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Linkage analysis of anther-derived monoploids showing distorted segregation of molecular markers

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Abstract Monoploids can be obtained from several diploid plant species by anther culture. Mapping of molecular markers using monoploids is greatly facilitated by the simple 1:1 segregation ratio expected from all heterozygous loci in the genome. Distorted segregation of molecular markers, however, appears to be a common phenomenon in many crop species and hinders the use of monoploids for mapping purposes. This report examines the segregation pattern of two marker genes linked together with one locus or separately with two independent loci which are responsible for the observed distortion. Each of the loci exhibiting distorted segregation has one of the two alleles which inhibits regeneration of the gametic cells *in vitro* and disrupts the expected segregation ratio of the linked markers. All possible situations in which linkage occurs between markers and distortion-causing genes are considered. Theoretical results outlining the segregation pattern among these linkage types indicate that the distinguishable distorted ratios can be used for mapping purposes. A protocol is given for the mapping of distorted gene markers based on existing gene mapping software. An example is presented of the mapping of distorted RAPD markers of monoploids obtained from a diploid potato genotype.

Key words Gene mapping · Potato · Segregation ratio · *Solanum tuberosum* L.

Introduction

Anther culture of diploid plants leads to the production of monoploids (Heberle-Bors 1985; Meyer et al. 1993; Rokka et al. 1995). Frequently, gametic cells in anthers of crop species show differential responses to anther culture (Bajaj 1983; Heberle-Bors 1985). While some genotypes do not show any growth or development (Uhrig 1985), others have different degrees of tissue development, which include callus without clear morphogenesis, aberrant tissue mass with roots, and normal-appearing plantlets (Bajaj 1983). All of them are expected to be monoploids (Heberle-Bors 1985; Kotch et al. 1992). Segregating molecular markers obtained from DNA extracted from these monoploid tissues are expected to show a 1 (presence) :1 (absence) ratio of banding pattern (Kotch et al. 1992; Rivard et al. 1996). Distortion of segregation ratios, however, was reported on several crop species (Bentolila et al. 1992; Cloutier and Landry 1994, Rivard et al. 1996; Yamagishi et al. 1996). No satisfactory explanation has been offered for this phenomenon. The present report investigates the possible underlying genetic mechanisms which may lead to this distortion phenomenon. A method is developed to map the loci responsible for the distortion.

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Theory

It is possible that (1) cell division and callus growth, (2) root, and (3) shoot morphogenesis are all controlled by separate genes. Let *I-i* be a pair of alleles of a locus regulating the response of gametic cells to anther culture. Gametic cells containing the gene *I* respond positively to the culture medium and undergo morphogenesis, whereas those with the gene *i* fail to respond in culture. Half of the gametic cells of a mother plant with the *Ii* genotype are expected to carry the allele *I* and thus able to regenerate into shoots or embryos. Let a marker of the configuration *A-O* be linked with *Ii* . *A* denotes presence and *O* absence of the *A*-marker band. In coupling phase, i.e. the genotype *IA/iO* , the monoploids are expected to segregate by the ratio *IA:IO = (1-r) : r* where *r* is the recombination frequency between the two loci *I-i* and *A-O*. Gametic cells with the genotype *iA* and *iO* fail to develop in culture. The segregation ratio is distorted when $r < 0.5$. In repulsion phase, i.e. the genotype *iA/IO*, the expected ratio *IA* : *IO = r : (1-r)* is again distorted when $r < 0.5$.

Consider another marker locus *B-O* which is linked with *A-O*. To detect linkage relationship between them, we have to consider four marker configurations of the mother plant: *IAB/iOO*, *IAO/iOB*, *IOO/iAB*, and *IOB/iAO*. The recombination frequencies between *I-i* and *A-O* and between *A-O* and *B-O* are r_1 and r_2, respectively. Gametic cells with the genotype $i\_\ \_$ are not capable of responding to tissue culture and therefore do not undergo morphogenesis.

