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Genetic linkage map of *Brassica campestris* L. using AFLP and RAPD markers*

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Abstract: A genetic linkage map comprised of 131 loci was constructed with an F_2 population derived from an inter-subspecific cross between *Brassica campestris* L. ssp. *chinensis* cv. 'aijiaohuang' and ssp. *rapifera* cv. 'qisihai'. The genetic map included 93 RAPD loci, 36 AFLP loci and 2 morphological loci organized into 10 main linkage groups (LGs) and 2 small groups, covering 1810.9cM with average distance between adjacent markers being approximately 13.8cM. The map is suitable for identification of molecular markers linked to important agronomic traits, QTL analysis, and even for marker-assisted selection in breeding programs of Chinese cabbage and turnip.

Key Words: Genetic map, AFLP, RAPD, *Brassica campestris* L. ssp. *chinensis*.
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INTRODUCTION

Chinese cabbage originated in China, plays important role in vegetable production as a member of genus *Brassica*. A linkage map is a first step towards detailed genetic analysis for potential application of the results to the cloning of specific genes. Genetic linkage maps of molecular markers in this genus have been constructed in 5 agriculturally important species including *B. oleracea*, *B. napus*, *B. juncea*, *B. nigra* and *B. campestris* (syn. *B. rapa*), but all reported genetic or molecular linkage maps of *B. campestris* L. are based on oilrapa, except four in Chinese cabbage (ssp. *pekinensis*) of F_2 segregating populations (Lu et al., 1999). There are currently no detailed genetic or molecular linkage maps for *B. campestris* ssp. *chinensis* and turnip. Mapping in Chinese cabbage is crucial for understanding of the genetic control of important agronomic traits and the improvement of the crops.

Most maps of *B. campestris* are based on RFLP markers, except 3 maps constructed with RAPD markers, but AFLP markers are not reported until now (Nozaki et al., 1997; Ajisaka et al., 1999). Das et al. (1999) assessed genetic variation within *Brassica campestris* cultivars using AFLP and RAPD markers. AFLP, as an efficient technique to generate high-density genetic map compared to other DNA marker systems, has been used to construct genetic linkage maps in many species. It is likely that RAPD markers in combination with AFLP markers will result in a more complete coverage of a genome (Debener et al., 1999). In the present study, we report on the development of a genetic linkage map of *B. campestris* L. using AFLPs and RAPDs markers, that could be used as a basic framework to obtain further genetic information for Chinese cabbage.

MATERIALS AND METHODS

1. Plant materials

A segregation F_2 population was derived from a cross between *B. campestris L.* ssp. *chinensis* cv. 'aijiaohuang' self-bred line 97-2 and ssp. *rapifera* cv. 'qisihai' self-bred line 97-24. These two parents representing two diverse groups in *B. campestris* L. are different in sev-

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eral morphological and physiological traits. Single plants from each parent were used for producing the F_1 hybrid, and then a single F_1 plant was selected and self-fertilized by bud pollination to produce F_2 mapping population. F_2 plants and parents were grown in greenhouse in the experimental farm of Zhejiang University in the autumn of 1997. For DNA isolation, young leaves were harvested from 120 individual F_2 plants and bulked from 12 individuals for each of the self-pollinated progenies of the parents. DNA was extracted from leaf tissue following the procedure described by Chen et al., (1999).

2. RAPD analysis

A single 10-mer oligonucleotide primer with an arbitrary sequence (Sangong Co., Shanghai) was used in each PCR amplification carried out using a method similar to that described by Chen et al. (1999). The amplification products were separated by electrophoresis (4v/min) through a 1.2% agrose gel containing ethidium bromide, then visualized and photographed under ultra-violet light with Kodak Digital Science DTM Camera System.

