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Segregation distortion in homozygous lines obtained via anther culture and maize doubled haploid methods in comparison to single seed descent in wheat (*Triticum aestivum* L.)

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ABSTRACT

Background: The quality of wheat grain depends on several characteristics, among which the composition of high molecular weight glutenin subunits, encoded by *Glu-1* loci, are the most important. Application of biotechnological tools to accelerate the attainment of homozygous lines may influence the proportion of segregated genotypes. The objective was to determine, whether the selection pressure generated by the methods based on *in vitro* cultures, may cause a loss of genotypes with desirable *Glu-1* alleles.
Results: Homozygous lines were derived from six winter wheat crosses by pollination with maize (DH-MP), anther culture (DH-AC) and single seed descent (SSD) technique. Androgenetically-derived plants that originated from the same callus were examined before chromosome doubling using allele-specific and microsatellite markers. It was found that segregation distortion in SSD and DH-MP populations occurred only in one case, whereas in anther-derived lines they were observed in five out of six analyzed combinations.
Conclusions: Segregation distortion in DH-AC populations was caused by the development of more than one plant of the same genotype from one callus. This distortion was minimized if only one plant per callus was included in the population. Selection of haploid wheat plants before chromosome doubling based on allele-specific markers allows us to choose genotypes that possess desirable *Glu-1* alleles and to reduce the number of plants in the next steps of DH production. The SSD technique appeared to be the most advantageous in terms of Mendelian segregation, thus the occurrence of residual heterozygosity can be minimized by continuous selfing beyond the F_6 generation.

1. Introduction

Wheat (*Triticum aestivum* L.) is the world's most important cereal crop. Wheat grains are a valuable source of proteins, which are the major component of the human diet. The quality of wheat end-products is dependent mainly on the composition of high molecular glutenin subunits (HMW-GS), encoded by *Glu-1* loci, and storage protein content. Allelic variation at these loci has been associated with flour and dough quality [1,2,3,4]. In conventional breeding of bread wheat, genotypes possessing desirable alleles at *Glu-1* loci are usually selected at the final stages of the breeding process when pedigree lines are nearly homozygous. The effectiveness of bread genotype selection can be increased by the application of biotechnological tools for the derivation of homozygous lines and identification of *Glu-1* alleles. Allele composition at *Glu-1* loci can be identified by analysis of their protein products, *i.e.*, HMW glutenin subunits, or directly by using allele-specific PCR-based markers [5,6].

Wheat homozygous lines have conventionally been produced by pedigree selection, *i.e.*, repeated selfing of hybrids to attain successive generations in connection with the selection of desirable genotypes, or by SSD technique. These conventional methods take 6–7 generations to produce homozygous lines. The SSD method was developed by Goulden [7]. This technique is based on a random selection of one seed from all individual plant in each generation, starting from F_2 hybrids. In F_6 or more advanced generations, all seeds from individual plants are harvested and the progeny of a single plant is treated as an SSD line. Currently, SSD populations are used in genetic and genomic studies (*e.g.*, [1,8]). Since the SSD technique requires only a small area for the production of successive generations, it is frequently performed

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in a greenhouse where at least 2 generations per year are obtained [9], whereas in the field only one generation per year is feasible in the central or north European climate conditions. In SSD lines, similar to breeding lines produced by pedigree, residual heterozygosity may occur even in highly advanced generations [10].

To shorten the time required to obtain homozygosity and new cultivars, doubled haploid (DH) system has been developed [11,12,13]. DH lines are of great interest to geneticists and breeders because of their complete homozygosity and the short duration (1–2 years) of their production cycle. In wheat, DH lines can be obtained in two ways: androgenesis via anther or isolated microspore culture, and wide hybridization with maize [13,14]. The DH system has been widely used in wheat breeding programs and numerous new wheat cultivars of DH origin were released in Europe, USA, Canada, Brazil and China [11,15,16]. DH populations are frequently used in phenotypic and genetic research including construction of molecular maps, localization of loci responsible for qualitative and quantitative traits (QTLs), and in genomics and other molecular studies [8,17,18,19,20,21].

In the present study, the frequency of alleles at *Glu-1* loci in winter wheat DH and SSD populations of the same pedigree was compared with the aim to assess whether the selection pressure generated by the methods applied might cause a loss of genotypes carrying desirable *Glu-1* alleles. The DH populations covered in this work were produced via anther culture and maize pollination. Androgenetically-derived plants obtained from the same callus before chromosome doubling were examined using allele-specific and microsatellite markers to determine if the development of more than one plantlet from a single callus may cause a distortion in the segregation ratios of *Glu-1* alleles.

2. Materials and methods

The research material consisted of F_1 winter wheat hybrids (*T. aestivum* L.) of six cross combinations, designed as CC1–CC6. The hybrids were produced in 2009–2010 by crossing wheat cultivars with advanced breeding lines bred in Poland: CC1—Buteo/CHD973, CC2—DED2097/Anthus, CC3—AT23/Turkis, CC4—POB41/Titlis, CC5—STHn1/STH3195, CC6—AT12/Tonacja. The wheat genotypes used for crosses differed in two (CC1, CC2, CC3 and CC6) or three (CC4 and CC5) *Glu-1* loci encoding high-molecular weight (HMW) glutenin subunits (Table 1). The plants used in crosses were tested for their HMW glutenin subunits. Homozygous lines were obtained from F_1 hybrids in three ways: (1) androgenesis via anther culture, (2) pollination with maize and (3) the single seed descent (SSD) technique in seasons 2009–2011.

