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J.-H. Xie · J. Lu · J.-Y. Zhuang · H.-X. Lin
H.-R. Qian · M.-W. Gao · K.-L. Zheng

Identifying different types of de-differentiated microspores from *indica-japonica* F_1 hybrids with subspecies-differentiating RFLP probes in rice

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Abstract The *indica*, *japonica* and intermediary types of de-differentiated microspores from *indica-japonica* F_1 hybrids were identified with 11 subspecies-differentiating RELP probes in rice (*Oryza sativa* L.). The results showed that the distribution of *indica*, *japonica* and intermediary types of de-differentiated microspores could be easily detected in a simple and quick way using the RFLP method. Moreover, the microspores from the same F_1 hybrid but inoculated onto different media, or microspores from different F_1 hybrids when inoculated onto the same medium, often displayed distinctive distribution curves of de-differentiated microspores types, indicating that the media employed in this experiment had high selectivity for the de-differentiation of certain types of microspores. The application of the RELP method to de-differentiated microspore identification is of great theoretical and practical significance in rice doubled-haploid breeding.

Key words De-differentiated microspore · *Indica-japonica* F_1 hybrid · Restriction fragment length polymorphism (RFLP) · Subspecies-differentiating probe · *Oryza sativa* L.

Introduction

Rice is one of the most important crops in China as well as in other parts of the world. Many breeders have tried *indica-japonica* intersubspecies crosses in order to increase variation and enrich the genetic germplasm pool in rice, but little progress has been made due to high sterility and the irregular segregation of traits in their progenies (Zhy et al. 1991). According to current studies, these problems could be partially overcome through the anther culture of intersubspecies F_1 hybrids. Li (1991) reported that the seed setting rate of plants derived from subspecies F_1 hybrid anther culture could reach 80%, which was 2–3 times higher that that of F_1 plants. Moreover, margin of segregation of *indica-japonica* F_1 pollen-derived plants was much higher than that of intercultivar F_1 plants in many characters, and some regenerants of intermediary type were obtained (Zhu et al. 1991). A number of researchers have pointed out that *japonica* rice cultivars are generally more responsive to anther culture than *indica* (Cho and Zapata 1990; Chen et al. 1991). The difference in culture response between *indica* and *japonica* could influence the de-differentiation level of various types of microspores of intersubspecies F_1 hybrids and the trait segregation of gameto-regenerants (Xue 1991). Previous findings demonstrated that most of the *indica-japonica* F_1 hybrid pollen cultures tended to regenerate *japonica* plants or intermediary type, and only a few of them were *indica* or an *indica*-like type, which refers to a type close to *indica* (Cai et al. 1983; Zhu et al. 1991). In addition, the medium itself to a certain extent had a selective effect on the de-differentiation of the different types of microspore (Shen et al. 1978). Therefore, it is of great practical and theoretical significance to study the selective effects of various media and to find a medium with neutral selectivity between subspecies. However, the low frequency of plant regeneration, with a large quantity of albinos, in rice microspore culture is still a limiting factor for studying the selectivity of the culture medium. Moreover, the traditional classification of rice subspecies based on morphological traits and electrophoretograms of isozymes (Cheng 1985; Glaszmann 1987; Oka 1992) requires the use whole plants, which is time and labor intensive. Misclassification could also occur due to changes of environmental factors or to differences in personal skill (Morishima et al. 1981).

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J. -H. Xie(✉) · M.-W. Gao
Institute of Nuclear Agriculture Sciences, Zhejiang Agricultural University, Hangzhou 310029, China

J. Lu · J.-Y. Zhuang · H.-X. Lin · H.-R. Qian · K.-L. Zheng
Biotechnology Department, China National Rice Research Institute, Hangzhou 310006, China

Restriction fragment length polymorphisms (RFLPs) are most widely used as molecular markers in plant genetic research (Saghai-Maroof et al. 1984; Tanksley et al. 1989). In the classification of rice subspecies, RFLP has been regarded as an effective way to distinguish varieties in a subspecies with high reliability and reproducibility (Zheng et al. 1994; Qian et al. 1995). Classifying rice types with RFLP provides abundant information on variation at the DNA level between *indica* or *japonica* rice. Isolated microspore culture combined with RFLP analysis could facilitate the classification of regenerated plants derived from *indica-japonica* F_1 microspores, since each callus originates from a single microspore. The distribution curve of the *indica*- or *japonica*-type of callus detected by RFLP could thus represent the distribution curve of the type of microspores de-differentiated, and so define the distribution curve of the type of regenerated plant.

