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Identification of *S* haplotypes in doubled haploid lines of non-heading Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino)

Tingting Ge, Li Wang, Aifen Zhang, Tongkun Liu, Jianjun Wang, Xilin Hou[1], Ying Li[1]*

Horticultural Department, Nanjing Agricultural University, Nanjing 210095, China; State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing, 210095, China; [1]Key Laboratory of Southern Vegetable Crop Genetic Improvement, Ministry of Agriculture, Nanjing 210095, China

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Abstract

A set of 24 Doubled haploid (DH) lines of non-heading Chinese cabbage obtained through microspore culture were used for identification of self-incompatibility (SI) and *S* haplotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis with S-locus receptor kinase (SRK)-specific primers. The DH lines were grouped into five *S* haplotypes. The SI of two of these haplotypes was strong, whereas that of the remaining haplotypes was weak. The results obtained were consistent with the self-compatibility indices found. PCR-RFLP analysis using DH lines could enhance selection efficiency and improve the application of SI in the hybrid breeding of non-heading Chinese cabbage.

Key words: Non-heading Chinese cabbage, doubled haploid, self-incompatibility, S-locus receptor kinase (SRK), PCR-RFLP

Introduction

Self-incompatibility (SI) is an important mechanism for plants to promote outcrossing resulting in the maintenance of genetic variation in species [1-3]. In *Brassica*, SI is controlled by a single *S*-locus that contains multiple alleles [4]. At least three genes closely linked with *S*-locus have been identified, including *S*-locus receptor kinase (*SRK*), *S*-locus glycoprotein (*SLG*), and *S*-locus protein 11/*S*-locus cysteine-rich protein [5-7]. *S*-locus specificity genes are defined as *S* haplotypes. *S* haplotypes in *Brassica* may be classified into two groups, namely, classes I and II. Class I haplotypes show dominant and strong SI, whereas class II haplotypes are recessive and have relatively weak SI [8, 9]. Previous studies have investigated a large number of *S* haplotypes in *Brassica* [10-13].

Self-incompatibility is a main breeding strategy in the hybrid seed production of non-heading Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino). Given the potential for heterosis presented by SI, the *S* haplotypes of cultivated species must be identified to ensure the high quality of hybrid seeds. However, existing methods, such as the conventional test-crossing method, are time-consuming and costly. Therefore, development of a convenient and efficient method for identifying *S* haplotypes is necessary.

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis is an effective method for identifying *S* haplotypes in vegetable crops [14]. Based on divergent *SLG* and *SRK* genes, the method can be employed to investigate different *S* haplotypes by analyzing PCR products after digestion with appropriate restriction endonucleases [15]. However, the results of PCR-RFLP could be affected by the heterozygosity of plants because of the complicated dominance and recessive relationships between different *S* haplotypes [16]. Homozygous plants show more stable levels of SI than heterozygous plants [17], which enables the identification of *S* haplotypes through doubled haploid (DH) plants. DH technology is generally accepted as the most effective method for ensuring the purity of hybrids [18, 19]. Consequently, the accuracy and reasonability of *S* haplotype identified.

*Corresponding author's e-mail: yingli@njau.edu.cn
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In the present study, we analyzed polymorphisms of the *SRK* gene in 24 DH lines of non-heading Chinese cabbage through PCR-PFLP analysis and identified their *S* haplotypes classes. The self-compatibility indices (SCIs) of the 24 DH lines were also tested to check the results of the proposed molecular method. Our study provides a reliable and rapid approach for applying SI in the hybrid breeding of non-heading Chinese cabbage.

Material and methods

Plant growth

Twenty-four DH lines of non-heading Chinese cabbage were obtained through microspore culture. All plants were planted in Jiangpu Horticultural Experimental Station, Nanjing, China**.**

Isolation of genomic DNA

Genomic DNA was extracted from young leaves using a modified CTAB method according to Park *et al*. [15]. DNA samples were stored at –20 °C.

Determination of SI phenotype

Flowers were bagged at bud stage, and already opened flowers and siliques were removed. After 3-5 d, self-pollination was performed manually on the pistils of freshly opened flowers. The SCI was calculated using the formula of Watts and Nieuwhof [20, 21] with slight modifications (i.e., SCI = total number of seeds obtained after self-pollination/total number of siliques self-pollinated). The SI phenotypes of plants were classified as strong (SCI less than 0.5), weak (SCI ranging from 0.5 to 3), or stable (SCI more than 3).

Amplification of DNA fragments of SRK

SRK specific primers were designed based on the conserved regions of the *Brassica oleracea SRK* gene (GenBank No.: AB024422.2). P1+P2 and P3+P4 were respectively designated as class I and class II *SRK* primer pairs (Table 1). Genomic DNA was used as the template in a 20 µL reaction mixture containing 1.0 µL of the template DNA (30-50 ng), 1.0 µL each of the forward and reverse primers (10 µM), 1.6 µL of deoxynucleotide triphosphates (2.5 µM), 2.0 µL of 10 x PCR buffer, 1.6 µL of $MgCl_2$ (25 mM), and 0.2 µL of *Taq* polymerase (5 U/µL). PCR amplification was initiated by a pre-denaturation stage at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C (P1+P2) or 60°C (P3+P4) for 1 min, and an extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. PCR products were determined by ethidium bromide staining under UV light after electrophoresis in a 1% agarose gel.

