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Evaluation of "sequence-tagged-site" PCR products as molecular markers in wheat

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Abstract. The polymerase chain reaction (PCR) is an attractive technique for many genome mapping and characterization projects. One PCR approach which has been evaluated involves the use of randomly amplified polymorphic DNA (RAPD). An alternative to RAPDs is the sequence-tagged-site (STS) approach, whereby PCR primers are designed from mapped low-copy-number sequences. In this study, we sequenced and designed primers from 22 wheat RFLP clones in addition to testing 15 primer sets that had been previously used to amplify DNA sequences in the barley genome. Our results indicated that most of the primers amplified sequences that mapped to the expected chromosomes in wheat. Additionally, 9 of 16 primer sets tested revealed polymorphisms among 20 hexaploid wheat genotypes when PCR products were digested with restriction enzymes. These results suggest that the STS-based PCR analysis will be useful for generation of informative molecular markers in hexaploid wheat.

Key words: Wheat – Molecular – Markers – Breeding

Introduction

Genetic markers have many applications in plant breeding. Restriction fragment length polymorphisms (RFLPs) may be used as genetic markers, and RFLPs have been used to construct maps and estimate genetic diversity for many crop plants. Two factors have been important in the use of RFLPs in wheat. First, somewhat limited numbers of polymorphisms have been observed among wheat lines. For example, Kam-Morgan et al. (1989) found that polymorphisms were detected at approximately one in three loci when a set of hexaploid wheats were assayed with four restriction enzymes. Chao et al. (1989) found that 38% of 45 loci showed RFLP among six hexaploid wheats. A second important factor in wheat RFLP analysis is that allohexaploid wheat tolerates aneuploidy, thereby allowing aneuploid stocks to be used for mapping experiments (Sears 1954). In particular, aneuploid stocks have been used to locate RFLP loci to chromosome arms (Sharp et al. 1989; Anderson et al. 1992). Using aneuploid analysis in conjuction with mapping populations derived from a cross between *Triticum tauschii* accessions, Gill et al. (1991) located 127 RFLP loci to seven D-genome linkage groups.

The polymerase chain reaction (PCR) (Saiki et al. 1985) offers the potential to lessen the time and expense of molecular mapping. In particular, randomly amplified polymorphic DNAs (RAPDs) involving the use of single DNA primer to direct amplification of discrete sequences (Williams et al. 1990) have shown promise in cereals (D'Ovidio et al. 1990; Weining and Langridge 1991; Devos and Gale 1992). A combination of RAPDs and denaturing gradient gel electrophoresis has been used to distinguish among wheat cultivars (He et al. 1992, Dweikat et al. 1993). However, RAPDs have their own problems of limited repeatability, with the confounding factor that repetitive DNA sequences are often amplified (Devos and Gale 1992).

In the human genome mapping project, genetic mapping has been augmented by using sequence-tagged-sites (STS) for PCR analysis (Olson et al. 1989). A STS is a short, unique sequence amplified by PCR that identifies a known location on a chromosome.

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D'Ovidio et al. (1990) and Weining and Langridge (1991) showed that PCR can be used to detect polymorphisms in cereals with primer sequences derived from the α-amylase and γ-gliadin genes. Tragoonrung et al. (1992) extended the approach to barley, using eight sequences previously mapped in the barley genome. For this report, we constructed primer sets and mapped the amplified fragments for 22 wheat RFLP clones, and then assayed the ability of the method to distinguish among wheat lines. Additionally, 15 primer sets previously tested in barley mapping were tested for utility in wheat.

Materials and methods

DNA extractions and Southern blots were conducted as previously described (Talbert et al. 1992). Aneuploid 'Chinese Spring' stocks developed by Sears (1954) were obtained from the USDA-ARS Midwest Area Plant Genetics Unit, Columbia, Mo. These stocks were used to map PCR products to chromosome arms. Exotic hexaploid wheat accessions were obtained from the USDA Small Grains Germplasm Research Facility, Aberdeen, ID (Table 1). These stocks were assayed along with adapted cultivars to determine whether the STS PCR primers revealed polymorphisms among hexaploid wheat lines.