The present paper reports the identification of the types of de-differentiated microspores from *indica-japonica* F_1 hybrids, and the distribution of calli of various types formed on two media among three hybrid populations with 11 subspecies-differentiating RFLP probes.

Materials and methods

Plant materials

Two *indica-japonica* F_1 hybrids, W36 (Zhenshan 97A/02428) and W42(Zhenshan 97A/Ballila), were used in this experiment. Zhenshan 97A is a male-sterile line of the *indica* type from China; Ballila from Italy and 02428 from China are, respectively, a *japonica* variety and line. Seeds of these two F_1 hybrids were kindly provided by Mr. Xie Xiaobo, China National Rice Research Institute, Hangzhou, China, and were grown in the field at Zhejiang Agricultural University experimental station (Hangzhou) during a normal growing season. Panicles were collected at the booting stage when the anthers contained microspores at the mid- to late-uninucleate stage. The panicles were pre-treated at 6 ± 1°C for 17 days, then surface sterilized with 75% ethanol before anthers were aseptically removed from the panicle for microspore isolation.

Callus induction

Calli were induced from microspores in liquid induction media I2 (Xie et al. 1995) or YP (Pescitelli et al. 1989). The combinations of media and plant materials employed are shown in Table 2. The microspsore callus-induction method was similar to that described previously by Xie et al. (1995), except that the anther pre-culture period was 10 days. After 2–3 weeks of subculture, mini calli derived from microspores were transferred to I2 or YP medium, respectively, in tubes solidified with 0.6% (w/v) agar. One callus was placed in each tube in order to ensure that each separated callus represented one single microspore. The calli were used in DNA extraction when they had reached a size of 0.4–1.2 g.

RFLP markers

Eleven RFLP markers, i.e. subspecies-differentiating probes (Qian et al. 1995), were used in the present study. Among them, clones RG256, RG345, RG351, RG358, RG375, RG462, RG482, RG620, RG667 and RG684 are random rice genomic DNA fragments from Dr. Tanksley of Cornell University, USA, and clone G318 was from Dr. Uchimiya of the Institute of Applied Microbiology, the University of Tokyo, Japan. All markers except RG684 have already been mapped on rice chromosomes (McCouch et al. 1988; McCouch and Tanksley 1991; Oba et al 1991; Uchimiya, personal communication).

RFLP detection

The genomic DNAs of the three parents and that of the three callus populations of intersubspecies F_1 hybrid were extracted. The moisture of the calli was absorbed partially by tissue paper before DNA extraction. Then the calli were ground into fine powder with arenaceous quartz in liquid nitrogen. The DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blotting were as described previously (Zheng et al. 1990; Lu and Zheng 1992) DNAs were digested with two enzymes, *Eco*RI and *Hin*dIII. The plasmids were hexamer labelled with ^{32}P-dCTP of high specific activity ($5–10 \times 10^8$cpm/ug) and used as probes on filters of rice DNAs. All enzymes and DNA labelling kits were obtained from GIBCO-BRL; Genescreen-plus nylon membranes came from the Dupont Company.

Data analysis

The hybridization patterns of each sample of the three populations and of the parantal materials were investigated for every probe-enzyme combination. The hybridization fragment that belonged to the *japonica* type was defined as "1" while that of the *indica* type was defined as "0". The mean of 11 markers for a single sample was calculated. The classification of each callus (*indica*- or *japonica*-type) was based on the mean of 11 marker hybridization results.

Results and discussion

Subspecies-differentiating hybridization patterns of three callus populations and their parents

The *indica* or *japonica* type of the calli in three hybrid populations and their parents were detected by two restriction enzymes, *Eco*RI and *Hin*dIII, in combination with 11 subspecies-differentiating probes. The results showed that the hybridization fragments of most probe-enzyme combinations differed obviously between the *indica* and *japonica* types and could easily distinguish between these types in both the parental materials and the three hybrid populations. The molecular weights of the *indica* and *japonica* hybridization fragments in various probe-enzyme combinations are shown in Table 1. In the *indica* type, only one band (4.0 kb) was seen in the RG351-*Eco*RI combination while two bands (4.0 and 3.0 kb) appeared in the *japonica* type in the same probe-enzyme combination. The lengths of the *indica* and *japonica* hybridization fragments of RG482-*Eco*RI were very close to each other (6.2 and 6.0 kb). Probes RG358, RG 375 and RG318 readily hybridized with the DNA of *indica* but not with the DNA of *japonica* and are considered as *indica*-specific probes. According to Qian et al. (1995), the genomic DNA sequences homologous to these probes have been deleted in *japonica*. With 11 probe-enzyme combinations, the paternal materials 02428 and Billila were of the *japonica* type, in full agreement with previous results (Zheng et al. 1994), while the maternal line Zheshan 97A is of the *indica* type. Figure 1 exhibits the Southern hybridization fragment

