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Development of NBS-related microsatellite (NRM) markers in hexaploid wheat

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Abstract *NBS* (nucleotide binding site) genes, one type of the most important disease-resistance genes in the plant kingdom, are usually found clustered in genome. In this study, a total of 2288 full-length NBS protein-coding sequences were isolated from the wheat (*Triticum aestivum* L.) genome, and 903 *TaNBSs* of which were found expressed in wheat. Meanwhile, 2203 microsatellite loci were detected within 1061 scaffolds containing *TaNBS*. The distribution of these microsatellite loci across wheat homologous groups (HG) is 20% HG2, 16% HG7, 15% HG1, 15% HG6, 12% HG4, 12% HG5 and 10% HG3. We developed 1830 NBS-related microsatellite (NRM) markers for the microsatellite loci on *TaNBS*-scaffold sequences.Among them, 342 NRM markers were developed for HG2 with the largest number of microsatellite loci, and 69 out of these markers were anchored to the wheat genetic map using mapping population. Then, a total of 26 2AS-NRM markers, nine 2BL-NRM markers and nine 2DL-NRM markers were integrated into the genetic maps carrying *Yr69*, *Pm51* and *Pm43*, respectively. Finally, candidate sequences, within the gene clusters where *Yr5* and *Sr21* located, were analyzed according to the genomic position information of *TaNBS* and NRM markers. These NRM markers have clear chromosome locations and are correlated with potential disease resistance sequences, which can be manipulated to mapping or adding linkage markers of disease-resistance genes or QTLs, especially for those in the *NBS* gene clusters.

Keywords Wheat · Disease resistance · *NBS* genes · NRM markers

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Introduction

NBS (nucleotide binding site) gene family is one of the largest and most important disease resistance gene families in plant. The NBS domain, encoded by *NBS* genes, contains several conserved motifs such as Kinase1, Kinase2, Kinase3 and HD residues (Meyers

et al. 2003), which can directly or indirectly mediate the pathogen recognition via binding ATP or GTP and therefore participate in signal transduction (Elmore et al. 2011; Krattinger and Keller 2016). To date, more than 140 disease resistance (R) genes were cloned and 80% of them are *NBS* genes (Shao et al. 2016). With the publications of more and more draft genome sequence of different plants, *NBS* gene families of 19 dicots and 11 monocots have been isolated from respective genome (Urbach and Ausubel 2017; Morata and Puigdomènech 2017). In dicots, some NBS domains are usually linked to N-terminal TOLL/interleukin 1 receptor (TIR) or C-terminal leucine-rich repeat (LRR) that associated with pathogen recognition; while not the TIR but a coiled-coil domain (CC), which associated with protein–protein interactions, is present in the NBS domain of monocots (Lee and Yeom 2015; Urbach and Ausubel 2017).

Bread wheat (*Triticum aestivum* L.) is the most widely cultivated food crop, which faces attacks from various pathogens during its growth. Of which, fungal diseases like powdery mildew and rust (stripe rust, leaf rust and stem rust) can cause severe damage to wheat production (Goutam et al. 2015). Extensively identifying and utilizing R genes for disease resistance breeding is the most cost-effective way to control wheat disease (Chen and Line 1995). So far, a total of 58 powdery mildew R genes (Wiersma et al. 2017), 76 stripe rust R genes (Xiang et al. 2016), 76 leaf rust R genes (Bansal et al. 2017) and 59 stem rust R genes (Rahmatov et al. 2016) were formally designated. However, only 24 out of them have been cloned, and 20 (83%) were *NBS* genes, namely: *Pm2a* (Sánchez-Martín et al. 2016), *Pm3a-g* (Yahiaoui et al. 2004; Tommasini et al. 2006), *Pm8* (Hurni et al. 2014), *Pm21* (Xing et al. 2017; He et al. 2017), *Yr10* (Liu et al. 2014), *Lr1* (Cloutier et al. 2007), *Lr10* (Sela et al. 2012), *Lr21* (Huang et al. 2003), *Lr22* (Thind et al. 2017), *Sr22* (Steuernagel et al. 2016), *Sr33* (Periyannan et al. 2013), *Sr35* (Saintenac et al. 2013), *Sr45* (Steuernagel et al. 2016) and *Sr50* (Mago et al. 2015). Moreover, *Yr5* (Smith et al. 2007) and *Sr21* (Chen et al. 2015) were mapped to the *NBS* gene clusters, and the expressed sequence tag (EST) of *Yr5* was also reported to encode partial NBS domain (Smith et al. 2007). Except for this, a large number of resistances genes and QTLs, formally designated or not, were only preliminarily mapped. Most of the linked molecular markers are often unable to be effectively used for molecular breeding because of their distant locations from the target genes or QTLs. Therefore, isolation of wheat *NBS* family with clear position information and molecular markers is valuable for both fine mapping the target genes or QTLs mentioned above and the candidate sequences screening.

Using the 454 sequencing data of the common wheat cultivar Chinese Spring (Rachel et al. 2012), 580 and 986 complete *NBS* sequences were isolated respectively and their types and structures were analyzed (Bouktila et al. 2014, 2015; Gu et al. 2015); however, genomic positions of these sequences are unknown. In this study, the wheat genome sequence based on sequencing isolated chromosome arms (International Wheat Genome Sequencing Consortium 2014) was employed to isolate wheat *NBS* sequences with genomic location information. In addition, a set of NBS-related microsatellite (NRM) markers were developed according to the microsatellite loci adjacent to the *NBS* sequences; meanwhile, NRM markers in wheat homologous group (HG) 2, which harbors the most microsatellite loci, were used to construct the genetic map via mapping population and were analyzed their linkage with R genes. These *TaNBS* sequences and corresponding molecular markers can be further used for R genes or QTLs mapping.

Materials and methods

Plant materials

A RILs population of 194 lines derived from a cross between wheat CH7034 and SY95-71 were recruited to construct NRM marker map; An F_2 population of 136 plants from CH5025 (carrying *Pm43*, He et al. 2009)/Taichang (TC) 29 and 92 $F_{2:3}$ lines derived from CH7086 (carrying *Pm51* and *Yr69*, Zhan et al. 2014; Hou et al. 2016)/TC 29 were used to test for marker-R gene association. DNA samples of the above mapping population and their parents as well as phenotype data (He et al. 2009; Zhan et al. 2014; Hou et al. 2016) were provided by Shanxi Key Laboratory of Crop Genetics and Molecular Improvement.

NBS sequences isolation and bioinformatics analysis

The wheat whole genome sequences and predicted protein sequences were downloaded from the URGI database (http://wheat-urgi.versailles.inra.fr/). The wheat predicted protein data were retrieved in HMMER 3.0 software (Mistry et al. 2013) using the NBS family Hidden Markov Model file (accession number PF00931, downloaded from http://pfam.xfam.org/); the search results were then examined for the conserved domains of NBS proteins via SMART (http://smart.embl-hei-delberg.de). The obtained NBS sequences were submitted to the CDD database in NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd) to further analyze characteristic domains such as CC, LRR that correlated with NBS. The corresponding coding sequences and scaffold sequences were extracted from the wheat genome data according to the protein accession number; and their position information was determined by retrieving the wheat genome; then, *TaNBSs* were assigned to corresponding chromosomes. Gene structures of *TaNBS* were determined by the GSDS2.0 (http://gsds.cbi.pku.edu.cn/). Expression profiles of *TaNBSs* were obtained from retrieving wheat transcriptome sequencing data (accession number PRJNA243835, downloaded from http://www.ncbi.nlm.nih.gov/sra/).

NRM markers development and PCR validation

SSRhurnter software (Li and Wan 2005) was used to search for the microsatellite loci within scaffolds where *TaNBSs* situated. The searching criteria were set as follows: the nucleotides per repeat unit were two to five, and the repeat times ≥ 5. Primers were designed using the Primer5 software (http://www.premierbiosoft.com/primerdesign/) for NRM loci.

