Botanical Studies (2008) 49: 93-99.

A genetic linkage map based on AFLP and NBS markers in cauliflower (*Brassica oleracea* var. *botrytis*)

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(Received March 26, 2007; Accepted October 4, 2007)

ABSTRACT. A genetic linkage map of cauliflower (*Brassica oleracea* var. *botrytis*) has been constructed based on AFLP and nucleotide binding site (NBS) markers, in order to identify potential molecular markers linked to important agronomic traits that could be useful for developing and improving the species. NBS profiling was first used to map resistance gene analogues (RGAs) in cauliflower (*Brassica oleracea* var. *botrytis*), which simultaneously allowed the amplification and mapping of genetic markers anchored in the conserved NBS encoding domain of plant disease resistance genes. At the same time, the AFLP method was also performed in this paper to construct an intervarietal genetic map of cauliflower. A total of 234 AFLP markers and 21 NBS markers were mapped in an F_2 population derived by self-pollinating a single F_1 plant (a hybrid between "AD White Flower" and "C-8") based on seventeen AFLP primer combinations and two degenerate primer/enzyme combinations. The markers were mapped into nine major linkage groups, spanning 668.4 cM, with an average distance of 2.9 cM between adjacent mapped markers. Each of the linkage groups contained from 12 to 47 loci, and the distance between two consecutive loci ranged from 0 to 14.9 cM. The AFLP markers were well distributed throughout the nine linkage groups, and eight linkage groups for the NBS markers. Most NBS markers mapped in this study were organized in clusters, indicating that most of them could be real RGAs. The maps we have generated provide a firm basis for mapping agriculturally relevant traits, which will then open the way for application of a marker-assisted selection breeding strategy in this species.

Keywords: AFLP; Cauliflower (*Brassica oleracea* var. *botrytis*); Genetic linkage map; NBS profiling.

Abbreviations: NBS-LRR, nucleotide binding site-leucine-rich repeat; **RGAs,** resistance gene analogues; **PCs,** primer combinations; **LGs,** linkage groups; **cM,** centiMorgans; ***R gene*,** resistance gene.

INTRODUCTION

Over the past two decades, several genetic maps of *Brassica oleracea* have been constructed. The first map was based on the segregation of 258 restriction fragment length polymorphism (RFLP) loci in a broccoli × cabbage F_2 population. The genetic markers defined nine linkage groups, covering 820 recombination units (Slocum et al., 1990). Subsequently, at least ten genetic linkage maps were developed (Landry et al., 1992; Kianian et al., 1992; Bohuon et al., 1996; Camargo et al., 1997; Voorrips et al., 1997; Cheung et al., 1997; Hu et al., 1998; Moriguchi et al., 1999; Sebastian et al., 2000; Chen et al., 2002; Howell et al., 2002; Wang et al., 2005; Wang et al., 2007). These maps were based on intraspecific or intersubspecific populations. An intervarietal genetic map of cauliflower (*Brassica oleracea* var. *botrytis*) was missing. The high level of genetic variability within an intraspecific population or the level of DNA polymorphism in the parents may explain this. Nevertheless, having an intervarietal linkage map is the most direct and efficient approach for cauliflower breeding in the future since the genetic information is derived from cauliflower.

Most studies have dissociated the isolation (cloning) of resistance gene analogos (RGAs) from their genetic mapping in segregating progenies. Most often, cloned RGAs are mapped using RFLPs, which is often time-consuming (Calenge et al., 2005). Modified amplified fragment length polymorphisms (AFLP) and nucleotide binding site (NBS) profiling were proposed as new strategies by Hayes and Saghai-Maroof (2000) and by Van der Linden et al. (2004) to generate, simultaneously, polymorphism and specifically amplify highly conserved motifs. Both methods are based on the simultaneous use of an adapter primer matching a restriction enzyme site

