Russian Journal of Genetics, Vol. 33, No. 1, 1997, pp.43-49. Translated from Genetika, Vol. 33, No.1, 1997, pp.53-60.

PLANT GENETICS

Use of Polymerase Chain Reaction Products to Map the Barley *(Hordeum vulgare* L.) Genome

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Received July 19, 1995, in final form, December 29, 1995

Abstract A self-pollinating F_8 barley population obtained by crossing Odesskii and Golf varieties was used to map the loci of polymorphic PCR products. The genes coding for isozymes α-amylase I, β-amylase I, and esterases 1 and 5 served as markers with known localization. Seventy-seven arbitrary single primers and 10 pairs of primers for directed amplification were used. Polymorphism was revealed with 12 arbitrary primers and two pairs of directed ones. On the basis of 19 PCR markers and two isozymes (β-amylase and esterase 5), six linkage groups covering one-sixth of the barley genome (237 cM) were determined. Among 21 polymorphic amplification products, a single one was found to be codominantly inherited. The PCR marker (P6#900) was closely linked to gene *Bmyl* coding for β-amylase. The theoretical aspects of PCR product application to genetic analysis are discussed.

INTRODUCTION

Molecular genetic markers acquire great importance in studying genome organization and variability. The following three systems are most common: protein polymorphic markers, RFLP (Restriction Fragment Length Polymorphism), and AFLP (Amplification Fragment Length Polymorphism) [1-4].

The first two markers are well-studied and used in genome mapping and development of effective technologies for improving plants. Only a small number of loci, containing primarily structural genes, can be detected with protein markers. The methods employing RFLP markers require a radioactive (or some other) label and are labor-consuming and expensive. Despite this, great progress 4n mapping many species genomes was achieved due to RFLP markers. Employment of AFLP, a novel class of molecular markers, is highly technologically effective and provides an opportunity to simultaneously evaluate the variability of a great number of loci. The latter possibility is very important for determining the level of genetic interaction between plant species and varieties and animal breeds. AFLP markers can also be used for genome mapping [4, 5].

Location of DNA amplification products on chromosomes is an important problem for the development of theory and practice of plant breeding. A homozygote cannot be distinguished from a heterozygote with dominant PCR products; thus, to determine the linkage between the products and traits, a homozygous population should be used [1,5].

Dihaploid populations containing no heterozygotes are often used for studying the barley genome as they are convenient for mapping [6]. A self-pollinating population in a recombinant saturation state, when the number of heterozygotes is small and the ratio between recombinant and parent classes is constant, is another kind of homozygous material obtained by a cross between two barley varieties, although it is used less frequently for mapping [7, 8].

In this work, the barley *(Hordeum vulgare* L.) genome was mapped with an AFLP marker in a self-pollinating barley F_8 population obtained by crossing the varieties Odesskii 115 and Golf.

MATERIALS AND METHODS

We studied 150 lines of self-pollinating population no. 106 (F_8, Odesskii 115 x Golf) and Chinese spring wheat lines supplemented with one of the barley chromosomes (except the fifth) of the Betzes variety.

Oligonucleotides were synthesized in an Applied Biosystems 380 B automatic DNA synthesizer by the cyanophosphoramidite method. After overnight dethrilling in 30% NH_4OH at 55°C, the ten-membered oligonucleotides were reprecipitated by *2%* $LiClO_4$ in acetone. DNA fragments more than 20 nucleotides in length were purified by passing through an OPC (Oligonucleotide Purification Cartridges) column. Seventy-seven primers containing from 10 to 22 nucleotides were used in this study. Among them, those listed in the table (Table 1) were variable.

Isolation of plant DNA. An etiolated seedling from each line of population no. 106 was homogenized in 0.5 ml of lysing solution (20 mM EDTA-Na_3, 100 mM Tris-HCl, pH 8.0 (25°C), 1.4 M NaCl, 2%) and kept for 1 h at 60°C. An equal volume of a chloroform-methanol (24:1) mixture was added, and the overall mixture was thoroughly stirred. DNA was precipitated with an equal volume of isopropanol from the water phase obtained after centrifugation of the mixture for 5 min at a maximum rate in an Eppendorf 5414 table centrifuge.

