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Wheat Wild Relatives as a Source of RGA Diversity for *Septoria nodorum* Blotch Resistance Breeding

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ABSTRACT

Septoria nodorum* blotch (SNB), caused by *Parastagonospora nodorum*, is a major foliar disease of wheat, and host resistance is controlled by many minor genes. The resistance gene analogue (RGA) TraesCS1B02G003100 was previously identified as a candidate gene in an SNB resistance QTL interval on chromosome 1BS. The aim of this study was to characterize the sequence diversity of this gene in wild and cultivated relatives of bread wheat. Thirty-two accessions of *Triticum* and *Aegilops* and the cultivar 'Dropia' were screened with the 1BR_RGA_4B primers, and a partial genomic fragment (about 0.8 kb) was Sanger-sequenced in 15 accessions. The fragment spanned the 3′ part of exon 2, intron 2 and the 5′ part of exon 3, and encoded the C-terminal end of the LRR domain and the C-terminal tail of the protein. Amplification was obtained only from accessions carrying a B, G or S genome. Within the 755-bp region covered by all sequences, 58 segregating sites and three indels were found, and the 14 unambiguous sequences were grouped into five haplotypes, with uncorrected p-distances of 0.57-5.99%. *T. timopheevii* and *Ae. speltoides* shared a single haplotype, the hexaploid wheats grouped with *Ae. kotschyi*, and *T. turgidum* carried the most divergent haplotype. A 1-bp deletion causing a frameshift was found in *T. sphaerococcum* and in the Chinese Spring reference genome, whereas the other accessions retained the full reading frame. These polymorphisms are candidates for the development of allele-specific markers for SNB resistance breeding.

Keywords: *Triticum timopheevii*, *Aegilops*, resistance gene analogue, *Septoria nodorum* blotch, haplotypes.

INTRODUCTION

Septoria nodorum blotch (SNB) is a foliar and glume disease of wheat caused by the necrotrophic fungus *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley & Crous, formerly known as *Septoria nodorum* (Berk.) Berk. and *Stagonospora nodorum* (Berk.) E. Castell. & Germano (Quaedvlieg et al., 2013). The disease reduces photosynthetic capacity, grain yield and grain quality (Downie et al., 2021). Reports of fungicide resistance, the spread of reduced tillage, the high evolutionary potential of the pathogen and a changing climate have kept genetic resistance to SNB a breeding priority in many wheat-growing regions (Downie et al., 2021). In Romania, *Zymoseptoria tritici* (Roberge ex Desm.) Quaedvl. & Crous (syn. *Septoria tritici* Roberge ex Desm.) was long regarded as the main *Septoria*-type pathogen of wheat, but *P. nodorum* has gained importance in recent years. This shift has been attributed to its ability to develop over a wider temperature range under a drier climate (Cană and Georgescu, 2019). The agronomic relevance of Septoria diseases in Romanian cereal production is also supported by studies published in Romanian Agricultural Research. Based on 22 years of winter wheat cultivar trials conducted in Brașov, Tican (2005) reported that disease scores for *Septoria* were significantly associated with grain yield in some years, emphasizing the importance of disease resistance when selecting cultivars adapted to specific agroecological conditions. Although the pathogen was not identified to species level in that study, the results provide relevant local evidence of the relationship between Septoria leaf blotch and wheat

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performance under Romanian growing conditions.

Host response to SNB is quantitative and controlled by many minor genes. Part of this response is explained by necrotrophic effector proteins secreted by the pathogen, which interact with the products of host sensitivity genes in an inverse gene-for-gene manner (e.g., SnToxA-*Tsn1*, SnTox1-*Snn1*). However, the contribution of these interactions to field resistance remains under debate, and residual resistance not associated with known sensitivity loci has been identified, which indicates that other host genes also contribute to disease response (Downie et al., 2021). By aligning SNB resistance QTL on chromosomes 1BS and 5BL with the wheat reference genome, Li et al. (2021) identified several candidate resistance genes (R-genes). On 1BS, a gene encoding a putative resistance gene analogue (RGA; TraesCS1B02G003100.1), represented by the marker 1BR_RGA_4B, was highly significantly associated with the QTL QSnl08.daw-1B (LOD = 10.0; $p < 0.001$). Notably, tiling-path primers designed for this gene from the Chinese Spring reference failed to amplify in the susceptible cultivar Millewa, which indicates deletion or substantial sequence divergence of the locus among wheat genotypes (Li et al., 2021).

Resistance gene analogues are a large class of potential R-genes that share conserved domains and structural features. The best-known family is the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family, and markers designed from RGAs are used for mapping, fine mapping and cloning of resistance genes (Sekhwal et al., 2015). The structural and allelic diversity of such genes is therefore directly relevant to resistance breeding.

