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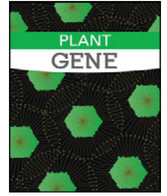

Genetic distance of inbred lines of Chinese cabbage and its relationship to heterosis

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ABSTRACT

Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) is an important vegetable in Asia. Most Japanese commercial cultivars of Chinese cabbage use an F_1 hybrid seed production system because of the high yielding cultivars produced. An efficient method for predicting hybrid performance in the parental generations is desired, and genetic distance between parental lines might be a good indicator of the level of hybrid vigor in a cross. Information concerning the genetic relationships among parental candidate inbred lines is useful for variety protection. The number of DNA markers available that can be used to assess the purity of inbred lines is limited in *B. rapa*. The aim of this study is to use DNA markers to assess the genetic distance between inbred lines to examine early developmental and yield heterosis so as to develop methods for selecting the best parental lines for the production of hybrids. We screened highly polymorphic SSR and CAPS markers to assess the genetic uniformity of inbred lines and characterize their genetic relationship. We examined the early size and yield heterosis in 32 F_1 hybrids of Chinese cabbage. There was a moderate correlation in mid-parent heterosis between leaf size at 21 days after sowing and harvested biomass but not in best-parent heterosis. In contrast there was no correlation between genetic distance and mid-parent or best-parent heterosis, indicating that genetic distance does not predict the heterosis phenotype.

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1. Introduction

Brassica rapa L. comprises commercially important vegetable crops consumed worldwide such as leafy vegetables including Chinese cabbage (var. *pekinensis*), pak choi (var. chinensis), and komatsuna (var. *perviridis*), root vegetables including turnip (var. *rapa*), and oilseed (var. *oleifera*). Chinese cabbage forms a head with large pale-green colored leaves and wide white midribs and is an important vegetable in Asia. As the genome sequence of Chinese cabbage (Chiifu-401-42) has been released and *B. rapa* is related to the model plant *Arabidopsis thaliana* (The *Brassica rapa* Genome Sequencing Project Consortium., 2011), detailed genetic and evolutionary studies have become possible.

In Japan, most commercial cultivars of Chinese cabbage are F_1 hybrids because of their agronomic benefits such as high yield, stress tolerance, disease resistance, and uniform phenotype. Hybrid breeding came from the discovery of heterosis or hybrid vigor, which is defined as the superior performance of hybrid plants over the parents (Crow, 1998). When breeding F_1 hybrid cultivars, breeders developed elite pure lines (inbred lines) as parents for hybrid production. About five to seven generations of selfing and selection based on traits concerned with the breeding objective are required for developing inbred lines as parental candidates. The level of heterosis of crosses of all possible combinations of the inbred lines is used to identify suitable parents for F_1 hybrid generation. An efficient method for predicting hybrid performance in the parental generations is desired as hybrid production can be expensive, time consuming, and labor intensive. Genetic distance between parental lines might be a good predictor, though the relationship between genetic distance and heterosis is controversial (Barth et al., 2003; Dreisigacker et al., 2005; Flint-Garcia et al., 2009; Geleta

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et al., 2004; Meyer et al., 2004; Yu et al., 2005). Information concerning the genetic relationships among parental candidate inbred lines is also useful for variety protection.

There are various types of DNA markers such as cleaved amplified polymorphic sequences (CAPS), amplified fragment length polymorphisms (AFLP), randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), and insertion/deletion polymorphism (InDel) markers. SSR markers have been widely used because of high polymorphism, reproducibility, co-dominant inheritance, and genome-wide coverage. In addition, SSR markers require only small amounts of DNA for PCR, and can be used for high-throughput analysis. SSR markers have been widely used for detecting genetic diversity and making genetic linkage maps, and many SSR markers are available for the genus Brassica (Guo et al., 2014; Hatakeyama et al., 2010; Lowe et al., 2004; Pino Del Carpio et al., 2011; Ramchiary et al., 2011; Suwabe et al., 2002; Suwabe et al., 2006). Sequencing technology enables us to identify SNPs easily, and SNPs are wide spread in the *B. rapa* genome (Metzker, 2010; Rafalski, 2002). SNPs detected by RNA-sequencing (RNA-seq) in coding regions are used for developing gene-based markers (Paritosh et al., 2013). Restriction-site associated DNA sequencing (RAD-seq) where the flanking region is sequenced from a specific restriction site, is useful for developing DNA markers and high-throughput genotyping (Baird et al., 2008).

The purpose of this study is to examine the possibility of using DNA markers to enhance breeding in *B. rapa*. We focused on the relationship between genetic distance and heterosis. We identified highly polymorphic DNA markers for calculating genetic distance or assessment of homozygosity in inbred lines of Chinese cabbage and examined the relationship between heterosis and genetic distance calculated by DNA markers. The information obtained in this study will be useful for breeding in Brassica.

2. Materials and methods

2.1. Plant materials and DNA extraction

The commercial F_1 hybrid cultivars of Chinese cabbage (Table S1) and F_2 populations produced by bud pollination of these F_1 hybrid cultivars were used as plant materials. A commercial cultivar of Chinese cabbage 'Chiifu', 31 Chinese cabbage inbred lines (RJKB-T01–T20, -T22–T24, -T26–T28, and -T30–T35), 3 turnip doubled haploid (DH) lines (Atsumi, Kisobeni, and BRA2209), 2 komatsuna inbred lines (YBCG-T01 and -T02) and 2 komatsuna DH lines (OSD2 and Wase Maruba) were also used in this study. We developed 31 F_1 hybrids by crossing between seed (RJKB-T01–T03, T07–T20, and -T26–T28) and pollen (RJKB-T30–T35) parents. Parental combinations were defined with regards to flowering time; RJKB-T33 and -T34 were early flowering, RJKB-T31 and -T32 were intermediate flowering, and RJKB-T30 and -T35 were late flowering.

