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ORIGINAL PAPER

Identification and mapping of a novel dominant resistance gene, *TuRB07* to Turnip mosaic virus in *Brassica rapa*

Mina Jin · Soo-Seong Lee · Lin Ke · Jung Sun Kim · Mi-Suk Seo · Seong-Han Sohn · Beom-Seok Park · Guusje Bonnema

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Abstract
Key message **A novel dominant resistance gene, TuRB07, was found to confer resistance to an isolate of TuMV strain C4 in B. rapa line VC1 and mapped on the top of chromosome A06.**
Abstract The inheritance of resistance to Turnip mosaic virus in *Brassica rapa* was investigated by crossing the resistant line, VC1 with the susceptible line, SR5, and genotyping and phenotyping diverse progenies derived from this cross. Both a doubled haploid population, VCS3M-DH, an F2 and two BC1 (F1 × VC1 and F1 × SR5) populations were created. Population tests revealed that the resistance to the TuMV C4 isolate in *B. rapa* is controlled by a single dominant gene. This resistance gene, *TuRB07* was positioned on the top of linkage group A06 of the *B. rapa* genome through bulk segregation analysis and fine mapping recombinants in three doubled haploid- and one backcross population using microsatellite markers developed from BAC end sequences. Within the region between the two closely linked markers flanking *TuRB07,* H132A24-s1, and KS10960, in the Chiifu reference genome, two genes encoding nucleotide-binding site and leucine-rich repeat proteins with a coiled-coil motif (CC-NBS-LRR), *Bra018862* and *Bra018863* were identified as candidate resistance genes. The gene *Bra018862* is truncated, but the gene *Bra018863* has all the domains to function. Furthermore, the analysis of structural variation using resequencing data of VC1 and SR5 revealed that *Bra018863* might be a functional gene because the gene has no structural variation in the resistant line VC1 when compared with Chiifu, whereas at the other NBS-LRR genes large deletions were identified in the resistant line. Allelic differences of *Bra018863* were found between VC1 and SR5, supporting the notion that this gene is a putative candidate gene for the virus resistance.

M. Jin and S.-S. Lee contributed equally to this work.

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M. Jin · J. S. Kim · M.-S. Seo · S.-H. Sohn · B.-S. Park
Department of Agricultural Biotechnology, National Academy of Agricultural Science, Rural Development Administration, 150 Suin-ro, Gwonseon-gu, Suwon 441-707, Korea

M. Jin (✉) · L. Ke · G. Bonnema (✉)
Wageningen UR Plant Breeding, Wageningen University and Research Centre, Droevendaalsesteeg 1, Wageningen, The Netherlands
e-mail: mina.jin@wur.nl

G. Bonnema
e-mail: guusje.bonnema@wur.nl

S.-S. Lee
BioBreeding Institute, Business Incubation, Chung-Ang University, Ansung, Gyounggi, Korea

Introduction

Turnip mosaic virus (TuMV) is one of the major diseases along with clubroot, blackleg and black rot, which can infect most plants of the family Cruciferae (Hatakeyama et al. 2013; Vicente and Holub 2013; Yu et al. 2013). It is a member of the genus *Potyvirus,* which is the largest genus in the *Potyviridae* family and was reported to have widely spread around the world, including Asia, Europe, and North America (Lehmann et al. 1997). TuMV has a wide host range, including not only many species in the Brassicaceae, but also legumes, ornamentals, and weedy plants (Green and Deng 1985). The most common symptom in infected

plants is a distinct mosaic of light and dark green colors in the leaves. Depending on the virus strain and the crop species, necrosis, chlorosis, and leaf malformation may also occur. Therefore, in the cultivation of *Brassica* crops with economic importance, such as cabbage, broccoli, cauliflower, Chinese cabbage, turnip, radish, and oilseed rape, it is a serious disease causing significant yield losses. The most effective way to control this virus disease is by introducing natural resistant varieties, because the use of insecticides against the aphids, which transmit the virus, is ineffective and is not environmentally friendly (Walsh et al. 1999).

To breed plants resistant against the virus, it is essential to understand the biological variation of TuMV isolates or strains. This variation is mainly classified into two systems, by Provvidenti (1980) and Walsh (1989). In the system defined by Provvidenti (1980), the strains C1–C8 are identified based on resistance responses of a variety of Chinese cabbage accessions (*B. rapa* ssp. *pekinensis*) gathered from mainly China, Japan, and Taiwan (Green and Deng 1985; Liu et al. 1990; Provvidenti 1980; Stobbs and Shattuck 1989). The classification system established by Walsh (1989) discriminates four groups of TuMV isolates using *Brassica napus* differentials. On the basis of this system, Jenner and Walsh (1996) developed a new system which integrated information from several systems, and could discriminate 12 different pathotypes by symptom interaction between *B. napus* differentials when screening 124 TuMV isolates, including the strains classified in the former system like C1–C4 by Provvidenti (1980) and Tu1-7 by Liu et al. (1990). Among numerous pathotypes, a few specific members of pathotype 1, 3, and 4 which are the most prevalent in Europe and Asia (Green and Deng 1985; Jenner and Walsh 1996), e.g., UK1 in pathotype 1, C4 (CHN4), C5 (CHN5), and CZE1 in pathotype 3, CDN1 in pathotype 4, have been principally used in studies to identify resistance gene loci or to investigate the interaction between host plants and TuMV.