Table 1 Probes with recommended enzymes for subspecies identification and the length of their hybridization fragments from *indica-japonica* F_1 hybrid microspore-derived calli

Probe	Chromosome	Enzyme	Length of hybridization fragment (kb)	
			indica	*japonica*
RG256	2	*Eco*RI	9.4	7.2
RG345	1	*Hin*dIII	14.0	8.8
RG351	7	*Eco*RI	4.0	4.0
RG358	9	*Hin*dIII	9.0[b]	–
RG375	1	*Eco*RI	8.5[b]	–
RG462	1	*Eco*RI	5.0	3.0
RG482	3	*Eco*RI	6.2	6.0
RG620	4	*Eco*RI	9.0	5.8
RG667	9	*Eco*RI	23.0	13.0
RG684	?[a]	*Hin*dIII	9.4	3.0
G318	12	*Eco*RI	5.8[b]	–

[a] ? Not yet mapped
[b] Absence of this band in some *indica* varieties

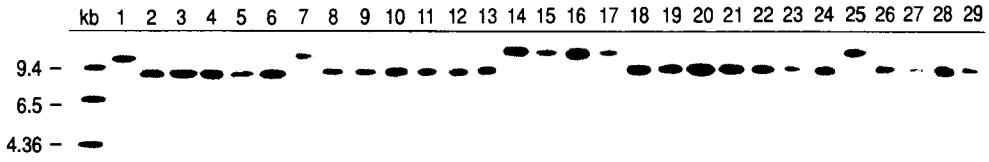

Fig. 1 The Southern-hybridization fragment of maternal line Zhenshan 97A(*1*), paternal Ballila(*2*) and 27 calli of W42-12 (*3–29*) using probe RG345 and the restriction enzyme *Hin*dIII

of Zhenshan 97A, paternal variety Ballila, and 27 calli of W42-I2 using RG345/*Hin*dIII. The *indica* parent Zhenshan 97A had a 14.0-kb fragment while the *japonica* parent Ballila had a 8.8-kb band. Among the 27 callus DNAs, 21 hybridization fragments were the same as that of Ballila, and the remaining six callus DNAs were identical to Zhenshan 97A. The hybridization patterns of the other groups of callus DNAs in the three populations were also either the same as that of the maternal line Zhenshan 97A, or as those of the paternal materials 02428 or Ballila. The length of the hybridization bands of *indica* or *japonica* were identical with those described in the previous report of Qian et al. (1995).

Since all the above results were readily reproducible, RFLP analysis provided accurate information on the gametic variation at the DNA level of the hybrids between *indica* and *japonica* rice.

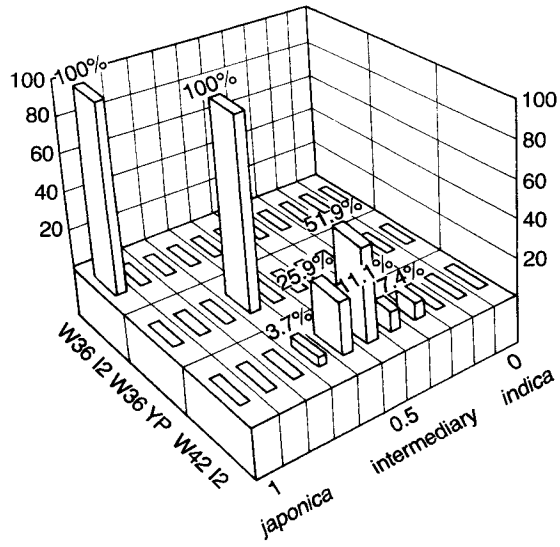

Fig. 2 Distribution of *indica-* or *japonica-type* calli in three populations

Distribution of the calli of *indica-* or *japonica*-type in three populations

In the present study, RFLP analysis with two restriction enzymes and 11 probes usually showed a biased distribution of the *indica* or *japonica* type of callus in the three populations each of which possesed its own distinctive distribution curve (Fig. 2). Theoretically, the microspores of the *indica-japonica* F_1 hybrids should segregate for at least five types: namely, a small percent of either the *indica* or the *japonica* type, a large intermediary type component, as well as types close to the *indica* or *japonica* parents. If every microspore had an equal ability to form a callus, then RFLP analysis of the calli derived from the *indica-japonica* F_1 hybrids would exhibit a normal distribution in terms of the segregation for these types. It is clear, however, that the distribution were far from normal, so that each microspore was assumed not to have same ability to form a callus under the present culture conditions, indicating the existence of strong selective factors in the media.

