

Identification of sequence-related amplified polymorphism markers linked to the red leaf trait in ornamental kale (*Brassica oleracea* L. var. *acephala*)

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ABSTRACT. Artistic diversiform leaf color is an important agronomic trait that affects the market value of ornamental kale. In the present study, genetic analysis showed that a single-dominant gene, *Re* (red leaf), determines the red leaf trait in ornamental kale. An F_2 population consisting of 500 individuals from the cross of a red leaf double-haploid line 'D05' with a white leaf double-haploid line 'D10' was analyzed for the red leaf trait. By combining bulked segregant analysis and sequence-related amplified polymorphism technology, we identified 3 markers linked to the *Re/re* locus. A genetic map of the *Re* locus was constructed using these sequence-related amplified polymorphism markers. Two of the markers, Me8Em4 and Me8Em17, were located on one side of *Re/re* at distances of 2.2 and 6.4 cM, whereas the other marker, Me9Em11, was located on the other side of *Re/re* at a distance of 3.7 cM. These markers could be

helpful for the subsequent cloning of the red trait gene and marker-assisted selection in ornamental kale breeding programs.

Key words: Ornamental kale; Sequence-related amplified polymorphism; Red leaf

INTRODUCTION

Ornamental kale (*Brassica oleracea* L. var. *acephala*) is a biennial herbaceous cabbage. It is considered more closely related to wild cabbage than to most domesticated forms (Wang et al., 1995). Many varieties of kale are referred to as flowering kales and are grown mainly for their ornamental leaves, which have brilliant white, red, pink, lavender, blue, or violet interiors or rosettes. They are important ornamental foliage crops and are widely used as potted plants or cut flowers (Wang et al., 2011).

Remarkable advancements have been made in the haploid breeding of ornamental kale (Lichter, 1989; Feng et al., 2007), but its unique ornamental characteristics have yet to be investigated in depth. The brunet of the leaf displays dominant inheritance for light color (Gu et al., 2002). Xie (2003) observed that the separation ratios of red and white leaves in the F₂ generations of 2 hybridized combinations are close to 3:1 and 15:1. Thus, leaf color is a qualitative trait controlled by 1 or 2 pairs of genes, as combinations of different parents give inconsistent separation ratios.

Molecular markers have advantages over morphological, cytological, and biological markers and have been applied extensively in the life sciences (Fang and Roose, 1997). DNA molecular markers have become effective tools in marker-assisted selection to improve agronomic traits (Lands and Thompson, 1990; Young, 1999; Xiao et al., 2009). However, molecular markers for the leaf color trait in ornamental kale have not been reported until now.

Sequence-related amplified polymorphism (SRAP) is a new and useful molecular marker system for tagging and mapping in *B. oleracea* (Li and Quiros, 2001). The SRAP system is easy to perform, gives stable and moderate yields and a high proportion of co-dominance, and ultimately simplifies the cloning and sequencing of target segments. The SRAP technique is an effective tool for constructing linkage maps (Yuan et al., 2008), carrying out comparative genomics, and analyzing genetic diversity (Ferriol et al., 2003; Li et al., 2003; Lin et al., 2004) because sequences are amplified against open reading frames, and sequences near the centromeres and telomeres of genomes are only rarely amplified. The SRAP technique has been applied successfully in studies of sweet potato, rape, and Chinese cabbage (Riaz et al., 2001; Wu et al., 2005; Yu et al., 2009).

The aims of the present study were to determine the genetic mechanism behind the red leaf trait in ornamental kale and identify molecular markers that are tightly linked to this trait by combining a bulked segregant analysis (BSA) (Michelmore et al., 1991) with SRAP marker technology. These data are expected to facilitate marker-assisted selection of the red leaf trait in kale breeding and advance studies for the map-based cloning of the red leaf trait gene.

MATERIAL AND METHODS

Plant material

A red leaf, double-haploid line, 'D05', and a white leaf, double-haploid line, 'D10', of

B. oleracea were used to construct an F_2 segregation population (Figure 1). These strains were obtained from isolated microspore cultures of ornamental kale. A cross (D05 x D10) was made to determine the inheritance of the leaf color trait, and F_2 and BC_1 populations were obtained. The F_2 population consisted of 500 plants, which were used for SRAP marker analysis. Plants were grown in a greenhouse under natural sunlight at Shenyang Agricultural University, and leaf color was determined by visual observation during the color development phase.

