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ORIGINAL PAPER

Genome-wide identification of NBS-encoding resistance genes in *Brassica rapa*

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Abstract Nucleotide-binding site (NBS)-encoding resistance genes are key plant disease-resistance genes and are abundant in plant genomes, comprising up to 2% of all genes. The availability of genome sequences from several plant models enables the identification and cloning of NBS-encoding genes from closely related species based on a comparative genomics approach. In this study, we used the genome sequence of *Brassica rapa* to identify NBS-encoding genes in the *Brassica* genome. We identified 92 non-redundant NBS-encoding genes [30 CC-NBS-LRR (CNL) and 62 TIR-NBS-LRR (TNL) genes] in approximately 100 Mbp of *B. rapa* euchromatic genome sequence. Despite the fact that *B. rapa* has a significantly larger genome than *Arabidopsis thaliana* due to a recent whole genome triplication event after speciation, *B. rapa* contains relatively small number of NBS-encoding genes compared to *A. thaliana*, presumably because of deletion of redundant genes related to genome diploidization. Phylogenetic and evolutionary analyses suggest that relatively higher relaxation of selective constraints on the TNL group after the old duplication event resulted in greater accumulation of TNLs than CNLs in both *Arabidopsis* and *Brassica* genomes. Recent tandem duplication and ectopic deletion are likely to have played a role in the generation of novel *Brassica* lineage-specific resistance genes.

Keywords *Brassica rapa* · NBS-encoding gene · Disease resistance · Genome diploidization · Evolution

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Introduction

Plants have developed a variety of defense systems to protect themselves from environmental pathogens and pests. One well-characterized defense mechanism is recognition of specific pathogens mediated by disease-resistance (R) proteins and initiation of defense responses, including localized cell death and systemic acquired resistance (Dangl and Jones 2001). The largest class of known R genes includes those that encode the nucleotide-binding site (NBS) domain showing similarity to eukaryotic cell death effectors and the leucine-rich repeat (LRR) domains. Most characterized R genes can be subdivided into two distinct types based on the structure of the N-terminal domain of the protein: the N-terminal domain is either a coiled-coil (CC) motif or a *Drosophila* Toll/mammalian Interleukin-1 Receptor (TIR) domain. Variant types with different domains or truncated structures also exist, but generally they can be classified into the CC-NBS-LRR (CNL) or TIR-NBS-LRR (TNL) types based on the NBS-coding sequences (Ameline-Torregrosa et al. 2008; Meyers et al. 2003).

NBS-encoding genes belong to one of the largest gene families in plant genomes, and have been identified in non-vascular plants to angiosperms. Sequencing of the genome of model plant species has enabled genome-level investigation of the NBS-encoding gene family in monocot and dicot species, including rice (Monosi et al. 2004; Zhou et al. 2004), sorghum (Paterson et al. 2009), *Arabidopsis thaliana* (Meyers et al. 2003; Tan et al. 2007), *Medicago truncatula* (Ameline-Torregrosa et al. 2008), poplar (Kohler et al. 2008), grape (Yang et al. 2008b), and papaya (Porter et al. 2009) (Table 1). According to these studies, approximately 0.6–1.8% of genes encoded by plant genomes are NBS-encoding genes that occur at a density of 0.3–1.6 genes per megabase, with the exception of papaya. In the papaya genome, which did not have its whole genome duplicated recently in contrast to other plants, only 0.2% of genes encode NBS domains at a density of 0.1 gene per megabase (Porter et al. 2009). Most NBS-encoding genes are unevenly distributed and clustered in specific chromosomal regions (Ameline-Torregrosa et al. 2008; Meyers et al. 2003; Monosi et al. 2004). These observations suggest that whole genome duplication (WGD) along with tandem and segmental gene duplication have generated the high number of NBS-encoding genes in the plant genome.

Many of the NBS-encoding genes are ancient in origin and orthologous relationships are difficult to determine owing to lineage-specific gene amplifications and deletions. Therefore, the spectrum of NBS-encoding genes present in one species is not characteristic of the diversity of NBS-encoding genes in other plant families (McHale et al. 2006). In general, CNL types are found widely in both monocot and dicot genomes, whereas no TNL type genes have been identified in monocot genomes, although a small number of TNL genes have been found in gymnosperms (Bai et al. 2002; Cannon et al. 2002; Liu and Ekramoddoullah 2003). Previous phylogenetic analyses suggested that the CNL group originated before the divergence of the monocots and dicots, whereas the TNL group expanded recently after the divergence of the two groups (McHale et al. 2006; Yang et al. 2008b). Consequently, the TNL group proteins are thought to be involved in recognizing "species-specific" pathogens while the CNL group proteins are predicted to have basic defense roles as a result of regional adaptation to the biotic environment (Yang et al. 2008b).

Brassica rapa is one of the most valuable vegetable crops worldwide. However, susceptibility to multiple pathogens, including *Plasmodiophora brassicae*, a clubroot fungus, *Pectobacterium carotovorum*, a bacterial pathogen of soft rot decay, turnip mosaic virus (TuMV), and *Meloidogyne* root knot nematodes, hinders cultivation and reduces produce yield and quality. One of the significant challenges in *B. rapa* breeding has been to introduce durable disease resistance into agronomically important cultivars. One approach that can be used to understand the fundamental basis of *Brassica* resistance and susceptibility is genomic evaluation of disease-resistance gene homologs. In an initial study to identify candidate disease-resistance genes in *Brassica* species, a comparative mapping approach was taken using the closely related *A. thaliana* genome sequence as a source (Sillito et al. 2000). However, the polyploid nature of *Brassica* genomes along with frequent genome rearrangements and high sequence similarity between homologs has hampered the identification of orthologous genes. Genome-wide, sequence-level comparative analysis of conserved sequence blocks between *A. thaliana* and *B. rapa* reveals that they shared three rounds of WGD events (hereafter referred to as 1R, 2R, and 3R,

Table 1 The number of predicted NBS-encoding R genes identified in sequenced plant genomes

	Br	*At*	*Mt*	*Pt*	*Vv*	*Cp*	*Os*
NBS-encoding genes							
CC-NBS-LRR	17	58	152	278	203	4	392
TIR-NBS-LRR	42	110	94	121	97	6	0
Others	33	39	87	3	159	44	81
Total	92	207	333	402	459	54	473
Genome size (Mbp)	≈100 (529)[a]	125	≈186 (500)[a]	485	487	372	389
Source		Meyers et al. (2003)	Ameline-Torregrosa et al. (2008)	Kohler et al. (2008)	Yang et al. (2008b)	Porter et al. (2009)	Monosi et al. (2004)

Br, *Brassica rapa*; *At*, *Arabidopsis thaliana*; *Mt*, *Medicago truncatula*; *Pt*, *Populus trichocarpa*; *Vv*, *Vitis vinifera*; *Cp*, *Carica papaya*; *Os*, *Oryza sativa*

Others represent NBS-encoding genes with unusual structures or unknown motifs

[a] The NBS-encoding genes of *Br* and *Mt* were identified based on partial genome sequences. Overall genome sizes are indicated in parentheses

which are equivalent to the γ, β, and α duplication events, respectively) before diverging from a common ancestor approximately 13–17 million years ago (MYA). Moreover, *Brassica* genomes contain triplicated homologous counterparts of corresponding segments of the *A. thaliana* genome due to a whole genome triplication (WGT) event (4R) approximately 11–12 MYA after speciation (Mun et al. 2009). Furthermore, an additional natural allopolyploidization event, which happened during the last 10,000 years and resulted in a change in chromosome number and genome size, has shaped the diversification of *Brassica* crops (Johnston et al. 2005; UN 1935). An alternative approach that can be used to identify *Brassica* NBS-encoding genes is mining of expressed sequence tag (EST) databases. However, although approximately 600K *B. napus* and 150K *B. rapa* ESTs are available in the dbEST database of the National Center for Biotechnology Information (NCBI), only a few R gene candidates are represented, indicating that not all R gene families are present in the current EST datasets. Recent progress in genome sequencing of *B. rapa*, however, allows genome-level identification of defense-related NBS-encoding genes.

Approximately 70 Mbp seed BAC sequences were recently made available on public databases and an additional 75 Mbp BAC sequences are accessible through the sequencing pipeline of the Korea *Brassica rapa* Genome Project (KBGP). The available genome sequences cover approximately 64% of the gene-rich euchromatic space of *B. rapa* based on estimation of the euchromatin amount as $\approx$220 Mbp (Mun et al. 2009). The main objective of this study was to use the available genome sequences to identify putative NBS-encoding gene candidates of *Brassica*. We determined the sequence characteristics of the NBS-encoding genes we identified, and examined the distribution of NBS-encoding genes across linkage groups. In addition, we performed phylogenetic analysis and calculated the number of substitutions per synonymous (K_s) and non-synonymous site (K_a) in *B. rapa* and *A. thaliana* sequences to gain insight into the evolution of NBS-encoding genes in *B. rapa*. Understanding of the genomic organization of NBS-encoding genes in *B. rapa* will allow genome-level insights into R gene evolution and inform genetic and breeding strategies to engineer disease resistance in *Brassica* crops.