The importance of incorporating disease resistance into cereal breeding programmes is further illustrated by Romanian research on triticale. Ittu et al. (2007) reported moderate resistance to Septoria in the Romanian triticale cultivars Gorun and Haiduc, evaluated across multiple environments. These findings support the value of assessing disease response alongside agronomic performance in cereal germplasm, although they do not establish resistance specifically to *P. nodorum*.

Wild and cultivated relatives of bread wheat are a major reservoir of this diversity. *Triticum timopheevii* (Zhuk.) Zhuk. (2n = 4x = 28, A^tA^tGG) belongs to the secondary gene pool of wheat. Its A^t genome derives from *Triticum urartu* Tumanian ex Gandilyan, and its G genome from an ancestor related to *Aegilops speltoides* Tausch. This species has already contributed genes for resistance to leaf rust (*Lr18*, *Lr50*), stem rust (*Sr36*, *Sr37*, *Sr40*) and powdery mildew (*Pm6*, *Pm27*, *Pm37*) to bread wheat, and has also been reported as a source of resistance to *Septoria* blotch (King et al., 2022). In a seedling screening of the VIR World Collection, accessions of *T. timopheevii* and of its wild form, *T. timopheevii* subsp. *armeniacum* (Jakubz.) Slageren (syn. *T. araraticum* Jakubz.), were resistant to *P. nodorum* (Tyryshkin et al., 2006). SNB resistance has also been found in partial amphiploids between wheat and *Thinopyrum ponticum* (Podp.) Z.-W. Liu & R.-C. Wang (Oliver et al., 2006). Under field conditions, several *Aegilops* species, including *Ae. speltoides*, showed complete resistance to a range of foliar pathogens (Stoyanov, 2014). Amphidiploids involving *T. timopheevii* and *Ae. speltoides* are valuable bridges for transferring resistance into bread wheat (Stoyanov, 2012), and wheat lines carrying alien genetic material, including from *Ae. speltoides*, have shown improved resistance to leaf diseases (Vyskub et al., 2023).

Despite this potential, sequence variation at SNB-associated candidate R-genes has so far been studied mainly in hexaploid bread wheat, and, to our knowledge, their diversity in wild relatives remains largely unknown. The aim of the present study was to sequence a partial genomic fragment (approximately 0.8 kb) of the candidate RGA gene identified on 1BS, amplified with the 1BR_RGA_4B primers, in 15 accessions of *Triticum* and *Aegilops*. We characterized its nucleotide and deduced amino acid variation and assessed the relationships among the accessions, in order to evaluate wheat wild relatives as a source of RGA diversity for SNB resistance breeding.

MATERIAL AND METHODS

Plant material

Thirty-two accessions of wild and cultivated relatives of bread wheat were used, obtained from IPK Gatersleben (Germany), IHAR (Poland), ICARDA (Lebanon), CIMMYT (Mexico), the National Small Grains Collection (USA), the National Agricultural and Food Centre - Research Institute of Plant Production (NAFC-RIPP, Piešťany, Slovakia), and Agrologica (Mariager, Denmark). The Romanian winter wheat cultivar 'Dropia' (*Triticum aestivum* L.) was used as reference National Agricultural Research and Development Institute Fundulea (NARDI Fundulea). All accessions and 'Dropia' were screened with the 1BR_RGA_4B primers. The accessions that did not produce an amplicon belonged to *T. timopheevii* (3), *Aegilops kotschyi* Boiss. (3), *Ae. tauschii* Coss. (3), *T. urartu*, *T. petropavlovskyi* Udacz. & Migush., *Ae. markgrafii* (Greuter) K. Hammer, *Ae. columnaris* Zhuk., *Ae. lorentii* Hochst., *Ae. searsii* Feldman & Kislev and *Ae. ventricosa* Tausch (one accession each). The 17 samples that produced a single amplicon were sequenced. Two of them, *T. dicoccoides* DIC4 and *T. turgidum* TRG54, were excluded because their reverse reads were of insufficient quality, so the final dataset comprised 14 accessions and 'Dropia' (Table 1). The plants were grown in the experimental field of NARDI Fundulea in the 2023-2024 season. Leaf tissue, collected from several plants per accession, was provided by the Phenotyping and Genotyping Laboratory of NARDI Fundulea as powder ground in liquid nitrogen, and was stored at -80°C until DNA extraction.

