AFLP MARKERS IN WHEAT AND BARLEY

Literature Review

A basic method to assign DNA markers or genes to specific chromosomes is by the use of aneuploids and chromosome addition and substitution lines (Sears 1991). In wheat and barley, wheat nullisomic-tetrasomic stocks (Sears 1954), wheat ditelosomic stocks (Sears 1954) and wheat-barley addition lines (Shepherd and Islam 1981) are very useful for mapping DNA markers or genes to specific wheat or barley chromosomes. Nullisomic-tetrasomic stocks and ditelosomic stocks of Chinese Spring wheat have been developed by Sears (1954). Each nullisomic-tetrasomic stock of wheat lacks one pair of homologous chromosomes in combination with the tetrasomic state of a pair of homoeologous chromosomes compensating the missing chromosomes. Each ditelosomic wheat stock lacks a pair of homologous chromosome arms. Nullisomic-tetrasomic stocks and ditelosomic stocks of the variety Chinese Spring have been used as the standards to map RFLP markers (Sharp et al. 1989, Gill et al. 1991, Anderson et al. 1992), STS-PCR markers (Talbert et al. 1994, Talbert et al. 1996), and microsatellites (Bryan et al. 1997)

to specific chromosomes. The successful wheat-barley addition lines were Chinese Spring-Betzes addition lines developed by Shepherd and Islam (1981). Each wheat-barley addition line has a pair of barley chromosomes added to the wheat genome. Wheat-barley addition lines have been used for determining the chromosomal location of protein and isozyme genes in barley (Hart et al. 1980, Powling et al. 1981, Islam and Shepherd 1981, Brown and Munday 1982) and to assign RFLP probes (Shepherd and Islam 1987) and STS-PCR markers (Tragoonrung et al. 1992) to particular barley chromosomes.

The assignment of genes and DNA markers to wheat and barley chromosomes is essential for genetic manipulation in wheat and barley improvement. While aneuploids and chromosome addition and substitution lines provided efficient methods to assign known DNA sequences to specific chromosomes, efforts should be made to explore the potential to identify novel chromosome-specific DNA sequences using these materials. PCR-based DNA fingerprinting techniques have brought about the possibilities to identify chromosome-specific DNA markers in these aneuploids and chromosome addition and substitution lines. Especially, several features of the newly developed AFLP technique indicate that AFLP is an efficient way to provide large numbers of reliable and reproducible polymorphisms and it should be a suitable method for genomic fingerprinting of aneuploids and identifying chromosome-specific DNA markers.

AFLP technique utilizes PCR to amplify a subset of restriction fragments from a complete digest of genomic DNA. Genomic restriction fragments are generated by use of two restriction endonucleas, a six-base cutter enzyme ('rare' cutter) and a four-base cutter enzyme ('frequent' cutter). The use of 'frequent' cutter is to generate small restriction

fragments with the sizes in the optimal ranges for PCR amplication. The use of 'rare' cutter is to reduce the number of restrition fragments to be analyzed since the design of AFLP-PCR conditions only allows the rare cutter/frequent cutter restriction fragments to be amplified and visualized. After digestion, double-stranded oligonucleotide adapters are ligated to both ends of the restriction fragments to create primer annealing sites for PCR-amplification. AFLP primers are designed according to the core sequences of the adapters and the sequences of the restriction sites, with 1-3 arbitrarily chosen selective nucleotides at their 3'-ends. These selective nucleotides are used to reduce the amount of the restriction fragments to be amplified. Amplification can only be achieved from those restriction fragments in which the 1-3 nucleotides adjacent to the restriction sites of the fragment exactly match the 1-3 selective nucleotides of the primers. Thus a subset of restriction fragments are seletively amplified by the use of selective primers. PCR products are resolved on denaturing polyacrylamide gels and visualized by autoradiography, silver-staining or fluorescent labelling. Typically 50-100 scorable amplification products are detected per gel, providing a tool of great potential to reveal multiplex polymorphisms (Vos et al. 1995).

The goals of the following experiments were to apply AFLP analysis on wheat nullisomic-tetrasomic stocks, wheat ditelosomic stocks and wheat-barley addition lines in order to identify chromosome-specific AFLP markers in wheat and barley and to obtain sufficient template materials for the consequent cloning and conversion experiments.

Materials and Methods

Plant materials

Twenty-one wheat nullitetrasomic stocks (NTs) of 'Chinese Spring' wheat (Sears 1954), three Chinese Spring wheat ditelosomic stocks (DTs) (Sear 1954), five wheat-barley addition lines (WBALs) (Shepherd and Islam 1981), wheat cultivar Chinese Spring and barley cultivar Betzes were used for AFLP analysis. WBALs for chromosomes 1, 2, 4, 6 and 7 were used, while WBALs for chromosomes 3 and 5 were not available.

Preparation of genomic DNAs

Total genomic DNA was extracted from young leaves of greenhouse-grown plants as described by Dellaporta et al. (1983). A single plant was used to represent a genotype. Approximately 1.0 g fresh young leaves from a single three-week old plant of each wheat stock was collected. Leaf tissue was ground in mortar and pestle with 15 ml extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH8.0, 100 mM NaCl, 1% SDS, and 10 mM mercaptoethanol). After grinding, leaf tissue extraction was transferred to a 30 ml Oakridge tube and incubated at 65°C in a waterbath for 10 minutes. 5 ml 5 M potassium acetate was then added to each sample followed by incubation on ice for 20 minutes. Tubes were centrifuged at 20,000 x G for 20 minutes. Supernatants were filtered into clean 30 ml tubes containing 10 ml cold isopropanol and 1 ml 5 M ammonium acetate. Samples were mixed well and incubated at -20°C for 30 minutes. DNA pellets were precipitated at 20,000 x G for 15 minutes. The supernatants were gently poured off and

DNA pellets were dried by inverting tubes onto paper towel for 10 minutes. DNA pellets were redissolved in 0.7 ml TE buffer (10 mM Tris-Cl, 1 mM EDTA pH 8.0) and transferred to 1.5 ml microfuge tubes. 75μl 3 M sodium acetate pH 7.0 and 500 μl cold isopropanol were added to each sample and mixed well. Microfuge tubes were centrifuged at 14,000 rpm for 5 minutes. Supernatants were discarded and DNA pellets were redissolved with 200 μl sterilized distilled water. DNA concentrations were determined by comparison with tomato DNA control (100 ng/μl) from AFLP Analysis System I, AFLP Start Primer Kit (Life Technologies ,Gaithersburg, MD) on 0.8% agarose gels in 1 x TBE buffer.

AFLP analysis

AFLP marker analysis was conducted using AFLP Analysis System I, AFLP Start Primer Kit (Life Technologies ,Gaithersburg, MD), as described by Vos et al (1995). A total of 250 ng of genomic DNA for each wheat stock was digested with *Eco*RI / *Mse*I in a total reaction volume of 25 μl at 37°C for 2 hours followed by 15 minutes at 70°C to inactivate the restriction endonucleases. *Eco*RI and *Mse*I adapters were ligated to the restriction fragments and the ligation was carried out at 20°C for 2 hours. A 1 : 10 dilution of the ligation mixture was prepared for using as template DNAs in subsequent preamplification reactions by transferring 10 μl of the reaction mixture to a 1.5 ml microcentrifuge tube and adding 90 μl sterilized distilled water. The dilutions and the unused original reaction mixtures were stored at -20°C. Preamplification reaction mixture contained 5 μl diluted template DNA from the ligation reaction, 40μl pre-amp primer mix

containing *Eco*RI/*Mse*I primers with one seletive nucleotide (Table 1), 5μl 10 x AFLP-PCR buffer plus Mg, and 1μl *Taq* DNA polymerase (5 units/μl). PCR conditions of preamplification were 20 cycles at 94°C for 30 s, 56°C for 60 s, 72°C for 60 s followed by hold at 4°C. A 1 : 10 dilution of the preamplification product of each sample were prepared for the subsequent selective AFLP amplification by using sterilized distilled water. Both diluted and undiluted preamplification products were stored at -20°C. For selective amplification, primer labeling was performed by end-labeling of the *Eco*R I primers with γ-^{32}P or γ-^{33}P ATP (NEN, Boston, MA) and T_4 kinase. The labeling reaction was carried out at 37°C for 1 hour followed by inactivation of the enzyme at 70°C for 10 minutes. Selective AFLP amplification was performed as follows: one cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s; twelve cycles at 94°C for 30 s, annealing temperature lowering 0.7°C each cycle, and 72°C for 60 s; twenty-three cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. Primers with three selective nucleotides were used for selective amplification (Table 1). After PCR, 20μl stop solution (98% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each reaction. Selective amplification products were heated at 90°C for 3 minutes before loading on 6% polyacrylamide denaturing sequencing gels (20 : 1 acrylamide:bis; 7.5 M urea; 1 x TBE buffer). Gels were run at 50w constant power for 3 - 4 hours, transferred to Whatman paper, dried, marked with radioactive ink or nicks in film corners for orientation purposes, and exposed to X-ray film (Kodak Biomax-MR) for 16 - 24 hours. Intense and well separated bands were scored.

Results and Discussion

Evaluation of different AFLP primer combinations

Seven wheat nullitetrasomic and ditelosomic stocks, NT3B, DT3BS, DT3BL, NT4B, NT4BS, NT5B, DT5BL, were used for AFLP analysis in this experiment with wheat cultivar Chinese Spring as control material. Fifty-eight selective *Eco*RI/*Mse*I primer combinations (Table 1) were examined to evaluate the resolutions of scorable bands and polymorphic bands for each combination in wheat stocks, in that guidelines for primer combination selection in wheat were not available. These selective *Eco*RI/*Mse*I primer combinations were made up from eight three-selective-nucleotide *Eco*RI primers and eight three-selective-nucleotide *Mse*I primers (Table 2). Each *Eco*RI primer, which was labeled with γ-^{32}P ATP, was used in combination with one of the eight *Mse*I primers for selective amplification. Gels were run at 50w constant power for 3 - 4 hours until xylene cyanol (slower dye) was approximately 3 inches to the bottom of the gel. This allowed AFLP bands with sizes ranging from 50bp to 700bp to be resolved on each gel. The exposure time of autoradiography for visualizing γ-^{32}P ATP-labeling selective amplification products was around 16 hours at room temperature. Intense bands present in Chinese Spring controls were considered as valid scorable bands. The resolution of each primer combination were determined according to the number of scorable bands, the separation of these bands and the number of polymorphic bands.

Table 1 EcoRI/MseI primer combinations used for evaluation.

E-A/M-C[a]			
E-AAC/M-CAG[b]	E-ACA/M-CAT	E-ACG/M-CTA	E-AGC/M-CAT
E-AAC/M-CAT	E-ACA/M-CTA	E-ACG/M-CTC	E-AGC/M-CTA
E-AAC/M-CTA	E-ACA/M-CTC	E-ACG/M-CTG	E-AGC/M-CTC
E-AAC/M-CTC	E-ACA/M-CTG	E-ACG/M-CTT	E-AGC/M-CTG
E-AAG/M-CAA	E-ACC/M-CAA	E-ACT/M-CAA	E-AGC/M-CTT
E-AAG/M-CAC	E-ACC/M-CAC	E-ACT/M-CAC	E-AGG/M-CAA
E-AAG/M-CAG	E-ACC/M-CAG	E-ACT/M-CAG	E-AGG/M-CAC
E-AAG/M-CAT	E-ACC/M-CAT	E-ACT/M-CAT	E-AGG/M-CAG
E-AAG/M-CTA	E-ACC/M-CTA	E-ACT/M-CTA	E-AGG/M-CAT
E-AAG/M-CTC	E-ACC/M-CTC	E-ACT/M-CTC	E-AGG/M-CTA
E-AAG/M-CTG	E-ACC/M-CTG	E-ACT/M-CTG	E-AGG/M-CTC
E-AAG/M-CTT	E-ACG/M-CAA	E-ACT/M-CTT	E-AGG/M-CTG
E-ACA/M-CAA	E-ACG/M-CAC	E-AGC/M-CAA	E-AGG/M-CTT
E-ACA/M-CAC	E-ACG/M-CAG	E-AGC/M-CAC	
E-ACA/ M-CAG	E-ACG/M-CAT	E-AGC/M-CAG	

[a] Preamplification primers

E-A: GACTGCGTACCAATTC-A
M-C: GATGAGTCCTGAGTAA-C

[b] Selective amplification primers

E-AAG: GACTGCGTACCAATTC-AAG
M-CAC: GATGAGTCCTGAGTAA-CAC

Table 2 Selective *Eco*RI and *Mse*I primers comprising different *Eco*RI/*Mse*I primer combinations.