To compare with the wheat molecular map constructed by wPt-DArT and SSR markers (Marone et al. 2013), 34 GBS-DArT markers, which located in the same loci (genomic distance < 10 kb) with the wPt-DArT markers in the HG2 of wheat, as well as 346 pairs of SSR markers were also recruited for NRM-map construction. PCR used for screening and mapping NRM and other SSR markers was performed in 15 μL reaction mixture containing 1 U Taq DNA polymerase (Takara Bio Inc. Dalian, China), 1.5 μL 10 × buffer, 0.2 mmol L^{-1} dNTPs, 0.25 μmol L^{-1} primers and 100 ng of genomic DNA. PCR products were separated in 8% non-polyacrylamide denaturing gel and visualized by silver staining. The GBS-DArT marker typing was performed by Diversity Arrays Technology (DArT) PL, Canberra, Australia. The linkage map was constructed using JoinMap 4.0 software (https://www.kyazma.nl/index.php/JoinMap/), and the parameters were set as follows: Node-population, Grouping-independence LOD and Mapping function-Kosambi's.

Results and analysis

Distribution, types and expression of wheat NBS family

Conserved domain check was conducted on sequences isolated from the wheat database, and a total of 2288 complete protein sequences containing NBS domain were obtained. The distribution of these sequences in genome A, B and D is 34.1, 37.7 and 28.2%, respectively; among them, chromosome 4A contained the highest number of protein sequences, up to 227; while 4D contained the lowest, only 24 protein sequences (Fig. 1a). The length of TaNBS varies considerably, from the shortest sequence Ta1a-sLoc007418.1, only 48aa to the longest Ta4al-Loc027793.2 for 1816aa (Table S1). Based on whether the CC and LRR domains are included, TaNBS sequences were classified into 4 types: CC-NBS-LRR (CNL), NBS-LRR (NL), CC-NBS (CN) and NBS (N); and the N-type was the largest, which comprises 1144 sequences, accounting for 50% (Fig. 1a). The gene length of *TaNBSs* range from 251 bp to 7762 bp and the introns contained varies from one to 13; a total of 903 (56.3%) *TaNBSs* expressed in wheat (Table S1); out of the 632 *TaNBSs* with genetic position information, 477 (75.5%) were clustered (Table S1).

TaNBS-related microsatellite loci analysis and marker development

In total, 2203 microsatellite loci were detected on 1061 scaffold sequences containing *TaNBS*, of which 1621 were dinucleotide repeats loci, accounting for 73.6%, while the five nucleotide repeats loci was the least, only six (Fig. 1b). The distribution of these

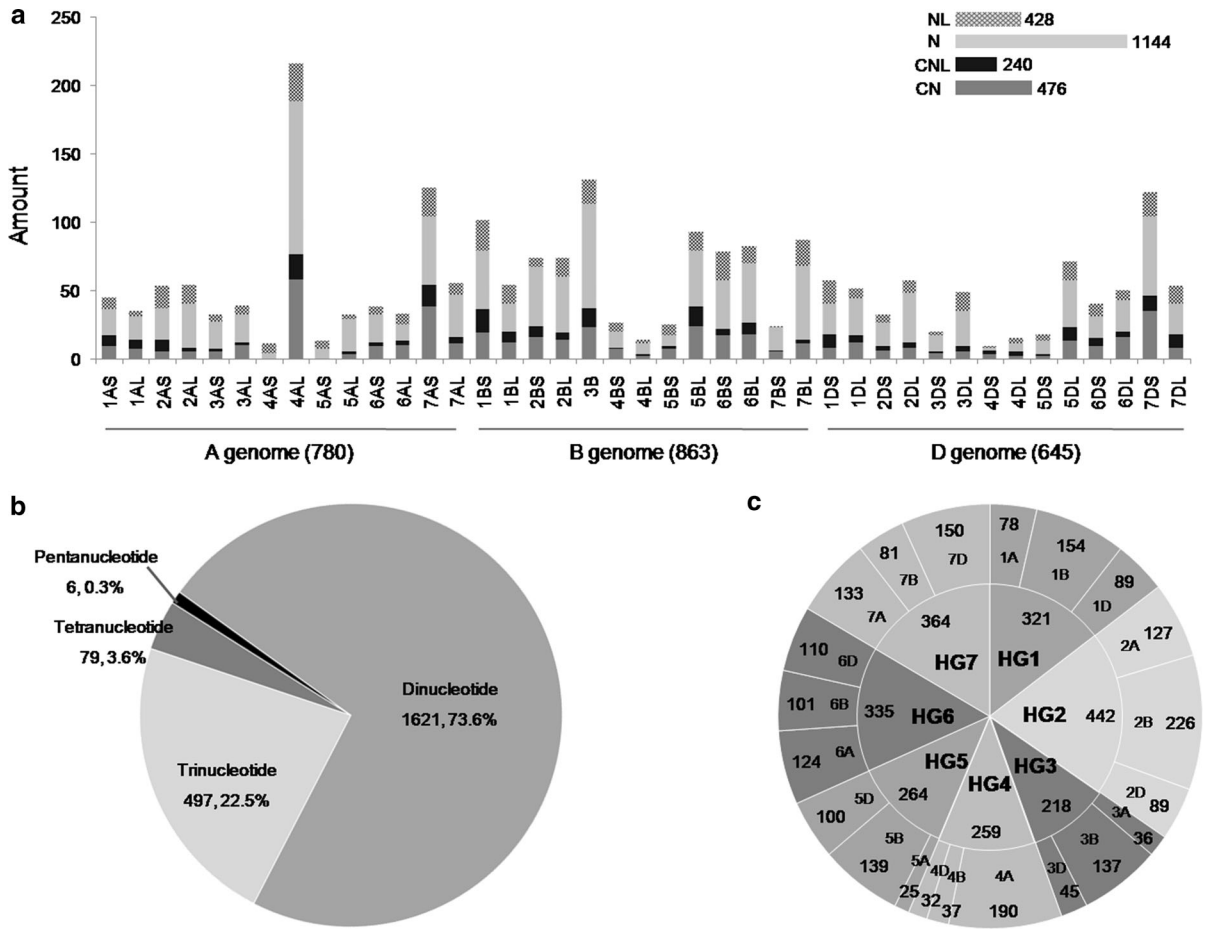

Fig. 1 Classification and chromosome distribution of TaNBS protein sequences (**a**) and NBS-related microsatellite loci (**b**, **c**) in wheat

microsatellite loci across the wheat HGs of is HG2 (20%), HG7 (16%), HG1 (15%), HG6 (15%), HG4 (12%), HG5 (12%) and HG3 (10%) (Fig. 1c). We totally developed 1830 pairs of NRM markers from *TaNBS*-scaffold sequences with microsatellite loci (Tables 1, S2). Among them, 342 pairs of NRM markers were developed on HG2 that contained the most microsatellite loci, including 49 2AS-NRM markers, 51 2AL-NRM markers, 71 2BS-NRM markers, 94 2BL-NRM markers, 34 2DS-NRM markers and 43 2DL-NRM markers (Table 1).