1022-7954/97/3301-0043$ 18.00 :) 1997 MAMK Наука/Interperiodica Publishing

Table 1. Sequence of primers used in genetic analysis

Primer	Sequence 5'-3'	Annealing temperature of PCR, °C*	Amplification products
P2	GACAGACAGACAGACA	50.1-51.5	P2#1300
P6	GAGCAAGTTCAGCCTGG	53.7-55.1	P2#604
			P6#900
P9	ACCACAGGCAGAGTAAGAGG	54.5 -55.9	P9#1150
			P9#680
P10	CCACCCTGCTTACAGCAATG	55.5-56.9	P10#1300
			P 10#580
P22	TTATGAAAACGACGGCCAGT	55.7-57.1	P66#2320
P66	GTAAAACGACGGCCAGT	54.0-55.4	P66#2320
P39	CCAGTTCGCC	50.1-51.4	P39#2300
			P39#1188
P44	GGACCCCGCC	52.0-53.4	P44#560
P52	AGGACTGGAC	48.1-49.5	P52#1190
			P52#9I2
			P52#700
P56	CGATTTGTCC	48.7-50.1	P56#840
P57	TCAGGACGCTAC	49.9-51 .2	P57#1000
P63	CTGCCGCACTTGATACGTTGTC	56.5-57.9	P63#700
P69	AGCCTGATGCC	50.5-51.8	P69#1200
			P69#840
P82	GAGCCGTGCTGCCGGAGC	55.0**	P82/83#450
P83	GCCGTGAAGCCTGATGCC		
P92	CCACCAAGCGTGGAGTC	55.0	P92/93#594
P93	GGGTGGCGTGGGGTG		

Notes: * Optimal annealing temperature in PCR for a product from 300 to 2000 hp with 50% GC-composition.
**Annealing temperature during all amplification cycles for this pair of primers.

The pellet of DNA was dissolved in 700 µl of TE solution (10 mM Tris-HCl, pH 8.0 and I mM of EDTA-Na_3) in the presence of 1 µg/ml of RNAase A. DNA concentration was determined in a TKO 100 (Hoefer Scientific Instrument) fluorometer.

Polymerase chain reaction. The polymerase chain reaction mixture of 20 µl volume contained 50 mM KC1, 20 mM Tris-HCl, pH 8.4 (25°C), 3 mM $MgCl_2$, 0.01 % Tween-20, 0.15 mM of each dNTP, 0.2 mkM of a primer, 5% glycerol, 20 ng of DNA, and 1 U Taq polymerase. The content of the tubes was overlaid with 40 µl of mineral oil. Amplification was carried out as follows: the first four cycles, 1 min at 93°C; 1.5 min at 40-45°C (depending on the primer), and 2 min at 72°C; during the following cycles, the annealing occurred at 47-55°C. Thirty-five cycles were performed. The last elongation continued for 10 min. The reaction was stopped by the addition of 60 µl of TE solution. One-fourth of the reaction mixture was used in electrophoresis.

Electrophoresis was in 2% agarose gel in 1 x TBE (50 mM Tris-H_3BO_3 and 2 mM of EDTA, pH 8.0) lasting 5-7 h at 50 V, and the bands were visualized by ethidium bromide treatment.

To obtain a template-primer duplex when the sequences were not completely complementary, the annealing during the first four cycles was at a temperature lower than the calculated optimum. When primers of more than 14 nucleotides in length were used, the temperature was 42-45°C during these initial cycles; for primers of 10-13 nucleotides, the temperature was 40°C [9-11].

The directed polymerase chain reaction with directed primers was carried out in a similar reaction solution supplemented with 1.5 mM $MgCl_2$. The annealing temperature was 55°C during 35 cycles.

Electrophoresis and isozyme identification of a- and B-amylases and also of esterases 1 and 5 were performed according to Netsvetaev [12-14].

The following isozyme loci of known localization served as markers: *EstS* localized in the short arm of chromosome I [14]; *Estl* marking the long arm of chromosome III [6]; *Amy I* localized in chromosome VI [6]; *Bmyl* marking the long arm of chromosome IV [12].

The appropriate software was used: PCR to select optimum primers and PCR conditions and MAP_QTL

to determine the linkage between AFLP loci and loci coding for isozymes.

The recombination frequency p was estimated by Fisher's method of maximum likelihood, which follows up the overall possible sample information on recombination frequency [8]:

$$p = \frac{a+b}{2(c+d)},$$

where a and b are the numbers of recombinant genotypes; c and d are the numbers of maternal genotypes.