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and of a degenerate primer targeting the NBS encoding region. Genetic variation is sampled in the gene region flanking the primer-binding site. Nevertheless, NBS profiling has some advantages that improve specificity. NBS profiling involves a two-step PCR procedure. The first step is a linear (asymmetric) PCR with a limited amount of the NBS specific primer. The asymmetric PCR is then followed by an exponential PCR with an NBS primer and an adapter primer. An amino linker effectively blocks extension of the 3′ end of the short arm in the subsequent PCR reaction. The adapter primer can thus only participate in the PCR reaction after extension of the selective NBS primer. Amplification depends highly on the selectivity and specificity of this NBS primer. To further increase the specificity of the PCR procedure, the actual amplification (exponential PCR) is preceded by linear (asymmetric) PCR with only the NBS primer. In the modified AFLP method, in the initial digestion and ligation steps, approximately 87.5% of the soybean genome is eliminated because *Mse/Mse* fragments are not detected in the preceding steps. Nonetheless, NBS profiling can be identified in all genome. NBS profiling was shown to be highly effective in generating polymorphic markers with high sequence homology for RGAs in several species (Van der Linden et al., 2004).

The purposes of this paper were: 1) To construct an intervarietal linkage map of cauliflower. This map could be used to identify more markers, which would eventually be linked to genes controlling important agronomic characters and provide a powerful tool to be used in marker-assisted breeding of cauliflower. 2) To demonstrate the reliability and efficiency of the NBS profiling approach for mapping homologous nucleotide binding site-leucine rich repeat (NBS-LRR) genes in cauliflower, and 3) to determine the distribution and characterization of NBS-LRR-like sequences in the cauliflower genome.

MATERIAL AND METHODS

Plant materials and DNA isolation

An F_2 population of 100 progenies was generated by self-pollination of a single F_1 plant from an intervarietal cross between "AD White Flower" as pollen parent and "C-8" as seed parent. The mapping population was supplied by the Tianjin Kernel Vegetable Research Institute. "AD White Flower" is an inbred line, developed from a general Chinese variety through several inbreeding generations. "C-8" is a self-incompatible line derived from an individual plant mutation of an exotic line.

Total genomic DNA of mapping progenies was isolated following the CTAB method (Murray and Thompson, 1980) with some modifications. Fresh leaves (0.2 g) of both lines were ground in liquid nitrogen. The frozen powder was directly added to 2 ml lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, 2% PVP and 1×CTAB). After incubation at 56°C for 30 min, 2 ml phenol: chloroform: isoamyl alcohol (25:24:1) was added. The supernatant was obtained by centrifugation at 10,000 *g* for 10 min and extracted once with an equal volume of chloroform: isoamyl alcohol (24:1). After centrifugation at 11,000 *g* for 15 min, a two-thirds volume of cold isopropanol was added to the supernatant. The mixture was incubated for 30 min at -20°C. DNA was pelleted by centrifugation at 11,000 *g* for 30 min at 4°C and dissolved in 100 µl TE (pH 8.0) buffer. RNA was removed from the DNA by treatment with 200 µg µl-1 RNase A for 1 h at 37°C.

AFLP molecular marker analysis

AFLP analysis was performed according to Vos et al. (1995) with minor modifications. Briefly, 100-150 ng of DNA was digested with 1.5 U of both *EcoR* I and *Mse* I (Shanghai Sangon, Shanghai China). After ligation and pre-amplification, selective amplification was conducted by combining 30 ng of an *Eco*R I primer containing two selective nucleotides and an *Mse* I primer containing three selective nucleotides. Thermocycling (Mastercycler Gradient 5331, Eppendorf Germany) was done with 35 cycles and included a 12-cycle touchdown (annealing temperature was reduced from 65°C to 56°C at 0.7°C per step for 12 cycles, and subsequently maintained at 56°C for 23 cycles). Amplification products were separated in 6% (w/v) denaturing polyacrylamide gels at 65 W for 2.5 h in 1 ×TBE buffer. After silver staining (Bassam et al., 1991), the gels were dried at room temperature and photographed.

NBS profiling protocol

NBS profiling of the progeny was performed essentially as described by Van der Linden et al. (2004). In brief, DNA was digested for 4 h with *Mse* I, using 400 ng DNA per individual. An adapter was ligated to the restriction fragments. Adapter sequences and the adapter primer were:

Adapter long arm: 5′-ACTCGATTCTCAACCCGAAA GTATAGATCCCA-3′

Adapter short arm: 5′-TGGGATCTATACTT-3′ (with 3′ amino group)

