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ORIGINAL PAPER

# Identification of the *BrRHP1* locus that confers resistance to downy mildew in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) and development of linked molecular markers

**Sunggil Kim · Young Ha Song · Ji-Yeon Lee · Su Ryun Choi · Vignesh Dhandapani · Chang Soon Jang · Yong Pyo Lim · Taeho Han**

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**Abstract** Inheritance of resistance to downy mildew (*Hyaloperonospora parasitica*) in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) was studied using inbred parental lines RS1 and SS1 that display strong resistance and severe susceptibility, respectively. $F_1$, $F_2$, and $BC_1F_1$ populations were evaluated for their responses to downy mildew infection. Resistance to downy mildew was conditioned by a single dominant locus designated *BrRHP1*. A random amplified polymorphic DNA (RAPD) marker linked to *BrRHP1* was identified using bulked segregant analysis and two molecular markers designated BrPERK15A and BrPERK15B were developed. BrPERK15B was polymorphic between the parental lines used to construct the reference linkage map of *B. rapa*, allowing the mapping of the *BrRHP1* locus to the A1 linkage group. Using bacterial artificial chromosome clone sequences anchored to the A1 linkage group, six simple polymerase chain reaction (PCR) markers were developed for use in marker-assisted breeding of downy mildew resistance in Chinese cabbage. Four simple PCR markers flanking the *BrRHP1* locus were shown to be collinear with the long-arm region of *Arabidopsis* chromosome 3. The two closely linked flanking markers delimit the *BrRHP1* locus within a 2.2-Mb interval of this *Arabidopsis* syntenic region.

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S. Kim · Y. H. Song · J.-Y. Lee · T. Han (✉)
Department of Plant Biotechnology, Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Korea
e-mail: hanth@jnu.ac.kr

S. R. Choi · V. Dhandapani · Y. P. Lim
Department of Horticulture, Genome Research Center, Chungnam National University, Kung-Dong 220, Yusong-Gu, Daejeon 305-764, Korea

C. S. Jang
HanKook Seed Co, Ansung-Si, Kyunggi-Do 456-841, Korea

## Introduction

Downy mildew, one of the most severe diseases affecting the Brassicaceae family, is caused by the obligate parasite *Peronospora parasitica* (Yerkes and Shaw 1959). Recently, the genus *Peronospora* was split into three genera, and the *Brassica*-infecting genus was renamed *Hyaloperonospora parasitica* (Constantinescu and Fatehi 2002). This disease causes worldwide damage to vegetable production of *Brassica* species during both seedling and adult-plant stages in cool and humid weather conditions. Endemic downy mildew is difficult to control unless the weather conditions change to hot and dry. Fungicides such as metalaxyl have been used to control downy mildew, but overuse of fungicides has adverse environmental effects and can be associated with the appearance of resistant pathogen isolates (Brophy and Laing 1992; Vishunavat et al. 1998). Breeding of resistant cultivars is an attractive and environmental friendly approach to control downy mildew.

Extensive studies on inheritance patterns of downy mildew resistance have been performed in *Brassica* species. Multiple sources of resistance and partially dominant conditioning genes have been reported in *Brassica napus* (Lucas et al. 1988; Nashaat et al. 1997). Several studies on resistance at the cotyledon, seedling, and adult-plant stages of *Brassica oleracea* indicate roles for a single dominant gene (Mahajan et al. 1995; Jensen et al. 1999; Coelho and Monteiro 2003b), two duplicate dominant genes (Monteiro et al. 2005), and recessive genes (Carlsson et al. 2004).

In addition, Monteiro et al. (2005) showed that resistance at the cotyledon and adult-plant stages is controlled by different resistance genes. Some resistance loci to downy mildew in *B. oleracea* were mapped using molecular markers (Giovannelli et al. 2002; Farinhó et al. 2004).

Gene-for-gene interactions between *Arabidopsis thaliana* and *Hyaloperonospora arabidopsidis* (formerly known as *Peronospora parasitica* or *Hyaloperonospora parasitica*), the pathogen responsible for downy mildew in *Arabidopsis*, are a model system for studying plant-pathogen interactions. Nearly 20 *RPP* loci (recognition of *Peronospora parasitica*) conferring resistance to different isolates of downy mildew have been identified in diverse accessions of *A. thaliana* (Holub et al. 1994; Slusarenko and Schlaich 2003), and six *RPP* genes or gene clusters have been cloned (Parker et al. 1997; Botella et al. 1998; McDowell et al. 1998; Bittner-Eddy et al. 2000; Sinapidou et al. 2004). In addition, the avirulence gene in *H. arabidopsidis* was isolated, and evidence of diversifying selection in the resistance and avirulence genes was presented (Allen et al. 2004).