Materials and methods

Identification of sequences that encode NBS motifs in the *B. rapa* genome

We used $\approx$145 Mbp phase 2 or 3 genome sequences from 1,199 BAC clones of *B. rapa* ssp. *pekinensis* cv. *Chiifu*, of which 568 BAC clones ($\approx$70 Mbp) were deposited in the HTGS database of NCBI and 631 BAC clones ($\approx$75 Mbp) were in the genome sequencing pipeline of KBGP. The 568 BAC clones were selected from the gene-rich euchromatic region of *B. rapa* based on BAC-end sequence comparison with the *A. thaliana* counterpart regions and assembled into approximately 70 Mbp of non-redundant sequence contigs (Mun et al. 2009). In addition, we also downloaded 75 Mbp of 631 BAC clones, generated mainly from sequencing of chromosomes A3 and A9, from the KBGP database (The Korea *Brassica rapa* Genome Project, http://www.brassica-rapa.org/BRGP/index.jsp). Because these sequences are individual BAC sequences that have not been assembled, several of them contain overlapping redundant sequences. In general, minimum tiled BAC clones in the sequencing pipeline of A3 and A9 are allowed approximately 30% overlap. Therefore, using the same proportion of sequence redundancy, we estimate that the sequences we used contained $\approx$100–145 Mbp of non-redundant sequence covering approximately 19–27% of the entire *B. rapa* genome (529 Mbp) and capturing almost half (47–66%) of the euchromatic gene space (220 Mbp).

To make a list of NBS-encoding genes from the BAC sequences, protein-coding genes were predicted ab initio using the FGENESH program with the parameters trained using the *B. rapa* matrix. To reduce sequence redundancy, self-BLAST comparison of gene models was conducted and the redundant gene models were collapsed into a single gene model if BLAST showed perfect matches and source BAC sequences overlapped with one another at the positions that the gene models predicted using Pipmaker (Schwartz et al. 2000). Candidate genes containing NBS domains were identified based on a BLASTP search (Altschul et al. 1997) at a cut-off value of $<E^{-20}$ using 207 *A. thaliana* NBS-LRR-encoding genes downloaded from the NIBLRRS server (NIBLRRS database, http://niblrrs.ucdavis.edu/data_links.php) as a query. To ensure that there were no additional related genes missing from the gene prediction, a TBLASTN search was also used against the nucleotide sequences at a cut-off value of $<E^{-10}$. Matches were considered to be genes encoding NBS motifs if the TBLASTN translation was not interrupted by stop codons in the open reading frame. Sequences found multiple times in the output were identified and manually removed based on BLASTN self comparisons of the initial candidates and sequence comparisons of BAC clones. The sequences used in this study can be found in the GenBank database under the accession numbers listed in Online Resource 1. To identify EST matches of the candidate NBS-encoding genes of *B. rapa*, 26,416 unigenes and 127,144 EST sequences of *B. rapa* ssp. *pekinensis* cv. *Chiifu* were downloaded from the KBGP database (The Korea *Brassica rapa* Genome Project). All unigenes and

ESTs were compared with the coding sequences of candidate NBS-encoding genes. We considered unigenes and ESTs to have a genome match if more than 90% of sequences matched with at least 95% identity in a BLASTN search (Altschul et al. 1997). If one unigene or EST matched with multiple candidate genes, only the gene with the best alignment score was considered.

Analysis of conserved domains and motif searches

Conserved domains and motifs were identified using hmmpfam comparison to Pfam v23 (Finn et al. 2008). Protein sequences of candidate NBS-encoding genes were compared to the Pfam v23 HMMs using HMMER 2.3.2. (Eddy 2003). To classify the candidate NBS-encoding R genes based on domain structure, each sequence was examined for N-terminal regions, the NBS domains, and the LRR domains plus C-terminal regions using the Multiple Expectation Maximization for Motif Elicitation (MEME) program (Bailey and Elkan 1995). Because Pfam and MEME analyses could not identify the CC motif in the N-terminal region, the region from the N-terminal to the beginning of the P-loop of the NBS domain was also examined using the MARCOIL program with a threshold probability of 90 (Delorenzi and Speed 2002).

Alignment and phylogenetic analysis of sequences

Nucleotide-binding site-encoding genes are known to be subdivided into CNL and TNL groups. However, we did not separate the groups in the phylogenetic analysis as we were interested in examining the divergence between groups based on phylogeny. Amino acid sequences of the NBS domain spanning the P-loop/Kin1 motif to the RNBS-D motif were used. The selected sequences were aligned using ClustalW (Thompson et al. 1994) with default options, and the alignment was manually corrected using the alignment editor Jalview (Waterhouse et al. 2009). Aligned sequences were trimmed at both ends to eliminate regions of poor alignment. A phylogenetic tree was constructed using the Bootstrap neighbor-joining (NJ) method in MEGA4 (Tamura et al. 2007). The stability of tree nodes was tested by bootstrap analysis with 1,000 replicates.

Chromosome assignment of NBS-encoding genes

The source BAC clones which contain the candidate NBS-encoding R genes were anchored on the 10 *B. rapa* chromosomes using sequence-tagged site markers (Kim et al. 2006) with additional information of assembled sequence contigs (The Korea *Brassica rapa* Genome Project), fluorescent in situ hybridization (FISH) of BAC clones, and physical map information (Mun et al. 2008).

K_s and K_a analyses of homologous sequences

The timing of a duplication event or divergence of homologous segments and selective pressure on duplicated genes were estimated by calculating the synonymous (K_s) and non-synonymous substitution (K_a) per site between homologous genes. To calculate the K_s and K_a value, the protein sequences of the gene models were aligned using the all-against-all alignment and the resulting alignment was used as a reference to align the nucleotide sequences. After the removal of gaps, the K_s and K_a values from pairwise alignments of homologous sequences were determined using the maximum likelihood method implemented in the CODEML (Goldman and Yang 1994) module of the PAML (Yang 2007) package using the F3 × 4 model, similar to the analysis described by Blanc et al. (2003). We compared the mode rather than the means of K_s distributions because the mode is not affected by bias due to incorrectly defined homolog pairs, which is partly responsible for overestimation of K_s values. Only gene pairs with a K_s estimate of <5 were considered for further evaluation and their K_s age distribution was represented at an interval of 0.1. The calculation of divergence time was based on the neutral substitution rate of 1.5×10^{-8} substitutions per site per year for chalcone synthase (*Chs*) (Koch et al. 2000).

Results

Identification of NBS-encoding genes and analysis of domains and motifs

The *B. rapa* NBS-encoding genes were identified based on BLAST searches of non-redundant gene models against the *A. thaliana* R gene family followed by a hmmpfam search. A total of 92 non-redundant NBS-encoding R gene candidates were identified from the genomic sequences we collected (Table 1; Online Resource 1). Among the candidate genes, 17 genes were identified as CNLs and 42 genes were identified as TNLs. In addition, 33 different predicted protein sequences that lack specific motifs or domains were classified into three distinct groups namely CN (2), NL (21), and TN (10); some of these genes are probably pseudogenes. TIR-only and TIR-X sequences were not indentified in this study.

Because typical NBS domains often contain conserved motifs that allow CNL and TNL sequences to be distinguished, the 33 genes lacking the full complement of domains were further divided into CNL or TNL according to their NBS signature. A previous study in *A. thaliana* identified eight major NBS motifs that differed in sequence between CNL and TNL proteins (Meyers et al. 2003).

As expected, MEME analyses identified consensus sequence motifs for the P-loop, kinase-2, NBS-A to -D, GLPL, and MHDV regions; these consensus sequence motifs are similar to those of *A. thaliana* with some slight modifications (Table 2). These motifs were relatively well conserved between CNL members. In contrast, the NBS-A and kinase-2 motifs were frequently deleted in the TNL members (Online Resource 1). In particular, the MHDV motif in TNL proteins was often modified into an MHSL motif, and several TNs (BrTN2, BrTN7, and BrTN10) also contained a modified MHDV motif that is not present in *A. thaliana* TNs, indicating two different origins of TNs in *Brassica*. We also examined proteins with multiple NBS domains and found three proteins (BrTNL14, BrTNL26, and BrTNL35) that have two domains within a single protein (TNLN). The NBS domains were similar in motif structure, but the first NBS domain was truncated. This domain type has only ever been detected in a single poplar R gene (Kohler et al. 2008).

