Mapping of Loci Affecting the Cultural Efficiency of Microspore Culture of *Brassica rapa* L. syn. *campestris* L. using DNA Polymorphism

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Summary

A linkage map of *Brassica rapa* was constructed based on random amplified polymorphic DNAs (RAPDs), isozymes and restriction fragment length polymorphism (RFLP), using an F₂ population derived from the crossing of a Chinese cabbage line, 'Homei 09' and a club-root-resistant turnip line, 'Siloga S2'. The map covered 851 cM, about half of the genome, and consisted of 16 linkage groups. 'Homei 09' is highly responsive to microspore culture unlike 'Siloga S2'. To identify the loci affecting embryogenesis in microspore culture, frequencies of RAPD in a microspore-derived population obtained from an F₁ of the same parentage were scored. Several parts of the linkage map showed a conspicuous distortion toward 'Homei 09' alleles. The microspores of another F₂ population from the same parentage were cultured. Embryogenic ability and RAPD genotype of the each F₂ plant were scored. Embryogenic ability of the plants for two marker types (presence and absence) was determined at each RAPD locus. In some parts of the linkage map, the plants carrying 'Homei 09' alleles showed a higher ability of embryogenesis than those of 'Siloga S2' homozygotes. Comparison of the distortion data with those in the embryogenesis of the F₂ suggested that at least one part of the linkage map affected the embryogenic process in microspore culture. Genes in these areas of 'Homei 09' may play a key role in triggering the embryogenesis or in embryo development at the early stage of microspore culture.

Key Words : *Brassica rapa* syn. *campestris*, linkage map, microspore culture, embryogenesis, embryogenic ability, genetic distortion, doubled haploid.

Introduction

Microspore culture and anther culture are important

methods in haploid breeding, which is widely used for genetic analysis and is applied to breed homozygous lines for hybrid seed production in *Brassica* (Chen and Beeversdorf 1990, Kuginuki *et al.* 1997a, Voorrips *et al.* 1997). Microspores of *Brassica* species exposed to thermal stimulation at the onset of the culture can directly be grown into embryos (Keller and Armstrong 1978, 1979, Sato *et al.* 1989b). However, a large difference in the embryogenic ability has been observed among cultivars and lines in *Brassica* crops. Most of the common type Chinese cabbages cultivars (*Brassica rapa* L. syn. *campestris* L. ssp. *pekinensis*) show a low responsiveness (Kuginuki *et al.* 1997b). A line with a low ability of embryogenesis can, therefore, be crossed with a highly responsive line to obtain a homozygous line with a desirable genotype through microspore culture. However, distortion in gene frequency was reported in doubled haploid (DH) lines obtained from the microspore culture of *Brassica napus* (Cloutier *et al.* 1995) and anther culture of maize (Bentolila *et al.* 1992). The distortion may interfere with the genetic analysis and also decrease the possibility of incorporating some traits from a line with a low responsiveness into the DH lines. This phenomenon is a shortcoming in the utilization of microspore culture for genetic analyses and breeding programs. It is considered that the distortion was derived from preferential incorporation of microspore culture-responsive genes and adjacent areas on the chromosome into DH lines. A number of studies have already been published on the distortion in microspore-derived plants or DH lines of maize and rice (Yamagishi *et al.* 1998, Bentolila *et al.* 1992, Wan *et al.* 1992). Analyzing the distortion of two microspore-derived populations, Cloutier *et al.* (1995) identified putative genes associated with the responsiveness in *B. napus*. However no detailed data on the cultural efficiency of segregating population were reported. Therefore, it remains to be determined whether the distortion observed in the DH population is derived from differences in the embryogenic ability of the parents. Moreover no genetic studies have been reported on *B. rapa*.

Here, we studied the distortion in gene frequency in a microspore-derived population, and the data on the distortion were compared with the embryogenic ability

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of plants in an F_2 population. Mapping of loci affecting the embryogenesis may provide valuable information for utilizing microspore culture in the breeding of *Brassica* crops and also for studying the physiological and genetic control of the embryogenesis of plants.