NRM markers map construction of the HG2 in wheat

The RILs population of CH7034/SY95-71 was amplified with 342 NRM markers of HG2, and 115 NRM markers showed polymorphism between the parents. Finally, 69 NRM markers, 20 SSR markers and 16 DArT markers were mapped to the genetic map (Fig. 2). The results showed that 31 NRM markers were assigned to chromosome 2A, 25 of them were on the short arm and mainly clustered in two regions; eight NRM markers were identified in the region of DArT marker *1088906-1138983*, and this region may contain disease resistance genes, such as *Yr69* (Hou et al. 2016) and *Yr17/Lr37/Sr38* (Helguera et al. 2003). In addition, 22 NRM markers were assigned to chromosome 2B, and three 2BL-NRM markers were detected in the region of SSR markers *Xgwm501-Xwmc332*, which may contain *Pm51* (Zhan et al. 2014), *Yr5* (Smith et al. 2007; McGrann et al. 2014), *Lr48* (Singh et al. 2011), *Sr9a* (Tsilo et al. 2007), *Sr28* (Rouse et al. 2012) and *QYraq.cau-2BL* (Guo et al. 2008). Sixteen NRM markers were assigned to chromosome 2D, and four 2DL-NRM markers were

Table 1 Sixty-nine NRM markers mapped to homologous group 2 of wheat in this study

NRM	Related-NBS	Scaffold	Location	SSR loci	Primers-F	Primers-R
2AS-NRM2	Ta2asLoc021433.1	5307566	2A:583433-585907	AT5	CAGCTGGCACCTGCTAGTT	CAAAGACCAAACACAAGCTCT
2AS-NRM4	Ta2asLoc002313.1	5185018	2A:630021-636069	AT5	GGTCTTGGTCACCCATGGT	CAACGGTGTCGTTGCCAGT
2AS-NRM6	Ta2asLoc002313.1	5185018	2A:630021-636069	AT5	GATGGACACACCAAGCTGAT	CTCCAGTTCTGCCACCATGT
2AS-NRM9	Ta2asLoc015225.5	5277207	2A:3638016-3625977	TGC5	GTCTTCAAGGGTATCAACCAT	GAGGAGATGAGCCACTCGT
2AS-NRM10	Ta2asLoc015225.5	5277207	2A:3638016-3625977	CT5	GGGATCTTCAGCCTCCTGT	CCGACGGAGATTGCTACAAT
2AS-NRM13	Ta2asLoc004895.1	5203038	2A:5956187-5955028	AT5	GAGATGCATTGAGGTGGCTT	CTGCAATCTTAGCACGCAGT
2AS-NRM14	Ta2asLoc004895.1	5203038	2A:5956187-5955028	TA5	GTATGGCTAGGCTGAAAGCT	CACTTGCTCATGTCGTAAGAT
2AS-NRM15	Ta2asLoc004895.1	5203038	2A:5956187-5955028	TC5	GATGTCCATAGCTGCAATGTT	GCAGATACTAGCAGCTTGAAT
2AS-NRM17	Ta2asLoc004895.1	5203038	2A:5956187-5955028	GA5	GCAGCAGATAGAGTAGGTCT	CTCTCACAGCCTCTCTTTCT
2AS-NRM18	Ta2asLoc007974.1	5225035	2A:5957047-5949810	TC5	GAGACCCATGCATGGCACT	CCTTGATGACCAAGATCTAGT
2AS-NRM19	Ta2asLoc009961.5	5239140	2A:12178511-12172412	AC7	GACAGATCTGGTTGGGAAAT	CATGCACAGTAGCAGGTTCT
2AS-NRM20	Ta2asLoc009963.1	5239140	2A:12178511-12172412	GC11 GT12	CGACACTCTTTCAATGTGGTT	GAAGCAACAGAGATGGACTAT
2AS-NRM22	Ta2asLoc004422.1	5199336	2A:12192281-12198261	AC9	GCAAGATGCCACTAGACTCT	CCGACGATCGTCACACAGT
2AS-NRM24	Ta2asLoc003361.1	5192461	2A:13812277-13817081	GCG7	CACAGCCTTACTTTCCAGGT	GCAGAGAGCTCAGAGTGCT
2AS-NRM26	Ta2asLoc008215.1	5227140	2A:13845027-13852773	AT5	GGGAGCAAGCTACAGTAGAT	GTGTTAGGTACACAGGCTGT
2AS-NRM27	Ta2asLoc009393.6	5234765	2A:16983922-16992949	AT6	GTCCTGTACAGGTGCTGGT	GAGAACTGCTCCACCTAGAT
2AS-NRM29	Ta2asLoc012209.4	5255452	2A:17033930-17025599	AT5	GAACCATACCATGAGGTCGT	CACATGTCCAAGTTGCACAAT
2AS-NRM32	Ta2asLoc007928.1	5224764	2A:17208106-17214103	TA6	GAGTCATTGGTCATAGCTAGT	CTCCTTTCCTCTCAGTGTGT
2AS-NRM33	Ta2asLoc016226.2	5283924	2A:17602503-17611133	AC6	CCTTGCGTCTGCTTTGCTTT	GATGTCAGACAACAACCAGAT
2AS-NRM34	Ta2asLoc016226.2	5283924	2A:17602503-17611133	AT6	CCGTCAAGCCTTAGGAGCT	GCTCGGTGATACGCACGTT
2AS-NRM35	Ta2asLoc016226.2	5283924	2A:17602503-17611133	CT5 CCA5	CACGAGAATCAGACTGTGGT	GGTTCGTCGGTCGATCCAT
2AS-NRM38	Ta2asLoc021823.1	5308525	2A:18281351-18278091	CCA5	GGCAAAGCTGTGACGGTGT	CCCTTCACGATCCAATCCAT
2AS-NRM39	Ta2asLoc010583.1	5243689	2A:18294996-18303844	AG5	CGAAGCATGCTTCATGTGGT	CGAAGCCTGCATTGACCTAT
2AS-NRM41	Ta2asLoc008879.1 Ta2asLoc013849.1	5267618	2A:18340931-18336309	CT5	CCTATGCTTGGCTGGGTGT	GAAGGTTACCTGAGAGAGCT
2AS-NRM47	Ta2asLoc020350.4	5305865	2A:72934552-72944884	TAC5 CAT5	CAGCTATCGAAGAGCTGTGT	GGGTTGAGTCTGCACTTGTT
2AL-NRM14	Ta2alLoc031092.1 Ta2alLoc031098.1	6439430	2A:718124257-718133644	AGG6	GAGCAGCAGAAGAACAGGAT	GGACAGACAGCCTGTGATTT
2AL-NRM25	Ta2alLoc016870.1	6380185	2A:762880801-762894894	TC9	CAACCTGATGAAGAGAGCACT	GCCAGCTATAGCCTTGCTAT
2AL-NRM28	Ta2alLoc027083.3	6433075	2A:762972271-762979431	TA5	CAGCTTTGTTTGCACCTGAT	GGACGTTTGACATGTGTTGT
2AL-NRM30	Ta2alLoc008041.2	6335704	2A:764664566-764657601	AT5	CGCTGTCTCTTGAACAAGAT	CATCTTCAGAGCCAAACACT
2AL-NRM32	Ta2alLoc002192.2	4144983	2A:764787482-764781784	GT34 TA5	GGTGATCGTTCTAGCAGAGT	GTGTGGAAGACTATTGCAGAT
2AL-NRM51	Ta2alLoc004920.1	6318795	2A:778565873-778563882	AT8	CTGAGATCTAACCACACCTTT	GATGACATCGACAACAAGGAT