Clones derived from the D genome diploid *Triticum tauschii* were obtained from B. S. Gill, Kansas State University (Gill et al. 1991). Approximately 200 bp were sequenced at both ends of the cloned sequence by the dideoxy chain termination method (Sanger et al. 1977). Primers approximately 20 base pairs in length were designed based on guidelines of Saiki (1990) from the sequenced clones (Table 2) and synthesized with a PCR-Mate 391 DNA synthesizer (Applied Biosystems) using standard phosphoramidate chemistry. Additional primer sets were obtained from the Montana State University barley genome mapping project (Tragoonrung et al. 1992). Primer concentrations were adjusted to 100 ng/ul. PCR reactions contained 50 m*M* KCl, 10 m*M* Tris-Cl, 0.1% Triton X-100, 50 u*M* of each dNTP, 1.5 m*M* $MgCl_2$, 400 nM each primer, 0.6 unit *Taq* polymerase, and 100 ng genomic DNA in a total volume of 50 µl. Approxi-

Table 1. Hexaploid wheats accessions tested for polymorphisms using STS PCR primers

Name or plant no.	Varietal group	Origin	Class[a]
Amidon	*aestivum*	USA	HRS
Newana	*aestivum*	USA	HRS
MT 8849	*aestivum*	USA	HRS
Hi-Line	*aestivum*	USA	HRS
Lew	*aestivum*	USA	HRS
Klasic	*aestivum*	USA	HWS
Owens	*aestivum*	USA	SWS
Penawawa	*aestivum*	USA	SWS
Plainsman V	*aestivum*	USA	HRW
Judith	*aestivum*	USA	HRW
Chinese Spring	*aestivum*	China	SWS
PI 15129	*aestivum*	Italy	
PI 372129	*aestivum*	USSR	
PI 428343	*vavilovii*	Sweden	
PI 221419	*spelta*	Yugoslavia	
PI 272577	*spelta*	Hungary	
PI 352302	*compactum*	Austria	
PI 352304	*compactum*	France	
PI 17731	*sphaerococcum*	Unknown	
CI 4528	*sphaerococcum*	India	

[a] HRS, Hard red spring; HWS, hard white spring; SWS, soft white spring; HRW, hard red winter

Table 2. Sequences of wheat STS primers.

Homoeologous chromosome group[a]	Primer Set[b]		Sequence (5'-3')
1	D14	L	CGCTTTTACCGAGATTGGTC
		R	CCAAAGAGCATCCATGGTGT
1	E8	L	TGCTCGGTTCAATTGACTGC
		R	TATGGGCCAGTGATTTCCAC
1	E11	L	GTTGCTAGAAACATGTCACAGC
		R	CCGTACGTTTGTGCAATCATG
1	Hor2	KV1	CCACCATGAAGACCTTCCTC
		KV2	ACCTTGCATGGGTTTAGCTG
1	Pst340	L	TAGCATCGGTAATCTCTCGC
		R	CCCTTTATATACACTGCCGA
2	D8	L	ACTGTCTGTGCCTTGTGATC
		R	GGATGTCTCATATGCATGCAC
2	D18	L	CCACTGTTAGGATTAGTGATCC
		R	GGACACTAAACTTTAGAGGC
2	E16	L	CACCATCGTGCAGATGGAGATC
		R	CAGACATACATAGATGGAGGC
3	E2	L	GTATTTCTACCATGGCTAGC
		R	CTGATTTAGTCCTGTGGCAC
3	G36	L	TGTCGCAACACTGTAGCACG
		R	GGACATTATCAGTTATCAGC

Table 2. (*Continued*)