***Eco*RI Primers**	***Mse*I primers**
E-AAC[a]	M-CAA[b]
E-AAG	M-CAC
E-ACA	M-CAG
E-ACC	M-CAT
E-ACG	M-CTA
E-ACT	M-CTC
E-AGC	M-CTG
E-AGG	M-CTT

[a] E-AAC: GACTGCGTACCAATTC-AAC
[b] M-CAA: GATGAGTCCTGAGTAA-CAA

The resolutions of the tested fifty-eight primer combinations showed that primers with three-selective-nucleotide were suitable for AFLP analysis in wheat stocks. Most tested primer combinations visualized distinctive and reproducible banding patterns (Figure 1, 2, 3). These AFLP banding patterns varied from combination to combination depending upon the selective nucleotides being used. In combination with the same *Eco*RI primer, the differences of one nucleotide on the 3' position of *Mse*I primers generated totally different AFLP patterns (Figure 1, 2, 3). In other words, the differences of one selective nucleotide effectively resulted in the selection of a different subset of the genomic

restriction fragments. An extreme example was shown by comparison of the binding patterns between combination E-ACC/M-CAC and combination E-ACC/M-CAT (Figure 3). Combination E-ACC/M-CAT provided high number of well-separated scorable bands. Whereas combination E-ACC/M-CAC resulted in very poor amplification. The difference of one selective nucleotide produced greatly diverged resolutions, indicating the high selectivity of the selective nucleotides in AFLP analysis. The evaluations for the fifty-eight primer combinations tested in this experiment were given in Table 3. This table would serve as a guideline for primer pair selection in the subsequent AFLP analysis experiments. Those primer combinations which produced the highest numbers of distinctively scorable bands were defined as strongly recommended primer pairs for AFLP analysis in wheat. Those primer combinations which produced middle numbers of distinctively scorable bands were defined as recommended primer pairs. Those which gave poor amplification products were defined as primer pairs not recommended.

Identification of chromosome-specific AFLP markers in wheat

A complete set of the nullisomic-tetrasomic wheat stocks of Chinese Spring were used for identification of chromosome-specific AFLP markers in wheat. The designations of these nullisomic-tetrasomic wheat stocks were: NT1A, NT1B, NT1D, NT2A, NT2B, NT2D, NT3A, NT3B, NT3D, NT4A, NT4B, NT4D, NT5A, NT5B, NT5D, NT6A, NT6B, NT6D, NT7A, NT7B, NT7D, with each stock for one of the 21 chromosomes. Cultivar Chinese Spring wheat was used as control material. Twelve primer combinations (Table 4) were chosen for AFLP analysis according to the guidelines obtained from the

Figure 1 AFLPs in Chinese Spring wheat (CS), nullitetrasomic 4B (NT4B), ditelosomic 4BS (DT4BS), nullitetrasomic 3B (NT3B), ditelosomic 3BS (DT3BS), and ditelosomic 3BL (DT3BL) (Ordering from left to right in each group). Each lane represents one wheat stock. Each group represents the results from one AFLP selective primer combination. From left to right, the combinations are E-AGC/M-CAC, E-AGC/M-CAG, E-AGC/M-CTG, E-ACG/M-CAC, E-ACG/M-CAG, E-ACG/M-CTG, and E-ACG/M-CAT. These primer combinations visualize distinctive and reproducible banding patterns. These AFLP banding patterns varied from combination to combination depending upon the selective nucleotides being used. In combination with the same *Eco*RI primer, the differences of one nucleotide on the 3' position of *Mse*I primers generated totally different AFLP patterns, indicating the high selectivity of the selective nucleotides in AFLP analysis.

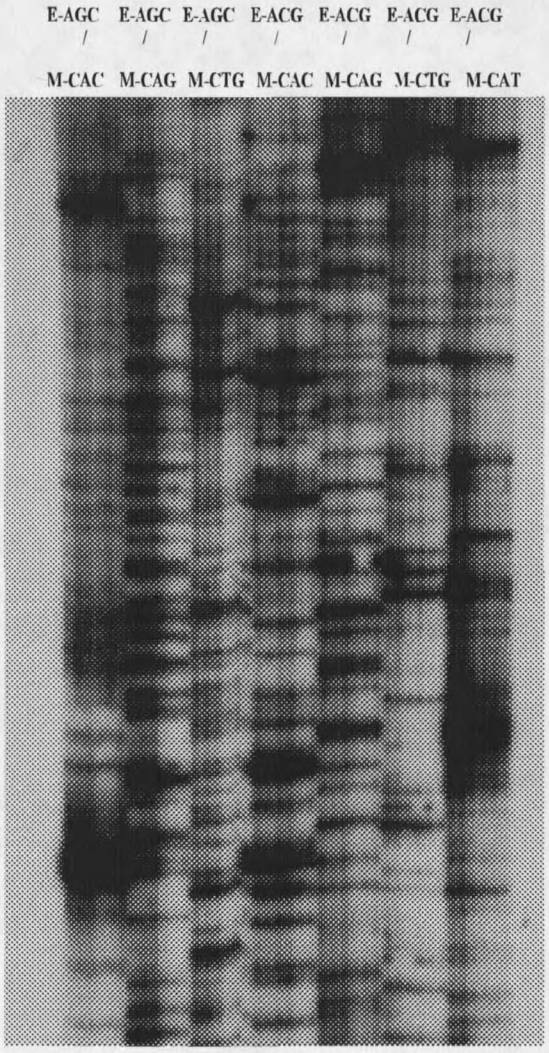


Figure 2 AFLPs in Chinese Spring wheat (CS), nullitetrasomic 4B (NT4B), ditelosomic 4BS (DT4BS), nullitetrasomic 3B (NT3B), ditelosomic 3BS (DT3BS), ditelosomic 3BL (DT3BL), nullitetrasomic 5B (NT5B), and ditelosomic 5BL (DT5BL) (Ordering from left to right in each group). Each lane represents one wheat stock. Each group represents the results from one AFLP selective primer combination. From left to right, the combinations are E-AAG/M-CAG, E-AAG/M-CAT, E-AAG/M-CTC, E-AAG/M-CTG, and E-AGC/M-CTC. Four of these primer combinations visualize distinctive and reproducible banding patterns, whereas combination E-AGC/M-CTC gives less scorable bands. These AFLP banding patterns varied from combination to combination depending upon the selective nucleotides being used. In combination with the same *Eco*RI primer, the differences of one nucleotide on the 3' position of *Mse*I primers generated totally different AFLP patterns, indicating the high selectivity of the selective nucleotides in AFLP analysis.

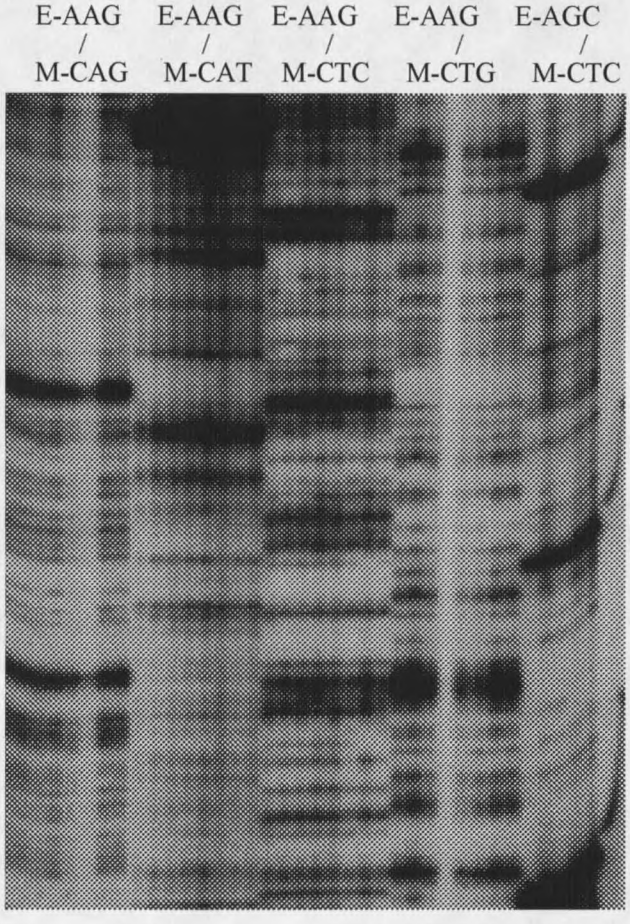


Figure 3 AFLPs in Chinese Spring wheat (CS), nullitetrasomic 4B (NT4B), ditelosomic 4BS (DT4BS), nullitetrasomic 3B (NT3B), ditelosomic 3BS (DT3BS),ditelosomic3BL (DT3BL), nullitetrasomic 5B (NT5B), and ditelosomic 5BL (DT5BL) (Ordering from left to right in each group). Each lane represents one wheat stock. Each group represents the results from one AFLP selective primer combination. From left to right, the combinations are E-ACC/M-CAC, E-ACC/M-CAT, E-ACC/M-CTA, E-ACC/M-CTC, and E-ACC/M-CTG. An extreme example is shown here by comparison of the binding patterns between combination E-ACC/M-CAC and combination E-ACC/M-CAT. Combination E-ACC/M-CAT provides well-separated scorable bands. Whereas combination E-ACC/M-CAC resulted in very poor amplification. The difference of one selective nucleotide produces greatly diverged resolutions, indicating the high selectivity of the selective nucleotides in AFLP analysis.

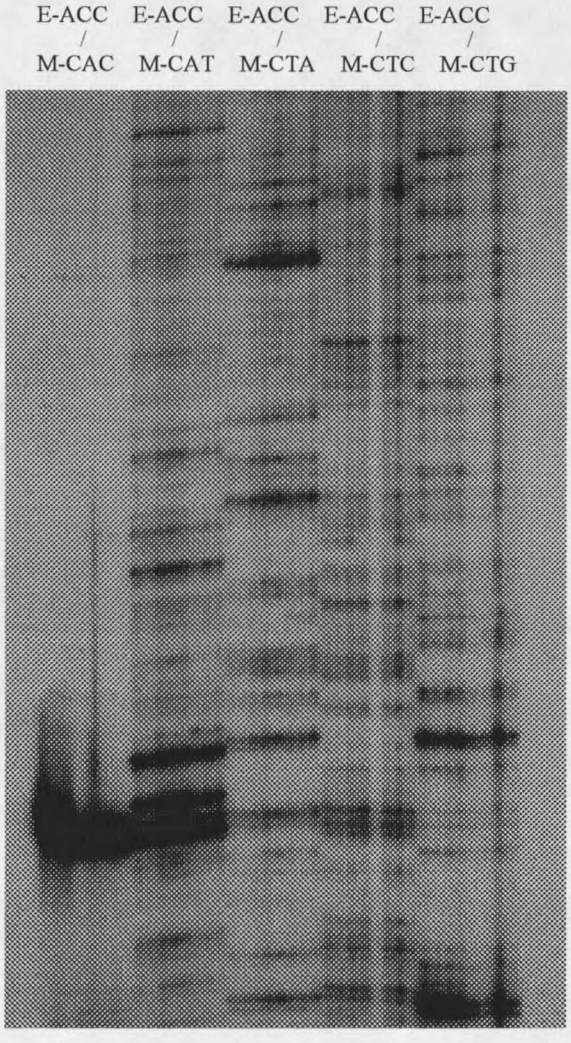


Table 3 Results of AFLP primer combination evaluation in wheat stocks.

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	n.d.	n.d.	++	-	-	-	n.d.	n.d.
E-AAG	+	+	+	+	+	++	++	+
E-ACA	+	+	+	+	+	+	+	n.d.
E-ACC	+	-	+	+	++	+	++	n.d.
E-ACG	+	++	++	++	++	++	++	+
E-ACT	+	++	+	+	+	++	++	-
E-AGC	+	-	++	+	+	+	++	-
E-AGG	+	+	+	++	+	+	+	+

++: Strongly recommended primer pair

+: Recommended primer pair

-: Primer pair not recommended

n.d. No data

previous experiments. These AFLP primer combinations revealed an average of 104 (+/- 30.9) scorable amplification products per combination in cultivar Chinese Spring wheat. Size range was from 50bp to 700 bp. Wheat chromosome-specific AFLP markers were identified as bands missing in only one NT stock but present in all other NTs and Chinese Spring (Figure 4, 5, 6). The numbers of AFLP markers assigned to each chromosome in each primer combination were scored (Table 5). A total of 461 wheat

Table 4 AFLP primer combinations used with nullisomic-tetrasomic wheat stocks of Chinese Spring.

E-A/M-C[a]
E-AAG/M-CAC[b]
E-AAG/M-CTA
E-AAG/M-CTC
E-ACC/M-CTA
E-ACC/M-CTG
E-ACG/M-CAC
E-ACG/M-CAG
E-ACG/M-CAT
E-ACG/M-CTC
E-ACT/M-CAC
E-AGC/M-CAG
E-AGC/M-CTG

[a] Preamplification primers

E-A: GACTGCGTACCAATTC-A
M-C: GATGAGTCCTGAGTAA-C

[b] Selective amplification primers

E-AAG: GACTGCGTACCAATTC-AAG
M-CAC: GATGAGTCCTGAGTAA-CAC

chromosome-specific AFLP markers were identified, accounting for 36.8% of the 1253 scored amplification products of the control cultivar Chinese Spring wheat (Table 6). The amplified products with no chromosome specificity may either be repetitive or low copy loci on more than one homoeologous chromosome. The number of AFLP markers

Figure 4 Identification of wheat chromosome-specific AFLPs using nullitetrasomic wheat stocks with primer combination E-ACG/M-CAC. The samples are as indicated on the picture, for example, CS stands for Chinese Spring wheat, NT3B stands for nullitetrasomic 3B, and etc. The arrows indicate wheat chromosome-specific AFLPs identified as bands missing in only one NT stock but present in all other NTs and Chinese Spring.