The error of recombination frequency *(Sp)* was determined as follows:

$$Sp = (1+2p)\sqrt{\frac{p}{2n}},$$

where n is a sample size $(a + b + c + d)$.

The χ^2_1 served as a significance criterion and was calculated from the following formula:

$$\chi^2_l = n - \frac{4(c+d)(a+b)}{n}.$$

Results with χ^2_1 values lower than seven were considered no significant. The maximum χ^2_1 value is equal to the sample size at 0% recombination.

RESULTS

Linkage analysis of DNA amplification products and enzymes demonstrated that the barley (β-amylase gene localized in the long arm of chromosome IV (4M) [12] is closely linked to the locus of the P6#900 (primer's designation # the size of the product in terms of nucleotides) product (Figs. 1 and 2). The other β-amylase gene marker, P9#680, localized at a distance of 1% recombination, was also determined. The gene *Est5*, determining the synthesis of esterase 5, was found to be linked with the chromosomal locus marked by the amplification products P92/03#594 and P9#1150, which were obtained by using a pair of primers P92 and P93 and an arbitrary primer P9 (Fig. 3). These genetic units - *EstS*, P92/93#594, and P9#1150 - are localized on the short arm of chromo-some I. The third linkage group includes five loci: P69#1050, P39#2300, P44#560, P52#912, and P52#l 190. The fourth linkage group includes P63#700, P66#2320, P10#1300, and P52#700. The fifth one includes P56#840, P69#840, P82/83#450, and P39#1188 (Fig. 4). The sixth linkage group includes two AFLP loci: P2#804 and P2#1300 amplified with P2 primer (Table 2, Fig. 5). Linkage analysis showed that the inheritance of *Estl* and *AmyI* loci is independent of any of the PCR products analyzed.

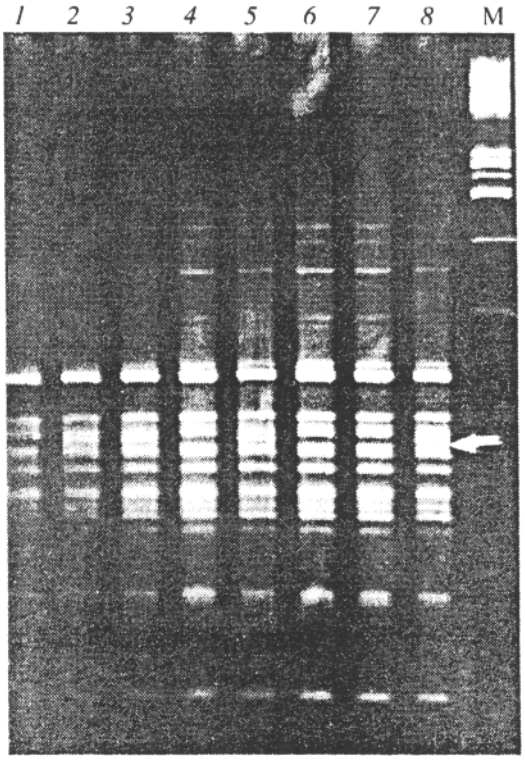

Fig. 1. Electrophoresis of DNA amplification products obtained with P6 primer on the parents (P1 and P2) and F'_8 Odesskii 115 x Golf lines. (/) P,, (2) L76, *(3) L75, (4)* L74, (5) L73. (6) L72, (7) L71, *(8)* P_2. M is T7 restricted by *Hpal* Arrow indicates the product P6#900).