In the populations of DH and SSD lines, segregation of alleles at *Glu-1* loci was analyzed by identification of HMW glutenin subunits in grains by SDS-PAGE. Additionally, the segregation of *Glu-1* alleles in haploid plants (before chromosome doubling) was analyzed in androgenetically-derived population of the CC6 cross combination using allele-specific PCR-based markers. Plants originating from calluses, possessing the same alleles at *Glu-1* loci were also analyzed by microsatellite (SSR) markers to evaluate their similarity/diversity at other loci. Segregation ratios were compared in two sets of androgenic plants: (A) comprising all plants developed *in vitro*, and (B) where only single plant per callus was included in the population.

2.1. Anther culture

Donor plants of winter wheat were grown in the greenhouse. Tillers were detached when most microspores were at the uninucleate stage. Then they were cold-treated at 4°C for 6–9 d in mineral salt medium N_6 with 2 mg l^{-1} 2,4-D. Next, spikes were surface-sterilized with 5% calcium hypochlorite for 8 min and subsequently washed several times with sterilized distilled water. Isolated anthers were transferred into Petri dishes containing C17 liquid induction medium [22] with 90 g l^{-1} maltose (instead of sucrose) and incubated in the dark at 28°C. Embryo-like structures were transferred to a solid regeneration medium 190–2 [23] and incubated under fluorescent light at 22°C with a 16/8 h day/night photoperiod. Green haploid plants were treated with colchicine solution (0.1%) to induce chromosome doubling.

2.2. Wheat × maize pollination

The standard method was applied as described by Laurie and Bennett [14]. Spikes of wheat were manually emasculated and pollinated with fresh pollen of maize. Maize cultivar Waza was the pollen donor. Pollinated spikes were treated with 2,4-dichlorophenoxyacetic acid (2,4-D). Immature embryos were dissected from seeds 15–18 d after pollination and cultured *in vitro* on B5 medium [24] in tubes. Haploid plants were vernalized for 8 weeks and then treated with colchicine solutions for chromosome doubling.

2.3. SSD technique

In F_2 populations, 120–170 plants from each cross combination were randomly selected to develop SSD lines. The assumption was to obtain *ca.* 100 lines for cross combinations with segregation at two *Glu-1* loci and 150 for cross combinations with segregation at three loci (20 plants were treated as a reserve). In accordance with the SSD technique, each individual was represented by one seed in the next generation. To accelerate the attainment of successive generations, *in vitro* culture of immature embryos was used. One spike from each plant was detached 15–20 d after flowering and single seed was dissected for *in vitro* culture of embryo. Embryos were cultured on B5 medium [24] in boxes (50 embryos per box). After 3–4 d of culture, boxes with developing plantlets were placed into a vernalization chamber for 8 weeks and then transferred *ex vitro* into pots in the greenhouse. This approach allowed us to produce at least 2 generations per year. In the F_6 generation, all seeds from each plant were harvested and accounted for the SSD line.

2.4. Identification of HMW glutenin subunits in grains

The HMW-GS composition was determined in grains of each line by SDS-PAGE using 11.5% (w/v) polyacrylamide gel. The electrophoretic

Table 1
HMW-glutenin subunits in parental genotypes (P_1 and P_2) of analyzed wheat cross combinations.

Cross combination	HMW glutenin subunit		Allele					
	P_1	P_2	*Glu-A1*		*Glu-B1*		*Glu-D1*	
			P_1	P_2	P_1	P_2	P_1	P_2
CC1	n/6 + 8/5 + 10	1/7 + 9/5 + 10	*c*	*a*	*d*	*c*	*d*	*d*
CC2	n/6 + 8/5 + 10	n/7 + 9/2 + 12	*c*	*c*	*d*	*c*	*d*	*a*
CC3	2*/7 + 9/2 + 12	n/7 + 9/5 + 10	*b*	*c*	*c*	*c*	*a*	*d*
CC4	n/7 + 8/5 + 10	1/7 + 9/2 + 12	*c*	*a*	*b*	*c*	*d*	*a*
CC5	n/6 + 8/3 + 12	1/17 + 18/5 + 10	*c*	*a*	*d*	*i*	*b*	*d*
CC6	n/7 + 9/2 + 12	2*/7 + 8/2 + 12	*c*	*b*	*c*	*b*	*a*	*a*

procedure and designation of the HMW glutenin subunits were performed according to Tohver [25].