Homoeologous chromosome group	Primer Set[b]		Sequence (5′-3′)
3	WG110	KV25	TCTGATACACACCTCCAGCG
		KV26	ACGGCGATCCGTCCACGAGC
3	His3	KV12	ATGGCCCGCAC(C/G)AAGCAGAC
		KV13	AGCTGGATGTCCTTGGGCAT
4	B5	L	CTCAACTAAGAAGCACCGGC
		R	AACCACCTACAACCGGCTTC
4	C2	L	ATGGAGAAGTCTTACCTCAGC
		R	TTTCGCTGTGGCACTTGTAC
4	D21	L	TCTTCCAGTTAGAGATCTCC
		R	TCGTTCGTACTAGTAGTACC
4	E6	L	TGCAGCATTCTGGAACATGC
		R	AATCAAGAAGGACAATGCC
4	E9	L	AAATCCAGCGGTATGCATGC
		R	TTCACAAAATTCACCCAAGC
4	G10	L	GTGTTGATGTCCTTGAGGCC
		R	TGTCCAGCTTCAGCGAGTAC
4	CMd	ST4	ATCCACAGCGGCTGTTCCAC
		ST6	TCTGGCCACCGTCATGGTCT
4	WG464	L	AGGACTGTGAAGATGCTACT
		R	AGTCCAAATGATGTCACAGG
5	A3	L	AACATGGTCCTCAGGGAATC
		R	GTATTATGGTCCATGTCTAC
5	D16	L	AAATCTGTCAGAGCCTGATGC
		R	GAAGAACACGTGCGTGCACC
5	G44	L	GTACTGATCAAGTTCTGTCATCG
		R	CAGCATTATACTGGACGATGC
5	Pst319	L	AGCTGAGCAAGCTTCTTTGG
		R	AACATGCTGGGCAACTCCCA
6	D1	L	CGGATCCTATAAAGTAGCGC
		R	CTGTCAAAATCTGTTGGGTC
6	D17	L	CAAACAAGCAGCCAGGTAG
		R	CTGCTTGGGTCATCATGCAA
6	F19	L	AAGGTGTCCTTTTGCAGGCAC
		R	ACTGTTATCTGGAGGTCTCC
6	G8	L	CCGTCGATTACTTGAGTAGAC
		R	ACCACAGAGTATTGGCTTGC
6	ABG458	L	AGTCTTGCGCATGGTGACAC
		R	CACCAATTGCATCAAAGCTC
7	A1	L	CAACAGAGATATTGCCGTAG
		R	AAGATTGTCAACAAGTGCC
7	His3	KV12	ATGGCCCGCAC(C/G)AAGCAGAC
		KV24	GACTTCCT(C/G)GCCGCCTGCAA
7	WG686	L	TCGCTTTACCACAATTTCAG
		R	GCTGTTCATATAAAAGGAGA

[a] Chromosomal locations of the RFLP fragments based on mapping experiments in wheat (Gill et al. 1991) or barley (Tragoonrung et al. 1992)

[b] Underlined primer sets were from the barley mapping project (Tragoonrung et al. 1992); all others from D genome RFLP clones (Gill et al. 1991)

mately 60 ul mineral oil overlayed each reaction mixture. The typical temperature conditions for PCR were 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1.2 min. Three primers had exceptional amplification protocols. G 10 had an annealing temperature of 50 °C, while primers sets KV 1/2 and KV 12/24 had an amplification protocol, after the initial 4-min-long denaturation step, of 30 cycles of 94 °C for 50 s, followed by 55 °C for 10 s, followed by a final extension step of 72 °C for 10 s. For all other primer sets, at the end of 30 cycles the temperature was set to 72 °C for 7 min prior to cooling to 4 °C. Reaction products were subsequently digested with approximately 1 unit of *Hin*fI, *Hha*I, *Dde*I, and/or *Rsa*I per reaction mixture for 1 h at 37 °C. Products were separated on a 7% polyacrylamide gel with a 0.5 × TRIS-borate buffer (22 m*M* TRIS, 22 m*M* boric acid, 0.5 m*M* EDTA). Gels were stained with ethidium bromide, and DNA was visualized with UV light.

Results

Map location of primer set products determined using aneuploid wheat stocks

Of the 22 sets of primers designed from D-genome RFLP clones (Table 2), 19 amplified products in 'Chinese Spring' wheat under our standard conditions. The remaining 3 primer sets (A3, C2, and E9) did not amplify easily discernible fragments. We also tested 15 primer sets developed by the barley genome mapping project, of which 10 resulted in clear amplification products in wheat (Table 2). The primer sets which did not give clear amplification were not tested further. It is possible that different amplification protocols may give different results with these primers.

The 29 primer sets which generated distinct bands were tested using 'Chinese Spring' nulli-tetrasomic stocks to determine if the amplified products mapped to expected chromosomes based on prior mapping experiments of the RFLP clones (Gill et al. 1991; Tragoonrung et al. 1992). Of the 29 primer sets 23 generated diagnostic sequences that were missing in at least one of the expected nullisomic-tetrasomic wheats (Table 3). Thus, it appeared that the primers generally amplified the expected sequences. The amplification products were localized to chromosome arms using ditelosomic stocks (Table 3). An example of a mapping experiment with primer set G36 is shown in Fig. 1. This figure shows that an amplified product is missing in nullisomic 3B (lane B) relative to 'Chinese Spring' (lane F). This product is present in ditelosomic 3BL (lane D) and absent in ditelosomic 3BS (laneE). This indicates that the 1700-bp fragment maps to the long arm of chromosome 3B. The origin of the fragments amplified by G36 that are present in all nullisomic stocks (lanes A, B, C) is not known. These may be from other homoeologous chromosomes. Alternatively, the fragments may represent conserved sequences on chromosomes 3A and 3D.