Adapter primer: 5′-ACTCGATTCTCAACCCGAAAG-3′

Two different degenerate primers, NBS2 (5′-GTWG TYTTICCYRAICCISSCAT-3′) and NBS5 (5′-YYTKR THGTMITKGATGAYGTITGG-3′), were then used for a two-step PCR procedure. These primers were designed from part of the conserved P-loop and Kinase-2 motif, respectively, belonging to the NBS encoding region of several plant disease resistance genes (Van der Linden et al., 2004). The first PCR was linear, and only the degenerate primer (either NBS2 or NBS5) was used. The second PCR was exponential and was performed with both the degenerate primer and the adapter primer. Both PCRs were performed with an annealing temperature of 60°C on a Mastercycler Gradient 5331 (Mastercycler Gradient 5331, Eppendorf Germany), using the following cycling

program: 30 cycles of 30 s at 95°C, 1 min 40 s at 60°C and 2 min at 72°C. The PCR products were separated in 6% (w/v) polyacrylamide gels for 2.5 h at 65 W, then dried at room temperature and photographed.

Genetic linkage map construction

The AFLP fragments were scored as dominant, presence versus absence of bands, and therefore for the parents, F_1 and F_2 generation markers were assigned to either parental allele for map construction. AFLP markers were designated by the PCs used, followed by a number reflecting the fragment length on the gel (e.g., M-AAGe-AC400 = *Mse* I +AAG/*Eco*R I +AC, band length is 400 bp). NBS markers were tested against the predicted 3:1 ratio, representing [homozygote + heterozygote]: [homozygotes], by the presence: absence of the marker band, respectively. NBS markers were named using the same protocol/logic as AFLP (e.g., NBS2M60 = NBS2/*Mse* I, band length is 60 bp).

Markers originating from each parent were scored according to the standard coding system using A, B, C, D and H of JoinMap, Version 3.0 (Van Ooijen and Voorrips, 2001). Ambiguous genotypes were resolved by assigning a blank score (-) to the individual locus for map construction. Chi-square (χ^2) tests of goodness-of-fit were performed on segregation data for all markers, with a 0.5% threshold level for significance. To determine marker order within a linkage group, the following JoinMap parameter settings were used: Rec = 0.40, LOD = 1.0, Jump = 5. Markers were assigned to linkage groups (LGs) by increasing the LOD score for grouping with steps of one LOD unit. No order was forced during the linkage analysis. Recombination frequencies were converted to map distances in centimorgans (cM) using the Kosambi mapping function (Kosambi, 1943).

RESULTS

Polymorphism analysis using AFLP markers

Initially, sixty-four AFLP PCs were screened for polymorphism by using two parents. Seventeen most informative *Eco*R I+ 2/*Mse* I+3 PCs (Table 1) were selected for mapping according to the number and reliability of the polymorphic marker. Approximate 1,700 bands were produced by the seventeen PCs in the mapping population with an average of 100 bands per PC. In total, 339 polymorphic markers were scored as dominant markers (Figure 1). The number of polymorphic AFLP markers per PC ranged from 13 to 26, with a mean of 20 markers per PC (Table 1). Chi-square analysis revealed 234 of the 339 markers (69.9%) fitted a 3:1 ratio. The remaining 105 (30.1%) showed segregation distortion within the population.

NBS profiling marker development in cauliflower

Two degenerate primer/enzyme combinations were tested in the progeny: NBS2/*Mse* I and NBS5/ *Mse* I. An average of 60-90 monomorphic bands per PC was obtained. Thirty-one polymorphic bands were identified with the two combinations and scored as dominant markers (Figure 1). Ten markers (32.3%) had a distorted segregation in the progeny as assessed by chi-square tests and were discarded from data. 21 polymorphic markers (68%) were reliably mapped on the linkage map (Figure 2). NBS markers were mapped to 8 of the 9 linkage groups of

Table 1. Primer combinations for AFLP and NBS markers are shown with the number of polymorphic markers generated for each of them.