3. AFLP analysis

All reagents required for AFLP analysis were obtained from Life Technology (Geithersburg, USA) as kits except for Taq polymerase from Promega. The protocol adopted for the generation of AFLP markers was essentially the same as described by Vos et al (1995). Genomic DNA (250 ng) was digested with EcoR I and MseI (2.5 u each). EcoR I and Mse I adapters were subsequently ligated to the digested DNA fragments. The adapter-ligated DNA was pre-amplified with AFLP primers each having one selective nucleotide using the following cycling parameters: 25 cycles of 30 s at 94℃, 60 s at 56℃ and 60 s at 72℃. The pre-amplified DNA was diluted (1:20) and an aliquot was used for selective amplification with the EcoR I and Mse I primers having three selective nucleotides at the 3'-ends. The following amplification procedures were used: 1 cycle of 30 s at 94℃, 30 s at 65℃ and 60 s at 72℃. The annealing temperature was then lowered by 0.7℃ per cycle during the first 12 cycles, and then 23 cycles were performed at 94℃ for 30 s, 56℃ for 30 s and 72℃ for 60 s. The reaction products were resolved on 5% polyacrylamide gels and then shown with silver-staining methods.

4. Segregation and linkage analyses

Bands generated by RAPD and AFLP analyses were scored as dominant markers. Chi-square was calculated to test for deviation from the 3:1 single gene segregation ratio and for deviation from independent inheritance of the markers. A linkage map was constructed using the Mapmaker/EXP version 3.0. Two-point linkage analysis was used to sort the loci into distinct groups. A minimum LOD threshold of 4.0 and a maximum recombination value of 0.30 were used to group the RAPD and AFLP loci into potential linkage groups. For each group, three point and multipoint analysis was used to find the most likely locus orders within a linkage group followed by the 'order' command. The linkage orders were determined with the 'ripple' command. Linkage maps were generated with the 'map' command using the Kosambi mapping function in centiMorgans (cM).

RESULTS

1. Screening of RAPD markers and segregation analysis in F_2 population

A total of 500 random 10-mer primers were pre-screened against the parents 'aijiaohuang' and 'qisihai'. Of total screened primers, 151 primers (30.2%) producing reproducible and easily scored polymorphisms were selected and these polymorphic primers were also tested on the F_1 plant and 20 F_2 plants. Only well-defined polymorphic bands were selected, and they were consistent and distinct. A rigorous screening of polymorphic fragments was performed to avoid the well-known problem of RAPD data reproducibility. 111 primers were finally selected for segregation analysis of the F_2 plants (Fig. 1).

The selected primers amplified a total of 169 putatively segregating marker loci, 78 from '*aijiaohuang*' and 87 from '*qisihai*' in the F_2 mapping population, which corresponded to 1.52 loci per primer, a similar efficiency of the polymorphic primer in detecting segregating RAPD loci had been observed in other *Brassica* crosses, for examples, 1.7 loci for *B. oleracea*

(Moriguchi et al., 1999) and 2.0 loci for *B. rapa* (Nazaki et al., 1997). *Chi-square tests showed that* 140 *markers out of* 169 *fit the* 3:1 *Mendelian ratio. Only* 29 *loci* (17.1%) *exhibited a distorted segregation.*