Relatively few studies on downy mildew resistance have focused on Chinese cabbage (*Brassica rapa*). Diverse accessions of Chinese cabbage were screened to identify resistance to downy mildew (Yuen 1991; Silue et al. 1996), and resistance genes were inherited as a single dominant gene (Niu et al. 1983). Recently, major quantitative trait loci (QTL) controlling resistance at seedling stages were identified using QTL mapping, and the position of a resistance locus, *BraDM*, was localized on the A8 linkage group of *B. rapa* (Yu et al. 2009).

Since the first linkage map of *B. rapa* was reported by Song et al. (1991), several others have been constructed using diverse molecular markers (Nozaki et al. 1997; Kim et al. 2006; Suwabe et al. 2006). The linkage map produced by Choi et al. (2007) using doubled haploid populations originating from the cross between inbred lines 'Chiifu-401-42' and 'Kenshin-402-43' was proposed by The Multinational Brassica Genome Project (http://www.brassica.info/) as a reference linkage map of *B. rapa*. An updated second generation reference linkage map was constructed in which each linkage group was matched to the 10 *B. rapa* chromosomes using fluorescence in situ hybridization (FISH) and bacterial artificial chromosome (BAC) clones as probes (Kim et al. 2009). A high-density reference linkage map (Li et al. 2010) along the first BAC-based physical map (Mun et al. 2008) was also constructed to support whole genome sequencing of *B. rapa* genomes. With the help of these sophisticated linkage maps, the QTL or loci governing economically important traits of *B. rapa* such as clubroot resistance (Piao et al. 2004; Saito et al. 2006), black rot resistance (Soengas et al. 2007), white rust resistance (Kole et al. 2002), level of self-incompatibility (Hatakeyama et al. 2010), and flowering time (Li et al. 2009) have been mapped.

In this study, we identified a novel locus, *BrRHP1*, conferring resistance to downy mildew at adult-plant stages, and localized it on the A1 linkage group of *B. rapa* using the reference linkage maps (Choi et al. 2007; Kim et al. 2009). In addition, we developed simple polymerase chain reaction (PCR) molecular markers linked to the resistance locus for marker-assisted breeding of downy mildew resistant cultivars in Chinese cabbage.

## Materials and methods

### Plant materials and evaluation of downy mildew resistance

An inbred line (RS1) showing strong downy mildew resistance in a field where downy mildew was endemic for several years was selected as a resistant parent, and an inbred line (SS1) showing the most severe symptoms of downy mildew was selected as a susceptible parent. A cross was made between RS1 and SS1 to produce $F_1$ hybrids, which were then self-pollinated to produce $F_2$ populations. The $F_1$ hybrids were also backcrossed with both parental lines to produce $BC_1F_1$ populations. The parental lines, $F_1$ hybrids, $F_2$, and $BC_1F_1$ populations were germinated in 128-cell plug trays measuring 54 × 28 × 8 cm, after which four- to six-leaf-stage seedlings were transplanted to plastic greenhouses with a spacing of 10 cm between plants on September 17, 2007 at Ansung-Si, Korea. The plastic greenhouses were maintained for years without any disinfection treatments for efficient natural infection of downy mildew.

Disease symptoms were evaluated by the visual examination at 70 days after transplanting. Disease severity was rated according to the following index (Fig. 1): 0 (no symptoms), 1 (one to three very small necrotic spots on the upper leaf surface), 2 (increased necrotic spots distributed on up to 25% of the upper leaf surface and weak sporulation on the lower leaf surface), 3 (necrotic spots distributed on 25–50% of the upper leaf surface and increased sporulation on the lower leaf surface), 4 (necrotic spots distributed on more than 50% of the upper leaf surface and heavy sporulation on the lower leaf surface). Disease indices 0 and 1 were considered as resistant while the others were considered as susceptible. The spores were examined under a microscope, and characteristic conidia and conidiophores of downy mildew were observed.

Since monogenic inheritance of resistance to downy mildew was observed in the $F_2$ and $BC_1F_1$ populations, for more reliable genotyping of the resistance locus, 95 lines of the $F_{3:4}$ population originating from $F_2$ population using

