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Analysis of Wheat Bread-Making Gene (*wbm*) Evolution and Occurrence in Triticale Collection Reveal Origin via Interspecific Introgression into Chromosome 7AL

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Abstract: Bread-making quality is a crucial trait for wheat and triticale breeding. Several genes significantly influence these characteristics, including glutenin genes and the wheat bread-making (*wbm*) gene. World wheat collection screening showed that only a few percent of cultivars carry the valuable *wbm* variant, providing a useful source for wheat breeding. In contrast, no such analysis has been performed for triticale (wheat (AABB genome) × rye (RR) amphidiploid) collections. Despite the importance of the *wbm* gene, information about its origin and genomic organization is lacking. Here, using modern genomic resources available for wheat and its relatives, as well as PCR screening, we aimed to examine the evolution of the *wbm* gene and its appearance in the triticale genotype collection. Bioinformatics analysis revealed that the wheat Chinese Spring genome does not have the *wbm* gene but instead possesses the orthologous gene, called *wbm-like* located on chromosome 7A. The analysis of upstream and downstream regions revealed the insertion of LINE1 (Long Interspersed Nuclear Elements) retrotransposons and Mutator DNA transposon in close vicinity to *wbm-like*. Comparative analysis of the *wbm-like* region in wheat genotypes and closely related species showed low similarity between the *wbm* locus and other sequences, suggesting that *wbm* originated via introgression from unknown species. PCR markers were developed to distinguish *wbm* and *wbm-like* sequences, and triticale collection was screened resulting in the detection of three genotypes carrying *wbm*-specific introgression, providing a useful source for triticale breeding programs.

Keywords: triticale; wheat bread-making gene; introgression; PCR markers

1. Introduction

Wheat is a global source of food and calories and the main ingredient in many products, with an annual world production volume of around 771 million tons as of 2017 (FAOSTAT, 2017). The bread-making quality is the most important trait for wheat breeding programs worldwide. However, the estimation of bread-making quality is challenging because it is costly and requires a substantial amount of seed for analysis [1]. To overcome these difficulties, researchers have searched for

the genetic determinants controlling the trait to enable marker-assisted selection (MAS) [2–4]. However, the genetic basis of the bread-making quality of wheat is not yet understood and the genes that have been found do not explain the broad variation in this characteristic. High molecular weight (HMW) glutenin genes (*Glu-A1*, *Glu-B1*, and *Glu-D1*) [2], located on the long arm of group 1 chromosomes, are the most well studied among the major genes controlling bread-making quality. A number of markers have been developed to distinguish different alleles of glutenin genes [5–10]. A gene, called wheat bread-making (*wbm*), was identified in wheat, and its elevated expression level in the endosperm during wheat grain development has been demonstrated [11]. Analysis of *wbm* expression in different wheat genotypes resulted in two contrast groups: (1) genotypes with high expression of *wbm* and (2) genotypes with the negligible expression of the gene. A high correlation of *wbm* expression and the values of some quality characteristics have been demonstrated [1,11]. The expression level of *wbm* along with the allelic composition of glutenin genes, and the presence of 1BL.1RS translocation is one of the key determinants of major wheat quality traits coupled with bread-making quality [1]. Analysis of variation in the upstream sequence of *wbm* allowed the identification of one variant, GWseqVar3, which was associated with high *wbm* expression in cultivars with good bread-making characteristics [11]. Based on this knowledge, PCR-based (NWP, [11]) and Kompetitive allele specific PCR (KASP, [10]) markers were developed to specifically identify GWseqVar3. These markers were employed for screening wheat genotypes in different world collections [1,10]. These studies found rare (0% to 36%) occurrences of GWseqVar3 of *wbm* in wheat collections, highlighting the importance of screening of germplasm on *wbm* to improve bread-making quality.

Despite the relative progress in wheat collection screening, the frequency of *wbm* occurrence in hexaploid triticale (×*Triticosecale* Wittmack) ($2n = 42$, AABBRR, wheat × rye hybrid) collections is unexplored. Triticale occupies 4 million hectares worldwide with an approximate production volume of 15 million ton per year, which is comparable to rye production. Triticale is a hardy crop, combining many beneficial traits inherited from both parents, including high yield, increased tolerance to biotic and abiotic stresses, and nutrient-use efficiency [12–18]. Although triticale is an important crop for biofuel production and forage, application of triticale grains in the bakery industry is limited, with no registered cultivars with good bread-making quality [19]. However, with the current growth in the number of health-conscious people, triticale is becoming more attractive as a human food source [20]. In this context, new breeding strategies are needed to improve triticale end-use characteristics and identification of valuable gene variants located in the A and B genomes.