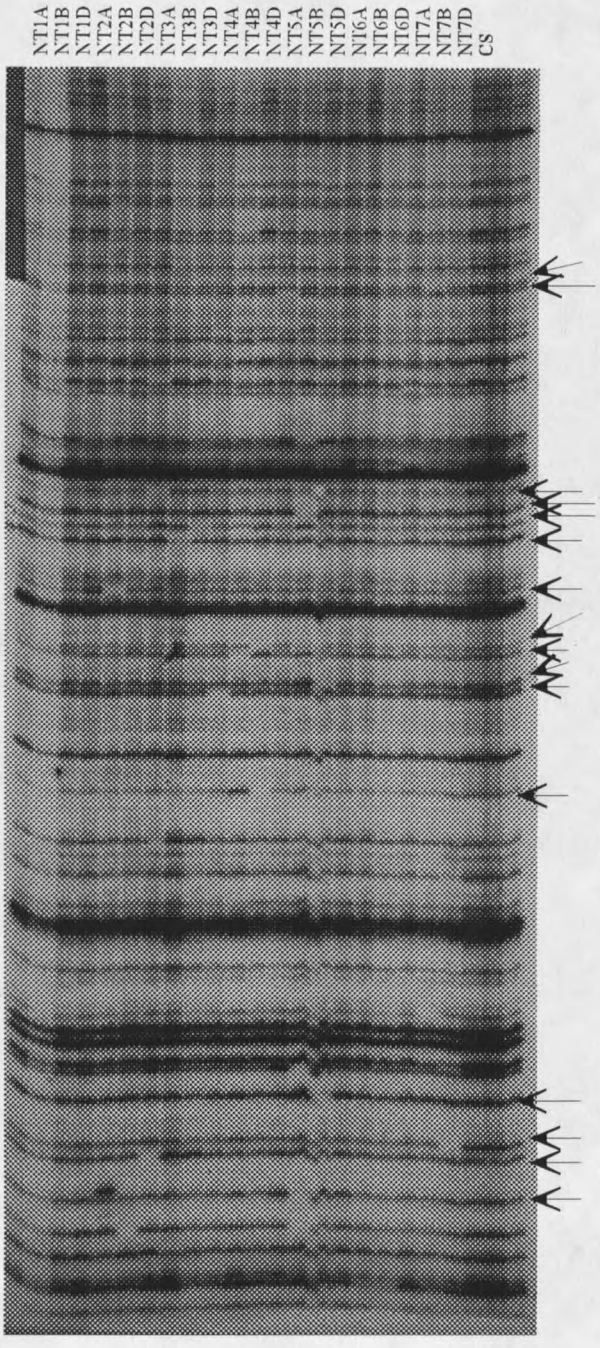


Figure 5 Identification of wheat chromosome-specific AFLPs using nullitetrasomic wheat stocks with primer combination E-AAG/M-CTC. The samples are as indicated on the picture, for example, CS stands for Chinese Spring wheat, NT3B stands for nullitetrasomic 3B, and etc. Wheat chromosome-specific AFLP markers were identified as bands missing in only one NT stock but present in all other NTs and Chinese Spring. The arrows indicate wheat chromosome-specific AFLPs.

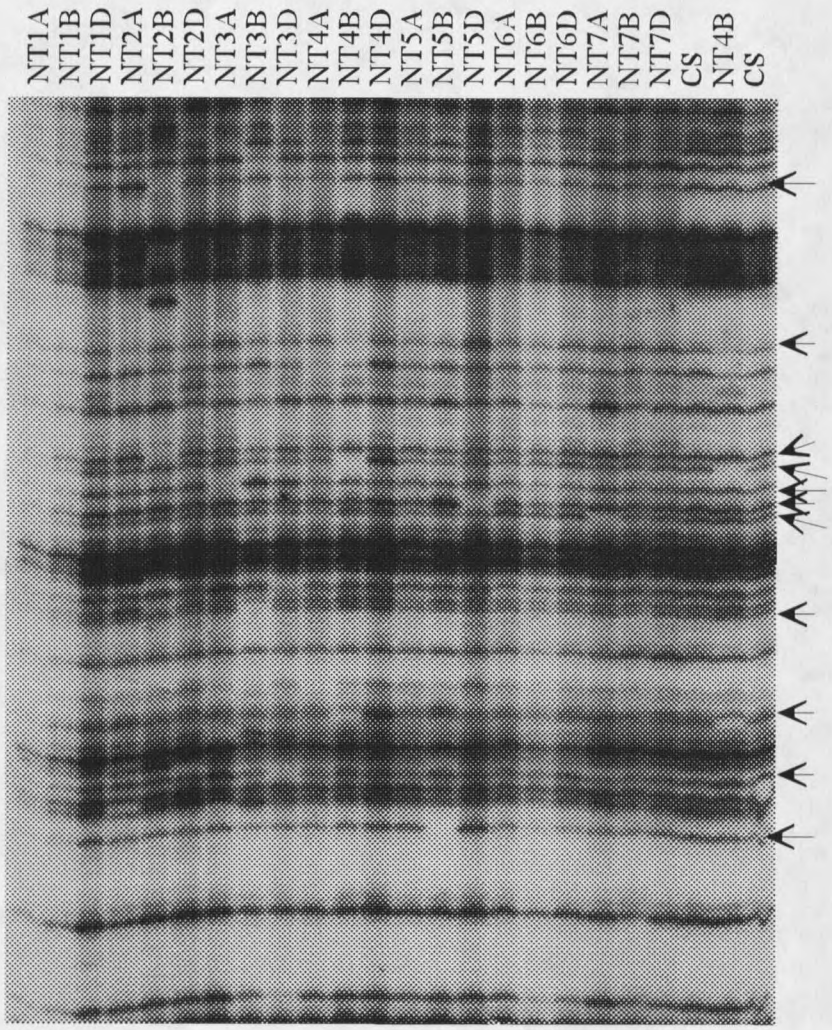
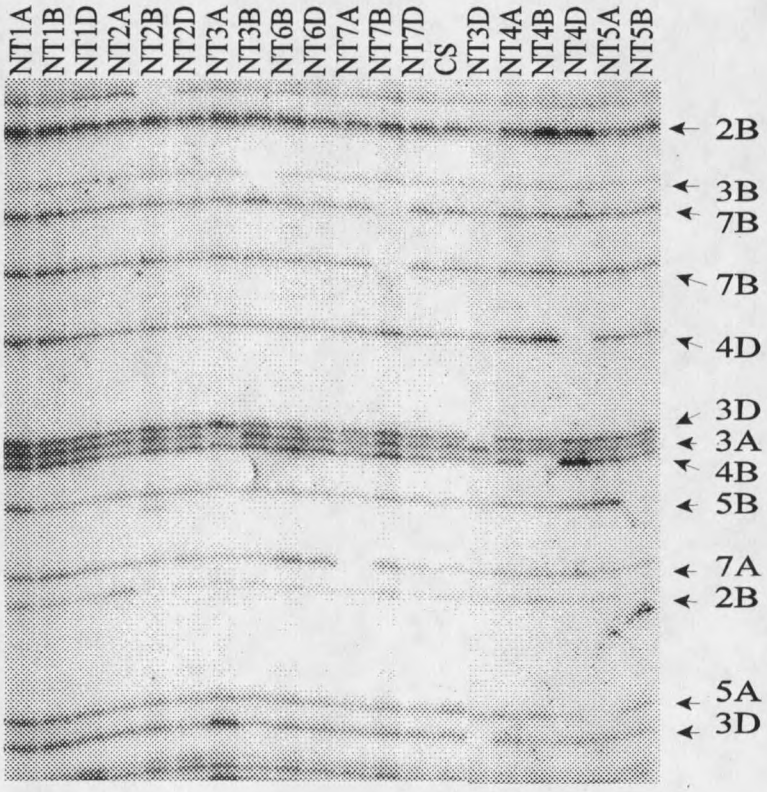


Figure 6 Identification of wheat chromosome-specific AFLPs using nullitetrasomic wheat stocks with primer combination E-ACG/M-CTC. The samples are as indicated on the picture, for example, CS stands for Chinese Spring wheat, NT3B stands for nullitetrasomic 3B, and etc. Wheat chromosome-specific AFLP markers were identified as bands missing in only one NT stock but present in all other NTs and Chinese Spring. The arrows indicate wheat chromosome-specific AFLPs.



assigned to each wheat chromosome were not evenly distributed, ranging from four for 1A to 32 for 3B and 5B (Table 6). The chromosome-specific markers were confirmed by repeating this AFLP analysis.

Further AFLP analysis was conducted using nullisomic-tetrasomic wheat stocks NT3B and NT4B, ditelosomic wheat stocks DT3BS, DT3BL and DT4BS to identify AFLP markers specific to chromosome arms 3BS and 4BS, since there is a shortage of PCR markers for 3BS and 4BS chromosome regions (Erpelding et al. 1996). Cultivar Chinese Spring wheat was used as the control material. Eighteen primer combinations were used for this experiment (Table 7), with *Eco*RI primers being end-labeled by γ-^{33}P ATP. γ-^{33}P ATP labeled primers resulted in better resolution of the amplification products on the gel after autoradiography, providing more and sharper scorable bands per combination (Table 7). 3BS specific AFLP markers were identified as bands missing in NT3B and DT3BL, but present in Chinese Spring and DT3BS (Figure 7). The same criterion was applied to identify 4BS and other chromosome arm specific AFLP markers (Figure 7). The numbers of polymorphic bands mapping to specific chromosome arms is given in Table 7. The chromosome arm specific markers were confirmed by repeating this AFLP analysis. Confirmation of their chromosome specific identity was conducted by refering back to the nullisomic-tetrasomic AFLP analysis data . Some of the confirmed 3BS and 4BS chromosome arm specific AFLP markers were selected for subsequent cloning experiments.

Table 5 Numbers of AFLP markers assigned to each wheat chromosome using nullisomic-tetrasomic stocks of Chinese Spring wheat for each tested primer combination.

	E-AAG/M-CAC	E-AAG/M-CTA	E-AAG/M-CTC	E-ACC/M-CTA
NT1A	0	1	0	1
NT1B	2	2	1	4
NT1D	1	3	2	4
NT2A	0	3	2	4
NT2B	3	3	6	3
NT2D	3	2	2	3
NT3A	0	2	3	6
NT3B	1	4	2	4
NT3D	1	0	2	1
NT4A	0	3	1	3
NT4B	4	4	2	3
NT4D	1	2	1	3
NT5A	3	3	0	3
NT5B	3	1	6	3
NT5D	0	1	4	0
NT6A	1	0	0	1
NT6B	n.d.	4	4	1
NT6D	1	5	3	1
NT7A	0	0	3	0
NT7B	2	0	1	3
NT7D	1	1	0	n.d.
Chinese Spring	152	122	117	136

Table 5 Continued.

	E-ACC/M-CTG	E-ACG/M-CAC	E-ACG/M-CAG	E-ACG/M-CAT
NT1A	0	1	0	1
NT1B	4	0	2	2
NT1D	2	1	0	1
NT2A	6	1	1	3
NT2B	3	3	2	2
NT2D	1	1	1	2
NT3A	4	5	1	1
NT3B	1	3	2	2
NT3D	2	1	4	2
NT4A	2	4	0	1
NT4B	6	6	0	5
NT4D	0	3	2	3
NT5A	2	1	1	2
NT5B	2	5	2	1
NT5D	0	1	0	4
NT6A	0	0	2	1
NT6B	4	1	n.d.	4
NT6D	0	1	1	1
NT7A	2	2	1	3
NT7B	1	3	2	3
NT7D	0	1	6	1
Chinese Spring	88	110	56	112

Table 5 Continued.

	E-ACG/M-CTC	E-ACT/M-CAC	E-AGC/M-CAG	E-AGC/M-CTG
NT1A	0	0	0	0
NT1B	0	0	0	4
NT1D	0	0	1	3
NT2A	1	1	5	3
NT2B	7	3	1	3
NT2D	0	2	3	7
NT3A	4	2	0	2
NT3B	7	1	3	2
NT3D	5	2	0	3
NT4A	0	1	2	3
NT4B	2	0	2	0
NT4D	2	0	0	1
NT5A	2	1	1	1
NT5B	1	1	4	3
NT5D	1	1	0	2
NT6A	1	0	0	2
NT6B	1	3	0	0
NT6D	1	1	0	0
NT7A	2	1	5	0
NT7B	3	1	1	2
NT7D	0	1	2	0
Chinese Spring	61	66	102	131

Table 6 Summary of the AFLPs observed in nullitetrasomic wheat stocks (NTs).

Wheat Stocks	Primer combinations tested	Total AFLPs scored	Chromosome specific AFLPs	% Chromosome specific AFLPs
NTs	12	1253	461	36.8
NT-1A			4	
NT-1B			21	
NT-1D			18	
NT-2A			30	
NT-2B			39	
NT-2D			27	
NT-3A			30	
NT-3B			32	
NT-3D			23	
NT-4A			20	
NT-4B			34	
NT-4D			18	
NT-5A			20	
NT-5B			32	
NT-5D			14	
NT-6A			8	
NT-6B			22	
NT-6D			15	
NT-7A			19	
NT-7B			22	
NT-7D			13	

Figure 7 Mapping chromosome-specific AFLPs to chromosome arms using ditelosomic wheat stocks. From left to right, the samples are: Chinese Spring wheat (CS), nullitetrasomic 3B (NT3B), ditelosomic 3BS (DT3BS), ditelosomic 3BL (DT3BL), nullitetrasomic 4B (NT4B), and ditelosomic 4BS (DT4BS). The primer combination was E-AGC/M-CAG. The band present in Chinese Spring and DT3BS but absent in NT3B and DT3BL indicates that this band marks wheat 3BS chromosome arm. The band present in Chinese Spring and DT4BS but absent in NT4B indicates that this band marks wheat 4BS chromosome arm. The arrows point to these chromosome-specific AFLPs.