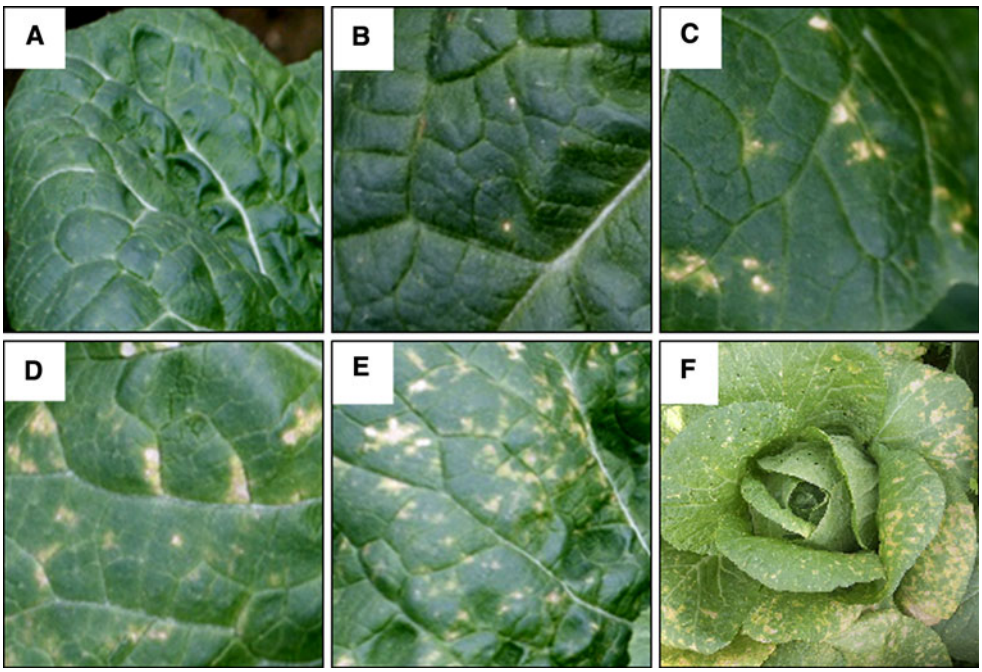


**Fig. 1** Responses of Chinese cabbages to infection with downy mildew at adult-plant stages. The disease index was determined depending on the size and numbers of necrotic spots and the degree of sporulation. **a** No symptoms (index 0), **b** one to three small necrotic spots on the upper leaf surface (index 1), **c** increased necrotic spots distributed on up to 25% of the upper leaf surface and weak sporulation on the lower leaf surface (index 2), **d** necrotic spots distributed on 25–50% of the upper leaf surface and increased sporulation on the lower leaf surface (index 3), **e** necrotic spots distributed on more than 50% of the upper leaf surface and heavy sporulation on the lower leaf surface (index 4). **f** An adult plant Chinese cabbage showing severe symptoms of downy mildew

the single seed descent (SSD) method were evaluated in the same plastic greenhouse in 2009. Each line consisting of 30 plants were partitioned into three replications, each of which was transplanted following a randomized complete block design. The rating of disease severity and classification of resistant and susceptible individuals were carried out the same as above.

## DNA extraction and bulked segregant analysis

Total genomic DNAs were extracted from the young leaf tissues of adult plants of the $F_2$ and $F_4$ populations after disease evaluation using a commercial DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN, Valencia, CA, USA), according to the manufacturer's manual. DNAs extracted from the 10 most resistant and 10 most susceptible $F_2$ individuals were pooled to make resistant and susceptible bulks for bulked segregant analysis (Michelmore et al. 1991). Random decamers (Operon Technologies, Alameda, CA, USA) were used to identify linked randomly amplified polymorphic DNA (RAPD) markers. PCR was performed in a 10 μL reaction mixture containing 0.05 μg of template, 1 μL of 10 × PCR buffer, 2 μL of decamer primer (10 μM), 1 μL of dNTPs (2.5 mM each), and 0.25 units of Taq polymerase (Prime Taq polymerase; GeNet Bio, Nonsan-Si, Korea). PCR amplification was carried out with an initial denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 37°C for 1 min, and 72°C for 2 min with a final 10-min extension at 72°C.

## Sequencing of RAPD fragment and determination of flanking sequences by genome walking

One RAPD fragment linked to the resistance allele was identified by PCR using the primer OPA08. The RAPD fragment was gel purified using a QIAquick PCR Purification kit (QIAGEN), cloned into a pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced. Sequencing reactions were carried out using Big Dye (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, and the sequences were obtained using an ABI 3700 Genetic Analyzer (Applied Biosystems).

The flanking sequences of the RAPD sequence were obtained by genome walking using the Universal GenomeWalker Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Genome walking libraries were constructed from the total genomic DNA of the resistant parent. Following PCR amplification, the PCR products were purified using a QIAquick PCR Purification kit (QIAGEN), and the purified PCR products were directly sequenced.

### PCR amplification of sequence characterized amplified region (SCAR) markers

For marker genotyping of the SCAR markers developed in this study, PCR was performed in a 10-μL reaction mixture containing 0.05 μg of template, 1 μL of 10 × PCR buffer, 0.2 μL of forward primer (10 μM), 0.2 μL of reverse primer (10 μM), 0.2 μL of dNTPs (10 mM each), and 0.1 μL of polymerase mix (Advantage 2 Polymerase Mix, Clontech). PCR amplification of some markers consisted of an initial denaturation step at 95°C for 5 min, 40 cycles at 94°C for 30 s, 68°C for 60 s, and 72°C for 60 s, and a final 10 min extension at 72°C. PCR amplification of other markers was performed with an initial denaturation step at 95°C for 5 min, 10 cycles at 95°C for 30 s, 65°C (0.8°C decrements in each cycle) for 30 s, and 72°C for 2 min, followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 2 min, and a final 10 min extension at 72°C. The PCR products were visualized on 1.5% agarose gel or 9% polyacrylamide gel electrophoresis after ethidium bromide staining. Primer sequences of the SCAR markers used in this study are presented in Table 1.