Table 1 continued

NRM	Related-NBS	Scaffold	Location	SSR loci	Primers-F	Primers-R
2BS-NRM20	Ta2bsLoc024802.1 Ta2bsLoc024805.1	5243837	2B:13717742_13713685	AT5	CATTCACCACTTTGCATCACT	CAGACATGTTGTGCTGAAACT
2BS-NRM28	Ta2bsLoc023785.1 Ta2bsLoc026043.2	5242540	2B:17933932_17939358	TC5	CCATCATCTTCGTGAGCTGT	CAACCAAGCAACAAAGCAACT
2BS-NRM36	Ta2bsLoc002494.1	5145377	2B:18171553_18169332	AC5 CG7 CA5	CGGATGGTATCTCAGTCTCT	GTGGAACAAGCTGTTGCACT
2BS-NRM39	Ta2bsLoc008488.1	5179295	2B:19418355_19412542	GA7	CACCACAACCCAGCAAGCT	GTCCTTGTGCATGCATGGAT
2BS-NRM45	Ta2bsLoc018202.1	5220288	2B:22321587_22316136	CGG5 AGC7	CTTCCACCTCGAAGCCTCT	CAGCATCTGCACAATGTCATT
2BS-NRM61	Ta2bsLoc007981.1	5177111	2B:43273402_43284263	CG5 AT11	GCTCGAGGACAAGCTGCAT	GATCCATACCAGAGTGTGCT
2BS-NRM68	Ta2bsLoc014218.1	5203447	2B:62200686_62205287	GCA5	GAGGAGGAGGTAAGCCACT	GTGCTCTTCCCTACTCTTCT
2BL-NRM11	Ta2blLoc026726.1 Ta2blLoc034108.1 Ta2blLoc034772.1	8061017	2B:683034934_683045924	AT5	GCCTGGAGACTCATACTTCT	GGATGGGTTCTCCCTGGTT
2BL-NRM12	Ta2blLoc032359.3	8086799	2B:683049791_683034934	AT5	GGATGGGTTCTCCCTGGTT	GCCTGGAGACTCATACTTCT
2BL-NRM17	Ta2blLoc023962.8	8045581	2B:685741229_685747793	CT7 TC16	GTTGCTACCATGCATGACCAT	CTCGATTGAGGTCCTAAGGT
2BL-NRM19	Ta2blLoc023962.8	8045581	2B:685741229_685747793	TG5	GCTTGGAGGCTTTGTCATTCT	CTCCTAACAGAACCTGAAACT
2BL-NRM27	Ta2blLoc017765.1	8009224	2B:703889622_703896056	TA5 TA5	CAATGGGTGCTGAACCAAT	GTCGTGGAAGGTGGTTGTT
2BL-NRM39	Ta2blLoc024018.1	8045859	2B:774710817_774701377	CT5	CTGCCATGCCTGTTTGTGAT	GCAACCTCCTACAGCAAACT
2BL-NRM42	Ta2blLoc027978.1	8068690	2B:775325818_775331540	GA20	GTCCTTCTCTTTCGCATGCT	GGGTTCTCGAGAGACAACAA
2BL-NRM43	Ta2blLoc002937.1	6805858	2B:776433393_776432210	TA6	GGAGCTCCCAGCGTTCGT	CCTTTGTGTGCCTGCTTTCT
2BL-NRM44	Ta2blLoc002937.1	6805858	2B:776433393_776432210	CT5	CAGCTCTCCTGCCAACATTT	CCATGGGTGATGCATGCAAT
2BL-NRM50	Ta2blLoc020549.1	8025387	2B:780018363_780019248	TA8	CCTCCTGTTACACCACTCAT	GTTGAGCTAGTCTGGCAAAC
2BL-NRM53	Ta2blLoc021454.2	8071109	2B:787964802_787955565	GGA5	CACTTTCTGCTTGTGCAATCT	GCAAACTCATCATCAGCAGGT
2BL-NRM56	Ta2blLoc021454.2	8071109	2B:787964802_787955565	AAG9	GTGGAGGACCACACCACAT	GCTACTGCCTGCTGGTCTT
2BL-NRM73	Ta2blLoc033622.1	8088888	2B:794414183_794408012	AT5	GCTTCTCACCAACGAACCAT	CAACTTCCATCCTCTACTCTT
2BL-NRM79	Ta2blLoc028467.2	8071656	2B:794591793_794600393	TGTA6	GAAGCGAACAAAGCGACCAT	TGAGCGACTCGCTATCGAAT
2BL-NRM81	Ta2blLoc028467.2	8071656	2B:794591793_794600393	CGA5 CGA13	GCGTCGTGATCCTCGACA	CGAGTTCGTTCAAACATAAACAT
2BL-NRM84	Ta2blLoc036149.6	8093398	2B:794667237_794675985	CTG7	CGTGTGGACCTATGTGGTAT	CATCGACGAGGCATTCCTCT
2DS-NRM6	Ta2dsLoc015487.1	5379495	2D:2554528_2547533	CAG5	CTGGTTGTAGAGGTGGATGT	CTCCTCCAGGAACAAGAGTT
2DS-NRM14	Ta2dsLoc014907.1	5376421	2D:7968579_7974310	GT5	GCTAGTCGGTTGTATGCACT	GTTGCTAGTCAGTGGTCTGT
2DS-NRM15	Ta2dsLoc014907.1	5376421	2D:7968579_7974310	GA5	GGTAACCACGTCGTCCAGT	CGACGGTATGCCTCGGATT
2DS-NRM26	Ta2dsLoc010644.1 Ta2dsLoc010646.1	5354297	2D:16356700_16331896	CT6	CATGCGACTGGAATCTCACT	CGTAGCATGCTTCATGTGGT
2DL-NRM7	Ta2dlLoc026152.1	9906982	2D:574424890_574417515	TC12	CCATCCTTCTTCTTCTTCCTT	CTTGTAAGTCTGTTGCTGCTT

Table 1 continued

NRM	Related-NBS	Scaffold	Location	SSR loci	Primers-F	Primers-R
2DL-NRM14	Ta2dlLoc024520.1	9900253	2D:580248099_580241389	TG11	GGAAAGTGTTGATGCCTGAT	CATGCTTGACTGCTTGAGTT
2DL-NRM16	Ta2dlLoc024520.1	9900253	2D:580248099_580241389	TCT11	GTGCGTCCTCTTCTCCTCT	GATGAAGTGAGAGCACGGAT
2DL-NRM17	Ta2dlLoc024520.1	9900253	2D:580248099_580241389	GT5	CGAGTGCTGTTCGCTTGGT	GAGAACAGTACACTGAACCAT
2DL-NRM18	Ta2dlLoc024520.1	9900253	2D:580248099_580241389	TG5	CTGATCTGATCTGATGTGTGT	CAAAGAAGAGCAAGTACCTGT
2DL-NRM23	Ta2dlLoc005806.1	9910373	2D:625145480_625153357	GT5	GCCTCGATTCTGGTTCACAT	GCTAGGAATCACCGAGGTAT
2DL-NRM28	Ta2dlLoc027460.1	9908568	2D:635879946_635872478	CTC5	GTTCCTGAGTACGAGATGCT	CTGACTTCCAGACCCATGAT
2DL-NRM29	Ta2dlLoc022402.1	9888942	2D:636910770_636914879	AG7	GACGAAGCATGATGTCTTAGT	GTTCGGACAGAAGTTTGATCT
2DL-NRM33	Ta2dlLoc017939.1 Ta2dlLoc017940.1	9865621	2D:644665988_644657917	TG6 TG5	CTCACATGACTAGCAAGCATT	GAGGAACATCTTCTATGGACT
2DL-NRM34	Ta2dlLoc011906.1	9833583	2D:646495012_646491356	TG5 GC6	GAAACAAGCCCAAACTGGATTT	GAATGAGAAGAGCAAACTGCAT
2DL-NRM42	Ta2dlLoc010152.1	9823729	2D:648551574_648554977	TC5 TCC7	GAGAAAGCCTGTTGATGGAGT	GATGCTCGTGACCTAGCATCT

in the region of DArT marker *1114628-1086188*. There might be *Sr6* (Tsilo et al. 2010) and *QPm.caas-2DL* (Lan et al. 2010) in this region.