Table 1. Nucleotide sequences of *SRK* gene specific primers for the amplification of class I and class II alleles

Primers	Nucleotide sequence (From5' to 3)	Length (bp)
Class I *SRK*		
P1	CTGCTGATCATGTTCTGCCTCTGC	24
P2	CAATCCCAAAATCCGAGATCT	21
Class II *SRK*		
P3	AAGACKATCATATTACCGAGC	21
P4	GAGGGCGA GA AGATCTTAATT	21

Cloning and sequence analysis of DNA fragments

DNA fragments amplified with *SRK* primer pairs were extracted from agarose gel using an agarose gel DNA purification kit (version 2.0; TaKaRa). DNA fragments were ligated directly into the pMD 18-T cloning vector (TaKaRa) and transformed into *Escherichia coli* DH5α cells. The presence of the target gene was verified by PCR with specific primers (Table 1) and sequenced in Nanjing GenScript BioTechnologies Co., Ltd.

Sequence similarity was blasted in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database, and multiple sequences were compared using DNAman software ver. 5.2.2 (Lynnon Biosoft).

PCR-RFLP analysis

Purified *SRK* products were digested with *Hinf I* (TaKaRa). For digestion, 8 µL of the PCR products was mixed with 2.5 µL of 10 x buffer and 1 µL of *Hinf I*. The final volume was 20 µL. The mixture was incubated at 37°C for 6 h. The digested DNA was subjected to electrophoresis in a 2% agarose gel at 100 V for 2 h, and DNA bands were detected with ethidium bromide staining under UV light.

Results and discussion

SI is a genetic strategy for promoting outcrossing and preventing inbreeding in flowering plants [1]. The loss and regain of *S*-alleles during evolution result in novel *S* haplotypes that are easily affected by external factors, such as high temperature and humidity [22, 23]. Under the present conditions of climate change, the identification of *S* haplotypes is necessary for

exploring new *S* haplotypes and accelerating the hybrid breeding process of vegetable crops, especially among Cruciferae [24, 25]. The identification of *S* haplotypes, for example, has indicated that the distribution of class I *S* haplotypes in broccoli is related to geographical regions [23]. However, few studies have identified *S* haplotypes in cabbage [26, 27]. Thus, *S* haplotypes in *B. campestris*, especially in non-heading Chinese cabbage, should be further examined. In this study, we analyzed SI phenotypes and *S* haplotypes in 24 DH lines of non-heading Chinese cabbage by PCR-RFLP analysis.

S haplotypes analysis of DH lines

Genomic DNA from 24 DH lines was amplified using class I (P1+P2) and class II (P3+P4) *SRK* specific primers. Electrophoresis results showed that approximately 1, 000 bp specific bands were amplified with P1+P2 in 6 lines (i.e., 04, 06, 07, 09, 20, and 22) (Fig. 1A) and P3+P4 in 14 lines (i.e., 01, 02, 03, 05, 08, 09, 10, 12, 14, 15, 16, 20, 21, and 22) (Fig. 1B). The amplified products from 24 DH lines were classified into four types: (i) three lines (i.e., 04, 06, and 07) with only one specific band through class I *SRK* primers; (ii) 11 lines (i.e., 01, 02, 03, 05, 08, 10, 12, 14, 15, 16, and 21) with one DNA fragment through class II *SRK* primers; (iii) three lines (i.e., 09, 20, and 22) with one band of class I and one band of class II haplotypes; and (iv) seven lines (i.e., 11, 13, 17, 18, 19, 23, and 24) with no band.

The four types of amplified products indicated that lines 04, 06, and 07 contain class I *S* haplotypes and that lines 01, 02, 03, 05, 08, 10, 12, 14, 15, 16, and 21 contain class II *S* haplotypes. Observation of two-band patterns, which combine one band of class I and one band of class II haplotypes, suggested that lines 09, 20, and 22 are hybrids of class I and class II *S* haplotypes.

The SCIs of DH plants were also evaluated by calculating the seed rates of each plant (Table 2). The

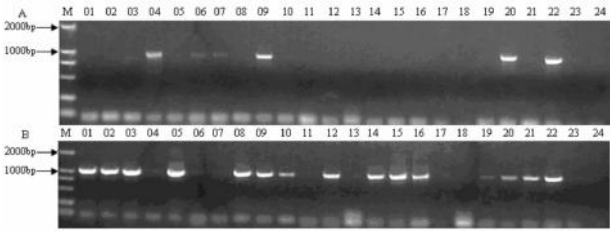

Fig. 1. DNA fragments amplified by PCR from genomic DNA of 24 DH lines through primers P1 + P2 (A) and P3 + P4 (B)

SCIs of lines 04, 06, and 07 were less than 0.5. Among these lines, the SCI of line 07 was the lowest (0.01), which suggested that the DH line 07 has a strong SI phenotype. Lines 01, 02, 03, 05, 08, 10, 12, 14, 15, 16, and 21 displayed weak SI, with SCIs ranging from 0.57 to 1.40 (Table 2). The SCI of three lines 09, 20, and 22, which featured mixed band patterns, were 0.40, 0.47, and 0.26, respectively; these results implied that all three lines also have strong SI.