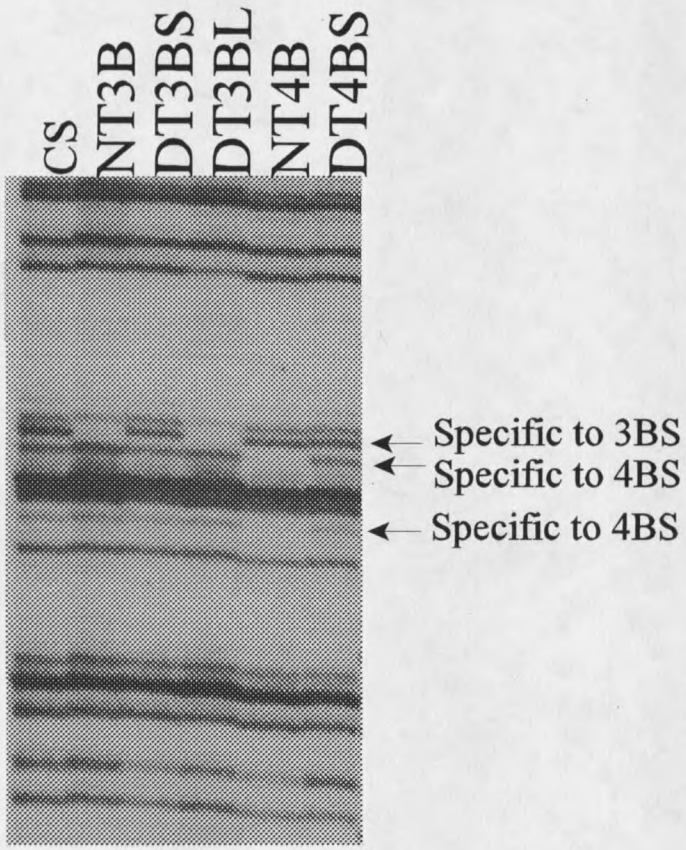


Table 7 Numbers of scorable bands revealed by AFLP primer combinations in wheat Chinese Spring and numbers of polymorphic bands mapped to specific chromosome arms by using wheat stocks: Chinese Spring, NT3B, DT3BS, DT3BL, NT4B, and DT4BS.

	Chinese Spring	NT 3B	DT 3BS	DT 3BL	NT 4B	DT 4BS	NT 4BL
E-AAG/M-CAT	152	3	2	1	2	2	0
E-AAG/M-CTC	133	5	3	2	4	3	1
E-AAG/M-CTG	128	2	2	0	4	2	2
E-ACC/M-CAT	135	2	1	1	6	5	1
E-ACC/M-CTA	134	5	2	3	3	0	3
E-ACG/M-CAC	112	3	2	1	5	2	3
E-ACG/M-CAT	117	3	1	2	5	3	2
E-ACG/M-CTA	73	2	0	2	1	0	1
E-ACG/M-CTC	80	4	4	0	1	1	0
E-ACG/M-CTG	48	3	1	2	1	0	1
E-ACT/M-CAC	144	3	2	1	1	1	0
E-ACT/M-CTC	127	2	1	1	0	0	0
E-ACT/M-CTG	102	1	1	0	2	1	1
E-AGC/M-CAG	128	7	3	4	2	2	0
E-AGC/M-CTG	119	4	3	1	2	0	2
E-AGG/M-CAT	167	3	2	1	2	1	1
E-AGG/M-CTA	117	6	3	3	3	1	2
E-AGG/M-CTG	133	3	2	1	3	1	2

Identification of barley chromosome specific AFLP markers

Five wheat-barley addition lines (WBALs), Betzes barley and Chinese Spring wheat, were used to identify barley chromosome-specific AFLP markers. These tested wheat-barley addition lines were designated as WBAL1, WBAL2, WBAL4, WBAL6, and WBAL7, with each number refering to the number of the corresponding barley chromosome. Fourteen primer combinations (Table 8) were used, with *Eco*RI primers labeled by γ-^{33}P ATP. An average of 56 (+/- 30.6) scorable barley-derived amplification products per primer combination was observed. A band present in Betzes and one WBAL but absent in Chinese Spring and other WBALs was considered as a barley chromosome-specific AFLP marker (Figure 8A, 8B). A summary of the numbers of the barley chromosome specific AFLPs observed in each wheat barley addition line for each primer combination was given in Table 9. A total of 174 barley chromosome-specific AFLP markers out of 781 barley-derived bands were scored (Table 10), with chromosome specific AFLP markers accounting for 22.3% of the total. The numbers of AFLP markers assigned to each barley chromosome were more evenly distributed than observed for wheat (Table 10). These barley chromosome-specific AFLP markers were confirmed by repetition of AFLP analysis on the WBALs. Barley chromosome-specific AFLP markers well-separated from surrounding AFLP fragments, ranging in size from 150- 650 bp, were chosen for subsequent cloning experiments.

In our experiments, the application of genomic DNA fingerprinting on wheat aneuploids and wheat-barley chromosome addition lines using AFLP technique revealed abundant chromosome-specific AFLP markers in wheat and barley. Our results also

showed the high efficiency, reproducibility of AFLP method for revealing polymorphisms among homoeologous chromosomes in wheat and barley. From these experiments, sufficient AFLPs of specific chromosomes were available to study on cloning of AFLPs and conversion of AFLPs to sequence specific PCR primers.

Table 8 AFLP primer combinations used with wheat-barley addition lines (WBALs).

E-A/M-C
E-AAG/M-CTA
E-AAG/M-CTC
E-ACG/M-CAC
E-ACG/M-CAG
E-ACG/M-CAT
E-ACG/M-CTA
E-ACG/M-CTC
E-ACG/M-CTG
E-ACT/M-CAC
E-ACT/M-CTC
E-ACT/M-CTG
E-AGC/M-CAG
E-AGC/M-CTA
E-AGC/M-CTG

[a] Preamplification primers

E-A: GACTGCGTACCAATTC-A

M-C: GATGAGTCCTGAGTAA-C

[b] Selective amplification primers

E-AAG: GACTGCGTACCAATTC-AAG

M-CAC: GATGAGTCCTGAGTAA-CAC

Figure 8 AFLPs in wheat-barley addition lines (WBALs), Chinese Spring wheat, and Betzes barley. Each lane represents one WBAL or one control stock (Chinese Spring wheat or Betzes barley), as indicated on the picture. Each group represents the results from one AFLP selective primer combination. The arrows indicate barley chromosome-specific AFLPs. Fig. 8A. A close-up look of some barley chromosome-specific AFLPs in the primer combination E-AGC/M-CTG.Fig. 8B: AFLPs in WBALs,Chinese Spring and Betzes using selective combinations E-AGC/M-CAG, E-AGC/M-CTA and E-AGC/M-CTG (From left to right).

Figure 8A

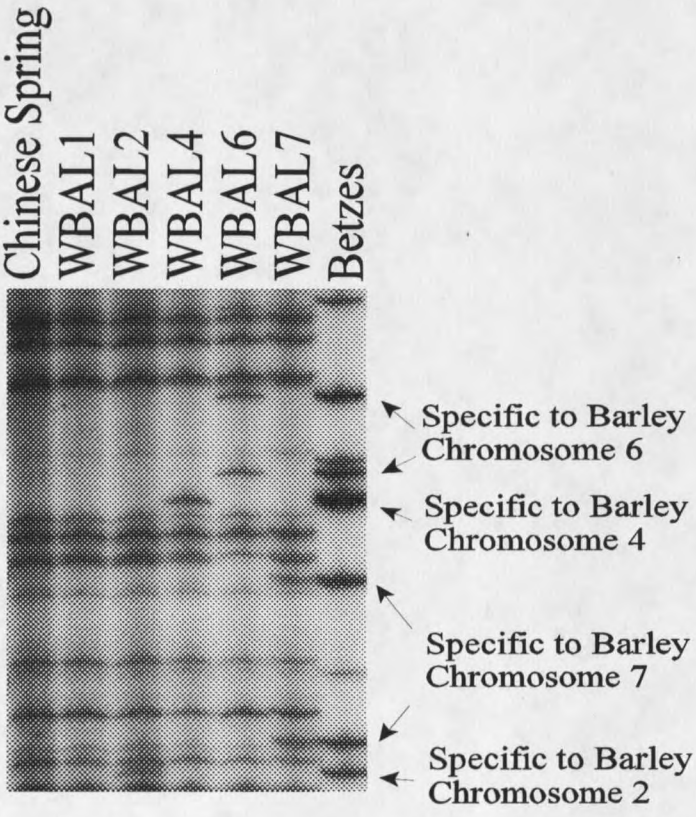


Figure 8B

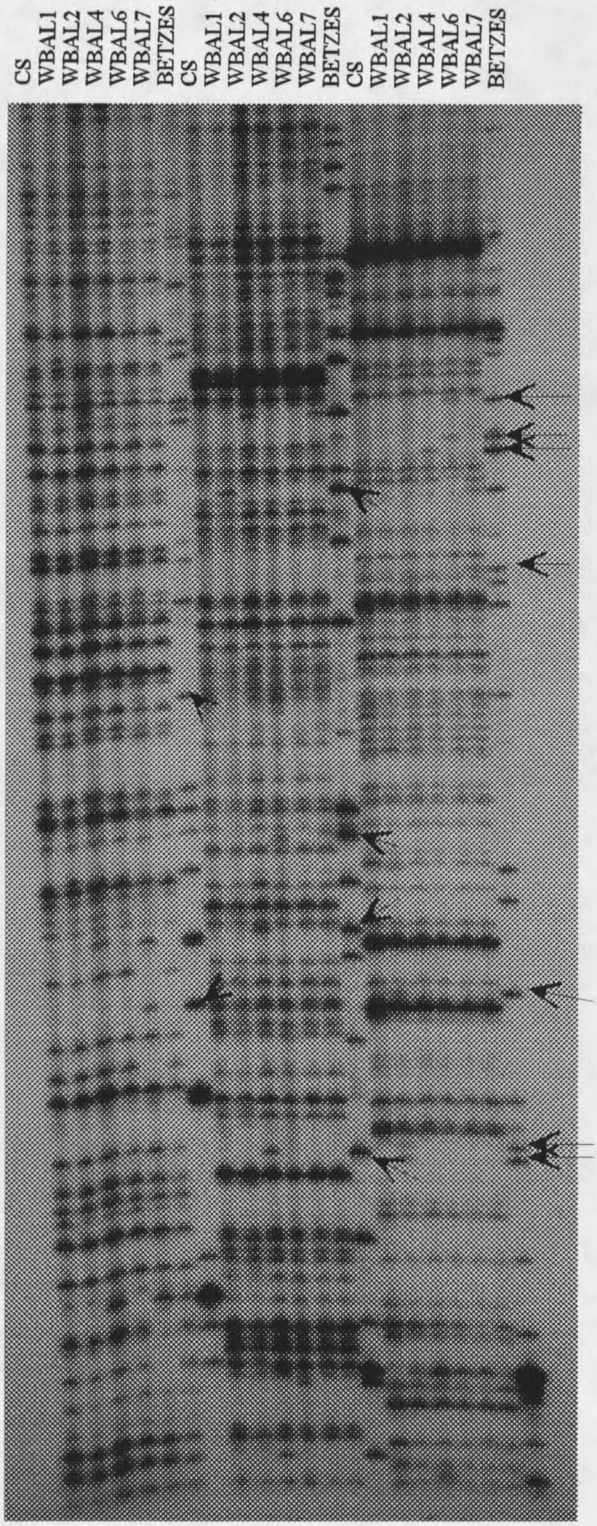

CS
WBAL1
WBAL2
WBAL4
WBAL6
WBAL7
BETZES
CS
WBAL1
WBAL2
WBAL4
WBAL6
WBAL7
BETZES
CS
WBAL1
WBAL2
WBAL4
WBAL6
WBAL7
BETZES

Table 9 Numbers of barley chromosome-specific AFLPs observed in each of the wheat barley addition lines (WBALs) and the total barley-derived bands observed in cultivar Betzes for each primer combination.

	WBAL 1	WBAL 2	WBAL 4	WBAL 6	WBAL 7	Betzes
E-AAG/M-CTA	2	4	6	1	2	74
E-AAG/M-CTC	4	7	9	5	2	68
E-ACG/M-CAC	3	3	2	0	1	31
E-ACG/M-CAG	3	2	1	1	3	25
E-ACG/M-CAT	3	2	0	7	2	119
E-ACG/M-CTA	6	2	3	5	1	91
E-ACG/M-CTC	1	2	2	2	5	67
E-ACG/M-CTG	0	0	1	1	0	95
E-ACT/M-CAC	6	5	5	2	4	50
E-ACT/M-CTC	3	1	2	3	1	40
E-ACT/M-CTG	1	3	0	1	1	29
E-AGC/M-CAG	1	0	0	3	1	18
E-AGC/M-CTA	3	4	2	1	2	45
E-AGC/M-CTG	4	2	2	2	3	29

Table 10 Summary of the AFLPs observed in wheat-barley addition lines (WBALs).