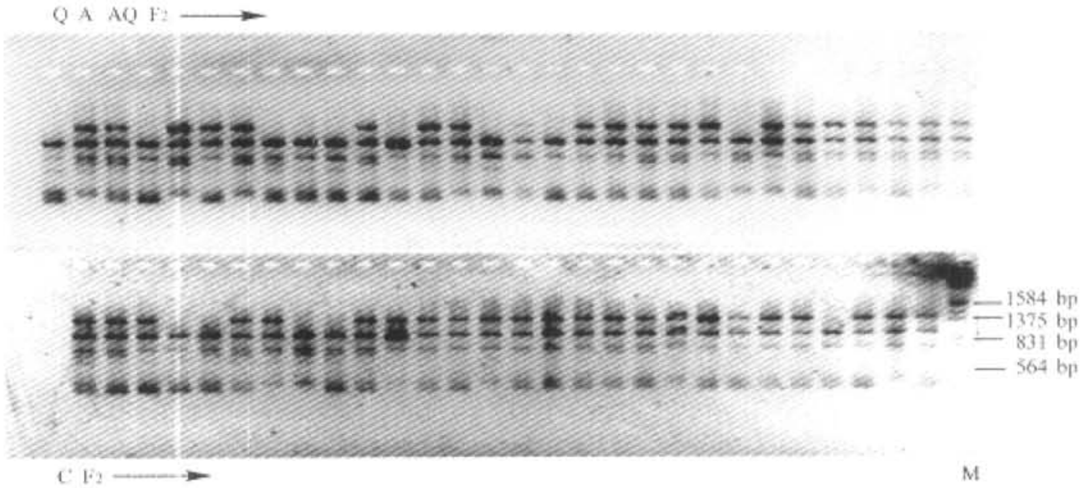

Fig.1 Detection of the segregation in F_2 population derived from the cross of *B. campestris* L ssp. *chinensis* and ssp. *rapifera* with RAPD markers.

Q: qisihai; A: aijiaohuang; AQ: the F_1 hybrid of cv. aijiaohuang × qisihai; C: control; M: Lambda DNA/EcoR I+HindIII markers, others all are F_2 plants.

2. AFLP markers and segregation analysis in F_2 population.

Eight EcoRI and MseI primers with three selective bases were used. A total of 64 primer combinations were tested against the parents and resulting 2504 amplified bands with average of 39.1 bands per primer pair. All of the primer pairs showed clear polymorphism, the number of polymorphic bands for each primer combination ranged from 1 to 10. Twelve primer pairs were finally used for mapping, which included 84 markers scored in the parents, but only 51 markers (61%) could be confidently scored and were consistently reproduced and used for genetic mapping in the F_2 population (Fig.2). The rest were neither well amplified, nor well segregated in the population, or too faint to be scored, although the parental bands were strong and easy to score. The average number of mapped AFLP markers was 4.3 loci per primer pair, which was higher than that of RAPDs. This result indicated that polymorphic AFLP primers were more efficient than RAPD primers in the detection of reproducible segregating loci. Most of the markers fitted the expected monogenic segregations. Only 7 markers (13.7%) deviated from the monogenic ratio at the 1% or the 5% level of significance.

3. Construction of the linkage map

Based on the frequency of recombination in the F_2 mapping population, 93RAPD loci, 36AFLP loci and two morphological loci including pubescence (Pub1) presence or absence and leaf surface character (LSM1) (smooth or wrinkled) were arranged into 10 main linkage groups (LGs) numbered 1~10 in descending order of their lengths in centiMorgans (Fig. 3). This number of major LGs agreed with the haploid chromosome number (n=10) (Fig.3) and previous reports of 10 major LGs in this species (Kole et al., 1997). In addition, there were 7 loci located on 2 smaller, unassigned segments (table 1). The total map distance was 1810.9cM with average distance of 13.8 cM between loci. The size of the LGs differed substantially between the maximum of 291.2 cM in LG1 and the minimum of 41.6cM in LG12. The numbers located on each linkage group also varied greatly from 3 to 20. LG6 was the most densely populated LG and showed average marker interval of 11.4 cM.