2.5. Identification of alleles in Glu-1 loci in androgenetically-derived plants

Leaves of androgenetical plants developed from individual calluses were subjected to AS-PCR marker analyses to identify allelic composition at *Glu-1* loci. For the identification of allelic composition at the *Glu-A1* locus AS1 and AS2 primer sets were used, identification of *Glu-B1* alleles was carried out with AS4, AS6 and AS8 primers, and AS9 and AS10 primers for identification of alleles at *Glu-D1* locus. Primer sequences used in the analysis are listed in Table 2. The amplification reaction was 25 μl and it contained 1× PCR buffer (Qiagen), 2 mM $MgCl_2$, 300 μg of each dNTP, 0.2 μg of each primer, 50 ng genomic DNA and 0.5 U HotStar Taq DNA polymerase (Qiagen). Amplification was performed in an Applied Biosystems thermal cycler. PCR conditions were specific for each primer. PCR products were analyzed on 1.5% (w/v) agarose gels, stained with ethidium bromide, and detected using UV light.

2.6. Microsatellite marker analysis

In CC6 cross combination, plants derived from calluses, which appeared to be identical at *Glu-1* loci, were characterized in more detail by the use of microsatellite (SSR) markers. Eighty microsatellite markers, selected earlier as polymorphic for this combination, were used for analysis.

Genomic DNA was extracted from leaves of haploid plants using Promega Kit. Extracts were diluted to 50 ng ml^{-1} and stored at -20°C. PCR reactions were performed in 25 μl volumes containing 250 nM of each primer, 0.2 mM of each of dNTP, 1× PCR buffer, 1.5 mM of $MgCl_2$, 1.0 unit of Taq DNA polymerase, and 50 ng of genomic DNA. Samples were denaturated at 94°C, and submitted to 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 55–60°C (depending on Tm of primers), and 2 min elongation at 72°C, with a final extension of 10 min at 72°C. For PCR amplifications, the Applied Biosystem thermal cycler was used. Microsatellite alleles were detected on an Applied Biosystems 3130 Genetic Analyzer.

2.7. Statistical calculations

The chi-square test was applied to examine the difference between the expected and observed number of lines with particular allele compositions at *Glu-1* loci, considering all segregating loci together and each locus individually in particular cross combinations. In addition, the chi-square test was used to study the regularity of segregation in three types of populations in all cross combinations.

Table 2
Allele-specific markers used for identification of alleles in *Glu-1* loci in winter wheat.

Primer	HMW glutenin subunit	Primer sequence	Reference
AS1	AxNull	F: ACGTTCCCCTACAGGTACTA R: TATCACTGGCTAGCCGACAA	Lafiandra et al. [37]
AS2	Ax2*	F: CCGATTTTGTTCTTCTCACAC R: CACCAAGCGAGCTGCAGAT	De Bustos et al. [38]
AS4	Bx7	F: ATGGCTAAGCGCCTGGTCCT R: TGCCTGGTCGACAATGCGTCGCTG	Ahmad [39]
AS6	By8	F: TTAGCGCTAAGTGCCGTCT R: TTGTCCTATTTGCTGCCCTT	Lei et al. [40]
AS8	By20, By8, By8*,By18 By9	F: TTCTCTGCATCAGTCAGGA R: AGAGAAGCTGTGTAATGCC	Lei et al. [40]
AS9	Dx5	F: GCCTAGCAACCTTCACAATC R: GAAACCTGCTGCGGACAAG	D'Ovidio, Anderson [41]
AS10	Dy10 Dy12	F: GTTGGCCGGTCGGCTGCCATG R: TGGAGAAGTTGGATAGTACC	D'Ovidio, Anderson [41]

3. Results

The composition of alleles encoding the HMW glutenin subunits in parental genotypes used for crossings is presented in Table 1. In the crosses CC1, CC2, CC3 and CC6, parental cultivars differed in two *Glu-1* loci, and in CC4 and CC5 in three loci. HMW-GS alleles present in the populations of DH and SSD lines are given in Table 3. In all three population types, classes of genotypes with segregated HMW allele compositions should be in equal proportions: 1:1 for segregation in one locus, 1:1:1:1 and 1:1:1:1:1:1:1:1 for 2 and 3 loci, respectively.

In the populations of DH lines, derived by interspecific hybridization with maize, significant distortion was found only in one out of six crosses, *i.e.*, CC3, in which the number of lines with the *Glu-A1c* allele (regardless of the alleles at *B1* and *D1* loci) was two times greater than the number of lines with the *A1b* allele. In the remaining crosses, the frequency of lines with particular allele compositions was in equal proportion, *i.e.*, 1:1.

In populations of doubled haploids obtained by androgenesis, the observed and expected number of lines with a given composition of HMW-GS alleles was similar only in one cross (CC1), whereas in the remaining crosses segregation was significantly distorted. The largest disturbance was recorded in CC4 where no line with a *Glu-A1c* allele (*A1c*/*B1b*/*D1d*, *A1c*/*B1c*/*D1d*, *A1c*/*B1b*/*Da*, *A1c*/*B1c*/*D1a*) was found. Moreover, almost 50% of the lines obtained in that cross were DHs carrying *A1a*/*B1c*/*D1a* alleles. In anther-derived CC2 populations, distortion in the frequency of particular classes of genotypes was caused by a strong deviation of the *D1a*:*D1d* segregation ratio, whereas the frequency of alleles in the *Glu-B1* locus did not differ significantly from the Mendelian 1:1 ratio.