Table 1. Accessions of *Triticum* and *Aegilops* analysed in this study

Sample ID	Accession code	Species	Source
Dropia	-	*Triticum aestivum* L. cv. Dropia	NARDI Fundulea
1	AKO6	*Aegilops kotschyi* Boiss. var. *palaestina* Eig	IPK, Germany
3	DIC5/24	*Triticum dicoccoides* (Körn. ex Asch. & Graebn.) Schweinf.	CIMMYT, Mexico
5	TIM45	*T. timopheevii* (Zhuk.) Zhuk. subsp. *timopheevii*	ICARDA, Lebanon
7	TIM25/24	*T. timopheevii*	IHAR, Poland
12	TIM44	*T. timopheevii* var. *viticulosum* Zhuk.	IPK, Germany
15	TIM47	*T. timopheevii* subsp. *timopheevii*	ICARDA, Lebanon
16	ASP21	*Aegilops speltoides* Tausch subsp. *speltoides* var. *speltoides*	IPK, Germany
17	SPH17	*Triticum sphaerococcum* Percival	Agrologica, Denmark
19	TIM23	*T. timopheevii*	IHAR, Poland
21	TRG55	*Triticum turgidum* L.	IHAR, Poland
26	TIM52	*T. timopheevii* subsp. *timopheevii*	NSGC, USA
27	TIM20	*T. timopheevii*	IHAR, Poland
28	TIM30	*T. timopheevii*	IHAR, Poland
32	TIM21	*T. timopheevii*	IHAR, Poland

IPK - Leibniz Institute of Plant Genetics and Crop Plant Research; IHAR - Plant Breeding and Acclimatization Institute; ICARDA - International Center for Agricultural Research in the Dry Areas; CIMMYT - International Maize and Wheat Improvement Center; NSGC - National Small Grains Collection; NARDI - National Agricultural Research and Development Institute. Sample IDs are used throughout the text and figures.

DNA extraction

Genomic DNA was extracted from 30-40 mg of powdered tissue with the innuPREP Plant DNA I Kit-IPC16 (Analytik Jena, Germany) on an InnuPure C16 automated system. Samples were lysed in 500 µl SLS lysis buffer with 20 µl proteinase K at 65°C for 60 min, filtered through prefilter columns, treated with RNase A and processed with the protocol Ext_Lysis_200_C16_04, with an elution volume of 50 µl. DNA concentration and purity were measured with a NanoDrop

1000 spectrophotometer (Thermo Fisher Scientific, USA).

PCR amplification and sequencing

A partial fragment of the candidate RGA gene TraesCS1B02G003100.1 was amplified with the primers 1BRF NRC 4BF (5'-CTGCCAAGGAAGGGATCG-3') and 1BRF NRC 4BR (5'-GACAGCCACTGCCAACATT-3'), which correspond to tile 4 of the tiling path designed by Li et al. (2021). Three other primer pairs of the same tiling path produced multiple amplicons and were not used further. Reactions of 10 µl contained 20 ng genomic DNA, 5 µl Platinum II Hot-Start PCR Master Mix (2X) (Invitrogen, USA) and 1 µl of each primer (10 µM). Cycling consisted of 94°C for 2 min, 35 cycles of 94°C for 15 s, 65°C for 30 s and 68°C for 1 min, and a final extension at 68°C for 2 min. The annealing temperature was optimised by gradient PCR (55-65°C) on 'Dropia' DNA. Amplicons were separated on 1% agarose gels in 1X TAE, stained with SYBR Green I and visualised with a PharosFX imager (Bio-Rad, USA), using the 1 Kb Plus DNA Ladder (Invitrogen) as size marker. Single-band amplicons were purified and sequenced in both directions by Sanger sequencing at CEMIA (Larissa, Greece).

Sequence processing and alignment

Low-quality ends of the forward and reverse reads were trimmed manually by inspection of the chromatograms, the reverse reads were reverse-complemented with EMBOSS revseq, and the two reads of each accession were merged into a consensus sequence with EMBOSS merger (Rice et al., 2000). Ambiguous positions in accessions 17 and 26 were checked on the raw forward and reverse chromatograms. Multiple alignments of the nucleotide sequences were produced in Jalview 2.11.5.2 (Waterhouse et al., 2009) with MAFFT (Katoh and Standley, 2013) and Clustal Omega (Sievers et al., 2011), run through the JABAWS web services (Troshin et al., 2011). The coding frame of each sequence was identified with NCBI ORFfinder, and the deduced amino acid sequences were aligned with the reference protein XP_037445307.1 using MAFFT L-INS-i in the same environment.

Sequence annotation

The identity of the fragment was assessed by BLASTX and TBLASTN searches (Altschul et al., 1997) restricted to *T. turgidum* (taxid 4571). Its position within the encoded protein was determined by mapping it onto the conserved-domain annotation of XP_037445307.1 from the NCBI Conserved Domain Database (Lu et al., 2020). The consensus sequences were compared with the Chinese Spring reference genome (IWGSC, 2018) by BLASTN 2.16.0+ (Altschul et al., 1997) in Ensembl Plants release 63, and exon–intron boundaries within the amplicon were defined from the gene model of TraesCS1B02G003100.1 and the canonical GT–AG splice sites. Figure 2 was drawn in Python with the Matplotlib library (Hunter, 2007).