The distribution of calli of the *indica* or *japonica* type in two populations which were induced from the same F_1 hybrid, W36 (Zhenshan 97A / 02428), but in two different induction media, were not identical (Table 2). In the W36 – I2 combination, the hybridization patterns of all microspore calli with the 11 probe-enzyme combinations were of the *japonica* type. This means that only microspores of the *japonica* type were able to enter into

Table 2 Number of calli of different types detected by subspecies-differentiating probes in three hybrid combinations of rice

Hybrid combination	Medium	No. of calli analyzed	Probe/enzyme[a]																			
			RG256/E		RG345/H		RG351/E		RG358/H		RG375/E		RG462/E		RG482/E		RG620/E		RG667/E		RG684/H		G318/E	
			i	j	i	j	i	j	i	j	i	j	i	j	i	j	i	j	i	j	i	j	i	j
W36 (Zhenshan 97A/02428)	YP	27	0	27	0	27	0	27	0	27	0	27	0	27	0	27	0	27	0	27	0	27	0	27
W36 (Zhenshan 97A/02428)	YP	29	29	0	0	29	0	29	0	29	0	29	0	29	0	29	0	29	29	0	0	29	29	0
W42 (Zhenshan 97A/Ballila)	YP	27	0	27	6	21	27	0	17	10	3	24	13	14	16	11	10	17	27	0	27	0	7	20

[a] E = *Eco*RI, H = *Hin*dIII, i = *indica* type, j = *japonica* type

the de-differentiation phase when W36 microspores were cultured on I2 medium. The situation was quite different when the microspores of W36 were cultured in YP medium; in this case, only *japonica*-like type microspores, which refers to the microspores close to the *japonica* type, could form calli. With 11 probes-enzymes combinations, the DNA hybridization patterns of G318-*Eco*R I, RG256-*Eco*RI and RG667-*Eco*RI were of all of the *indica* type. As for the other eight probe-enzyme combinations, all the calli of W36 microspores in YP medium were of the intermediary and *japonica*-like types, with an average of 0.73 which was close to the *japonica* type.

Moreover, the culture response of different microspores from F_1 hybrids in the same I2 medium was also different (Table 2). In the W42-I2 population, all the hybridization patterns of the RG256-*Eco*RI combination were of the *japonica* type, whereas those of the RG351-*Eco*RI, RG667-*Eco*RI and RG684-*Hin*dIII combinations were of the indica type. The hybridization patterns of the seven other probe-enzyme combinations varied in respect of their distribution curves: 51.9% of the calli of W42-I2 belonged to the intermediary type, the other 48.1% calli represented a normal distribution around the intermediary type while all calli of the W36-I2 population were typical *japonica* types (Fig. 2). However, no parental type callus could be detected in the W42-I2 population. The *japonica* or *japonica*-like type microspores responded well to in vitro culture, and formed calli easily in the population. This phenomenon was mainly caused by the strict selectivity of the medium, the mechanism of which needs to be further investigated. In W36 and W42 populations, the maternal lines are the same, Zhenshan 97A, but paternal lines were different. So, the difference both in the de-differentiation of F_1 hybrid microspores and in callus formation may be attributed to the different paternal lines. For instance, in the male parent Ballila microspores of the *japonica* type are more capable of forming calli on I2 induction medium.

Both the genetic background of the donor plants and other factors, such as tissue culture conditions, could influence the development of isolated microspore cultures. The culture medium also has a selective effect on isolated microspore cultures. Therefore, changing the medium composition might eliminate or alleviate the selectivity for certain microspores to de-differentiate. The present experiment supports the previous finding at the DNA level that a majority of the pollen plants from intersubspecies F_1 hybrids had a tendency to be either of the *japonica* type or else to be intermediary or *japonica* like (Shen et al. 1978; Cai et al. 1983; Zhu et al. 1991). Some differences existed in the composition of the I2 and YP media; the predominant role of hormones in plant growth and development suggests that hormone involvement in tissue culture competence is of major importance (Arnison et al. 1990). In our experiment, the 1 mg/l 2,4-dichlorophenoxyacetic acid, and 1 mg/l naphthaleneacetic acid in the I2 medium, compared with the 1 mg/l 2,4-dichlorophenoxyacetic acid and the 2 mg/l

phenylacetic acid in YP medium might play an important role in the differences of selectivity which the media exhibited.

Because the induction medium has a strict selective effect on microspore de-differentiation, it is essential that media employed in the anther and microspore culture should be modified according to the different materials used.

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