Molecular characterization of the *Wx-B1* allelic variants identified in cultivated emmer wheat and comparison with those of durum wheat

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Abstract

Emmer wheat is a neglected crop that could be used in the breeding of modern durum wheat for quality. One important aspect of this quality is the starch composition which is related to the waxy proteins. A collection of 87 accessions of Spanish emmer wheat was analysed for *waxy* protein composition by SDS-PAGE. No polymorphism was found for the *Wx-A1* gene. However, for the *Wx-B1* gene, three alleles were detected, two of them being new. The whole gene sequence of these alleles was amplified by PCR in three fragments, which were digested with several endonucleases to determine internal differences in the sequence. These variants were also compared with the *Wx* alleles present in durum wheat. Differences in size and restriction sites were detected. DNA sequence analysis confirmed that the alleles found in emmer wheat are different from those of durum wheat. The first data suggested that these alleles showed a different influence on the amylose content of these lines. The variation found could be used to enlarge the gene pool of durum and emmer wheat, and design new materials with different amylose content.

Keywords: amylose content; emmer wheat; genetic polymorphism; waxy proteins.

Introduction

A characteristic in which there has been increasing interest for wheat quality is the starch and the proteins involved in its synthesis, mainly the *waxy* (Wx) proteins that are involved in amylose synthesis. The starch component affects starch gelatinization, pasting and gelation properties of wheat (Zeng et al. 1997). The Wx proteins are granule-bound starch synthases (GBSS) synthesised by *waxy* genes, which are located in the *waxy* locus on the group-7 chromosomes of each genome (Chao et al. 1989; Ainsworth et al. 1993). *Wx-A1* is located on chromosome 7AS, *Wx-B1* on 4AL (a segment of chromosome 7BS that has been translocated), and *Wx-D1* on 7DS in common wheat (*Triticum aestivum* ssp. *aestivum* L. em. Thell). Tetraploid wheat is composed of A and B genomes, and thus carries two Wx proteins, Wx-A1 and Wx-B1.

The food industry demands different kinds of starch for different processes. Wheat *waxy* proteins have been amply studied in recent years due to their effect on the amylose content of the grain. Diverse studies about the polymorphism of *waxy* proteins have also been carried out (Yamamori et al. 1994, 1995; Rodriquez-Quijano et al. 1998; Nieto-Taladriz et al. 2000). All of them show that the polymorphism of these proteins is low, even more when compared with other proteins of wheat grain such as the storage proteins (glutenins and gliadins). Yamamori et al. (1994, 1995) found five alleles for the *Wx-A1* gene in bread wheat and other six alleles have been described for the *Wx-B1* gene in common and durum wheats -*T. turgidum* ssp. *durum* Thell. em. Desf.- (Rodriquez-Quijano et al. 1998; Nieto-Taladriz et al. 2000). On the other hand, in more recent studies (Caballero et al. 2008) about *waxy* proteins in ancient wheats and related species, the diversity reported has been higher. These ancient wheats include the hulled wheats, the main species of which are einkorn (*T. monococcum* L. ssp. *monococcum* ; 2n = 2× = 14, AA), emmer (*T. turgidum* ssp. *dicoccum* Schrank; 2n = 4× = 28, AABB) and spelt (*T. aestivum* ssp. *spelta* L. em. Thell.; 2n = 6× = 42, AABBDD). In Spain, these species were widely cultivated until the first part of the 20th Century and were phased out towards the late 1960s, when these crops were displaced by the semi-dwarf wheats bread by the International Maize and Wheat Improvement Center (CIMMYT). Fortunately, the great majority of these materials were conserved in Germplasm Banks.

Due to the increase of interest in these species, a programme for the evaluation of

variability in Spanish emmer wheat has recently been initiated, its main goal being to reintroduce and/or to safeguard the lines of interest for quality (Pflüger et al. 2001; Alvarez et al. 2007); together with the evaluation of new genes that could enlarge the gene pool of durum wheat.

The aim of this study was the analysis of the polymorphism of the waxy proteins in Spanish cultivated emmer wheat, as well as its molecular characterization and comparison with those present in durum wheat.