Genetic studies towards resistance to TuMV have been primarily performed in the Brassicaceae species, including the model plant *Arabidopsis*. Most results showed that the resistance was under qualitative control by one or two genes and only a few studies reported that quantitative trait loci were involved in resistance (Walsh and Jenner 2002; Zhang et al. 2008). A number of resistance genes have been mapped in *B. napus*, most of which are dominant, such as *TuRB01* (Walsh et al. 1999), *TuRB04-05* (Jenner et al. 2002), and *TuRB03* (Hughes et al. 2003), and in *Brassica rapa TuRB01b* (Rusholme 2000) and *TuRBCH01* (Wang et al. 2009), and in *Arabidopsis thaliana TuNI* (Kaneko et al. 2004). Recently, a few recessive resistance genes have been identified in *B. rapa*, which are *retr01* (Rusholme et al. 2007), *retr02* (Qian et al. 2013), and *rnt1-2* (Fujiwara et al. 2011). Rusholme et al. (2007) revealed that a dominant gene, *ConTR01* and a recessive gene, *retr01*, which is epistatic to the dominant gene, together control resistance to TuMV in *B. rapa*.

Studies of the interactions between host plants and TuMV have identified pathogenic determinants from the virus for some resistance genes mapped in *Brassica* crops. The cylindrical inclusion protein of the virus was found to be the avirulence determinant breaking the resistance genes to the UK1 strain (pathotype 1), e.g., dominant resistance genes in *B. napus*, *TuRB01* and *TuRB05* (Jenner et al. 2000, 2002) and one recessive resistance gene, *rnt1-2* in *B. rapa* (Fujiwara et al. 2011). The P3 protein of TuMV was also reported to be associated with the avirulence when interacting with *TuRB03* (a dominant resistance gene to CDN1, pathotype 4) and *TuRB04* in *B. napus* (Hughes et al. 2003; Jenner et al. 2002).

Using an *Arabidopsis* mutant with decreased susceptibility, Lellis et al. (2002) identified a recessive resistance gene to TuMV, *lsp1* and isolated the gene encoding the protein *eukaryotic initiation factor 4E* (*eIF4E*) as a candidate resistance gene by map-based cloning. They suggested that the *eIF4E* gene functions in translation initiation of viral RNA. In *B. rapa* and *B. napus*, the genes encoding *eIF4E* or *eIF4G* were predicted as candidates for recessive resistance genes *retr01* (Rusholme et al. 2007) and *retr02* to TuMV (Qian et al. 2013). Complementation of the *Arabidopsis* mutant with *eIF4E* and *eIF(iso)4E* isolated from *B. rapa* showed that indeed the TuMV can use these genes from *B. rapa* for replication (Jenner et al. 2010). The molecular mechanism of dominant resistance to TuMV in *Brassica* crops is less well characterized. Only in one study by Kaneko et al. (2004) genes encoding a lipase-like protein and a CC-NBS-LRR protein, were suggested as candidate resistance genes, as they map physically close to the dominant resistance locus *TuNI* to TuMV.

To breed durable resistant *Brassica* varieties to TuMV, it is important to combine resistance genes effective against many pathotypes or isolates of the virus. TuMV strain C4, a member of pathotype 3 (Jenner and Walsh 1996) is the most widespread strain in Korea, Taiwan, and China (Suh et al. 1995). This study describes the identification of a novel resistance to a TuMV C4 isolate in *B. rapa*, genetic inheritance of the resistance, and the genetic map position of the resistance gene in several mapping populations. In addition, a candidate resistance gene was predicted based on the structural variation in the parental lines of NBS–LRR genes physically located between the two markers flanking the resistance gene.

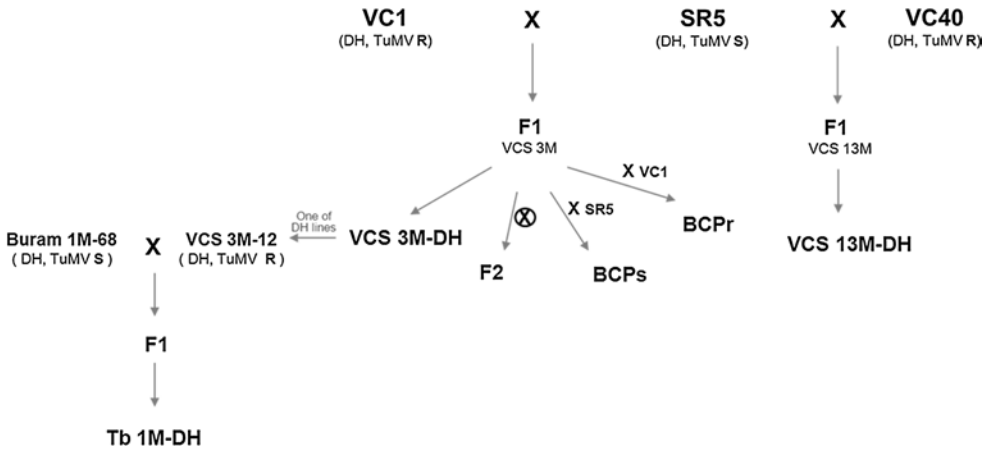

Fig. 1 Pedigree of plant materials used to assess reisistance/susceptibility to TuMV in this study. The Chinese cabbage, VC1, and VC40 DH lines were resistant to the strain C4 isolate; these lines were derived from microspore culture of a single-resistant breeding line. They were individually crossed with the susceptible SR5DH line and two double-haploid populations were made using their F1 plants, VCS3M and VCS13M. VCS3M was selfed and back-crossed to each parental line. One resistant line of VCS3M-DH population, VCS3M-12 was crossed to another susceptible line, Buram 1M-68, and using its F1 plant a double-haploid population was made through microspore culture

Materials and methods

Plant materials

Two Chinese cabbage lines, VC1 and VC40 were used as resistant parent, both doubled haploids derived from microspore culture of one breeding line resistant to a TuMV C4 isolate. The VC1 and VC40 DH lines were each crossed with susceptible parent SR5, and the former crossing F1 (VC1 × SR5) was named VCS3M and the latter crossing (VC40 × SR5) was named VCS13M (Fig. 1). For the disease assays, doubled haploid populations, VCS3M-DH ($n = 110$) and VCS13M-DH ($n = 87$) were developed from microspore culture of each F1 following the method by Lee et al. (2011). To examine the inheritance of the resistance and map the resistance gene, the VCS3M F1 was selfed (F2 = 186) and crossed with each parental line, VC1 (R) and SR5 (S) to produce backcross populations. To study the resistance in different susceptible backgrounds, another susceptible double-haploid line, Buram 1 M-68 was crossed with one resistant line of the VCS3M-DH population, VCS3M-12. From microspore culture of the F1, the Tb1 M-DH population ($n = 140$) was developed.