Wheat Stocks	Primer combinations tested	Total AFLPs scored	Chromosome specific AFLPs	% Chromosome specific AFLPs
WBALs	14	781	174	22.3
1			40	
2			37	
4			35	
6			34	
7			28	

CHAPTER 3

CONVERSION OF AFLP MARKERS INTO SEQUENCE-SPECIFIC PCR MARKERS IN WHEAT AND BARLEY

Literature Review

Conversion of different DNA marker types is often necessary for efficient applications. Olson et al. (1989) proposed the conversion of RFLP and RAPD markers to STS-PCR markers for developing a physical map of the human genome and since then the conversion strategy has been widely used in many genetic studies. In most cases, sequence-specific PCR-based markers, such as STS markers, were chosen as the targeted DNA marker type for conversion, since these simplified consensus PCR-based markers are more convenient and economical in applications involving large populations. Conversion between different DNA marker types have been reported by many researchers (Bradshaw et al. 1994; Salentijn et al. 1995; Brady et al. 1996; Talbert et al. 1996; Cheung et al. 1997), including the conversion of RFLP, RAPD and microsatellite markers into sequence-specific PCR markers, such as STS markers, for various applications.

Amplified fragment length polymorphism (AFLP) is a multiplex fingerprinting technique that displays a high degree of polymorphisms on DNA samples of any origin and complexity (Vos et al. 1995). This degree of polymorphism is often difficult to achieve using

morphological, biochemistry, and other DNA marker techniques. However, as a new technique, there are still areas to be explored for its utilities. AFLP markers are presence/absence-based dominant DNA markers identified by a side-by-side comparison of similar-sized, comigrating amplification products among DNA samples. AFLP markers may not be transferable in between different populations or between different labs. Waugh et al (1997) reported that similar-sized AFLP markers in three different mapping populations of barley could be mapped to the same genetic locus. They suggested that the size of AFLP product could be a reliable indicator of homology and at least within the cultivated barley gene pool that AFLP markers mapped in one population could be transfered to another population based on size if they also segregated in that population. Jones et al (1997) reported that a standardised AFLP genetic screening package prepared from one lab was distrbuted to other collaborators in order to achieve consistent AFLP data between labs.

An alternative strategy to circumvent the disadvantage of the non-transferability of AFLP markers may be the conversion of them to their sequence-specific counterparts, such as RFLP probes or sequence-specific PCR-based markers. Since AFLP is a multiplex DNA fingerprinting technique, it detects large numbers of polymorphic DNA fragments simultaneously, and it also visualizes abundant non-polymorphic DNA fragments at the same time. The conversion of the polymorphic fragments identified by AFLP technique to more specific simplex types of DNA markers would be useful for many genetic applications.

To date, only a few cases regarding cloning of AFLP fragments for conversion to other marker types have been reported. Meksem et al (1995) reported cloning of two AFLP markers cosegregating with the *R1* locus on chromosome V of potato and their conversion

to STS-PCR markers and RFLP probes. Neither of the STS-PCR markers allowed identification of alternative alleles. One RFLP probe revealed repetitive fragment patterns on a genomic Southern blot. The other RFLP probe detected a single-copy sequence in potato, cosegregating with *R* allele. Because the AFLP markers they cloned were 120 bp and 80 bp in length, respectively, they postulated that the STS-PCR products were too small to detect polymorphisms. Qu et al (1998) described cloning of six AFLP fragments corresponding to the Chinese Spring *ph1b* deletion and their conversion to PCR-based markers. One primer generated a product from Chinese Spring but not from the *ph1b* deletion line. These results showed that after conversion, only a few of the corresponding RFLP or sequence-specific PCR markers retained the specificity indicated by the original AFLP markers.

From our previous experiments, sufficient chromosome-specific AFLP markers in wheat and barley have been identified by using wheat nullitetrasomic stocks and wheat-barley addition lines. The goals of the following experiments were to determine the feasibility and the efficiency of cloning and converting chromosome-specific AFLPs to sequence-specific PCR markers in wheat and barley.

Materials and Methods

AFLP fragment isolation

After autoradiography, dried gels and films were aligned up using radioactive ink marks or nicks to isolate targeted AFLP fragments. A needle was used to punch around the band of interest through the film to the dried gel. A sharp, clean razor blade was used to excise

the selected piece of gel. The DNA-containing gel piece was placed into a 1.5 ml microcentrifuge tube with 100 µl of sterile water for 10 min at room temperature. The water was decanted and replaced with 100 µl extraction buffer (0.5M ammonium acetate, 5mM EDTA). The gel piece was crushed with a pipet tip, boiled for three minutes, and tubes were centrifuged at 14,000 rpm for 5 minutes. The supernatant was transferred to a new tube and 100 µl 8M ammonium acetate, 5 µg tRNA and 700 µl cold 100% ethanol were added to precipitate the DNA. Tubes were incubated at - 70°C for 30 minutes then centrifuged for 5 minutes. The supernatant was removed and pellets washed with cold 80% ethanol. Samples were centrifuged, the supernatant was removed, and pellets were resuspended in 10 µl sterile water. One - four ul were used for PCR.

Cloning of AFLP fragments

Standard PCR protocol was performed in a 50 µl reaction (94°C - 4min; 30x of 94°C-1 min, 50°C-1 min, 72°C-1.2 min; 72°C - 7 min; 4°C-hold) on each extracted AFLP fragment. Primers were the corresponding unlabeled selective AFLP primers using 0.2 µg per 50 µl reaction. A 10 µl sample of each PCR product was electrophoresed on 2% agarose gel. The size of each band was compared with that estimated from the AFLP gel. Cloning of PCR products was done using the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA).

Conversion of AFLPs to sequence-specific markers

After cloning, ten to 20 white colonies from each transformation were selected and each colony was cultured overnight in 100 µl LB broth with 50 µg/ml ampicillin. A four µl aliquot

of each culture was amplified by PCR as described above using the same set of unlabeled AFLP selective primers. PCR products were digested with two to four restriction enzymes and screened on 2% agarose or 7% polyacrylamide gels. Selected colonies were sequenced using the Sequenase version 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, Ohio). Based on the sequences of cloned fragments, new primers internal to the AFLP selective primers were designed using the OLIGO program (Rychlik and Rhoads 1989). These primers were used to amplify genomic DNAs of wheat nullitetrasomic stocks with Chinese Spring wheat as the control or wheat barley addition lines with Chinese Spring wheat and Betzes barley as the controls. PCR products were examined on 2% agarose gels or 7% polyacrylamide gels to determine whether the primers amplified a fragment from the same chromosome indicated by the corresponding AFLP markers.

Colony lifting and hybridization

An alternative method for identifying target colonies after cloning for the WBAL experiment was by colony lifting and hybridization. The colonies on the plates were replica-plated, followed by colony-lifting and hybridization. Probes were made from total unlabeled AFLP selective amplification products, with the positive control being the WBAL of the targeted chromosome and the negative control being a WBAL for a nontargeted chromosome. Magnacharge Nylon membrane (Micron Separations Inc) was used following the manufacturer's instructions. Prime-It®II, Random primer labeling kit (Stratagene, La Jolla, CA) was used for probe labeling.

Results

Strategies for improvement of precision in cloning AFLPs

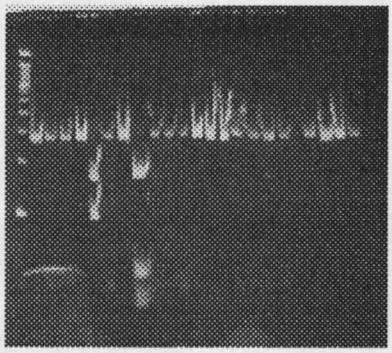
Amplification products of AFLP were resolved on sequencing gels (6% polyacrylamide gels in our experiments). In order to accurately extract and clone the targeted AFLP fragments, several aspects were taken under considerations. The first consideration concerned excising the AFLP bands accurately. For this purpose, asymmetric isotopic ink marks as well as punched holes were used for lining up the autoradiograph and the dried gel. The edges of the target bands were punched out carefully to keep the gel pieces to be cut as narrow as possible. After cutting, the gel was exposed to a second X-ray film overnight. The AFLP bands having been cut out should no longer show up in the second autoradiographs if the cutting was accurately. The second consideration concerned the reamplification of the target AFLP fragments. After extraction, the exact corresponding unlabeled AFLP selective primers were used for PCR to recover the target fragments. Reamplification products were examined on 2% agarose gels along with a molecular marker and the size of each band was compared with that estimated from the AFLP gels. The third consideration concerned the screen of the heterogeneous colonies. We found that when reamplifing a target AFLP band, even if the exact corresponding AFLP selective primers were used, unexpected fragments were often amplified from the gel piece, resulting in a heterogeneous mixture of fragments. Thus inserts in the colonies from a single transformation event were typically not identical. Fifty colonies from each transformation were chosen to perform PCR with the corresponding

unlabeled AFLP selective primers. The amplified inserts were digested with two to four four-base cutter restriction enzymes and resolved on 2% agarose gels. Generally one restriction pattern was shared by a majority of inserts. Some had two or three restriction patterns occurring at similar frequencies. A few of them had no predominant pattern with colonies showing high heterogeneity (Figure 9). It was therefore necessary to screen mixed colonies for the target AFLP fragment before sequencing. The strategies to screen the mixed colonies in our experiments were either by restriction pattern analysis or by colony hybridization, which will be explained in more details in the following experiments.

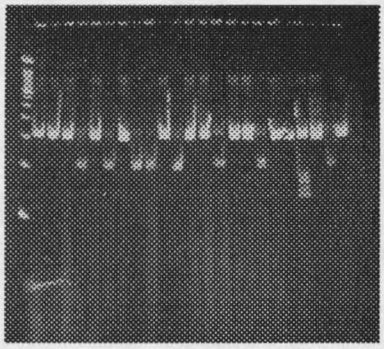
Conversion of barley chromosome -specific AFLPs to sequence-specific PCR markers

An initial set of cloning experiments was done with barley chromosome-specific AFLP markers. Ten AFLP fragments (Table 11) selected to be well-separated from surrounding fragments, marking specific barley chromosomes, were isolated from the AFLP gels, reamplified, and cloned into the pCR 2.1-TOPO vectors. Colonies were screened prior to sequencing, either by restriction analysis or by colony hybridization. Restriction analysis involved selecting twenty colonies from each plate and performing PCR on each colony, using the original set of unlabeled AFLP selective primers. Amplified products were digested with restriction enzymes and the banding patterns were resolved on 2% agarose gels or 7% polyacrylamide gels. One colony representing the majority banding pattern was selected and sequenced. The alternative strategy to screen the mixed colonies was by colony hybridyzation. This strategy involved screening all colonies from each plate. The colonies on the plates were replica-plated, followed by colony-lifting and hybridization. Probes were made from total unlabeled AFLP selective amplification products, with the positive control being

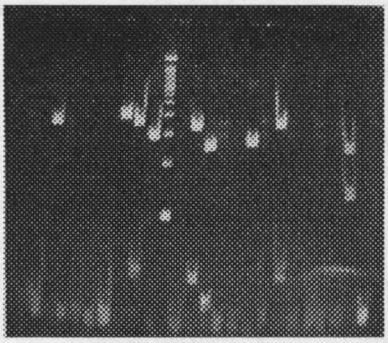
Figure 9 Examples showing the extent of heterogeneous colonies seen in AFLP fragment cloning experiments. Generally one restriction pattern was shared by a majority of the inserts (Panel A). Some had two or three restriction patterns occurring at similar frequencies (Panel B). A few of them had no predominant pattern with colonies showing high heterogeneity (Panel C).



Panel A



Panel B



Panel C

Table 11 Summary of ten barley chromosome specific AFLP fragments isolated from acrylamide gels.

AFLP marker designation	AFLP primer combinations	Chromosome specificity	Size
D1	E-ACT/M-CAC	4	300 bp
D2	E-ACT/M-CAC	4	200 bp
D3	E-ACT/M-CAC	6	350 bp
D4	E-ACT/M-CAC	7	250 bp
D5	E-ACT/M-CTC	4	450 bp
D6	E-ACT/M-CTC	1	350 bp
D7	E-ACT/M-CTG	4	400 bp
D8	E-AGC/M-CTA	2	400 bp
D9	E-AGC/M-CTA	4	170 bp
D10	E-AGC/M-CTA	4	170 bp

the WBAL of the targeted chromosome and the negative control being a WBAL for a nontargeted chromosome. Colonies which hybridized to the positive control (WBAL of the targeted chromosome) but not the negative control (WBAL of a nontargeted chromosome) were selected and sequenced. Two positive colonies from each of ten plates were chosen for sequencing. Fifteen of the 20 colonies selected by colony hybridization were identical to those identified by the restriction analysis strategy. All ten sequences identified through restriction fragment analysis were identified by the colony hybridization method. This analysis suggests that the restriction pattern analysis strategy was as efficient as the more laborious method of colony hybridization. The restriction analysis strategy has been used successfully

to identify target sequences cloned from differential display gels (Zhao et al., 1996). Primers were designed for the ten sequences identified by both methods (Table 12). Amplification with these sequence-specific PCR primers on the genomic DNA of the wheat-barley addition lines revealed four distinct results: 1) Five primer sets amplified a similar sized fragment on all barley chromosomes but no fragment in wheat (Figure 10A). 2) Three primer sets amplified fragments specific to the predicted barley chromosome. (Figure 10B). 3) One primer set amplified a similar sized fragment in both wheat and barley. Polymorphisms existed between wheat and barley upon digestion of these amplified products with *Rsa*I or *Hinf*I (Figure 11). 4) One primer set gave no amplification.