Diversity and phylogenetic analyses

Segregating sites, indels and haplotypes were determined over the 755-bp region covered by all sequences (positions 41-795 of the nucleotide alignment). Positions with ambiguous bases were not counted, and accession 26, which carried numerous ambiguous positions, was not assigned to a haplotype. Uncorrected p-distances between haplotypes were calculated over the same region in MEGA 12 (Kumar et al., 2024), with pairwise deletion of gaps and ambiguous positions. A neighbour-joining tree (Saitou and Nei, 1987) of all 15 sequences was constructed in Jalview from percentage identity (PID) over the full alignment.

RESULTS AND DISCUSSION

Amplification, sequencing and structure of the fragment

The 1BRF NRC 4BF/4BR primers produced a single amplicon at an annealing temperature of 65°C in 16 of the 32 accessions screened and in cv. Dropia (Figure 1). After quality control, consensus sequences of 771-823 bp

were obtained for 15 accessions. MAFFT and Clustal Omega produced almost identical alignments of 828 positions, which differed only in the placement of a few gaps.

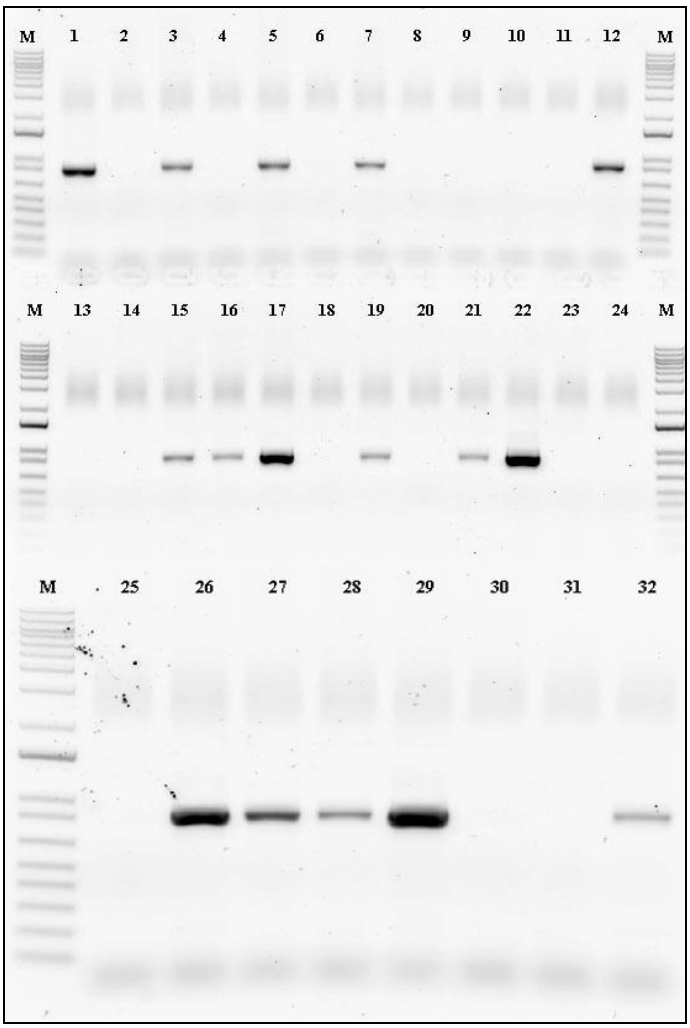


Figure 1. Amplification of the 1BR_RGA_4B fragment with the primers 1BRF NRC 4BF/4BR in the 32 accessions screened. Lane numbers correspond to the Sample IDs in Table 1. M - 1 Kb Plus DNA Ladder (Invitrogen).

BLASTX and TBLASTN searches identified the fragment as part of an NLR-type resistance gene. The best BLASTX hit was VAH11946.1 (63.41% identity, E = 4e-67), and the hits also included RGA1/RGA2/RGA3, RPP13-like proteins and the stem rust resistance protein Sr9e. In TBLASTN, hits included *cre3* and the *Pm68-NLR2* variants. The deduced amino acid sequences aligned with residues 951–1118 of the *T. dicoccoides* RGA2-like protein XP_037445307.1 (1125 aa). The fragment therefore covers the C-terminal end of the LRR region (annotated up to residue 974) and the C-terminal tail of the protein (Figure 2a). At the nucleotide level, the amplicon comprised the 3′ part of exon 2 (about 500 bp), intron 2 (138 bp, bounded by canonical GT-AG splice sites) and the 5′ part of exon 3, which contains the last seven codons and the stop codon followed by part of the 3′ untranslated region (about 150 bp), in agreement with the three-exon IWGSC gene model of TraesCS1B02G003100.1 (Figure 2b). BLASTN searches against the Chinese Spring reference genome returned, for all 15 sequences, a best hit in this gene on chromosome 1B (1B:2,003,754-2,004,577), whereas all other hits were short (≤138 bp) partial matches to other NLR genes. The sequence of *T. sphaerococcum* (17) was identical to this region over its full length (818 bp), whereas ‘Dropia’ differed from it by seven substitutions and a 1-bp indel.

