African Journal of Biotechnology Vol. 10(41), pp. 7962-7968, 3 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.239
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A random amplified polymorphic DNA (RAPD) molecular marker linked to late-bolting gene in pak-choi (*Brassica campestris* ssp. *chinensis* Makino L.)

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Accepted 24 June, 2011

Bulked segregant analysis (BSA) and random amplified polymorphic DNA (RAPD) methods were used to analyze F_2 individuals of P-27 × P-28 to screen and characterize the molecular marker linked to late-bolting gene in pak-choi (*Brassica campestris* ssp. *chinensis* Makino L.). A total of 200 random primers were used for RAPD analysis. One RAPD marker $S265_{750}$ was identified to be co-segregating with the late-bolting gene, and the genetic distances between $S265_{750}$ and late-bolting gene was 3.14 cM. The results of the study can be seen as a starting point for future researches on the pak-choi bolting gene mapping and molecular marker assisted breeding.

Key words: Pak-choi, late bolting, random amplified polymorphic DNA (RAPD), bulked segregant analysis (BSA).

INTRODUCTION

Pak-choi (*Brassica campestris* ssp. *chinensis* Makino L.) originated from China, and is one of the most important vegetable crops in China and worldwide that has the largest planting area and total yield (Liu et al., 1998). Naturally, bolting is interrelated greatly with the yield and quality of Pak-choi cultured in spring, and has been reported as a quantitative trait, which is mostly influenced by the environment (Wang and Paterson, 1994; Wang et al., 2008; Xu and Wang, 2008). In recent years, bulked segregant analysis (BSA) method has been reported to make great progress in some important properties of marker gene location selection in various plants. Gu et al. (2006) reported a bitter Bt gene in cucumber. Lei et al. (2006) reported bacterial wilt resistance genes in potatoes. Leng et al. (2007) reported a downy mildew disease gene in Chinese cabbage (*Brassica rapa* ssp. *Pekinensis*).

Methods for the localization of molecular markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and sequence characterized amplified regions (SCAR), have been used extensively to localize genes of interest and in molecular-assisted plant breeding (Zhu et al., 2004). RAPD is one of the simplest and fastest molecular marking technologies (Gabriel et al., 2005; Zhang et al., 2008), and bulked segregant analysis (BSA) is also one of the effecttive methods in finding a linked marker of a gene (Michelmore et al., 1991). In recent years, many important quality trait genes of main crops have been marked (Zhu et al., 2004). Hidetoshi et al. (2001) identified one QTL RA1255 (530 bp) controlling bolting in cabbage, and the gene position in BN007-1. Du et al. (2007) reported two SRAP markers closely linked to late-bolting in cabbage. Zhang (2007) reported one SSR marker linked to late-bolting gene in pak-choi.

Using tightly linked molecular markers to target genes to screen progenies is one of the most effective methods to carry out artificial selection in pak-choi. But until now, there have been no scientific reports focusing on the late-bolting gene linked to a molecular marker in pak-choi, except one SSR marker (Zhang, 2007). In this paper, BSA and RAPD methods were used to analyze F_2 individuals of P-27 × P-28 to screen and characterize the molecular marker linked to late-bolting gene, and the preliminary results of identification of RAPD marker tightly

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Table 1. Sequences of part RAPD primers used in this study.

Name of primer	Sequence of primer (5'→3')	Name of primer	Sequence of primer (5'→3')
S106	ACG CAT CGC A	S265	GGC GGA TAA G
S129	CCA AGC TTC C	S271	CTG ATG CGT G
S151	GAG TCT CAG G	S273	CAC AGC GAC A
S241	ACG GAC GTC A	S275	ACA CCG GAA C
S243	CAT TGC CGA C	S277	GTC CTG GGT T
S248	GGC GAA GGT T	S279	CAA AGC GCT C

linked to late-bolting gene is presented.

MATERIALS AND METHODS

P-28 (P_1) was used as the early-bolting parent and P-27 (P_2) was used as the late-bolting parent. The F_2 (255 individuals) population was constructed by the progeny of P-28 × P-27. The materials were obtained from the Horticultural Research Institute, Shanghai Academy of Agricultural Sciences. The seedlings of 85 P_1, 144 P_2 and 255 F_2 individuals were grown in a glasshouse at 25 ± 2°C after 40 days low temperature treatment at 5 ~ 10°C, during the experiments. The parental lines were different in bolting traits. F_2 plants were grown in greenhouse with photoperiod of 10/14 (day/night) and in rosette stage, leaf samples were harvested from individual plants for DNA extraction.