### Linkage analysis

The BrPERK15B marker identified in this study was integrated into the reference linkage map of *B. rapa* (Choi et al. 2007; Kim et al. 2009) using JoinMap version 4.0 (Stam 1993) to position the downy mildew resistance locus in the reference linkage map. The linkage relationship among molecular markers developed in this study and the downy mildew resistance locus were calculated using JoinMap version 4.0, and the map distances (cM) were calculated from recombinant frequencies using the Kosambi function (Kosambi 1944). Arabidopsis chromosomal regions showing synteny with flanking sequences of the resistance locus were identified using BLAST search with linked BAC clone sequences as queries.

**Table 1** Primer sequences of the molecular markers developed in this study

| Primer names | Primer sequences (5′–3′) |
|---|---|
| OPA08 | GTGACGTAGG |
| A-F1 | CTGGTTTCTTCCTTGCATTGCCCGATA |
| B-F1 | AGTTCATCGGTTTGAACCGGCTTGTTG |
| Co-R1 | GACGCCGGCCTGTTGGTAAATCACAT |
| BN-F1 | TCTGAGCTCCCGTCTAAGTTG |
| BN-R1 | TGTCCAACATTCAGCAAAGC |
| G17-F | GCGGGTTGACCCCTAGTAAT |
| G17-R | TGCAAGTTGTGTCGGACAAT |
| M22-F | ATACCAAAAGCAACGGCAAC |
| M22-R | TGGGGAAGAAGGTTTGTTTG |
| N18-F | GAGGCAAGAACCTTCTCCAG |
| N18-R | TTGCTCAACATCATCGGTCT |
| M05-F | ACAACATTAGCAACGCACCA |
| M05-R | CTTTTCTATCGCGCCTGAAC |
| J11-F | TGTGGGAGAGATAGGGTTGG |
| J11-R | TTTGTCGGAGGGATCAAAAA |
| A03-F | AGGTTCGACCACCATGACTC |
| A03-R | TGGGGTGTTTACACAAAGCTC |

## Results

### Inheritance pattern of resistance to downy mildew in Chinese cabbage

Inbred *B. rapa* lines showing strong resistance (RS1) and severe susceptibility (SS1), to endemic downy mildew were selected for an inheritance study. $F_1$ hybrids were produced by cross-pollination between the two lines, and $F_2$ and $BC_1F_1$ segregating populations were produced from the $F_1$ hybrids. Germinated seedlings were transplanted to plastic greenhouses where downy mildew was endemic. Necrotic spots appeared on susceptible plants when seedlings reached maturity. The disease index was scored at 70 days after transplanting.

The parental RS1 line and $F_1$ hybrids showed no symptoms, but all susceptible parental line plants showed severe necrotic spots covering most leaves (Fig. 1f and Table 2). The phenotypes of the $F_2$ population segregated, and the resistant to susceptible ratio fit a 3:1 Mendelian ratio, indicating that one dominant gene controlled resistance to downy mildew in this population. The $BC_1F_1$ population originating from the cross between the $F_1$ hybrid and RS1 showed all resistant phenotypes, except for one plant showing moderate necrotic spots on some of its leaves. The $BC_1F_1$ population from the cross between the $F_1$ hybrid and SS1 showed a 1:1 ratio of resistance to susceptibility, confirming single-gene inheritance of downy mildew resistance (Table 2). We designated this resistance locus as *BrRHP1* (*Brassica rapa* recognition of *Hyaloperonospora parasitica*).

### Identification of RAPD markers linked to *BrRHP1* and conversion into SCAR markers

DNAs were extracted from 10 resistant $F_2$ plants and the 10 most susceptible $F_2$ plants and pooled to make resistant and susceptible DNA bulks for bulked segregant analysis (Michelmore et al. 1991). RAPD analysis was carried out using two DNA bulks as templates, and 913 random decamers were screened to identify polymorphisms between the two bulks. One decamer (OPA08) yielded a

**Table 2** Inheritance patterns of resistance to downy mildew in segregating populations originating from the cross between resistant (RS1) and susceptible inbred lines (SS1)

| Population | Observed ratio (R:S)[a] | Expected ratio (R:S) | $\chi^2$ | *P* value |
|---|---|---|---|---|
| RS1 | 35:0 | 1:0 | 0 | 1 |
| SS1 | 0:40 | 0:1 | 0 | 1 |
| $F_1$ (RS1 × SS1) | 45:0 | 1:0 | 0 | 1 |
| $F_2$ | 369:114 | 3:1 | 0.50 | 0.48 |
| $BC_1F_1$ ($F_1$ × RS1) | 458:1 | 1:0 | 0.002 | 0.96 |
| $BC_1F_1$ ($F_1$ × SS1) | 233:245 | 1:1 | 0.30 | 0.58 |

[a] *R* resistant, *S* susceptible

polymorphic PCR product with a size of approximately 650 bp. The polymorphic PCR product appeared in the resistant parent, $F_1$ hybrid, and in resistant $F_2$ individuals, and not in susceptible plants (data not shown).

