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Segregation distortion at marker loci: variation during microspore embryogenesis in maize

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Abstract In the present study, we analyzed the segregation distortions of markers during in vitro androgenesis in maize. This was based on four segregating populations derived from the A188×DH7 one-way-cross. These populations consisted of very young androgenetic embryos, well-developed calluses, haploid regenerated plantlets and spontaneous diploid plantlets. These structures all represented different developmental stages, from that of microspores to the regenerated plantlets. This study complemented a previous one by Murigneux et al. 1994, where distorted segregations of RFLP markers were detected in a single-seed-descent population and in a doubled-haploid population derived from the same cross. The weakly biased SSD maize genetic map was used as a reference to locate 145 AFLP loci whose allelic segregations were also analyzed in the androgenetic segregating populations. Segregation distortions were determined based on chi-square analysis ($P<0.01$ and $P<0.001$). Regions on chromosomes 2 and 8 showed distortions from the beginning of embryo formation, with large effects throughout the process. Regions on chromosomes 3, 4, 6 and 10 could control callus formation from microspores. Other deviations of marker genotypes on chromosomes 1, 4, 6 and 10 could be associated with the regeneration phase. Moreover, the statistical method of Cheng et al. for mapping a lethal factor locus inside segments of linked distorted markers was used to estimate the position of seven partial lethal androgenetic factors on chromosomes 1, 2, 8 and 10. These factors could represent selective genes actively involved in maize androgenesis.

Keywords Microspore embryogenesis · Regeneration · AFLP · Maize · Segregation distortion · Partial lethal androgenetic factor

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Introduction

The development of permanent mapping populations including recombinant inbred lines (RIL) or doubled-haploid lines (DH) is important for plant breeding and the genetic dissection of agronomic traits (Snape et al. 1984; Burr and Burr 1991). The main advantages of DH over single seed descent (SSD) are a quicker recovery of complete homozygosity from segregating material and a higher frequency of extreme recombinant lines (Gallais 1988; Murigneux et al. 1993). However, in various crops, the production of DH lines remains largely dependent on techniques and genotypes. In maize, anther culture is one of the most widespread systems for generating in vitro derived DH plants (Genovesi et al., 1982; Barloy et al., 1989).

Maize DH lines derived from anther culture have been shown to be highly homogeneous, with infrequently somaclonal variation (Murigneux et al. 1993). Nevertheless, some studies on different cereals such as wheat (BjOrnstad et al. 1993; Henry et al. 1993), barley (Powell et al. 1986; Schön et al. 1990; Graner et al. 1991; Heun et al. 1991; Thompson et al. 1991; Zivy et al. 1992; Kleinshof et al. 1993), rye grass (Hayward et al. 1990), rice (Guiderdoni et al. 1989; Guiderdoni 1991; Yamagishi et al. 1996) and maize (Bentolila et al. 1992; Murigneux et al. 1993) have reported that androgenesis can induce significant segregation distortion of morphological, biochemical, or molecular markers. Distorted segregation of such markers may be attributed to a wide range of selective factors, resulting from the partial or total elimination of gametes or zygotes (Lyttle 1991).

It may be involved in androgenesis through selective processes acting at the gametophytic or at the haploid developing sporophytic phase. A probable relation between genes or quantitative trait loci (QTLs) affecting androgenesis and the selective factors causing segregation distortion in DH populations has been proposed by (Wan et al. (1992) and Yamagishi et al. (1996). To reach a maximum efficiency in plant breeding applications, due to linkage drift, such distorted segregation is undesirable if its extension over the map genome is too significant.

The present study was carried out to characterize major segregation distortions occurring during the different developmental stages of microspore embryogenesis in maize. Populations of (1) early induced androgenetic embryos, (2) calluses derived from embryos, (3) haploid plants regenerated from embryos, as well as (4) spontaneous regenerated diploid plantlets, were studied and compared to classical SSD and DH lines.