Construction of more densely populated map with NRM markers

NRM markers were employed to enhance the map-density of three disease resistance genes *Yr69* (2AS, Hou et al. 2016), *Pm51* (2BL, Zhan et al. 2014) and *Pm43* (2DL, He et al. 2009) in HG2, respectively. The results showed that 26, nine and nine NRM markers were integrated into the genetic maps where *Yr69*, *Pm51* and *Pm43* sited, respectively. Among them, eight NRM markers were integrated into the original *X2AS33*-1.9 cM-*Yr69*-3.1 cM-*Xmag3807* region of *Yr69*, which narrowed the region to *X2AS-NRM34*-0.4 cM-*Yr69*-1.3 cM-*X2AS-NRM31*, inferring that *Yr69* may locate in the *NBS* gene cluster (Fig. 3a). In addition, a marker *X2DL-NRM05* (Fig. 3c), which is closer to *Pm43*, was obtained.

Identification of candidate sequences within gene clusters by *TaNBS* and NRM markers

Yr5 (Smith et al. 2007) and *Sr21* (Chen et al. 2015) are two disease resistance genes that have been confirmed to be located in the *NBS* gene cluster. The EST (Genbank number JN631792) of *Yr5* encoded partial NBS structure, and its co-segregation marker *TaAffx.65234.1.S1_at* as well as its flanking linkage marker *S23M41-310* were also *NBS* sequences, in which *S23M41-310* is orthologous to the rice *NBS* gene *OsXa1* (Smith et al. 2007; McGrann et al. 2014). In this study, *Yr5* was assigned to a gene cluster, consisting of 11 *TaNBSs* and 11 NRM markers, in the region 693,372,707-732,340,422 of chromosome 2B. The *TaNBS* sequence Ta2blLoc006115.1 includes the whole *Yr5*-EST (100% sequence similarity) and can express in wheat (Table S1); its co-segregation marker *TaAffx.65234.1.S1_at* and flanking marker *S23M41-310* correspond to *Ta2blLoc008215.1* and *Ta2bl-Loc034091.1* in the gene cluster, respectively (Fig. 4).

In addition, we assigned seven linkage markers of *Sr21* to chromosome 2A with the closest flanking markers corresponded to a 1.28 Mb region 709,765,601-711,049,068, which contained four *TaNBS* sequences and two NRM markers (Fig. 5). Multiple sequence alignment results showed that the

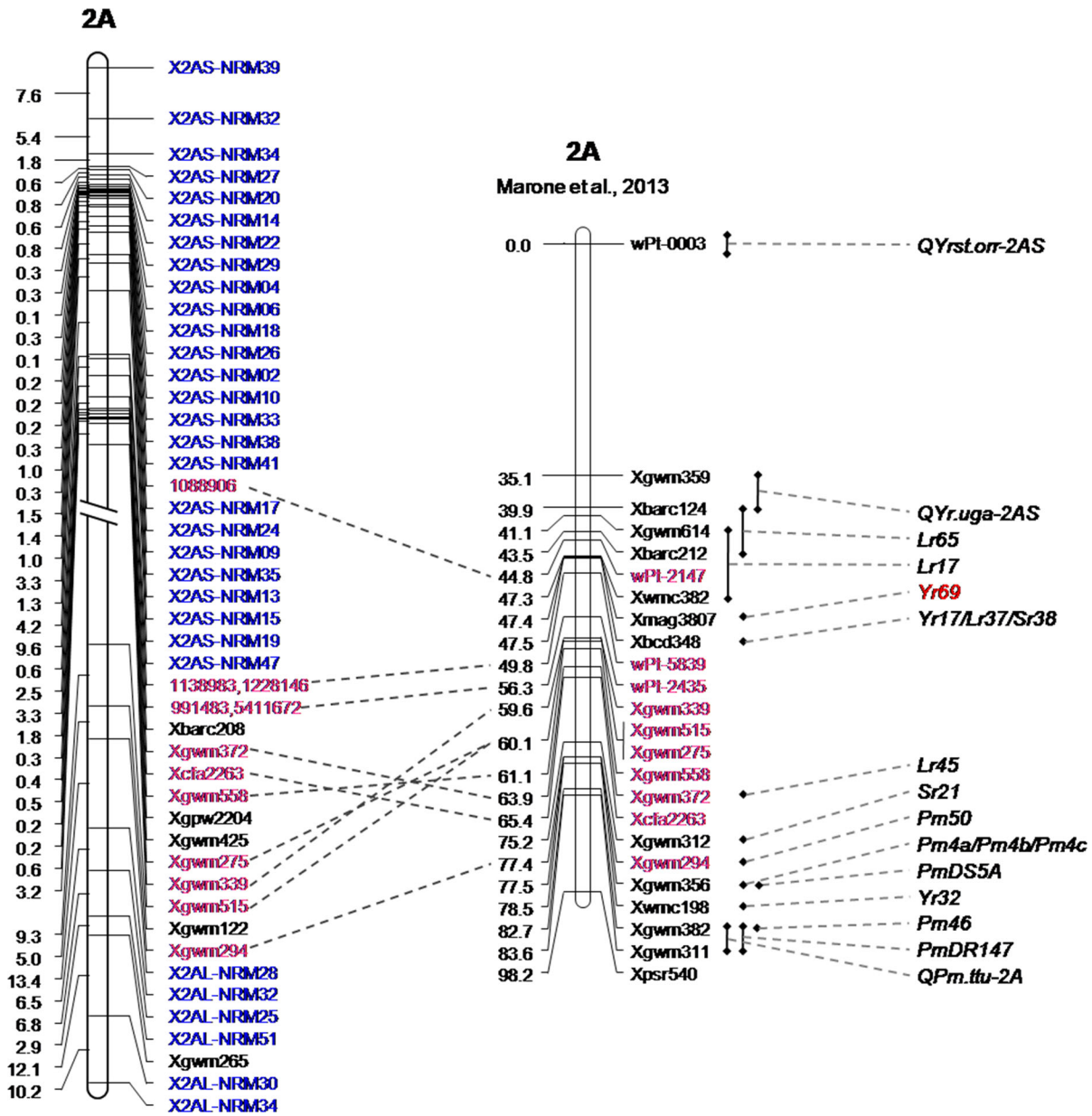

Fig. 2 Genetic map of NRM markers in homologous group 2 of wheat constructed by CH7034/SY95-71 RILs population. The NRM markers are labelled in blue, the SSR or DArT markers appearing in both linkage maps are labelled by pink, and the genes for the next enhancing map-density are labelled with red. (Color figure online)

TaNBS protein sequences Ta2blLoc019062.3, Ta2blLoc029872.3 and Ta2blLoc029875.1 exhibited highly similar motifs to those within the NBS domain of cloned disease resistance genes (Fig. S1), such as P-loop (GGxGKTT) for ATP/GTP binding, Kinase2 (LLVLDDxW), Kinase3 (GxxxLxTxR) and HD residues. Moreover, Ta2blLoc019062.3 expressed in wheat (Table S1), which means it may participate in the process against pathogens, this requires subsequent validation in the mapping population using its marker*2AL-NRM05*.