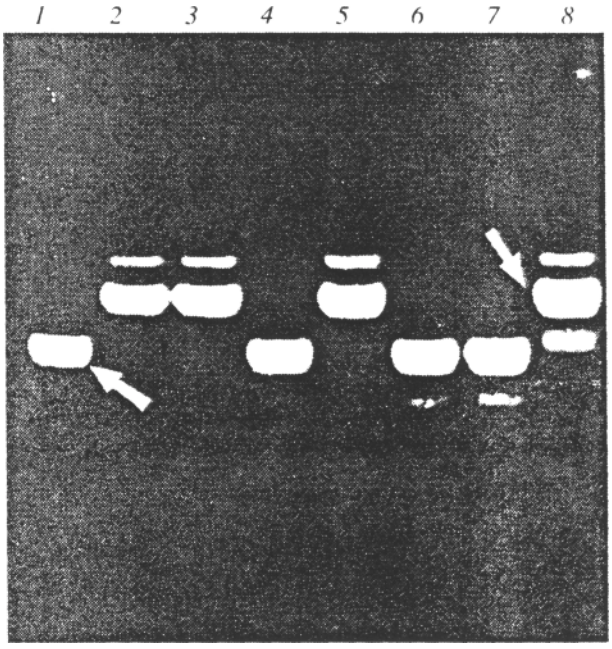

Fig. 2. PAAG-electrophoresis of (B-amylase isozymes from mature barley seeds of F_8 Odesskii x Golf line: (/) P1, (2) L76, *(3)* L75, *(4)* L74, (5) L73, (6) L72, (7) L71, *(8)* P2. Arrows indicate polymorphic markers.

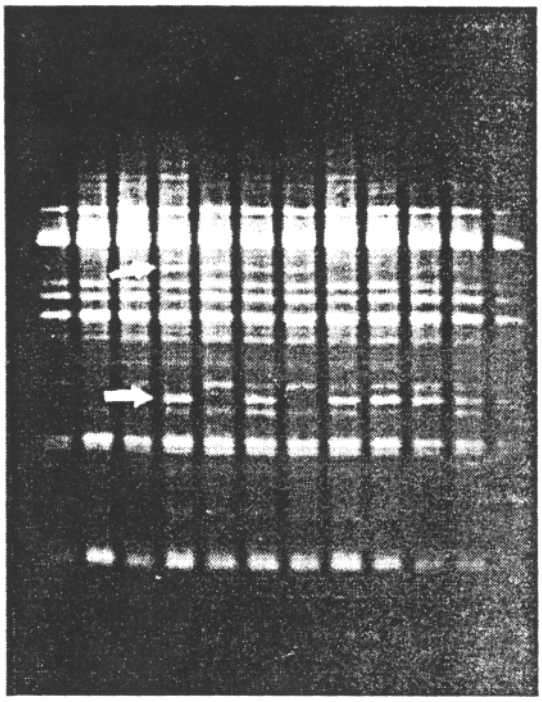

Fig. 3. Electrophoresis of DNA amplification products obtained with P9 primer in population no. 106. Arrows indicate PCR products P9#l 150 and P9#680.

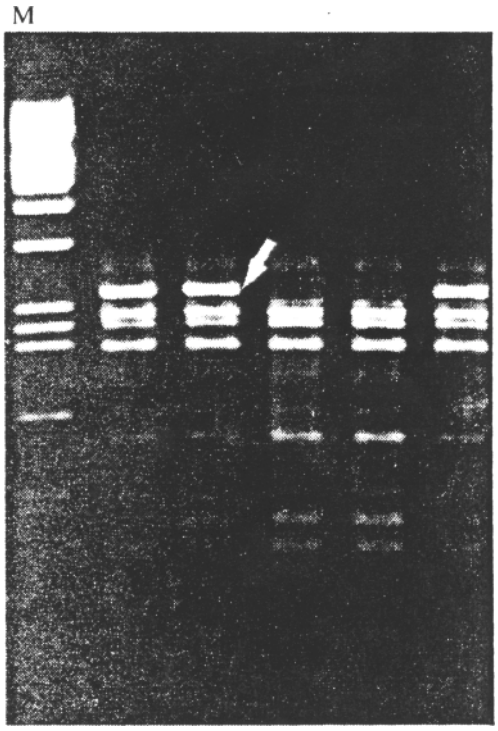

Fig. 4. Electrophoresis of DNA amplification products obtained with P56 primer in population no. 106. M is T7 restricted by *HpaI.* Arrow indicates to the P56# 1050 product.

In 12 of 77 arbitrary primers, the polymorphism of no. 106 population lines was revealed. Two of 10 directed primer pairs appeared to be polymorphic. A total of 25 polymorphic markers (4 isozymes and 21 AFLP markers) were identified in population no. 106. Among these, 21 markers formed six linkage groups covering 189% of recombination, i.e., 237 cM or one-sixth of the barley genome (the size of the barley genome is about 1300 cM [6]). Most markers are located near one another. No linkage was detected between the amplification products and the genes of a-amylase and esterase I or between two AFLP markers (P57#1000 and P10#1300).

