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ORIGINAL ARTICLE

Fine mapping of powdery mildew resistance genes *PmTb7A.1* and *PmTb7A.2* in *Triticum boeoticum* (Boiss.) using the shotgun sequence assembly of chromosome 7AL

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Abstract

Key message **A novel powdery mildew resistance gene and a new allele of *Pm1* were identified and fine mapped. DNA markers suitable for marker-assisted selection have been identified.**

Abstract Powdery mildew caused by *Blumeria graminis* is one of the most important foliar diseases of wheat and causes significant yield losses worldwide. Diploid A genome species are an important genetic resource for disease resistance genes. Two powdery mildew resistance genes, identified in *Triticum boeoticum* (A^bA^b) accession pau5088, *PmTb7A.1* and *PmTb7A.2* were mapped on chromosome 7AL. In the present study, shotgun sequence assembly data for chromosome 7AL were utilised for fine mapping of these *Pm* resistance genes. Forty SSR, 73 resistance gene analogue-based sequence-tagged sites (RGA-STS) and 36 single nucleotide polymorphism markers were designed for fine mapping of *PmTb7A.1* and *PmTb7A.2*. Twenty-one RGA-STS, 8 SSR and 13 SNP markers were mapped to 7AL. RGA-STS markers *Ta7AL-4556232* and *7AL-4426363* were linked to the *PmTb7A.1* and *PmTb7A.2,* at a genetic distance of 0.6 and 6.0 cM, respectively. The present investigation established that *PmTb7A.1* is a new powdery mildew resistance gene that confers resistance to a broad range of *Bgt* isolates, whereas *PmTb7A.2* most probably is a new allele of *Pm1* based on chromosomal location and screening with *Bgt* isolates showing differential reaction on lines with different *Pm1* alleles. The markers identified to be linked to the two *Pm* resistance genes are robust and can be used for marker-assisted introgression of these genes to hexaploid wheat.

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Introduction

Powdery mildew (PM) caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) often occurs in regions with cool and humid climate and can cause severe yield losses (Bennett 1984; Everts and Leath 1992; Kapoor and Singh 1993). Breeding cultivars with inherent resistance is the most promising approach to manage this disease. However, development of powdery mildew resistant cultivars and their durability relies on the availability of diverse sources of resistance. Till date, more than 73 powdery mildew resistance genes/alleles have been identified at 50 loci (*Pm1–Pm54*; *Pm18* = *Pm1c*, *Pm22* = *Pm1e*, *Pm23* = *Pm4c*, *Pm31* = *Pm21*) in wheat and its wild relatives (McIntosh et al. 2013; Xiao et al. 2013; Mwale et al. 2014; Ouyang et al. 2014; Zhan et al. 2014; Hao et al. 2015; Petersen et al. 2015). Of the 50 loci, 11 have been mapped on the A genome, 26 on the B

genome and 13 on the D genome of wheat. Thirty-four of the 73 alleles at 50 loci were derived from *Triticum aestivum* and the remaining genes were introgressed from species belonging to the primary (such as *T. monococcum, T. turgidum, Aegilops tauschii*), secondary (*T. timopheevii, T. araraticum*, *T. carthlicum*, *Aegilops speltoides, Aegilops longissima*) or tertiary gene pools (*Ae. ovata, Ae. umbellulata, Secale cereale, Haynaldia villosa, Elytrigia intermedium, Thinopyrum ponticum*). The diploid 'A' genome species, *Triticum monococcum* ssp *monococcum* (*T. monococcum*—A^mA^m), *T. monococcum* ssp *aegilopoides* (*Triticum boeoticum*–A^bA^b) and *Triticum urartu* (A^uA^u), harbour useful variability for many economically important genes, including resistance to diseases (Feldman and Sears 1981; Dhaliwal et al. 1993; Hussien et al. 1997; Miranda et al. 2007; Singh et al. 2007a; Chhuneja et al. 2008; Singh et al. 2010). PM resistance genes/alleles *Pm1b* and *Pm4d* have been transferred from *T. monococcum* (Hsam et al. 1998; Schmolke et al. 2012) and *Pm25* has been transferred from *T. boeoticum* (Shi et al. 1998). Other two genes designated as NCA6 *Pm* and *PmU* have been transferred form *T. monococcum* and *T. urartu*, respectively (Qiu et al. 2005; Miranda et al. 2007). Both these genes map close to *Pm1* locus, hence these are either different alleles of *Pm1* or a novel loci tightly linked to *Pm1*.

In wheat, transfer of genes from diploid progenitor species is relatively easy due to free recombination but these have not been used extensively for the identification of new alleles and genes useful for wheat improvement. Two powdery mildew resistance genes were identified in *T. boeoticum* acc. pau5088 by our group. These were mapped on the long arm of chromosome 7A, and tentatively designated as *PmTb7A.1* and *PmTb7A.2* (Chhuneja et al. 2012). The *PmTb7A.2* gene was mapped to the region where complex locus *Pm1* maps, indicating that *PmTb7A.2* might be a new allele of *Pm1* or a new gene closely linked to *Pm1*. *PmTb7A.1* maps proximal to *PmTb7A.2* at a distance of ~48 cM, in a region where no *Pm* gene has been reported so far. Both the genes are effective against powdery mildew isolates present in Switzerland (Chhuneja et al. 2012) as well as in India (Elkot et al. 2015). These genes were mapped on 7AL using simple sequence repeat (SSR), sequence-tagged sites (STS) and diversity arrays technology (DArT™) markers. Due to low density of mapped SSR markers in these regions, DArT markers were found to be closely linked to both the genes, however, DArT markers being dominant in nature were not suitable for marker-assisted introgression of these genes from *T. boeoticum* to *T. aestivum*.

Recent advances in chromosome arm-based shotgun sequencing of wheat genome have generated immense genomic resources for development of large number of new markers (IWGSC 2014). Wheat chromosome 7AL is anchored with more than 35,000 contigs which present a huge resource for marker development. Gene-containing contigs identified by homology search of orthologous *Brachypodium distachyon* chromosome(s) were used to identify disease resistance genes followed by marker development. Here, we report further fine mapping of two PM resistance genes and identification of closely linked STS markers suitable for marker-assisted selection.

Materials and methods

Plant material

The plant material used for mapping of the powdery mildew resistance genes consisted of a set of 148 recombinant inbred lines (RILs) derived from *T. boeoticum* acc. pau5088/*T. monococcum* acc. pau14087. For brevity, the *T. boeoticum* acc. pau5088 and *T. monococcum* acc. pau14087 will hereafter be referred to as *Tb*5088 and *Tm*14087, respectively. Detailed information on these accessions and a molecular linkage map generated using this population was described by Singh et al. (2007b) and is also available at http://wheat.pw.usda.gov/ggpages/map_summary.html.