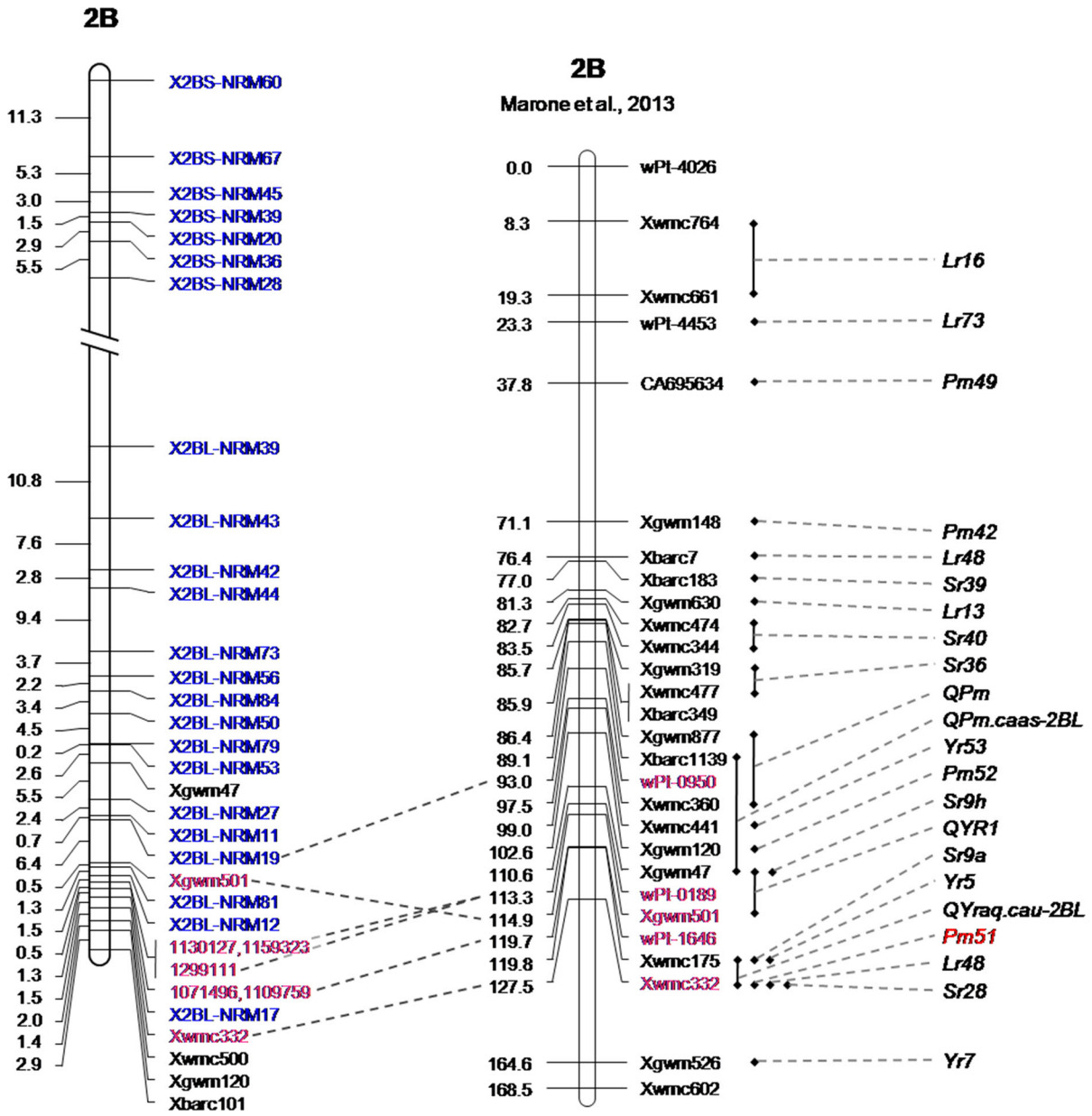

Fig. 2 continued

Discussion

Size of the wheat *NBS* family

NBS, the largest disease resistance gene family, has been surveyed in various plants, such as bryophytes, lycopodiums, gymnosperms and angiosperms (Elmore et al. 2011; Krattinger and Keller 2016; Shao et al. 2016; Urbach and Ausubel 2017; Lee and Yeom 2015). The fungal bloom during the Cretaceous-Paleogene boundary (~ 66 MYA) may trigger the intensive expansion of *NBS* genes in plants (Shao et al. 2016), which suggests the important role of *NBS* genes in the fight against fungal disease during evolution. In cotton, the *NBS* expansion in *Gossypium raimondii* enhanced its resistance to *Verticillillm dahliae*, while

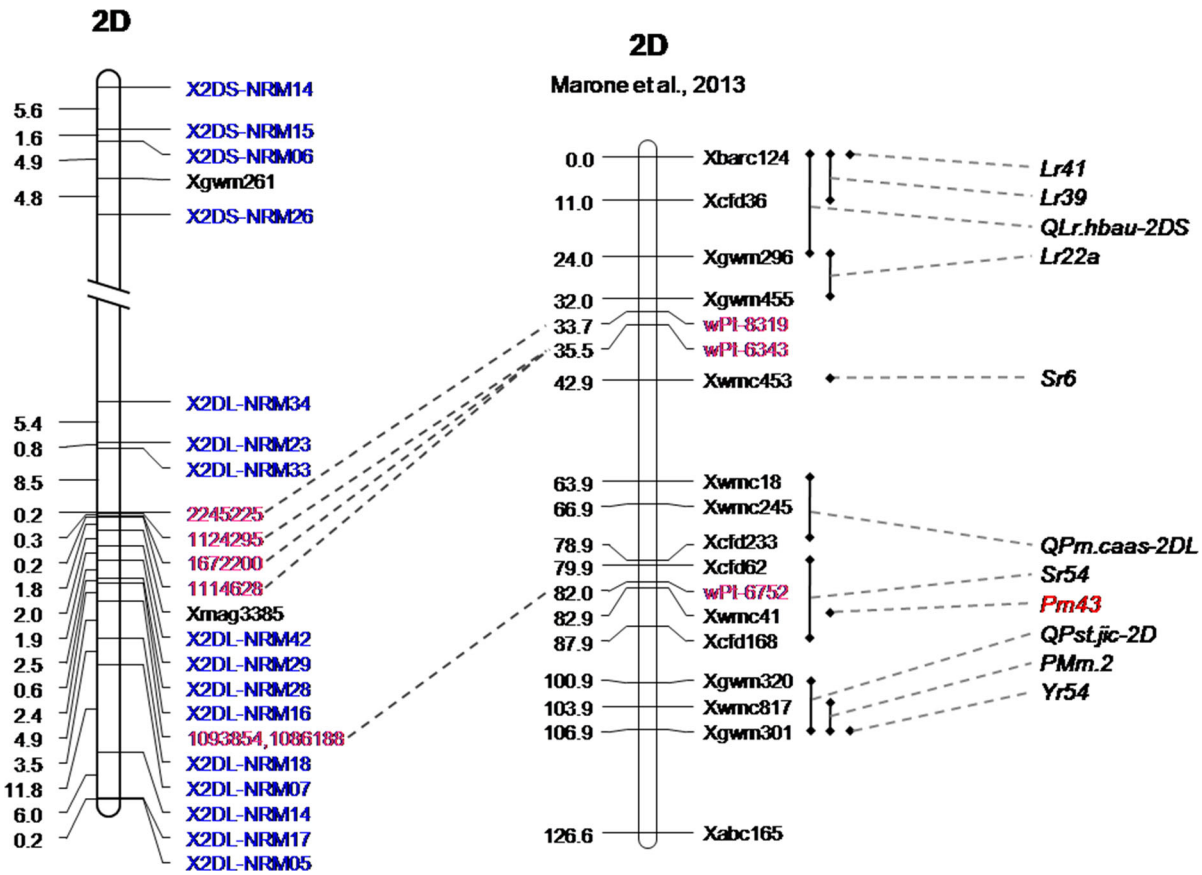

Fig. 2 continued

G. arboreum without *NBS* expansion was easily susceptible (Li et al. 2014). After undergoing three independent whole-genome duplications (WGD), however, only 117 *NBS* genes were included in banana genome, which may explain its susceptibility to pathogen attacks (D'Hont et al. 2012). Therefore, isolation and analysis of plant *NBS* gene families can help us better elucidate the underlying disease resistance mechanisms.

