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ORIGINAL ARTICLE

The tandem repeated organization of NB-LRR genes in the clubroot-resistant *CRb* locus in *Brassica rapa* L.

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Abstract To facilitate prevention of clubroot disease, a major threat to the successful cultivation of Chinese cabbage (*Brassica rapa* L.), we bred clubroot-resistant (CR) cultivars by introducing resistance genes from CR turnips via conventional breeding. Among 11 CR loci found in *B. rapa*, we identified *CRb* in Chinese cabbage cultivar 'CR Shinki' as a single dominant gene for resistance against *Plasmodiophora brassicae* pathotype group 3, against which the stacking of *Crr1* and *Crr2* loci was not effective. However, the precise location and pathotype specificity of *CRb* have been controversial, because *CRa* and *Rcr1* also map near this locus. Previously, our fine-mapping study revealed that *CRb* is located in a 140-kb genomic region on chromosome A03. Here, we determined the nucleotide sequence of an approximately 64-kb candidate region in the resistant line; this region contains six open reading frames (ORFs) similar to NB-LRR encoding genes that are predicted to occur in tandem with the same orientation. Among the six ORFs present, only four on the genome of the resistant line showed a strong DNA sequence identity with each other, and only one of those four could confer resistance to *P. brassicae* isolate No. 14 of the pathotype group 3. These results suggest that these genes evolved through recent gene duplication and uneven crossover events that could lead to the acquisition of clubroot resistance. The DNA sequence of the functional ORF was identical to that of the previously cloned *CRa* gene; thus, we showed that the independently identified *CRb* and *CRa* are one and the same clubroot-resistance gene.

Keywords *Brassica rapa* · *Plasmodiophora brassicae* · Clubroot · NB-LRR · Resistant gene · Duplication

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Introduction

Crop production is constantly threatened by a variety of diseases caused by pathogen attack. Plants have evolved a sophisticated mechanism comprising two lines of defense against pathogen infection (Dangl and Jones 2001; Marone et al. 2013). The first is the basic recognition by the innate immune system of generic molecular motifs referred to as pathogen-associated molecular patterns. Activation of the second line of defense occurs with the direct or indirect recognition of pathogen avirulence effectors by the products of plant resistance (*R*) genes, resulting in a strong resistance reaction called effector-triggered immunity (Dangl and Jones 2001). Many of these *R* genes encode intracellular proteins with an N-terminal Toll–interleukin 1 receptor (TIR) or a coiled-coil (CC), central nucleotide-binding (NB) site, and a C-terminal leucine-rich repeat (LRR), and form the complex clustered *R*-gene families, which are

considered to promote allelic diversities and race specificities of *R* genes (Leister 2004; Marone et al. 2013).

Clubroot disease is a major threat to the cultivation of *Brassica* vegetable and oil crops, including Chinese cabbage, cabbage, broccoli, cauliflower, and canola; the causal agent *Plasmodiophora brassicae* produces galls on the roots of infected plants, preventing the uptake of water and soil nutrients. The resultant stunting or killing of plants leads to yield reductions (Dixon 2009). Because agricultural practices, such as crop rotation and liming, are insufficient to protect crops from clubroot disease, most experts consider that breeding of resistant cultivars is one of the most effective control measures (Hirai 2006; Diederichsen et al. 2009; Piao et al. 2009). However, growers observe a rapid loss of resistance on the part of clubroot-resistant (CR) Chinese cabbage due to the existence in the field of multiple pathogenic genotypes of *P. brassicae* (Manzanares-Dauleux et al. 2001; Hatakeyama et al. 2004). Therefore, stacking of multiple CR genes with different pathotype specificities might prove a promising strategy to increase the durability of resistance.

Genetic mapping analysis of CR European fodder turnips and CR Chinese cabbage cultivars identified 11 CR loci in *B. rapa*: *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRa*, *CRb*, *CRc*, *CRk*, *Rcr1*, *PbBa3.1*, and *PbBa3.3* (Matsumoto et al. 1998; Suwabe et al. 2003, 2006, 2012; Hirai et al. 2004; Piao et al. 2004; Sakamoto et al. 2008; Chen et al. 2013; Chu et al. 2014). *Crr1*, *Crr2*, *Crr4*, and *CRc* are located on *B. rapa* chromosomes A08, A01, A06, and A02, respectively, whereas all the remaining seven loci are on chromosome A03. Although *Crr1* was originally identified as a single locus, fine-mapping study reveals that this locus comprises two loci: *Crr1a* with a major effect and *Crr1b* with a minor effect (Hatakeyama et al. 2013). For *CRa* (Ueno et al. 2012) and *Crr1a* (Hatakeyama et al. 2013), the responsible genes were cloned and shown to encode a TIR-NB-LRR class disease-resistance protein that triggers the effector-triggered immunity response. Based on its expression in stele and cortex of hypocotyl and root, Hatakeyama et al. (2013) suggest that the Crr1a protein inhibits the development of plasmodia during the secondary infection phase, but the molecular mechanism of resistance remains unclear.