The LRR regions were characterized by LRRs that were variable in sequence, size, and occurrence per gene. The average number of LRR domains in TNL (5.2 ± 2.4 SD, range 2–12) was more than twofold higher than in CNL (2.4 ± 0.9 SD, range 1–4). Pfam and MEME analyses identified three conserved LRR motifs and two of them showed group specificity (Online Resource 2). LRR motif 1 (FNPxxLVELNxxNSKLEKLWEGxxPLTxL), which is similar to *A. thaliana* TNL-LRR motif 4, was common in TNL proteins. In contrast, LRR motif 3 (SLxYLxLSGxSIx xLPSxLKx) was frequently identified in CNL proteins. However, the LRR motif 2 (SNxTxLExLDLSxCxS LVELPSSxxNLKK), which corresponds to the *A. thaliana* LDL-LRR motif, showed relatively low group specificity. Furthermore, duplicated repeated motif patterns were frequently observed in TNLs, suggesting that duplication of the LRRs of TNL accounts for their higher length variation in *Brassica*. A putative nuclear localization signal (NLS) has been described in the C-terminal domains of the *A. thaliana* RRS1 (Deslandes et al. 2003) and RPS4 (Wirthmueller et al. 2007) proteins and the N-terminal of five poplar TNL proteins (Kohler et al. 2008). We identified two proteins containing a NLS at the N-terminal (BrNL16) and C-terminal (BrCNL16), respectively. The C-terminal NLS of BrCNL16 (PRCRRKKRK) was similar to that of the *A. thaliana* RRS1 protein (KKRKRS), suggesting that this signal may target the protein to the nucleus. However, the putative N-terminal NLS of BrNL16 (RRRRLEFKLR FINSPRPVVFTYSNIKAK) appears to be a unique variant or a spurious domain, because it does not have sequence similarity to any R protein N-terminal NLS sequence.

In summary, we found 30 CNL and 62 TNL genes in *B. rapa* showing 1:2 ratio of CNL to TNL, which is similar to what is found in *A. thaliana* (Table 1) (Meyers et al. 2003). The average number of exons in NBS-encoding genes was 4.2 in *B. rapa* (Online Resource 1). This exon estimate was less than that in all predicted genes (4.7 per gene). In addition, the average number of exons per CNL genes (2.3 per gene) was significantly lower than that

Table 2 Consensus sequences of major NBS domain motifs found within the predicted *B. rapa* CNL and TNL proteins

Class	Motif	Consensus sequence[a]
TIR-NBS-LRR	TN linker	TPSKDFDDLVGIEAHIEKLKS
	TNL P-loop/Kin1	VRMVGIWGPAGIGKTTIARALYNQLSSRF
	TNL RNBS-A	DDYGLKLRLQENFLSQIFNQK
	TNL Kin2	DIEVRHLGRAQEMLSDKKVLVVLDEVDNWWQLE
	TNL RNBS-B	LKDKKVLVVLDDVDDLEQLEALAGETQWFGPGSRIIITTRD
	TNL RNBS-C	IDHIYEVxFPSEDEALQIFCQYAFGQKSPPDGF
	TNL GLPL	LAREVTKLAGNLPLGLRVLGSYLRGMSKEEWIEALPRLRTS
	TNL RNBS-D	LDGEIESTLRFSYDALDDNEKTLFLHIACFFNGEKVDYVKS
	TNL MHDV	MHSLLQQMGREIVR
CC-NBS-LRR	CNL P-loop/Kin1	VIGIYGMGGIGKTTLARQIYN
	CNL RNBS-A	VIWVVVSQDLTIHKIQDDIAQ
	CNL Kin2	WDKKEExEKAxDIHNVLRRKKFVLLLDDIWEKVDLSEIGVP
	CNL RNBS-B	FPTKENGCKVVFTTRSKEVCGRMGVDDPM
	CNL RNBS-C	VKCLDTDKAWDLFKKKVGENTLKSHPDIPELARKVAGKCRG
	CNL GLPL	VAEKCRGLPLALNVIGETMSCKRTVQEWRHAVDVLTSSAAEFSGMEDKILPILKYSYD
	CNL RNBS-D	KSCFLYCSLFPEDYKIDKEELIEYWICEG
	CNL MHDV	MHDVVREMALWIAS

[a] Consensus amino acid sequence was derived from MEME. Underlined residues indicate consensus sequences identical to the motifs detected in the *Arabidopsis* NBS domains by Meyers et al. (2003). x indicates a non-conserved residue

in TNL genes (5.2 per gene; *t* test, $P < 0.001$) and 43% of CNL genes were encoded by a single exon. This result is consistent with what is found in *A. thaliana*. In *A. thaliana*, NBS-encoding genes contain 4.2 exons, but CNLs and TNLs have 2.2 and 5.3 exons per gene on average, respectively (Meyers et al. 2003).

Genomic organization of NBS-encoding genes in the *B. rapa* genome

To determine the distribution of *B. rapa* NBS-encoding genes in the genome, genetic mapping information, namely the position of source BACs, was used. Most of the source BAC sequences have been anchored on two existing "Jangwon" and "VCS" genetic maps (Kim et al. 2006; The Korea *Brassica rapa* Genome Project). Seventy-nine *B. rapa* NBS-encoding genes were distributed across linkage groups except A4, whereas 13 genes did not map to any characterized BACs (Online Resource 3). Of the 79 NBS-encoding R genes located on linkage groups, almost 57% of the genes are encoded on chromosomes A3 and A9 (Fig. 1; Online Resource 3), primarily due to the higher representation of sequences from these two chromosomes in this study. Additionally, 48% (44/92) of NBS-encoding genes form physically homogenous clusters and are unevenly distributed in the genome as tandem arrays. There were more TNL tandem duplicates (53%) than CNL tandem duplicates (37%). Uneven distribution of NBS-encoding genes on chromosomes appears to be common in plants (Kohler et al. 2008; Meyers et al. 2003; Porter et al. 2009; Yang et al. 2008b; Zhou et al. 2004). Interestingly, TNL genes are generally widely distributed across the *B. rapa* chromosomes except for chromosomes A4 and A5, whereas 60% of CNL genes are located on chromosome A9.

As the *B. rapa* genome has not been fully sequenced, we attempted to estimate how many NBS-encoding R genes exist in the *B. rapa* genome using the *B. rapa* unigene sets (The Korea *Brassica rapa* Genome Project) with a BLASTN similarity *E*-value cut-off of E^{-20} to *B. rapa* and *A. thaliana* NBS-encoding genes. Applying this criterion, we found 98 NBS-like expressed sequences in the 26,416 unigenes. We identified 34 *B. rapa* NBS-encoding genes that showed a nearly identical match to the unigene sequences. Therefore, we estimate that the current genome sequence contains roughly 35% (34/98) of *B. rapa* NBS-encoding genes. Taken together with the approximately 47% euchromatic genome coverage of sequences used in this study, we estimate that the overall number of NBS-encoding genes in the *B. rapa* genome is at least $\approx$200–270, which is relatively lower than other sequenced model species with similar genome sizes (Ameline-Torregrosa et al. 2008; Kohler et al. 2008; Monosi et al. 2004; Yang et al. 2008b). Because *B. rapa* NBS-encoding genes are not evenly distributed in the genome, it is possible that major NBS-encoding gene clusters lie in the regions of genome that is not analyzed.