The RAPD fragment was sequenced to allow development of a SCAR marker. A 655-bp sequence was obtained, and used to design four pairs of primers for PCR. Single PCR products were obtained from both resistant and susceptible parents (data not shown); however, direct cycle sequencing of the PCR products revealed mixed peaks in the chromatograph from the resistant parent indicating the presence of two PCR products. Sequences flanking the RAPD fragment were obtained by genome walking. The 3′ flanking region contains a partial sequence of a gene showing homology (80% nucleotide identity of coding regions) with the *Arabidopsis* gene encoding proline-rich receptor-like protein kinase (PERK15; Fig. 2). In the 5′ flanking regions, two completely different sequences were identified 613 bp upstream from the stop codon of the gene (Fig. 2b). Hereafter, the sequence containing the RAPD sequence is designated BrPERK15A, and the second partially homologous sequence is BrPERK15B.

When PCR was performed using multiple primers complementary to the sequence unique to BrPERK15A (including primer A-F1) and to the common region (Co-R1), one band appeared in the resistant parent, $F_1$ hybrid, and in resistant $F_{3:4}$ lines, and no PCR product was detected in the susceptible parental line and $F_{3:4}$ lines. These data suggested that the sequence corresponding to BrPERK15A might have been deleted in the susceptible lines. When PCR was carried out using Co-R1 and a primer unique to BrPERK15B, a single band was produced in both parental lines, but sequencing of the PCR products revealed polymorphisms. Specifically, the BrPERK15B sequence of the susceptible parental line contained 7 and 12-bp insertions, which were absent in BrPERK15A in the resistant line (Fig. 2b).

A molecular marker for detection of BrPERK15B was developed that uses a pair of primers (BN-F1 and BN-R1)

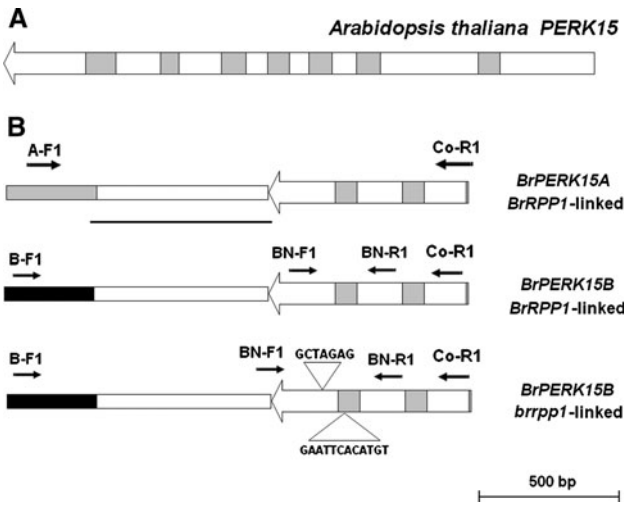


**Fig. 2** Conversion of RAPD marker into SCAR markers. **a** An *Arabidopsis* gene encoding proline-rich receptor-like protein kinase (PERK15). *Arrow-shaped boxes* indicate the 5′–3′ direction. *Filled and empty boxes* indicate introns and exons, respectively. **b** Sequences containing partial sequences of *B. rapa* genes, which are homologous to the *Arabidopsis PERK15* gene. These sequences are linked to the *BrRHP1* locus. The *thick line* under *BrPERK15A* indicates the region amplified by the linked RAPD marker. *Gray and black boxes* on the 5′ ends indicate completely different sequences. *Horizontal arrows* indicate primer-binding sites. Sequences on the *empty triangles* indicate insertions

complementary to sequences flanking the two insertions. To prevent amplification of the BrPERK15A sequence, primary PCR products amplified with B-F1 and Co-R1 were diluted 10 times and used as template in a nested PCR in which primers BN-F1 and BN-R1were used. Interestingly, the marker BrPERK15B was found to be linked to the BrPERK15A marker. There was one recombinant between the BrPERK15A and BrPERK15B markers in 95 $F_{3:4}$ lines. Unlike the BrPERK15A marker, BrPERK15B marker was shown to be a co-dominant marker through which heterozygous genotypes could be detected.

## Identification of chromosomal location of *BrRHP1* locus and development of more reliable linked markers

To identify the chromosomal location of the newly identified downy mildew resistance locus, parental lines of mapping populations of the reference linkage map (Choi et al. 2007; Kim et al. 2009) were screened for polymorphisms using the BrPERK15A and BrPERK15B markers. The BrPERK15A marker was monomorphic, but the BrPERK15B marker was polymorphic between the parental lines. The BrPERK15B marker genotypes of 78 DH lines were integrated with other marker genotypes (Choi et al. 2007; Kim et al. 2009) to construct a new linkage map. The map shows that the BrPERK15B marker is positioned on the A1 linkage group (Fig. 3), placing

the downy mildew resistance locus *BrRHP1* on the A1 linkage group as well.