Plants were grown in a glasshouse at 20–22 °C in Ansung, South Korea, with natural day length (14 h) during the experiments.

TuMV strain C4 isolate and disease assays

A single TuMV isolate, which belongs to strain C4, was used in this study and it was kindly provided by the National Institute of Horticultural and Herbal Science, Korea. The virus was originally given by Dr. Green of the Asian Vegetable Research and Development Center, AVRDC, Taiwan (Green and Deng 1985; Suh et al. 1995; Yoon et al. 1993). The virus was maintained in leaves of a susceptible *Brassica juncea* line.

To prepare virus inoculum, 10 g of infected mustard (*B. juncea*) leaves was homogenized with 0.1 M potassium phosphate buffer (pH 7.5) and the homogenate was subsequently filtered through three layers of cheesecloth (Yoon et al. 1993). Plants were mechanically inoculated twice at 2 and 3 weeks after sowing (Suh et al. 1995). The two youngest leaves for first inoculation and a new leaf for the second inoculation were dusted with carborundum and rub inoculated with the prepared inoculum. For the double-haploid populations, five plants per line were grown, three of which were inoculated, while the remaining two plants acted as controls to detect environmental effects. In case DH lines showed an ambiguous phenotype in the first assay, the same procedure as for the first trial was carried out again.

Symptoms were observed by visual assessment 4 weeks after the second inoculation. Plants were considered as susceptible, if any leaves of the inoculated plants showed mosaic, necrotic, or chlorotic symptoms, while only plants without symptoms at all were considered as resistant.

Genetic marker analysis

Genomic DNA was extracted from young leaf tissue of all the plants from DH, BC1, or F2 populations, using the method of Kim et al. (2006), and stored at −20 °C. For bulked segregant analysis (BSA), one resistant and one

susceptible bulk were made by mixing genomic DNA of each of the 12 resistant and 12 susceptible lines selected from the VCS3M-DH population.

The primer sequences for a total of 238 SSR markers of *B. rapa* were obtained from the Korean *Brassica rapa* genome project (http://www.brassica-rapa.org/BRGP/geneticMap.jsp), which were developed from the sequences of bacterial artificial clones (BACs) of the *B. rapa* inbred line 'Chiifu' (Mun et al. 2009a). A genetic map for the VCS3M-DH population was already constructed using these genetic markers (Seo et al. 2013). PCR amplification was carried out in 20 μl volumes, containing 1 U of *Taq* polymerase (Genetbio, Daejeon, Korea), 5 pmol of each primer, 250 μM dNTPs, 2.0 mM $MgCl_2$, 1× *Taq* buffer and 50 ng of genomic template DNA. The PCR program was as follows: 5 min at 95 °C, followed by 35 cycles with 30 s of DNA denaturation at 95 °C, 30 s of annealing at the 54–58 °C of appropriate temperature, and 1 min of extension at 72 °C and final extension at 72 °C for 10 min. PCR was carried out in GeneAmp PCR system 9700, Applied Biosystems. All the PCR products were size separated in 2.5 % agarose gel or 5 % polyacrylamide gel.

Development of additional SSR markers to narrow down the resistance locus

In 2010, before the whole genome sequence of *B. rapa* inbred line 'Chiifu' was released, comparative analysis between *B. rapa* and the model plant *Arabidopsis* was used to develop markers closer linked to the resistance locus. Pairwise sequence comparison between the sequences of *B. rapa* BAC clones located around the three SSR markers selected from BSA and the *A. thaliana* genome was conducted by BLASTN and PipMaker (http://www.pipma

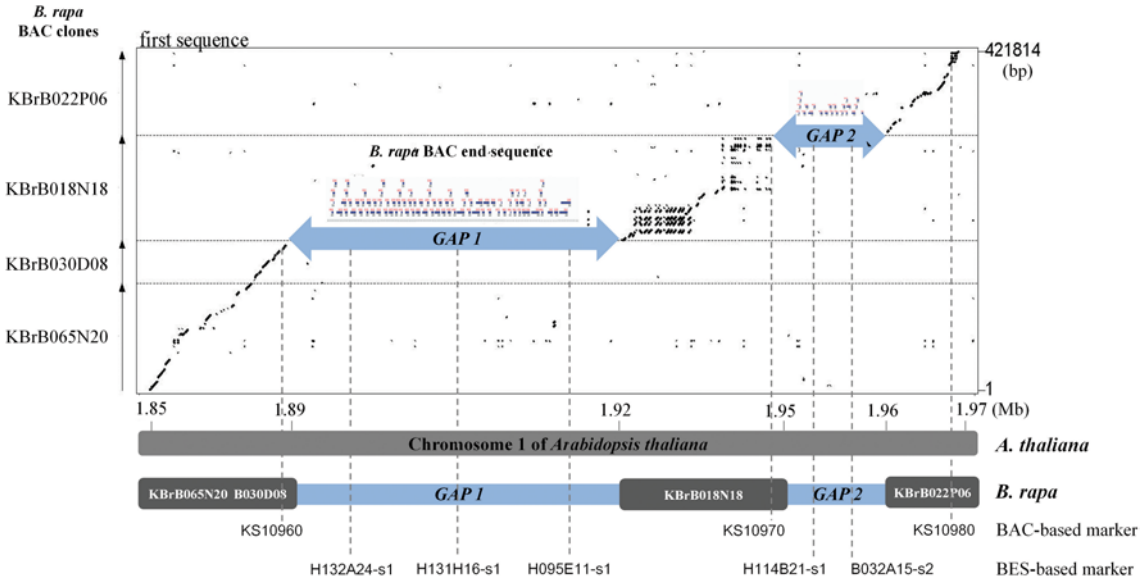

Fig. 2 Dot-plot analysis between four *B. rapa* BAC clones harboring SSR markers selected by BSA and the counterpart syntenic region in *Arabidopsis*. To develop additional SSR markers in the two gaps, about 91,000 BES were aligned on to their counterpart region in *Arbidopsis*. Five primer pairs were designed using microsatellite in the BESs matched on the gaps