Another case requiring consideration is the situation in which two makers are linked with two independent distortion-causing loci. Let *A-O* be linked with I_1 - i_1 and *B-O* with I_2 - i_2. Again r_1 represents the recombination rate between the first pair whereas r_2 represents that between the second pair of loci. There are also four possible marker configurations.

Segregation ratios of monoploids from each of the eight mother genotypes (Table 1) show the expected segregation ratios of markers linked with the distortion causing loci. The degree and pattern of distortion of the ratio depends on the mother genotype and the intensity of linkage of the markers with the loci. Consider the two corresponding mother genotypes *IAB/iOO* and I_1A/i_1O I_2B/i_2O. The segregation ratio of monoploid genotypes from both mother genotypes is *AB:AO:OB:OO* = 1:1:1:1 when $r_1 = 0.5$ and $r_2 = 0.5$. The proportions of *AB* and *AO* are increased whereas those of *OB* and *OO* decreased when $r_1 < 0.5$. Neither *A-O* nor *B-O* show the expected ratio of *A:O* = *B:O* = 1:1. From the mother genotype *IAB/iOO*, *OO* is expected to have a much larger fraction than that of *OB* when $r_1 < 0.5$ and $r_2 < 0.5$. The opposite is true for monoploids obtained from the mother genotype I_1A/i_1O I_2B/i_2O, i.e., *OO* has a much smaller fraction than that of *OB*. The expected difference between *OO* and *OB* is $r_1(1–2r_2)$ and - $r_1(1–2r_2)$ for the mother genotypes *IAB/iOO* and I_1A/i_1O I_2B/i_2O, respectively. As a double crossover rarely occurs when the linkages between *I-i* and *A-O*, and those between *A-O* and *B-O* are tight, the fraction of *OB* from the mother genotype *IAB/iOO* can be much lower than the expected fraction r_1r_2, and thus the difference of fractions between *OO* and *OB* is even larger than the expected value .

The above hypothesis can be applied to detection of the distorted pattern of segregation ratios of monoploids obtained from the other three pairs of corresponding mother genotypes in Table 1. A protocol is presented here to detect and map a series of markers which are located beside a distortion-causing locus:

1) Segregation data of all markers from the monoploids obtained from a mother genotype are tested for distortion from the expected ratio 1:1. This is achieved by carrying out a statistical test such as the χ^2 goodness-of-fit test for each of the individual segregating markers.
2) A marker with the highest degree of distortion is identified. A 2×2 contingency table is formed between this marker and every other distorted marker. Linkage between two markers is detected when the statistical test for goodness-of-fit is significant and the fraction of the double recombinant monoploid is lower than that of the single recombinant one (e.g. *OO* >*OB* from the mother genotype *IAB/iOO*). A group of markers linked with a putative distortion-causing locus is identified.
3) Genotype of the group of markers from each of the scored monoploids is used to simulate a hypothetical “mirror image” monoploid which possesses the other alleles of the markers. This is aimed at restoring those gametic cells which are not able to undergo morphogenesis to form monoploids because of the distortion-causing gene. The segregation ratio is reestablished for each of the markers in the group to the expected 1:1 ratio in the combined sample of observed and hypothetical monoploids. The genotype of the putative distortion-causing locus is simulated as *I* for all scored monoploids and *i* for all simulated monoploids.
4) Segregation data obtained from (3) are used to map the group of markers and the putative distortion-causing locus. This can be accomplished by means of an established software for gene mapping such as MAPMAKER (Lander *et al.* 1987) with the F_2 backcross option.
5) The procedure is repeated for another highly distorted marker which is not included in the above group.