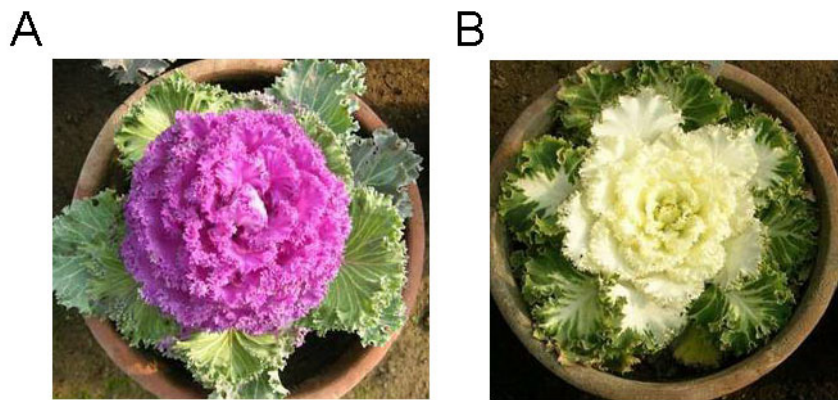


Figure 1. Double-haploid lines of ornamental kale. **A.** Red leaves (D05). **B.** White leaves (D10).

DNA extraction and BSA design

Genomic DNA of the parent plants and the F_2 segregating population was extracted from leaves using a cetyltrimethylammonium bromide extraction protocol (Clark, 1997), with minor modifications. The purity and concentration of the DNA were checked on 1% (w/v) agarose gels with an ultraviolet spectrophotometer. DNA concentration was adjusted to 50 ng/ μ L and stored at -20°C until used. Equal amounts of DNA from 8 red leaf and 8 white leaf plants were selected at random from the F_2 population and were pooled to construct 2 DNA bulks for BSA.

SRAP analysis

A total of 99 SRAP primer combinations from the studies of Li and Quiros (2001), Ferriol et al. (2003), and Li et al. (2003) were used to screen polymorphisms in the 2 parents and 2 DNA bulks. Table 1 shows the SRAP primer sequences used in the present study. All primers were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). Primers that amplified polymorphic bands in the BSA bulks were tested in the 16 individual plants that made up the 2 bulks and then checked further for their linkage to the red leaf gene in the F_2 population (500 individuals). Each polymerase chain reaction (PCR) was performed in a total volume of 10 μ L made up of 0.8 μ L 2.5 mM deoxyribonucleotide triphosphate mix, 1 μ L 10X PCR buffer, 0.2 μ L 2.5 U/ μ L *Taq* polymerase, 1 μ L 3 μ M/ μ L forward and reverse primers, 0.5 μ L DNA template, and 6.5 μ L double-distilled H_2O . Each reaction was started at 94°C for 3 min, followed by 8 cycles at 94°C for 30 s, 35°C for 30 s, and 72°C for 1 min; 35 cycles at

94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were separated on a 5% denaturing polyacrylamide gel. After electrophoresis, the gel was stained with AgNO₃ in solution (Bassam et al., 1991). SRAP analysis was repeated at least twice for each DNA sample.

Table 1. Primer sequences of polymorphic SRAP markers used in this study.

Forward primer name	Sequence (5'-3')	Reverse primer name	Sequence (5'-3')
Me1	TCAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTGTC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTGGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTAG	Em7	GACTGCGTACGAATTCAA
Me8	TGAGTCCAAACCGGAAC	Em8	GACTGCGTACGAATTCTG
Me9	TGAGTCCAAACCGGAGT	Em9	GACTGCGTACGAATTCGA
		Em10	GACTGCGTACGAATTCAG
		Em11	GACTGCGTACGAATTCGA

Linkage analysis and marker nomenclature

Linkage analysis was carried out using Join Map 3.0 (Van Ooijen et al., 2001), and the linkage group was drawn using MapChart (Voorrips, 2002). Recombination percentages were converted to genetic distances using the Kosambi mapping function (Kosambi, 1944). The polymorphic markers were named according to their corresponding primer combination codes.