Table 1 Sequence of SSR markers used for mapping the *R* gene

Marker name	BAC or BES	Forward sequence	Reverse sequence	SSR motif
KS10960	KBrB030D08	TCTTCACGCAATGGCTTT	TCCCCATTAATGACACGC	ACC
KS10970	KBrB018N18	GTGGGATCGATGGTGATCTA	GTGTGGCTTTCACAAATTCC	AT
KS10980	KBrB022P06	CCATACCAAAGCAGTACTCGAA	GAAAACTTTAAAACACTCACACAG	AG
H132A24-s1	KBrH132A24R	TCCAGCTTTATCTGCATCG	GTGTTGCCTTTGAGCCAG	AAG
H131H16-s1	KBrH131H16F	GCATCCACTGAGGCATGT	CCGCACCGCAGTTAATAG	AT
H095E11-s1	KBrH095E11R	ATCCACGCCCATTTTCTT	CAGAATTCACGGCGAGAG	AG
H114B21-s1	KBrH114B21R	TGAACTTCATTCAAATCGTTACTG	TTCAAATTGATGACCGCA	AAT
B032A15-s2	KBrB032A15F	GGCAGATTCATCAAGATCCA	TGCTCTTCAAGGCAAGGA	AC

ker.bx.psu.edu/pipmaker/) (Fig. 2). To develop SSR markers in the two gaps between the counterpart region of *A. thaliana* to *B. rapa* BAC sequences, around 91,170 BAC end sequences (BES) of *B. rapa* inbred line 'Chiifu' were in silico mapped relative to the two gaps on the Arabidopsis chromosome by BLASTZ at a cutoff of <E-6. Five additional SSR primer pairs were designed from the BESs matching those gaps and their actual map position was determined on the VCS3M-DH genetic map. The sequences of all primers used to map the resistance locus were listed in Table 1.

Statistical analysis and linkage mapping

Segregation data of resistant and susceptible responses for the F2, backcross and doubled haploid generations was analyzed by Chi-square for goodness of fit to each expected segregation ratio.

To map the TuMV C4 resistance locus on different populations, genetic linkage analysis was performed using JoinMap version 4.0. Recombination frequencies were converted to map distances using Kosambi's mapping function and linkage maps were visualized using MapChart (Voorrips 2002).

Structural variation of genes in region around *TuRB07* of both parental lines

The VC1 DH line was re-sequenced using Illumina HiSeq 2000 with a depth of 17× genome coverage. The re-sequenced raw reads of VC1 were aligned to the latest version of the *B. rapa* reference genome (version 1.2) downloaded from BRAD using BWA 0.5.9 with default settings. The aligned sam file was later sorted by chromosome coordinates and converted to bam format with PCR duplicates removal at the region from 850,000 (above KS10980) to 1,850,000 (below KS10950) on Chromosome 6 using samtools (0.1.18). This region encompasses the physical distance between KS10980 and KS10950, and includes an additional linked region with 3 NBS-LRR genes on A06 (Fig. 3). The generated bam file was used to detect the structural variation against the same reference genome downloaded before with DELLY (0.0.9).

The assembled contigs of SR5 were obtained from the National Agricultural Bioinformatics Information Center (http://www.nabic.rda.go.kr/) and aligned to the reference genome used in VC1 analysis (version 1.2) by nucmer. The output was filtered at the same candidate region (chromosome 6 from 850,000 to 1,850,000) as in the VC1 analysis.