For RAPD primers (Shanghai Sangon Biotech Co., Ltd, China) (Table 1), PCR reaction, the amplification profile and PCR product detection published by Anthony et al. (2008) was used.

DNA bulking and DNA extraction

To isolate RAPD markers that were tightly linked to the late-bolting locus in pak-choi, early-bolting and late-bolting bulks were used, all of which were derived from the F_2 population of P-27 × P-28. Every ten plants were randomly selected from early-bolting and late-bolting segregants, and leaf DNA was isolated from individual plants.

The BSA approach was used to compare two pooled DNA samples of the individuals (Michelmore et al., 1991). In order to perform BSA for identification of markers closely linked to late-bolting gene, we selected ten early-bolting and ten late-bolting F_2 individuals to construct early-bolting bulk and late-bolting bulk, respectively. The young leaves of parental lines and F_2 plants were selected to extract genomic DNA using the CTAB method (Murray and Thompson, 1980; Saghai-Maroof et al., 1984; Rogers and Bendich, 1988).

Random amplified polymorphic DNA (RAPD) analysis

The RAPD exploration and PCR protocol were adopted from the method described by Li et al. (2001). Amplifications were carried out in 20 µl of reaction mixture, including 20 ng of template DNA, 20 ng of each random primer (Table 1), 2.5 mM $MgCL_2$, 0.2 mM dNTPs, 1.0 U Taq polymerase and 10×PCR buffer (Shanghai Sangon Biotech Co., Ltd, China).

The cycling parameters were denatured for 5 min at 94°C, followed by 45 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 90 s. The PCR was terminated following incubation at 72°C for 10 min and then stored at 4°C until the PCR products were analyzed.

The quality and quantity of DNA extracted were estimated by electrophoresis on agarose gel (0.8 %) containing ethidium bromide using 1×TAE buffer and by UV spectrophotometry, respectively. The concentration of extracted DNA was adjusted to 10 to 20 ng/µl and samples were stored at - 20°C.

Bulk segregant analysis (BSA)

BSA (Michelmore et al., 1991) was finally performed for each individual on the bulks of DNA of ten early-bolting plants or late-bolting plants. Primers which amplified a DNA fragment in only one of the bulks were confirmed on the same set of bulk, followed by PCR on ten individual early-bolting and ten late-bolting plants. RAPD markers with the best linkage to late-bolting gene were evaluated further on an additional number of individual plants.

Marker scoring and linkage analysis

The banding patterns obtained from RAPD were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity.

Among the 200 RAPD primers, the polymorphic primers were identified between the two parents and the F_1 hybrid. For each polymorphic primer, the RAPD bands of P_1, P_2 and 255 F_2 individuals were treated as dominant markers and scored as either present (1) or absent (0). Clearly distinguishable bands ranging from 100 to 2000 bp were used in the genetic and marker analysis. Linkage between DNA markers and the late-bolting locus was determined by the recombination rate. Recombination rate (cM) was calculated as follows: recombinator / (no. of individual of F_2 generation × 2) × 100 %, where 2 stand for diploid genome (Liu et al., 2002).

RESULTS

DNA testing

The PCR products were separated on 0.8% agarose gel according to the lengths of amplified fragments and stained with ethidium bromide (Jiang et al., 2006). The results of the inspection was electrophoresis strip clear and consistent, neat zone type and no degradation phenomenon (Figure 1), which indicates that the extraction of DNA can satisfy the RAPD amplified reactions needed.

The field performance investigation of F_2 individuals

255 F_2 plants derived from P-27 and P-28 crosses were

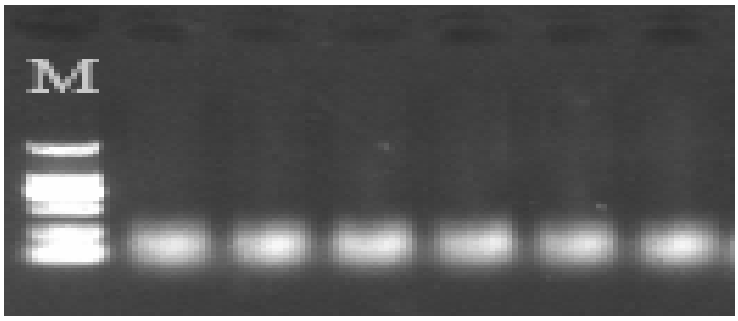

Figure 1. Examination of isolated DNA on agarose gel.