Three CR loci of *B. rapa*, *CRa*, *CRb*, and *Rcr1*, map near each other around 24 Mb on the *B. rapa* chromosome A03. *CRa*, identified from *Brassica* host ECD 02 of European Clubroot Differentials (ECDs), is reported to be a single dominant gene for resistance to race 2 of *P. brassicae* based on the differential sets of Williams (1966) (Matsumoto et al. 1998). Piao et al. (2004), using a resistant DH line derived from 'CR Shinki', identified *CRb* as a single dominant gene for resistance to *P. brassicae* race 4 of Williams (1966). Using an F_2 population derived from each of the CR cultivars 'Akiriso' and 'CR Shinki' and based on the differential sets of Hatakeyama et al. (2004), Kato et al. (2012) demonstrated that *CRb* is a single dominant gene conferring resistance to the *P. brassicae* group 3 pathotype. Kato et al. (2013) subsequently delimited the candidate region of *CRb* to 140 kb on the Chinese cabbage Chiifu-401-42 reference genome (Wang et al. 2011). *Rcr1* as identified from pak choy (*B. rapa* ssp. *chinensis*) is a single dominant gene for resistance to *P. brassicae* pathotype 3 based on Williams's (1966) classification (Chu et al. 2014). Zhang et al. (2014) demonstrated that *CRb* identified by Piao et al. (2004) is located in a region 500–800 kb away from the position of *CRb* defined by Kato et al. (2013) (referred to as CRb^{Kato} in Zhang et al. 2014). Kato et al. (2012) considered *CRb* to be a useful locus to improve durability of resistance, because it is effective against *P. brassicae* pathotype group 3, against which stacking of *Crr1* and *Crr2* is not effective. However, the differences among genetic resources and pathogens used for mapping have led to uncertainties about the relationship among the three CR loci mapped around 24 Mb on linkage group A03, thereby hindering understanding of the relationship between CR loci and pathotypes of the clubroot pathogen.

In this study, to clarify the relationships between *CRa* and *CRb*, we determined the sequence of about 64 kb of the *CRb* genome covered by two fosmid clones screened with markers developed by Kato et al. (2013). We show that *CRb* is a complex locus comprising at least six ORFs similar to NB-LRR genes in tandem with the same orientation. We demonstrate that compared with the Chinese cabbage reference genome, four of these ORFs were present only in the genome of the resistant line. The transgenic plants harboring one of these predicted NB-LRR genes were resistant to *P. brassicae* isolate No. 14 of pathotype group 3.

Materials and methods

Plant materials

A clubroot-resistant F_3 line 1074RR and the susceptible F_3 line 1866HS were obtained by selfing of a clubroot resistant and a susceptible F_2 plant, respectively; both of these lines were derived by selfing of an F_1 Chinese cabbage 'CR Shinki' (Takii Seed Co., Kyoto, Japan) (Kato et al. 2012, 2013). 'CR Ryutoku' (Watanabe Seed Co., Kogota, Japan), which is considered to carry *CRb* (Kato et al. 2013) or *CRa* (Ueno et al. 2012), was used as a positive control in the clubroot test for the transgenic *B. rapa*.

Pathogen and inoculation test

We used the *P. brassicae* field isolate No. 14 classified into pathotype group 3 based on the differential sets

of Hatakeyama et al. (2004), because we had previously defined *CRb* as a clubroot-resistance gene effective against this isolate (Kato et al. 2012, 2013). Differential sets of Williams (1966) showed different responses against the isolates classified into group 3. No. 2 was defined as race 4 (Hatakeyama et al. 2004), but Rokunohe-01 and No. 14 were not clearly defined, because 'Jersey Queen' and 'Wilhelmsburger' showed partial resistance against Rokunohe-01 (Kuginuki et al. 1999) and all differential hosts showed partial resistance against No. 14 (unpublished results). 'CR Shinki' harboring *CRb* showed partial resistance against all three isolates classified into group 3: Rokunohe-01, No. 2, and No. 14 [mean disease index (DI) = 1.0 to 1.4; Hatakeyama et al. 2004; Kato et al. 2012].