Study of the *wbm* gene has focused on practical aspects, whereas *wbm* gene origin, evolution, and genomic organization studies are lacking. In their initial publication, Furtado et al. [11] performed PCR screening of wheat progenitor species (*Triticum monococcum*, *Triticum urartu*, and *Triticum turgidum*) to address *wbm* origin but obtained controversial results. The PCR products of the expected size were amplified in some samples of *T. monococcum* (AA genome) and *T. urartu* (AA genome) genomic DNA but not in *T. turgidum* (AABB genome). Because no D-genome donor species (*Aegilops tauschii*) have been used for PCR analysis, the location of the *wbm* gene on wheat A or D genomes was proposed. Rasheed et al. [10], using a Basic Local Alignment Search tool (BLAST) search, proposed the location of the *wbm* gene on chromosome 7AL; whether *wbm* gene can also be found in the D genome has not been studied. Thus, no consistent information about the genomic organization of *wbm* has been reported.

The objective of this study was to examine *wbm* evolution and genomic organization and to use this information in the identification of triticale lines carrying *wbm* for further improvement of the bread-making quality of this crop. From several lines of evidence, we proved the location of *wbm* gene chromosome 7AL and demonstrated considerable diversity in the *wbm* protein sequence between closely related species. Using available genomic data for grasses and PCR analysis with newly developed markers, we found that *wbm* is not an allelic variant but was introgressed from unknown species into the wheat genome. Chinese Spring cultivar has an orthologous *wbm* gene (*wbm-like*), which has negligible expression in grain, and its evolution was accompanied by mobile element insertion.

Location of *wbm* in the A genome provides an opportunity for screening the collection of triticale lines possessing A, B, and R genomes, and allowed us to identify three triticale lines possessing this gene.

2. Material and Methods

*2.1. Identification of wbm-*Like *Gene*

To identify the *wbm* location on a wheat chromosome, a 75-amino acid (aa) sequence of *wbm* protein [11] was employed as a query for tBLASTn search against the expressed sequence tags (EST) collection of NCBI, and an EST clone (BQ606004.1, https://www.ncbi.nlm.nih.gov/nuccore/BQ606004.1/) was found. This EST has a 225-base pair (bp) open reading frame predicted by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), and the corresponding protein sequence was identical to original *wbm* protein. Using this EST sequence, we next conducted a nucleotide BLAST search against the IWGSC RefSeq v1.0 ([21]) reference genome assembly of wheat Chinese Spring (https://urgi.versailles.inra.fr/blast/, [22]) and the NCBI WGS database.

2.2. Multiple Alignment of wbm and Related Protein and Nucleotide Sequences

To compare *wbm* and *wbm-like* with the related sequences from other species and cultivars nucleotide BLAST [23] was performed against the latest assembly of their genomes (wheat cultivars: https://wheatis.tgac.ac.uk/grassroots-portal/ [24], http://www.10wheatgenomes.com/; *S. cereale*: https://webblast.ipk-gatersleben.de/ryeselect/, other species: https://plants.ensembl.org/Triticum_aestivum/ [25], https://urgi.versailles.inra.fr/blast/blastresult.php) using default parameters. The *wbm*-similar sequences were retrieved, and open reading frames (ORFs) were predicted using ORFfinder software (https://www.ncbi.nlm.nih.gov/orffinder/). Corresponding ORF sequences overlapping with *wbm* hits and deduced protein sequences were aligned by mafft [26] with standard parameters and visualized using UGene (Version 33, UNIPRO, LLC, Novosibirsk, Russia,) [27].

2.3. Phylogenetic Tree Construction

Phylogenetic trees were build based on multiple alignments of protein or nucleotide sequences of *wbm*, *wbm-like,* and *wbm* similar genes in UGene software [27] using the PhyML maximum likelihood method with bootstrap branch support computed from 100 replicates.

*2.4. wbm-*Like *Expression Analysis*

The wheat expression atlas (wheat-expression.com [28]) database was mined by the gene corresponding to the *wbm-like* sequence (TraesCS7A02G531903 [21]) in the CS genome. The expression matrix was retrieved only for grain, where *wbm* was found to be expressed [11]. Data were visualized (Supplementary Figure S1) by bar plot build by R programming language using the ggplot2 package [29].

