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DETECTION OF ANDROCLONAL VARIATION IN ANTHER-CULTURED RICE LINES USING RAPDS

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Summary

Random amplified polymorphic DNA analysis was used to determine the occurrence and extent of variation in rice (*Oryza sativa* L.) plants regenerated from anther culture. Androclonal variation in morphologically uniform progenies was detected using 40 10-mer oligonucleotide arbitrary primers. Among 27 plants from nine anther culture-derived lines, variation was detected in three plants from two lines by two primers, namely UBC 160 and UBC 209. Primer UBC 160 amplified a polymorphic band on one of the three progenies from DH-34, while UBC 209 detected polymorphisms on two out of three progenies from line DH-58. Apart from these, the amplification products were monomorphic across all the regenerants from anther culture-derived plants. Out of 40 tested primers, no difference in the banding pattern was observed in three seed-derived plants. The significance of possible androclonal variation at the DNA level in rice doubled haploid breeding and genetic mapping is discussed.

Key words: double haploid; genetic variation; *Oryza sativa*; polymorphism.

Introduction

Rice (*Oryza sativa* L.) is widely cultivated and provides the main source of food for more than 50% of the world's population. It is also the most promising cereal for physical mapping because of its small genome size (430 Mb), about three times that of *Arabidopsis*, as well as the low proportion of repetitive sequences in its genome (Arumuganathan and Earle, 1991).

Doubled haploids (DHs) have become a promising tool for significantly shortening the breeding cycle in some important crops. This system is fully compatible with other biotechnological approaches such as mutation techniques for gene character identification or genetic engineering. DH lines are ideal materials for molecular analysis because they are highly homozygous (Nagamura et al., 1997). In rice, DHs have been produced in many genotypes using anther and microspore culture (Raina, 1989). Much of the gene mapping work has been carried out using DH lines derived from rice anther culture (Zhu et al., 1993; Huang et al., 1994; Yamagishi et al., 1996; Maheswaran et al., 1997; Nagamura et al., 1997; Subudhi and Huang, 1999).

Since somaclonal variation was first defined (Larkin and Scowcroft, 1981), it has been widely documented in tissue culture-raised plants at the morphological, chromosomal, biochemical and molecular levels in many plant species and extensively reviewed (Brown, 1991; Karp, 1991). The extent of somaclonal variation using DNA markers has been assessed in tissue culture and primary regenerants of rice (Muller et al., 1990; Godwin et al., 1997). Polymorphism at the DNA level among the somaclonal families which were phenotypically normal was reported in *Triticum* (Brown, 1991), in rice (Godwin et al., 1997) and in *Populus deltoides* (Vijay et al., 1995). These alterations included gene methylation changes, DNA rearrangements and alterations in copy number. The phenotypic variants recovered from the anther culture-derived plants, e.g. short growth duration, increased grain yield, superior grain quality, have been used to develop new rice cultivars (Raina, 1989). However, silent genetic changes without any phenotypical expression might interfere with directed genetic manipulation and generate misunderstandings of the results of gene mapping or mutation induction using DH lines. Moreover, for induced mutation studies, highly uniform materials, such as DH lines, are desirable. Therefore, it is important to ascertain genetic purity of the anther culture-derived lines before they are used for molecular studies.

RAPD (random amplified polymorphic DNAs) is a powerful technique for the identification of the genetic variation (Welsh and McClelland, 1990). It has the distinct advantage of being technically simple and quick to perform, requiring only small amounts of DNA compared to the restriction fragment length polymorphism (RFLP) analysis (Welsh et al., 1991). Using RAPDs we examined genetic variation in phenotypically uniform plants of rice cv. Taipei 309 raised from anther culture.

Materials and Methods

Callus induction. *Japonica* rice (*Oryza sativa*. L) var. Taipei 309 was used in the present study. Plants were grown in pots in the greenhouse and maintained at 30/20°C day/night temperature and a minimum relative humidity of 70% during the day. Panicles were harvested from uniform donor plants at the booting stage when the distance between the subtending leaf and the flag leaf was 8–12 cm. They were subjected to cold

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pretreatment at 8°C for 8 d. After that, the panicles were surface-sterilized by immersing in 70% ethanol for 20 s and then in 10% Clorox (commercial bleach containing 5.2% w/v, NaOCl) for 10 min, and washed three times with sterile distilled water before plating. The anthers were plated on N6 semi-solid medium (Chu et al., 1975) supplemented with 2 mg l^{-1} (9.05 μM) 2,4-dichlorophenoxyacetic acid, 60 g l^{-1} sucrose (pH 5.8) and incubated in the dark at 25°C for callus induction for 6–8 wk.