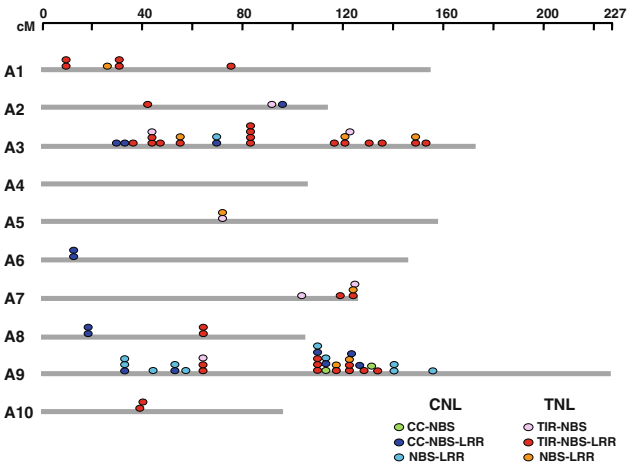


Fig. 1 Distribution of predicted NBS-encoding genes on the ten linkage groups of *B. rapa*. *Color ovals* above each linkage group (*gray bars*) indicate the approximate locations of each gene. For more details, including the BAC clone identities and associated markers, see Online Resource 3

Phylogeny construction and *in silico* analysis of gene expression

A phylogenetic analysis of 90 NBS-encoding sequences was performed to classify subgroups and identify relationships between genes. Two genes (*BrCNL1* and *BrNL7*) were excluded due to more than 50% deletion of the NBS domain. Because a previous study showed that phylogenetic analyses of NBS domains allow subgroups of R genes to be distinguished (Meyers et al. 1999), we constructed a composite phylogenic tree for all candidate genes using NBS consensus sequences. Both distance and parsimony algorithms produced similar trees (data not shown). Figure 2 shows a phylogeny of *B. rapa* NBS-encoding genes with the chromosomal origin of genes and expression support indicated. Six subgroups, comprising three CNL subgroups and three TNL subgroups, are present in the *B. rapa* genome. There was a clear separation between the CNL and TNL groups, reflecting ancient differentiation of NBS-encoding genes into two major groups in the *B. rapa* genome. Among the CNL and TNL subgroups, TNL-3 was paraphyletic; phylogenetic and motif analyses of the NBS domain suggested that the TNL-3 subgroup was derived from the other two TNL subgroups by deletion or recombination of motifs (Fig. 2; Online Resource 1). Most of the CNL genes clustered in the two distinct major subgroups, CNL-1 and CNL-2, with the exception of two genes, *BrCNL8* and *BrCNL15*, which clustered in the CNL-3

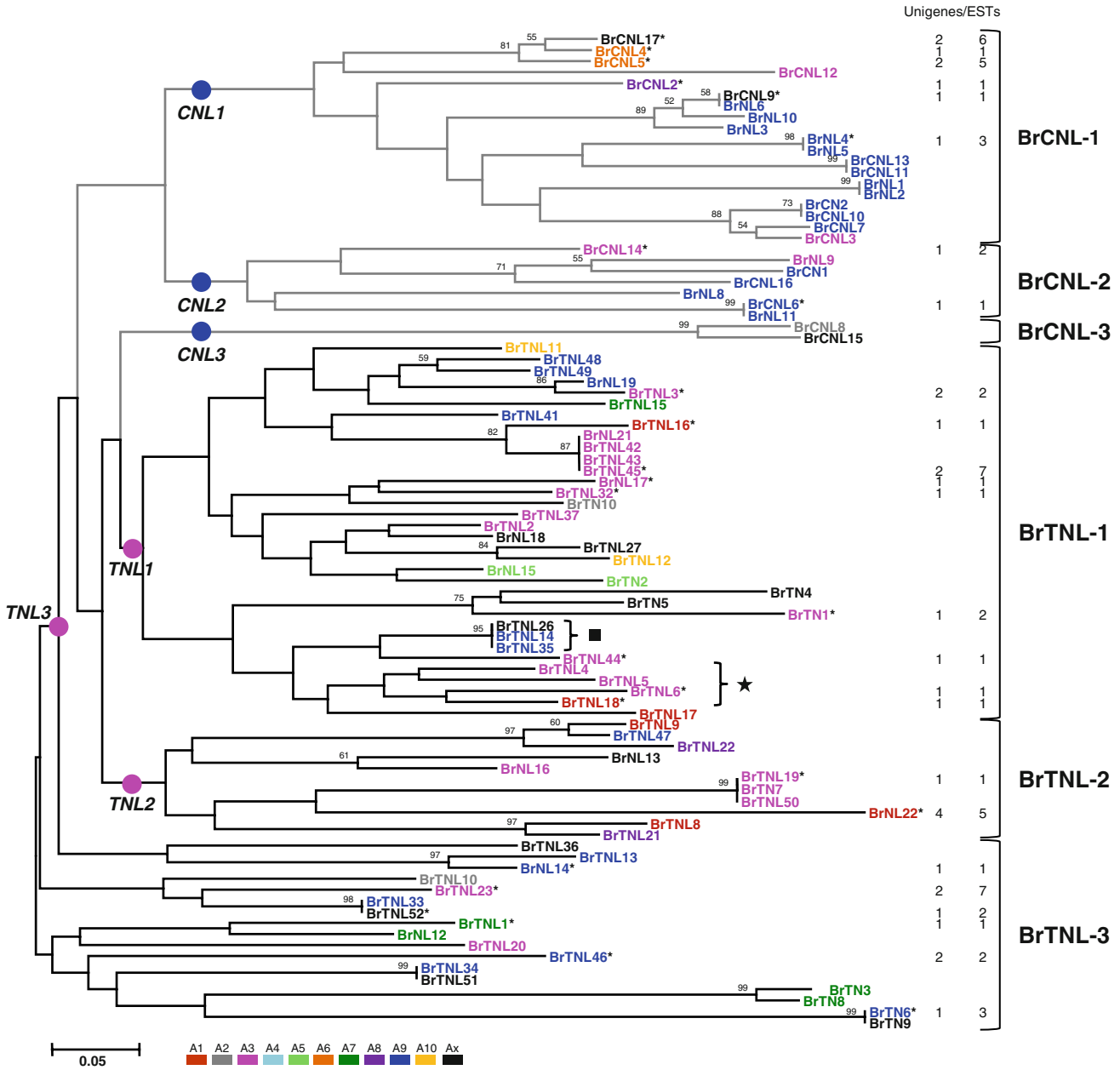


Fig. 2 A phylogenetic tree of 90 NBS-encoding genes of *B. rapa* based on neighbor-joining analysis. CNL and TNL groups are represented as *gray* and *black bars*, respectively. Bootstrap values are indicated on the *branches* and the branch length reflects the estimated number of substitutions per 100 sites; bootstrap results were not reported if the support was <50%. To visualize genomic location better, the names of genes located on the same chromosome are indicated in the same *color*. Gene names are intended to represent domain configurations. *Asterisks* indicate the gene models with expression support. The number of matched unigenes and ESTs for each gene is indicated on the *right margin*. *Brackets* on the *right* of the tree indicate subgroup names. A *brace* with *square* indicates genes containing two NBS domains (TNLN); the second complete NBS was used for phylogeny construction. A *brace* with *asterisk* denotes TNL members that were generated by recent lineage-specific events, as shown in Figs. 3 and 4. For all other relevant information, please see Online Resource 1

subgroup due to deletion of the kinase-2, NBS-B, and NBS-C motifs in the NBS domain. NBS sequences from the truncated R genes (TN or NL) were dispersed among the different subgroups, indicating a diverse origin rather than a monophyletic origin due to domain loss. Local expansion of specific types was also apparent. For example, CNLs on chromosome A9 comprised 65 and 71% of CNL-1 and CNL-2 subgroups, respectively. Similarly, TNLs on chromosome A3 comprised approximately 40% of TNL-1 and TNL-2 subgroups. *In silico* analysis of gene expression indicated that only 25 predicted genes (28%) have EST support with an average of 2.4 ESTs

per expressed NBS-encoding gene. These genes were expressed in a wide range of cDNA libraries, including those constructed from various development stages, tissue types, and abiotic stress-treated tissues. There was no difference in the ratio of expressed genes between CNL (29%) and TNL (27%) groups. However, expression patterns vary between subgroups and even between highly similar genes within the same subgroup. For example, CNL-3 is not expressed and the TNL-2 subgroup has only 18% of the expression. Moreover, among the four highly similar genes in the TNL-1 subgroup (*BrNL21*, *BrTNL42*, *BrTNL43*, and *BrTNL45*), *BrTNL45* displayed seven ESTs while the other genes had no ESTs.