It can be seen that in three crosses (CC2, CC3, CC4), lines with genotypes *A1c*/*B1c*/*D1a* had the lowest frequency (Table 3).

Populations of lines derived from F_2 hybrids by the single seed descent technique were characterized by a similar frequency of *Glu-1* alleles. In all crosses, the chi-square test showed no significant differences between the observed and expected numbers of genotypes with particular allele compositions at *Glu-1* loci. Lines that were not fully homozygous were identified in all crosses. The percentages of heterozygous lines ranged from 3.06 in CC3 to 10.34 in CC5, an average of 6.53% of SSD lines appeared to be heterozygous at least in one of *Glu-1* loci (Table 3).

Segregation of alleles at individual *Glu-1* loci, examined for all 6 cross combinations collectively is presented in Table 4. Results of the chi-square test for populations derived by maize pollination revealed significant (at $P = 0.01$) distortion only in the segregation ratio (1:1) of *A1b* and *A1c* alleles. Segregation ratios in AC populations were distorted in five out of seven examined pairs of alleles, and only the *B1c*:*B1d* and *D1a*:*D1d* ratios did not deviate from Mendelian segregation. Segregation of *Glu-1* alleles in SSD populations appeared to be in equal proportion as no significant distortion was found.

3.1. Analyses of androgenetical plants derived from calluses

Alleles at *Glu-1* loci of androgenetical plants derived from CC6 F_1 hybrids were analyzed before chromosome doubling with the use of allele-specific markers AS1, AS2, AS4, AS6, AS8, AS9 and AS10. This approach allows the determination of the *Glu-1* allele composition in green plants developed from individual calluses, which may have been lost during colchicine treatment (Table 5). The AS1 primer amplified 920 bp band in four haploids derived from the calluses CC6/2 and CC6/5, indicating the presence of an *A1c* (AxNull) allele. The AS2 primer gave a PCR amplification product of 2652 bp for all the remaining haploids, which demonstrated the occurrence of an *A1b* (Ax2* subunit) allele. The AS4 primer amplified 2373 bp bands

Table 3
Segregation of *Glu-1* alleles in wheat DH and SSD populations.

Cross combination	*Glu-1* genotype	DH lines		SSD lines	
		Maize pollination	Anther culture	Homozygous	Heterozygous (loci)
		no.			
CC1	*A1a/B1d/D1d*	18	14	23	5 (*B1d/B1c*)
	A1a/B1c/D1d	16	17	27	
	A1c/B1d/D1d	11	11	28	
	A1c/B1c/D1c	13	9	20	
Total		58	51	98	5 (4.85%)
χ^2 (1:1:1:1)		2.00	6.02	1.67	
–A1a/B1d:A1a/B1c:A1c/B1c:A1c/B1d					
χ^2 (1:1)		1.72	2.37	0.04	
–A1a:A1c					
χ^2 (1:1)		0.00	0.02	0.16	
–B1c:B1d					
CC2	*A1c/B1d/D1d*	21	22	23	9 (*B1d/B1c*)
	A1c/B1c/D1d	19	25	26	
	A1c/B1d/D1a	18	15	21	
	A1c/B1c/D1a	17	3	24	
Total		75	65	94	9 (8.74%)
χ^2 (1:1:1:1)		0.47	17.65**	0.55	
–B1c/D1a:B1c/D1d:B1d/D1a:B1d/D1a					
χ^2 (1:1)		0.12	1.25	0.38	
–B1c:B1d					
χ^2 (1:1)		0.33	12.98**	0.17	
–D1a:D1d					
CC3	*A1b/B1c/D1d*	10	10	23	3 (*D1a/D1d*)
	A1b/B1c/D1a	10	46	27	
	A1c/B1c/D1d	23	34	21	
	A1c/B1c/D1a	17	0	24	
Total		60	90	95	3 (3.06%)
χ^2 (1:1:1:1)		7.87*	59.87**	0.79	
–A1b/D1a:A1b/D1d:A1c/D1a:A1c/D1d					
χ^2 (1:1)		6.67**	5.38*	0.26	
–A1b:A1c					
χ^2 (1:1)		0.60	0.04	0.51	
–D1a:D1d					
CC4	*A1a/B1b/D1d*	19	10	18	5 (*B1b/B1c*)
	A1a/B1c/D1d	17	22	19	
	A1a/B1b/D1a	17	11	17	
	A1a/B1c/D1a	19	41	19	
	A1c/B1b/D1d	19	0	16	
	A1c/B1c/D1d	16	0	21	
	A1c/B1b/D1a	18	0	17	
	A1c/B1c/D1a	16	0	16	
Total		141	84	143	5 (3.38%)
χ^2 (1:1:1:1:1:1:1:1)		0.67	143.24**	13.70	
–A1a/B1b/D1d:A1a/B1c/D1d:A1a/B1b/D1a:A1a/B1c/ D1a:A1c/B1b/D1d:A1c/B1c/D1d:A1c/B1b/D1a:A1c/B1c/D1a					
χ^2 (1:1)		0.06	84.00**	0.06	
–A1a:A1c					
χ^2 (1:1)		0.18	21.00**	0.34	
–B1b:B1c					
χ^2 (1:1)		0.01	4.76*	0.17	
–D1a:D1d					
CC5	*A1a/B1i/D1b*	15	39	22	6 (*B1d/B1i*)
	A1a/B1i/D1d	16	4	15	4 (*D1b/D1d*)
	A1a/B1d/D1d	10	14	15	5 (*B1d/B1i*)/(*D1b/D1d*)
	A1a/B1d/D1b	17	13	13	
	A1c/B1i/D1d	8	22	16	
	A1c/B1i/D1b	6	24	17	
	A1c/B1d/D1d	14	18	16	
	A1c/B1d/D1b	14	18	16	
Total		100	152	130	15 (10.34%)
χ^2 (1:1:1:1:1:1:1:1)		14.07	38.00**	11.68	
–A1a/B1i/D1b:A1a/B1i/D1d:A1a/B1d/D1d:A1a/B1d/ D1b:A1c/B1i/D1d:A1c/B1i/D1b:A1c/Bid/D1d:A1c/B1d/D1b					
χ^2 (1:1)		2.56	0.95	0.00	
–A1a:A1c					
χ^2 (1:1)		0.82	4.45*	0.77	
–B1d:B1i					
χ^2 (1:1)		0.16	8.53**	0.28	
–D1b:D1d					
CC6	*A1b/B1c/D1a*	13	33	19	2 (*A1b/A1c*)
	A1b/B1b/D1a	17	15	26	7 (*B1b/B1c*)
	A1c/B1c/D1a	20	12	28	
	A1c/B1b/D1a	14	17	25	