2.5. Plant Material

A total of 107 forms and cultivars of spring triticale were used, including selection forms obtained at the Department of Genetics, Russian State Agrarian University, and accessions from the VIR collection (Supplementary Table S1).

2.6. DNA Isolation and PCR Analysis

PCR amplification was conducted using specific primers (Table 1) designed using Primer 3.0 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, [30]). The PCR conditions for custom primers were 94 °C for 1 min, 35 cycles: 94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min; and final elongation: 72 °C for 3 min. For NWP primers [11], the annealing temperature was 55 °C.

Table 1. Primers designed in this study and the expected length of the PCR product.

Primer ID	Primers 5′-3′	Expected Length of PCR Product (bp)
pro	F: TTGAAGAGAAGTGGCCAGCG R: GTTCATGCGATGCAGAGAGC	988
L1	F: ACAACATCACAAGTGGAAAGGC R: AGGTTGGTAGTAATTCCAAATTCAAGT	2200
wbm	F: TGTGTGTTGCTACCATCATGG R: GGCAGCTCCCATGTTGTACA	219
wbm-like	F: TTACTACGCACGGGCAGTTT R: GTGTGTTGCCACCATCATGG	225

For verification, PCR products of three selected lines were purified and sequenced using ABI 3130×l Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

3. Results

3.1. *wbm* Located on Chromosome 7A

We aimed to obtain insight into the genome organization of *wbm* in wheat. For this, we identified the genomic location of *wbm* using corresponding EST and protein sequences [11]. We did not find any genomic sequences with absolute identity with the *wbm* sequence in the wheat Chinese Spring (CS) genome reference data. Hits located on chromosomes 7A and 7D were the most significant (81% identity and 100% coverage, E-value $3e \times 10^{-59}$ for both hits). The hit located on chromosome 7A overlaps a gene, (TraesCS7A02G531903, *wbm-like* hereafter), whereas the hit located on chromosome 7D (*wbm_hit_7D*) partially overlapped with the 5′ end of the annotated non-coding RNA (STRG_Seed.132206.1). We also performed a BLAST search with the Tag-A sequence (CATGTTGTTCCGTGTAGTACC), which was used for *wbm* identification [11]. We found that this sequence had near-perfect similarity (only one mismatch) with the downstream region of *wbm-like*. To obtain additional evidence of *wbm* location on chromosome 7A, we applied the recently released wheat 10+ genomes project (http://www.10wheatgenomes.com/). Using *wbm-like*, *wbm*, and their promoter sequences, as well as the *wbm_hit_7D* sequence, as a query, we performed a similarity search against de novo genome assembly of 10 wheat cultivars. We found one wheat cultivar, Mace, which shows absolute similarity with *wbm* and its promoter sequence, whereas no absolute similarity with *wbm-like* was observed, pointing to the absence of *wbm-like* in the genome of this cultivar and presence of *wbm*. The similarity search for *wbm_hit_7D* resulted in a hit with 100% similarity in the Mace cultivar located on distinct chromosome from *wbm* gene hit, further supporting that *wbm_hit_7D* is less related to *wbm* gene than *wbm-like*. Thus, the obtained results show that *wbm* and *wbm-like* are orthologous and located in the long arm of chromosome 7A.

3.2. *Comparative Analysis of wbm and wbm*-Like

To trace the origin of *wbm* and *wbm-like* (cultivar CS), we conducted multiple alignment of their nucleotide and protein sequences with sequences of *Triticum urartu* (A genome donor), *Aegilops speltoides* (B genome donor), *A. tauschii* (D genome donor), *T. monococcum*, and *Secale cereal*, followed by phylogenetic tree construction. *wbm_hit_7D*, located on chromosome 7D, and *wbm* sequences from two additional cultivars (Mace, which has *wbm*, and Julius, which has *wbm-like*) were also involved in the multiple sequence alignment. Comparison of the nucleotide sequences resulted in distinct cluster generated by the reference and Mace *wbm* genes (Cluster 1) and three clusters formed by *S. cereale* (Cluster 2), *A. speltoides* (Cluster 3), and *wbm-like* sequences from other species (Cluster 4) (Figure 1A,C). We next assess whether the comparison of translated protein sequences will show similar phylogenetic picture. Prediction of protein sequences from *wbm_hit_7D* and *A. tauschii* revealed a premature termination codon at the same position, indicating that sequences may not encode proteins.