Amplified fragment length polymorphism (AFLP) markers covering the maize genome were used to identify chromosomal regions with distorted segregations. Moreover, a recent statistical method developed by Cheng et al. (1998) for estimating the recombination values between a partial lethal factor locus and linked molecular markers, was used to more-accurately map putative selective factors considered to be active along the androgenesis pathway. Relationships between these partial lethal factors and the published QTLs affecting anther culture ability identified in maize were examined (Armstrong et al. 1992; Cowen et al. 1992; Wan et al. 1992; Murigneux et al. 1994; Beaumont et al. 1995).

Materials and methods

Plant material and segregating populations

Six different segregating populations were derived from the same one-way cross between the pure lines A188 and DH7. Line A188 exhibits a very high somatic embryogenic tissue-culture response but no anther-culture ability. Conversely, DH7 is a dihaploid androgenetic line derived from a Chinese population (Barloy et al. 1989) with a high ability to form androgenetic embryos but poor somatic tissue ability.

Sixty recombinant inbred F_6 lines (SSD population) and 109 doubled-haploid progeny (DH population) are those employed in a previous analysis using restriction fragment length polymorphism (RFLP) markers (Murigneux et al. 1993). For the present study, four other populations were generated from this same A188×DH7 cross. These androgenetic populations corresponded to samples of genotypes obtained during the principal different phases of androgenesis, including callus structures (CAL population), well-formed and young androgenetic bipolar embryos (EMB population), regenerated haploid plantlets (NPL population) and regenerated spontaneous diploid plants (2NPL population). They contained 94, 107, 91 and 98 genotypes, respectively.

In vitro technique

Anther-culture methods and media were described by Barloy and Beckert (1993). Six weeks after plating, different morphological androgenetic structures were easily observed at the macroscopic level. Small embryos and calluses were collected under a binocular magnifying glass according to their developmental stage. Samples of embryos were cultured to promote their regeneration. The ploidy level of regenerated plantlets was estimated using a flow cytometer (Partec II), according to the method given in Antoine-Michard and Beckert (1997).

Figure 1 summarizes the developmental stages analyzed during microspore embryogenesis, from the induced microspores to the regenerated plantlets. In our in vitro regenerating procedure, both callus structures and the haploid plantlets were dead ends because neither plant regeneration nor selfed progeny occurred beyond these developmental stages.

Molecular analysis

Additional RFLP analyses were performed to enhance the density of the previous SSD map, according to the procedure described in Murigneux et al. (1993), using 26 RFLP markers provided by the Brookaven National Laboratory (Upton, N.Y.) and the University of Missouri (Columbia, Mo.) (See Table 1).

In the present study, obtaining large amounts of DNA from the very small androgenetic structures was a limiting factor for achieving efficient RFLP mapping. Consequently, AFLP markers were used in order to saturate the SSD RFLP map and to analyze the androgenetic populations. AFLP analyses were carried out according to the protocol described by Vos et al. (1995).

A first pre-amplification was performed using selective primers with a single extension: *Eco*R1 primer (E01) 5′-GACTGCGTACCAATTC(A)-3′ and Mse1 primer (M02) 5′-GATGAGTC-

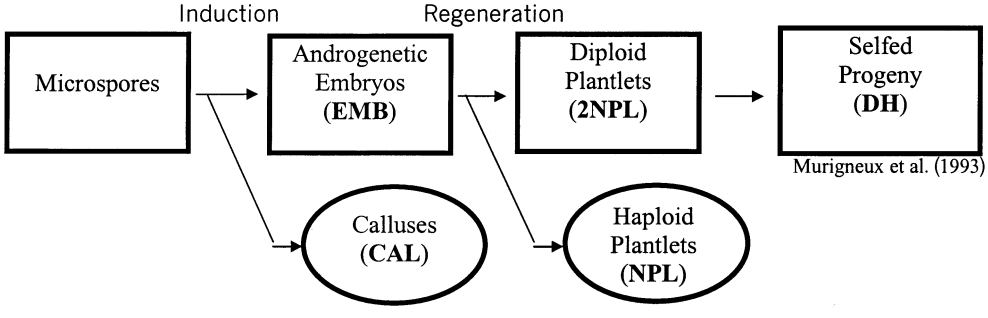

Fig. 1 Morphological development of the embryogenetic microspores to the DH progeny