Localization of AFLP products with the aid of wheat lines supplemented with barley chromosomes proved to be difficult. Certain polymorphic PCR products of the varieties Odesskii 115 and Golf were not found in the Betzes variety; also, some polymorphic PCR products of these varieties were present in the Chinese spring wheat genome. The P6#900 amplification product was detected in the electrophoretic DNA spectrum of the Betzes variety and localized on chromosome IV; the allele of the P57#1000 product 900 nucleotides in length was found both in wheat and the Betzes variety (Fig. 6). The P44#560 product was identified on chromosome IV, which allows the third linkage group to be assigned to this chromosome (Fig. 7).

Ten pairs of primers were used for directed amplification. Up to 20 reproducible amplification products were obtained in PCR; they can serve as molecular markers for mapping (data not shown).

Only two of these pairs (P82/P83 and P92/P93) allowed us to reveal polymorphism between the Odesskii 115 and Golf varieties. The pair of primers P82 and P83 was selected for the PTaq546 sequence of wheat genome DNA at coordinates 2356 and 3897. This DNA region contains a tandem repeat, 5'-TAAG-3', and a longer inverted repeat. Up to 30 reproducible amplification products were formed when this pair of primers was used to amplify barley and wheat genome DNA under stringent conditions (1.5mM $MgCl_2$ in the reaction mixture and an annealing temperature of 55°C). These amplification products in barley varieties were 50% polymorphic. Polymorphic products containing these primers are not allelic and are inherited independently from each other because this pair of primers flanks different chromosomal regions. Only the product P82/83#450 was polymorphic among 25 PCR products amplified with this pair of primers in the samples of population no. 106. The amplified locus of the product P82/83#450 appeared to be associated with the loci of the products P56#840 and P69#840 of the fifth linkage group.

Information about the pair of primers P92 and P93 was obtained from report [6], where it was used for

Table 2. Inheritance analysis of PCR products and associated biochemical markers in plants of the F_8 Odesskii x Golf population

Linkage group	Genetic factors *A x B*	Phenotypes	Number of families		χ^2	Recombination, %	Chromosome
			BB	*bb*			
1		AA	*6*	*26*			I
	Est5 x P92/93#594	aa	37	9	29.5	11.9 ±3.4	
		AA	12	74			
	P9#1150xP92/93#594	aa	61	1	100.6	4.8 ±1.4	
		AA	34	12			
	Es/5xP9#1150	aa	4	28	27.1	12.9 ±3.6	
2		AA	0	66			IV
	Bmyl x P6#900	aa	74	0	140.0	0.0 ± 0.2	
		AA	2	63			
	Bmyl x P6#680	aa	72	2	123.5	1.4 ±0.7	
		AA	2	63			
	P6#900 x P9#680	aa	72	2	123.4	1.4 ±0.7	
3		AA	38	26			IV
	P39#2300xP69#1050	aa	22	41	7.6	30.4 ±5.6	
	V	AA	7	58			
	P39#2300 x P44#560	aa	55	17	57.8	10.6 ±2.3	
		AA	65	26			
	P52#912xP44#560	aa	6	48	45.2	14.1 ±2.8	
		AA	25	45			
	P52#1190xP52#912	aa	68	10	41.1	15.5 ±2.9	
4		AA	21	59			Not identified
	P66S2320 x P63#700	aa	64	5	63.1	10.6 ±2.3	
		AA	54	20			
	P66#2320xP10#1300	aa	25	49	22.7	21.8±3.9	
		AA	52	14			
	P10#1300xP53#700	aa	19	44	30.8	17.2 ±3.5	
5		AA	3	88			V
	P56#840 x P69#840	aa	52	0	131.2	1.0 ±0.6	
		AA	29	61			
	P69#840xP82/83#450	aa	41	9	29.3	18.6 ±3.5	
		AA	47	17			
	P39#1188xP82/83#450	aa	15	44	28.3	17.6 ±3.6	
6		AA	47	6			
	P2#1300xP2#604	aa	16	37	36.2	13.0 ±3.1	Not identified

mapping the Morex/Steptoe barley genome. The locus of the product P92/93#580 flanked by these primers was linked to the gene coding for esterase 5.

Two arbitrary primers, P22 and P66, were identical in their first 15 nucleotides at the 3'-end (Table 1). The amplification products obtained by these primers were identical, suggesting that primers of no more than 15 nucleotides in length are sufficient to reveal polymorphism. Increasing primer size by several nucleotides causes no significant alterations.