Evaluation of selected RILs for powdery mildew resistance

Details of the screening of the RIL population against three powdery mildew isolates were described in Chhuneja et al. (2012). In the present study, 12 RILs possessing *PmTb7A.2* and 12 RILs with *PmTb7A*.1 (Table 1), selected based on flanking markers identified in Chhuneja et al. (2012), were screened with nine *Bgt* isolates available at the Institute of Plant Molecular Biology, University of Zurch, Switzerland. These nine isolates exhibit differential reactions for known *Pm1* alleles (Hurni S– unpublished). Six wheat lines Axminster/8*Cc, MocZlatka, Weihestephan M1N, WW and Virest, carrying alleles *Pm1a, Pm1b, Pm1c, Pm1d,* and *Pm1e*, respectively (Table 1) were included to investigate whether the *PmTb7A.2* is one of the existing alleles of *Pm1*, a new allele of *Pm1* or indeed a different locus.

Marker development from shotgun sequence data

DNA from individual wheat chromosome arms was isolated from double ditelocentric stocks of wheat line Chinese Spring (Sears and Sears 1978) using the flow-sorting technique (Dolezel et al. 2007). Shotgun sequence data of flow-sorted 7AL, generated using the Illumina platform by the International Wheat Genome Sequencing Consortium (IWGSC) (IWGSC 2014, http://wheat-urgi.versailles.inra.fr/Projects) were used to design 7AL-specific markers. In this, the 7AL contigs were aligned against genic sequences

of the orthologous chromosome regions of *Brachypodium* and the wheat contigs covering 7AL uniformly were selected for further analysis. The selected contigs were analysed for presence of genes using FGenesh (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). Conserved motifs in disease resistance genes, i.e. NB-ARC and LRR were screened from protein sequences of predicted genes using the online tool SMART (http://smart.embl-heidelberg.de/help/smart_about.shtml) and confirmed with the LRRfinder (http://www.lrrfinder.com/). The Prot_Map (http://linux1.softberry.com/berry.phtml?topic=prot_map&group=programs&subgroup=xmap) was used to align protein sequences with their corresponding nucleotide sequences. The contigs with significant hits for conserved sequences of resistance gene analogues (RGA) were considered and used for primer design using PerlPrimer v.1.1.21. The identified RGA-STS (Sequence-Tagged Sites) markers were further analysed, using in house perl scripts, for estimating melting temperature of the primers. The selected contigs were also used to find SSRs through MIcroSAtellite (MISA) identification tool (Thiel et al. 2003) and SSR primers were designed using PerlPrimer v.1.1.21 (Marshall 2004). The primer sequences and amplification conditions are presented in Supplementary Table S1. All forward primers were synthesized with M13 tail sequence.

Mapping of SSR and RGA-STS markers

DNA from parents and the RIL population was extracted following the CTAB method as described in Singh et al. (2007b). Forty sequence-tagged microsatellite (STMS) or SSR marker primer pairs were designed from selected contigs and 73 STS primer pairs were designed from the sequences flanking NBS/LRR domains in the selected contigs. All primers were tested for amplification in Chinese Spring (since primers were designed from shotgun sequence data of Chinese Spring), *Tb*5088 and *Tm*14087. PCR were performed in 20 µl aliquots containing 35 ng and 60 ng of template DNA, for SSRs and RGA-STS markers, respectively, 0.125 mM of each dNTPs, 1X buffer (10 mMTris-HCl - pH 8.3, 50 mMKCl, 1.5 mM $MgCl_2$), 0.25 µM of each primer and 0.5U of Taq DNA polymerase. Thermal cycling conditions were: 94 °C for 4 min followed by 34 cycles of 94 °C for 60 s, 55–60 °C (depending on the primer combination) for 60 s, 72 °C for 90 s for SSR and 150 s for STS primers, followed by an elongation step of 7 min at 72 °C. Amplification was tested by resolving PCR products in 1.5–2.0 % agarose gel. Primers showing amplification in the *Tm*14087 and *Tb*5088 were then analysed in a LI-COR 4200 DNA analyser (http://biosupport.licor.com/docs/Applications_Manual). PCR in 10 µl aliquots was performed with 0.05 µM M13 tailed forward primer, 0.2 µM reverse primer and 0.05 µM M13-IRD 700 or M13-IRD 800. The reaction mix was run in 6 % polyacrylamide gels after diluting the original PCR product to 10:1 with formamide tracking dye.

Conversion of RGA-STS to CAPS markers

The RGA-STS markers that were not polymorphic in the parental lines *Tb5088* and *Tm14087* were converted to cleaved amplified polymorphic sequence (CAPS) markers. In this, the amplicons from *Tb5088* and *Tm14087* were sequenced directly on ABI 3730 sequencing machine. CLC genomics workstation (http://www.clcbio.com/products/clc-genomics-workbench/) was then used to identify differential restriction sites in the amplicons of *Tb5088* and *Tm14087*. Restrictions were performed in 25 µl aliquots containing 10 µl PCR product and 15 µl of restriction enzyme cocktail (2.5 µl 10× buffer, 5U restriction enzyme) for overnight at 37°C. The restriction products were resolved on 1.5 % agarose after adding 5 µl loading dye.

SNP discovery and HRM analysis

SNPs between *Tb5088* and *Tm14087* were detected from the shotgun sequencing data of *T. boeoticum* acc. pau5088 (15×) and *T. monococcum* acc. pau14087 (25×) generated at Kansas State University. Chinese Spring gene-containing sequence contigs which mapped on 7AL were repeat masked and BLAST searched against *T. monococcum* assembly and the best BLAST hits were then aligned to *T. boeoticum* contigs for SNP calling. The SNPs were filtered for depth (5 reads) and base consensus ratio (0.9). Out of 37 contigs, 32 showed good BLAST hits and a total of 49 SNPs were identified between *Tb*5088 and *Tm*14087. Primers for these SNPs were designed and used for mapping through high-resolution melting (HRM) curve analysis in a Bio-Rad CFX96 Touch Real Time PCR Detection System. Parental survey for HRM was conducted in 12 µl PCR aliquots containing 30 ng template DNA, 200 nM of forward and reverse primers, 6 µl of SsoFast™ EvaGreen® supermix (Bio-Rad). SNPs showing differential melt curves were then analysed on the RIL population.