Clubroot resistance was tested in *Arabidopsis thaliana* and *B. rapa*; root symptoms evaluated as described in Hatakeyama et al. (2013). Briefly, *A. thaliana* T_2 seedlings carrying the transgene were selected on MS medium containing kanamycin, transplanted on soil, and inoculated by the injection of 2–4 mL of a resting spore suspension. The DI of each T_2 plant was scored on a scale of 0–3, and the mean DI of each T_1 line was expressed as the mean of two or three clubroot tests (9 T_2 seedlings per test). The clubroot test for *B. rapa* was carried out by the insertion method using ~8 T_1 seedlings derived from an independent transgenic line (T_0). Before evaluation of resistance, genomic DNA was isolated from the T_1 seedlings and PCR analysis was carried out to check for the presence of the transgene.

Screening of fosmid library and construction of shotgun library

A fosmid library with pCC1FOS vector (Epicentre, Madison, WI, USA) was constructed with DNA extracted from leaves of the CR 1074RR line by means of a NucleoBond AXG 100 Column (TaKaRa Bio, Kusatsu, Japan). More than 75,000 clones were initially screened by PCR using the markers reported in Kato et al. (2013): the positive clones obtained were extracted using NucleoBond XtraBAC (TaKaRa Bio) and fragmented by sonication. After size fractionation by agarose gel electrophoresis, approximately 1.5–3.0-kb bands were excised from the gel, blunted with KOD DNA polymerase (Toyobo, Osaka, Japan), and ligated into the Hinc II–digested pUC118 vector (TaKaRa Bio). More than 768 independent clones were isolated from a single fosmid clone and sequenced at both ends by Sanger sequencing. We used MacVector with the Assembler software (MacVector, Inc., Apex, NC, USA) and CodonCode Aligner software (CodonCode Co., Centerville, MA, USA) to assemble the sequences obtained from the shotgun clones, predict the ORFs, and compare the sequences. The genome sequence of the 1074RR was compared with the Chinese cabbage Chiifu-401-42 reference genome by means of the *Brassica* database (http://www.brassicadb.org). To map the DNA markers reported in Ueno et al. (2012) on the genome sequence of the 1074RR, fragments GC11250-2 and GC30 flanking *ORF1* were amplified from the genomic DNA of the 1074RR and fosmid clones. Amplified fragments were cloned into pCR2.1 vector (ThermoFisher Scientific, Waltham, MA, USA) and sequenced.

Vector construction and transformation

Genomic DNA fragments of candidate ORFs were amplified from the isolated fosmid DNA using KOD-Plus-DNA polymerase (Toyobo) with primers (described in Table S1) and inserted downstream of the $Crr1a^{G004}$ promoter sequence (Hatakeyama et al. 2013) via the In-Fusion Cloning system (TaKaRa Bio), except for *CRb*_α, which was cloned with the restriction endonucleases Sma I and Sal I. At least three clones were sequenced to verify whether the sequence of the amplified ORF coincided with that of the fosmid DNA. Ligation of the gene cassette into the plant binary vector and transformation of *A. thaliana* and *B. rapa* were carried out as described by Hatakeyama et al. (2013). For *B. rapa* transformation, the commercial F_1 cultivar of leafy vegetable 'Gokurakuten' (Takii Seed Co.) was used as recipient.

Expression analysis of candidate genes by RT-PCR

Total RNA was extracted from leaves and roots with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and then converted to first-strand complementary DNA using SuperScript III First-Strand Synthesis System for RT-PCR (ThermoFisher Scientific). PCR was performed with ExTaq HS polymerase (TaKaRa Bio) according to the following program: 2 min at 94 °C; 30 cycles of 10 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C; and a final extension of 3 min at 72 °C.

Results

Physical map of the *CRb* locus

In a previous study, we used an F_2 population derived from selfing of a CR F_1 Chinese cabbage 'CR Shinki' to delimit *CRb* to the 140-kb genomic region between markers KB59N07 and B1005 on the Chinese cabbage reference genome (Wang et al. 2011): we found that two candidate ORFs, *Bra019410* and *Bra019413*, encode proteins similar to TIR-NB-LRR class disease-resistance proteins (Kato et al. 2013; Fig. 1a). Here, to clone the region containing these candidate ORFs, we screened a fosmid