(continued on next page)

Table 3 (*continued*)

Cross combination	*Glu-1* genotype	DH lines		SSD lines	
		Maize pollination	Anther culture	Homozygous	Heterozygous (loci)
		no.			
Total		64	77	98	9 (8.41%)
χ^2 (1:1:1:1) *–A1b/B1b:A1b/B1c:A1c/B1b:A1c/B1c*		1.88	13.75**	1.84	
χ^2 (1:1) *–A1b:A1c*		0.25	4.86*	0.65	
χ^2 (1:1) *–B1b:B1c*		0.006	2.19	0.16	
Total numbers of lines		498	519	658	46 (6.53%)

* $P < 0.05$.
** $P < 0.01$.

in all analyzed plants, which indicated the presence of Bx7 subunit. Primer AS6 gave a product of 527 bp for three plants derived from the CC6/1 callus, which identified the By8 subunit, while no product was detected in other plants. AS8 amplified the 662 bp fragment, specific for By9 subunit, in all the remaining haploid plants. Thus, the examined haploids had Bx7 + By8 (CC6/1) and Bx7 + By9 (CC6/2–CC6/6) HMW glutenin subunits. Parental genotypes of the CC6 cross combinations did not differ at the *Glu-D1* locus as both had Dx2 + Dy12 subunits, confirmed by allele-specific markers. AS9 primer did not amplify any DNA product, indicating a subunit other than Dx5 (*e.g.* Dx2, Dx3, Dx4). In all samples, AS10 primer gave 612 bp fragments, corresponding to Dy12 subunit in all plants examined. Due to the fact that the parental genotypes possessed Dx2 subunit, this indicated the presence of Dx2 + Dy12 subunits in all haploid plants.

Since parental genotypes of the CC6 cross combination differed at *Glu-A1* and *Glu-B1* loci, four classes of genotypes were expected to occur in equal proportions in this haploid population. As shown in Table 6, the number of plants with particular allele compositions appeared to be different and the differences between the expected and observed numbers were statistically significant ($X^2 > X^2_{0.05}$) (CC6/A in Table 6). In the next step, only one plant per callus was included in the population (CC6/B in Table 6). This approach resulted in changes in the proportion of individual genotypic classes, so that it was closer to the expected ratio of 1:1:1:1, which was confirmed by non-significant results of X^2 test.

Results of the application of allele-specific PCR markers to identify alleles at *Glu-1* loci showed that among six sets of androgenetical plants developed from individual calluses, only haploids derived from callus CC6/5 possessed a different allele composition at *Glu-1* loci (Table 5).

Anther-derived haploid plants listed in Table 5 were also analyzed using microsatellite markers. Among six analyzed sets of androgenetical plants, in four of them all plants developed from single callus gave the same SSR products. In the CC6/1 set, two plants had the same products for all primers and in one plant, products of different length were found for six out of 18 primers (Table 7). In CC6/5 set, two pairs of plants differed in *Glu-1* allele composition, but each pair appeared to have all SSR markers of the same size. These pairs of haploids varied in 13 out of 18 SSR loci. Fig. 1 presents microsatellite products amplified by gwm102 and cfd43 primer pairs in four plants developed from callus CC6/6.