Materials and methods

Plant materials

A clubroot-resistant (CR) European fodder turnip cultivar, 'Siloga', was selected for the resistance, then selfed two times and used as a parent ('Siloga S2'). Since 'Siloga' is a valuable source of CR, microspore culture of this cultivar was carried out several times, but the response was always very poor. The present parent 'Siloga S2' is, therefore, considered to lack microspore culture-responsive genes unlike 'Homei'. Another parent was a DH line, 'Homei 09', obtained through microspore culture of a Chinese cabbage cultivar of the tropical type, 'Homei'. This cultivar was found to be highly responsive to anther culture or microspore culture, and has been extensively used for microspore culture (Sato *et al.*, 1989a, Kuginuki *et al.*, 1997b). Three populations derived from the same parentage were used in this study. A linkage map was constructed using 95 F_2 plants (population I). A total of 63 regenerated plants derived from microspore culture of the F_1 were used for scoring the frequency of RAPD (population II). The chromosome numbers of these regenerants were not examined. Therefore this population may consist of a mixture of both haploids and DH. Natural chromosome doubling was, however, common in microspore culture of 'Homei' in our preliminary experiments (data not shown). Another F_2 population consisting of 56 plants, was used to score the embryogenic activity of the individuals (population III).

DNA analysis

True leaves of field- or greenhouse-grown plants were used for the DNA extraction as described by Murray and Thompson (1980).

Random amplification of genomic DNA was conducted as described previously (Hashizume *et al.* 1993). Random primers used in the present study were as follows: RA1014 to RA1035: Designed and synthesized at NIVOT, 10 mers. RA1211-RA1284: Designed and synthesized at NIVOT, 12 mers. OPG: Operon (Operon Technologies Inc., Alameda) primers G series, 10 mers. WA to WG: Wako primers A to G series (Wako pure chemicals industries, Ltd., Osaka), 12 mers. A total of 449 primers were screened in the present study. Only clear and reproducible RAPDs were used for the mapping and the genetic study. The RAPD bands were recovered from an agarose gel block using Suprec 01 (Takara Shuzo Co., Ltd., Kyoto), then ligated to a pCR vector (Invitrogen, Leek). The plasmid was used to transform *Escherichia coli* JM109. The inserts of the plasmids were used as a probe for the RFLP detection.

Five microgram of genomic DNA was digested with a restriction enzyme (*Bam*H I, *Bgl* II, *Eco*R I, *Hae* III or *Xba* I), separated by electrophoresis on agarose gel, then blotted onto a Nylon membrane (Sambrook *et al.* 1989). ECL system (Amersham international plc, Amersham) was used to detect RFLP according to the manufacturer's instructions. The probes were denoted by three-digit numbers with a BN prefix (ex. BN003).

Isozyme analysis

Acid phosphatase (EC 3.1.3.2) (ACP) and leucine amino peptidase (EC 3. 4. 1. 1) (LAP) were extracted from leaves and separated by PAGE (Hirai and Kajiura 1987), then stained (Vallejos 1983).

Data analysis

A software MAPMAKER/EXP Version 3.0 (Lander *et al.* 1987) was used to construct linkage maps with Kosambi function (Kosambi 1994). The parameters of the analysis were 4.0 and 37.2 as the thresholds of LOD and map distance between markers, respectively. The difference between the embryogenic ability of the two groups of F_2 plants categorized as harbouring or lacking RAPD markers, was tested by the non-parametric method described by Wilcoxon (1945).

Nomenclature of loci

RAPD loci were denoted by the primer name plus a letter (ex. RA1035C). Since some of the probes detected more than one locus, the RFLP loci were denoted by probe name and a number after a dash (ex. BN064-1 and BN064-2).

Microspore culture

Microspore culture was conducted as previously described (Kuginuki *et al.* 1997b). The flower buds from each F_2 plant were classified into two groups according to the bud-length; from 2.4 to less than 2.8mm and from 2.8 to less than 3.2mm. The microspores obtained from the buds were cultured in a liquid medium at a density of 2×10^5 per ml to score the embryogenic ability. Two to three plates containing 2.5 ml medium were prepared for each class of each plant, and then cultured for 3 weeks. The number of embryos obtained was counted at the end of the culture and then the higher number of embryos in the two classes of each F_2 plant was used.

Results

Linkage map of *Brassica rapa*

In a preliminary analysis, segregation data were collected for about 190 RAPD and 2 isozyme loci in 95 F_2 plants (population I). Based on these data, a map covering 1189 cM with 16 linkage groups was constructed using MAPMAKER with the default parameters (data not shown). To reinforce the map with codominant markers, 34 RAPD bands were cloned and then used for

the probes of RFLP. Among them, 3 clones showed too intense signals such as highly repetitive sequences and could not be used for the detection of RFLP. Some of the probes did not show clear codominant alleles. Finally 19 probes detected 21 loci. From the accumulated data, loci showing a pronounced distortion were omitted for the construction of the map. A new map was constructed based on recombination values of 2 isozymes, 144 RAPDs and 21 RFLPs using LOD 4.0 as the threshold for grouping. The map consisted of 16 linkage groups, and covered 851 cM (Fig. 1). Among the linkage groups, 4 covered more than 100 cM, while 6 covered less than 30 cM. Three markers (WE22B, WE49B and RA1275A), which had been found to be linked to a major CR gene (Kuginuki *et al.* 1997a), were linked to each other in linkage group 2. These three RAPD bands were cloned and were used for the detection of the RFLP locus. Among them WE49B detected two independent codominant loci designated BN064-1 and BN064-2, while the other two did not show clear codominant loci. These markers, WE22B, RA1275A and BN064-1 were tightly linked to each other within a distance of 3.1 cM. A marker for the late bolting trait of Chinese cabbage, RA1255C (Ajisaka *et al.* 1997) was

located in linkage group 12. The cloned fragment detected a codominant locus BN007-1 (Fig. 1).