Multiple alignment of all other predicted proteins (Figure 1B) and phylogenetic tree construction resulted in three clusters, generated by *wbm* (Cluster 1), *wbm-like* (Cluster 2), and *wbm* similar sequences from *S. cereale*, *T. urartu*, *A. speltoides*, and *T. monococcum* (Cluster 3). Thus, both phylogenetic trees show the distinct phylogenetic position of *wbm* gene. Interestingly, with a high divergence rate between the sequences from different clusters, N-terminus, corresponding to the signal peptide of the *wbm* protein [11], is well conserved.

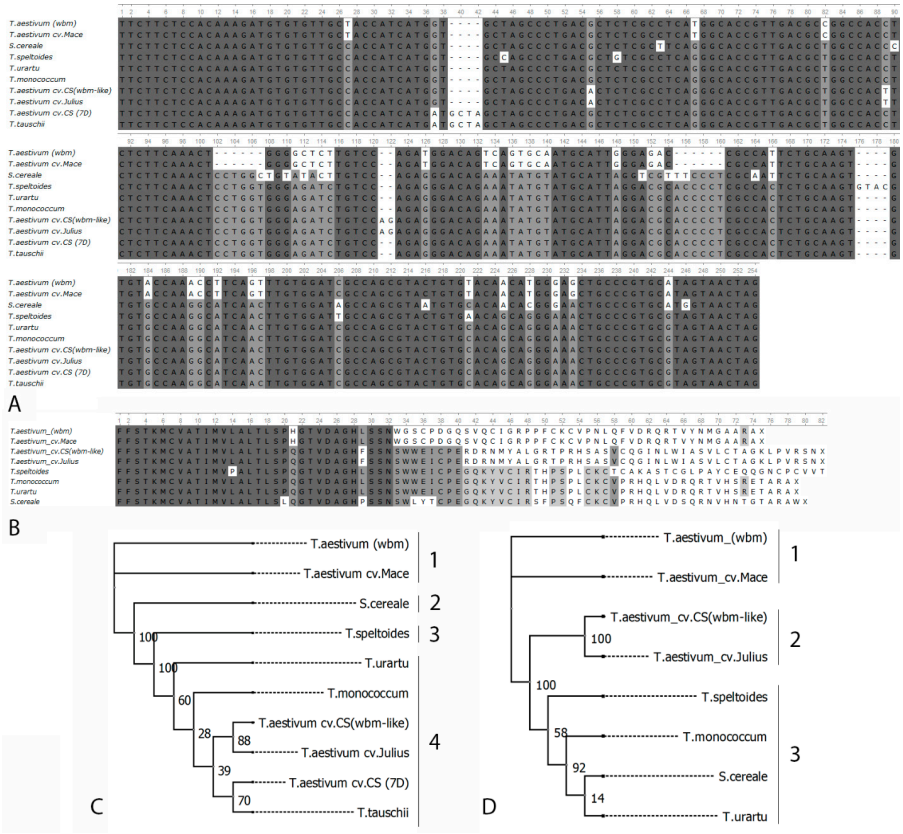

Figure 1. Conservation analysis of *wbm* and *wbm-like*. Multiple alignment of *wbm* and *wbm-like* (**A**) nucleotide and (**B**) amino acid sequences with similar sequences from *Triticum urartu*, *Aegilops speltoides*, *Triticum monococcum*, *Secale cereale*, and *Aegilops tauschii*. (**C**) Phylogenetic tree based on multiple alignment of *wbm*(*-like*) (**C**) nucleotide and (**D**) amino acid sequences. Bootstrap values are indicated.

Taken together, the comparative analysis indicated that *wbm* has a distinct origin from wheat genome donor species, supporting its introgressive origin in wheat. In addition, *wbm*, *wbm-like*, and *wbm* similar sequences from analyzed species demonstrate signatures of diversifying selection with high conservation in the N-terminus of protein.

3.3. Screening of Triticale Collection in the Presence of wbm

The established location of *wbm* and *wbm-like* on the chromosome 7A (Figure 2A) provided the possibility of checking for the presence of the genes in triticale possessing A, B, and R genomes but not D genome. The previously designed PCR marker (NWP, [11]) on *wbm* was dominant, resulting in amplification only in *wbm*-carrying genotypes. Therefore, we designed new *wbm-like*-specific primers on the upstream region (pro primers, Figure 2B) and CDS (*wbm-like* primers, Figure 2B) of the gene (Figure 2). In addition, *wbm*-specific primers on the CDS region of the gene were also designed (*wbm* primers, Figure 2B). In this study, we used the triticale germplasm collection consisting of 107 lines, most of which were obtained in Russia (Supplementary Table S1). Using the primer sets, we conducted PCR screening of this collection. Screening of this collection with *wbm* specific primers (NWP and

wbm) revealed amplification of the PCR product of expected size (961 bp for NWP and 988 for pro, Figure 2C) only in three triticale lines, L8665, P13-5-13, and P13-5-2. The results of PCR screening with *wbm-like* specific primers showed that the expected PCR products were obtained for all lines except three (L8665, P13-5-13, and P13-5-2; Figure 2D), which do not possess *wbm-like*. Thus, we identified three triticale lines carrying *wbm* that can be further used in plant breeding programs to improve the bread-making quality of triticale.