Six *B. rapa* BAC clones (KBrH003N18, KBrB036M22, KBrB042J11, KBrB078A03, KBrB085G17, and KBrB08 9M05) linked to BrPERK15B were used to develop more reliable markers linked to *BrRHP1*. These BAC clones were previously anchored in the A1 linkage group using Simple Sequence Repeat (SSR) markers (Kim et al. 2009). Initially, six SSR markers, one for each BAC clone, were tested for polymorphisms in our mapping population. All six were either monomorphic or displayed multiple non-specific band patterns (data not shown). Next, we divided each BAC clone sequence into three parts, designed primer pairs for each section, and screened for polymorphisms between resistant and susceptible parental lines by PCR and sequencing. In addition to many single nucleotide polymorphisms, several indels were identified. Simple PCR co-dominant markers were developed using PCR primers to flanking regions of relatively large indels in KBrB 089M05 (221 bp), KBrB078A03 (63 bp), KBrH003N18 (19 bp), KBrB042J11 (13 bp), and KBrB036M22 (9 bp). In the case of the KBrB085G17 clone, multiple pairs of primers failed to yield PCR products indicating that more than 3 kb might have been deleted. Marker names were assigned using last one alphabet and two digit numbers. PCR results show that homozygous and heterozygous individuals were clearly distinguished by these five markers. A linkage map was constructed after analyzing $F_{3:4}$ populations (Fig. 3). Syntenic regions of the six BAC clone sequences on the *Arabidopsis* genomes were searched and we found that four markers (A03, N18, M05, and J11) flanking the *BrRHP1* locus were collinear with sequences on the long arm of *Arabidopsis* chromosome 3, while two markers (G17 and M22) were collinear with sequences on the chromosome 4 (Fig. 3).

## Discussion

### Inheritance patterns of resistance to downy mildew in Chinese cabbage

A novel locus controlling downy mildew resistance at adult-plant stages was identified in this study. To our knowledge, this is the first report of a locus involved in downy mildew resistance at adult-plant stages in *B. rapa*. Niu et al. (1983) reported several resistant accessions and showed that resistance at cotyledon stages is conditioned by single dominant genes. Yuen (1991) and Silue et al. (1996) also reported resistant accessions, but inheritance was not examined in segregating populations. In the absence of allelism tests or linkage mapping, it is impossible to determine whether genetic systems of downy mildew resistance in *B. rapa* are controlled by a common single gene or multiple genes.

Yu et al. (2009) recently reported a major QTL controlling downy mildew resistance at the seedling stages in *B. rapa*, and the major locus, *BraDM*, was mapped to the A8 linkage group. A minor QTL was also identified on the A6 linkage group in their study, but the effect of the minor QTL disappeared when resolution of QTL analysis increased. These data are consistent with a single major gene conferring resistance to downy mildew, but detailed inheritance results using segregating populations were not reported. Since the *BrRHP1* resistance locus identified in our study was located on the A1 linkage group, the resistance gene mapped by Yu et al. (2009) to the A8 linkage is likely to be a separate control locus. The resistance locus reported by Yu et al. (2009) may confer resistance at seedling stages while the *BrRHP1* locus confers resistance at adult-plant stages or field resistance.

Different genetic systems of resistance to downy mildew at seedling stages and adult-plant stages have been reported in *B. oleracea*. Some *B. oleracea* accessions are resistant at cotyledon stages but susceptible at adult-plant stages or vice versa (Coelho and Monteiro (2003a). Monteiro et al. (2005) showed that the response to downy mildew inoculation at cotyledon stages of $F_2$ segregating populations is independent from the response at adult-plant stages of the same $F_2$ individuals. These data indicate the existence of independent genetic systems for seedling and adult-plant resistance in *B. oleracea*. In apparent contrast, Wang et al. (2000) showed that all broccoli accessions displaying resistance at cotyledon stages are also resistant at three to four true-leaf stages. However, three to four true-leaf seedlings are still young and the resistance of adult plants may differ from that of seedlings (Coelho and Monteiro 2003a).

Yuen (1991) showed that inoculation of downy mildew isolates on detached cotyledons in vitro is a useful technique for screening downy mildew resistance in Chinese cabbage. However, adult-plant or field resistance is more important than cotyledon resistance in commercial Chinese cabbage production. More Chinese cabbage accessions need to be tested for differential responses to downy mildew inoculation between cotyledon and adult-plant stages. The resistance locus identified in this study will be a useful resource for the development of resistant Chinese cabbage cultivars to downy mildew.

### Development of reliable molecular markers for selection of downy mildew resistance

To convert the RAPD marker linked to the *BrRHP1* locus into a SCAR marker, we sequenced the RAPD product and isolated flanking sequences by genome walking.