Table 1 Additional RFLP markers

BNL5.10; BNL5.21; BNL5.59; BNL6.06; BNL6.22; BNL7.20; BNL8.35; BNL9.08; BNL10.38; BNL13.05;UMC3; UMC4; UMC26; UMC66; UMC82; UMC85; UMC104; UMC105; UMC119; UMC137; UMC141; UMC140; UMC151; UMC153; UMC158; UMC167

CTGAGTAA(C)-3′. A second amplification was performed using five primer combinations containing three selective nucleotides: (1) *Eco*R1+AAG (E33)/*Mse*1+CCA (M51); (2) *Eco*R1+ACA (E35)/*Mse*1+CCA (M51); (3) *Eco*R1+AGA (E39)/*Mse*1+CAC (M48); (4) *Eco*R1+ATG (E45)/*Mse*1+CAC (M48); (5) *Eco*R1+ATT (E46)/*Mse*1+CTA (M59).

In order to avoid scoring errors insofar as possible, ambiguous bands were discarded or scored as "data missing" and suitable markers were scored twice among the populations. Polymorphic AFLP markers were named according to the primer combination (for example, E33 for *Eco*R1 and the selective combination AAG), and serially numbered beginning with the lowest fragment.

Linkage analysis

Linkage analysis was performed for the SSD segregating population previously analyzed by Murigneux et al. (1993), using combined RFLP and AFLP marker data sets and MAPMAKER 3.0b computer software (Lander et al. 1987). The SSD saturated genetic map was constructed using the Haldane mapping function (Haldane 1919).

The androgenetic populations revealed high levels of distorted segregation for numerous markers. Moreover MAPMAKER is based on the absence of distorted segregations. Thus, the recombination fraction and the map distances were estimated only for adjacent makers in the SSD reference map, used as a reference for AFLP marker orders and locations in the four other androgenetic populations specifically developed for this study. The amplification products generated in the different populations, from the same initial A188×DH7 cross, were assumed to be identical alleles on the basis of their molecular weight.

Detection of segregation distortion and lethal factor mapping

For each population, a 1:1 single-factor segregation was tested at each locus using a chi-square test at the P=0.01 and P=0.001 levels. A chromosomal region was considered to be associated with a factor leading to segregation bias if at least two closely linked markers deviated from the expected Mendelian ratio. Single loci showing allelic distortions at P=0.001 were also retained, when adjacent loci without distortion were at a distance of at least 15 cM.

Genomic regions containing putative lethal factors were firstly identified according to the hypothesis that the tighter the linkage between a marker locus and the genetic factor, the greater the segregation distortion. We then identified more-specific locations using the method described in Cheng et al. (1998). The differential viability "t" and the putative recombination value "r1" between a partial lethal factor (L/l) and marker A, as well as their standard errors, were estimated between each pair of flanking markers that exhibited distorted segregation at the P=0.001 level. The most likely position of the partial lethal factor within a distorted linkage group was determined from the most-significant t values (inferior to 0.33 or greater than 3; indicating that LL genotypes are three times more viable than ll genotypes). The map distances between neighboring markers and partial lethal factor loci were estimated using the Haldane mapping function: $x(r)=-1/2\ln(1-2r1)$ (Bailey 1961).

Results

Addition of RFLP and AFLP markers to the previous framework SSD map

Starting from the SSD map generated by F6RIL from a A188×DH7 cross (Murigneux et al. 1993), 26 additional probes were used to map 39 additional RFLP loci. A framework of 140 loci was generated. The RFLP marker orders are consistent with those reported in the UMC and BNL maize genetic maps (Fig. 2).

In order to complete this SSD map and to analyze the androgenetic populations, AFLP data was generated using five primer combinations. The number of polymorphic bands generated by each combination in this cross ranged from 21 (E46M59) to 38 (E33M51). A total of 145 segregating AFLP markers were integrated into the ten linkage groups of the RFLP reference map and subsequently analyzed across the segregating androgenetic populations. The integration of AFLPs into the RFLP map generated a new map covering a genetic distance of 1886 cM. The distribution of AFLPs was not uniform across the whole SSD maize map. The centromeric region gathered numerous AFLP loci, and several chromosomal arms were not mapped by AFLP markers (end of 1L, 2 S, end of 3 S, end of 5 S, end of 6L, end of 7L, 9 S and 10 S). However, the five restricted combinations were not chosen to increase the global level of saturation of the SSD RFLP map, but above all to provide a reasonable framework of markers allowing a good coverage of the maize genome. Considering the UMC 98 map as a reference, we estimated that about 70% of the maize genome was covered.