Relationship between NBS-encoding genes of *B. rapa* and *A. thaliana*

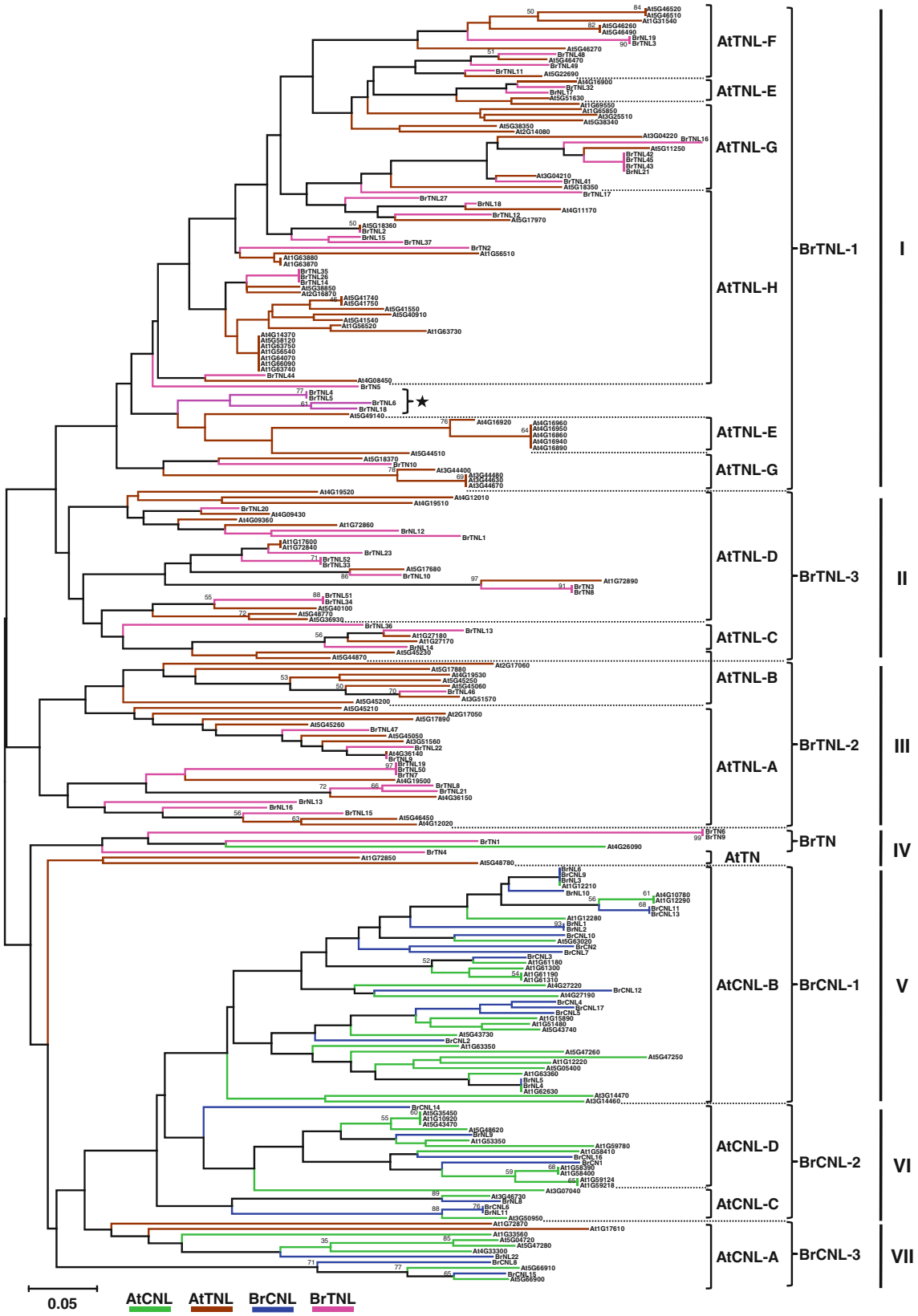
Further phylogenetic reconstruction using the conserved NBS domains of *A. thaliana* and *B. rapa* NBS-encoding genes was performed to assess the support for R gene subgroups and to investigate the evolution of *B. rapa*-specific NBS-encoding genes (Fig. 3). Because *A. thaliana* CNL and TNL groups have been categorized (Meyers et al. 2003), we constructed a composite phylogenetic tree for the two groups. The analysis separated CNL and TNL type sequences, and each group was divided into three major clades: clades I–III for TNL, and V–VII for CNL. Clade IV was a paraphyletic group that contained TN genes; four from *B. rapa* (*BrTN1*, *BrTN4*, *BrTN6*, and *BrTN9*) and two from *A. thaliana* (*At1G72850* and *At5G48780*). These proteins were characterized by a missing MHDV motif and are considered to have an ancient origin based on a previous report that TN proteins are encoded in the genomes of conifers and grasses, and that two *A. thaliana* TN proteins are extremely well conserved in rice (Meyers et al. 2002). Although bootstrap values were low for the branches, the topology of the composite tree for *A. thaliana*-*B. rapa* NBS-encoding genes was consistent with that of the *B. rapa* NBS-encoding gene tree. All *B. rapa* subgroups present in Fig. 2 were present in the composite phylogenetic tree in Fig. 3, which supports the categorization of *B. rapa* NBS-encoding genes into six subgroups. In addition, the tree indicates syntenic relationships between *A. thaliana* and *B. rapa* subgroups. *B. rapa* subgroups were clustered together with one or two distinct *A. thaliana* subgroups except clade I in which BrTNL-1 were paraphyletic with AtTNL-E to -H, suggesting possible further division of BrTNL-1 members. Further identification of NBS-encoding genes from the *B. rapa* genome will enable the reconstruction of a more robust phylogeny.

The phylogenetic distribution of *A. thaliana* and *B. rapa* NBS-encoding genes does not indicate a major *B. rapa*-specific expansion. Rather, the local expansion of particular types was detected. Four genes in the BrTNL-1 subgroup located on chromosome A1 (*BrTNL18*) and A3 (*BrTNL4*, *BrTNL5*, and *BrTNL6*) formed a local cluster separated from the AtTNL-E subgroup, suggesting that these genes might have been generated recently after the divergence of *A. thaliana* and *B. rapa*. We compared the microsyntenic structure of *B. rapa* source BAC clones containing the four genes and syntenic counterpart regions of *A. thaliana* (Fig. 4a; Online Resource 4). This comparison revealed that the *B. rapa* region is shrunken in size compared to the syntenic *A. thaliana* region as reported by Mun et al. (2009) and that *BrTNL4*, *BrTNL5*, and *BrTNL6* are tandem duplicates without homologous *A. thaliana* counterparts. Estimation of K_s in these genes indicated that they are recent duplicates that occurred after the split of *A. thaliana* and *B. rapa* (Fig. 4c). Because *BrTNL17* and *BrTNL18* have homologous counterparts of ancient origin in the AtTNL-G subgroup (*At5G18350*, *At5G18360*, and *At5G18370*) and frequent ectopic translocation of NBS-encoding genes among different chromosome does not occur in *A. thaliana* (Baumgarten et al. 2003), loss of synteny for *BrTNL4*, *BrTNL5*, and *BrTNL6* suggests gene deletion in the *A. thaliana* genome. This result suggests that recent tandem duplications and ectopic deletions may play a role in the generation of novel lineage-specific *Brassica* R genes.

Fig. 3 Phylogenetic relationships between NBS-encoding genes in the *B. rapa* and *A. thaliana* genomes. The neighbor-joining tree was constructed using the NBS domains of 90 *B. rapa* and 140 *Arabidopsis* NBS-encoding proteins reported by Meyers et al. (2003) using MEGA4 software. CNL and TNL groups of each species are represented by *color bars*. Bootstrap values are indicated on the *branches* and the branch length reflects the estimated number of substitution per 100 sites; bootstrap results were not reported if the support was <50%. *Brackets* and *braces* in the tree indicate the subgroup names of *Arabidopsis* as reported by Meyers et al. (2003) and the *B. rapa* groups identified in Fig. 2, respectively. On the right margin, clades I–III represent the TNL group; clade IV represents a mixed TN group; and clades V–VII represent the CNL group. An example of local expansion in BrTNL-1 is indicated with an *asterisk* ▶

Duplication and differential selection of the *B. rapa* NBS-encoding gene family

Similar to *Arabidopsis*, TNL genes also out-number CNL genes in *B. rapa*, indicating either amplification of TNL genes or loss of CNL genes. To investigate the evolution of NBS-encoding genes and to estimate the approximate timing of duplication, we compared the distribution of synonymous substitutions (K_s) between CNL and TNL genes in *A. thaliana* and *B. rapa*. As shown in Fig. 5a–c, CNL and TNL groups in both the *A. thaliana* and *B. rapa* genomes appear to have diverged after a single ancient