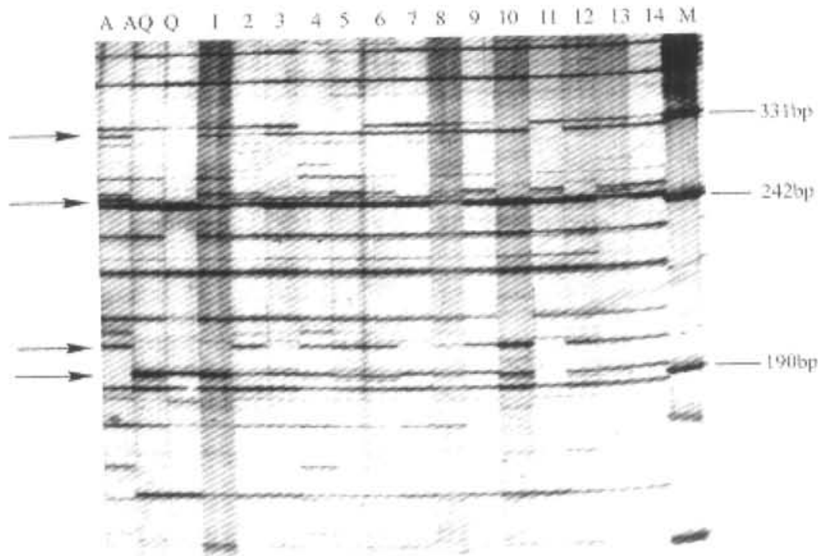

Fig. 2 Segregation of AFLP markers in F_2 population derived from the cross of *B. campestris* L. ssp. *chinensis and* ssp. *rapifera*.

A: aijiaohuang; AQ: the F_1 hybrid of cv. aijiaohuang × qisihai; Q: qisihai, 1 − 14: F_2 plants; M: pUCmix DNA marker; The primer is ACT/CTT

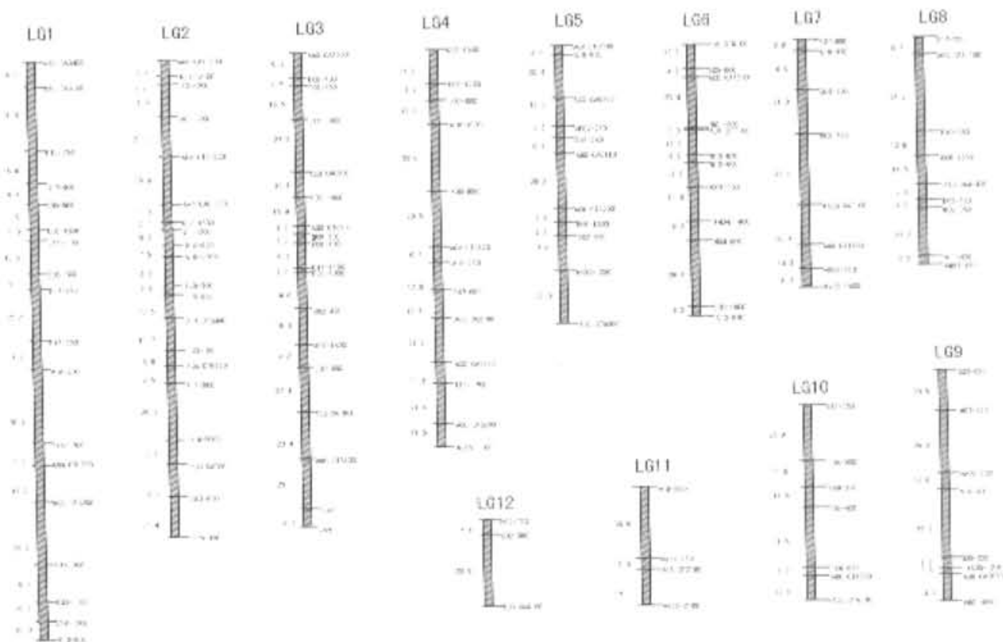

Fig. 3 A genetic map of *Brassica campestris* L. constructed from the F_2 population derived from the cross between *B. campestris* L. ssp. *chinensis and* ssp. *rapifera*. with RAPD and AFLP markers.

Linkage group (LG) numbers are indicated on top, loci are listed on the right-hand side of the LGs and the Kossami centiMorgan distances on the left-hand side. * distorted marker at 5% level.