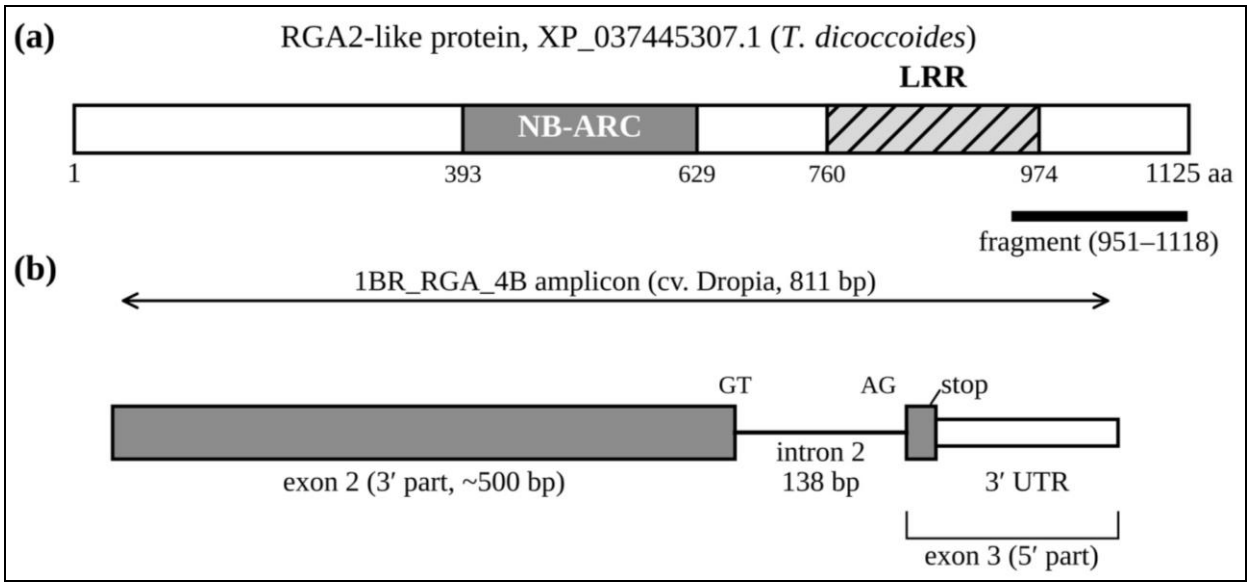


Figure 2. Position of the sequenced fragment. (a) Domain organisation of the RGA2-like protein XP_037445307.1 (*T. dicoccoides*; NCBI CDD annotation) and the region covered by the deduced amino acid sequences; (b) structure of the 1BR_RGA_4B amplicon in cv. Dropia, following the exon-intron organisation of the IWGSC gene model TraesCS1B02G003100.1

The primers of Li et al. (2021) were designed to target genome-specific polymorphisms of the candidate genes, and the results obtained here are consistent with the amplification of a single B-genome locus. Each accession produced a single amplicon, all 15 sequences had their best BLASTN hit in TraesCS1B02G003100, and the sequence of *T. sphaerococcum* was identical to the Chinese Spring reference. All amplified accessions carry a B, G or S genome, whereas no product was obtained from species lacking such a genome (*T. urartu*, *Ae. markgrafii*, *Ae. tauschii*, *Ae. ventricosa*, *Ae. columnaris* and *Ae. lorentii*). Amplification also failed in three *T. timopheevii* accessions, three of the four *Ae. kotschyi* accessions, *Ae. searsii* and *T. petropavlovskyi*, which suggests polymorphism at the primer-binding sites or presence-absence variation of the gene. Li et al. (2021) mapped an RGA from this candidate gene in a 1BS QTL interval linked to *Snn1*, which was associated with SNB resistance in one environment, and reported marked genome rearrangements on 1BS between the cultivars they studied and Chinese Spring. The sequenced fragment covers the C-terminal end of the LRR domain and the C-terminal tail of the protein. The LRR domain of NBS-LRR proteins is highly variable and mediates the protein–protein interactions involved in pathogen recognition (Sekhwal et al., 2015), so the variation described below concerns a region potentially relevant to resistance specificity. The NB-ARC domain and most of the LRR region remained outside the analysed fragment.