4. Discussion

Various biotechnological tools have been designed to accelerate the development of homozygous breeding lines and shorten the development of new wheat cultivars. In the present studies, DH and SSD populations of the same pedigree, comprising a total of 1721 lines, were compared in the frequency of segregants at *Glu*-1 loci encoding HMW glutenin subunits that determine bread-making quality in wheat. Analysis of HMW glutenin subunits in DH lines revealed that the frequency of *Glu-1* alleles was significantly distorted in androgenetically-derived populations when compared with populations produced by maize pollination and the SSD technique. This distortion was manifested not only in significantly different number of particular segregants within cross combinations, which are expected to be similar in populations of randomly-derived homozygous lines, but also in the complete absence of certain genotypes. It was additionally confirmed when segregation in individual loci was considered in all cross combinations. This segregation distortion was not a product of small sample size as, *e.g.*, distortion in the *A1a*:*A1c* ratio found in AC populations, was based on the analysis of 287 DH lines.

Segregation distortion is defined as deviation from Mendelian segregation. It is a phenomenon that has been observed in many plant populations (*e.g.*, [26,27,28,29]). Segregation distortion of molecular markers has been observed in the populations of homozygous lines produced via androgenesis, among others, by Xu et al. [30] in rice, Devaux et al. [31] in barley, and Guzy-Wróbelska and Szarejko [27]

Table 4
Chi-square test for 1:1 segregation ratio of alleles in *Glu-1* loci in populations of winter wheat lines produced by maize pollination (MP), anther culture (AC) and SSD technique (over 6 cross combinations).

Glu-1 allele ratio	DH-MP		DH-AC		SSD	
	No. of lines	χ^2 (1:1)	No. of lines	χ^2 (1:1)	No. of lines	χ^2 (1:1)
A1a:*A1c*	299	0.27	287	24.00**	359	0.80
A1b:*A1c*	124	4.64*	141	9,71**	193	0.05
B1d:*B1c*	133	0.07	116	0.55	192	0.02
B1b:*B1c*	205	1.10	135	19.27**	241	0.04
B1d:*B1i*	100	1.00	152	4.45*	130	0.77
D1a:*D1d*	276	0.36	239	0.71	332	0.19
D1b:*D1d*	100	0.16	152	8.52**	130	0.28

* $P < 0.05$.
** $P < 0.01$.

Table 5
HMW-GS allele composition in wheat androgenetic plants derived from individual calluses in CC6 cross combination.

Callus no.	No of plants	Allele composition in *Glu-1* loci	HMW glutenin subunits
CC6/1	3	*A1b/B1b/D1a*	2*/7 + 8/2 + 12
CC6/2	2	*A1c/B1c/D1a*	n/7 + 9/2 + 12
CC6/3	6	*A1b/B1c/D1a*	2*/7 + 9/2 + 12
CC6/4	2	*A1b/B1c/D1a*	2*/7 + 9/2 + 12
CC6/5	2	*A1c/B1c/D1a*	n/7 + 9/2 + 12
	2	*A1b/B1c/D1a*	2*/7 + 9/2 + 12
CC6/6	7	*A1b/B1c/D1a*	2*/7 + 9/2 + 12

Table 6
Frequency of genotype classes in populations of wheat androgenetically-derived plants in CC6 cross combination.

Cross combination	Genotype class	CC6/A[a]	CC6/B[2]
		No.	
CC6	*A1b/B1c/D1a*	33	20
	A1b/B1b/D1a	15	13
	A1c/B1c/D1a	12	10
	A1c/B1b/D1a	17	17
Total		77	60
X^2 (1:1:1:1)—*A1b/B1b*: *A1b/B1c*:*A1c/B1b*:*A1c/B1c*		13.75**	3.87
X^2 (1:1)—*A1b*:*A1c*		4.86*	0.60
X^2 (1:1)—*B1b*:*B1c*		2.19	0.00

[a] Population consisting of all androgenetic plants; [2] population covering only one plant per callus.
* $P < 0.05$.
** $P < 0.01$.

in wheat. Deviation from the expected number of segregants in androgenetical populations may be a result of various pre- and post-zygotic causes, of both genetic and/or *in vitro* culture origin [26,30]. In our study, in the CC6 cross combination, the number of DH lines with *Glu-1A1b/B1c/D1a* allele composition significantly outnumbered other genotypes and when only one plant per callus was left in the population, the distortion was eliminated. This confirms that one of the reasons for the segregation distortion in androgenetically-derived populations may be the development of several meristematic centers within a callus, stemming from one microspore, and from which several identical plantlets can grow. In anther culture, the development of more than one plantlet from single microspore in a callus may be the result of growth hormone application, *e.g.*, 2,4-D. SSR marker analysis in plants originating from the same callus confirmed their identity. There were only two cases (CC6/1 and CC6/5) where one or two haploid plants appeared to have different SSR alleles. This might suggest that two calluses developed from two microspores in close proximity, and thus they were difficult to distinguish during the course of subsequent steps of *in vitro* culture.