RESULTS

Genetic analysis of the red leaf color trait in kale

The results of the genetic analysis are shown in Table 2. All 30 plants obtained from the cross (D05 x D10) had red leaves, indicating that the red leaf trait was dominant. Chi-square tests of the cross (D05 x D10) confirmed a ratio of 3:1 (red leaf:white leaf) in the F₂ population, and 1:1 (red leaf:white leaf) in the BC₁ backcross population (using the white leaf line as the recurrent parent). These results indicate that a single-dominant gene controls the red leaf trait. The dominant and recessive alleles of this gene are herein referred to as *Re/re*.

Table 2. Segregation analysis of the red leaf trait in F₁, F₂ and BC₁ populations of the cross (D05 x D10).

Population	Total No. of individuals	D05 x D10		Expected ratio	χ^2
		No. of red leaf plants	No. of white leaf plants		
F ₁	30	30			
F ₂	500	376	124	3:1	0.011
BC ₁	123	61	62	1:1	0.008

$$\chi^2_{0.05} = 3.84.$$

Detection of genomic DNA purity and integrity

Genomic DNA from leaves was extracted, and the integrity of DNA was deemed acceptable with 1% agarose gel electrophoresis, as the DNA bands were distinct and showed no dispersed appearance (Figure 2). The ratios of optical density $(OD)_{260} : (OD)_{280}$ were always between 1.819 and 1.954.

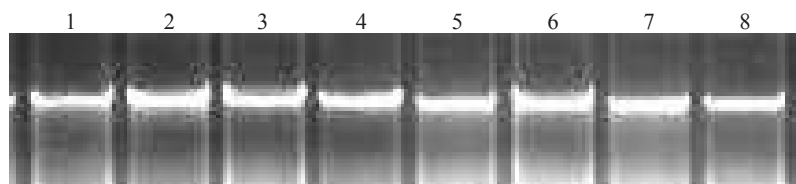


Figure 2. Electrophoresis of genomic DNA. Lanes 1 to 8 = individuals selected randomly from F_2 population.

Detection of SRAP markers linked to the *Re/re* locus

To identify markers linked to the *Re/re* gene, we used 99 SRAP primers to observe polymorphism. Of these, 41 primer combinations (41.4%) were polymorphic in both parent plants (D05 and D10). After further testing with the 2 DNA bulks and 16 individuals that made up these 2 DNA bulks, 3 SRAP primer combinations (Me8Em4, Me8Em17, and Me9Em11) were found to exhibit polymorphic bands between the 2 bulks and 16 individuals. We used these 3 markers to test the recombination ratio in the 124 individuals with the recessive *re* allele. This analysis showed that the numbers of recombinant plants in this population were 10, 13, and 11, respectively, giving recombination ratios of 4.0, 5.2, and 4.4%, respectively. The amplification results of the 3 primers in the recessive plants are shown in Figure 3.

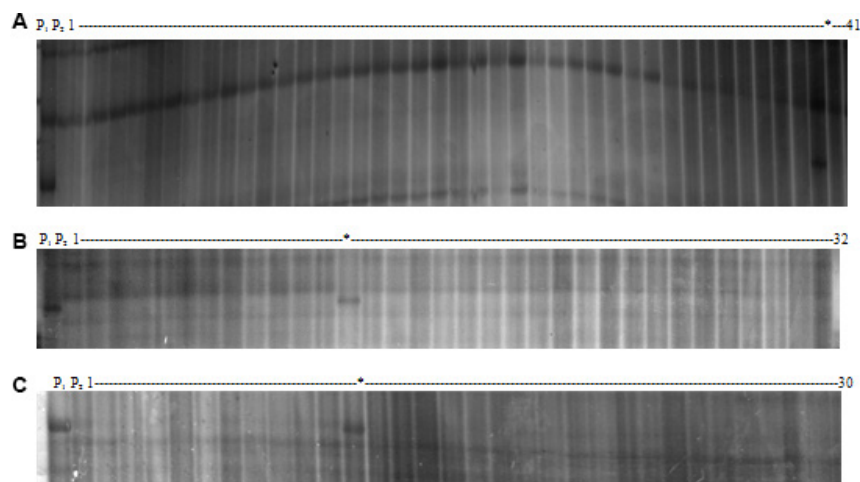


Figure 3. **A.** Amplification of Me8Em4 in parental lines and white leaf individuals. **B.** Amplification of Me8Em17 in parental lines and white leaf individuals. **C.** Amplification of Me9Em11 in parental lines and white leaf individuals. Lane P_1 = D05; lane P_2 = D10; lanes 1 to 41 (A); lanes 1 to 32 (B); lanes 1 to 30 (C) = all white leaf individuals; (*) = recombinants.