It is noted that the χ^2-test for the 2×2 contingency table of two markers may also show significance because they

Table 1 Expected segregation ratios in monoploids of two marker loci *A-O* and *B-O* linked with one common locus *I-i* (first four cases) or two separate and independent loci I_1 -i_1 and I_2 -i_2 from eight diploid mother genotypes

	Monoploid genotype			
	IAB	*IAO*	*IOB*	*IOO*
IAB/iOO	$(1-r_1)(1-r_2)$	$(1-r_1)r_2$	r_1r_2	$r_1(1-r_2)$
IAO/iOB	$(1-r_1)r_2$	$(1-r_1)(1-r_2)$	$r_1(1-r_2)$	r_1r_2
IOO/iAB	$r_1(1-r_2)$	r_1r_2	$(1-r_1)r_2$	$(1-r_1)(1-r_2)$
IOB/iAO	r_1r_2	$r_1(1-r_2)$	$(1-r_1)\ (1-r_2)$	$(1-r_1)r_2$
	I_1AI_2B	I_1AI_2O	I_1OI_2B	I_1OI_2O
$I_1A/i_1O\ I_2B/i_2O$	$(1-r_1)(1-r_2)$	$(1-r_1)r_2$	$r_1(1-r_2)$	r_1r_2
$I_1A/i_1O\ I_2O/i_2B$	$(1-r_1)r_2$	$(1-r_1)(1-r_2)$	r_1r_2	$r_1(1-r_2)$
$I_1O/i_1A\ I_2O/i_2B$	r_1r_2	$r_1(1-r_2)$	$(1-r_1)r_2$	$(1-r_1)(1-r_2)$
$I_1O/i_1A\ I_2B/i_2O$	$r_1(1-r_2)$	r_1r_2	$(1-r_1)(1-r_2)$	$(1-r_1)r_2$

Table 2 Markers with distorted segregation ratios from the expected 1:1 ratio based on χ^2 goodness-of-fit test

Code	Marker	Number of monoploids	No-band condimuration (%)	Band (%)	Probability
m1	OPA002-0.67	19	10.53	89.47	<0.01
m2	OPA002-0.76	19	15.79	84.21	<0.01
m3	OPA002-0.93	19	73.68	26.32	0.04
m6	OPB010-0.36	25	12	88	<0.01
m9	OPB010-0.96	25	8	92	<0.01
m13	OPC007-1.84	19	21.05	78.95	0.01
m15	OPC013-0.56	23	26.09	73.91	0.02
m20	OPC015-0.39	25	16	84	<0.01
m24	OPC015-0.71	25	28	72	0.03
m26	OPC015-1.00	25	72	28	0.03
m27	OPC015-1.16	25	20	80	<0.01
m28	OPC015-1.47	25	16	84	<0.01
m33	OPH008-1.00	23	13.04	86.96	<0.01
m34	OPH008-1.26	23	13.04	86.96	<0.01
m35	UBC008-1.00	25	84	16	<0.01
m37	UPC131-1.55	25	80	20	<0.01
m38	UBC153-0.64	25	8	92	<0.01
m39	UBC184-0.59	23	26.09	73.91	0.02
m42	UBC291-0.67	23	78.26	21.74	0.01
m43	UBC291-1.14	23	8.7	91.3	<0.01
m44	UBC308-0.67	25	76	24	0.01
m45	UBC308-0.96	25	8	92	<0.01
m49	UBC504-1.03	25	20	80	<0.01
m53	UBC533-0.93	23	21.74	78.26	0.01
m54	UBC533-1.27	23	8.7	91.3	<0.01

are tightly linked with the distortion-causing locus located between them. This makes, for example, the frequency of *OO* from the mother genotype *AIB/OiO* lower than that of *OB*, a situation opposite to that from the mother genotype *IAB/iOO*. But the frequency of *OO* is lower than the expected $r_1\, r_2$ because of the low frequency of double crossover between the markers, which is different from the expected outcome from the mother genotype $I_1A/i_1O\ I_2B/i_2O$.