In the present study, in the CC4 cross combination, the frequency of androgenetical DH lines carrying *Glu-1* alleles from parent P_2 (cv. Titlis) exceeded the number of DH lines with alleles from parent P_1 (POB41) (48.8% and 0%, respectively), whereas SSD and DH lines obtained by maize pollination segregated regularly according to the Mendelian 1:1 distribution. This suggests that the observed segregation distortion is connected with *in vitro* anther culture. It was found that in androgenetical DH populations preferential transmission of alleles can occur from one parent that is highly responsive to anther culture [26,32]. In our experiments, both parents of CC4 hybrids showed a similar response to anther culture (data not shown). Predominance of lines with the HMW-GS allele composition derived from cv. Titlis could instead be attributed to the process of chromosome doubling. The ability of haploid plants to survive the colchicine treatment may result in segregation distortion in the doubled haploid population, as suggested by Devaux [33]. On the other hand, the zero-frequency of four classes of DH genotypes in that cross combination, all carrying *A1c* allele, could be caused by the high frequency of albino plantlets observed in that population. Their alleles coding HMW glutenin subunits were not analyzed, but it cannot be excluded that some of albino plants could be segregants possessing the lacking alleles. Furthermore, the observed distortion could have been the result of the insufficient number of androgenetical lines studied in this population.

In DH populations produced via maize pollination, significant differences (at $P = 0.05$) between the expected and observed number of particular classes of genotypes were recorded only in CC3. This was caused mainly by the deviation of the *A1b*:*A1c* ratio from the expected Mendelian 1:1 ratio. Segregation distortion in DH populations obtained by wide-hybridization has been observed, *e.g.*, by Devaux [34] in DH populations of barley acquired through the *Hordeum bulbosum* technique, and Guzy-Wróbelska and Szarejko [27] in wheat DHs derived by maize pollination. Comparison of the androgenesis and wide-hybridization methods for the development of DH lines in wheat and barley has revealed that the frequency of segregation distortion is markedly higher in populations obtained through androgenesis, both

Table 7
SSR marker products amplified in DNA samples of haploid plants developed from two calluses (no. 1 and 5) in anther culture of CC6 cross combination.

SSR primer	PCR product (bp)								
	P_1	P_2	CC6/1			CC6/5			
	(TA12)	(Tonacja)	1	2	3	1	2	3	4
gwm102	137	141	137	137	141	141	137	141	137
cfd43	161	165	161	161	165	165	161	165	161
gwm515	131	128	128	128	128	131	128	131	128
wmc177	184	186	184	184	184	184	186	184	186
cfd51	155	185	185	185	155	155	185	155	185
cfd36	191	196	191	191	191	196	191	196	191
wmc790	194	154	194	194	194	154	194	154	194
gdm33	146	154	146	146	154	146	146	146	146
cfd92	252	254	254	254	254	252	254	252	254
wmc522	198	186	186	186	186	198	186	198	186
cfd56	259	248	259	259	259	259	248	259	248
gwm234	237	233	237	237	237	237	233	237	233
barc75	105	108	108	108	105	108	108	108	108
cfd189	279	281	279	279	279	279	279	279	279
gwm296	164	162	162	162	162	164	162	164	162
gwm469	170	166	166	166	166	166	166	166	166
barc124	247	249	249	249	249	247	249	247	249
cfd65	193	191	193	193	191	193	193	193	193