In this study, 2288 complete *TaNBS* sequences were isolated from the hexaploid wheat genome, which is significantly higher than that of other gramineous crops evolved from the same grass ancestor (50–70 MYA) (Salse et al. 2008), such as 535 of rice (Zhou et al. 2004), 420 of barley, 316 of *Brachypodium distachyon* (Gu et al. 2015), 274 of sorghum (Cheng et al. 2010) and 109 of maize (Cheng et al. 2012). We also isolated 463 (Liu et al. 2017) and 701 complete *NBS* sequences (the results were not listed) from *Triticum urartu* and *Aegilops tauschii*, respectively. It was hypothesized that the *NBS* families of three wild ancestral species of wheat were integrated into the genome of the hexaploid wheat after two polyploidization events (0.8 and 0.4 MYA, IWGSC 2014); then with the propagation of wheat, the *TaNBS* family expanded again to adapt to the infection of various pathogens in different planting areas, which eventually led to this gene family with large number of members. The majority of these *TaNBS* members (about 70%) are clustered in genome, which is also true for *NBS* family members in plant species like *Arabidopsis thaliana* (71.1%, Meyers et al. 2003), rice (76%, Zhou et al. 2004) and potato (73%, Jupe et al. 2012). The presence of the *TaNBS* gene clusters as well as the loss of some subgenomic copies in the evolutionary process (Comai 2005; Otto 2007) resulted in an uneven distribution of *NBSs* across chromosomes in HGs; For example, there were

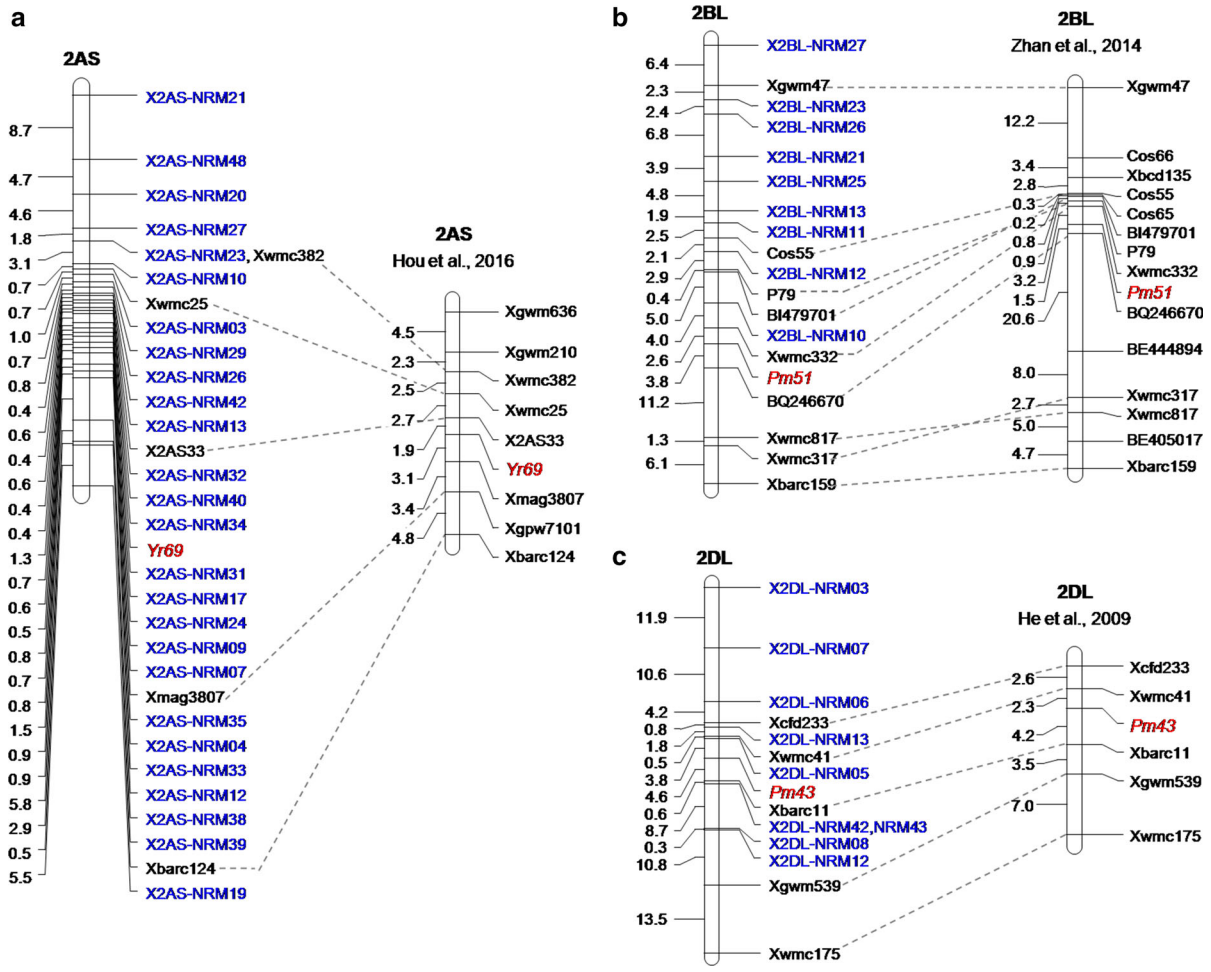

Fig. 3 Construction of more densely populated maps of *Yr69* (**a**), *Pm51* (**b**) and *Pm43* (**c**). The disease resistance genes and their linked NRM markers were labelled in red and blue, respectively. (Color figure online)

248 *TaNBS* genes on chromosome 4A, while only 40 and 24 were on chromosome 4B and 4D, respectively. This may explain that the cloned disease resistance genes are often one copy rather than 'triplet gene' (Pfeifer et al. 2014).

TaNBSs provide reference for homology-based and map-based cloning

To date, some disease resistance-related *NBS* genes were cloned from wheat using a homology-based cloning strategy. In the case of resistance to powdery mildew, there are genes such as *TmMla1* (Jordan et al. 2011), homologous to barley *HvMla1* (78% sequence similarity), from diploid wheat; *TdRGA-7Ba* (Gong et al. 2013), a *Pm3b* homologue (sequence similarity > 90%), from durum wheat; and *TaRGA* from common wheat (Wang et al. 2016), which is homologous to multiple plant disease resistance genes. Many *TaNBS* isolated in this study exhibited high sequences similarity with those cloned disease resistance genes. For example, both Ta1bsLoc017427.1and Ta1bsLoc003202.1 shared over 70% identity with barley powdery resistance genes *HvMla1* (Zhou et al. 2001), *HvMla6* (Halterman et al. 2001) and *HvMla13* (Halterman et al. 2003) at the protein level. These *TaNBSs* can provide candidate gene sequences for cloning wheat disease-resistance genes using homology-based method. Normally, genes with similar domains may possess similar functions. The 19 cloned wheat *NBS*-encoding protiens are all CNL-type, and the number of TaNBS proteins with CNL

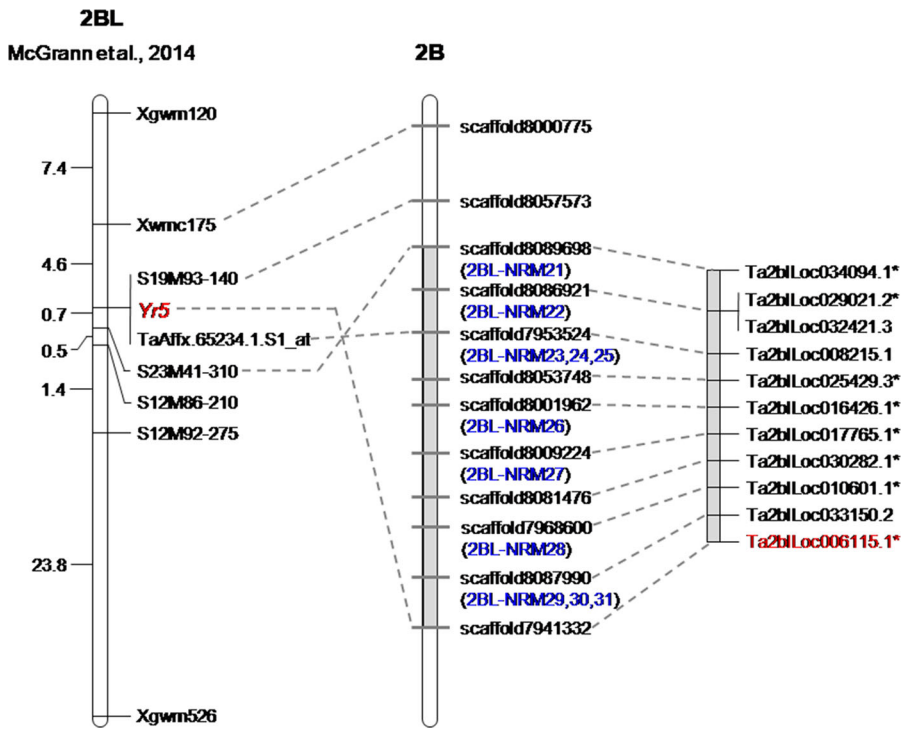

Fig. 4 Identification and analysis of *NBS* cluster of *Yr5*. *Yr5* and the NRM markers in this cluster were labelled in red and blue, respectively. (Color figure online)