DISCUSSION

Only unique genome structures are used for mapping. High repeats can also form such structures in the case of their unique location in the genome. Unique hybridization bands can be revealed by means of RFLP analysis even if a probe for hybridization is highly repetitive. This depends on the location of restriction sites.

Amplification with a single arbitrary primer produces a DNA fragment flanked by the inverted primer's sequence.

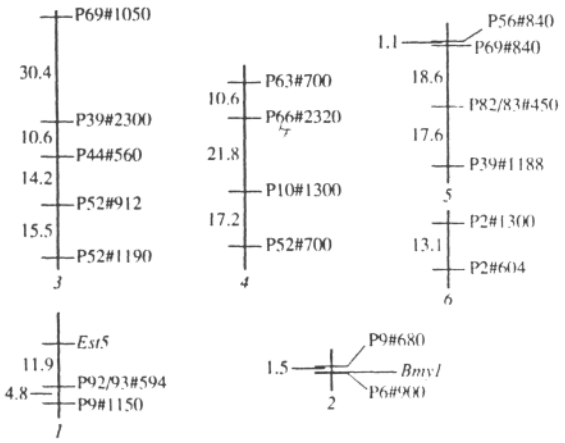

Fig. 5. Six linkage groups revealed in self-pollinating F_8 Odesskii 115 x Golf population.

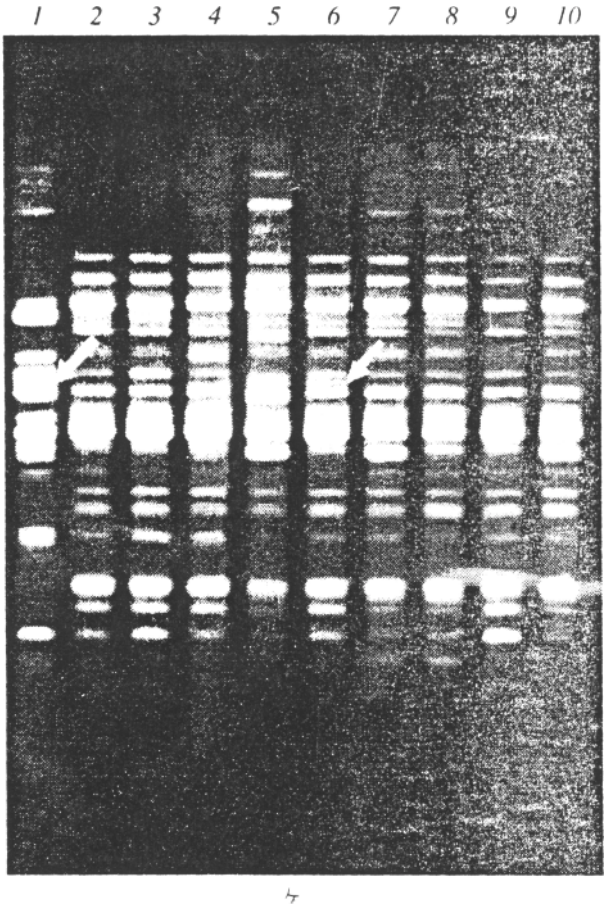

Fig. 6. Electrophoresis of amplification products obtained with P6 primer in Chinese spring wheat lines supplemented with barley chromosomes: *(1)* Betzes barley, *(2)* Chinese spring; *(3)* chromosome 1, *(4)* chromosome II, *(5)* chromosome III, *(6, 7)* chromosome IV, *(8, 9)* chromosome VI, *(10)* chromosome VII. Arrows indicate P6#900 product.

The middle region of this structure is most often composed of repeats [1], but the product as a whole is unique due primarily to its terminal sequences (i.e., the inverted sites or priming). Polymorphism of this PCR product confirms that it is unique.

AFLP marker polymorphism is usually manifested by the presence or absence of a band (product) on the electrophoregram. In this case, the product is quite probably a unique structure. The absence of PCR product is most likely a result of mutation in the region of priming. If there are repeats in the latter, then the mutations in a single or several copies do not cause the band to disappear, but do change its intensity. The probability of simultaneous mutations in all copies of this region is small. Insertion or deletion in the internal part of the amplification product will result in a change in the product size.

Codominant inheritance was observed for the fragment amplified with primer P57. Alleles of its locus had a size of 1000 and 980 nucleotides. In the remaining cases, studying genetic polymorphism of population no. 106 revealed the presence or the absence of a PCR product.