Conversion of wheat chromosome -specific AFLPs to sequence-specific PCR markers

The second set of AFLP fragments cloned was wheat chromosome-specific markers. Twelve and four AFLP fragments specific to wheat 3BS and 4BS chromosome arms, respectively, were isolated and cloned (Table 13) and the colonies were defined as colonies of positive cloning reaction. Since 3BS specific AFLP markers were identified as bands missing in NT3B and DT3BL, but present in Chinese Spring and DT3BS, the corresponding gel pieces of the band missing positions in NT3B were also cut out and used as negative controls for colony screening. DNAs were amplified from some of these gel pieces which appeared to be blank area. These amplified DNA fragments were also cloned and the colonies were defined as colonies of negative cloning reaction. The negative controls of 4BS were prepared using the same method. We only used the restriction analysis method for colony screening on this group because this method proved to be as efficient as the colony hybridization method in screening the mixed colonies in previous experiments. A single

colony representing the major banding pattern from each positive cloning reaction was chosen for sequencing. The colony restriction pattern of each negative cloning reaction, if available, was also examined for eliminating the unexpected background bands.

Primers were designed from the sequence data (Table 12) and used for PCR on wheat genomic DNAs. Out of twelve AFLP wheat 3BS markers cloned, three primer sets amplified fragments specific to the wheat 3BS chromosome arm (Table 12). Two of these primer sets also amplified an additional fragment that was not chromosome-specific (Figure12). One primer set amplified a single, chromosome-specific band (Figure 13). Eight primer sets amplified fragments with no specificity. One primer set gave no amplification. Of the four AFLP 4BS markers cloned, three primer sets amplified fragments on all wheat NT stocks tested, whereas one primer set gave no amplification.

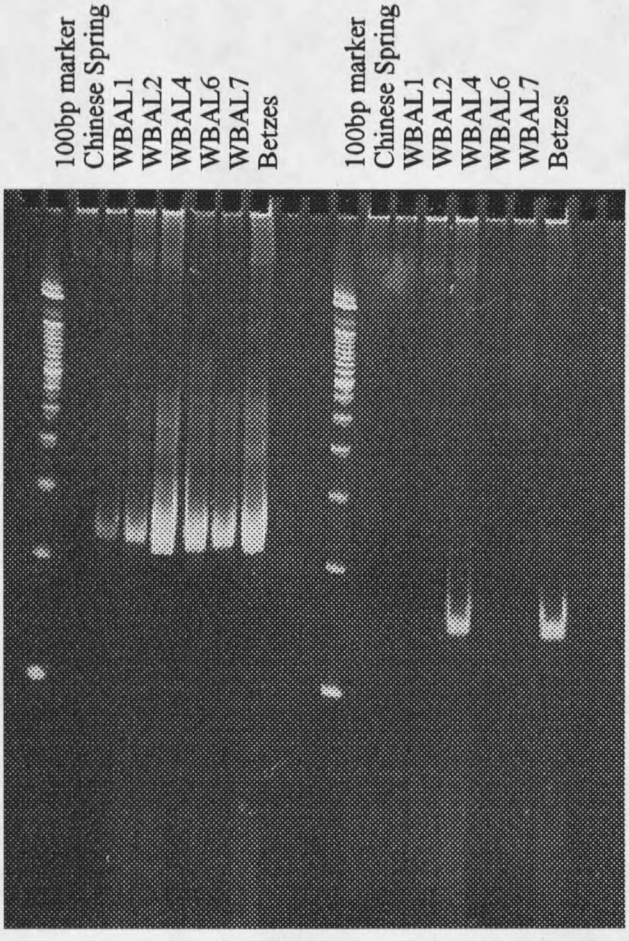
Segregation ratio tests of sequence specific PCR markers

While chromosome-specific primer sets were obtained through these studies, it is not clear that a single locus is amplified. To test this, we searched for polymorphisms in two wheat mapping populations and one barley mapping population for the wheat and barley-derived primer sets, respectively. The amplified products were not polymorphic in the populations for most of the primer sets, even after digestion with a battery of restriction enzymes. The exception to this was primer set XJ28, in that the amplified products revealed polymorphisms among parental genotypes of the Opata 85-Altar 84 wheat mapping population (Nelson et al., 1995). However, observed segregation for parental types among recombinant inbred lines derived from the cross was 25:12. This does not fit the expected 1:1 ratio, and we are unable to conclude that XJ28 amplifies a single locus.

Table 12 PCR primers specific to wheat or barley designed from AFLP markers

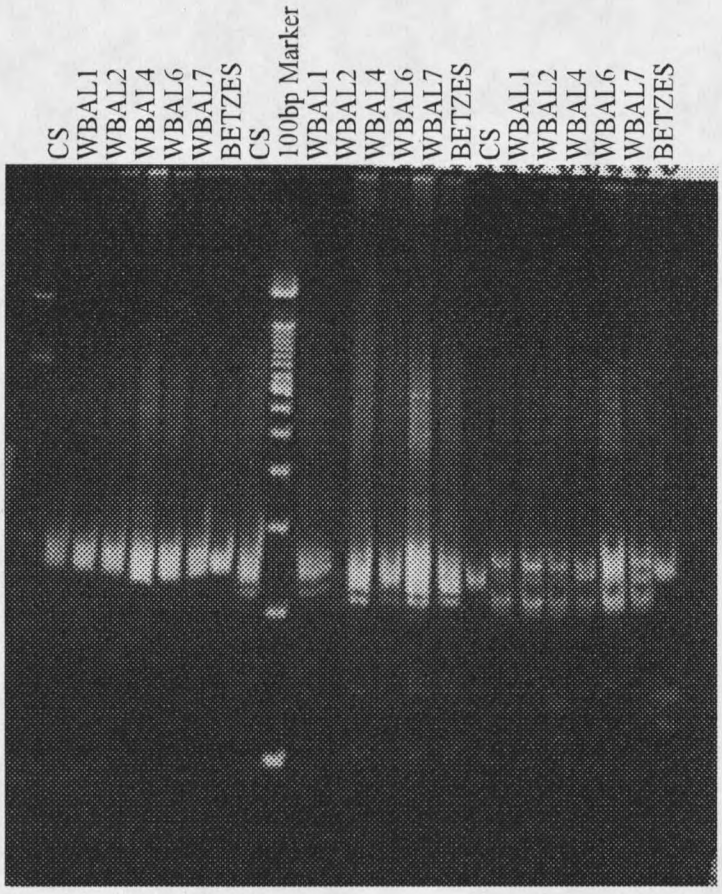
Primer Designation	Specificity	Sequence (5'-3')
XD1	Barley	U: AACAAGCTGAGACCCTACTG
		R: CTACTTGTATCGGTTTATCG
XD2	Barley chromosome 4	U: GTACCAGTATGGCACTCCTC
		R: GCCCGTAATGCTCATCTTTA
XD3	Barley	U:AGACTTTGATTGGTCATGGC
		R:TCAAGTGACGAAAAGGAACG
XD4	Barley chromosome 7	U:GGGTTGATGTTTTTGACATG
		R:AACACATTTGGGGAGAAGAG
XD5	Barley chromosome 4	U:TTGGGAGATGGTGAGGTTAG
		R:CAAAATCACATGCAACCCAC
XD6	Barley	U:CTCGATGCAACATCATATGC
		R:CCTAACATATACCTCGTGCA
XD7	Barley (Digest with *Rsa*I)	U:CACACTGCCACAGCATATTA
		R:ACTCCTCACTGATTCTTCGG
XD8	Barley	U:CATCCCATACATCCAATACA
		R:AATTCAGCGATATTGTTGGA
XD9	Barley	U:GTTATGCACCTGGAGATGTG
		R:CGAGCTCCTAGTAGTTGTTG
XJ5	Wheat 3BS	U:GACTCGTGATCGAAATCTTT
		R:AGAGTGTGAATGCTTCAAGA
XJ26	Wheat 3BS	U:TTGCCTAGTCAATCACTAGT
		R:TTCTGAATACCAGCATTAGC
XJ28	Wheat 3BS	U:TGTGGAGCAAATCTGCTATT
		R:AGAGATTCCCGAGATTACAT

Figure 10 Gel picture of two primer sets designed from barley chromosome 4 AFLP markers. Panel A: Primer set XD1 amplifies a similar sized fragment in all wheat barley addition lines but not in Chinese Spring wheat, indicating that it is specific to barley but not to any particular chromosome. The 100bp marker contains bands of size range 100-1500 bp. Panel B: Primer set XD2 amplifies a fragment in WBAL4 and Betzes barley but not in any other WBAL or Chinese Spring wheat, indicating that it maps to barley chromosome 4.



Panel A Panel B

Figure 11 Gel picture of primer set XD7 designed from a barley chromosome 4 AFLP marker. XD7 amplifies a similar sized fragment in all wheat barley addition lines, Betzes barley and Chinese Spring wheat (Panel A). Polymorphisms existed between wheat and barley upon digestion of these amplified products with *Hinf* I (Panel B) and *Rsa*I (Panel C). The 100bp marker contains bands of size range 100-1500 bp.



Panel A Panel B Panel C

Table 13 AFLP fragments specific to wheat 3BS and 4BS chromosome arms isolated from acrylamide gels.

AFLP markers designation	AFLP primer combinations	Chromosome arm specificity	Size
J4	E-AAG/M-CTC	4BS	250 bp
J5	E-AAG/M-CTG	3BS	500 bp
J7	E-ACC/M-CAT	4BS	450 bp
J8	E-ACC/M-CTA	3BS	450 bp
J14	E-ACG/M-CAC	4BS	220 bp
J15	E-AAG/M-CAT	3BS	500 bp
J19	E-ACG/M-CAT	3BS	300 bp
J20	E-ACG/M-CTC	3BS	400 bp
J21	E-ACG/M-CTC	3BS	350 bp
J22	E-ACG/M-CTC	4BS	200 bp
J26	E-ACT/M-CTG	3BS	170 bp
J27	E-AGC/M-CAG	3BS	250 bp
J28	E-AGC/M-CAG	3BS	150 bp
J29	E-AGC/M-CTG	3BS	170 bp
J32	E-AGG/M-CTA	3BS	300 bp
J35	E-AGG/M-CTG	3BS	280 bp

Discussion

From our previous experiments, abundant chromosome specific AFLPs in wheat and barley were identified and available for conversion of AFLPs to sequence-specific PCR primers. While the high reproducibility, reliability and efficiency of AFLP technique were demonstrated

in fingerprinting of wheat aneuploids and wheat barley addition lines, little was known regarding the cloning of AFLPs and conversion of them to other types of DNA markers. From the experiments of this chapter, we wished to address some issues by directly cloning and sequencing the AFLP fragments and converting them to sequence-specific PCR markers. These experiments may also allow to interpret some of the attributes of AFLP markers from a different perspective.

The cloning of AFLPs and their conversion to simple sequence PCR markers has been described in only a few reports (Meksem et al.1995, Qu et al. 1998, Koebner et al. 1998). For instance, Qu et al (1998) found that out of six AFLP markers converted, one primer pair generated a product retaining the expected specificity indicated by the original AFLP marker (Qu et al. 1998). Our experiments showed that after cloning 26 wheat or barley chromosome specific AFLP markers and converting them to sequence-specific primers, six primer sets retained the specificity indicated by AFLP markers. We found that the difficulties in cloning of AFLPs and conversion of them to sequence specific PCR markers may result from both technical considerations and the nature of AFLP polymorphisms.

Technical problems may affect the effectiveness of AFLP cloning and conversion. In our experiments, we found that reamplification of apparently chromosome-specific AFLP bands resulted in a heterogeneous mixture of similar-sized fragments. This caused mixed colonies from a single transformation event. Therefore, it is necessary to screen the mixed colonies before sequencing. We used two screening methods to improve the precision in cloning the target AFLPs. A restriction analysis strategy involved screening 20 colonies from each plate. One colony representing the majority digestion pattern of each plate was sequenced. A

Figure 12 Gel picture of primer set XJ5 designed from a wheat ditelosomic 3BS AFLP marker. XJ5 amplifies two fragments. One fragment is amplified in all nullitetrasomic stocks (only nullitetrasomic chromsomes 1 and 3 are shown) except NT 3B. Additionally it amplifies the same fragment in ditelosomic 3BS but not 3BL. This indicates that XJ5 maps to wheat chromosome arm 3BS. Primer set XJ5 also amplifies an additional fragment that is not chromosome-specific. The 100bp marker contains bands of size range 100-1500 bp.