An example

A diploid clone 9507–04 derived from *Solanum tuberosum* haploid × *S. chacoanse* was used for anther culture (Aziz et al. 1999). The ploidy of the anther-derived roots and plantlets from this clone was determined by flow cytometric analysis. The monoploids were used for random amplified polymorphic DNA (RAPD) analysis. A total of 25 monoploids were used for the analysis and data from 25 RAPD markers showing distorted segregation were used in the present study. Details of the experiment are provided in Aziz (1998).

Distortion from the expected 1:1 segregation ratio for the presence and absence of banding configuration of each of the 56 markers was tested by the χ^2 goodness-of-fit test. A total of 25 markers were found to exhibit distorted segregation (Table 2). These distorted markers were pooled together in a new data base. The degree of distortion varied among the markers. Markers m9, m38, and m45 had the highest degree of distortion with 8% of the monoploids showing the no-banding configuration. With respect to markers m43 and m54 each, 8.7% of the monoploids showed the no-band configuration, m1 10.53%, m33 and m34 each 13.04%, m2 15.79%, and m20 16%; whereas with m35 16% of the monoploids showed banding configuration. All these markers but m34, m35, and m43 were used as "seed" to establish linkage groups. Marker m34 had an identical segregation pattern as m33, and m35 and m43 were identified as members of an established linkage group. These markers were thus not used as "seed". Of the 25, markers not linked with an established linkage group but only having a moderate degree of distortion were not used to identify distortion-causing genes.

Each of the seed markers was used to form a 2 × 2 contingency table with all other markers. As the sample size was rather limited (i.e., 19–25 monoploids), both χ^2 goodness-of-fit test and Fisher's exact test were used to test whether a pair of markers were independent to each other or linked. Two markers were deemed linked when χ^2 was significant at $P = 0.05$ and the exact test at $P = 0.10$. The statistical analyses were carried out by the software SYSTAT version 7.0. Table 3 presents pairs of markers showing significant results.

Genotype of markers in the linkage group for each of the observed monoploids was then used to simulate putative monoploids which have the other alleles, i.e., *AB, AO, OB,* or *OO* of an original genotype is changed to *OO, OB, AO,* or *AB,* respectively. Segregation ratios of every individual marker in the combined sample is exactly in a 1:1 ratio. Data of markers in each of the established linkage groups were then input into MAPMAKER to construct a linkage map. A LOD threshold of 3.0 was used, and order and distance between markers were obtained by the multipoint analysis.

Table 3 Results of linkage test on distorted markers based on χ^2 and Fisher's exact test

Marker pairs	Phase of pairing	Number of monoploids	*AB*	*AO*	*OB*	*OO*	χ^2 (Probability)	Exact-test
m1 & m2	Coupling	19	84.21	5.26	0	10.53	0.001	0.035
m 1 & m13	Coupling	19	78.95	10.53	0	10.53	0.004	0.035
m1 & m35	Repulsion	19	5.26	84.21	10.53	0	0.001	0.018
m1 & m37	Repulsion	19	15.79	73.68	10.53	0	0.012	0.058
m9 & m6	Coupling	25	88.00	4.00	0	8.00	<0.001	0.010
m 9 & m13	Coupling	19	78.95	15.79	0	5.26	0.047	0.211
m 28 & m45	Coupling	25	84.00	8.00	0	8.00	0.001	0.020
m 38 & m28	Coupling	25	84.00	8.00	0	8.00	0.001	0.020
m 38 & m43	Coupling	23	91.30	4.38	0	4.38	0.001	0.087
m20 & m42	Repulsion	23	13.04	73.91	8.70	4.35	0.040	0.110
m 42 & m 26	Coupling	23	17.39	4.35	13.04	65.22	0.007	0.017
m 34 & m26	Repulsion	23	17.39	69.57	13.04	0	0.005	0.020
m 54 & m53	Coupling	23	78.26	13.04	0	8.70	<0.001	0.040