Seeds were sown on soil and plants were grown in growth chambers under a 16-h/8-h light/dark cycle at 22 °C. Young leaves harvested from the F_1 and F_2 seedlings were used for genomic DNA extraction. Total genomic DNA was isolated by the Cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980).

2.2. Evaluation of heterosis phenotype

For examining the heterosis phenotype of early developmental stages, plants were grown in plastic dishes containing Murashige and Skoog (MS) agar medium supplemented with 1.0% sucrose (pH 5.7) in growth chambers under a 16-h/8-h light/dark cycle at 22 °C. Cotyledons at 6 days after sowing (DAS), and 1st and 2nd leaves at 14 DAS were fixed in a formalin/acetic acid/alcohol solution (ethanol:acetic acid:formalin = 16:1:1). The image of the whole cotyledon or leaf was photographed under a stereoscopic microscope, and sizes were determined with Image-J software (http://rsb.info.nih.gov/ij/).

For examining the yield under field conditions, seeds were sown on multi-cell trays on 21st August 2014 and grown in a greenhouse. At 6 DAS, cotyledons were photographed, and the area of the cotyledons was determined with Image-J software (http://rsb.info.nih.gov/ij/). On 3rd September 2014, seedlings were transplanted to the field at Osaki, Miyagi, Japan (38°57′N, 141°00′E). Thirty plants per plot were transplanted and plot size was 13.5×0.7 m. Row spacing is 70 cm and planting distance is 40 cm. At 21 DAS, leaf lengths and widths of the first and second largest leaves were measured. On 13th and 14th November 2014, plants were harvested. Statistical comparisons of cotyledon area, leaf size, fresh weight of total biomass and harvested biomass were performed using Student's *t*-test ($p < 0.05$).

The ratio of heterosis performance between F_1 and mid parent value (MPV) (termed rMPV) is calculated as follows, rMPV = F_1 (mean)/MPV (mean of two parents). The ratio of heterosis performance between F_1 and better parent value (BPV) (termed rBPV) is calculated as follows, rBPV = F_1 (mean)/BPV (mean of better parent).

2.3. Detection of DNA polymorphism with SSR markers

A total of 321 SSR markers, "BRAS", "BRMS", "BnGMS", "CB", "KBr", "Na", "Ni" and "Ol", were used to screen for polymorphisms among F_2 individual plants derived from the F_1 hybrid cultivar 'W77' (Table S2). The PCR reaction was performed using the following conditions; 1 cycle of 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 3 min. The PCR products were electrophoresed on 10% or 13% polyacrylamide gel using NA-1040 or NA-1118 (NIHON EIDO, Japan). The gel was stained with Gelstar solution (0.1 μl/10 ml; Takara Bio Inc., Japan). Primer sequences used in this study are shown in Table S3.

2.4. Detection of DNA polymorphism with CAPS markers

A total of 38 CAPS markers were used for examining the genetic distances among Chinese cabbage inbred lines. The PCR reaction was performed using the following conditions; 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 3 min. Amplified DNA digested by *Afa* I, *Hae* III, *Hha I*, *Hinf* I, *Mbo* I, *Msp* I, or *Hae* III restriction enzymes were electrophoresed on 13% polyacrylamide gel. The gel was stained with Gelstar solution (0.1 μl/10 ml; Takara Bio Inc., Japan). Primer sequences used in this study are shown in Table S3.

2.5. RAD-seq

Genomic DNA was digested using two restriction enzymes, *Bgl* II and *Eco* RI. The digested DNA fragments and two adapters (Bgl II adapter and Eco RI adapter) were ligated. The digestion and ligation were simultaneously performed at 37 °C for 16 h. The reaction mixture consisted of 20 ng of genomic DNA, 5 units of *Bgl* II (NEB), 10 units of *Eco* RI-HF (NEB), 1× NEB buffer2 (NEB), 1× BSA (NEB), 0.2 μM Bgl II adapter, 0.2 μM Eco RI adapter, 1 mM ATP (Takara), 300 units of T4 DNA ligase (Enzymatics). The ligation product was purified by the AMpureXP (Beckman coulter) according to manufacturer's instructions. One tenth of the purified DNA was used in the PCR enrichment with the KAPA HiFi HS ReadyMix (KAPA biosystems). Sequences of adaptors and primers used in this study are shown in Table S4. Approximately 350 bp fragments of the PCR product was selected by the E-Gel size select 2% (Life technologies). Single end 50 bp and index sequence of the library was sequenced by the HiSeq2500 (Illumina) with the TruSeq v3 chemistry. Preprocessing of the sequence data was performed by the trimmomatic-0.32 with the following parameters: ILLUMINACLIP TruSeq3-SE.fa:2:30:10 LEADING:19 TRAILING:19 SLIDINGWINDOW:30:20 AVGQUAL:20 MINLEN:51 (Bolger et al., 2014). The preprocessed sequences were analyzed by the Stacks program with default parameters (Catchen et al., 2013). We selected 288

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