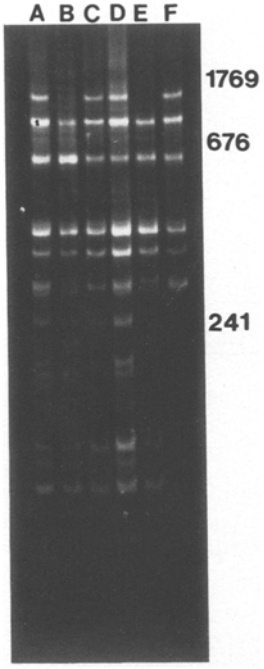

Fig. 1. Polyacrylamide gel of PCR amplification products using primer set G36 followed by digestion with *Hha*I. *Lane A* nullisomic 3A, tetrasomic 3D; *B* nullisomic 3B, tetrasomic 3A; *C* nullisomic 3D, tetrasomic 3A; *D* ditelosomic 3BL; *E* ditelosomic 3BS; *F* 'Chinese Spring'

Table 3. Map locations of amplified products based on analysis of nulli-tetrasomic and ditelosomic aneuploids of 'Chinese Spring'

Primer Set	Location[a]	Primer Set	Location
D14	1BS (b, c)	E9	poor ampl.
E8	1BL, 1DL (a, b)	G10	4A, 4BL, 4DL(b)
E11	ND	CMd	4AL, 4BL (b)
Hor2	1BS, 1DS (c)	WG464	4BL, 4DL (a)
Pst340	1BL, 1DL (b, c)	A3	poor amplification
D8	2BS (a)	D16	ND
D18	2AS	A1	ND
E16	2AL (a, b)	G44	5BL, 5DL (a, d)
E2	3BS (c)	Pst319	5BL (b)
G36	3BL (c)	D1	6DL (b, c)
WG110	3DL (d)	D17	6DL (a, b)
His3(KV12/13)	3AS (b, c)	F19	ND
B5	4DL (c)	G8	6BS, 6DS (b)
C2	poor ampl.	ABG458	6BL (b, c)
D21	4DL (a, b, c)	His3(KV12/24)	7AS (b)
E6	ND	WG686	7AL, 7BL (b)

[a] Enzyme which gave informative band indicated parenthetically, where a = *Rsa*I, b = *Hin*fI, c = *Hha*I, and d = *Dde*I
[b] ND, Chromosome-specific markers were not detected with any of the four enzymes

For those primer sets not giving bands which were absent from any of the nullisomic-tetrasomic stocks, we conducted Southern blots of the amplification products hybridized with the labelled RFLP clone. For E11 and F19, little or no hybridization was observed between amplified products and the original clone. This suggests either a high degree of sequence divergence between the cloned sequence from *T. tauschii* and those in 'Chinese Spring' or that the primers did not amplify sequences homologous to the RFLP clone. Stronger hybridization occurred with RFLP clones A1, D16, D18, and E6 and their respective amplification products, suggesting that homologous sequences were amplified. However, the fact that no missing bands were observed with any of the nullisomic stocks may suggest that no polymorphisms exist between the A, B, and D genome homoeologues with the restriction enzymes we tested.

STS primers reveal polymorphisms among hexaploid wheat genotypes

We assayed 16 sets of STS PCR primers for their ability to reveal differences among a set of 20 diverse hexaploid wheat genotypes (Table 1). Of the 16 primer sets 9 gave products which generated polymorphic banding patterns upon digestion with either *Hin*fI or *Hha*I. Two examples are shown in Fig. 2. Primer set G8 (Panel 1) generated more banding patterns than any other primer set (7), while primer set E16 (Panel 2) is more typical and generated two basic banding patterns. The number of banding patterns observed among the 20 lines were 7 with G8, 5 with D18, 4 with D16, and 2 with ABG458, WG464, D8, Pst340, G36, and E16. Eight of the nine informative primer sets also differentiated among the hard red spring wheats included in this study. This is evident in Fig. 2, where hard red spring wheats 'Amidon' (lanes A), 'Hi-Line' (lanes B), 'Lew' (lanes E), 'Newana' (lanes F), and 'MT 8849' (lanes J) show variation for G8 (Panel 1) and E16 (Panel 2). In fact, the number of polymorphic bands within the hard red spring wheat group was similar to that observed between hard red spring wheats and the other wheat classes. For instance, 'Hi-Line' hard red spring wheat had an average of 6.5 polymorphic bands relative to the other hard red spring wheats, while this figure was 6.9 when 'Hi-Line' was compared to the other 15 wheat lines in our study.