Primer combination	Number of markers	Primer combination	Number of markers
M50/E11	18	M48/E13	25
M60/E12	19	M60/E13	13
M59/E13	21	M49/E12	26
M62/E13	20	M60/E11	21
M47/E12	20	M49/E11	23
M47/E11	24	M61/E11	20
M48/E11	25	M48/E12	15
M61/E12	23	NBS2/*Mse*I	13
M62/E12	19	NBS5/*Mse*I	8
M61/E13	17		
		Total	370

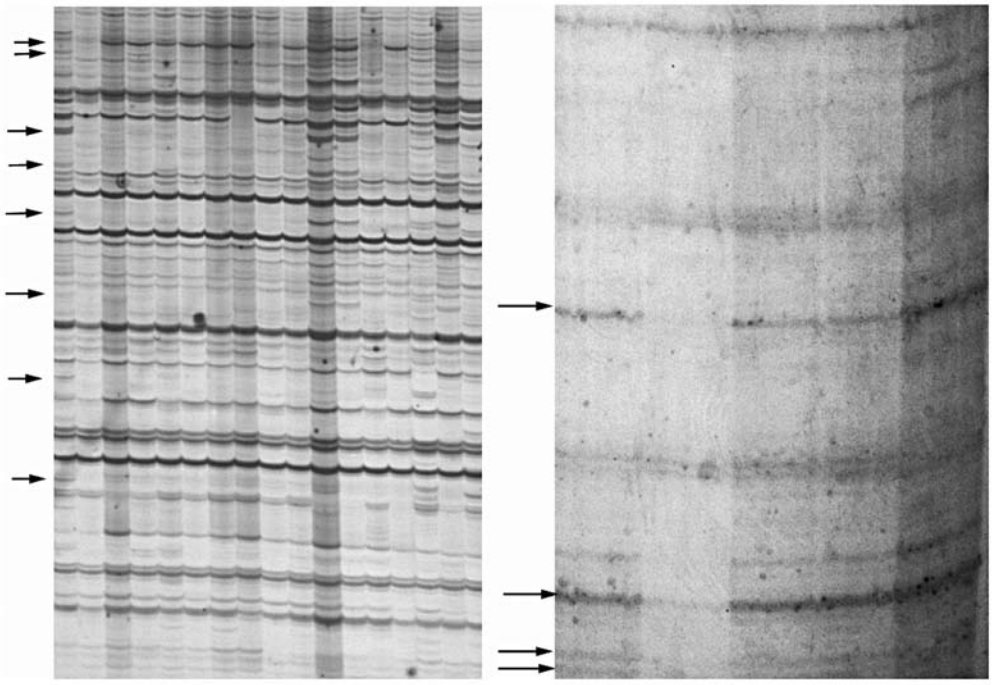

Figure 1. AFLP and NBS markers in a F_2 mapping population. Markers used for this study are indicated by arrow. Primer combinations for AFLP and NBS profiling were M-CTT/E-AG and NBS5/*Mse*I, respectively.

the genetic maps (Figure 2). Most markers were organized in more or less wide clusters of 0-18 cM.

Linkage analysis and map construction

The 234 AFLP markers and 21 NBS markers were used to construct a genetic map. Altogether, 255 of the 370 polymorphic markers were assigned to nine linkage groups (LGs) (Figure 2) ranging in size from 12 to 60 markers each. Map distances ranged from 42.6 to 113.0 cM per LGs (Table 2). The 255 markers (229 loci) gave a total map length of 668.4 cM and an average genetic distance between adjacent mapped loci as 2.9 cM (Figure 2). Linkage relationships of the 255 segregating markers were established at a $3.0 \leq \text{LOD} \leq 10.0$ and a recombination fraction smaller than 0.5 Map distances were converted to cM using the Kosambi mapping function (Kosambi, 1943). According to Figure 2, we can see the names of markers are shown at the right and their map position (cM) at the left. All the markers appear to be distributed randomly along all LGs, except LGs 3, 4 and 5, which show rather few markers.

DISCUSSION

Several genetic linkage maps of *B. oleracea* have been produced in the past using intraspecific or intersubspecific mating systems. It is generally believed that the degree of polymorphism is lower in an intervarietal population than in an intraspecific or intersubspecific population. The high level of genetic variability within an intraspecific population or the level of heterozygosity of the parents may explain this. However, Hu et al. (1998) compared three previous independent *B. oleracea* linkage maps and pointed out that the distances between markers often varied from map to map. This demonstrated that sequence rearrangement is a distinct feature of this genome. Slocum et al. (1990) reached the same conclusion. Their study suggested that a fairly high degree of genetic rearrangement has occurred in the evolution of *B. oleracea.* With respect to cauliflower breeding, we believe that intervarietal crosses should be better than intersubspecific. Results of this study suggest that an intervarietal cross allows better map resolution and even distribution in LGs.