isolated from this study is 240 (Fig. 1). In addition, some TaNBSs contain other distinct domains, such as Ta2bsLoc003709 and Ta2bsLoc014737, in HG2, contian ABC (ATP-binding cassette) transport protein domain; Ta2alLoc020734 and Ta2dlLoc021209 contain Jacalin domain; while Ta2alLoc012596 and Ta2blLoc002392 contain zinc finger domian; these domains have been proved to play an important role in disease resistance process (Krattinger et al. 2009; Ma et al. 2013; Guo et al. 2013). ABC is also the function domains of *Lr34* (Krattinger et al. 2009). Studies have shown that these domains may interact with the NBS domain and against pathogen invasion together (Deslandes et al. 2002). Hence, it is necessary to further explore these TaNBSs in depth.

Furthermore, *TaNBSs* can provide reference sequences for map-based cloning of disease-resistance genes that in the *NBS* gene cluster. It has been reported in previous studies that if the comparative genome analysis showed the region of a mapped wheat resistance gene corresponded to the *NBS* gene cluster of rice, *B. distachyon* or other model plants, then often this gene is in the wheat *NBS* gene cluster and is a *TaNBS* gene, like both *Sr35* (Saintenac et al. 2013) and *Sr50* (Mago et al. 2015) are *TaNBS* genes in the *NBS* gene cluster. In this study, we analyzed the genomic location of *NBS* gene cluster where *Yr5* sited and identified a *TaNBS* sequence Ta2blLoc006115.1 containing *Yr5*-EST, which indicated the accuracy of the genomic location of *TaNBS* family. Then, *Sr21*, which is also in the *NBS* gene cluster, was analyzed and anchored its linkage marker to genomic map. Finally, four candidate *TaNBS* sequences were found in the target region.

NRM markers in gene mapping and molecular breeding

Extensively identification and cloning disease resistance genes are the foundations for wheat disease resistance breeding. So far, among nearly 300 powdery mildew and rust resistance genes were formally designated in wheat, only few of them can be used for wheat improvement and most of them are facing the risk of resistance loss due to pathogens variation before being used for breeding. This situation could be

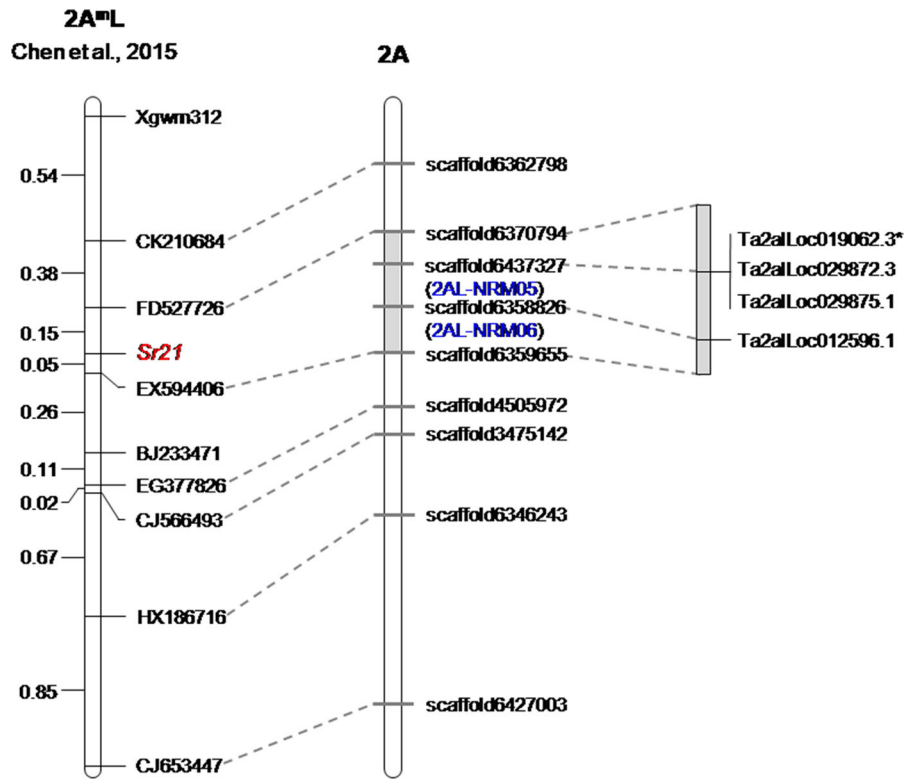

Fig. 5 Identification and analysis of *NBS* cluster of *Sr21*. *Sr21* and the NRM markers in this cluster were labelled in red and blue, respectively. (Color figure online)

attributed to that the linkage marker of the R gene cannot be efficiently used for marker-assisted selection (MAS). Since the common wheat contains three sub-genomes and highly repetitive sequences (80%, IWGSC et al. 2014), most of the SSR markers, routinely used for R gene mapping, have a low distribution density across the wheat genome, which resulted in their often faraway locations from the gene of interest. Besides, it is not easy to develop markers in the linkage region due to the unclear genome location of these SSR markers. Thus, gene recombination may occur between linked markers and target genes and result in the marker missing, which leads to breeding failure.

With the rapid development of sequencing technology, markers with high density and precision like SNP, DArT have been developed, which greatly improved mapping accuracy and narrowed the distance between mark and gene. Meta analysis showed a number of DArT markers that in the same loci as the R genes or QTLs are *NBS* sequences, such as *PmHNK54/wPt-5865*, *QPm.inra.2A/wPt-6064*, *Pm23/wPt-7024* and *Pm42/wPt-2600* in wheat HG2 (Marone et al. 2013). This could be explained by the fact that many R genes are located in the *NBS* gene cluster, thus their linked DArT markers may also *NBS*-related sequences. However, relatively high chip scanning costs makes it unsuitable for large population screening in breeding process for now. Aiming at improving the effectiveness of routinely used molecular markers, we developed 1830 NRM markers, each of which lay in the same scaffold sequence with *TaNBS*. Of all the NRM markers is *7DL_NRM59* the farthest from *TaNBS* sequence Ta7dlLoc025447 with the longest genetic distance of 38589 bp, which is approximately equal to 0.007 cM based on the ratio of physical and genetic distance on chromosome 7DL (5.41 Mbp/cM; IWGSC, 2014). The remaining genetic distances between NRM markers and *TaNBSs* are below the value. Since most NRM markers were clustered in some regions with the *NBS* genes, the polymorphism of NRM markers is low in the genome regions that do not contain disease resistance loci. If R gene and multiple NRM markers are anchored to the same loci, this gene may locate in a *NBS* gene cluster; such as *Yr69* in this study. Moreover, when a *TaNBS* is confirmed to be associated with disease resistance, the NRM marker(s) on its scaffold can be directly used as

a co-segregation marker, which will improve the MAS efficiency in breeding process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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