Linkage mapping

A linkage analysis of polymorphic marker loci in the RILs was conducted with MAPMAKER (Lander et al. 1987; Lincoln et al. 1993). Recombination frequencies were converted to centi Morgans (cM) using the Haldane mapping function (Haldane 1919). Multipoint analysis was used at a LOD threshold of 5.0 and maximum recombination fraction of 0.30. Polymorphic markers were ordered using the 'compare' command. Additional markers were added afterwards on this frame using the 'try' command. The final

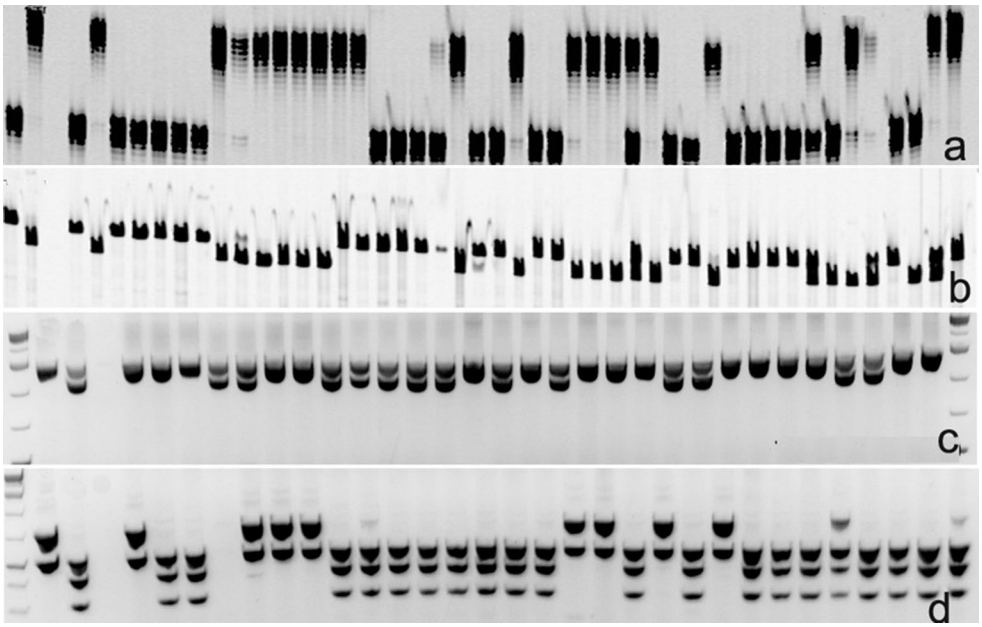

Fig. 1 PCR amplification profile of markers developed from shotgun sequence data of 7AL. **a** Ta7AL-4554964 (SSR), **b** Ta7AL-4556232 (STS), **c** Ta7AL-4437049 (RGA-STS-CAPS), **d** Ta7AL-4444971(RGA-STS-CAPS). *Lanes 1*, *2* and *3* in all the four panels represent *T. monococcum*, *T. boeoticum* and negative control and the remaining lanes represent the RILs

order was verified with the 'ripple' command with a window size of five and LOD threshold of 5.0. The linkage map was finally drawn using the software Mapchart, Version 2.1 (Voorrips 2002).

QTL mapping

Since the population segregated for two powdery mildew resistance genes, Mapmaker could not be used for mapping of the powdery mildew resistance genes in this population. In QTL analysis, phenotypic data for three *Bgt* isolates (reported in Chhuneja et al. 2012) were included along with the genotypic data of the RILs. Linkage map of chromosome 7A consisting of 101 markers was used for mapping QTL using algorithm WinQTL Cartographer v.2.5 (Wang et al. 2012). The positions and effects of the QTL were determined using composite interval mapping (CIM). The significant threshold logarithm of the odds (LOD) scores for detection of the QTL was calculated based on 1000 permutations at $P \leq 0.05$ (Churchill and Doerge 1994). The proportion of observed phenotypic variation explained due to a particular QTL was estimated by the coefficient of determination (R^2) using maximum likelihood for CIM.

Comparative mapping

To validate the prediction of RGAs identified from 7AL Chinese Spring contigs and used to design primers, the selected sequences were BLAST searched against *Brachypodium* and *Oryza* genomes. Also, to understand syntenic relationship of *T. monococcum* and *T. boeoticum* with *T. urartu* sequences, protein data set of *T. urartu* (http://gigadb.org/dataset/100050) was downloaded and BLAST searched against the RGA sequences of *Tm14087* and *Tb5088* using BLASTX. Orthologous genes identified from *T. urartu* were run with Interproscan (Jones et al. 2014) to identify different domains and compared these with the *T. boeoticum* and *T. monococcum* sequences.

Results

PM reaction of selected RILs

The RIL population generated from the cross involving PM-resistant *Tb*5088 and PM susceptible *Tm*14087 showed segregation for two powdery mildew resistance genes. Both genes mapped on the long arm of chromosome 7A. The two powdery mildew resistance genes, designated as *PmTb7A.1* and *PmTb7A.2*, provided complete resistance to three *Bgt* isolates (used in our earlier study—Chhuneja et al. 2012) individually as well as in combination. *PmTb7A.2* was proposed to be allelic to *Pm1* as it mapped to the same marker region as *Pm1*. However, it could not be determined if it was a new allele of *Pm1* or new gene closely linked to *Pm1*. To resolve this, PM-resistant RILs with either *PmTb7A.1* or *PmTb7A.2* gene, distinguished based on linked flanking markers, were screened against nine *Bgt*

Table 1 The reaction of *T. boeoticum/T. monococcum* RILs to nine *Bgt* isolates showing differential reaction against different *Pm1* alleles

S. no.	Line	*Pm* gene/allele	*Bgt* isolate								
			07230	Asosan 95.9	98229	07201	07298	97011	07286	07302	C3-1
1	Axminster/8*Cc	*Pm1a*	V	V	V	V	V	V	A	A	A
2	MocZlatka	*Pm1b*	V	V	V	V	V	A	A	A	A
3	Weihestephan M1N	*Pm1c*	V	V	V	V	A	V	A	A	A
4	WW	*Pm1d*	V	V	V	V	V	V	A	A	A
5	Virest	*Pm1e*	V	V	V	V	V	V	A	A	A
6	RIL4	*PmTb7A.2*[a]	A	A	A	A	A	A	A	A	A
7	RIL9	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
8	RIL17	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
9	RIL19	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
10	RIL20	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
11	RIL62	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
12	RIL80	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
13	RIL102	*PmTb7A.2*	A	A	–	A	A	A	A	A	A
15	RIL106	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
16	RIL107	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
17	RIL117	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
18	RIL127	*PmTb7A.2*	A	A	A	A	A	A	A	A	A
19	RIL2	*PmTb7A.1*[a]	A	A	A	A	A	A	A	A	A
20	RIL11	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
21	RIL14	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
22	RIL15	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
23	RIL29	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
25	RIL49	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
26	RIL64	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
28	RIL65	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
29	RIL72	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
30	RIL94	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
31	RIL124	*PmTb7A.1*	A	A	A	A	A	A	A	A	A
33	RIL128	*PmTb7A.1*	A	A	A	A	A	A	A	A	A