3.4. Genomic Region of *wbm*-Like Lacks Conservation with *wbm* and Surrounded by Transposon Insertions

To understand the conservation rate of the genomic regions near *wbm* and *wbm-like*, promoter sequences (~−1 Kb region) of the genes were sequenced. Comparison of the obtained sequences demonstrated only partial similarity of the 67 bp region upstream of *wbm* and *wbm-like*. Analysis of the *wbm* genome locus in the CS genome assembly showed that the gene is surrounded by two transposable element (TE) insertions, and none of them were observed in the promoter of *wbm*. Of them, the TE located downstream (ca. 350 bp) belong to mutator DNA transposons family, whereas the TE located ca. 270 bp upstream of *wbm* (promoter) is L1-like retrotransposon (L1). To verify that the presence of L1 elements is unique to lines carrying *wbm-like*, we designed a primer pair (*wbm*_L1) flanking the L1 insertion. The screening of the triticale collection with this primer pair showed that the PCR product of expected size was obtained only in lines possessing *wbm-like*; no amplification was observed with DNA of lines possessing *wbm* (Figure 2C). Comparison of the *wbm-like* promoter with the *T. urartu* genome resulted in the finding of a highly similar sequence that also contains LINE1 insertion.

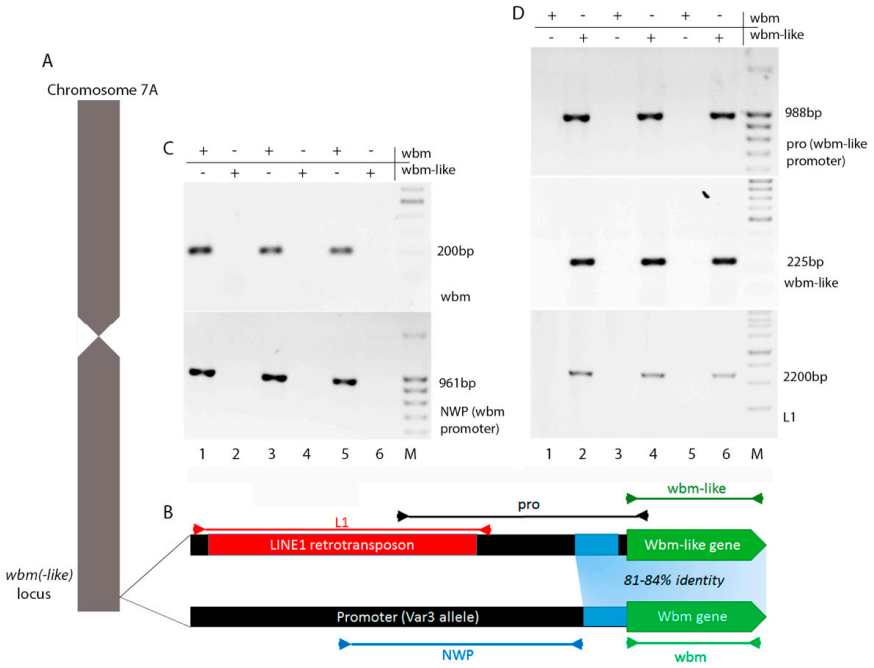

Figure 2. Genomic organization of *wbm-like* and *wbm* in genome of Chinese Spring. (**A**) Schematic representation of Chromosome 7A with marked position of *wbm*(*-like*) gene position. (**B**) Schematic organization of *wbm* and *wbm-like* and their promoter regions. The blue box depicts regions with high similarity (81% to 84%) and red box depicts Long interspersed nuclear element (LINE1) retroelement insertion. Horizontal lines show positions of primers. Gel electrophoresis of PCR products obtained with DNA of triticale lines (6 lines are represented here), and the primers specifically designed for (**C**) *wbm* gene (NWP (Furtado et al. [11]), *wbm* (specific for *wbm* open reading frame (ORF))) and (**D**) *wbm-like* gene (pro (promoter region of *wbm-like*), L1 (flanking LINE1 insertion in promoter region of *wbm-like*), and *wbm-like* (specific for *wbm-like* ORF)). 1–6 lanes of the gels corresponding to the 3 *wbm* positive lines (lane 1: L8665, lane 3: P13-5-13, and lane 5: P13-5-2) and 3 randomly selected *wbm-like* positive lines (lane 2: 131/7, lane 4: C 235, and lane 6: Yarilo).