Distortion of gene frequency in the microspore-derived population

Since the parent 'Siloga S2' is not a pure line, and these three populations were derived from different F_1 individuals, genetic analysis was performed only on markers common to the populations compared. Frequencies of 83 RAPD loci and one isozyme locus in population II, which consists of a population of the microspore-derived lines ($n=63$), were scored. Several parts of the linkage groups 1, 3, 5, 6, 11 and 12 together with several unlinked loci showed a significant distortion toward 'Homei 09' alleles from the expected ratio of 1:1. Fig. 2A illustrates these RAPD loci and some of the neighboring loci. A large part of the linkage group 12 showed a pronounced distortion toward the 'Homei 09' alleles. Since the frequencies of these RAPDs in the F_2 population (population I) were not significantly skewed (data not shown), the observed distortion towards 'Homei 09' alleles in this microspore-derived population was considered to be mainly derived from the difference in the embryogenic ability of the parental lines, while two

Fig. 1. Linkage map of *Brassica rapa* using an F_2 population (population I) derived from the crossing of a Chinese cabbage line, 'Homei 09' and a turnip line, 'Siloga S2'.

Data of 195 markers were processed by MAPMAKER/EXP ver. 3.0 with a minimum LOD score of 4.0 and a maximum map distance of 37.2 cM. Then a total of 2 isozymes, 144 RAPDs and 21 RFLPs were mapped in 16 linkage groups.

RAPD markers were denoted by the name of the primer plus a letter, as WE22B or RA1255C. Codominant RFLP markers were denoted a three-digit number with a BN prefix. A number after a dash was used to distinguish duplicated loci. Two isozyme markers were as follows: LAP: leucine amino peptidase, ACP: acid phosphatase. Original RAPD locus and the RFLP locus detected by the probe cloned from the RAPD band were both illustrated in the linkage map. The small gap observed between the loci could be due to the instability of the RAPDs.

parts showed a distortion toward Siloga S2 alleles (data not shown).

Segregation of the embryogenic ability of the F_2 individuals.

Embryogenesis in microspore culture of each F_2 plant was scored using population III ($n=56$). The score was expressed as the number of embryos obtained per 10^5 microspores cultured. The cultural efficiency obtained in the present study was somewhat lower than that obtained in the previous study (Kuginuki *et al.* 1997b). RAPD genotypes of the F_2 plants were also analyzed. To analyze the loci affecting the embryogenesis, the number of embryos obtained was calculated for two marker types (presence or absence) at each RAPD locus. A total of 42 RAPDs were analyzed. A difference in the number of embryos obtained was found in several parts of the linkage map. About half of the parts showed that the number of embryos in the plants having 'Homei 09' alleles was higher than that having the 'Siloga S2' genotype. Among them, two parts, RA1267B in linkage group 6 (Fig 2B) and WE22B in linkage group 2 (Fig. 1, not shown in Fig 2B) showed significant differences at 5 % level using the non-parametric test (Wilcoxon 1945). The plants having 'Siloga S2' alleles at several loci including the RA1035C locus in linkage group 5 showed a higher embryogenic ability than those with the 'Homei 09' genotype (Fig.2B). The differences were, however, not significant based on the non-parametric test.

The distortion of RAPD frequencies in population II and the segregation of the embryogenic ability in population III were compared with each other. A RAPD locus, RA1267B in linkage group 6 showed the matching of the data (Fig. 2A and 2B). On the contrary, segregation of WE22B in the microspore-derived population was not distorted from the expected ratio of 1:1 (data not shown).