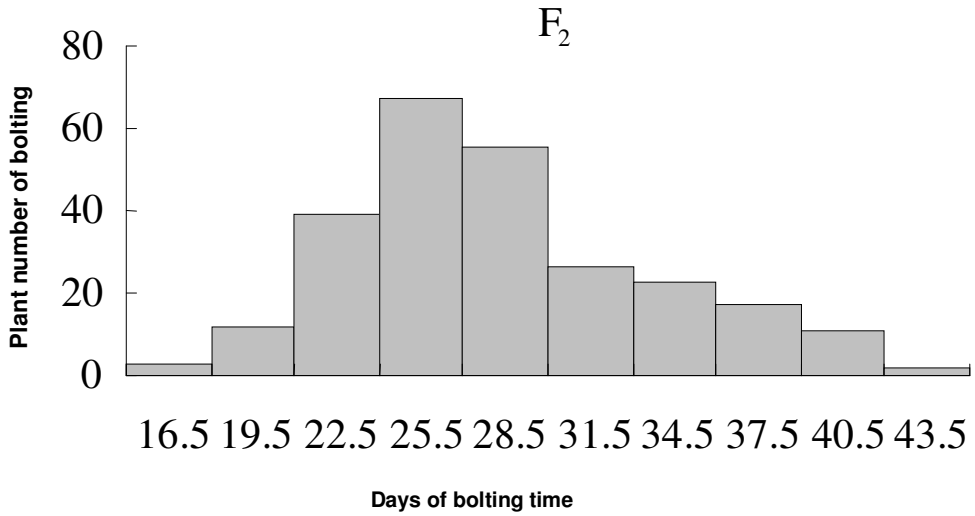

Figure 2. Frequency of bolting time distribution of F_2 population.

cultivated in the field. Investigation on the field performance of each F_2 individual plant was significant, making the phenotypic identification easier. The study showed that the separation of bolting time was obvious in the F_2 generation of P-28 × P-27 (Figure 2) and it was close to a Skewness distribution, according to quantitative traits genetic characteristic.

RAPD primer screening

According to the polymorphism between P_1, P_2 and F_1, 90 RAPD polymorphic primers were selected in 200 random primers. The 90 RAPD polymorphic primers provided a clear polymorphism in early-bolting bulk and late-bolting bulk (Figures 3 and 4). One RAPD primer S265 amplified about 750 bp band (Figure 4), which was then named $S265_{750}$.

To identify RAPD marker linked to late-bolting genes, DNA bulks were composed of the latest bolting and earliest bolting plants of the segregating families of P-27 and P-28. For each set of bulks, 200 random primers were screened (Figure 3), and 90 (45%) polymorphic RAPD primers between parents were used to screen 255 individuals from F_2 population. Between S101 to S300, primers amplified RAPD markers either in the early-bolting or in the late-bolting bulk. These primers were examined further on F_2 individual plants. A polymorphic fragment was obtained from plant P-27 and late-bolting plants, but no corresponding band was obtained from P-28 and early-bolting plants (Figure 4). The primer S265 (5'-GGCGGATAAG-3'), which produced one RAPD marker $S265_{750}$ with about 750 bp, generated DNA fragments that were found to be linked to the late-bolting locus in F_2 population. The presence of the RAPD markers $S265_{750}$ was analyzed in the entire population of F_2 (Figures 4 and 5).

Linkage map of late-bolting gene marker

A total of 200 RAPD markers scattered on the whole pak-choi chromosomes were used to determine the linkage to late-bolting. 90 RAPD polymorphic primers were