V virulent, *A* avirulent

[a] Gene designations are as per Chhuneja et al. (2012)

isolates which show differential reaction on the lines with different *Pm1* alleles (Table 1). Four *Bgt* isolates *Bgt*07230, *Bgt* Asosan95.9, *Bgt*98229 and *Bgt*07201 virulent to all five designated *Pm1* alleles were avirulent on all RILs with *PmTb7A.2* (Table 1). Indeed, all the nine *Bgt i*solates were avirulent on the RILs carrying *PmTb7A.2* (Table 1), thereby conclusively proving that *PmTb7A.2* is either a new allele of *Pm1* or a new locus very closely linked to *Pm1*.

Enrichment of the genetic map of chromosome arm 7AL

Forty SSR and 73 RGA-STS markers were used for amplifying genomic DNA of Chinese Spring, *Tm14087* and *Tb5088*. Out of the 40 SSR primer pairs tested, 36 showed amplification in Chinese Spring, whereas only 19 of these showed amplification in both *Tm14087* and *Tb5088*, and three primers showed amplification in *Tb5088* but not in *Tm14087*. Overall, 22 SSR markers showed amplification in *Tm14087* and/or *Tb5088* but only 10 were polymorphic between *Tb5088* and *Tm14087* (Fig. 1a) and eight of these were mapped on chromosome 7A. Likewise, out of the 73 RGA-STS primers tested, 67 showed amplification in Chinese Spring but only 36 of these showed amplification in both *Tm14087* and *Tb5088*. Four primer pairs showed amplification only in *Tb5088* and two showed amplification only in *Tm14087* and hence could be mapped as dominant markers. Out of the 36 primers amplifying in both the parental lines, five were mapped as co-dominant markers after resolving on LI-COR 4200 Genotyping Platform (Fig. 1b) and 11 primers were mapped as CAPS markers on the RIL population (Fig. 1c, d). Overall, 21 RGA-STS markers could be mapped

on 7AL. Each of the RGA-STS markers mapped here depicted the location of a putative disease resistance gene. In addition, 13 SNP markers were analysed on the population using HRM and were mapped on 7AL. An integrated linkage map of 7AL incorporating new SSR, RGA-STS and SNP markers to the map reported in Singh et al. (2007b) and Chhuneja et al. (2012) is presented in Fig. 2. The marker sequences, annealing temperatures, resolving media and restriction enzymes used for the mapped markers are summarised in supplementary Table S1.

Fine mapping of the *Pm* genes

As many as 101 markers on chromosome 7AL, including SSR, SNPs, DArT, STS, and RGA-STS based on shotgun sequence data were used for fine mapping of the *Pm* genes, identified by Chhuneja et al. (2012). The CIM module of WinQTL cartographer detected the same chromosomal regions associated with PM resistance on 7AL as reported in Chhuneja et al. (2012) but with more refined intervals and better markers which are suitable for MAS). *PmTb7A.1* mapped in the region flanked by DArT marker *wPt860777*and *wPt0961* in a 0.1 cM interval with the closest RGA-STS marker *Ta7AL-4556232_rga* at a distance of 0.6 cM with high LOD score and R^2 values for all the three *Bgt* isolates (Table 2; Fig. 3). *PmTb7A.2* mapped in a 0.3 cM marker interval flanked by DArT markers *wPt375859* and *wPt3004* (Table 2; Fig. 3). The STS markers *Xmag1759* and *Xmag2185*, reported to be linked with *Pm1* (Yao et al. 2007), map 0.6 cM from left flanking marker *wPt375859* and 0.3 cM from the right flanking marker *wPt3004*, respectively.

Since our initial linkage map was based on 90 RIL only, the closest RGA markers *7AL-4556232_rga* for *PmTb7A.1* and *7AL-4426363_rga* and other co-located markers for *PmTb7A.2* were then analysed on a population consisting of 144 RILs (Table 3) so as to identify markers which can be readily used for MAS. Out of 99 PM-resistant RILs, 32 showed *Tb5088* specific segments harbouring both the *Pm* genes, 33 had *Tb5088* segments with *PmTb7A.1* only and 29 had *PmTb7A.2* segment only, three were heterozygous and two did not have *Tb5088* specific at either of the loci, thereby indicating recombination between the gene and the marker (Table 3). Out of 45 susceptible RILs, two were heterozygous for *PmTb7A.2*, whereas none of the susceptible RILs showed the signature for the presence of *PmTb7A.1* at *7AL-4556232_rga*.

Comparative mapping of *T. monococcum* and *T. boeoticum* RGA loci with *Oryza* and *Brachypodium* genomes

Twenty-three Chinese Spring sequence contigs of chromosome 7AL from which RGA-STS and SSR markers were developed and nine *T. monococcum* contigs carrying SNP markers were used as queries for searching syntenic regions in *Brachypodium* and *Oryza* genomes. Twenty-four *Brachypodium* genes were orthologous to these contigs and the majority of these genes were located on *Brachypodium* chromosomes 4 and 1 (Supplementary Figure 1). Four genes on *Brachypodium* chromosome 2 and three on chromosome 3 were also orthologous to 7AL sequences. Similarly, Chinese Spring contigs showed orthology with 21 rice sequences for chromosomes 2, 5, 6, 8, 10 and 11 and one sequence each on rice chromosomes 1, 4 and 12. The Chinese Spring contigs encoding NBS-LRR domains showed homology with *Brachypodium* and *Oryza* orthologues encoding NBS-ARC domains characteristic of disease resistance proteins (Supplementary Table 2). All RGA-STS markers showing amplification in *Tm14087* and *Tb5088* and mapped on 7AL were sequenced. The amplicon sequences from *Tm14087* and *Tb5088* were then aligned with the *T. urartu* draft sequence (Ling et al. 2013) using BLASTx and BLASTn. The corresponding sequences in *T. urartu* were used for gene prediction. *Tm14087* and *Tb5088* amplicon sequences from 19 RGA-STS markers identified common homologues in *T. urartu* sequences. The identities of homologous *T. urartu* sequences and corresponding gene annotations are summarised in Table 4. Comparative analysis of the sequences of chromosome 7AL of hexaploid wheat and diploid A genome species *T. boeoticum* and *T. monococcum* with the genomes of *T. urartu*, *Brachypodium* and *Oryza* revealed that the RGA sequences were conserved across the species. This also validated predicted RGA sequences from the draft survey sequence of chromosome 7AL of hexaploid wheat.