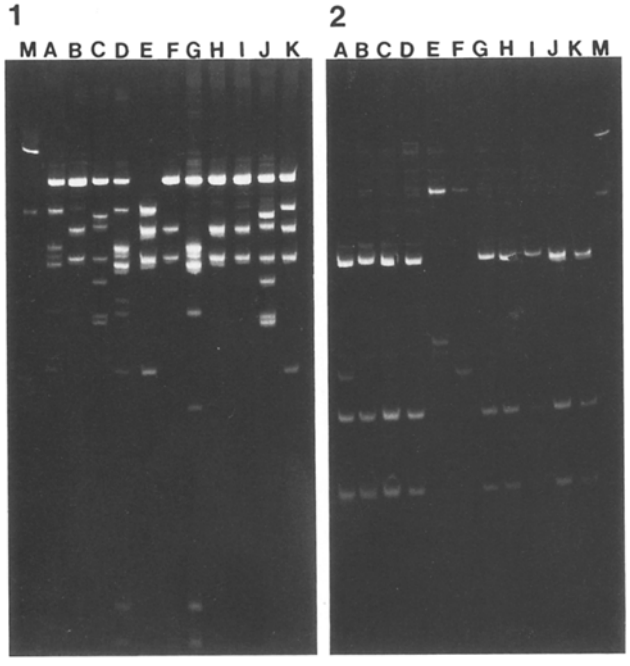

Fig. 2. Polyacrylamide gel of digested DNA amplified from hexaploid wheat lines. *Lanes M* pUC 18 digested with *Rsa*I to give fragments of 1769 bp, 676 bp, and 241bp. *A* 'Amidon', *B* 'Chinese Spring', *C* 'Hi-Line', *D* 'Judith', *E* 'Lew', *F* 'Newana', *G* 'Penawawa', *H* 'PI 352302', *I* 'CI 4528', *J* 'MT 8849', *K* 'PI 372129'. **Panel 1** Products of primer set G8 digested with *Hha*I; **panel 2** products of primer set E16 digested with *Hin*fI

Discussion

We wished to test the possibility that primers designed from mapped low-copy RFLP clones could be used as a tool for genome analysis in wheat. Possible advantages of the technique include safety and efficiency over traditional RFLP analysis and the elimination of confounding results due to repetitive DNA sequence amplification by RAPDs PCR. Additionally, once primers are developed and tested, published sequences can be easily shared with other researchers without the trouble and expense of handling and shipping recombinant RFLP clones (Olson et al. 1989).

We tested a total of 37 primer sets designed from mapped RFLP clones. Of these, 29 directed successful amplification of wheat genomic DNA and, of these 29, 23 primer sets amplified products that mapped to the expected homoeologous chromosome group. Of the 6 primer sets that could not be mapped, 4 hybridized on Southern blots to the RFLP clone from which they were designed. These data suggest that in general, primer sets designed from RFLP clones will result in effective amplification in wheat. These results are analogous to results in humans (Olson et al. 1989) and barley (Tragoonrung et al. 1992), and point to a general utility of the STS approach for genome analysis.

A second factor important for the applied use of STS PCR primers is that polymorphisms exist among wheat cultivars and lines. We tested 16 of the 29 primer sets by digesting amplified products from 20 hexaploid wheat lines with the restriction enzymes *Hha*I and

*Hin*fI. Nine of the primer sets revealed polymorphisms. One of the lines included in this study ('PI372129') is a major source of Russian wheat aphid resistance (Quick et al. 1991) and is used in many wheat backcross breeding programs. This line showed abundant polymorphisms relative to the cultivated wheats, suggesting that marker-based selection may be possible to increase the efficiency of the backcrossing programs. One unexpected result was the number of polymorphisms observed within hard red spring wheat cultivars. These five cultivars were developed either in Montana or North Dakota, all are currently scheduled for release or are already in commercial production, and all could legitimately be considered to be elite hard red spring wheat germ plasm in the Northern Great Plains. Perhaps marker-assisted selection and/or parental assessment may be informative even within germplasm groups.

A short-term disadvantage for the STS PCR primers as compared to RAPDs primers is the need for sequence analysis before primers can be designed. However, this has to be accomplished only once, after which STS primer sequences may find general utility for wheat genetic mapping and applied plant breeding studies. Empirical investigation will certainly be required to assess possible applications.

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