Sequence variation and haplotypes

Within the 755-bp region covered by all sequences, there were 58 segregating sites, 32 of them singletons (ambiguous positions of accession 26 not counted), and three indels. First, there was a 50-bp deletion within the intron of *Ae. kotschyi* (1). Second, there was a 3-bp in-frame deletion in exon 3, shared by the *T. timopheevii* accessions, *T. dicoccoides*, *Ae. speltoides* and *T. turgidum* but absent from ‘Dropia’, *T. sphaerococcum* and *Ae. kotschyi*. Third, there was a 1-bp deletion in *T. sphaerococcum* (17), which shifts the reading frame downstream of reference residue 984. The 1-bp deletion was supported by clean, concordant forward and reverse chromatograms and is also present in the Chinese Spring reference genome, where the annotated TraesCS1B02G003100.1 protein (901 aa) terminates in the same shifted reading frame. All other accessions, including ‘Dropia’, retain the reading frame of the *T. dicoccoides* RGA2-like protein.

The 14 unambiguous sequences were grouped into five haplotypes (Table 2). Ten accessions shared one identical haplotype (H1): *T. dicoccoides* (3), *Ae. speltoides* (16) and all eight unambiguous *T. timopheevii* accessions, so that no variation was found among them. 'Dropia' (H2), *T. sphaerococcum* (H3) and *Ae. kotschyi* (H4) each had a distinct haplotype, but the three formed a closely related group, sharing 10 substitutions not found in any other accession. *T. turgidum* (H5) had the most divergent haplotype, with 28 haplotype-specific substitutions. Accession 26 was not assigned to a haplotype.

The complete identity of the fragment, including the intron, in the eight *T. timopheevii* accessions and in *Ae. speltoides* is consistent with the origin of the G genome from a species related to *Ae. speltoides*, and the absence of variation among *T. timopheevii* accessions from three genebanks suggests that this region is highly conserved in the species. In contrast, the grouping of the *T. dicoccoides* accession (3) in H1 was unexpected. The B and G genomes diverged about 2.5-3.5 million years ago (Czajkowska et al., 2019), and the B-genome sequences of 'Dropia' and *T. sphaerococcum* differed from H1 by 3.3-3.9%. Hulled tetraploid wheats with AB and A^tG genomes are morphologically similar, and misclassified accessions have been reported in germplasm collections (Czajkowska et al., 2019). The genome constitution of accession 3 should therefore be confirmed, for example with a B/G discriminatory marker, before H1 is interpreted as shared between the AB and A^tG wheats.

Table 2. Haplotypes of the 1BR_RGA_4B fragment identified in the accessions analysed

Haplotype	Sample IDs	Haplotype-specific substitutions	Indels	Residue 995[a]	C-terminal end[a]
H1	3, 5, 7, 12, 15, 16, 19, 27, 28, 32	10	3-bp deletion (exon 3)	D	…KAMEDV
H2	Dropia	0	–	Y	…KAVYDDV
H3	17	3	1-bp deletion (frameshift)	–[b]	…PAIMS[b]
H4	1	1	50-bp deletion (intron)	Y	…KAVYDDV
H5	21	28	3-bp deletion (exon 3)	D	…KAVEDV

[a] Numbering and C-terminal end of the reference protein XP_037445307.1: residue 995 = Y, C-terminal end …KAVYDDV. [b] Reading frame altered downstream of residue 984; the same frameshift and C-terminal end occur in the Chinese Spring protein TraesCS1B02G003100.1. Substitutions shared by more than one haplotype: H2-H4 (10 sites), H2-H3-H5 (3), H2-H3 (2), H3-H5 (1). Accession 26 combines the H1 and H5 sequences and was not assigned to a haplotype.

Accession 26

The sequence of *T. timopheevii* subsp. *timopheevii* accession 26 contained 41 ambiguous positions (IUPAC codes), with the same double peaks in the forward and reverse reads. All 41 coincided with segregating sites of the dataset, and each resolved into the H1 allele plus the allele carried by *T. turgidum* (H5); at 30 of these positions, the second allele was found only in *T. turgidum*. The sequence of accession 26 therefore corresponds to a mixture of two templates, one identical to H1 and one identical or closely related to H5.

Because DNA was extracted from leaves pooled from several plants, the mixture of two templates most likely reflects heterogeneity within the accession, resulting from a mixed seed lot or from outcrossing. Analysis of DNA from individual plants would be needed to distinguish between these possibilities.

Genetic distances and relationships

Uncorrected p-distances between haplotypes ranged from 0.57% (H2 vs. H4) to 5.99% (H3 vs. H5) (Table 3). H1 differed from the H2–H4 group by 2.99-3.86% and from H5 by 5.59%, while H5 was the most divergent haplotype, differing from all others by 5.59-5.99%. In the neighbour-joining tree, 'Dropia' (H2), *T. sphaerococcum* (H3) and

Ae. kotschyi (H4) formed a separate cluster from H1 and H5, and accession 26 was placed next to *T. turgidum* (H5) (Figure 3).