Locations and directions of skewness in androgenetic populations

The percentages of markers showing distortions observed in the androgenetic populations were similar (31% in EMB) or slightly greater (between 40 and 45% in the CAL, NPL and 2NPL populations) than the one observed in the previous doubled-haploid population (about 31%; Murigneux et al. 1993). In the 2NPL population, exhibiting the highest level of distorsion, these distorted regions represented about 29% of the present maize SSD map.

A total of 14 chromosomal regions, located on eight chromosomes, showed significant segregation distortion, excluding chromosomes 5 and 7 at P<0.01. Of all the distorted AFLP loci, 80% were clustered in five genomic regions on chromosomes 1, 2, 8 and 10. Major distorted areas common to the different populations were found on chromosomes 1, 2, 4, 6, 8, 9 and 10. Deviations from the expected 1:1 segregation ratio towards the DH7 allele were extremely important on chromosomes 1, 2 and 8. The most significant χ^2 value (81.4) was observed in the 2NPL population on chromosome 8 at the E45M48.12 locus.

Population-specific distortions were also observed on chromosomes 3, 6 and 10. CAL population-specific distorted chromosomal regions were found on chromosomes 3 C and 10L towards the DH7 alleles, and on chromosome 6L towards the A188 alleles. An NPL population-specific distorted region was found on chromosome 3L towards the A188 alleles. Two distorted regions towards the A188 alleles were detected in the 2NPL pop-