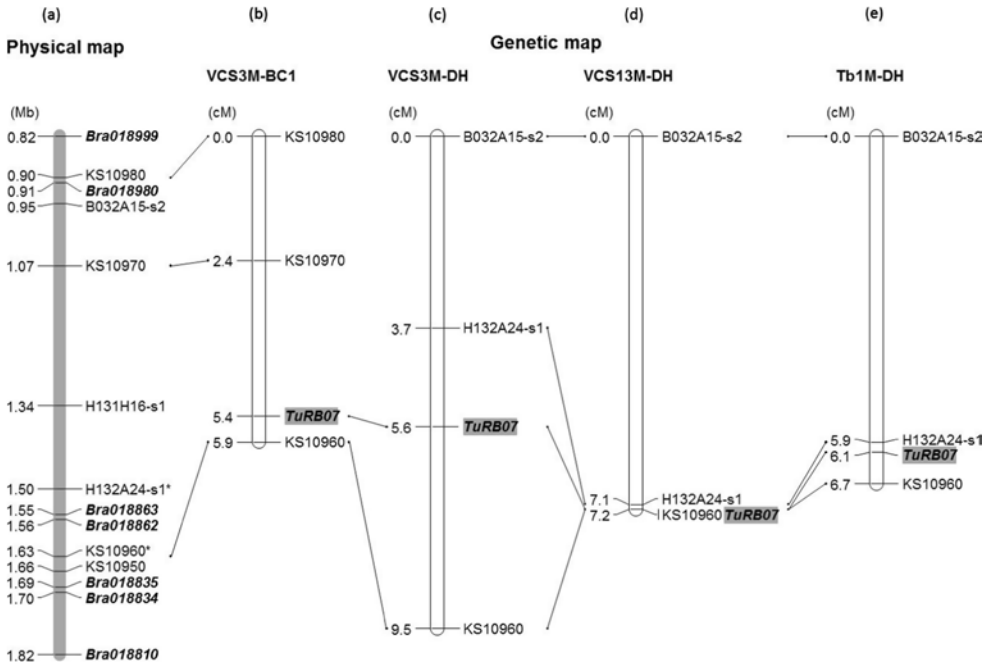

Fig. 3 *TuRB07* locus on four genetic maps and the physical map of the reference genome Chiifu. The position of the SSR markers used to map the resistance locus is shown on the physical map of 'Chiifu'; the two markers closely linked to *TuRB07* have an *asterisk* (**a**). The genes in bold represent predicted NBS-LRR genes linked to the resistant locus, *TuRB07*. *TuRB07* locus was mapped on linkage group A06 of genetic maps of four different populations (**b**–**d**). First, three SSR markers selected from BSA were mapped in the VCS3M-BC1 population ($n = 187$) to map the *R* gene locus. For the reproducible genetic analysis, disease inoculation, and the marker assays, including BES-based SSR markers were performed on three double-haploid populations, VCS3M-DH ($n = 110$), VCS13M-DH ($n = 87$), and Tb1M-DH ($n = 140$) markers. Genetic distances were calculated using Kosambi's mapping function in JoinMap v 4.0

Table 2 Inheritance of resistance to TuMV C4 strain in different Chinese cabbage populations

Name	Population	Number of Plants			Expected ratio (*R*:*S*)	Goodness of fit	
		Total plants	Resistant (*R*)	Susceptible (*S*)		χ^2	*P* value
VCS3M	VC1 (R)	20	20	0			
	SR5 (S)	48	0	48			
	F1 (VCS3)	48	48	0			
	F2	186	145	41	3:1	0.303	0.582
	BC1R (F1 × VC1)	150	150	0	1:0		
	BC1S (F1 × SR5)	182	112	70	1:1	4.455[a]	0.035
	DH	109	46	63	1:1	1.038	0.308
VCS13 M	DH	83	40	43	1:1	0.006	0.938
Tb1 M	DH	136	47	89	1:1	6.026[a]	0.014

[a] Significant at the 5 % level

Detected structural variations from both comparisons were filtered by excluding the inter-chromosome translocation and large size structural variation (>10,000 bps). The genes with structural variations identified were further investigated to detect whether the structural variation was likely to affect gene function.

Sequence variation of candidate genes

In the *Brassica* database (BRAD, http://brassicadb.org/brad/), the candidate resistance genes were selected that mapped physically between the SSR markers flanking the TuMV resistance locus. Specific oligonucleotides were designed to amplify these genes using the reference genome of *B. rapa*, including the attB1 and attB2 sites upstream of the 5′ and 3′ gene-specific priming sequences for the gene cloning using the gateway vector (G63F; AAAAAGCAGGCTTGAGCTAAGATGGGAGGCTG, G63R; AGAAAGCTGGGT TCATTCATCAACTTCATTAC). PCR products of about 2.5 kb amplified by Ex *Taq* polymerase (Takara) were purified with the PCR purification kit (Inclone). BP clonase recombination reactions were done with the purified PCR products and pDONR221 for 4 h in 25 °C. After transformation into DH5α competent cells, plasmids were isolated and candidate gene sequence was validated by ABI 3730xl sequencer (Supplementary data 1). Sequence alignment was performed by Clustal Omega, EBI and GeneDoc.

Results

Genetic inheritance of the resistance against TuMV strain C4

All F1 plants derived from the cross between VC1 (R) and SR5 (S) and the whole progeny of the backcross with the resistant parent (F1 × VC1) were resistant to the used TuMV C4 isolate, indicating that TuMV resistance is dominantly inherited (Table 2). The observed segregation for virus resistance in the F2 generation significantly fitted the expected segregation ratio 3R:1S. Furthermore, disease response in two double-haploid populations VCS3M-DH and VCS13M-DH significantly fitted the expected ratio 1:1. This suggests that a single dominant gene is involved in the resistance to the TuMV strain C4 isolate. However, the segregation data in the backcross population to the susceptible parent (F1 × SR5) did not fit the expected segregation ratio of 1:1, as expected for a Mendelian model for monogenic dominant inheritance, as 112 plants were resistant and 70 were susceptible. In the Tb1M-DH population, having a different susceptible parent, the disease response did also not fit the expected segregation ratio 1:1 for a single dominant gene, as many more susceptible (89) than resistant (47) DH lines were detected. These data mean that other factors, either environmental or genetic, could be involved in the expression of resistance to the TuMV C4 isolate.

Bulk segregation analysis of the resistance against TuMV C4 isolate in *B. rapa*

For bulked segregant analysis (BSA), 12 resistant and 12 susceptible DH lines were selected from the VCS3M-DH population to construct a resistant bulk (RB) and a susceptible bulk (SB), respectively. Among 238 SSR markers screened over RB and SB, 3 SSR markers (KS10960, KS10970, and KS10980) showed clear polymorphism between these two bulks. To map the resistance locus against TuMV in *B. rapa*, these three SSR markers were applied to the 187 plants of the backcross population to the susceptible parent and the genotypic data was used to map the TuMV resistance locus between KS10960 (0.5 cM) and KS10970 (3 cM) (Fig. 3b). The resistance gene to the TuMV C4 isolate was named *TuRB07* for *TuMV*

RESISTANCE in Brassica 07, in line with the previously described resistance loci.