Identification of markers for marker-assisted selection

Despite mapping 42 additional SSR, RGA-STS and SNP markers, the nearest flanking markers for the two PM resistance genes were still the DArT markers, which are not suitable for MAS until converted to SCAR markers. However, the nearest RGA-STS and SNP markers were close enough to be used for marker-assisted selection. RGA-STS marker *Ta7AL-4556232_rga* is linked to *PmTb7A.1,* with a recombination distance of 0.6 cM. Marker *Ta7AL-4556232_rga* amplified a fragment of 1200 bp in *Tm14087* and this amplicon had a 19 bp InDel in the *Tb5088* and also a number of SNPs (Supplementary Figure 2). The *Ta7AL-4556232_rga* sequence of *Tm14087* also had a restriction site for *Dra*I, whereas *Tb5088* lacked this restriction site. When the PCR amplicons were restricted with *Dra*I, it generated 900 and 300 bp fragments in the *Tm14087* (Fig. 4b). On the other hand, in various *T. aestivum* and *T. durum* cultivars, this marker amplified a fragment which is higher in

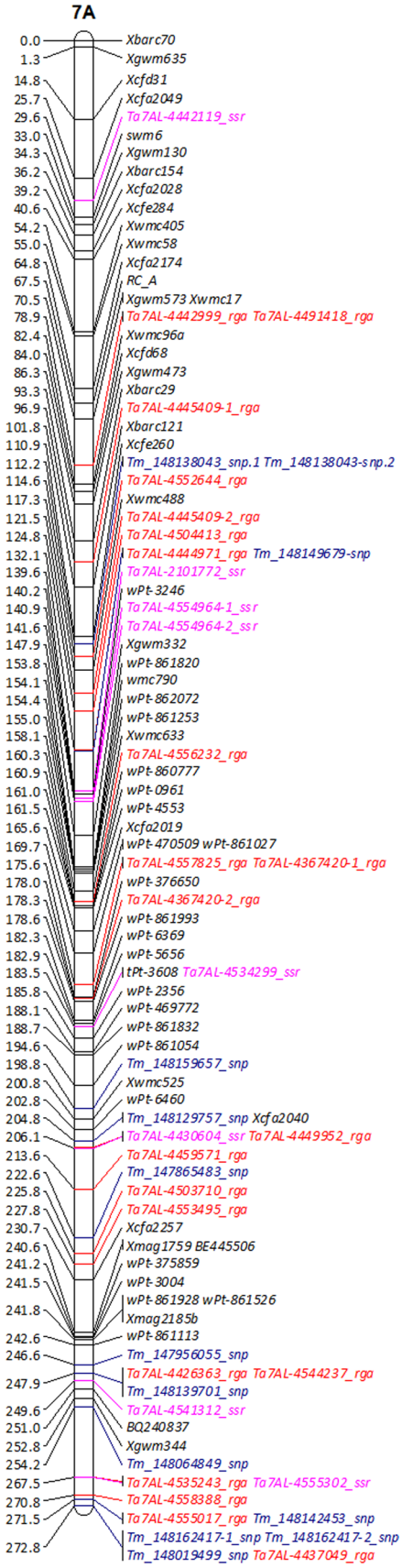

Fig. 2 Integrated linkage map of 7AL consisting of markers developed from shotgun sequence assembly; Markers in *red* colour are RGA-STS, in *magenta* SSRs and in *blue* are those mapped as SNPs. Markers in *black* represent the ones from Singh et al. (2007b) and updated in Chhuneja et al. (2012)

molecular weight than that of *Tm14087* and *Tb5088* amplicons and showed polymorphism in agarose gel. It can also be used as CAPS marker for marker-assisted introgression of the *PmTb7A.1* from *Tb5088* to bread wheat.

Likewise, for the PM resistance gene *PmTb7A.2*, the nearest flanking markers were *wPt-375859* and *wPt-3004* (Table 2, Fig. 3). The nearest STS, STS-RGA or SNP markers were *Xmag1759, Xmag2185, Tm_147956055_snp*, *Ta7AL-4426363_rga* and *Ta7AL_4544237_rga* encompassing a 7.3 cM region. The *PmTb7A.2* is putatively a novel allele of *Pm1*; hence, markers linked to *Pm1* can also be used for MAS. However, we identified new markers, i.e. the RGA-STS markers *Ta7AL-4426363_rga* and *Ta7AL-4544237_rga* which carried number of SNPs in addition to the restriction site for enzymes *Hph*I and *Taq*I which were used for detecting polymorphism between parental lines (Fig. 4). The primer sequences and resolution conditions of the markers identified for MAS are presented in Table 5. These two RGA-STS markers also exhibited polymorphism with different bread and durum wheat cultivars. The new set of linked RGA-STS markers for both the powdery mildew resistance genes *PmTb7A.1 and PmTb7A.2* have been used for marker-assisted transfer of the two PM resistance genes from *Tb5088* to hexaploid wheat (Elkot et al. 2015).

Discussion

Sufficient variability exists in wild species of wheat, especially for disease resistance genes, however, many of the genes could not be used commercially due to linkage drag (Olson et al. 2010) but fine mapping in wild species gives us leverage for precise transfer of the genes from wild to cultivated types (Periyannan et al. 2011). Once mapped in wild progenitor species, these genes can be transferred to hexaploid wheat with minimum of the linked alien chromatin to ensure its commercial utility. *T. urartu*, the A genome donor of bread wheat and other related A genome species *T. monococcum* and *T. boeoticum*, have been a rich source for powdery mildew resistance genes. The long arm of wheat chromosome 7AL is known to carry several disease resistance genes including *Pm1*, *Pm9, Pm37, Lr20, Sr15, and Sr22* (McIntosh et al. 2013, Ji et al. 2008). In addition, many more undesignated *pm* genes NCA6*Pm*, from *T. monococcum* (Murphy et al. 1999), *mlRD30* from *T. aestivum* (Singrun et al. 2004), *PmU* from *T. uratu* (Qui

Table 2 Summary of composite interval mapping of powdery mildew resistance genes in *T. boeoticum/T. monococcum* RIL population based on an enriched 7AL map