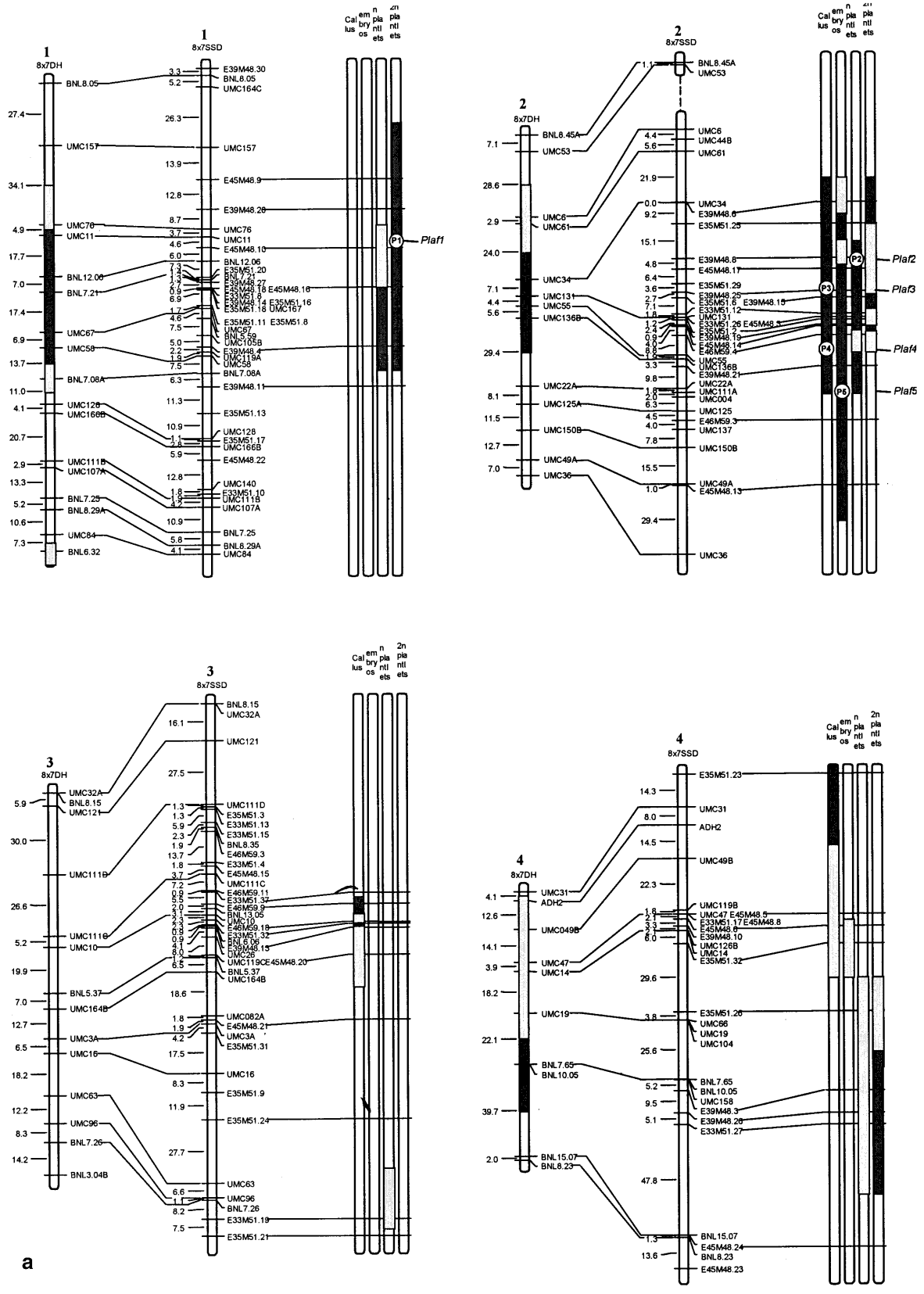

Fig. 2 Locations of segregation distortions and putative partial lethal androgenetic factors (*plfa* loci) in genetic maps of SSD, DH populations (Murigneux et al. 1993) and the four androgenetic populations developed for the present study. Distortions are visualised by *boxes* on chromosomes; towards DH7 alleles at the P=0.01 ■ and P=0.001 ■ levels, towards A188 alleles at the P=0.01 ■ and P=0.001 ■ levels