Development of additional markers to narrow down the TuMV resistance locus

Around *TuRB07* four BAC clones, KBrB065N20 (132 Kb), KBrB030D08 (53 Kb), KBrB018N18 (135 Kb), and KBrB022P06 (102 Kb), from which the SSR markers, KS10950, KS10960, KS10970, and KS10980 were derived, were aligned to the *Arabidopsis* genome. They showed a homologous match colinear to a syntenic region on *Arabidopsis* chromosome 1 (18.5–19.7 Mb) at a significance level <1e-6 (Fig. 2). However, two gaps (gap 1, 18.95–19.20 Mb; gap 2, 19.42–19.53 Mb) were found of about 365 Kb in length, with no *B. rapa* BAC sequences that matched the syntenic *A. thaliana* sequence. Through a blastZ search of 91,179 *B. rapa* BES against *Arabidopsis*, 36 BES and 8 BES were allocated to gap 1 and gap 2, respectively, and seven primer pairs were designed to develop three and four microsatellites for gaps 1 and 2, respectively. Out of seven primer pairs tested, five primer pairs showed polymorphism between the parents VC1 and SR5. Three of those microsatellites, H132A24-s1, H131H16-s1, and B032A15-s2 mapped on the top of linkage group A06 in the VCS3M-DH genetic map; nearby, the three markers identified through the BSA test (KS10960, KS10970, and KS10980), while the other microsatellites, H095E11-s1 and H114B21-s1 were mapped on linkage group A05 which is syntenic to the telomeric region of the short arm of A06 (Supplementary data 2).

To confirm the genetic locus of the TuMV C4 isolate resistance gene and to identify markers closer to the *TuRB07* gene, the BES-SSR markers on linkage group A06 and the closest marker KS10960 were scored on all DH lines from three double-haploid populations VCS3M-DH, VCS13M-DH, and Tb1M-DH (Fig. 3c, d; Supplementary data 3). The *TuRB07* was mapped between the BES-based SSR, H132A24-s1 and KS10960 on all these genetic maps. H132A24-s1 was closer to the resistance locus than KS10960 on the genetic maps of the VCS3M-DH and Tb1M-DH populations.

Candidate resistance gene and allelic variation between resistant (VC1) and susceptible (SR5) parents

The *B. rapa* region between the two markers flanking *TuRB07* and the syntenic *A. thaliana* blocks were investigated for the presence of gene models that represent candidate virus resistance genes. Two markers close to resistance locus *TuRB07*, H132A24-s1, and KS10960 were located physically at 1.50 and 1.63 Mb of chromosome A06. Twenty-eight genes were predicted between these two markers (130 Kb) in BRAD, six of which are unknown genes and 22 genes with annotation, from which two genes named *Bra018863* and *Bra018862* encode a nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins with a coiled-coil (CC) motif in the N-terminal domain (CC-NBS-LRR) (Fig. 3). These two CC-NBS-LRR genes were tandem arranged, but the *Bra018863* sequence encompassed a full-length CC-NBS-LRR protein while the *Bra018862* sequence was truncated.

After analysis on of a region of one megabase (0.85–1.85 Mb) around *TuRB07* in both parental genomes aligned against the reference genome, among 187 genes predicted in the region, 32 genes in VC1, and 36 genes in SR5 were suspected to be affected by structural variation (Supplementary data 4). In particular, for both *Bra018862* and *Bra018863,* no structural variations were detected between the reference genome Chiifu and the resistant parent VC1, while two duplication events on *Bra018863* and 13 bps insertions followed directly by one duplication event on *Bra018862* were detected between SR5 and the reference genome. In this genomic region, three other NBS-LRR genes were detected, *Bra018835*, *Bra018834*, and *Bra018810*; in both parental lines, these three genes contained structural variations as compared to the Chiifu reference genome. In these three genes, VC1 had large deletions, including a whole gene deletion for *Bra018810*, and SR5 had duplications and insertions in *Bra018835* and *Bra018834* and a duplication in *Bra018810* (Supplementary data 4).

To look at the sequences of CC-NBS-LRR gene *Bra018863* in more detail, a specific primer pair was designed amplifying the majority of the open reading frame of *Bra018863* in both the resistant VC1 line and the susceptible SR5 line. As expected in the analysis of structural variation, sequencing revealed that one gene was obtained in the resistant line VC1 and two duplicated genes, one full length and the other truncated as a result of a stop codon at the 390th amino acid sequence, were obtained in SR5. When compared with *Bra018863* from Chiifu, the sequences of the full length genes *Bra018863* between VC1 and SR5 had 89 and 87 % homologies at the nucleotide sequence level and both 74 % identity at the amino acid sequence level. On the other hand, comparisons between the full length sequences of the *Bra018863* genes from VC1 and SR5, showed homologies of 99 % in nucleotides and 97 % in amino acid sequences. Nucleotide insertions and deletions between parental lines were revealed at 14 different positions that gave rise to 24 predicted amino acid changes. These amino acid changes were identified in three parts of the CC-NBS-LRR gene, N-terminus, NBS domain, and C-terminus (Fig. 4), which shows that these resistant and susceptible parental lines have different alleles.