Variation in the intensity of electrophoretic bands is apparently not associated with differences in the copy number per genome but results from annealing conditions, which are optimal for the intensely amplified products but not for poorly amplified ones.

Heterozygous plants were indistinguishable from dominant homozygotes in the amount of amplified product. Amplification is usually carried out up to saturation; i.e., when the reaction reaches a plateau, the amount of the amplification product increases linearly rather than exponentially during the subsequent reaction cycles. Amplification of PCR products proceeds at a rate close to 2^n-2n, where n is the number of cycles [10]. The number of template copies in heterozygotes is twice less than in homozygotes and, respectively, the reaction attains a plateau one cycle later. Deficit in the amount of the product in heterozygotes is easily covered by additional amplification. This makes AFLP analysis in F_2 slightly less informative than other methods because heterozygotes cannot be distinguished from dominant homozygotes with the use of dominant AFLP markers. The proportion of heterozygotes in a population *(H)* is reduced by half in each following generation:

$$H=2^{1-n},$$

where n is the number of generations; in F_2, $H = 0.5$. These facts were taken into account during analysis of the almost homozygous F_8 population.

We showed previously that a self-pollinating population of the senior generations in the state of recombinant saturation could be used for mapping [7, 8]. Such a situation appears by the seventh or eighth generation. Note that in the state of recombinant saturation, the ideal self-pollinating population provides considerable

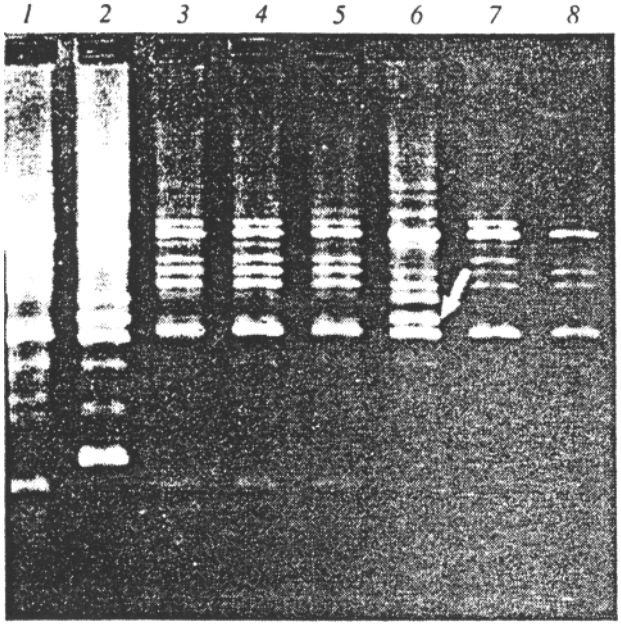

Fig. 7. Electrophoresis of amplification products obtained with P44 primer in *Chinese spring* wheat lines supplemented with barley chromosomes: (/) Betzes barley, *(2) Chinese spring, (3)* chromosome I, *(4)* chromosome II, *(5)* chromosome III, *(6)* chromosome IV; *(7)* chromosome VI; *(8)* chromosome VII. Arrow indicate P44#560 product.

information about the p value, especially if the latter is low. For example, at $p < 40\%$, the population analyzed is more informative than F_2 at the repulsion stage under complete dominance; at p<20%, it is more informative than F_2 at the attraction stage under complete dominance, and at $p < 15\%$, it is more informative than the test cross.

The locus of the product P6#900 is shown to be closely linked to the gene coding for (β-amylase. This product is possibly a structural part of the b-amylase gene. This was indirectly confirmed by the fact that the presence or absence of the P6#900 products in the samples was associated with the expression of less or more mobile isozyme forms (of greater or smaller molecular mass), respectively (Figs. 1 and 2). When comparing "fast" and "slow" β-amylase isozymes, encoded by the alleles *BmylBr* and *BmylAr,* respectively [13], the "fast" one was found to show less amylolitic activity due to the point mutation in the structural part of the isozyme, which resulted in alteration of its amino acid composition and reduction of activity [15]. The primer sequence possibly interacts with only this region of the p-amylase gene. If a mutation occurred in the region complementary to the P6 primer, the synthesis of the P6#900 product was not initiated.

ACKNOWLEDGMENTS

This work was supported by the International Science Foundation (grant no. PSU054057) and by the Foundation for Fundamental Research of the State Science and Technology Committee of Ukraine (project no. 5.3/466).

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