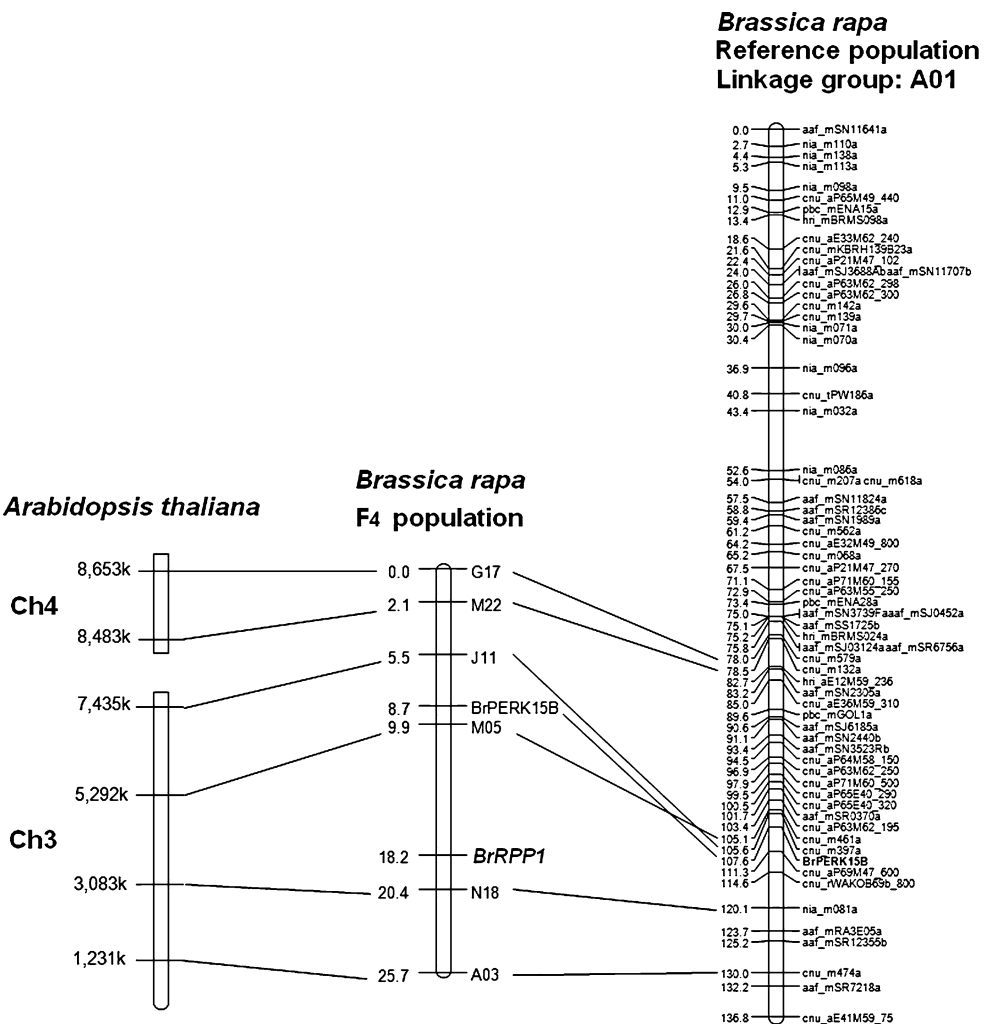


**Fig. 3** Linkage maps of molecular markers linked to the *BrRHP1* locus. The linkage map at center was constructed using the mapping population analyzed in this study. The map on the right was constructed using the mapping population of the reference linkage map of *B. rapa* (Choi et al. 2007; Kim et al. 2009). Cumulative genetic distances (cM) are shown to the left of the maps, while marker loci are shown to the right of the maps. The marker loci on the center map are connected with lines to the corresponding BAC clones from which each marker was developed. The BAC clones were previously anchored to the reference linkage map using SSR markers shown on the right of the map (Kim et al. 2009). The physical map of *Arabidopsis* chromosomes showing synteny is positioned on the left. The coordinates to the left of the map are shown in kilobase pairs (kb)

Interestingly, only the dominant *BrRHP1* allele contained the gene with homology to the *Arabidopsis PERK15* gene. The BrPERK15A marker based on that sequence is a dominant marker, which is less efficient than a co-dominant marker in marker-assisted selection. The partially homologous BrPERK15B marker exists on both dominant and recessive *BrRHP1* alleles and showed polymorphisms including 7 and 12-bp indels, however, in practice, the high homology with BrPERK15A sequence interfered with BrPERK15B-specific amplification.