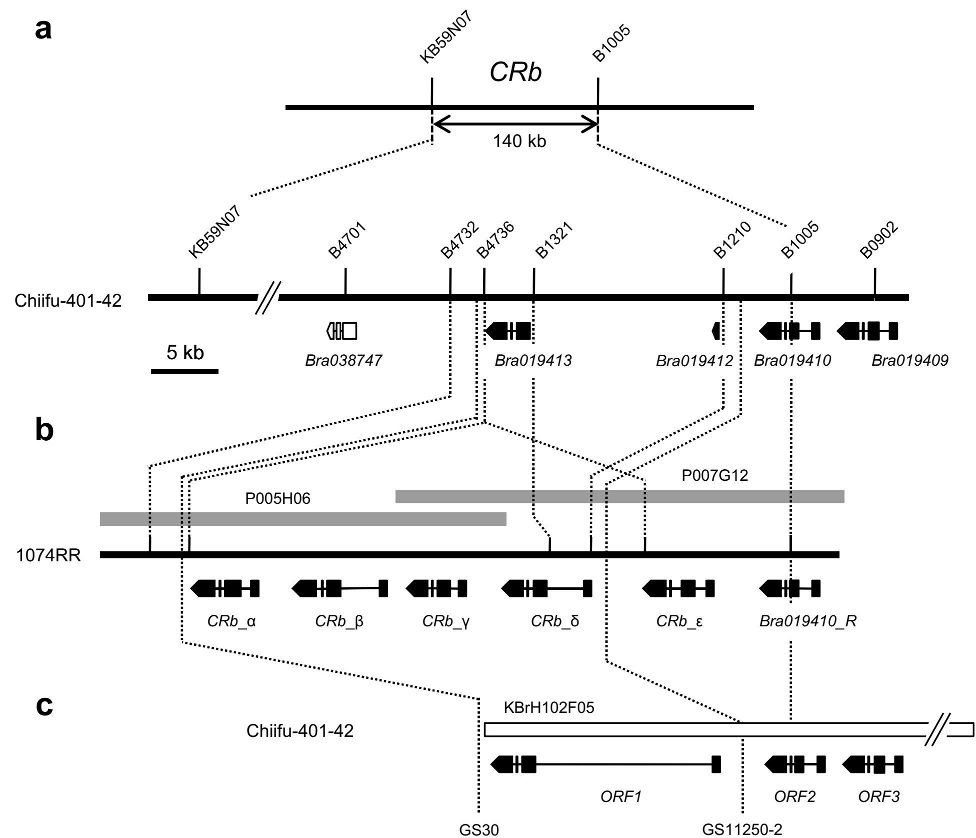

Fig. 1 **a** Physical maps of the *CRb* locus for the *B. rapa* reference genome (Chiifu-401-42). *Black boxes* indicate ORFs with NB-LRR structure. **b** Contig of resistant 1074RR fosmid clones (*gray bars*) spanning the region between markers B4732 and B1005. **c** Physical map of *CRa* locus reported by Ueno et al. (2012). *White bar* indicates BAC clone. The sequences of fosmid clones P007G12 and P005H06 have been deposited in the GenBank database (accession nos. LC155799 and LC155800)

library constructed from the CR F_3 line 1074RR, with marker B4732, which is located between *Bra038747* and *Bra019413*. Analysis of the positive clones with other PCR markers suggests that two clones, P007G12 and P005H06, span the region between the markers B4732 and B1005 (Fig. 1a, b). We determined the sequences of these two clones by assembling the sequences derived from the shotgun clones and found that they overlapped by 9794 bp, providing a contiguous sequence of 63,852 bp. Comparison of the resultant contig with the reference genome revealed that the order of PCR markers developed based on the reference genome sequence was well conserved (Fig. 1b). In the 1074RR genome, six ORFs encoding the TIR-NB-LRR class of resistance protein resided in tandem with the same orientation. We designed five ORFs as *CRb*_α, _β, _γ, _δ, and _ε, and one ORF located on B1005, which we identified as *Bra019410_R*. Based on their locations on the genome, we considered that *CRb*_α, _β, _γ, and _ε were present only in the 1074RR genome. PCR analysis using primers CRb_F and CRb_α_RsaI in Table S1 confirmed that amplicons corresponding to *CRb*_α and _γ were obtained from 'CR Ryutoku' (data not shown), which is considered to carry *CRb* (Kato et al. 2013) or *CRa* (Ueno et al. 2012). Because it was speculated that *CRb* (analyzed in this study) and *CRa* are closely linked CR genes (Zhang et al. 2016; Fredua-Agyeman and Rahman 2016), we compared the sequence of BAC clone KBrH102F05, including *CRa* (Ueno et al. 2012) with the reference sequence (Fig. 1c): we found that this BAC clone was perfectly aligned with the region between markers B4736 and B0902 on the reference genome. DNA markers GC11250-2 and GC30, flanking *ORF1* on the KBrH102F05 (Ueno et al. 2012), were mapped at the 5′ region of *CRb*_δ and the 3′ region of *CRb*_α, on the 1074RR genome, respectively (Fig. 1c).