AtTNL-F
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AtTNL-A
AtTN
AtCNL-B
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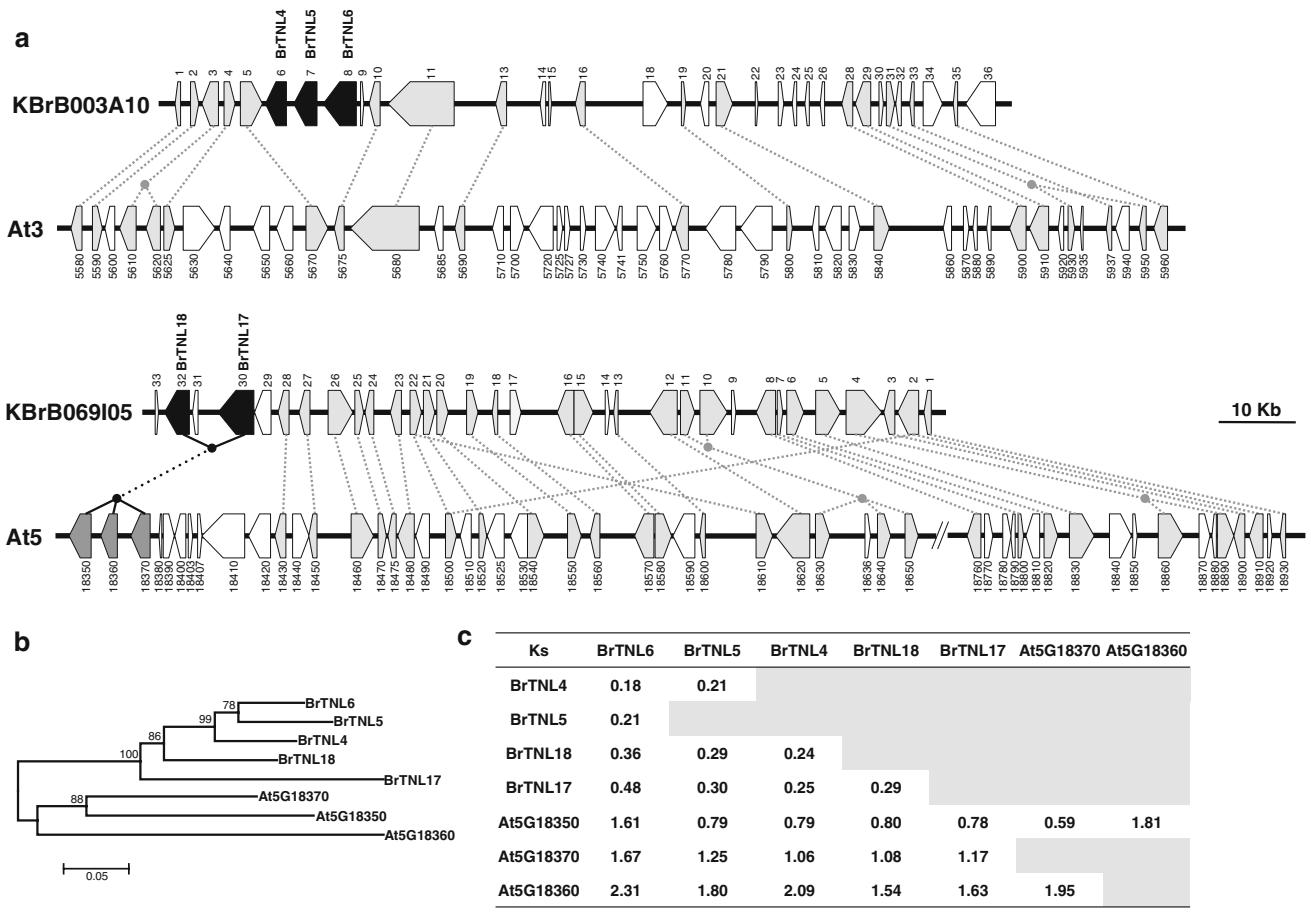

c

Ks	BrTNL6	BrTNL5	BrTNL4	BrTNL18	BrTNL17	At5G18370	At5G18360
BrTNL4	0.18	0.21					
BrTNL5	0.21						
BrTNL18	0.36	0.29	0.24				
BrTNL17	0.48	0.30	0.25	0.29			
At5G18350	1.61	0.79	0.79	0.80	0.78	0.59	1.81
At5G18370	1.67	1.25	1.06	1.08	1.17		
At5G18360	2.31	1.80	2.09	1.54	1.63	1.95	

Fig. 4 An example of lineage-specific expansion of *B. rapa* TNL genes. **a** Microsynteny between syntenic regions of *B. rapa* and *Arabidopsis* showing unique amplification of *B. rapa BrTNL4*, *BrTNL5*, and *BrTNL6* due to tandem duplication and/or deletion of counterparts. *Lines* indicate homologous pairs. Supporting data and gene annotations are provided in Online Resource 4. **b** A neighbor-joining tree showing phylogenetic relationship among the NBS-encoding genes in the syntenic regions. **c** K_s estimates for the NBS-encoding genes in the syntenic regions

duplication event 115–125 MYA based on K_s modes of 2.4–2.6, which might be resulted from the oldest WGD (1R). The ancient duplication of NBS-encoding genes in the *A. thaliana* and *B. rapa* genomes is supported by the K_s distribution of each CNL and TNL group (Fig. 5d–i). The age distributions of CNL and TNL indicate that each group has been generated by several rounds of duplication events that may be related to the WGDs. The K_s distributions for the CNL and TNL groups between *A. thaliana* and *B. rapa* are characterized by three major peaks at $K_s = 2.5–2.8$, 1.3–1.7, and 0.5–0.8, which roughly correspond to three WGD events (1R, 2R, and 3R, respectively) in the common ancestor of *Arabidopsis* and *Brassica* (Fig. 5d, g). In particular, note that the TNL group has a reduced K_s peak for the ancient duplication event (1R) compared to the CNL group, suggesting that the relatively recent duplication in the TNL group resulted from the old duplication event (2R) or that more TNL genes were deleted after the ancient duplication. A comparison of the K_s mode for the paralogs of each group in both *A. thaliana* and *B. rapa* identified differences in the duplicated genes retained in the two genomes. For CNL group, two peaks representing 1R and 2R are evident, but 3R peak has reduced (Fig. 5e, f). However, for TNL group, the peak representing 2R are evident, but the 1R and 3R peaks have collapsed (Fig. 5h, i). Surprisingly, in the *B. rapa* genome, a peak (mode $K_s = 0.2–0.3$) for WGT event (4R) has also been underestimated in both types (Fig. 5f, i). Microsynteny comparison of selected paralogous regions showed none of NBS-encoding genes conserved in the duplicated or triplicated regions (Fig. 6; Online Resource 5). Taken together, these findings suggest that duplicated R genes produced by the 3R or 4R events were widely lost in the triplicated *B. rapa* genome.

To test whether different selection pressures can explain the differential accumulation of the two groups in the Brassicaceae genome, K_a/K_s ratios were calculated. In both *A. thaliana* and *B. rapa* genomes, the overall K_a/K_s ratios for CNL (0.404 and 0.387, respectively) were higher than