Some published AFLP linkage maps show clustering of these markers in centromeric regions, due to an excess of repeats in this area and suppressed recombination shrinking the genetic map relative to the DNA content (Jeuken et al., 2001). In this study, do not find any mark on centromeric regions of any LG (Figure 2). Chromosome centromeric regions are usually conserved and may not be polymorphic when self-pollinated. This is in contrast to the clusters of markers mapped on an interspecific segregating population (Pearl et al., 2004; Lan et al., 2008).

It was previously demonstrated that the NBS profiling method allows the generation of polymorphic markers among different cultivars of potato, tomato, barley, and lettuce (Van der Linden et al., 2004). The present investigation is the first to test this new method in cauliflower and the first to demonstrate its utility for

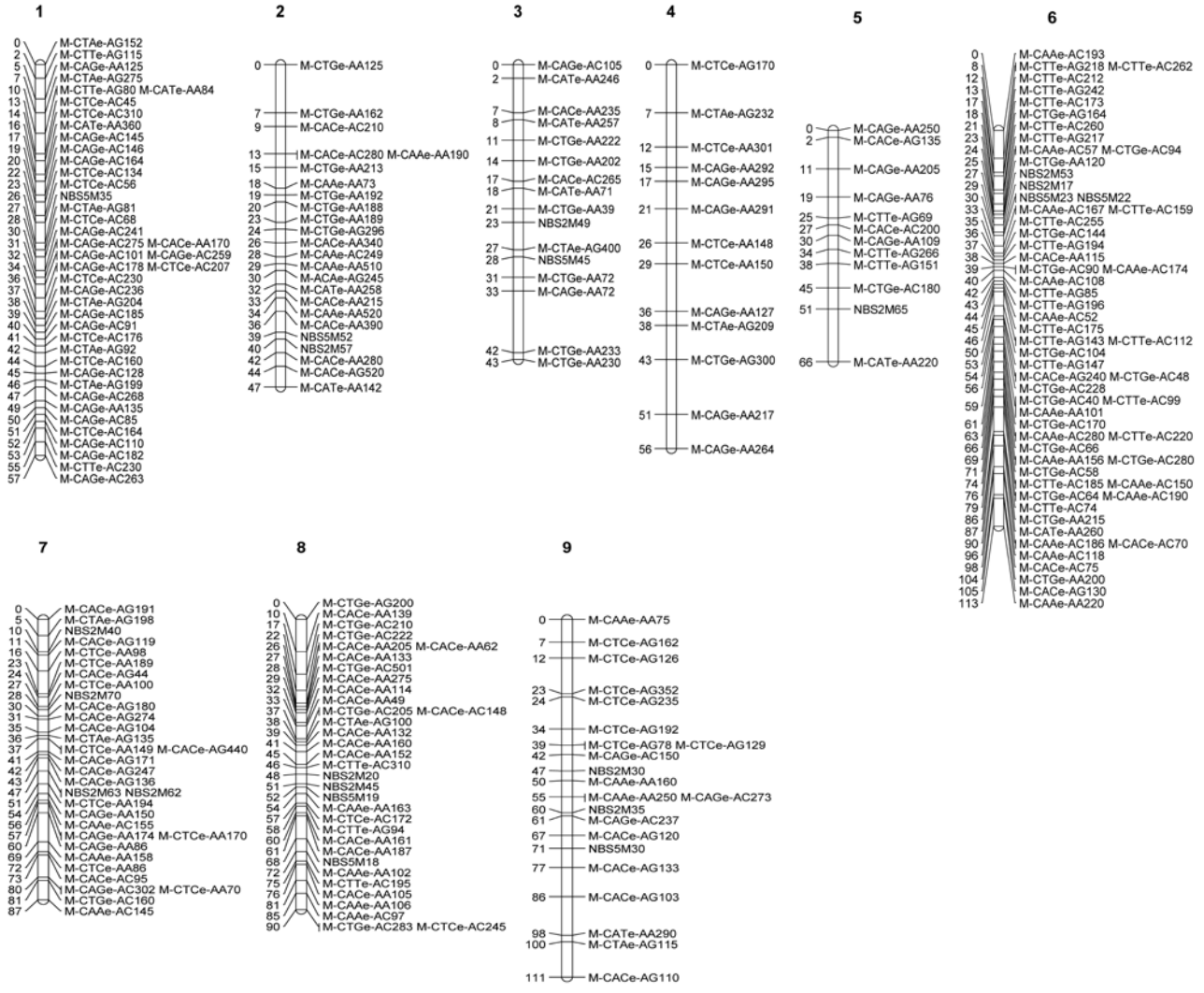

Figure 2. Genetic linkage map of cauliflower (*Brassica oleracea* var. *botrytis*). Linkage groups are numbered LG1 through to LG9. Markers are indicated at the right of each linkage group. Recombination distances are in Kosambi's cM and are indicated at the left of each linkage group.