Functional analysis of the predicted ORFs

To determine the functionality of the predicted ORFs on the 1074RR genome, we cloned the genomic DNA fragments containing the entire region for five candidate ORFs into the region downstream of the *Crr1a* promoter and transformed the resultant constructs into the clubroot-susceptible *Arabidopsis* wild-type Col-0. From independent T_1 lines, we derived T_2 seedlings that were homozygous or heterozygous for the transgene; these T_2 seedlings were inoculated with *P. brassicae* field isolate No. 14. All T_1 lines transformed with the *CRb*_β, _γ, _δ, and _ε were rather susceptible (mean DI of each T_1 line >2.0) (Fig. 2). In contrast, four T_1 lines of transgenic plants containing *CRb*_α were strongly resistant (mean DI < 1.0) (Figs. 2, 3b, d, e), whereas the remaining plants were moderately

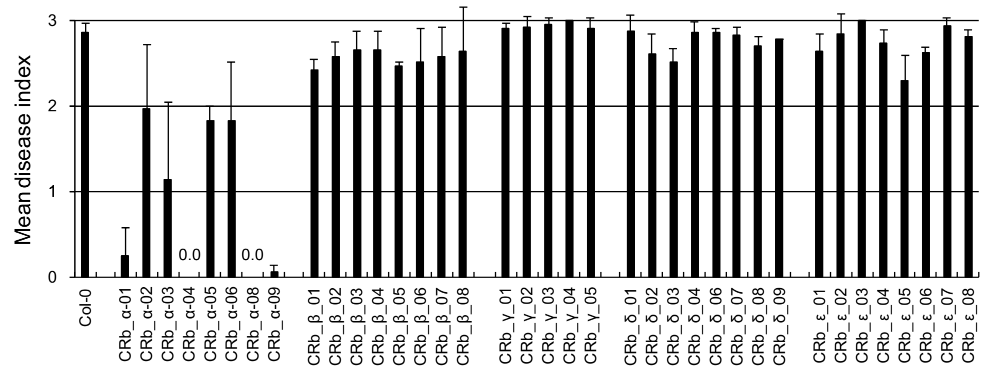

Fig. 2 Functional analysis of the candidate ORFs predicted on the *CRb* locus. Results of clubroot test for *Arabidopsis* wild-type Col-0 and the transgenic T_1 lines. Root symptoms of the inoculated plants were evaluated according to Hatakeyama et al. (2013), and mean disease indexes (±SD) of the T_1 lines were calculated based on the average of two-to-three tests (~nine T_2 plants per test)

Fig. 3 Typical root phenotype of the inoculated *Arabidopsis* wild-type Col-0 (**a**) and T_2 plants derived from T_1 lines with *CRb*_α transgene, *CRb*_α_01 (**b**), *CRb*_α_02 (**c**), *CRb*_α_04 (**d**), and *CRb*_α_08 (**e**). *Scale bar* indicates 20 mm

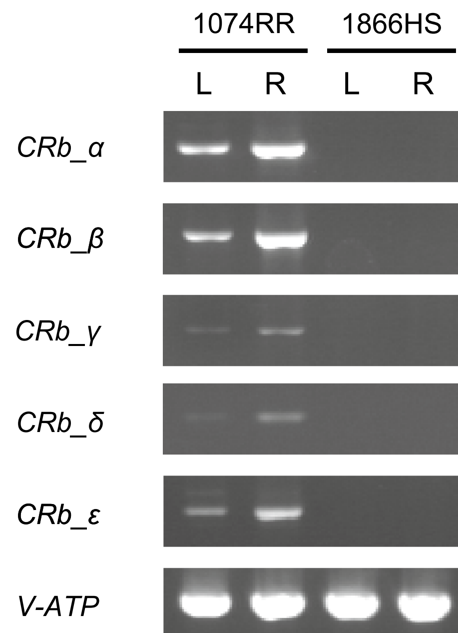

Fig. 4 Expression analysis by RT-PCR followed by gel electrophoresis of the five predicted ORFs. Leaves (L) and roots (R) of the resistant (1074RR) and susceptible (1866HS) lines were analyzed; vacuolar H^+-ATPase gene (*V-ATP*) was the control

resistant (1.0< mean DI < 2.0) (Figs. 2, 3c). For further verification, we transformed the same construct into *B. rapa*: from 12 independent T_0 lines, we derived T_1 seedlings that were homozygous or heterozygous for the transgene; these T_1 seedings were inoculated with the isolate No. 14. Among 12 T_0 lines, 9 showed strong resistance to No. 14 (mean DI < 1.0) and 3 were intermediate (mean DI > 1.0) (Table S2; Fig. S1). All plants without the transgene segregated in T_1 were completely susceptible (DI = 3) (Fig. S1). However, the level of resistance tended to be slightly lower than that in clubroot-resistant Chinese cabbage 'CR Ryutoku,' which is totally resistant to the *P. brassicae* field isolate No. 14 (Hatakeyama et al. 2004). These results indicate that *CRb*_α is the functional gene responsible for clubroot-resistant *CRb*.