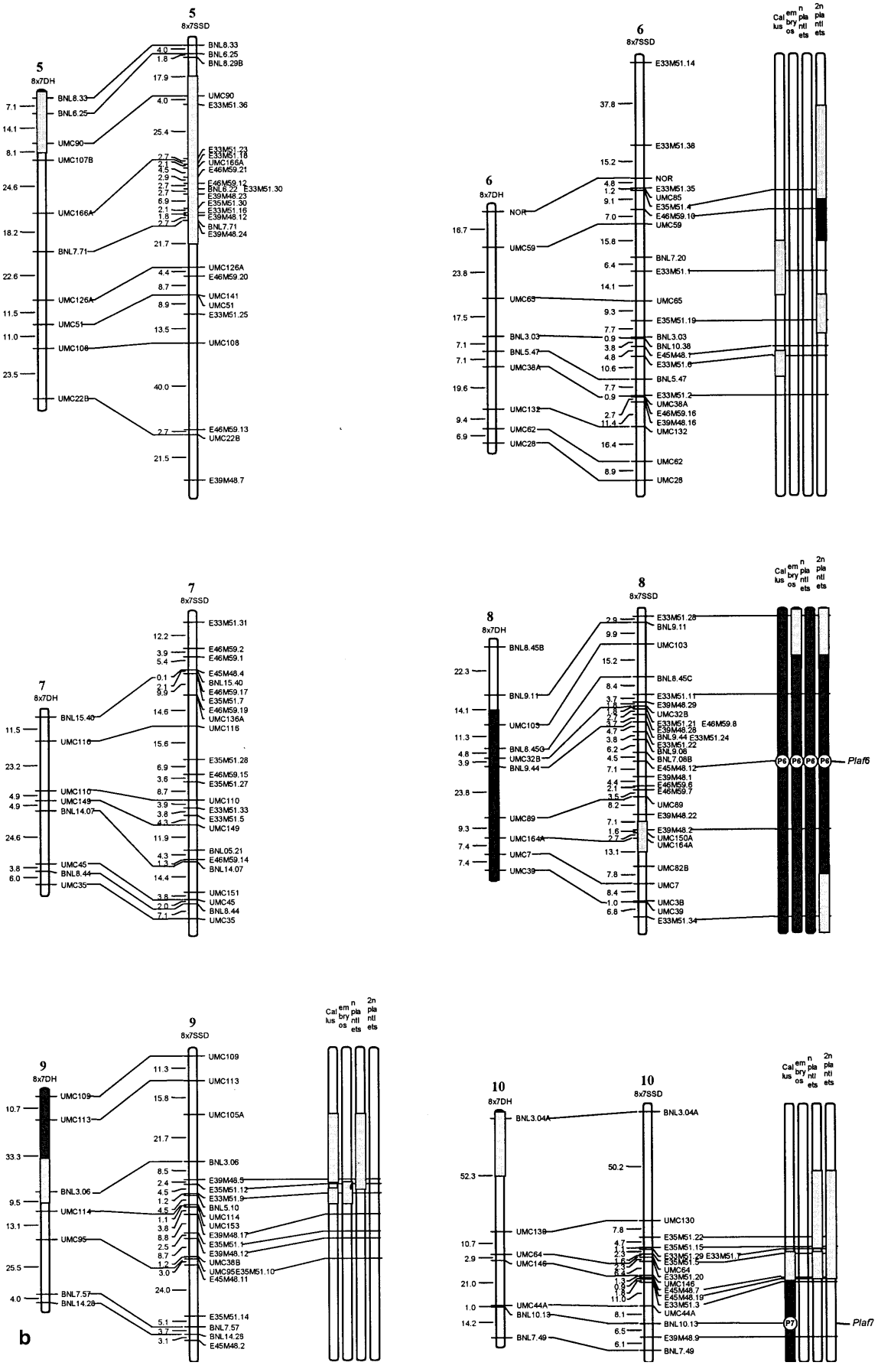

Fig. 2 (continued)

Table 2 Distorted segregation of AFLP markers linked to putative partial lethal factor (*PLAF*) loci

Chromosome populations	Plaf locus closest marker	Chi-square test (1:1)	Segregations A188:DH7 AA–AB–BA–BB	Recombination values:r1±SE	Viabilities: t±SE
C1 2NPL	*Plaf1* E45M48.10	45.43	E39M48.20 10–18–4–58	0.018±0.014	0.166±0.05
C2 NPL	*Plaf2* E39M48.8	26.18	E45M48.17 19–1–3–65	0.003±0.006	0.298±0.076
C2 CAL	*Plaf3* E39M48.25	22	E35M51.29 19–4–2–60	0.014±0.013	0.315±0.08
C2 CAL	*Plaf4* E46M59.4	19.9	E39M48.21 14–7–8–57	0.087±0.03	0.235±0.064
C2 EMB	*Plaf5* E39M48.21	22.25	E46M59.3 18–5–9–56	0.041±0.021	0.315±0.079
C8 All populations	*Plaf6* E45M48.12	81.4 (2NPL)	E39M48.1 18–4–12-233	0.0131±0.007	0.076±0.018
C10 CAL	*Plaf7* E39M48.9	20.05	E33M51.3 16–7–11–54	0.073±0.028	0.283±0.073

ulation on chromosome 6L, whose location was different from the distortions observed in the CAL population. The extent of the distortions in these specific areas was fairly limited and χ^2 values varied from 7.19 to 20.05.

When the direction of distortion was conserved at the same locations across the different androgenetic populations, the direction changed between more-distant chromosomal areas. On chromosome 3, distortions in the E46M59.9–E45M48.20 segment skewed towards DH7 alleles in the CAL population, while segregation at the E33M51.19 locus skewed towards allele A188 in the NPL population.

Location of major partial lethal androgenetic factors

Based on the locations of the markers showing the most-pronounced segregation distortions and using the method described in Cheng et al. (1998), seven putative major lethal androgenetic factors (*plaf1–plaf7*) were identified in the different androgenetic populations, i.e. they expressed a differential viability of gametes or sporophytic plant-derived gametes at least three times lower than that of normal gametes or regenerated plants.