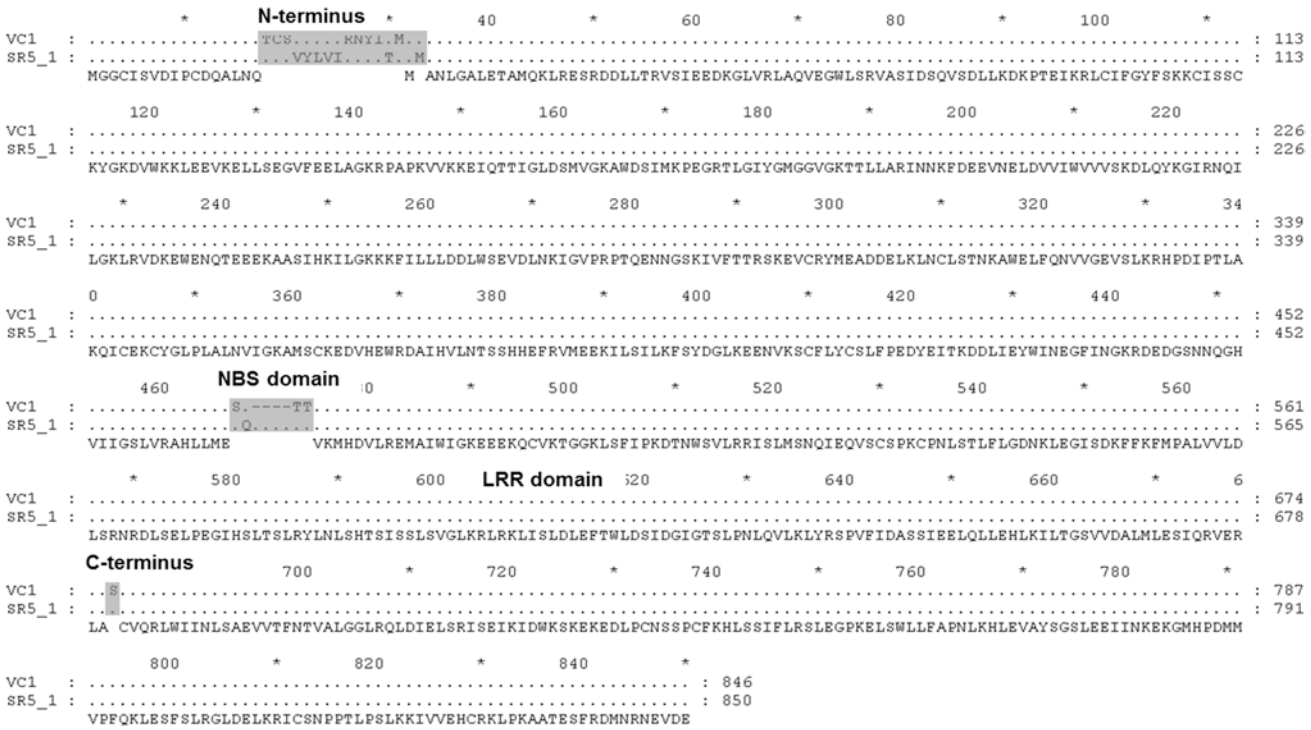

Fig. 4 Alignment of *Bra018863* gene from VC1 (resistant) and SR5 (susceptible). The variation at amino acid level was identified in three domains of the CC-NBS-LRR resistance gene protein sequence, N-terminus, NBS domain, and C-terminus. *CC* coiled coil, *NBS* nucleotide-binding site, *LRR* leucine-rich repeat

Discussion

A single genetic locus was found to confer resistance to the TuMV strain C4 isolate used in this study in *B. rapa* line VC1 and this gene, *TuRB07,* was positioned on the telomere of the short arm of chromosome A06. The resistance inherited dominantly. Different inheritance mechanisms of resistance to TuMV have been reported depending on the strains tested, resistant and susceptible parents used and the sensitivity of the virus resistance tests (Walsh and Jenner 2002). Recessive, dominant, and quantitative resistances have been described to strain C4 (Qian et al. 2013; Suh et al. 1995; Yoon et al. 1993; Zhang et al. 2008). The single dominant gene *TuRB07* identified in this study might be the same as the resistance described in the line 0–2 by Suh et al. (1995). Furthermore, our study suggests that there might be other factors affecting the resistance response (genetic or environmental), besides the single dominant gene, *TuRB07*, because segregation for disease resistance did not fit the expected ratio in a subset of the populations evaluated (VCS3M-BC1S and Tb1M-DH). In the VCS3M-BC1S population, there were many more resistant than susceptible lines and in the Tb1M-DH population more susceptible lines were observed.

Walsh and Jenner (2006) suggested that resistance to TuMV in the family *Brassica* may be broad spectrum to a wide range of isolates or effective against only a few isolates. In the present study, it is difficult to estimate whether *TuRB07* can render broad-spectrum resistance because most broad-spectrum resistance was known to be induced by recessive gene(s) (Qian et al. 2013; Rusholme et al. 2007; Walsh et al. 2002). However, in *B. rapa*, a number of resistance sources were identified, both narrow and broad spectrum (Hughes et al. 2002; Suh et al. 1995; Walsh et al. 2002; Yoon et al. 1993), while in *B. napus* only narrow resistant spectra were reported (Hughes et al. 2002, 2003). Furthermore, most of the *B. rapa* lines showing resistance to pathotype 3 (isolates CZE1 and CHN5) or pathotype 4 (isolate CDN1) also showed resistance to other pathotypes, whereas some of the lines having resistance to pathotype 1 (UK 1) were susceptible to pathotype 3 (isolates CZE1 and CHN5) and/or pathotype 4 (isolate CDN1) (Hughes et al. 2002; Walsh et al. 2002). Therefore, whether *B. rapa* VC1 with resistance to an isolate of the strain C4 (pathotype 3) displays broad-spectrum resistance effective to different pathotypes should be tested.

A number of dominant and recessive genes were mapped in *Brassica* species. The single dominant resistance genes, *TuRB01* and *TuRB03* in *B. napus,* and *TuRB01b* in *B. rapa* were mapped on chromosome A06 in a similar interval and were expected to be members of a resistance gene cluster or even possibly allelic (Hughes et al. 2003; Rusholme et al.