Locus	Marker interval (cM)	Flanking markers	*Bgt*07201		*Bgt*97011		*Bgt*07302	
			LOD (3.3)[a]	R^2 (%)	LOD (3.3)	R^2 (%)	LOD (3.2)	R^2 (%)
PmTb7A.1	0.1	*wPt-860777–wPt-0961*	13.6	33.2	13.1	31.3	18.6	38.0
PmTb7A.2	0.3	*wPt-375859–wPt-3004*	12.5	23.4	10.6	21.1	12.5	21.2

[a] values in parentheses are LOD threshold values

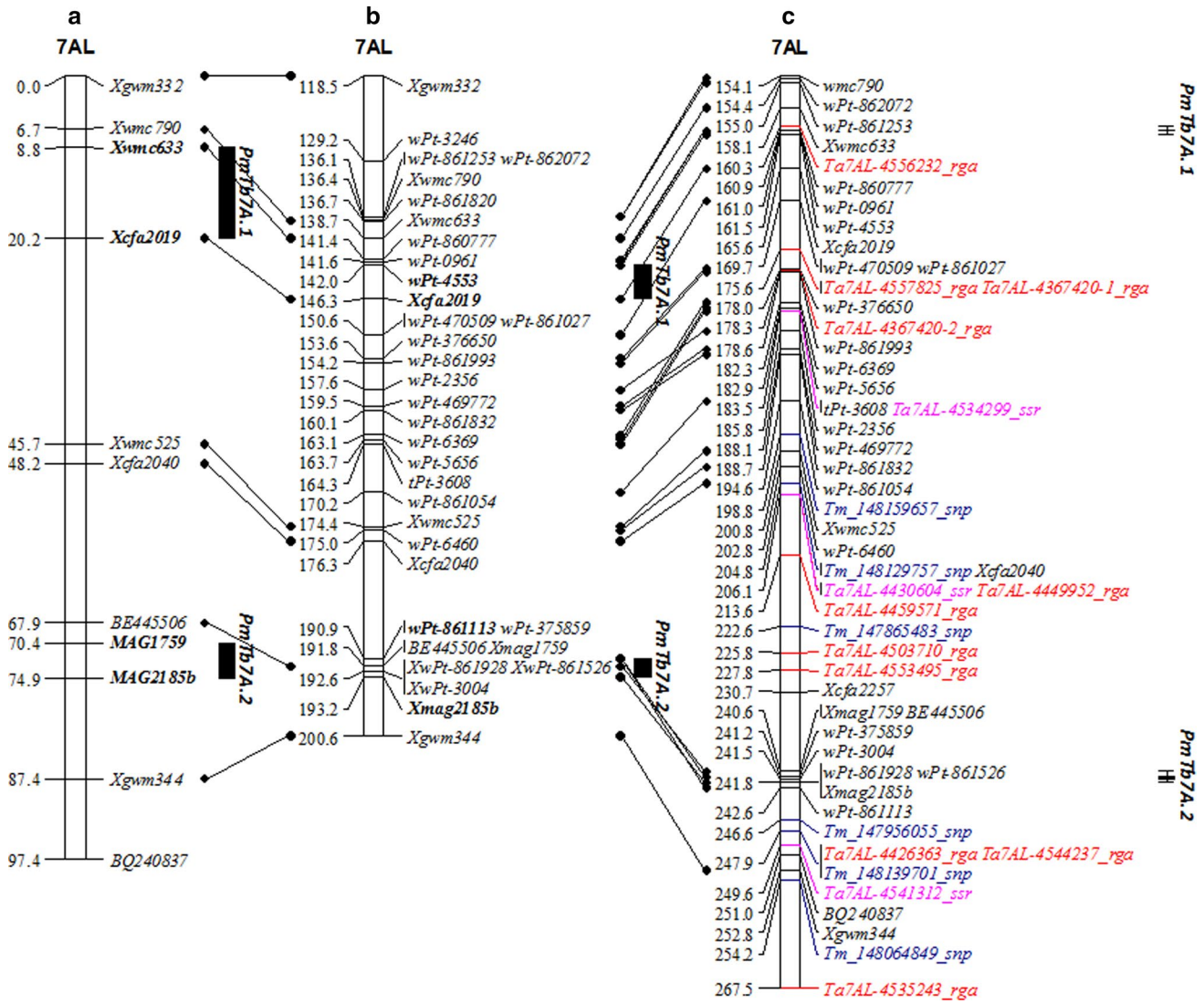

Fig. 3 Comparative partial linkage maps of chromosome 7AL based on SSR, DArT, STS and RGA –STS markers **a** SSR and STS **b** SSR, STS and DArt (Chhuneja et al. 2012) and **c** SSR, STS, DArT, RGA and SNP markers showing chromosomal location of significant QTL for powdery mildew resistance in *T. boeoticum/T. monococcum* RIL population

et al. 2005), *Mlm2033* and *Mlm80* from *T. monococcum* (Yao et al. 2007), *mllw72*, *MIWE18*, and *PmG16* from *T. dicoccoides* (Ji et al. 2008; Han et al. 2009; BenDavid et al. 2010) have been mapped on 7AL. In an earlier study, we identified two powdery mildew resistance genes *PmTb7A.1* and *PmTb7A.2* in *T. boeoticum* acc. pau5088 and mapped these on chromosome 7AL using SSR, EST and DArT markers (Chhuneja et al. 2012). However, closely linked markers suitable for marker-assisted selection could not be identified. In the present study, we developed a series

Table 3 Distribution of the *T. boeoticum/T. monococcum* RILs at two *Pm* resistance gene regions depicted by the closest RGA-STS markers

Marker allele	*Ta7AL-4556232_rga*			
	Resistant			Susceptible
	B[a]	H	A	A
Ta7AL-4426363_rga and *Ta7AL-4544237_rga*				
B	32	–	29	–
H	1	–	–	2
A	33	2	2	43
Total	66	2	31	45

[a] B, A, and H indicate *Tb5088*, *Tm14087* and heterozygous alleles, respectively

of SSR, RGA-STS and SNP markers from the shotgun sequence assembly of chromosome 7AL (IWGSC 2014) and mapped eight additional SSR markers from gene-containing contigs, 21 RGA-STS and 13 SNP markers to the linkage map of 7AL. All the RGA-STS markers mapped on 7AL represent putative disease resistance genes.

