Distorted segregation of RFLP markers in regenerated plants derived from anther culture of an F_1 hybrid of rice

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To examine whether parental alleles were randomly transmitted from heterozygous donor plants to anther culture (AC)-derived plants, two AC-derived populations and one F_2 population in rice were compared for 50 RFLP markers distributed on the rice chromosomes. Two populations which were developed through different anther culture methods (the ordinary method and the direct regeneration method) and the F_2 population were produced from an F_1 hybrid between distantly related cultivars of Nipponbare (*japonica* type) and Milyang 23 (*indica* type). RFLP analysis revealed that ten and eleven of the 50 markers in the two AC-derived populations showed distorted segregation ratios from the theoretical ratio of 1:1. Parental alleles were not randomly transmitted from the F_1 plant to the AC-derived plants. Additionally, the segregation ratios of seven and six RFLP markers, respectively, were distorted both from the 1:1 ratios and from the observed ratios in the F_2 population. The chromosomal regions involving these markers were on chromosomes 1, 3, 7, 10, 11 and 12. Some of the regions were different in the two AC-derived populations. Non-random assortment of parental alleles might be influenced by anther culture methods.

INTRODUCTION

Doubled haploid (DH) lines generated from heterozygous plants through the anther culture method or *Hordeum bulbosum* method are useful materials for plant breeding and genetics. Homozygous DH lines have been produced from F_1 plants in a short time (Foroughi-Wehr and Friedt, 1984; Ye et al., 1987; Zapata et al., 1991; Henderson and Pauls, 1992; Shimada et al., 1994), and RFLP maps have been constructed using DH lines for barley (Graner et al., 1991; Heun et al., 1991; Kleinhofs et al., 1993), maize (Bentolila et al., 1992; Murigneux et al., 1993), and pepper (Lefebvre et al., 1995). A random assortment of parental alleles from heterozygous plants to DH lines is desirable for the use of DH lines in breeding and genetics. Therefore, one important question to be an-

swered is whether or not parental alleles are transmitted with the theoretical Mendelian ratio from F_1 plants to DH populations.

Distorted segregation of molecular markers from the theoretical segregation ratios in DH populations has been reported in maize (Bentolila et al., 1992; Murigneux et al., 1993), barley (Graner et al., 1991; Heun et al., 1991; Thompson et al., 1991; Zivy et al., 1992; Kleinhofs et al., 1993), broccoli (Orton and Browsers, 1985), pepper (Lefebvre et al., 1995), and *Solanum chacoense* (Rivard and Saba-El-Leil, 1994). In rice, segregation distortion of isozyme markers in anther culture (AC)-derived plants has been reported (Guiderdoni, 1991). However, the number of isozyme markers examined was not sufficient to cover the whole rice genome.

Recently, RFLP linkage maps have been constructed for rice (McCouch et al., 1988; Saito et al., 1991; Causse et al., 1994; Kurata et al., 1994) and have enabled to study the parental allele frequency in the AC-derived populations using RFLP markers which cover all 12 chromosomes of rice. The present study was undertaken to compare the

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segregation of RFLP markers in two AC-derived populations obtained through different anther culture methods and in one F₂ population, developed from the same F₁ hybrid.

MATERIALS AND METHODS

Plant materials. AC-derived plants were obtained through anther culture of F₁ plants derived from a cross between a *japonica* rice cultivar 'Nipponbare' and an *indica* rice cultivar 'Milyang 23'. F₂ plants were obtained through selfing of the F₁ plants. Nipponbare is highly responsive to anther culture and Milyang 23 is poorly responsive. Two AC-derived populations were developed through the different anther culture methods; the direct regeneration method (DR population) and the ordinary method (OR population). In the DR method, callus induction and subsequent plant regeneration occur on the same medium consisting of inorganic and organic salts of N₆ (Chu, 1978), 10⁻⁷ M 2,4-dichlorophenoxyacetic acid (2,4-D), 5 × 10⁻⁶ M naphthalene acetic acid (NAA), 1 g/l yeast extract, 70 g/l sucrose and 10 g/l agar (Nakamura et al., 1985). On the other hand, in the OR method, calli derived from microspores on an N₆ medium containing 4 mg/l 2,4-D, 50 g/l sucrose and 4 g/l gelrite are transferred to a regeneration medium consisting inorganic and organic salts of Linsmaier and Skoog (1965), 2 mg/l NAA, 1 mg/l kinetin, 2 g/l casein acid hydrolysate, 1 g/l 2-morpholinoethanesulfonic acid monohydrate (MES), 30 g/l sucrose, 30 g/l sorbitol and 4 g/l gelrite. Fifty plants of each of the AC-derived populations and the F₂ population were used to score genotypes for RFLP markers.

RFLP analysis. The AC-derived plants and the F₂ plants were grown in a green house or a paddy field. Total DNA was extracted from 5 to 10 g of fresh leaves using a CTAB method (Murray and Thompson, 1980). The DNA was then digested with *Bam*H I, *Bgl* II, *Eco*R I, *Eco*R V, *Hind* III, or *Kpn* I, followed by blotting onto nylon membranes (positive charge, Boehringer) by the alkaline method (Reed and Mann, 1985). Fifty mapped probes were selected from two rice RFLP linkage maps (Saito et al., 1991; Kurata et al., 1994). They were labelled, hybridized and detected using the ECL gene detection system (Amersham) according to the supplier's instructions.