One factor (*Plaf1*; t=0.166±0.05) affecting the plant regeneration process was located close to E45M48.10 between the UMC11 and BNL12.06 markers on chromosome 1. Four factors were found on chromosome 2. The first one (*Plaf2*; t=0.298±0.076) effective in the NPL population was located close to E39M48.8; The second factor (*Plaf3*; t=0.315±0.08) effective in the CAL population was located close to E39M48.25. The third factor (*Plaf4*; t=0.235±0.064) affecting the CAL structures was located between E46M59.4 and E39M48.21. The fourth factor (*Plaf5*; t=0.315±0.079) effective in the EMB population was mapped between E39M48.21 and E46M59.3. Considering the restricted population sizes, the difference in location, on the one hand, between *Plaf2* and *Plaf3*, and on the other hand between *Plaf4* and *Plaf5* may arise from sampling errors. Therefore, these four loci could correspond only to two different partial lethal factors on chromosome 2, the first of which was between E39M48.8 and E39M48.25, and the second one close to E39M48.21.

The most important factor (*Plaf6*) able to bring about considerable segregation distortions in all populations, showed an average differential viability (t) of gametes (l) at least 14 times lower than that of normal gametes (L) (t=0.076±0.018). It was located very close to E45M48.12 on chromosome 8. The last major factor (*Plaf7*; t=0.283±0.073), effective in the CAL population was located between E39M48.9 and E33M51.3, close to the UMC44 and BNL10.13 markers on chromosome 10. For all these *plaf* loci, the DH7 allelic form was more competitive than that of A188.

Discussion and conclusion

Segregation distortion can be defined as a deviation from the expected frequencies in a given phenotypic or genotypic class within a segregating population. As a result of the development of molecular maps, numerous examples of segregation distortions have been reported in a large number of linkage maps in plants. Some studies focused on doubled haploid progeny populations obtained by in vitro androgenesis, known to induce significant segregation distortions. However, these DH lines provided only a restricted and final view of the distortions induced during the androgenetic process.

In the case of in vitro microspore embryogenesis, segregation distortions may reflect several independent selection pressures exerted on linked genes, during the process of embryogenesis, from the extraction of micro-

spores to plant regeneration. The comparison between SSD, DH and in vitro androgenetic populations derived from the same parental combination made it possible to examine the specificity of segregation distortions, or else to identify whether selection took place at an early stage of microsporogenesis, during embryo formation, conversion from embryo to plantlet, plant development, or during the fertilization.

No common distortion loci were observed between the androgenetic populations and the SSD population developed by Murigneux et al. (1993). The two major deviations observed in the SSD population (chromosomes 5 and 8) totally contrasted with those observed in the androgenetic derived populations. Apparently, the A188×DH7 combination did not express partial lethal factors that promote differentially reduced pollen viability during microsporogenesis. The numerous distorted markers observed in our DH and androgenetic populations were consequently actively selected during embryogenesis and plant regeneration.

A relation between the difference in the anther culture ability of parental lines and the direction and extent of the deviations has been already observed in barley (Graner et al. 1991) and maize (Murigneux et al. 1993). Similarly, it can be suggested that observed similarities in the expression of distortion across several populations correspond to genetic factors conferring significant selective fitness at different steps of androgenesis. Concerning the occurrence of segregation distortion during the course of androgenesis, distortions reported on chromosomes 2, 8 and 9 started to appear with embryo induction or callus formation and continued during plantlet formation. This confirms the assumption that variations occur early at the start of the embryogenic phase and have considerable effects later on DH plant regeneration and production. The most relevant example was the highly distorted chromosome 8, the genomic region 8.04 of which carried one major lethal androgenetic factor *Plaf6*. This area seems to confer a greater selective advantage during anther culture where major QTLs for anther culture ability and the induction of embryo-like structures were observed in maize by Murigneux et al. (1994) and Beaumont et al. (1995).