2007; Walsh et al. 1999). In this study, *TuRB07* mapped on the telomere of short arm of chromosome A06 (1.5 Mb), which is a different genetic region when compared with the above-described dominant genes. Two SSR markers, sS1949, and sNRB93, flank *TuRB03*, the resistance locus to TuMV isolate CDN1 (pathotype 4) in *B. napus* (Hughes et al. 2003), and map at 105.5 and 116.2 cM in the BnaDYDH_A06 linkage map (Wang et al. 2011). These two markers were genetically closely linked to the public SSR markers Na10E02 (97.3 cM), Na12B08 (97.3 cM), and BRAS115 (121.2 cM). These marker sequences were blasted to the *B. rapa* genome and mapped around 20 Mb on chromosome A06. Therefore, the *TuRB07* is a novel resistance gene, different from other dominant genes identified as pathotype or isolate-specific resistance genes in *B. napus*.

Resistance mechanisms to pathogens in plants depend on the type of pathogen. Mechanisms induced by plant resistance (*R*) genes, most of which encode NBS-LRR proteins, are initiated by the recognition of pathogen-associated molecular patterns in plant transmembrane receptors, thereby inducing active defence responses (DeYoung and Innes 2006; Marone et al. 2013). On the other hand, passive resistance is induced by the absence of susceptibility factors in plants, such as eukaryotic initiation factor proteins *eIF4E*, *eIF(iso)4E*, *eIF4G,* and *eIF(iso)4G,* which can be required for pathogen replication (Gomez et al. 2009; Qian et al. 2013). In the passive mechanism, recessive alleles would confer resistance, whereas active resistance is often conferred by dominant alleles. Thus, the resistance by the single dominant gene, *TuRB07* is likely an active response mediated by an *R* gene. Furthermore, this *TuRB07* controls a typical response being accompanied by *R* gene-conferred resistance mechanism, extreme resistance, which does not show any necrotic local lesions at the primary infection site.

The region around *TuRB07* included several CC-NBS-LRR protein genes. NBS-LRR proteins exhibit narrow recognition specificity against pathogens. To recognize a wide range of pathogens, several NBS-LRR genes often occur in clusters that originate from gene duplications followed by local rearrangements and gene conversion (Meyers et al. 2003). The region where *TuRB07* is located on A06 was reported as one of the NBS-LRR gene clusters in *B. rapa* (Mun et al. 2009b). Among NBS-LRR genes in this cluster, *Bra018810*, *Bra018834*, and *Bra018835* genes had large deletions in VC1 as compared to Chiifu and the gene *Bra018862* was truncated in Chiifu and VC1. These four genes thus cannot be the functional genes in resistant parent VC1. However, the full length gene *Bra018863* containing all the motifs for CC-NBS-LRR proteins is a putative candidate resistance gene to the TuMV strain C4 isolate used in this study. We cannot exclude the possibilities that VC1 contains an insertion harboring a TuMV resistance gene that was not detected by aligning its re-sequenced genome to the Chiifu reference genome.

We do not have functional evidence to prove that the gene *Bra018863* is the resistance gene. However, many studies have demonstrated the role of NBS-LRR genes in resistance against diverse pathogens (Kachroo et al. 2006). Specifically, most of the cloned viral *R* genes, e.g., *Rx* to Potato virus X (PVX) in *Solanum tuberosum*, *Sw-5* to Tomato spotted wilt virus (TSWV) and $Tm2^2$ to Tomato mosaic virus (ToMV) in *Lycopersicon esculentum*, *HRT* to Turnip crinkle virus (TCV) and *RCY1* to Cucumber mosaic virus (CMV) in *A. thaliana*, belong to the CC-NBS-LRR class *R* genes, and only the *N* gene to Tobacco mosaic virus (TMV) in *Nicotiana glutinosa* encodes a TIR-NBS-LRR protein (Bendahmane et al. 2002; Brommonschenkel et al. 2000; Cooley et al. 2000; Lanfermeijer et al. 2003; Takahashi et al. 2002; Whitham et al. 1994).

The alleles of the CC-NBS-LRR gene, *Bra018863* were different from the resistant parent VC1 and the susceptible parent SR5, which fits the hypothesis that this is the resistance gene. Allelic differences were confined to the N-terminal domain with CC motif, NBS domain, and C-terminal domain. All the domains in NBS-LRR proteins are required to mediate resistance to pathogens in plants, but play different roles in the resistance response (DeYoung and Innes 2006). The initiation of a resistance response is believed to be controlled by CC or TIR (for Toll and Interleukin-1 Receptor) domain in the N-terminus (Joshi and Nayak 2011). These domains act to mediate recognition of pathogen determinants, such as fungal and bacterial effectors or viral proteins (Burch-Smith et al. 2007; Rairdan et al. 2008; Sacco et al. 2007). The NBS region between N-terminal and LRR domain is known to be responsible for ATP hydrolysis and subsequent activation of downstream signaling (Tameling et al. 2002). Many mutational analyses indicate that the NBS domain plays a key role in the function of *R* genes (Sekine et al. 2006; Tao et al. 2000; Tornero et al. 2002). The C-terminal LRR domain is thought to be involved in the specific recognition of pathogen effector molecules and to regulate signal transduction positively or negatively (Rairdan and Moffett 2006; Weaver et al. 2006). No difference was observed between LRR domains of VC1 and SR5 alleles. Further analysis and functional studies are required to prove that *Bra018863* is indeed the resistance gene and how different allelic variants act on resistance.

In conclusion, a novel resistance gene, *TuRB07*, rendering *B. rapa* resistant to a TuMV C4 isolate, was identified and localized on the top of chromosome A06. Two NBS-LRR genes were found between the two closely linked markers flanking *TuRB07*. Structural and sequence variation of these genes between parental lines and the reference genome suggested that one of these genes, *Bra018863*

might be a candidate gene for TuMV resistance. As resistance controlled by single dominant genes is often broken by the mutation of virus isolates, combining several resistance genes by introgression using molecular markers linked to *R* genes in elite germplasm, will increase durability, as well as provide broad-spectrum resistance.

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Conflict of interest We declare that we have no conflict of interest.

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