Plant regeneration and maintainance. Callus pieces of at least 2 mm size were transferred to basal MS semi-solid medium (Murashige and Skoog, 1962), supplemented with 1 mg l^{-1} (4.44 μM) 6-benzyladenine (BA), 0.5 mg l^{-1} (2.69 μM) α-naphthaleneacetic acid, 30 g l^{-1} sucrose (pH 5.8), and incubated under a 12-h light photoperiod with 66 μmol m^{-2} s^{-1} supplied by cool-white fluorescent lamps at 25°C for plant regeneration. Regenerated plants (R_0) were grown in pots to maturity and assigned a line number (1–21). Seeds were harvested from each plant individually for raising the R_1 generation. Twenty-one lines of three seeds each were grown for visual evaluation of morphological characters. Three seeds from control plants were also grown. Nine lines which were phenotypically identical to the three control and their sister plants for their plant height, plant type, leaf shape, seed size and panicle type were selected for RAPD analysis.

DNA extraction. DNA of 27 phenotypically uniform plants from nine androgenic lines and three control plants was extracted from immature plants following the method described by Lu and Zheng (1992).

Polymerase chain reaction (PCR). Forty 10-mer oligonucletide primers (University of British Columbia, Vancouver, Canada) were randomly chosen for the study. Each 30 μl reaction contained 15 ng of DNA template, 1.25 units of *Taq* DNA polymerase (Roche Diagnostics GesmbH, Mannheim, Germany), 0.2 μmol primer, 0.17 mmol dNTPs and PCR buffer (Roche) to a final concentration of 67 mM Tris–HCl, 1.7 mM $MgCl_2$, 167 mM KCl, pH 8.4.

PCR was initiated by a denaturation step at 94°C for 4 min, then the reaction was subjected to 45 cycles of 93°C for 20 s, 36°C for 40 s, 72°C for 60 s with a final elongation step of 4 min at 72°C. PCR was performed on a Cetus 9600 geneAmp PCR system (Perkin Elmer, Weiterstadt, Germany).

Amplification products were resolved by electrophoresis on a 1.2% agarose gel with ethidium bromide in Tris–borate electrophoresis (TBE) buffer and visualized under UV illumination. Since the RAPDs technique is very sensitive to even slight modifications of the reaction conditions, the reactions were repeated to establish reproducibility of results under strict control of the reaction conditions when variations were observed.

Results and Discussion

Twenty-seven plants coming from nine androgenic lines were phenotypically uniform with their sister plants and three seed-derived control plants. These plants were selected for RAPD analysis. Most primers used in the present study produced PCR products in the size range of 500–3000 bp. About 160 RAPD bands were scored from 40 10-mer arbitrary primers with an average of four bands per primer. Among the primers studied, all 40 generated the same banding patterns (Fig. 1) among the three control plants (Table 1) indicating that the seed-derived plants were uniform.

Generally, phenotypically identical plants derived from anther culture are believed to be truly homozygous. However, in the present study, among 27 plants from nine DH lines, variation at the molecular level was detected among three plants from two lines with two primers (Table 1). Primer UBC 209 (5′-TGCACTGGAG-3′) detected polymorphisms on two out of three progenies from line DH-58 (Fig. 2), while UBC 160 (5′-CGATTCAGAG-3′) amplified a polymorphic band on one of the three progenies from DH-34 (Fig. 3). In this study, the variation frequency of the DH lines was 22% (two out of nine), that of tested samples was 0.28% (two out of 1080, a total of three replicates from nine lines with 40 primers). The remaining plants of DH-58 and of DH-34 had the same banding patterns as the three seed plants and the other 24 anther culture plants. Our results showed that the variation at the molecular level

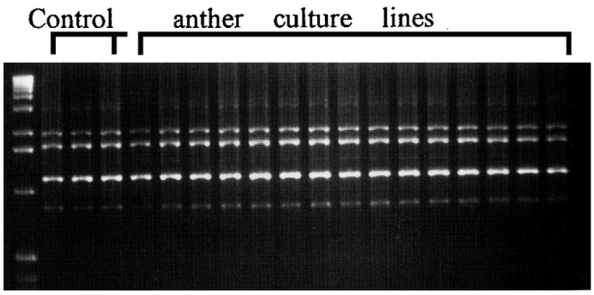

Fig. 1. Monomorphic bands among three control and anther-cultured lines of rice with primer UBC 206.