The insertion of transposable element nearby genes often results in silencing of the gene expression. The expression of *wbm-like* in the CS cultivar was almost undetectable (wheat-expression.com).

Thus, the results show that upstream (promotor) regions of *wbm* and *wbm*-like genes have substantial differences supporting the introgressive origin of *wbm* gene. Further studies are needed to establish the borders and the size of the introgression.

4. Discussion and Conclusions

Genomes of modern wheat cultivars and landraces are shaped by inter- and intra-species introgression events [31]. The functional consequences of the introgressions and their effect on phenotypic variation and traits beneficial to humans, such as bread-making quality, have been poorly explored. Our study showed that *wbm* is a part of genomic introgression of unknown scale into the long arm of chromosome 7A. The protein and nucleotide sequences of *wbm* are phylogenetically distant from the homologous sequence of the A genome donor species, pointing to its distinct origin from unknown species. Concluding whether the introgression was a result of interspecies hybridization during the wheat breeding process or if it existed before the origin of wheat is difficult. Furtado et al. [11] reported the presence of *wbm* only in some genotypes of *T. monococcum* and *T. urartu*, suggesting that both scenarios are possible. With such a high divergence rate of *wbm* locus, the resequencing and read mapping to the reference genome (CS cultivar) will not allow identifying the origin of *wbm,* as it requires substantial similarity between reference and target genomes. Therefore, ongoing de novo sequencing and genome assembly of wheat cultivars (10+ Genome Project, http://www.10wheatgenomes.com/) and closely-related species are essential to identify the introgression similar to *wbm*.

The function of *wbm* protein remains elusive. Based on the specific spatiotemporal expression pattern and cysteine content of *wbm* protein, Furtado et al. [11] hypothesized that *wbm* protein can influence breadmaking quality through the generation of inter and intramolecular disulfide bonds. Notably, *wbm* protein is encoded by a small open reading frame (sORF). sORFs underwent rapid genomic evolution and may occur de novo in the genomes [32]. Comparison of the *wbm* protein sequence demonstrated high divergence between closely related species, consistent with general patterns of sORF evolution. The length of putative *wbm*-like proteins from the CS cultivar and closely related species also differ from the *wbm* protein. Although the molecular mechanism of *wbm* function is unknown, whether the sequence divergence is coupled with changes in *wbm* function is a topic that requires future research.

The established location of *wbm* on the A genome enabled the screening of a collection of triticale genotypes possessing A, B, and R genomes, and we found only three lines possessing *wbm*. Although a number of desirable traits in triticale have been inherited from both parents [13,14], the genes controlling desirable dough properties are located on the D genome and therefore are not present in hexaploid triticale [14]. Hence, new genetic resources are required to produce triticale producing strong gluten with high extensibility. Storage proteins of triticale, secaloglutenins, include a combination of HMW and LMW glutenins from the A and B genomes, secalins from the R genome, and α-, β-, and γ-gliadins [33,34]. Triticale lines possessing *wbm* may have unique gluten properties if *wbm* is capable of generating links between different storage proteins. However, further testing of this hypothesis requires a substantial amount of seeds to perform the baking test for these lines, which is the goal for our future research.

Here, we showed that GWseqVar3, previously described as an allelic variant of *wbm* [11], is orthologous to *wbm-like* located on chromosome 7A of wheat. In turn, *wbm-like* the CS cultivar is most probably a pseudogene surrounded by insertion of mobile elements, which has a negligible expression in grain. We found that *wbm* previously introgressed into the genomes of some wheat and triticale cultivars from unknown species. Elucidating the evolutionary relationships between *wbm* and its homologous sequences in wheat and closely related species allowed us to demonstrate that *wbm* protein is highly diverged, except its N-terminal. Finally, the location of *wbm* in the A genome

offered the opportunity to select three triticale lines carrying this gene that can be further exploited in the future for improving the bread-making quality of this crop.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/12/854/s1, Table S1: the list of triticale genotypes used in this study.

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Conflicts of Interest: The authors declare no conflicts of interest.

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