Table 3. Uncorrected p-distances (%) between the haplotypes of the 1BR_RGA_4B fragment

	H1	H2	H3	H4
H2	3.32			
H3	3.86	0.66		
H4	2.99	0.57	1.28	
H5	5.59	5.72	5.99	5.98

Calculated over the 755-bp region covered by all sequences, with pairwise exclusion of gaps.

The cluster of 'Dropia', *T. sphaerococcum* and *Ae. kotschyi* included the two hexaploid wheats, whose sequences are expected to represent the B-genome copy of the gene, as supported by the identity of *T. sphaerococcum* with Chinese Spring. The close similarity of *Ae. kotschyi* to this group (0.57% from 'Dropia'), rather than to *Ae. speltoides*, was unexpected, and could not be examined further because the other three *Ae. kotschyi* accessions screened did not produce an amplicon. *T. turgidum* (H5), although expected to carry a B-genome copy similar to that of the hexaploid wheats, was the most divergent haplotype (5.72-5.99% from 'Dropia' and *T. sphaerococcum*). It shared the 3-bp deletion and aspartate at residue 995 with H1, but carried 28 haplotype-specific substitutions. As only one *T. turgidum* accession was analysed, it remains unclear whether H5 represents a divergent allele of this gene, a related copy absent from the reference genome, or an accession of uncertain genome constitution (Czajkowska et al., 2019).

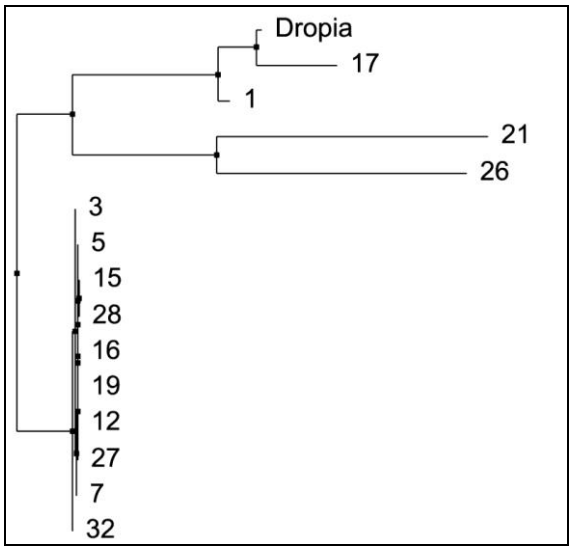


Figure 3. Neighbour-joining tree of the 15 sequences of the 1BR_RGA_4B fragment, calculated in Jalview from percentage identity (PID) on the full MAFFT alignment (828 positions). Branch lengths are proportional to percentage sequence difference. Samples are numbered as in Table 1.

Deduced amino acid variation

Among the accessions with an intact reading frame (all except 17 and 26), 14 amino acid positions were variable within the region covered by all sequences (Figure 4). Thirteen substitutions were found only in *T. turgidum*. At residue 995 of the reference protein, 'Dropia' and *Ae. kotschyi* carried tyrosine, as in XP_037445307.1, whereas H1 and *T. turgidum* carried aspartate. The 3-bp deletion in exon 3 changed the predicted C-terminal end of the protein from …KAVYDDV, in the H2–H4 group and the reference, to …KAMEDV in H1 and …KAVEDV in *T. turgidum* (Table 2).