Linkage group construction of the *Re/re* locus

The preliminary linkage analysis was performed using Join Map 3.0, revealing that the 3 linked SRAP markers flanking the *Re/re* locus were Me8Em4 at 2.2 cM, Me8Em17 at 6.4 cM, and Me9Em11 at 3.7 cM. The linkage group constructed with the markers linked to the *Re/re* locus is shown in Figure 4. Two markers - Me8Em4 and Me8Em17 - are located on one side of the *Re/re* locus, whereas the other marker (Me9Em11) is on the other side of this locus.

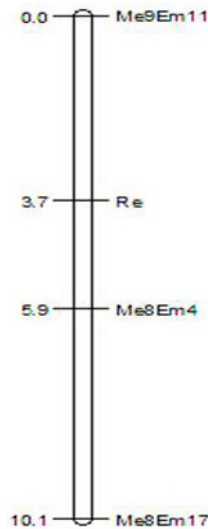


Figure 4. Genetic linkage map of the *Re* locus generated in the recombinant population (500 individuals) from the cross (D05 x D10).

DISCUSSION

Ornamental kale displays a range of leaf colors and shapes (Zhao et al., 2009). This kale has a longer ornamental period owing to its tolerance of cold temperatures, and it is also amenable to large-scale cultivation (Zhao et al., 2009). Because of these desirable characteristics, the plant is used widely for landscaping in cities and has become an important landscape plant during cold seasons. In the present study, of 500 individuals in the F_2 population, 124 individuals had white leaves, giving a segregation ratio of 3:1 ($\chi^2 = 0.011 < \chi^2_{0.05} = 3.841 = 3.841$). This segregation ratio confirmed that the red leaf trait is controlled by a single-dominant gene (Gu et al., 2002).

SRAP has been used widely in genetic studies of plant species including *B. oleracea* (Li et al., 2003), *Cucurbita pepo* (Ferriol et al., 2003), and *Brassica rapa* (Rahman et al., 2007). Compared with other randomly amplified markers, SRAP markers are distributed more evenly throughout the whole genome. Owing to the random combination of forward and reverse primers, SRAP markers may reduce the cost of primer synthesis and greatly improve efficiency by using only a small amount of primer to generate a large number of combinations (Liu et al., 2009). Both the polymorphism ratio and polymorphic fragment number of each

primer pair of SRAP markers are greater than those yielded by random-amplified polymorphic DNA (RAPD). SRAP has the same operation convenience as RAPD but shows improved stability over RAPD (Liu et al., 2004). In addition, SRAP markers require no complex mix of enzymes for cutting, connection, and pre-amplification compared with that needed for amplified fragment length polymorphism markers, which makes SRAP a more effective molecular marker system for genome studies (Xu and Zhao, 2009).

Of the 99 SRAP primer combinations tested in this study, 41 pairs (41.4%) were polymorphic between the 2 parents. BSA involves the mixing DNA of plants from a population with the same phenotype, which creates 2 gene bulks with the same genetic background except for the target gene loci (Chen et al., 2007). We screened polymorphisms of 99 SRAP pairs in the 2 parent plants and 2 DNA bulks by combining BSA and SRAP molecular marker approaches, and this technique identified 3 SRAP markers linked to the red leaf trait. Specifically, these markers were Me8Em4, Me8Em17, and Me9Em11, found at genetic distances of 2.2, 6.4, and 3.7 cM, respectively.

Fine mapping of a gene is essential for map-based cloning (Tanksley et al., 1995), and some genes of carotenoid mutants have been isolated using a map-based cloning technique (Ronen et al., 2000; Isaacson et al., 2002). Thus, the information presented in this paper may be useful for further fine-mapping studies and ultimately for map-based cloning of *Re*. We are currently preparing to fine map *Re* after the construction of a large-scale segregating population.

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