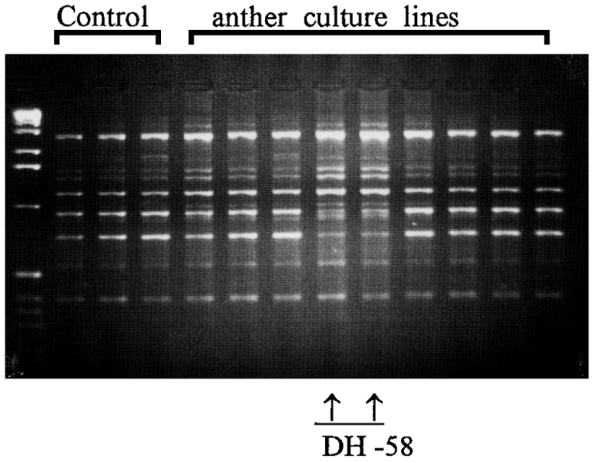

Fig. 2. Primer UBC 209 detected polymorphisms on two out of three progenies from *line* DH-58 of rice.

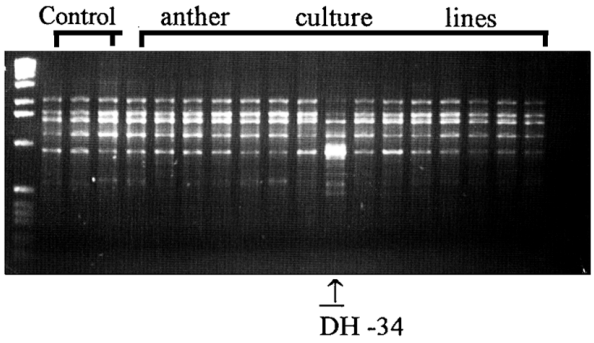

Fig. 3. Primer UBC 160 amplified a polymorphic band on one of the three progenies from DH-34 of rice.

was high among the phenotypically uniform progenies of anther-cultured DH lines. A high level of detectable polymorphisms was also found in genomic DNA of phenotypically normal R_0 *Triticum tauschii* regenerants (Brown, 1991). In rice (Godwin et al., 1997) and in *Populus deltoides* (Vijay et al., 1995) differences in RAPD profile among phenotypically identical somaclonal families was reported. Two possible explanations were described (Godwin et al., 1997): either plants were only assessed in the glass house for major phenotypic alteration or a significant proportion of RAPD loci were found in regions of highly repeated sequences which do not relate to