BLAST search analysis revealed that the nucleotide sequence of *CRb*_α is identical to the genome sequence of the previously cloned *CRa* gene (accession no. AB751516, Ueno et al. 2012), which includes 877 bp of the 5′ upstream region from the ATG codon and 2279 bp of the 3′ downstream region from the TAA codon (Fig. S2). Comparison of the amino acid sequence of CRa (CRb_α) with that of the previously cloned Crr1a revealed that the TIR and NB domains are relatively conserved (76.9 and 65.8%), although several amino acid substitutions were found in motifs characteristic of the NB domain (Fig. S3a, b). In contrast, the LRR domains were highly variable between CRa and Crr1a. The LRR region of the predicted Crr1a protein contains at least 11 imperfect LRRs consistent with the consensus motif IxxLxxLxxLxLxx(N/C/T)xxLxxLPxx recognized in the cytoplasmic resistance protein (Jones and Jones 1997), whereas that of CRa protein was predicted to contain five additional LRRs as well as numerous amino acid substitutions (Fig. S3c).

Expression and comparisons of the predicted ORFs

We analyzed the expression of the five putative ORFs (*CRb*_α, _β, _γ, _δ, and _ε) by RT-PCR using primers

Fig. 5 Comparison of nucleotide sequences among exons of nine ORFs found in the genome of 1074RR and Chiifu-401-42. *CRb*_α (*CRa*), _β, _γ, _δ, and _ε, and *Bra019410R* are from 1074RR. *Bra019412*, *Bra019413*, and *Bra019410* are from Chiifu-401-42. On the assumption that *Bra019412* (encoding the TIR domain) and *Bra019413* (encoding NB and LRR domains) form a single NB-LRR gene, we used *Bra019412* for comparison with exon 1 and *Bra019413* for comparison with the other three exons

Exon 1	1074RR					Chiifu-401-42	
	β	γ	δ	ε	*Bra019410R*	*Bra019412*	*Bra019410*
α (*CRa*)	100	100	89.9	97.4	82.9	90.7	84.4
β		100	89.9	97.4	82.9	90.7	84.4
γ			89.9	97.4	82.9	90.7	84.4
δ				89.6	78.6	99.2	79.9
ε					83.7	90.4	85.2
Bra019410R						79.4	95.3
Bra019412							80.6

Exon 2	1074RR					Chiifu-401-42	
	β	γ	δ	ε	*Bra019410R*	*Bra019413*	*Bra019410*
α (*CRa*)	97.7	93.2	85.6	98.6	86.9	83.0	87.8
β		92.8	86.5	96.4	86.9	83.8	87.8
γ			84.1	92.2	91.9	81.3	90.3
δ				85.6	87.0	96.0	83.5
ε					87.7	82.9	88.1
Bra019410R						88.0	99.3
Bra019413							81.1

Exon 3	1074RR					Chiifu-401-42	
	β	γ	δ	ε	*Bra019410R*	*Bra019413*	*Bra019410*
α (*CRa*)	88.0	88.6	88.2	99.3	87.2	86.2	87.6
β		94.8	92.3	88.0	89.3	96.3	89.7
γ			91.9	88.6	89.2	92.2	89.6
δ				88.2	90.5	91.2	90.9
ε					87.9	86.6	88.2
Bra019410R						87.5	99.7
Bra019413							87.8

Exon 4	1074RR					Chiifu-401-42	
	β	γ	δ	ε	*Bra019410R*	*Bra019413*	*Bra019410*
α (*CRa*)	68.0	79.5	68.7	91.8	61.1	72.4	60.4
β		73.7	81.2	63.7	79.2	76.2	78.9
γ			78.4	82.2	62.0	74.2	62.7
δ				69.4	78.9	75.2	78.2
ε					64.3	71.4	64.8
Bra019410R						73.3	95.3
Bra019413							73.6

specific for each ORF and confirmed that they were transcribed in leaves and roots of the resistant 1074RR (Fig. 4). Expression levels of all ORFs tended to be higher in roots than in leaves. In contrast, we could not detect the transcripts of these ORFs in leaves and roots of the CR-susceptible 1866HS line.

We predicted the exon–intron junctions of the ORFs on the *CRb* genomic region by an alignment with the genome sequence of *CRa*; the general structures of these junctions were well conserved. Comparison of exon sequences of nine ORFs, including *Bra019413*, *Bra019412*, and *Bra019410* found in the Chiifu-401-42 reference genome, revealed that exon 1 encoding the TIR domain and exon 2 encoding the NB domain were highly conserved. Nucleotide sequences of exon 1 among *CRb*_α, _β, _γ, and _ε were >97% identical to one another; most notably, identity was 100% among *CRb*_α, _β, and _γ (Fig. 5). Sequence identity among the nucleotide sequences of exon 4, encoding

the LRR domain, was lower than those for the other exons: 60–80%, except for the identity between *CRb*_α (*CRa*) and *CRb*_ε (91.8%). *CRb*_δ showed the highest sequence identity to *Bra019412* in exon 1 and *Bra019413* in exons 2–4.