Table 2. Marker number and map distance per linkage group.

Linkage group	Marker Num. (loci)	Marker Num AFLP/NBS (loci)	Length (cM)	Mean length (cM)	LOD
LG1	42(38)	41(37)/1(1)	56.7	1.5	10.0
LG2	24(23)	22(21)/2(2)	46.5	2.0	10.0
LG3	16(16)	14(14)/2(2)	42.6	2.7	10.0
LG4	13(13)	13(13)/0	56.1	4.3	7.0
LG5	12(12)	11(11)/1(1)	65.5	5.5	6.0
LG6	60(47)	56(44)/4(3)	113.0	2.4	6.0
LG7	33(29)	29(25)/4(4)	86.7	3.0	5.0
LG8	34(31)	30(27)/4(4)	90.5	2.9	4.0
LG9	21(20)	18(17)/3(3)	110.8	5.4	3.0
Total	255(229)	234(209)/21(20)	668.4	2.9	

mapping studies using a segregating progeny. Thirty-one polymorphic markers were observed in the intravarietal progenies across the two-primer/enzyme combinations tested, of which 21 markers could be mapped. Ten unmapped markers had a distorted segregation in the progeny, which could explain why they could not be reliably mapped. Fewer NBS profiling markers were obtained than AFLP markers. This is an inherent property of such markers, as each in principle corresponds to a member of the NBS-LRR RGA gene family that is present in far lower numbers in the plant genome than restriction sites (AFLP) (Syed et al., 2006). The usefulness of NBS markers is their close linkage to potentially important resistance genes. The NBS profiling molecular marker methods have been validated for cauliflower.

In our map, most markers were assembled in clusters. The best example is the cluster on linkage group LG6, which includes four markers (Figure 2). Therefore these markers correspond either to the same gene locus or to very tightly linked loci. The clustering of RGAs of the NBS-LRR type has previously been observed in numerous studies. Most NBS-LRR and other *R gene*-like sequences reside in large, extended arrays (Young, 2000). This clustering of RGAs is not surprising, considering that plant *R genes* often, though not always, belong to gene families with evolutionarily related tandemly repeated genes or to allelic series (Hulbert et al., 2001). The clustering of the markers mapped in this study adds to the evidence that most of them could be real RGAs.

Acknowledgements. The authors thank Ms Lili Liu from the Tianjin Kernel Vegetable Research Institute of China for planting and tending the mapping population. Financial support for this study was provided by the National Science Foundation of Tianjin (No.06YFJMJC10000).

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花椰菜 (*Brassica oleracea* var. *botrytis*) 遺傳圖譜的構建及 NBS-LRR 類抗性同源基因在圖譜中的定位

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利用 AFLP 和 NBS profiling 技術，以花椰菜自交系 "AD 白花" 與高代自交不親和系 "C-8" 雜交得到的 F_1 代自交產生的 F_2 代分離群體為材料，構建了第一個花椰菜遺傳連鎖圖譜。該圖譜由 234 個 AFLP 標記和 21 個 NBS 標記構成了 9 個連鎖群，總圖距為 668.4 cM，標記間平均距離為 2.9 cM。每個連鎖群包含的位點數從 12 到 47 個，相鄰兩標記之間的距離範圍是 0-14.9 cM。NBS 標記分佈在 8 個連鎖群中，這些標記大部分聚在一起。本研究的目的是構建花椰菜遺傳圖譜為以後的基因定位及重要農藝性狀的分析提供框架圖，此外，研究 NBS profiling 方法在花椰菜中的穩定性和有效性及 NBS-LRR 類 RGA 在花椰菜基因組中的分佈和特點。

關鍵詞：AFLP；花椰菜；遺傳連鎖圖譜；NBS profiling。