Insertion or deletion of BrPERK15A might have resulted from retrotransposon-mediated rearrangement. We identified an interesting chimeric sequence consisting of a 5′ part showing homology with the 5′ region unique to BrPERK15B and a 3′ part showing homology with an *Arabidopsis* retrotransposon (GenBank accession number: ABW81018). The position of the breakpoint between the 5′ homologous sequence and 3′ retrotransposon-like sequences was exactly the same as that of the breakpoint on the 5′ region between the BrPERK15A and BrPERK15B sequences (Fig. 2b). This retrotransposon-like sequence was also detected in two *B. rapa* BAC sequences in GenBank (KBrB080J15 and KBrB041L12). They all contained completely different 5′ sequences (data not shown). These results indicate that repeat sequences such as retrotransposons, which are implicated in genome triplication events in *B. rapa* since its divergence from the common ancestor of *B. rapa* and *Arabidopsis* (Mun et al. 2009a), might also contribute to other genome rearrangements.

Simple co-dominant markers linked to the *BrRHP1* locus were developed using the *B. rapa* reference linkage map (Choi et al. 2007; Kim et al. 2009). The *BrRHP1* locus was located on the A1 linkage group, and that location was confirmed by the presence of the full-length BrPERK15B sequence in a 2-Mbp scaffold sequence positioned in the A1 linkage group (data not shown). Once the BrPERK15B marker was integrated into the reference linkage map, the flanking BAC sequences were available for the marker development. Although the SSR markers used to anchor the BAC sequences on the reference linkage map (Kim et al. 2009) were either monomorphic or not detectable in our populations, the BAC sequences allowed the

identification of useful indels for the development of simple PCR co-dominant markers. Mining BAC sequences for ubiquitous indel polymorphisms could be a good strategy for integrating more BAC sequences into the reference linkage map and for the development of more simple PCR markers.

## Functional markers on the basis of the *BrRHP1* gene cloning

The most ideal molecular marker for marker-assisted selection is the so-called functional marker, designed based on functionally characterized sequence motifs affecting phenotypic variation (Andersen and Lübberstedt 2003). To develop a functional marker for downy mildew resistance, the *BrRHP1* gene must be isolated. Synteny between the *B. rapa* and *Arabidopsis thaliana* genomes would be informative for *BrRHP1* gene cloning. Since the collinear and syntenic regions between the flanking sequences of the *BrRHP1* and *Arabidopsis* genome were identified on the long arm of chromosome 3 (Fig. 3), sequence intervals between 3.1 and 5.3 Mb of Arabidopsis chromosome 3 could be used for saturation of molecular markers around the *BrRHP1* locus. Another resource is the well-studied genotype-specific interactions between *Arabidopsis thaliana* accessions and *H. arabidopsidis* responsible for downy mildew in *Arabidopsis*. More than 20 *RPP* loci have been reported based on differential interactions (Holub et al. 1994; Slusarenko and Schlaich 2003). *RPP5* (Parker et al. 1997), *RPP1* (Botella et al. 1998), *RPP8* (McDowell et al. 1998), *RPP13* (Bittner-Eddy et al. 2000), *RPP2* (Sinapidou et al. 2004), and *RPP7* (unpublished; Eulgem et al. 2007) were cloned by map-based cloning strategies, and all encode proteins containing nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. Combined association and linkage mapping studies have revealed that the majority of *RPP* loci are positioned in six clusters on the *Arabidopsis* chromosome (Nemri et al. 2010). No cluster was found on the long arm of chromosome 3 where the syntenic region of the *BrRHP1* flanking sequences was identified, but one cluster including *RPP1* and *RPP13* genes were positioned on the short arm of chromosome 3. The absence of RPP gene clusters in the corresponding regions of the *BrRHP1* flanking sequences implies that downy mildew resistance genes might have rapidly evolved after the divergence between *A. thaliana* and *B. rapa*.

A similar pathosystem to that of *Arabidopsis* and *H. arabidopsidis* might exist between *B. rapa* and *H. parasitica*, although the *H. arabidopsidis* that infects *Arabidopsis* does not infect *B. rapa* (Slusarenko and Schlaich 2003), since *B. rapa* is a relative of *Arabidopsis* and the pathogen causing downy mildew in both plant species belongs to the same genus. Therefore, the most plausible candidate genes responsible for downy mildew resistance in *B. rapa* would be those encoding proteins containing NBS-LRR domains. A recent genome-wide survey of genes encoding NBS-LRR domains in *B. rapa* BACs identified 92 genes (Mun et al. 2009b), but none in the vicinity of the *BrRHP1* locus. This may be due to the low coverage of *B. rapa* genome with BAC sequences (Mun et al. 2009b). Upon release of complete genome sequences of *B. rapa*, the candidate genes for the *BrRHP1* gene could be identified using molecular markers developed in this study, and a functional marker for selection of downy mildew resistance in Chinese cabbage could then be developed.

The flanking markers N18 and M05 developed in this study are closely linked to the *BrRHP1* locus and are simple PCR co-dominant markers. Thus, these markers would be useful to select downy mildew resistant plants in Chinese cabbage breeding programs. Other linked markers can be used as recombinant selection markers in marker-assisted backcrossing of downy mildew resistance. Furthermore, these markers can be used as fundamental resources in constructing a high-density linkage map of the *BrRHP1* locus for cloning of the resistance gene.

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