Discussion

We demonstrated that *CRb* is a complex locus composed of at least six NB-LRR genes in tandem with the same orientation (Fig. 1). Tandem repeated organization of NB-LRR genes might promote unequal recombination of paralogs to change the number of genes and generate a new recognition specificity of resistance genes (Leister 2004; Bulgarelli et al. 2010). Genome analysis of the *CRb* locus demonstrated that *CRb*_α, _β, _γ, and _ε are present only in the 1074RR genome, and the sequences of *CRb*_α, _β, _γ, and _ε showed a strong identity with each other; in particular, 100% identity was found among the exon 1 sequences of *CRb*_α, _β, and _γ (Fig. 5). Gain-of-function analysis revealed that only *CRb*_α encodes *CRb* specificity (Fig. 2). These results suggest that *CRb*_α, _β, _γ, and _ε might have evolved by relatively recent gene duplication and unequal crossover events, which would lead to the acquisition of clubroot resistance. In contrast, the tandem array localization of NB-LRR genes was not found in another cloned CR gene, *Crr1a* (Hatakeyama et al. 2013); this difference suggests that although they were considered to be derived from the same region of an ancestral *Brassica* genome (Hirai 2006), these CR genes subsequently evolved by different mechanisms.

The fact that the nucleotide sequence of the functional *CRb*_α is completely identical with that of the previously cloned *CRa* (Ueno et al. 2012) indicates that *CRb* cloned in this study and *CRa* are one and the same clubroot-resistant allele. The genome structure of the *CRa* locus in the resistant T136-8 line is unknown, because *CRa* was cloned by PCR using primers developed based on the sequence around *ORF1* on KBrH102F05 (Ueno et al. 2012). Ueno et al. (2012) report that *ORF1* is a *CRa* susceptible allele disrupted by a 16-kb insertion on the Chiifu-401-42 genome (Fig. 1). However, based on its location and sequence similarity, it is plausible that *CRb*_δ is allelic to *ORF1*, composed of *Bra019412* encoding the TIR domain and *Bra019413* encoding the NB and LRR domains (Figs. 1c, 5). Because *CRb*_α (*CRa*), _β, _γ, and _δ are located within the region between markers GC30 and GC11250-2 flanking *ORF1*, the resistant and susceptible alleles of *CRa* might have derived from *CRb*_δ by recent duplication and large insertion, respectively.

Three CR genes of *B. rapa* (*CRa*, *CRb*, and *Rcr1*) and two of canola (*B. napus*) are reported to be located near each other at around 23–24 Mb on *B. rapa* chromosome A03 (Piao et al. 2004; Kato et al. 2012, 2013; Chu et al. 2014; Zhang et al. 2014, 2016; Fredua-Agyeman and Rahman 2016). Although *CRb* has been identified in 'CR Shinki' independently by Piao et al. (2004) and Kato et al. (2013), Zhang et al. (2014) demonstrated that *CRb*, identified by Piao et al. (2004), may reside 500–800 kb apart from a small region of 24.200–24.543 Mb occupied by *CRa* and *CRb* defined by Kato et al. (2013) (they referred to it as CRb^{Kato}). Fredua-Agyeman and Rahman (2016) demonstrated that a CR gene derived from canola 'Mendel' was associated with the genomic region that included *CRa* and CRb^{Kato}. Our finding that *CRb* cloned in this study (CRb^{Kato}) and *CRa* are the same clubroot-resistant allele is consistent with these previous mapping studies. Because the target region of *Rcr1* included *Bra019409*, *Bra019410*, *Bra019412*, and *Bra019413* (Chu et al. 2014), *CRa* is a candidate for *Rcr1*. Zhang et al. (2016) found the CR locus linked to *CRa* in the canola (*B. napus*) resistant genotype '12-3'; they demonstrated that amplicons corresponding to NB and LRR regions using *CRa* targeted primers showed high sequence identity to *CRa*, but one of the fragments specific to the resistant parent was not associated with CR phenotype. Although the precise localization of this CR locus in canola genotype '12-3' remains unknown, this lack of association for one of the '12-3' fragments probably arose, because the resistance genes in this canola line form a complex cluster of highly homologous NB-LRR genes arranged in a tandem array, as do those of the *CRb* locus in *B. rapa*.