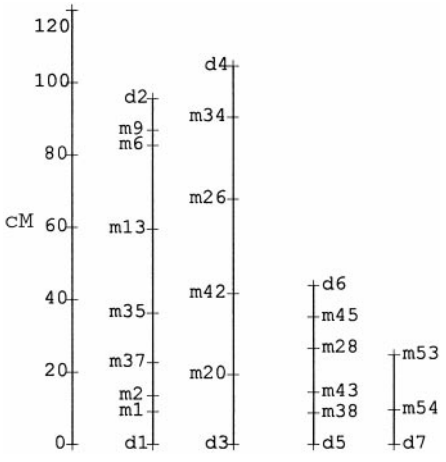

Fig. 1 Linkage groups of markers together with distortion-causing loci

Four linkage groups were obtained (Table 3). The first group included seven markers with distortion-causing loci at both ends of the linkage map. The second and third groups each had four markers with also distortion-causing loci at both ends of the map. The fourth group had two markers with only one distortion-causing locus at one end. The four linkage maps were 95.6 cM, 104.6 cM, 43.9 cM and 24.7 cM long, respectively, and are shown in Fig. 1.

Discussion

Monoploids or doubled haploids can represent the simplest way to obtain linkage maps because all segregating genes follow the 1:1 ratio. The approach becomes complex because a large percentage of molecular markers of many plant species have been reported to yield distorted segregation (Cloutier and Landry 1994; Cloutier et al. 1991; Rivard et al. 1996; Yamagishi et al. 1996). Surviving monoploids, however, show a unique change of segregation pattern between pairs of linked loci when compared with the undistorted situation (Aziz 1998). Information obtained from the segregation of distorted markers can therefore be used to sort them into linkage groups. Recombination frequencies between the marker loci in a group can then be estimated by the maximum likelihood method based on the expected segregation outcome in Table 1 and a linkage map subsequently constructed based on these estimates. An alternative technique is, however, available. A convenient way to map the marker loci in a linkage group is to simulate the missing data based on the fact that all segregating markers should have the expected ratio 1:1. The linkage map can be easily constructed by means of an existing mapping software such as MAPMAKER (Lander et al. 1987).

The mapping procedure should be started with the marker which shows the highest degree of distortion because this marker locus should theoretically be located closest to a distortion-causing locus ("seed marker"). Other distorted markers showing a lack of independence from this seed marker based on χ^2 test are then pooled together as a linkage group. The combined observed and simulated marker data are then used for mapping. The genotypes of the distortion-causing locus are simulated with distortion and non-distortion alleles segregated by the expected ratio 1:1 with all observed individuals possessing the non-distortion allele. Markers with a mild degree of distortion and not included in the linkage groups already established are questionable when used as seed to locate the distortion-causing locus and thus should be avoided for the mapping purposes.

A total of 25 markers showed distorted segregation in the present study. Seventeen of them were members from four linkage groups (Table 3). Three groups had distortion-causing loci at both ends of the map, and represented integrated segments of the chromosomes. It is interesting to note that 10 out of 12 recombination analyses in Table 3 had one of the four genotypes missing. The remaining two analyses also showed very low frequencies of one genotype. All of the recombinants are

the double crossover genotypes which are expected to show low frequencies when they are linked with a distortion-causing locus (Table 1). A comparison of genome structures between potato and tomato (Bonierbale et al. 1988) indicated that there were nearly identical linkage maps but that the potato had a much shorter total map length than the tomato, suggesting that the potato had a lower frequency of double crossovers between neighbourhood loci than that of the tomato.

Use of monoploids and their corresponding doubled haploids will unquestionably enhance the proficiency of gene mapping after the obstacle of segregation distortion is removed. Specifically, monoploids have the potential for locating genes showing segregation for characteristics of valuable economic traits such as disease and pest resistance, quality, and yielding ability of agronomic crops.

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