Previous studies have reported that most *S* haplotypes in cabbage, Chinese cabbage, and broccoli belong to class I and that only a few of these haplotypes belong to class II; however, class II has an extensive distribution [14, 28, 29]. The low amplification number in non-heading Chinese cabbage with class I *SRK* primers could be mainly due to differences in the materials used or quantity limitations in the materials. Interestingly, the results revealed three lines with a combined pattern of both classes of *S* haplotypes and strong SI, as determined by the SCI results. This finding suggested that class II S haplotypes are determined by class I haplotypes because the phenotype expressed is that of the class I S haplotype.

PCR-RFLP analysis of SRK

Digestion of the PCR products with *Hinf I* revealed polymorphisms of the *SRK* fragment (Figs. 2 and 3). Class I *SRK* genes from three lines were further divided into two types: (i) line 04 with one band and (ii) lines 06 and 07 with two bands (Fig. 2). Class II *SRK* genes from 11 lines were divided into three types: (i) lines 01, 02, 03, 10, and 21 with one band approximately at 300 bp; (ii) lines 05, 14, 15, and 16 with two bands approximately at 300 and 350 bp; and (iii) lines 08 and

Table 2. The self-compatibility index of 24 DH lines

DH lines	SC index	DH lines	SC index
1	0.87	13	0.43
2	0.60	14	0.57
3	1.40	15	1.20
4	0.01	16	0.75
5	0.90	17	1.10
6	0.03	18	0.36
7	0.11	19	0.89
8	1.20	20	0.47
9	0.40	21	0.73
10	0.70	22	0.26
11	1.02	23	1.30
12	0.93	24	0.76

12 with no specific band above 250 bp.

Five types of *S* haplotypes in 17 lines were identified after the digestion of *SRK* fragments, and the low number of *S* haplotypes is believed to be related to the distribution of non-heading Chinese cabbage, which originates from China and mainly grows in the southern part of the country [30, 31]. The seven other lines with no amplified products of *SRK* are likely due to the divergence of nucleotide sequences in the primer binding sites. Therefore, based on the diversity of *S* haplotypes, other *SRK* primers should be developed to differentiate unclassified DH lines.

DH lines are cultivated from isolated microspores and generated from completely homozygous lines [32]. Thus, the *S*-allele in DH plants is theoretically homozygous. However, class I and class II *S* haplotypes were both observed in at least part of the DH lines. This phenomenon may be attributed to the effects of physical and chemical factors, such as culture temperature, medium components, and low temperature pretreatment, during isolated microspore culture [18]. In addition, the structure and number of chromosomes frequently alter through rearrangement, deletion, or insertion, which may increase the likelihood of mutation in regenerated plants. Wang [32] observed a similar phenomenon and speculated that such an occurrence may result from microspore mother cells

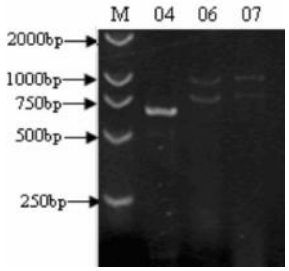

Fig. 2. PCR-RFLP analysis of *SRK* fragments of three DH lines through primer pair of P1 + P2, and digested with *Hinf* I

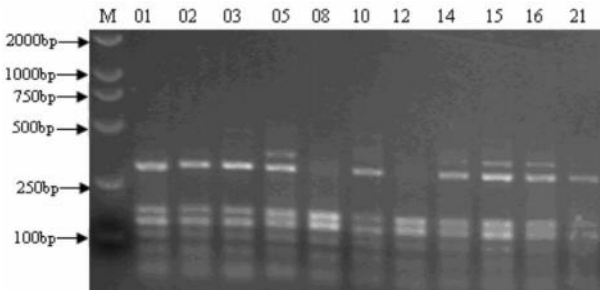

Fig. 3. PCR-RFLP analysis of *SRK* fragments of 11 DH lines through primer pair of P3 + P4, and digested with *Hinf* I

that do not undergo meiosis or the incomplete removal of somatic fragments during microspore culture. Finally, these defective plants remained heterozygous and required constant inbreeding or re-development by microspore culture. The PCR-RFLP analysis could be effectively applied in the identification of *S* haplotypes in non-heading Chinese cabbage. The present study provides a reliable and rapid approach for SI hybrid breeding in non-heading Chinese cabbage. Combined with the application of DH technology, PCR-RFLP analysis can help increase selection efficiency and improve the application of SI in non-heading Chinese cabbage hybrid breeding.

Acknowledgements

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