Discussion

Linkage map of *Brassica rapa*

Linkage maps of *B. rapa* were already published (Song *et al.* 1995, Chyi *et al.* 1992). The total length of these maps was about 1800 cM and 10 linkage groups were detected. Compared to those, the present map covers only half of the whole genome range. The number of linkage groups did not match the haploid chromosome number, 10. Since the present map was mainly based on dominant markers, calculation of the genetic distance between markers in repulsion is difficult, and this situation may disturb the construction of a map covering the whole genome. However, genes controlling clubroot resistance, and late bolting have already been mapped in the present map (Ajisaka *et al.* 1997). Therefore, the present map will be used for incorporating late bolting and/or clubroot resistance into vegetable crops in *B. rapa*. Addition of more codominant markers may enlarge and improve the present linkage map. Although

Fig. 2. Segregation ratio of RAPDs in the microspore-derived lines (population II) and embryogenic ability of the F_2 plants (population III).

The loci showing a significant distortion toward 'Homei 09' alleles from the Mendelian ratio of 1:1 in the microspore-derived population and some of the adjacent loci were only illustrated. The figure on the left (A) shows the segregation ratio with the listed numbers and their significance. * and ** show a significant distortion toward 'Homei 09' allele at 5 % and 1% levels, respectively. The figure on the right (B) shows a comparison of the embryogenic ability of F_2 plants for marker types (presence or absence) at the locus. * shows a significant difference at 5 % level in embryogenic ability analyzed by the non-parametric method (Wilcoxon 1945).

extensive duplication of chromosome segments was reported from RFLP studies on *Brassica* genomes (McGrath and Quiros 1991, Song *et al.* 1995), only 3 (BN009, BN021 and BN064) out of the 19 probes used in the present study detected two loci. Since RAPDs markedly distorted from the Mendelian ratio (3:1) in population I had not been used for mapping, some of the

duplicated areas might be eliminated in the present map.

Embryogenesis in microspore culture

One of the present parents, 'Homei 09' showed a high ability of the embryogenesis from microspore (Sato *et al.* 1989a, Kuginuki *et al.* 1997b), while 'Siloga S2' showed a very low embryogenic ability. The present study indicates that at least one part of the linkage map around RA1267B affects the embryogenesis. In this region, it is suggested that the 'Homei 09' alleles promoted embryogenesis. Although RA1255C and adjacent RAPDs showed a significant distortion in population II, and F₂ plants having 'Homei 09' alleles showed higher embryogenic ability, the differences were not significant. More detailed studies should be carried out to determine whether this part affects the embryogenic process. Although, the RA1035C locus in linkage group 5 showed a pronounced distortion toward the 'Homei 09' allele, the data on the embryogenesis did not match it. Since the present map covers only half of the whole genome, and several unlinked RAPDs were significantly distorted (Fig.2A), it is possible that other loci may affect the embryogenesis in microspore culture.

In this study distortion in the microspore-derived population did not always correspond to the embryogenic ability in the F₂ population. The microspore-derived plants had been cultured *in vitro* to plantlets for 3 to 4 months, then they were acclimatized in a greenhouse environment and were transplanted to soil. The embryogenic ability was assessed only 3 weeks after the microspore culture, when the embryos were still at the torpedo stage. Therefore, chromosomal areas effective for the growth *in vitro* and the acclimatization were not visible in the data on microspore culture, but were only manifested as genetic distortion in the microspore-derived population. The region found in the present study is, therefore, considered to be effective during the very early stages of embryo development. Since thermal treatment is essential for triggering embryogenesis in microspore culture of *Brassica* species (Keller and Armstrong 1979, Sato *et al.* 1989a), it is suggested that the region found in this study may be involved in the triggering phenomenon. It also possible that the region may be involved in embryo development in the early stage, since in some genotypes of *B. rapa* cell division ceases at the early stage. Analyzing the distortion in DH lines, Cloutier *et al.* (1995) showed that putative genes associated with the responsiveness to microspore culture were mapped in linkage groups 1 and 18 in *Brassica napus* using 'Topas' as a responsive parent. Although they briefly indicated the cultural efficiency of the segregating population, detailed data were not given. More detailed studies on segregation of the embryogenic ability may enable to identify the genetic factors controlling embryogenesis.

Similar studies were reported for anther culture of rice. Loci controlling callus formation and green plant regeneration were assumed to be located on chromo-

somes 1 and 10, respectively (Yamagishi *et al.* 1998). In the case of rice anther culture, both green and albino plants were regenerated through callus. Since the latter plants cannot be used for breeding purposes, the cultural efficiency of rice largely depends on the ratio of green plants together with the rate of callus formation from anther. The loci affecting regeneration in anther culture of rice may act differently from the loci identified in the present study for *Brassica*. In maize, genetic studies enabled to identify chromosomal regions controlling the formation of embryo-like structures in anther culture (Wan *et al.* 1992, Beaumont, *et al.* 1995, Cowen *et al.* 1992, Murigneux *et al.* 1994). It is suggested that the loci in the present study in *B. rapa* could expect effects similar to those observed in the maize genome.

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