The PM resistance gene *PmTb*7A.2 (Chhuneja et al. 2012) mapped closer to markers *Xmag2185, Xmag1759* and *BE445506*. These three markers are also linked to *Pm1* (Yao et al. 2007) thus indicating that *PmTb*7A.2 might be allelic to *Pm1*. However, *Pm9* identified in *T. aestivum* and *Pm37* identified in *T. timopheevi* are also closely linked to *Pm1* (Perugini et al. 2008). *Pm1* has multiple alleles *Pm1a, Pm1b, Pm1c, Pm1d and Pm1e* (McIntosh et al. 2013). *Pm1b* and *Pm1c* were identified in *T. monococcum (*Hsam et al. 1998), *Pm1d* in *T. spelta* (Hsam et al. 1998) and *Pm1c* and *Pm1e* in *T. aestivum* (Sears and Briggle 1969; Singrun et al. 2004). Qui et al. (2005) mapped *PmU*, transferred from *T. uratu*, on 7AL flanked by the markers *Xcfa2040* and *Xcfa2257*. Marker *Xcfa2257* mapped 10.8 cM proximal to marker wPt-375859 in our linkage map (Figs. 2 &3, 4) which flanks left of *PmTb7A.2* and *Xcf2257* maps in deletion bin 7AL-17 (Sourdille et al. 2004). Thus, the PM resistance genes *Pm1*, *Pm9*, *Pm37*, *PmU* and *PmTb7A.2* might be a gene complex very closely linked to each other.

In the present study, screening of a set of RILs carrying only *PmTb*7A.2 against nine *Bgt* isolates, having differential virulence/avirulence reaction on lines carrying *Pm1a, Pm1b, Pm1c, Pm1d and Pm1e*, established that *PmTb7A.2* is different from the earlier reported alleles of *Pm1*. Not conclusively, but most evidences from mapping and phenotypic reaction support that it might be an additional allele of *Pm1* but different from *Pm1a* already identified from *T. monococcum*. This new allele of *Pm1* confers resistance against the *Bgt* isolates present in North India in addition to the Swiss isolates. The *PmTb7A.2* is a potential source of resistance for PM in India and Europe and has been transferred to hexaploid wheat using linked RGA-STS-markers identified in the present investigation (Elkot et al. 2015). Markers *Xmag2185, Xmag1759* and *BE445506* which map closer to *PmTb7A.2* (Yao et al. 2007; Chhuneja et al. 2012) at a distance of 0.6 and 0.3 cM on either side are not usable for MAS, as these yield multiple bands. Enrichment of the region with STS and SNP markers led to identification of robust markers for MAS. SNP marker *Tm_147956055_snp* developed from *T. monococcum* and *T. boeoticum* sequence, and two co-located RGA-STS markers *Ta7AL-4426363_rga* and *Ta7AL-4544237_rga* (mapped as CAPS) at a distance of 5.1 and 6.4 cM, respectively, could be used for MAS (Elkot et al. 2015).

The second gene *Pm7A.1* was mapped between DArT markers *wPt-86077* and *wPt-0961* (Chhuneja et al. 2012). However, with the additional markers developed from NextGen shotgun sequence assembly (IWGSC 2014), we identified a robust RGA-STS marker *Ta7AL_4556232_rga* which is only 0.6 cM from *PmTb7A.1*. Thus, *PmTb7A.1* clearly represents a new gene as no other designated PM resistance gene is mapped in this region, except for NCA6*Pm* from *T. monococcum* (Murphy et al. 1999) which maps on chromosome 7AL flanked by the markers *Xcfa2019* and *Xbarc121* (Miranda et al. 2007). *PmTb7A.1* in the present study maps 4.6 cM proximal to *Xcfa2019* (Fig. 3), whereas NCA6Pm co-segregated with *Xcfa2019* as reported by Miranda et al. (2007). The linkage map of chromosome 7A generated from the *T. boeoticum/T. monococcum* RIL population in the present study spans 272 cM, which is larger compared to 7A map of hexaploid wheat being 131 (Somers et al. 2004), 146.7 (Song et al. 2005), 129.7 cM (Jing et al. 2009). This might be because of higher recombination rate in diploid A genome species compared to the A genome of the hexaploid wheat (Singh et al. 2007a). Thus, the markers identified for MAS of *PmTb7A.1* and *PmTb7A.2* may show less recombination in hexaploid wheat compared to the diploid wheat background.

All newly mapped RGA loci on 7AL represent the location of putative candidate disease resistance genes encoding NBS-LRR domains as ascertained from orthologous gene annotations in *Brachypodium*, *Oryza* and *T. urartu.* Genes containing these domains are reported to be the most abundant disease resistance genes in higher plants. For example, *Ta7AL-4556232_rga* maps between the SSR markers that are closely linked to *PmTb7A.1* and stem rust resistance gene *Sr22*. It is orthologous to *Brachypodium* gene *Bradi2g39537* that encodes an NBS-ARC domain and rice gene Os11g14380.1 coding for a protein similar to the wheat LR10 resistance protein (Feuillet et al. 2003). Thus, *Ta7AL-4556232_rga* could be one of these disease resistance genes. Similarly, the co-localized RGA-STS markers *Ta7AL-4426363_rga* and *Ta7AL-4544237_rga*

Table 4 Comparative mapping of *T. monococcum* and/or *T. boeoticum* resistance gene analogue sequences against *T. urartu* draft sequence (Ling et al. 2013)

S. No.	Sequence ID[a]	*T. urartu* contig	Functional domains of the *T. urartu* gene
1	Tm7AL-4426363_rga	TRIUR3_01038	Leucine-rich repeats (2 copies)/NB-ARC domain
	Tb7AL-4426363_rga		
2	Tm7AL-4442999_rga	TRIUR3_26800	Leucine-rich repeats (2 copies)/NB-ARC domain
	Tb7AL-4442999_rga		
3	Tm7AL-3234936_rga	TRIUR3_07080	NB-ARC domain
	Tb7AL-3234936_rga		
4	Tm7AL-4367420_rga	TRIUR3_25914	A receptor for ubiquitination targets/F-box-like
	Tb7AL-4367420_rga		
5	Tm7AL-4426363_rga	TRIUR3_01038	Leucine-rich repeats (2 copies)/NB-ARC domain
	Tb7AL-4426363_rga		
6	Tm7AL-4437049_rga	TRIUR3_33250	NB-ARC domain
	Tb7AL-4437049_rga		
7	Tm7AL-4442999_rga	TRIUR3_26800	Leucine-rich repeats (2 copies)/NB-ARC domain
	Tb7AL-4442999_rga		
8	Tm7AL-4444971_rga	TRIUR3_03897	NB-ARC domain
	Tb7AL-4444971_rga		
9	Tm7AL-4445409_rga	TRIUR3_12795	Leucine-rich repeat - CC (cysteine-containing) subfamily/NB-ARC domain
	Tb7AL-4445409_rga		
10	Tm7AL-4480992_rga	TRIUR3_33250	NB-ARC domain
	Tb7AL-4480992_rga		
11	Tm7AL-4491418_rga	TRIUR3_03454	NB-ARC domain/Leucine-rich repeats, typical (most populated) subfamily
	Tb7AL-4491418_rga		
12	Tm7AL-4504413_rga	TRIUR3_32850	NB-ARC domain
	Tb7AL-4504413_rga		
13	Tm7AL-4526534_rga	TRIUR3_07964	NB-ARC domain
	Tb7AL-4526534_rga		
14	Tm7AL-4543800_rga	TRIUR3_09524	NB-ARC domain
	Tb7AL-4543800_rga		
15	Tm7AL-4552501_rga	TRIUR3_24303	NB-ARC domain
	Tb7AL-4552501_rga		
16	Tm7AL-4552644_rga	TRIUR3_23725	Reverse transcriptase (RNA-dependent DNA polymerase)
	Tb7AL-4552644_rga		
17	Tm7AL-4553759_rga	TRIUR3_17279	NB-ARC domain
	Tb7AL-4553759_rga		
18	Tm7AL-4555017_rga	TRIUR3_33250	NB-ARC domain
	Tb7AL-4555017_rga		
19	Tm7AL-4557311_rga	TRIUR3_06241	NB-ARC domain
	Tb7AL-4557311_rga		