Because *CRb* and *CRa* are known to function as a single dominant gene (Matsumoto et al. 1998; Piao et al. 2004; Kato et al. 2012), breeders use them for clubroot resistance in developing many CR Chinese cabbage cultivars released in Japan (Aruga et al. 2013; Kato et al. 2013). However, *Crr1a* is incompletely dominant, and the reason for the differences in mode of inheritance between these genes remains unknown. Compared with the 1074RR genome, we found that the allele of *CRb*_α was not present in the Chiifu-401-42 genome (Fig. 1). Gain-of-function analysis revealed that the *CRb*_α transgene in *Arabidopsis* and *B. rapa* behaved as a dominant gene despite it being driven by the *Crr1a* promoter (Fig. 2; Table S2). These properties of *CRb*_α are unlike those of *Crr1a*, in which a highly homologous allele was present and was expressed in the susceptible A9709 line. The *Crr1a* transgene behaves as an incompletely dominant gene in transgenic *B. rapa*, as observed in genetic analysis of a *B. rapa* F_2 population, whereas it behaves as a dominant gene in transgenic *Arabidopsis* (Hatakeyama et al. 2013). Deslandes et al. (2002) observed a similar phenomenon in the *Arabidopsis RRS1-R* gene for resistance against *Ralstonia solanacearum*: *RRS1-R* is genetically defined as a recessive allele but behaves as a dominant resistance gene in transgenic plants; they

suggested that the truncated allele acts as a dominant-negative regulator of *RRS1-R* function in heterozygous plants. Stirnweis et al. (2014) demonstrated that suppression of *Pm3*-mediated resistance in wheat also is based on dominant-negative, post-translational effects among closely related NB-LRR alleles. Considering these findings with our results suggests that a highly homologous loss-of-function allele may interfere with the function of the resistance allele and that dominant expression of the *CRb* gene would be due to the absence of such alleles in the susceptible genome.

None of the genes *CRb*_β, _γ, _δ, and _ε individually was effective against the clubroot pathogen, although they were expressed in root and leaves (Fig. 4). Eitas and Dangl (2010) showed that a pair of linked NB-LRR genes functions together in disease resistance. In this study, we found that the level of resistance in transgenic *B. rapa* plants with *CRb*_α alone tended to be slightly lower than that in the clubroot-resistant Chinese cabbage 'CR Ryutoku' (Table S2). Therefore, NB-LRR genes linked to *CRb*_α may contribute to confer stable resistance to clubroot. Another possibility is that these genes are required for disease resistance against different pathogens as demonstrated in the *Arabidopsis R* genes *RPS4* and *RRS1* (Gassmann et al. 1999; Deslandes et al. 2002; Narusaka et al. 2009).

Two cloned CR genes, *CRb*_α (*CRa*) and *Crr1a*, possess differing specificities against pathotypes classified by the differential sets in Hatakeyama et al. (2004). In the present study, we demonstrated that *CRb*_α (*CRa*) was effective against isolate No. 14 of pathotype group 3. Hatakeyama et al. (2013) reported that *Crr1a* is effective against pathotypes groups 2 and 4, but not against groups 1 and 3. Comparison of both the predicted proteins shows that the TIR and NB domains are relatively conserved, whereas the LRR domains are highly variable. In particular, five additional consensus repeats were characteristic for the LRR region of the CRb_α protein (Fig. S3c). Because Ellis et al. (2007) consider the LRR domain to be the major determinant of resistance protein specificity, the high polymorphism of the regions of the LRR domain recognized between these two proteins is a candidate for the determination of specificity. Further investigation of the roles of the TIR, NB, and LRR domains of CR genes is necessary to understand the molecular mechanisms of resistance to clubroot.

In conclusion, we cloned and sequenced the approximately 64-kb genomic region, including the *CRb* locus identified in Chinese cabbage cultivar 'CR Shinki', and demonstrated that this is a complex locus composed of at least six NB-LRR genes in tandem with the same orientation; gain-of-function analysis demonstrated that one of the NB-LRR genes (*CRb*_α) encodes the *CRb* specificity. The fact that the nucleotide sequence of *CRb*_α is identical to the genome sequence of the previously cloned *CRa* gene strongly indicates that *CRb* (*CRb*^{Kato}) and *CRa* are one and the same clubroot-resistance allele. The complex nature of the relationship between these CR genes interferes with both attempts to evaluate the genetic basis of variations in pathogenicity and use of resistance genes to facilitate efficient CR breeding. Resolving this complexity is difficult because of the differences among the genetic resources and pathogens used for mapping. Although map-based cloning is not straightforward, acceleration of CR gene cloning will help to clarify the precise relationships among CR genes mapped in close proximity to each other, thereby improving the race classification system and marker-assisted selection.

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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