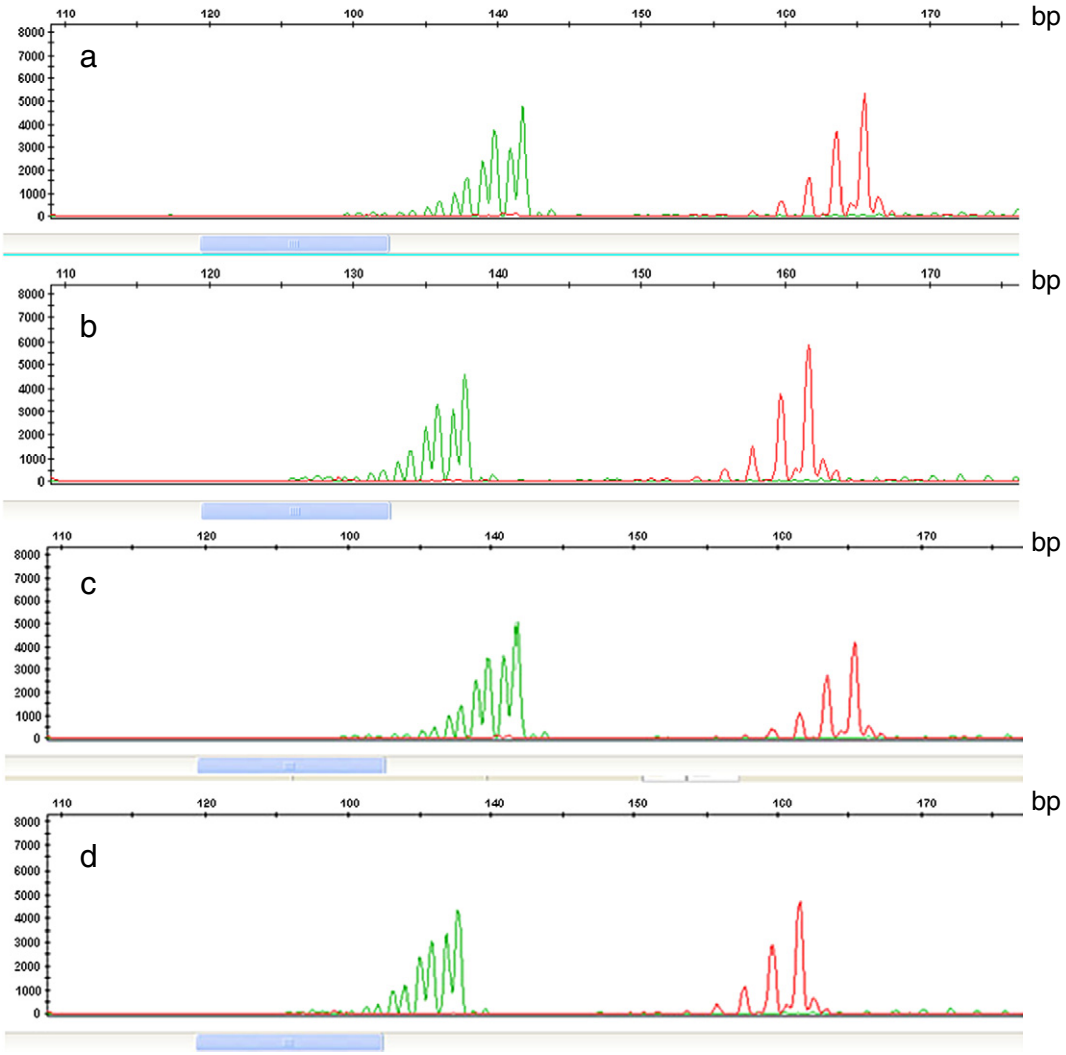

Fig. 1. Microsatellite products amplified by markers gwm 102 (green)—137/141 bp and cfd43 (red)—161/165 bp for wheat androgenic DH lines derived from calluses: (a) CC6/5/1; (b) CC6/5/2; (c) CC6/5/3; (d) CC6/5/4 (see Table 7).

via anther and microspore cultures, than in those obtained by interspecific hybridization [12,26,27].

Populations of DH lines can be applied in genetic research when segregation of markers/genes is not distorted. Therefore, statistical methods for QTL mapping have been developed for populations, in which markers segregate in a Mendelian fashion. Distortion of the frequency of markers/genes is also important from the breeding point of view, because genotypes with desirable alleles can be lost. In naturally existing plant populations, segregation distortion is under genetic control of segregation distortion loci (*SDL*), causing deviation from Mendelian segregation ratios. They affect competition among gametes, which leads to the preferential fertilization and abortion of male or female gametes. Distortion of segregation ratios may occur both in early generations and in the populations of homozygous lines produced in a natural way, *i.e.*, by self-pollination of successive generations [26,35]. In the present work, no segregation distortions were observed in SSD populations, which may be considered as the most similar to populations obtained in a natural way. This result confirms that the observed segregation distortion in anther-derived DH populations was not affected by *SDL* loci, but was the result of anther culture and/or chromosome doubling.

In the present studies, the SSD technique combined with *in vitro* culture of immature embryos allowed for the attainment of winter wheat homozygous lines (F_6) in a relatively short time (3 years). The main advantage of such an approach is the ability to obtain populations with segregation ratios close to the expected Mendelian distribution. This is important for the construction of genetic maps and for identification of chromosomal locations containing quantitative trait loci (QTL) or genes responsible for the traits of interest. In contrast, the main disadvantage of the SSD technique is potential occurrence of residual heterozygosity. In the current study, a total of 6.53% of SSD(F_6) lines were heterozygous at one or two *Glu-1* loci, which appeared to be close to that calculated according to the Michaelis formula [36] for 3 segregating loci in the F_6 generation (6.15%). It should be noted that this result was obtained on the basis of all 658 analyzed SSD lines; in particular cross combinations, where the number of lines was relatively low (94 to 143), the percentage of heterozygous lines ranged from 3.06 to 10.34. This indicates that in genetic studies where complete homozygous lines are required, the SSD technique should be continued to more advanced generations.

5. Concluding remarks

A comparison of three types of wheat populations, *i.e.*, produced by wide hybridization, anther culture and the SSD technique, shows that DH lines obtained via maize pollination are the most useful both for research studies and for the breeding of new wheat cultivars. The probability of segregation distortion and loss of desirable genotypes

in these populations is markedly lower than in anther-derived populations. Segregation distortion in androgenetically derived population can be minimized if only one plant per callus is included in the population. Due to the high probability that segregation distortion will occur in AC population, significantly higher than the planned number of lines should be produced. Selection of wheat haploid plants before chromosome doubling based on allele-specific markers allows for the selection of genotypes carrying desirable *Glu-1* alleles and a reduction in the number of plants devoted to the next steps of DH production. The technique of single seed descent is most advantageous in terms of Mendelian segregation, thus the presence of residual heterozygosity can be minimalized by continuous selfing beyond the F_6 generation, which can also reduce the occurrence of unfavorable linkage disequilibrium.

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