Chi-square (χ^2) analysis was performed between observed segregation ratios of each RFLP marker and the theoretical ratios (1:1 in the AC-derived populations, and 1:2:1 or 3:1 in the F₂ population). Additionally, the allelic frequencies of each of markers other than two dominant markers in the F₂ population were calculated from the segregation ratios, and chi-square test was also used for analysis between the allelic frequencies in the F₂ population and the observed segregation ratios in the two AC-derived populations.

RESULTS

Fifty RFLP markers distributed on 12 chromosomes of rice were selected to cover the rice genome (Fig. 1). Almost all AC-derived plants showed homozygous phenotypes.

Table 1. Segregation of RFLP markers in two anther culture derived populations (DR, OR) and one F₂ population, each of which were produced from a 'Nipponbare' (N.) × 'Milyang 23' (M.) F₁ rice

Chromosome	Markers	Segregation		
		DR N.:M.	OR N.:M.	F ₂ N.:H.:M.
1	<i>XNpb346</i>	25:24	30:20	13:28:8
	<i>XNpb393</i>	29:20	31:19	14:22:12
	<i>XNpb364</i>	31:19 ^{@@}	35:15 ^{*@@@}	9:25:15
	<i>C161</i>	27:22	23:26	9:25:14
2	<i>XNpb298</i>	23:26	30:20 [@]	9:26:14
	<i>XNpb45</i>	21:28	29:21	15:22:12
	<i>XNpb227</i>	20:29	22:25	11:23:13
	<i>XNpb349</i>	17:33 [*]	18:32 [*]	7:28:14
3	<i>XNpb48</i>	25:22	25:24	11:23:15
	<i>C136</i>	22:28	23:27	13:22:14
	<i>XNpb40</i>	26:24	25:25	15:22:13
	<i>XNpb144</i>	34:15 ^{**@@}	29:21 ^{@@}	6:19:24 ^{**}
	<i>XNpb74</i>	35:15 ^{**@@}	32:18 ^{**@@}	9:20:21 ^{**}
4	<i>XNpb279</i>	28:21	29:21	16:21:12
	<i>XNpb203</i>	28:22	25:25	16:20:13
	<i>XNpb271</i>	30:19	20:27	13:26:10
	<i>XNpb331</i>	30:19	20:27	13:26:10
5	<i>XNpb197</i>	30:19	20:27	13:26:10
	<i>R569</i>	24:26	25:25	8:24:15
	<i>XNpb105</i>	19:31	15:35 [*]	5:24:20 ^{**}
6	<i>XNpb25</i>	29:19	23:27	10:39 ^b
	<i>XNpb209</i>	29:20 [@]	22:26	11:19:19 [*]
	<i>C235</i>	22:27	21:28	9:21:19 [*]
7	<i>XNpb12</i>	22:27	25:25	6:25:18 [*]
	<i>XNpb342</i>	19:30	15:32 [*]	5:24:19 [*]
	<i>C615</i>	23:27	13:37 ^{*@@@}	15:27:8
8	<i>XNpb338</i>	19:30 ^{@@}	18:31 ^{@@}	17:22:10
	<i>XNpb152</i>	23:27	19:31 ^{@@}	18:22:10
	<i>XNpb117</i>	24:25	19:30 ^{@@}	20:19:10 [*]
	<i>C213</i>	24:25	23:26	7:14:5
	<i>XNpb56</i>	21:29 [@]	27:23	14:29:7
9	<i>G1073</i>	32:17 [*]	26:23	15:25:9
	<i>XNpb104</i>	32:17 [*]	24:24	14:24:12
	<i>XNpb38</i>	29:20	23:24	12:26:10
	<i>XNpb278</i>	28:22	22:28	10:25:14
	<i>XNpb36</i>	28:22	27:23	12:23:15
10	<i>XNpb160</i>	23:26	25:24	8:23:18 [*]
	<i>XNpb385</i>	22:28	22:28	9:22:16
	<i>XNpb293</i>	19:31	16:34 [*]	29:21 ^{**c}
	<i>G1084</i>	37:12 ^{**@@}	34:15 ^{**}	14:27:7
11	<i>XNpb37</i>	36:10 ^{**@@}	38:11 ^{*@@@}	13:24:13
	<i>XNpb127</i>	21:25	33:16 ^{**@@}	10:23:15
	<i>XNpb186</i>	21:28	22:28	15:23:11
12	<i>XNpb257</i>	4:46 ^{**@@}	19:31	13:23:13
	<i>XNpb179</i>	6:44 ^{**@@}	18:32 ^{*@}	14:26:10
	<i>XNpb169</i>	24:26	23:27	12:25:13
	<i>XNpb148</i>	27:22	22:27	8:25:16
12	<i>XNpb307</i>	32:17 ^{*@}	26:24	11:25:13
	<i>XNpb124-1</i>	31:18 ^{@@}	24:25	9:24:14
	<i>XNpb193</i>	25:24	23:27	11:25:13

Segregation deviations from the theoretical ratios (1:1, 1:2:1 or 3:1) are indicated by * ($P < 0.05$) or ** ($P < 0.01$), and from the parental allele frequency in an F₂ population calculated using the observed segregation ratios are @ ($P < 0.05$) or @@ ($P < 0.01$).