Table 1 The genetic map of *B. campestris* ssp. *chinensis* and linkage groups with F_2 population derived from the cross of *B. campestris* ssp. *chinensis* and ssp. *rapifera*

Linkage groups	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9	LG10	LG11	LG12	Total
Marker numbers	18	20	18	13	11	12	8	9	8	7	4	3	131
Map distance (cM)	291.2	257.5	236.2	196.2	138.9	136.6	122.1	112.8	119.4	98.5	59.6	41.6	1810.9
Mean distance (cM)	16.2	12.9	13.1	15.1	12.6	11.4	15.3	12.5	14.9	14.1	15.0	13.9	13.8

DISCUSSION

B. campestris is diploid with base chromosome number of 10 and has a genome size based on a nuclear DNA content of 1.33 ± 0.05 pg DNA/2C (Arumuganathan et al., 1991). The present investigation identified 10 main linkage groups and the total map distance was in good agreement with the predicted map size and similar to those in previous studies of *B. rapa* where Teutonico (1995) and Song (1991) identified 1785 cM and 1850 cM, respectively with F_2 populations. However, the distance of present linkage map was greater than those in Nozaki (1997) and Kole (1997)'s reports (733 cM with 52RAPDs, 890 cM with 144RFLPs). The differences may be attributed to the population used, the loci number and the better coverage of the *B. campestris* genome with the 131 loci detected in our investigation.

In our investigation, 17.1% of the RAPDs loci and 13.7% of AFLP loci were distorted from normal ratios. The total percentage of loci with distorted segregation was comparable to that reported previously in this species. Work with other *Brassica* populations also revealed a similar level of distortion. However, greater proportions of loci with deviant segregation had been reported in other investigation. More than 20% were observed in inter- or intraspecific crosses in *B. campestris* with RIL or DH population. Twenty-seven percent of the RFLP markers deviated from the Mendelian segregation ratio in *B. juncea* with DH population (Kole et al., 1997). In *Brassica oleracea*, the deviation could reach to 64% with DH population, but lower than 12% with F_2 population (Kianian et al., 1992). RAPD loci displaying skewed segregation in the present material coincide with the skewed segregation of neighboring AFLP loci; which suggests that the skewed segregation is not due to faults in scoring caused by a variable expression of the alleles.

An interesting aspect of the deviant loci in this study was that they were placed in linkage group with a preponderance of 'aijiaohuang' alleles. Most of the biased loci located mainly on LG5, LG7, LG9, LG11, on the ends of the linkage groups. LG5 and LG7 shared the most prominent cluster of segregation loci. This observation is similar to that reported by Moriguchi et al. (1999), in which most of them were located on LG7 or LG8. The observation that distorted loci are located in a specific region of one linkage group suggests that a biological cause may be involved. A possibility is the presence of a pollen killer or gamete eliminator, which had been identified in crosses between distantly related taxa of rice.

In this study, all AFLP markers used detected duplicate loci and 40% RAPD markers used for segregation analysis detected duplicate segregating loci which were lower in number than the duplicate segregating loci in other reports because of the strict selection of the polymorphic bands. The number of duplicate loci detected may vary in different species and parents used in each case.

The number of linkage groups being greater than the number of chromosomes in *B. campestris* ($n = 10$) is probably due to incomplete coverage of the entire genome. This may be

a consequence of the fairly small population size used in this study. In a long run the order of assigning markers will be resolved when more progenies are used and more markers are developed. Furthermore, the AFLP markers can be converted to either primers or RFLP probes to facilitate integration with other linkage maps (Sebastian et al., 2000). Also, some real linkage association may have been separated by use of higher LOD threshold for a few large groups. However, this approach could have resolved some spurious associations, and is a more conservative representation of the linkage groups. As more markers are added to the map, complete linkage groups will be resolved. The current molecular linkage map for Chinese cabbage can be used efficiently for the identification of molecular markers linked to important agronomic traits, for QTL analysis, and even for marker-assisted selection in breeding programs (the data will be shown in other papers).

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