7AL and number represent location of the marker and the contig ID and rga indicates the marker was designed from the NBS-LRR domain

[a] Tm and Tb denotes whether amplicon sequence was from *T. monooccum* or *T. boeoticum*

mapped in the region that carries the gene block *Pm1-Lr20-Sr15* and possibly also *Pm37*. *Ta7AL-4426363_rga* and *Ta7AL-4544237_rga* showed perfect alignment with NB-ARC domain containing orthologs of *Brachypodium* genes *Bradi4g04657* and *Bradi3g60337*, respectively. These RGAs might be representing one of the genes which can be resolved by developing high-resolution mapping populations. Selected RILs carrying either *PmTb7A.1* or *PmTb7*A.2 have been crossed to susceptible *T. monococcum* to develop high-resolution mapping populations with the aim of cloning these two powdery mildew resistance genes.

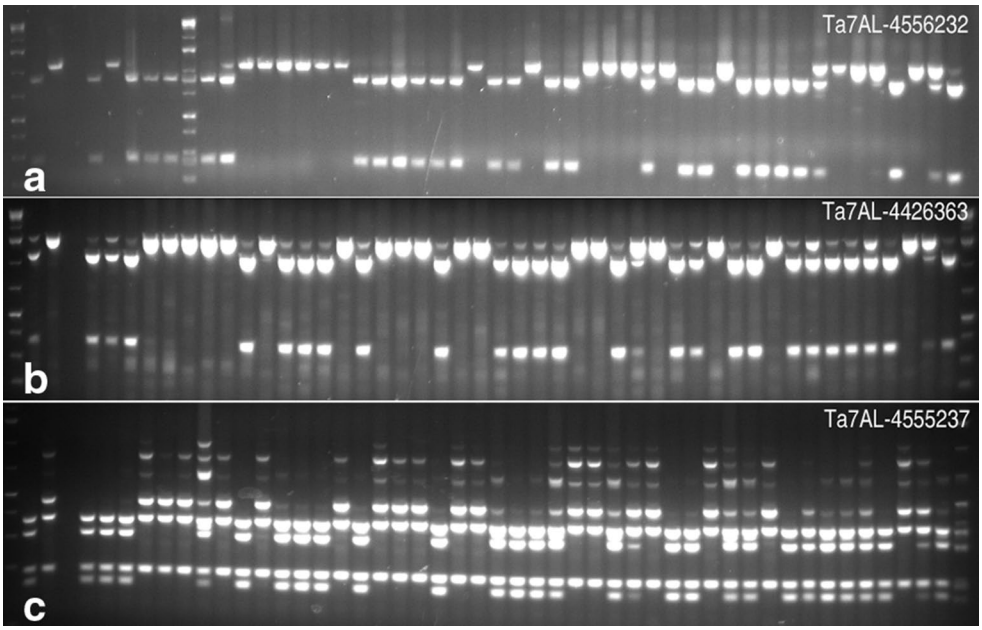

Fig. 4 Amplification profile of the RGA-STS markers linked to *PmTb7A.1* and *PmTb*7A.2 resolved as CAPS markers **a** *Ta7AL-4556232* restricted with RE *Dra*I **b** *Ta7AL-4426363* restricted with RE *Hph*I **c** *Ta7AL-4544237* restricted with RE *Taq*I. Lanes 1, 2 and 3, represent *Tm*, *Tb* and negative control (no DNA), respectively.

Table 5 Primer sequences and amplification conditions of the RGA-STS markers suitable for MAS of *T. boeoticum* powdery mildew resistance genes

Marker ID	Primer	Primer sequence	Marker type	Restriction enzyme
Ta7AL-4556232_rga	F	TTTCAAATAACGGCTTCTGG	19 bp InDel[a]	*DraI*
Ta7AL-4556232_rga	R	GAGACGAGCAAATAGATATGG	CAPS[b]	
Ta7AL-4426363_rga	F	GAATCCTCCAAAGCCTCCAC	CAPS[b]	*HphI*
Ta7AL-4426363_rga	R	GGCATATCTCATGTGAAGAACTG		
Ta7AL-4544237_rga	F	CACTACAATGATGGTAAGCGA	CAPS[b]	*TaqI*
Ta7AL-4544237_rga	R	GCAAGAAGAAACAAGGAGAG		

[a] Resolved on denaturing PAGE in Licor genotyping platform

[b] Resolved on 1.5 % Agarose. Annealing temperature for all the primers is 55 °C and the extension time is 150 s

Author contribution statement PC and KS—Generation of financial resources, development of RIL population, study concept and design, acquisition, analysis and interpretation of data, statistical analysis, preparation of the manuscript; BY–Bioinformatic analysis, primer designing; DS and SH–involved in powdery mildew screening, approval of final draft of the manuscript; SK–maintenance of the RIL population; AFE–data acquisition; BK–study concept and design, supervision and approval of final draft of the manuscript; TW–7AL shotgun sequence data analysis, identification of the contigs and bioinformatics analysis; SS and BS–generated shotgun sequence data of parental accessions and provided the SNP data.

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Compliance with ethical standards

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

Ethical standards All the authors are committed to upholding the integrity of the scientific records and hereby affirm that the manuscript has not been submitted to any other journal. However, it is continuation of an earlier work which is presented in detail in the background information as well as Material and Methods

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