^a H.: heterozygous for parental alleles

^b N.:M. (dominant marker, the theoretical ratio is 1:3)

^c N.:M. (dominant marker, the theoretical ratio is 3:1)

OR populations due to an excess of the *japonica* allele. On the other hand, the *indica* allele of the marker was overrepresented in the F_2 population. On chromosome 7, the segregation of a marker *C615* was distorted from the 1:1 ratio only in the OR population. Among three markers from chromosome 10, a marker *XNpb37* represented the highest distortion in segregation in the two AC-derived populations. On chromosome 11, the segregation of two markers *XNpb257* and *XNpb179* was highly distorted in the DR population. On chromosome 12, the segregation distortion of a marker *XNpb307* was specific to the DR population. The segregation ratios of these markers in the AC-derived populations were also significantly different ($P < 0.05$) from those in the F_2 population.

DISCUSSION

Fifty RFLP markers distributed on the rice chromosomes provided a precise description of the parental allele frequency in AC-derived populations over the whole rice genome. Ten, eleven and ten of fifty RFLP markers showed distorted segregation in the two AC-derived populations and the F_2 population, respectively. In F_2 populations derived from various crosses between distantly related cultivars, segregation distortion of genetic markers have been often reported (Nakagahra, 1972; Oka, 1974; Sano, 1990). In this experiment, distorted segregation was also observed in the F_2 population derived from a cross between Nipponbare and Milyang 23. Thus, to estimate the chromosomal regions showing distorted segregation caused by the anther culture, segregation ratios of distorted loci observed in AC-derived populations must be compared with that of the same loci in F_2 populations. Some markers (e.g.; *XNpb105*, *XNpb342*, *XNpb293*) distorted toward the same alleles both in the AC-derived populations and in the F_2 population. This distortion may be due to the selection of microspores before the mid-uninucleate stage of microspores when anthers were isolated to culture. Such markers may be linked to the factors that generate sterile pollen or reduce pollen viability, and be less transmitted to the AC-derived populations as well as the F_2 population. On the other hand, seven and six markers in the two AC-derived populations, which were located on chromosomes 1, 3, 7, 10, 11 and 12, were distorted both from the 1:1 ratio and from the segregation ratios in the F_2 population. The chromosomal regions involving these markers might be selected during anther culture.

In maize DH populations, Bentolila et al. (1992) and Murigneux et al. (1993) indicated that distortions in segregation were strongly affected by a difference in anther culturability of the two parental lines and showed that 72 and 52% of RFLP markers were distorted in DH populations derived from crosses between lines highly responsive to anther culture and lines poorly responsive to it. In barley DH lines derived from anther culture of an F_1

plant one parent of which showed a better response to *in vitro* culture, Graner et al. (1991) also showed that 43% of RFLP markers were distorted. However, in this study, only about 20% of RFLP markers showed segregation distortion in the two AC-derived populations although the populations had derived from a cross between a highly responsive and a poorly responsive cultivar. The percentages of the markers showing segregation distortion in AC-derived populations of rice were lower than those in maize and barley. Additionally, the percentage of distorted markers in the AC-derived populations was similar to that in the F_2 population. Thus, distortion in segregation does not appear to be a major drawback for the use of AC-derived populations for rice breeding and genetics.

The number and the loci of the markers showing distorted segregation in DH populations were varied with parent combinations of F_1 plants (Guiderdoni, 1991; Murigneux et al., 1993; Lefebvre et al., 1995), and were different between an AC-derived population and a *Hordeum bulbosum* method-derived population (Powell et al., 1986). However, the interaction between different methods of anther culture and the number and the loci of distorted markers has not been examined. In this study, some of the markers showing segregation distortion were different in the two populations produced through different methods of anther culture. A marker *C615* on chromosome 7 and a marker *XNpb307* on chromosome 12 showed distorted segregation only in the OR and DR populations, respectively. Moreover, the segregation of markers *XNpb257* and *XNpb179* on chromosome 11 was highly distorted in the DR population. These findings indicate that the distorted region might be affected by anther culture methods. In other words, when other anther culture methods are applied, the distorted region will change. Therefore, the distorted region may be partially controlled through the anther culture technique itself.

In maize and barley, the chromosomal loci involving the genes favorable for tissue-culture responses have been mapped by RFLP markers (Cowen et al., 1992; Armstrong et al., 1992) and isozyme markers (Komatsuda et al., 1993). Additionally, diallel analysis of anther culturability in rice implied that the traits of callus induction and plant regeneration were controlled by nuclear genes (Quimio and Zapata, 1990). Hence, genes controlling anther culture are thought to be present, and the six regions selected during anther culture were expected to involve those gene(s). Further analyses will be necessary to determine the effects of the six regions for anther culturability.

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