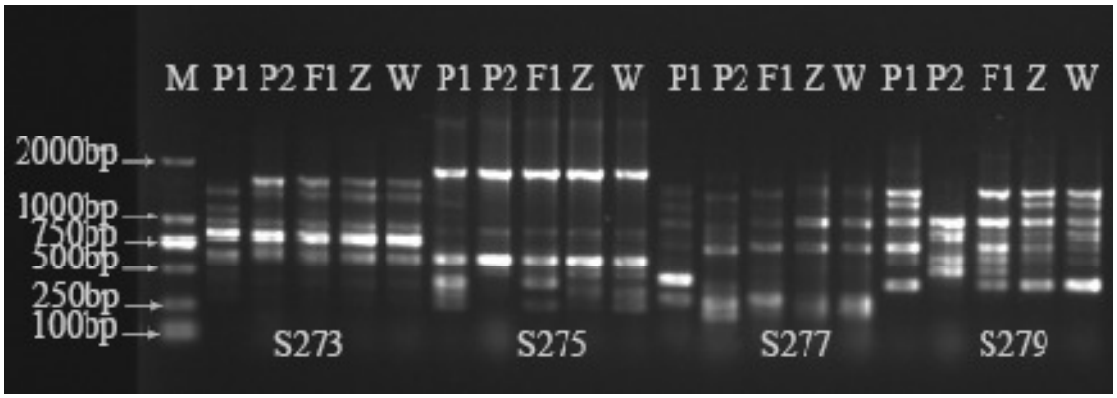

Figure 3. Amplification result of the four primers under P_1, P_2, F_1 early and late bolting pool optimized RAPD system. M, DL2000 DNA marker; P_1, mother; P_2, father; F_1, the hybrid of P_1 and P_2; Z / W, early / late bolting pool.

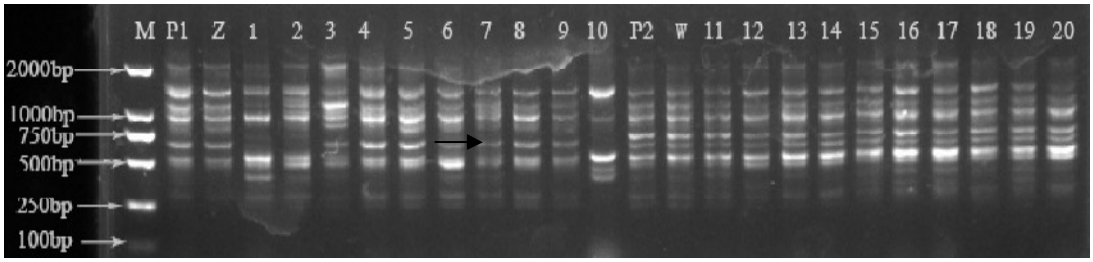

Figure 4. The amplification of primer S265 in the parents and two bolting bulks. M, DL2000 DNA marker; P_1, mother; P_2, father; Z / W, early / late bolting bulk; 1 to 10, early-bolting parents; 11 to 20, late-bolting parents.

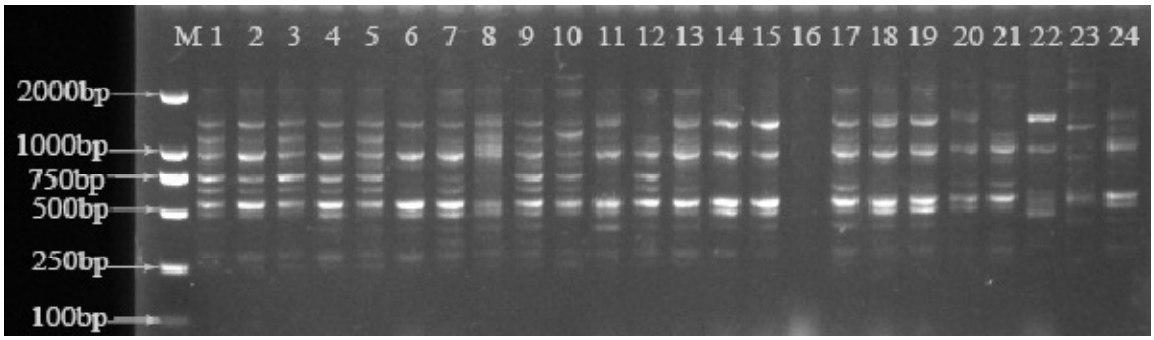

Figure 5. The amplification result of the primer S265 among part of the F_2 population. M, DL2000 DNA marker; 1 to 24, number of F_2 individuals.

employed to amplify P_1, P_2 and 255 F_2 plants. All clearly distinguished polymorphism bands ranging from 100 to 2000 bp were treated as dominant markers and scored as either present (1) or absent (0) (Table 2). According to Table 2, there was the ratio of 52:6:10:187 among late-bolting with belt mark, late-bolting without belt mark, early-bolting with belt mark and early-bolting without belt mark. So, there were 16 recombinants in 255 F_2 individuals. Linkage between DNA markers and the late-bolting locus was determined by the recombination rate. The genetic distance between $S265_{750}$ and late-bolting gene was about 3.14 cM, calculated as described in materials and methods.

DISCUSSION

Bolting traits belong to quantitative traits. Mero and

Table 2. Evaluation of bolting in F_2 individuals.