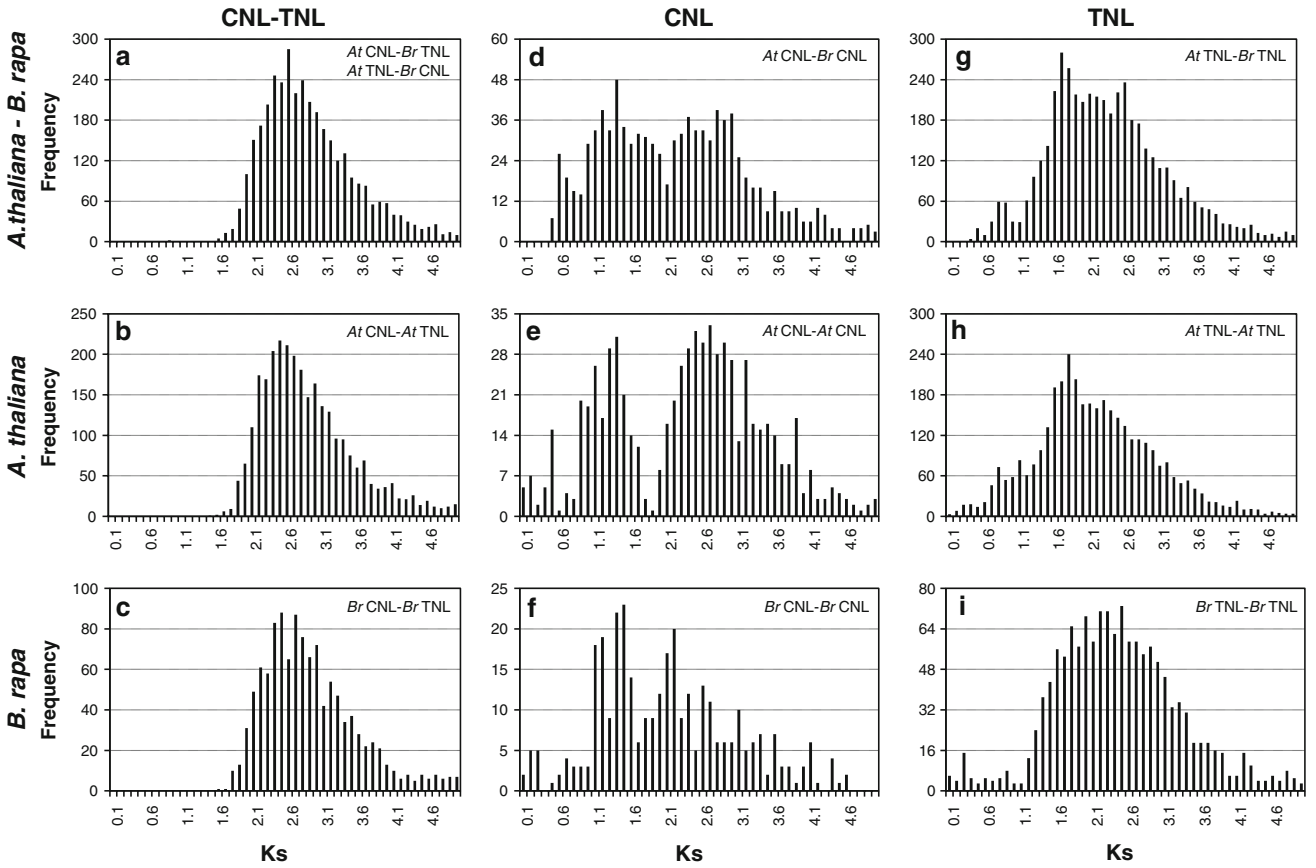

Fig. 5 Polyploidy events in the *A. thaliana* and *B. rapa* genomes viewed through the frequency distribution of relative K_s modes for NBS-encoding genes. Distributions of K_s values were obtained from homologous CNL and TNL genes (**a**–**c**), paralogous CNL genes (**d**–**f**), and paralogous TNL genes sets (**g**–**i**) in the *Arabidopsis* and *B. rapa* genomes. The *vertical axes* indicate the frequency of paired sequences and the *horizontal axes* denote K_s values at 0.1 intervals. The *black bars* depict the positions of the modes of the K_s distribution obtained from homologous or paralogous gene pairs. *At*, *A. thaliana*; *Br*, *B. rapa*

those for TNL (0.356 and 0.353, respectively). The difference between CNL and TNL was highly significant in a two-tailed *t* test ($P < 0.001$). This result is consistent with previous studies (Cannon et al. 2002; Yang et al. 2008b), suggesting higher selective constraints and purifying selection in the TNL group. Intriguingly, the distribution of K_a/K_s ratios according to K_s value for most homologs in each group indicated a significant change in selection pressure on the CNL and TNL groups at a specific time point. As shown in Fig. 7, the CNL group had a higher average K_a/K_s than the TNL group after the ancient duplication event (1R), suggesting that CNL genes were positively selected after the ancient WGD event in both the *A. thaliana* and *B. rapa* genomes. However, soon after the old duplication (2R), around $K_s = 1.6$, the TNL group genes had a higher K_a/K_s ratio than recent duplicates in the CNL group. This result suggests relaxation of selective constraint in the TNL group than the CNL group, which might have resulted in recent amplification of TNL genes in both the *Arabidopsis* and *Brassica* genomes. The difference in K_a/K_s ratios between the CNL and TNL groups in the *B. rapa* genome is smaller than that seen in *A. thaliana*; this may be because the whole genome sequence of *B. rapa* is not yet available for analysis.

Discussion

Brassica crop species have long been cultivated worldwide as vegetables, oilseed crops, forages, and condiments. The past couple of years have seen the development of abundant genetic and genomic resources for these crop species. In particular, various genomics tools for *B. rapa* have greatly facilitated our understanding of the structure and evolution of the *Brassica* genome. The value of this species has been elevated by its close relationship not only with *A. thaliana* but also with its diploid sibling species (*B. oleracea* and *B. nigra*) and allopolyploid hybrids (*B. napus*, *B. carinata*, and *B. juncea*), which is reflected by similar polyploidy genome structures and conserved phenotypes such as enlarged vegetative or floral meristems. Because *Brassica* species have a similar genome content

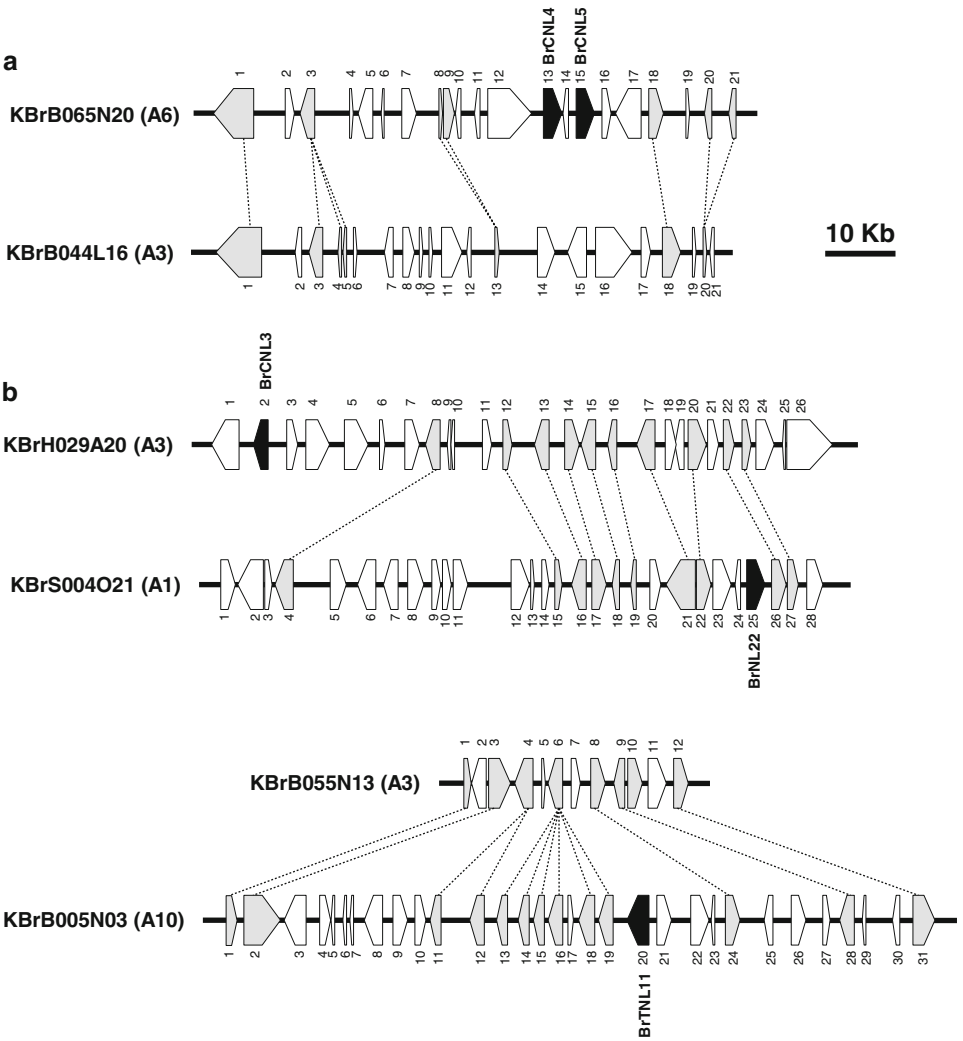

Fig. 6 An example of microsynteny relationship between paralogous BAC pairs of *B. rapa* showing loss of redundant NBS-encoding genes in the duplicated (**a**) or triplicated regions (**b**). Paralogous BAC pairs containing NBS-encoding genes were selected based on the report of Mun et al. (2009). *Lines* indicate paralogous pairs. Assigned chromosome of BAC clone was indicated in *parenthesis*. Supporting data and gene annotations are provided in Online Resource 5

and genome structure, *B. rapa* can serve as an alternative to *A. thaliana* for cloning the orthologs of many economically important genes in other *Brassica* crops. Likewise, because most *B. rapa* pathogens also infect other vegetable-type *Brassica* crop cultivars, *B. rapa* can be used as a source to identify R genes for common pathogens of *Brassica* crops. Furthermore, the long cultivation history of *Brassica* crops in a wide diversity of environmental conditions suggests that R gene products for diverse disease phenotypes can be discovered in the *B. rapa* genome. In such cases, R genes with novel specificities that are discovered in *B. rapa* could be directly applied to other cultivated *Brassica* crops using simple transformation techniques. A successful example of a translational strategy to introduce disease resistance from a model organism into a crop species is the introduction of the *RCT1* gene from *M. truncatula* into alfalfa cultivars for anthracnose resistance (Yang et al. 2008a). Therefore, a better understanding of the genomic organization of NBS-encoding genes in the *B. rapa* genome and characterization of their genetic and molecular mechanisms is highly desirable.