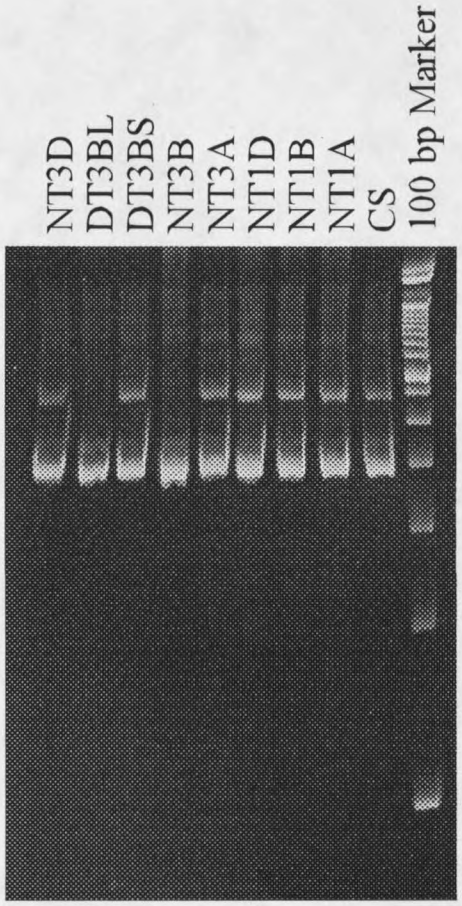
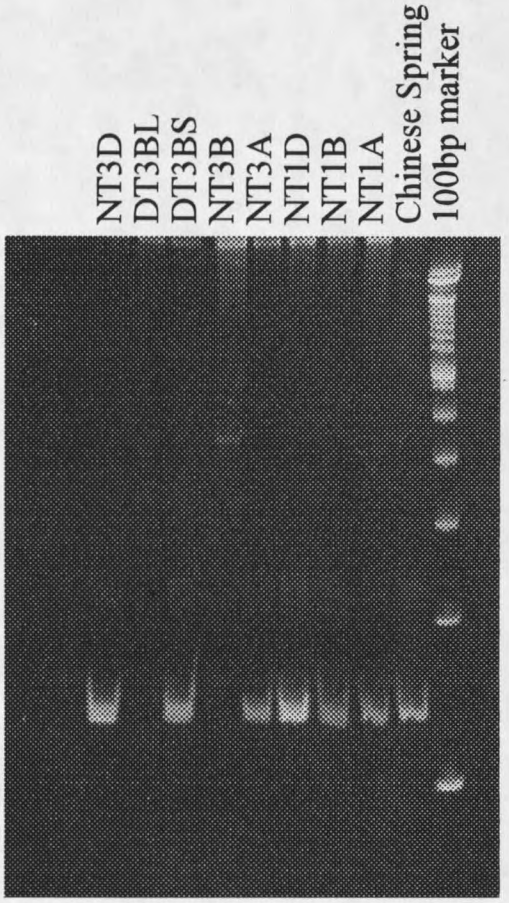


Figure 13 Gel picture of primer set XJ28 designed from a wheat ditelosomic 3BS AFLP marker. XJ28 amplifies a single copy fragment in all nullitetrasomic stocks (only nullitetrasomic chromsomes 1 and 3 are shown) except NT 3B. Additionally it amplifies the same fragment in ditelosomic 3BS but not 3BL. This indicates that XJ28 maps to wheat chromosome arm 3BS.The 100bp marker contains bands of size range 100-1500 bp.



colony hybridization strategy involved screening all colonies from each plate. Two colonies which hybridized only to positive control were sequenced. Both strategies revealed the same sequences from selected colonies. This indicated that the restriction analysis strategy was an efficient and precise enough method to screen the mixed colonies. Our findings of mixed colonies coincided with Koebner et al. (1998). They referred it as the bottleneck to identify true target sequence from the heterogeneous clones resulted from AFLP cloning. Various strategies were attempted by them to establish an effective, general procedure. These strategies included: 1. Reject *Mse*I/*Mse*I clones; 2. Compare gel mobility of PCR product of clones amplified with AFLP primers with the original AFLP band on a sequencing gel; 3. Group remaining clones by restriction profile with at least three 4bp cutters; 4. Hybridize dot blots of the AFLP reaction of critical genotypes with non-radioactively-labelled representatives of each group of clones based on restriction profiles; 5. Sequence candidate clones and check specificity by PCR.

Another possible reason for the lack of efficient conversion may lie with the nature of AFLP polymorphisms. In our experiments, we found that after conversion, many primers designed from chromosome-specific AFLPs amplified similar-sized fragments on each of the genomic chromosomes. These results may be explained by some basic features of AFLP technique. AFLP primers are designed according to the sequences of the adapters and the restriction sites with two or three selective nucleotides at the 3'-end. These selective nucleotides are arbitrarily chosen to determine the restriction fragments to be amplified. Only those restriction fragments in which the starting nucleotides next to the restriction site exactly match the 3'-end selective nucleotides of the primers can be

amplified. Thus, restriction sites and selective nucleotides are predominant determinants of the polymorphisms. Given a certain pair of restriction enzymes, the polymorphisms of AFLP are actually generated by the use of the selective nucleotides. These selective nucleotide-generated polymorphisms revealed by AFLP may largely affect the effectiveness of AFLP cloning and conversion.

Therefore although AFLP detects polymorphisms of genomic restriction fragments, at this point, analogous to RFLPs, because of the features of AFLP we mentioned above, differences exist in the polymorphisms detected by these two systems. Polymorphisms appearing on an AFLP gel may have three possibilities: i) The polymorphisms could be due to presence or absence of the target sequences. Sequence-specific PCR primers were generated from sequence internal to the AFLP primers. PCR markers converted from this kind of AFLPs could retain the specificity indicated by the AFLP markers. ii) The polymorphisms may be due to restriction site differences similar to RFLP. These AFLP polymorphisms related to *Eco*RI or *Mse*I restriction site differences may not be reflected in primers developed from internal sequence. However, polymorphisms may exist upon digestion with restriction enzymes. And iii) The polymorphisms could be due to differences corresponding to the selective nucleotides used in amplification. In combination with the same *Eco*RI primer, the difference of one nucleotide on the 3' position of *Mse*I primers could generate totally different AFLP patterns. In other words, the difference of even one selective nucleotide could result in the selection of a different subset of the genomic restriction fragments. Therefore, nucleotide differences specific to the AFLP primers may have resulted in the chromosome specificity of AFLP bands. This

specificity would be lost when internal primers are derived. If PCR primers are converted from this kind of AFLPs, they may amplify a sequence from all chromosomes. This complicates the conversion of AFLPs to sequence specific PCR markers.

CHAPTER 4

IDENTIFICATION OF CHROMOSOME-SPECIFIC AFLPS USING *PSTI/MSEI* PRIMER COMBINATIONS AND CONVERSION OF THEM INTO SEQUENCE-SPECIFIC MARKERS IN WHEAT AND BARLEY

Literature Review

In the previous chapters, our AFLP experiments were conducted using the restriction enzymes *Eco*RI/*Mse*I. In AFLP, the selection of different restriction enzyme combinations may be important for determination of the different portions of the genome to be scrutinized, in that the cleavage frequency of the restriction enzymes is variable. AFLP uses two restriction enzymes, a rare cutter (six-base or eight-base) and a frequent cutter (four-base), to generate genomic restriction fragments. Vos et al (1995) investigated the performance of several different rare cutter enzymes , including *Eco*RI, *Hin*dIII, *Pst*I, *Bgl*II, *Xba*I, and *Sse8387*I, in combination with a frequent cutter, either *Mse*I or *Taq*I, for AFLP analysis. They reported that other rare cutters generally worked equal to *Eco*RI and therefore, generally speaking, *Eco*RI is preferred because of its low cost. As for the frequent cutter, *Mse*I is preferred because it cuts more frequently than *Taq*I and generates fragments more evenly distributed in most eukaryotic genomes. Therefore, the combination *Eco*RI/*Mse*I is a reliable, efficient method which detects high

levels of polymorphism evenly distributed throughout the genome. In fact, despite the different applications of the AFLP technique reported so far, the combination *Eco*RI/*Mse*I was chosen in more cases than any other restriction enzyme combinations.

The combination *Pst*I / *Mse*I is a second commonly used combination of restriction enzymes. In contrast with methylation insensitive combinations such as *Eco*RI/*Mse*I, methylation sensitive combinations like *Pst*I / *Mse*I may allow targeting of AFLP analysis to only the hypomethylated portions of the genome.

Methylated DNA comprises a considerable portion of higher plant genomes, with the amount of 5-methyl cytosine accounting for up to 30% of cytosines in different species (Shapiro, 1976). Heavily methylated DNA has been found to be associated with unexpressed regions of genomes (Miller et al. 1974). There are also reports demonstrating that DNA methylation plays a role in the repression of transcriptional regulation and inhibition of gene expression (Naveh-Many et al.1981; Cedar 1988). Gruenbaum et al. (1981) reported that approximately 80% of CG sequences or CAG sequences in wheat and potato were methylated. Unmethylated CpG and CpXpG islands were reported to be associated with active genes in higher plants (Antequera et al. 1988; Klaas et al. 1989). Therefore, when genomic DNA is digested with methylation sensitive enzymes, which only recognize and cleave at the unmethylated CpG and CpXpG sequences , the digested portion of the plant genome may contain high levels of active genes. As a consequence, if AFLP profiles were generated by using methylation sensitive enzymes, the polymorphisms detected may contain relatively high proportion of sequences representing active genes, which would be more likely to be single or low copy DNAs.

Such a difference in the constitution of polymorphisms could be utilized for applications in AFLP fingerprinting.

A few cases have been reported regarding the utility of methylation sensitivity restriction enzymes for AFLP analysis. Donini et al. (1997) described that AFLP profiles generated by the combination *Sse8387I/Mse*I revealed polymorphisms between DNAs from different plant organs. *Sse8387*I is a methylation sensitive restriction enzyme (5'CCTGCAGG3'). *Sse8387I/Mse*I AFLP analyses were conducted on cultivars of bread wheat (*Triticum aestivum*). Polymorphic bands were detected between bulked seed DNA and leaf DNA of the same accession. When five individuals from each of three wheat accessions were examined, the same organ-specific polymorphisms were observed between DNA from the endosperm half of each grain and DNA from the leaf emerging from the embryo half of the same grain. Further experiments were conducted on two wild relatives of wheat, *Aegilops mutica* and *Aegilops speltoides.* Organ specific AFLP bands were detected between DNAs from the root and the shoot of the same seedling. They postulated that DNA methylation may very likely be the source of the organ-specific AFLPs. Barrett et al.(1998) compared the results of AFLP-based genetic diversity estimates (GDEs) using methylation sensitive combination *Pst*I/*Mse*I and using methylation insensitive combination *Eco*RI/*Mse*I, respectively. They found that *Pst*I-based GDEs had better correlations with pedigree-based GDEs than *Eco*RI-based GDEs did. They also indicated that AFLP markers obtained by use of methylation sensitive enzymes may better reflect the polymorphisms among the expressed genes. The mean diversity level detected by *Pst*I was significantly lower than by *Eco*RI. Blake et al.

(Montana State University,personal communication) evaluated the distribution of previously mapped markers on barley chromosomes by comparison with the locations of AFLP markers generated by the methylation sensitive enzymes. They found that the markers mapped by the North American Barley Genome Mapping Project were mainly assigned to undermethylated genomic regions.

In the experiments of this chapter, we wished to examine the performance of the methylation sensitive enzyme combination *Pst*I/*Mse*I for the identification of chromosome specific AFLP markers in wheat nullisomic-tetrasomic stocks and their conversion to sequence-specific PCR markers.

Materials and Methods

AFLP analysis using the *Pst*I/*Mse*I combination

AFLP analysis was conducted as described by Vos et al (1995), with some modification. A total of 500 ng genomic DNA for each wheat stock was digested with *Pst*I / *Mse*I in a reaction volume of 50 μl [5 μl 10x NEBuffer 2 (New England Biolabs Inc.), 5 u *Pst*I , 5 u *Mse*I (New England Biolabs Inc.)] at 37°C for 3 hours followed by 15 minutes at 70°C to inactivate the restriction endonucleases. Oligonucleotide sequences for *Pst*I and *Mse*I adapters and primers are listed in Table 14. *Pst*I adapter was made by combining equimolar quantities of ADT-*Pst*IA and ADT-*Pst*IB in ddH_2O with a final concentration of 5 pmol/μl. *Mse*I adapter was made by combining equimolar quantities of ADT-*Mse*IA and ADT-*Mse*IB in ddH_2O with a final concentration of 50 pmol/μl. The

Table 14 Oligonucleotide sequences for *Pst*I and *Mse*I adapters and primers.