Chromosome 2 showed more complex patterns of distortion. We located four putative viability factors, but since sampling errors may also cause segregation distorsions, major distorsions in chromosome 2 could be caused only by two independent *Plaf* factors. Both *Plaf2* and *Plaf3*, active in the haploid and callus population respectively, were closely located in genomic segment 2.04. The factors *Plaf4* and *Plaf5* affecting the callus and embryo structures were mapped in genomic region 2.06, that contains the *emb5* gene locus, and which play a role at the transition to the early coleoptilar stage.

If we now consider chromosome 9, the lack of markers certainly led to underestimating distortions on the S arm. Segregation distortions towards DH7 alleles were observed in the DH population on this entire S arm, and were less effective during the first three stages of anther culture (genomic regions 9.02 and 9.03). This chromosomal region might be more involved at the final steps of the whole process. A QTL for the induction of embryo-like structures (Cowen et al. 1992) and the emb2 locus controlling embryo development were located in this genomic region.

Specific deviations also occurred independently within different populations during androgenesis, indicating the existence of putative selective factors independently active either during embryogenesis and callus formation or during the regeneration phase. Markers showing specific distortions towards DH7 from the NPL and the 2NPL populations were located on chromosome 1, indicating that DH7 alleles might also specifically stimulate plantlet regeneration. This area concerns segments 1.02 to 1.05, in which QTLs for anther culture ability and induction of embryo-like structures were previously identified in maize by Cowen et al. (1992) and Beaumont et al. (1995). The partial lethal factor *Plaf1* was located in segment 1.03, which contains numerous *emb* loci.

Two other striking examples were illustrated in chromosomes 4 and 10, where two neighbouring regions each presented one distorted marker towards DH7 either during the early androgenetic anther response or plant regeneration. On chromosome 4, one first segment, involved in the microspore ability to generate embryos or calluses, deviated in genomic regions 4.00 to 4.05. QTLs for anther culturability and plantlet regeneration were observed in this area of the maize genome by Murigneux et al. (1994). The second deviated segment involved in the ability to regenerate plantlets was observed in regions 4.05 to 4.09, and probably corresponds to the distortion similarly reported in the aforementioned DH population by Murigneux et al. (1993). On chromosome 10, a first segment showed distorsions within regions 10.03 and 10.04 during plant regeneration and diploidization, in which area the lack of markers probably led to underestimating distortions on the 10S arm of androgenetic populations. The Plaf7 factor was mapped in the second segment deviated during callus formation, which corresponds to genomic regions 10.05 and 10.06.

Finally, noticeable segregation distortions towards A188 were also specifically observed during the ultimate step of the androgenetic process in the NPL and 2NPL populations. The A188 line is recalcitrant to anther culture; nevertheless, deviation at the E33M51.19 locus on the chromosome 3 confirms that A188 alleles may influence the regeneration of haploid plantlets. Moreover, distortion of markers located on chromosome 6 between genomic regions 6.01 and 6.02, also may in this case indicate that the non-responsive A188 line has positive traits and essential alleles for spontaneous diploidization.

Our experiment was aimed at gaining information on expressed segregation distortions and at mapping potentially important chromosomal regions from a developmental point of view during androgenesis in maize.

AFLPs proved to be valuable markers for investigating such a segregation bias in androgenetic material. The analysis of several populations enabled us to emphasize the following points:

(1) In the parental combination used, no distortion involving early gametophytic selection, i.e. occuring before induced androgenesis and in vitro culture, was observed.
(2) Common segments with segregation distorsions on chromosomes 2, 4, 8 and 9 between callus and embryo populations gave evidence that selection occurred at the early steps of embryogenesis.
(3) Specific distorted segments in the callus population (chromosomes 3, 6 and 10) could be related to genes leading to cell multiplication without differentiation.
(4) Common segregation distorsions on chromosomes 1, 4, and 10 between haploid and dihaploid populations, gave evidence of selection during plantlet regeneration and spontaneous chromosome doubling. The latter step was also specifically observed to influence the allelic distribution on chromosome 6.

A linkage between distortion at marker loci and genes controlling microspore embryogenesis was anticipated. Unfortunately, little is known about the general genetic control of induced microspore embryogenesis, development and plantlet regeneration, except for the fact that they are probably complex and determined by independently inherited genes. Further progress will require genetic and molecular studies on these distortor genes, which play a critical role in androgenesis.

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