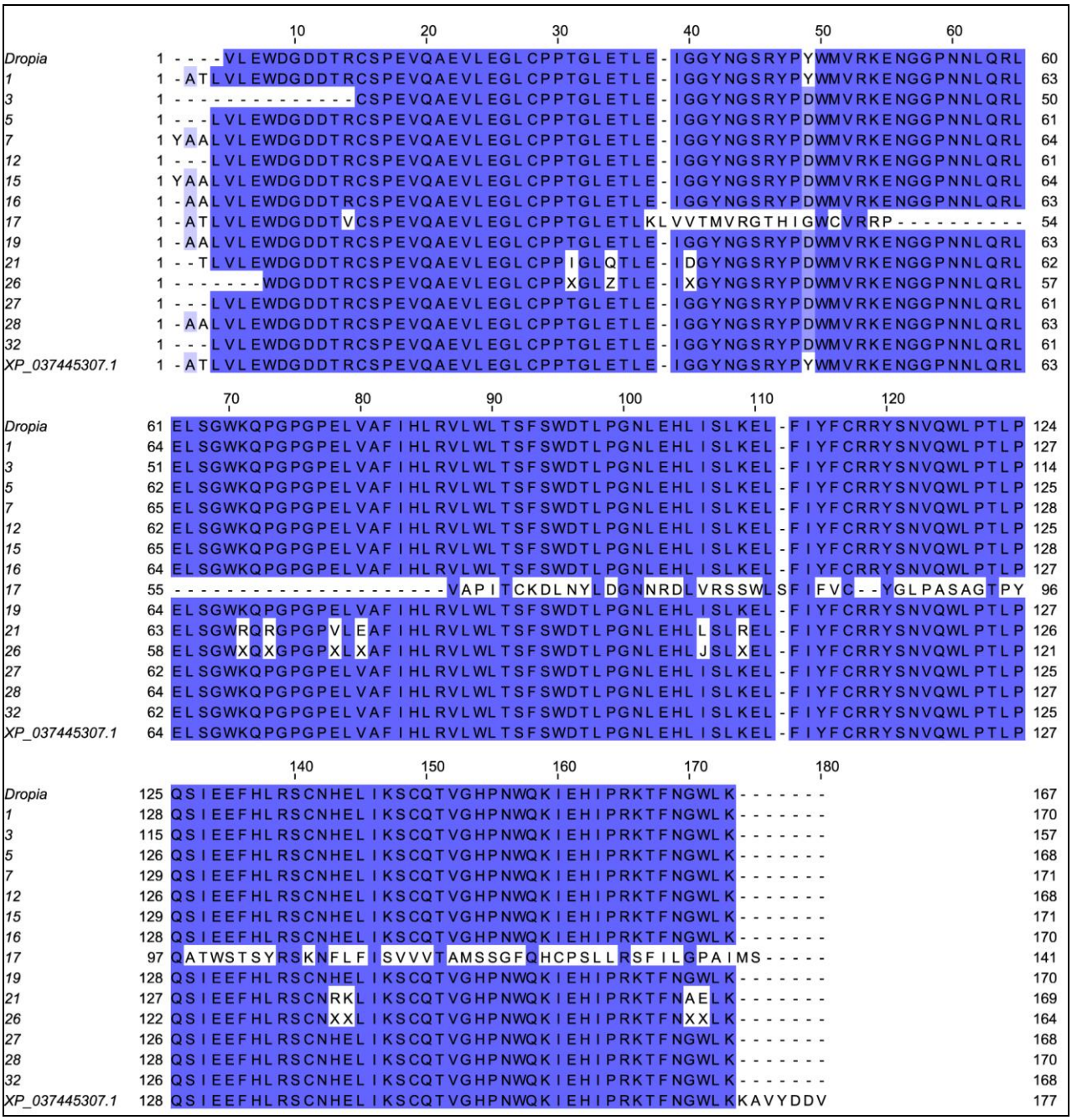


Figure 4. Alignment of the deduced amino acid sequences of the 1BR_RGA_4B fragment with the reference protein XP_037445307.1 (*T. dicoccoides*), produced with MAFFT L-INS-i in Jalview. Samples are numbered as in Table 1. Residues are shaded by percentage identity; X, Z and J denote ambiguous residues in sample 26. Column numbers refer to the alignment; residue i of the reference fragment shown corresponds to residue i + 948 of XP_037445307.1.

The 1-bp deletion found in *T. sphaerococcum* is also present in Chinese Spring, whose annotated protein ends in the shifted frame, whereas all the other accessions, including the cultivar 'Dropia', retain the reading frame of the *T. dicoccoides* protein. The Chinese Spring gene model may therefore not represent the protein encoded by this gene in other wheats, which illustrates the limitation of a single reference genome for identifying functional R-genes in modern cultivars noted by Li et al. (2021). Apart from *T. turgidum*, amino acid variation was limited to residue 995 and the C-terminal end, both located downstream of the annotated LRR region. Whether the substitution of tyrosine by aspartate at residue 995 and the altered C-terminal ends of H1 and H5 affect protein function cannot be inferred from sequence data alone.

CONCLUSIONS

A partial fragment of the candidate RGA gene TraesCS1B02G003100, covering the C-terminal end of the LRR domain and the C-terminal tail of the encoded protein, was amplified only from accessions carrying a B, G or S genome and showed five haplotypes in 14 accessions of *Triticum* and *Aegilops*. *T. timopheevii* and *Ae. speltoides* shared a single haplotype, the hexaploid wheats formed a closely related group with *Ae. kotschyi*, and *T. turgidum* carried the most divergent haplotype. A 1-bp deletion causing a frameshift was found in *T. sphaerococcum* and in the Chinese Spring reference, whereas 'Dropia' and the other accessions retained the full reading frame of the *T. dicoccoides* protein.

From a breeding perspective, the 1-bp and 3-bp deletions and the substitutions shared by the H2-H4 group are candidate polymorphisms for the development of allele-specific markers, such as KASP markers, which could be used to track these variants in segregating populations and to test their association with SNB response. The present study has several limitations: only a partial fragment (about 0.8 kb) of the gene was sequenced, most species were represented by a single accession, no data on SNB response were available for the accessions analysed, and the genome constitution of some accessions remains to be confirmed.

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