TABLE 1

THE DETECTED PCR BANDS AND POLYMORPHISMS AMONG SEED PLANTS AND ANTHER-CULTURED LINES OF RICE

Code	Primer no.	Sequence	Bands	Polymorphisms among seed plants	Polymorphisms among anther lines and seed plants
1	UBC 95	5′-GGGGGGTTGG-3′	3	No	No
2	UBC 96	5′-GGCGGCATGG-3′	4	No	No
3	UBC 97	5′-ATCTGCGAGC-3′	3	No	No
4	UBC 98	5′-ATCCTGCCAG-3′	1	No	No
5	UBC 141	5′-ATCCTGTTCG-3′	1	No	No
6	UBC 142	5′-ATCTGTTCGG-3′	3	No	No
7	UBC 143	5′-TCGCAGAACG-3′	2	No	No
8	UBC 149	5′-AGCAGCGTGG-3′	6	No	No
9	UBC 150	5′-GAAGGCTCTG-3′	4	No	No
10	UBC 151	5′-GCTGTAGTGT-3′	2	No	No
11	UBC 152	5′-CGCACCGCAC-3′	9	No	No
12	UBC 153	5′-GAGTCACGAG-3′	4	No	No
13	UBC 154	5′-TCCATGCCGT-3′	3	No	No
14	UBC 155	5′-CTGGCGGCTG-3′	5	No	No
15	UBC 156	5′-GCCTGGTTGC-3′	1	No	No
16	UBC 157	5′-CGTGGGCAGG-3′	6	No	No
17	UBC 159	5′-GAGCCCGTAG-3′	5	No	No
18	UBC 160	5′-CGATTCAGAG-3′	5	No	Yes
19	UBC 167	5′-CCAATTCACG-3′	3	No	No
20	UBC 168	5′-CATGATGTGC-3′	5	No	No
21	UBC 178	5′-CCGTCATTGG-3′	1	No	No
22	UBC 180	5′-GGGCCACGCT-3′	4	No	No
23	UBC 181	5′-ATGACGACGG-3′	4	No	No
24	UBC 182	5′-GTTCTCGTGT-3′	2	No	No
25	UBC 184	5′-CAAACGGCAC-3′	8	No	No
26	UBC 185	5′-GTGTCTTCAC-3′	3	No	No
27	UBC 186	5′-GTGCGTCGCT-3′	2	No	No
28	UBC 195	5′-GATCTCAGCG-3′	2	No	No
29	UBC 196	5′-CTCCTCCCCC-3′	3	No	No
30	UBC 203	5′-CACGGCGAGT-3′	7	No	No
31	UBC 204	5′-TTCGGGCCGT-3′	6	No	No
32	UBC 205	5′-CGGTTTGGAA-3′	1	No	No
33	UBC 206	5′-GAGGACGTCC-3′	4	No	No
34	UBC 208	5′-ACGGCCGACC-3′	7	No	No
35	UBC 209	5′-TGCACTGGAG-3′	5	No	Yes
36	UBC 212	5′-GCTGCGTGAC-3′	7	No	No
37	UBC 303	5′-GCGGGAGACC-3′	4	No	No
38	UBC 304	5′-AGTCCTCGCC-3′	4	No	No
39	UBC 309	5′-ACATCCTGCG-3′	6	No	No
40	UBC 310	5′-GAGCCAGAAG-3′	5	No	

TABLE 2

INFORMATION ON POLYMORPHISMS OF ANTHER-CULTURED LINES OF RICE

DH lines	Donor plant number	No. of plants observed	Polymorphism comparing with control	No. of plants with polymorphism
DH-3	1	3	No	0
DH-32	2	3	No	0
DH-34	3	3	Yes	1
DH-46	4	3	No	0
DH-54	5	3	No	0
DH-58	5	3	Yes	2
DH-59	5	3	No	0
DH-64	6	3	No	0
DH-66	6	3	No	0

any phenotypic variation. In wheat it has been reported that RAPD bands were in high copy sequences and not in the transcribed DNA (Devos and Gale, 1992). Similarly, a significant proportion of RAPD variation detected in rice may not lead to any change in expressed sequences. Thus, even the phenotypically normal DH lines may have cryptic changes. The widespread assumption that a normal phenotype after regeneration reflects a lack of variation must now be reconsidered.

Although three lines DH-54, DH-58, and DH-59 were derived from the same donor plant no. 5, no variation of DH-54 and DH-59

was observed with the 40 primers (Table 2) used. Since plants obtained from the same donor plant showed variation, they may have been initiated from different cells, or it could be that the variation was induced at a later stage. After analyzing maize callus cultures, Edallo et al. (1981) proposed that any single callus should be regarded as an assembly of genetically different cells. The genetic changes in plants derived from tissue and cell cultures may be induced by changes in chromosome number and structure (Armstrong and Phillips, 1988; Fourre et al., 1997), transposable element activation (Brettell and Dennis, 1991; Shimamoto, 1995), copy number alterations (Deumling and Clermont, 1989), methylation changes, point mutations (Muller and Brown, 1990; Brown, 1991; Kaeppler, 1992; Smulderms et al., 1995), DNA deletion (Kawata et al., 1995) and re-arrangements (Levall et al., 1994). Ziauddin and Kasha (1990) concluded that the possible origins of variation in haploid cell cultures could be variability in the explant material, variation induced by callus induction, and the effect of tissue culture components.

Anther culture is a valuable tool for conventional breeding, mutation induction, gene transformation, and genetic studies. In order to get the correctly expressed transferred/mutated gene, the recipient plants should obviously be identical to control non-tissue culture-derived plants, to enable their participation in breeding programs. However, variation induced by anther culture will influence the results of expression of a transferred/mutated gene. For genetic map construction, DH are propagated because of the genetic identity of the material from anther culture. The existing androclonal variation in DH lines may seriously interfere with mapping results; especially, silent genetic changes of DH lines could lead to misinterpretation of gene mapping results obtained with DH lines.

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