This study is the first report of genome-wide discovery of NBS-encoding R genes in the *Brassica* genome. Although the complete genome of *B. rapa* has not yet been fully sequenced, the availability of BAC sequences covering approximately 50% of the euchromatic genome of *B. rapa* enabled identification of NBS-encoding R genes in the *Brassica* genome. We estimated that *B. rapa* possesses smaller number of NBS-encoding genes recorded than sequenced crop species with similar genome sizes ($\approx$400–500 Mbp) with the exception of papaya. This finding is surprising, because the *B. rapa* genome has evolved as a triplicated hexaploid from the common ancestor of *A. thaliana*, and thus is expected to have high levels of genomic redundancy. However, the estimated number of NBS-encoding genes in the *B. rapa* genome is slightly higher than that in *A. thaliana*. This result suggests that roughly half of the NBS-encoding genes generated as a

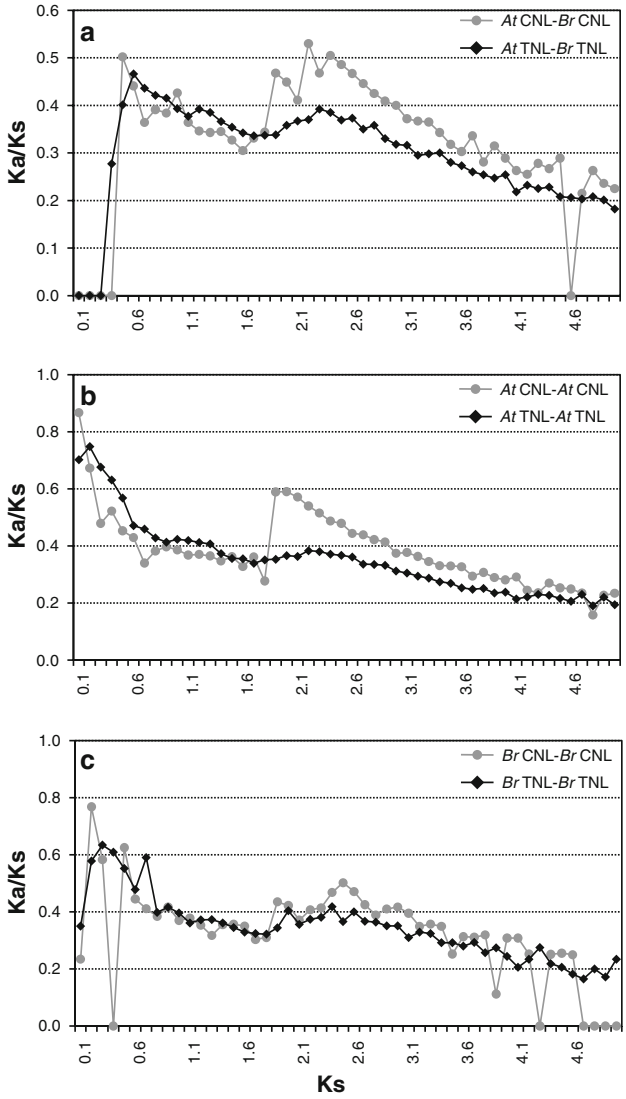

Fig. 7 The relationship between K_a/K_s and K_s of paralogous NBS-encoding gene sets between the *A. thaliana* and *B. rapa* genomes (**a**) or in each individual genome (**b**, **c**). The *vertical axes* indicate the average K_a/K_s ratio in CNL (*gray line*) and TNL (*black line*) groups. The *horizontal axes* denote a K_s value at 0.1 intervals

result of WGT approximately 11–12 MYA were lost in the current *B. rapa* genome, demonstrating a rapid birth and death dynamic in the *Brassica* R gene family.

One possible explanation for the low number of NBS-encoding genes despite the WGT event is the overall downsizing of the euchromatic gene space of the *B. rapa* genome, related to the diploidization process after the WGT event. A synteny comparison of *B. rapa* seed BAC sequence contigs with their homologous counterparts in the *A. thaliana* genome indicated that the *B. rapa* genome shrunk by ≈30% due mainly to the deletion of roughly one-third of the redundant proteome as well as transposable elements in the euchromatic regions (Mun et al. 2009). In addition, as shown in Fig. 6, loss of redundant genes in the paralogous regions generated by WGD or WGT was frequently observed in the *B. rapa* genome. Similar genome-wide deletion of redundant NBS-encoding gene components has also been reported in other plant genomes. None or only a small number of NBS-encoding genes have been identified in the WGD blocks in rice (Yu et al. 2005), grape (Yang et al. 2008b), *A. thaliana* (Nobuta et al. 2005), and poplar (Yang et al. 2008b), indicating that most duplicated NBS-encoding genes were lost soon after WGD. Functional redundancy or artificial selection during domestication could accelerate the deletion or silencing of duplicated NBS-encoding genes that have not functionally diversified in the polyploid *Brassica* genome. The remaining copies are likely to retain their original function, thus *B. rapa* may contain sufficient NBS-encoding genes to defend against pathogens. Moreover, gene loss in polyploid genomes is known to be non-random (Blanc and Wolfe 2004; Seoighe and Gehring 2004), and polyploidy followed by the genome-wide removal of some but not all redundant genomic material can result in species-specific differences in the function of homologous genes (Adams and Wendel 2005). Therefore, some of the NBS-encoding genes of *B. rapa* that survived may have become more specific or gained novel disease resistance. Furthermore, almost 50% of NBS family members were detected as tandem arrays within homogenous clusters, and this percentage is expected to increase when complete sequence scaffolds are analyzed. This result is consistent with previous reports from other plants (Ameline-Torregrosa et al. 2008; Nobuta et al. 2005; Yang et al. 2008b; Yu et al. 2005), suggesting that tandem duplication in combination with polyploidy played an important role in the expansion of NBS-encoding genes in the *Brassica* genome. The tandem-clustered R gene family could provide a source of lineage-specific R genes with taxonomically restricted functionality, though identification of the entire NBS-encoding gene family in the *B. rapa* genome is required to evaluate if this hypothesis is correct.

The availability of *B. rapa* genome sequences enabled genome-wide evolutionary analysis of NBS-encoding genes in the *Arabidopsis–Brassica* lineage. We found that the CNL and TNL groups in the *Arabidopsis* and *Brassica* genomes shared a single ancient duplication event (Fig. 5a–c). NBS-encoding genes in the *A. thaliana* and *B. rapa* genomes showed unique patterns of CNL and TNL expansion based on K_s analysis; these patterns were related to several WGD events. In addition, genes of each group that were retained in individual genomes had a specific K_s distribution pattern, suggesting that the two groups experienced different selection pressures. The relatively high K_a/K_s ratio found in ancient duplicates in the CNL group implies that relaxation of purifying selection on the ancient duplicated CNL genes might have resulted in ancient

diversification (accumulation of degenerative mutations) of this group before the second WGD event (2R), which is estimated to have occurred ≈53 MYA. In contrast, the K_s distributions for TNL genes in the *A. thaliana* and *B. rapa* genomes have relatively high frequencies for the old duplication event (2R) and some skew toward ancient (1R) and recent duplication events (3R). This pattern of K_s distributions indicates that the 2R event contributed significantly to the evolution of the TNL group. Analysis of K_a/K_s ratio indicates that the relatively higher relaxation of selective constraints on the TNL genes after the 2R event was sustained until separation of the *Arabidopsis* and *Brassica* lineages, resulting in amplification of the TNL group genes in the common ancestor of *Arabidopsis* and *Brassica*. These data are in good agreement with the previous finding that TNL group genes are more homogenous and have been amplified more recently in the *A. thaliana* genome than CNL group genes (Meyers et al. 2003). Interestingly, ancient tandem duplicates of CNL are very rare compared to those of TNL genes in the *B. rapa* genome; tandem duplicates of TNL genes appear to have originated over a long period of time (Online Resource 6). These results collectively indicate that greater deletion of ancient duplicate CNL genes and relatively strong relaxation of selective constraints of TNL genes after the 2R event may be responsible for greater amplification of TNL group genes than CNL group genes in the *Brassica* genome. Duplications to distant positions were also observed. Some of these rearrangements could be associated with segmental duplications of entire chromosomal regions, which are frequently observed in the *Brassica* genomes (Town et al. 2006; Yang et al. 2006).

Lineage-specific amplification of NBS-encoding genes in the *Brassica* genome is an ongoing process as emphasized by the local expansion of *B. rapa* TNL genes. The detection of R genes not present in *A. thaliana* (TNLN) indicates that domain acquisition has occurred in the *Brassica* lineage, which may result in biological innovation. The uneven and clustered distribution of NBS-encoding genes and the discovery of recently duplicated redundant genes provide insight into how novel pathogen-resistant genes can evolve via additional gene duplication events, ectopic recombination, unequal crossing over, and diversifying selection (Michelmore and Meyers 1998). We anticipate that further analysis of complete genome sequences with accurate annotation supported by manual verification will provide more insight into the evolution of NBS-encoding genes in the *B. rapa* genome.

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