Number of plant	Type of bolting	$S265_{750}$	Number of plant	Type of bolting	$S265_{750}$
1	Late	1	43	Early	0
2	Late	1	44	Late	0*
3	Late	1	45	Early	0
4	Late	1	46	Early	0
5	Late	1	47	Late	1
6	Early	0	48	Late	0*
7	Early	1*	49	Early	0
8	Early	0	50	Early	0
9	Late	1	51	Late	1
10	Late	1	52	Early	1*
11	Early	0	53	Early	0
12	Late	1	54	Late	1
13	Early	0	55	Late	1
14	Early	0	56	Early	0
15	Late	0*	57	Early	0
16	Early	-	58	Early	0
17	Early	0	59	Late	1
18	Early	0	60	Late	1
19	Early	0	61	Late	1
20	Early	0	62	Late	1
21	Late	0*	63	Late	1
22	Early	0	64	Early	0
23	Late	1	65	Late	1
24	Early	0	66	Early	0
25	Early	0	67	Late	1
26	Late	1	68	Late	1
27	Late	1	69	Early	0
28	Early	1*	70	Early	0
29	Early	1*	71	Late	1
30	Early	0	72	Late	1
31	Early	0	73	Early	0
32	Late	1	74	Early	0
33	Early	0	75	Late	1
34	Early	0	76	Late	1
35	Early	1*	77	Early	0
36	Early	1*	78	Late	1
37	Early	0	79	Late	1
38	Late	0*	80	Early	0
39	Early	0	81	Late	1
40	Early	0	82	Early	0
41	Early	0	83	Late	1
42	Late	0*	84	Late	1
85	Late	1	120	Late	0*
86	Early	0	121	Early	0
87	Late	1	122	Early	0
88	Late	1	123	Early	0
89	Late	1	124	Early	0
90	Late	1	125	Early	0
91	Early	0	126	Early	0
92	Late	1	127	Early	0
93	Early	0	128	Early	0

Table 2. contd.

94	Late	1	129	Early	0
95	Late	1	130	Early	0
96	Late	1	131	Early	0
97	Late	1	132	Early	0
98	Late	1	133	Early	0
99	Early	0	134	Early	0
100	Early	0	135	Early	0
101	Late	0*	136	Late	1
102	Early	0	137	Early	0
103	Late	1	138	Early	0
104	Early	0	139	Early	0
105	Late	1	140	Early	0
106	Late	1	141	Early	0
107	Late	1	142	Early	0
108	Late	1	143	Early	0
109	Late	1	144	Early	0
110	Early	0	145	Early	0
111	Late	0*	146	Early	0
112	Late	1	147	Early	0
113	Early	0	148	Early	0
114	Early	0	149	Early	0
115	Early	0	150	Early	0
116	Late	0*	151-255	Early	0
117	Early	0		Total	255
118	Early	0		Recombinant individuals	16
119	Early	0		Genetic distance	3.14 cM

1 = Having exceptional fragment; 0 = not having exceptional fragment; * = recombinant individuals; - = not having fragment.

Honma (1985) first reported that the hybrid between early-bolting or late-bolting varieties in Chinese cabbage showed early-bolting with an advantage, but late-bolting percentage can be effected by modify genes. Li and Cho, (1997) indicated that bolting traits is in accordance with dominant and addition genetic model, in which the addition effect played a more important role, while the dominant effect only had performance. Recessive genes were more than the dominant allele, and dominant allele only control part of dominant characters. Our study showed the separation of bolting time obviously in the F_2 generation of P-28 × P-27 (Figure 2), and it was close to a Skewness distribution, according to quantitative traits genetic characteristic.

In this study, the BSA method provided a rapid and simple alternative technique to identify one RAPD marker linked to late-bolting. S265 (5'-GGCGGATAAG-3'), one of the 200 random primers screened, reproducibly produced polymorphism $S265_{750}$ (about 750 bp) among parents and the two bolting bulks. The results confirm primarily that late-bolting gene is linked with the RAPD marker $S265_{750}$. The amplified products of S265 showed that there were 16 recombinants in 255 F_2 individuals. Genetic distance between $S265_{750}$ and late-bolting gene was about 3.14 cM, calculated as described in materials and methods.

The RAPD marker $S265_{750}$ in this study can be regarded as a marker tightly linked to a quantitative-character, and it can be used in the molecular-assisted breeding procedures. Molecular-assisted breeding of late-bolting in pak-choi plants can identify the late-bolting of plants in the early stage of breeding, increasing selection veracity and efficiency, and can accelerate reasonable and rapid utilization of the late-bolting gene, shortening the breeding cycle.

ACKNOWLEDGEMENT

This research was supported by the project of Science and Technology Commission of Shanghai Municipality (No. 06DZ19103 and 09391911400).

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