Oligo for *Pst*I adapters and primers	**Oligo for *Mse*I adapters and primers**
*Pst*I adapters ADT-*Pst*IA: CTCGTAGACTGCGTACATGCA ADT-*Pst*IB: TGTACGCAGTCTAC	*Mse*I adapters ADT-*Mse*IA: GACGATGAGTCCTGAG ADT-*Mse*IB: TACTCAGGACTCAT
*Pst*I preamplification primer XSPA: GACTGCGTACATGCAG XSPB: GACTGCGTACATGCAGA	*Mse*I preamplification primer PAP-M-C: GATGAGTCCTGAGTAAC
*Pst*I selective amplification primers XSP1: GACTGCGTACATGCAGACA XSP2: GACTGCGTACATGCAGACG XSP3: GACTGCGTACATGCAGCGA XSP4: GACTGCGTACATGCAGGCT	*Mse*I selective amplification primers XSM2: GATGAGTCCTGAGTAACAC XSM3: GATGAGTCCTGAGTAACAG XSM4: GATGAGTCCTGAGTAACAT XSM5: GATGAGTCCTGAGTAACTA XSM6: GATGAGTCCTGAGTAACTC XSM7: GATGAGTCCTGAGTAACTG

combined single stranded oligonucleotides were then annealed by incubating at 90°C for 5 minutes followed by gradual return to room temperature. For ligation reaction, a 10 μl solution [1 x T_4 DNA ligase beffer, 5 pmol *Pst*I adapter, 50 pmol *Mse*I adapter, 1U T_4

DNA ligase] was added to each of the digestion reaction mixtures and incubated at 37°C for 2 hours followed by 15 minutes at 70°C to inactivate the enzyme. A 1 : 5 dilution of the digestion/ ligation mixture was prepared with sterilized distilled water for using as template DNAs in preamplification reactions. The dilutions and the unused original reaction mixtures were stored at -20°C. Preamplification primers were XSPA/B and PAP-M-C (Table 14). Preamplification reaction mixture contained 2 μl template DNA from the 1:5 diluted digestion/ ligation reaction, 1 x PCR buffer (without Triton X-100), 50 ng XSPA/B primer, 50 ng PAP-M-C primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.8 U Taq polymerase, in a total volume of 40μl. The PCR protocol of preamplification was 94°C 2 min, 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 60 s followed by 72°C 5 min and hold at 4°C. A 1 : 5 dilution of the preamplification product of each sample were prepared for the subsequent selective AFLP amplification by using sterilized distilled water. Both diluted and undiluted preamplification products were stored at -20°C. Selective amplification primers are listed on Table 14. Primer labeling was performed by end-labeling of the *Pst*I primers with γ-^{33}P ATP (NEN, Boston, MA) and T_4 kinase. The labeling reaction was carried out at 37°C for 1 hour followed by inactivation of the enzyme at 70°C for 10 minutes. Selective AFLP amplification was performed in a total volume of 20 μl [5 μl of 1:5 dilution of preamplification products, 1 x PCR buffer (without Triton X-100), 5 ng of labeled *Pst*I selective primer, 30 ng of *Mse*I selective marker, 0.2 mM of each dNTP, 0.2 U Taq polymerase] as such a protocol: one cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s; twelve cycles at 94°C for 30 s, annealing temperature lowering 0.7°C each cycle, and 72°C for 60 s; twenty-three cycles at 94°C

for 30 s, 56°C for 30 s, and 72°C for 60 s. Primers with three selective nucleotides were used for selective amplification (Table 14). All amplifications were conducted in a Perkin Elmer GeneAmp® 9600 thermocycler. After PCR, 20μl stop solution (98% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each reaction. Selective amplification products were heated at 90°C for 3 minutes before loaded on 6% polyacrylamide denaturing sequencing gels (20 : 1 acrylamide:bis; 7.5 M urea; 1 x TBE buffer). Gels were run at 50w constant power for 3 - 4 hours, transferred to Whatman paper, dried, marked with radioactive ink or nicks in film corners for orientation purposes, and exposed to X-ray film (Kodak Biomax-MR) for 16 - 24 hours. Intense and well separated bands were scored.

Results and Discussions

Identification of chromosome-specific AFLP markers in wheat using *Pst*I / *Mse*I primer combinations

Twenty-one wheat nullitetrasomic stocks (NTs) of 'Chinese Spring' wheat (Sears 1954), with wheat cultivar Chinese Spring as control material, were used for AFLP analysis using the *Pst*I/*Mse*I combination. Seven selective primer combinations (Table 15) were tested for AFLP analysis. Figure 14 shows the AFLP binding pattern resulting from

Figure 14 Identification of wheat chromosome-specific AFLPs using nullitetrasomic wheat stocks with primer combination P-ACG/M-CAG. The samples are as indicated on the picture, for example, CS stands for Chinese Spring wheat, NT3B stands for nullitetrasomic 3B, and etc. Wheat chromosome-specific AFLP markers are identified as bands missing in only one NT stock but present in all other NTs and Chinese Spring. The arrows indicate wheat chromosome-specific AFLPs.

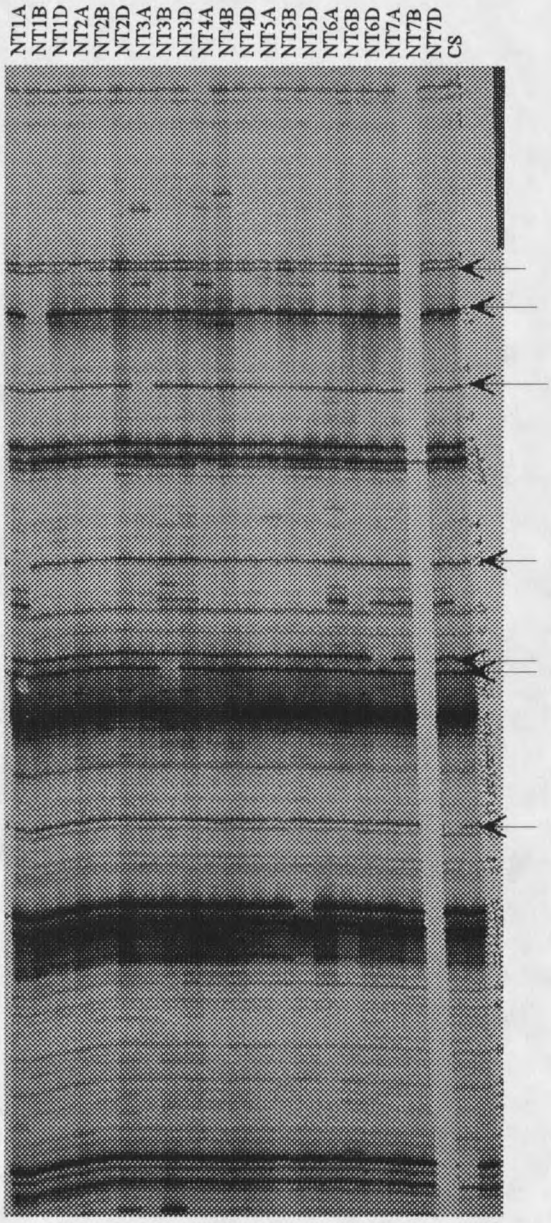


Table 15 *Pst*I/*Mse*I primer combinations used with nullisomic-tetrasomic wheat stocks of Chinese Spring for AFLP analysis.

P-0/M-C[a]
P-A/M-C
P-ACA/M-CAG[b]
P-ACA/M-CTA
P-ACG/M-CAG
P-ACG/M-CTA
P-CGA/M-CTA
P-CGA/M-CAG
P-CGA/M-CTC

[a] Preamplification primers

P-0: GACTGCGTACATGCAG
P-A: GACTGCGTACATGCAGA
M-C: GATGAGTCCTGAGTAA-C

[b] Selective amplification primers

P-ACA: GACTGCGTACATGCAG-ACA
M-CAG: GATGAGTCCTGAGTAA-CAG

one of the *Pst*I/*Mse*I combinations tested (P-ACG/M-CAG). These primer combinations revealed an average of 96 (+/- 19.35) scorable amplification products per combination in cultivar Chinese Spring wheat. The number of AFLP markers assigned to each chromosome for each *Pst* I / *Mse*I selective primer combination are in Table 16. A total of 143 wheat chromosome-specific AFLP markers were identified in this experiment,

accounting for 21.3% of the 671 scored amplification products in Chinese Spring wheat (Table 17). The number of AFLP markers assigned to each wheat chromosome were not evenly distributed, ranging from zero for 2D to 14 for 4A and 5B (Table 17).

Conversion of wheat chromosome -specific AFLPs to sequence-specific PCR markers

Ten wheat chromosome-specific markers were cloned in this experiment (Table 18) using pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). We only used the restriction analysis method for colony screening on this group because this method proved to be efficient in previous experiments. Two colonies representing the major banding pattern from each cloning reaction was chosen for sequencing. Sequencing was conducted on ABI PRISM™ automated sequencer (Perkin-Elmer, Foster City, CA) using ABI PRISM™ dye terminator cycle sequencing ready reaction kit (With AmpliTaq DNA polymerase,FS) (Perkin-Elmer, Foster City, CA). The sequences were analyzed using Chromas program (Griffith University, Brisbane, Queensland, Australia). Four of these sequences were chosen to design PCR primers using Oligo program (Rychlik et al. 1989). These primers were used for PCR on wheat nullitetrasomic genomic DNAs.

Amplification with these four sequence-specific PCR primers revealed that: one primer set amplified a similar sized fragment retaining the chromosome specificity being indicated by its original AFLP marker; one set amplified a similar sized fragment without the expected specificity; and two sets failed to amplify distinct bands.

From our experiments, we found that 21.3% of the scorable *Pst*I/*Mse*I generating

Table 16 Number of AFLP markers assigned to each wheat chromosome using nullisomic-tetrasomic stocks of Chinese Spring wheat for each tested *PstI/MseI* selective primer combination.

	P-ACA/M-CAG	P-ACA/M-CTA	P-ACG/M-CAG	P-ACG/M-CTA
NT1A	3	0	3	0
NT1B	1	0	1	2
NT1D	2	0	0	2
NT2A	0	0	1	0
NT2B	5	0	1	0
NT2D	0	0	0	0
NT3A	1	0	3	1
NT3B	0	1	4	2
NT3D	2	0	0	4
NT4A	0	0	1	2
NT4B	0	0	0	0
NT4D	1	0	0	0
NT5A	2	1	0	0
NT5B	3	0	2	0
NT5D	0	1	0	4
NT6A	0	2	0	1
NT6B	3	0	0	3
NT6D	1	0	1	0
NT7A	1	0	0	0
NT7B	0	1	0	0
NT7D	1	0	3	0
Chinese Spring	108	65	80	91

Table 16 Continued.

	P-CGA/M-CTA	P-CGA/M-CAG	P-CGA/M-CTC
NT1A	1	0	1
NT1B	0	0	1
NT1D	0	4	1
NT2A	0	0	0
NT2B	2	1	4
NT2D	0	0	0
NT3A	4	1	0
NT3B	2	2	0
NT3D	2	1	0
NT4A	7	3	1
NT4B	2	3	0
NT4D	0	1	0
NT5A	0	0	0
NT5B	3	2	4
NT5D	1	3	0
NT6A	0	3	0
NT6B	0	0	1
NT6D	0	1	0
NT7A	0	0	0
NT7B	0	3	4
NT7D	0	1	0
Chinese Spring	99	118	110

Table 17 Summary of the chromosome-specific AFLPs observed in nullitetrasomic wheat stocks (NTs) using *Pst*I/*Mse*I selective primer combinations.

Wheat Stocks	Primer combinations tested	Total AFLPs scored	Chromosome specific AFLPs	% Chromosome specific AFLPs
NTs	7	671	143	21.3
NT-1A			8	
NT-1B			5	
NT-1D			9	
NT-2A			1	
NT-2B			13	
NT-2D			0	
NT-3A			10	
NT-3B			11	
NT-3D			9	
NT-4A			14	
NT-4B			5	
NT-4D			2	
NT-5A			3	
NT-5B			14	
NT-5D			9	
NT-6A			6	
NT-6B			7	
NT-6D			3	
NT-7A			1	
NT-7B			8	
NT-7D			5	

Table 18 Summary of ten wheat chromosome-specific AFLP fragments isolated from acrylamide gels in the *Pst* I / *Mse* I experiment.

Name of the target AFLP markers	AFLP primer combinations	Chromosome specificity	Size
K1	P-ACA/M-CAG	1D	150 bp
K4	E-ACG/M-CAG	4A	400 bp
K5	P-ACG/M-CAG	1A	300 bp
K6	P-ACG/M-CAG	1B	250 bp
K7	P-CGA/M-CAG	5D	350 bp
K8	P-CGA/M-CAG	3D	320bp
K9	P-CGA/M-CAG	4B	300 bp
K10	P-CGA/M-CAG	5B	150 bp
K11	P-CGA/M-CTC	7B	120 bp
K12	P-CGA/M-CTC	2B	300 bp

AFLPs could be assigned to specific chromosomes. In contrast with the results from the previous experiments, in which 36.8% of the scorable *Eco*RI/*Mse*I generating AFLPs were chromosome-specific, we found that the percent chromosome-specific AFLPs detected by methylation sensitive *Pst*I/*Mse*I combinations in the hypomethylated portion of wheat genomic DNA was lower than the percent chromosome-specific AFLPs detected by methylation insensitive *Eco*RI/*Mse*I combinations throughout the whole wheat genome.

These results were in agreement with Barrett et al (1998). In their comparison between the results of AFLP-based genetic diversity estimates (GDEs) using methylation sensitive combination *Pst*I/*Mse*I and using methylation insensitive combination *Eco*RI/*Mse*I, they found that the mean diversity level detected by *Pst*I was significantly lower than by *Eco*RI. However, we currently cannot explain our observations.

Our experiments showed the feasibility of the cloning of methylation sensitive *Pst*I/*Mse*I AFLPs. In fact, in the research reported by Qu et al. (1998) one out of six sets of primers converted from AFLPs in wheat retained the expected specificity, using *Pst*I/*Mse*I enzyme combinations. This may indicate that due to the allopolyploid nature of wheat, the difficulty of conversion AFLPs to sequence-specific PCR markers may not be easily circumvented by the use of methylation sensitive enzyme combinations. The few reported researches regarding the conversion of AFLPs to simple PCR-based markers were only in wheat and potato, thus the performance and differences of the conversion of methylation sensitive AFLPs and methylation insensitive AFLPs in other plants are unknown yet. An alternative way to improve the efficiency of the conversion may be to convert mapped AFLPs. However, the conversion experiments reported by Meksem et al. (1995) was on mapped AFLPs in potato. Neither of the two STS-PCR markers allowed identification of alternative alleles. Again, because of the lack of references in other plants, there are still many things to be explored in the conversion of mapped AFLPs. From our experiments, we found that conversion of AFLPs to sequence-specific PCR markers was feasible, promising, and yet not efficient so far in wheat and barley.

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