Materials and Methods

Plant materials

Eighty seven lines of Spanish emmer wheat derived by single seed selection from an equal number of accessions obtained from the National Small Grain Collection (Aberdeen, USA), Center for Genetic Resources (Netherlands) and the Centro de Recursos Fitogenéticos-INIA (Alcalá de Henares, Spain), were analysed. These plants were grown under field conditions during 2007. Several spikes per plant were protected to avoid random crosses. Durum wheat cultivars Langdon and Mexicali, along with the KU 4213D line were used as standards. The KU 4213D line was kindly supplied by Dr. Endo Takashi from National BioResource Project (Kyoto, Japan).

Starch extraction and electrophoretic analysis

Twenty milligrams of flour were mixed with 1 ml of distilled water and incubated at 4°C for 24 h. The homogenate was filtered through Miracloth and centrifuged at 14000 g for 1.5 min. The pellet was washed with 1 ml of buffer A [55 mM Tris-HCl pH 6.8, 2.3% (w/v) sodium dodecyl sulphate, 2% (w/v) dithiotreitol, 10% (v/v) glycerol], according to Echt and Schwartz (1981). Then 1 ml of buffer A was added to the pellet and left for 30 min at room temperature. The pellet was washed three times with distilled water, once with acetone and then air-dried. The residue was mixed with 80 μl of buffer A, heated in a boiling bath for 2 min, cooled in ice and centrifuged.

Aliquots of supernatant (20 μl) were loaded in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at a polyacrylamide concentration of 12% (w/v, C: 0.44%). The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel at 18°C, continuing for 4

hours after the tracking dye migrated off the gel. Protein bands were visualised by silver staining.

DNA extraction and PCR amplification

For DNA extraction, approximately 100 mg of young leaf tissue was excised, immediately frozen in liquid nitrogen and stored at -80ºC. DNA was isolated using the DNAzol® method (Invitrogen, Carlsband, CA, USA).

The primers designed by Monari et al. (2005) were used to amplify the waxy genes in durum and emmer wheat: WxF3 (5´-TCTGGTCACGTCCCAGCTCGCCACCT-3´), WxVT1R (5´-ACCCCGCGCTTGTAGCAGTGGAAGT-3´), WxBAF (5´-ACTTCCACTGCTACAGCGCGGGGT-3´), WxBAR (5´-GCTGACGTCCATGCCGTTGACGATG-3´), WxVT1F (5´-CATCGTCAACGGCATGGACGTTCAGC-3´), and WxVTR (5´CCAGAAGCACGTCCTCCCAGTTCTTG-3´). Each 15-μl reaction included 50 ng DNA, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM dNTPs, 1.5 μl 10x PCR Buffer and 0.75U DNA polymerase (Biotools). The region spanning the first to the third exon was amplified using the primers WxF3 and WxVT1R; the pair WxBAF and WxBAR was used to amplify the region spanning the third to the sixth exon; and the pair WxVT1F and WxVTR for the region spanning the sixth to the eleventh exon. The PCR conditions included an initial denaturation step of 3 min at 94°C followed by 35 cycles as follows: for WxF3/WxVT1R, 40 s at 94°C, 1 min at 62°C then 1 min at 72°C; for WxBAF/WxBAR, 45 s at 94°C, 2 min at 62°C then 1 min 5 s at 72°C; and for WxVT1F/WxVTR, 40 s at 94°C, 1 min at 62°C then 1 min 30s at 72°C. After the 35 cycles all reactions included a final extension of 5 min at 72°C.

Analysis of PCR products

Amplification products were fractionated in vertical PAGE gels with two different polyacrylamide concentrations: 8% (w/v, C: 1.28%) and 6% (w/v, C: 1.28%), and the bands were visualized by ethidium bromide staining. Additionally, the PCR products were restricted with the endonucleases *Bgl*II, *Rse*I and *Stu*I (Roche) following the supplier's instructions. Digested fragments were analysed by polyacrylamide gel electrophoresis in discontinuous Tris-HCl buffer system (pH: 6.8/8.8) with different polyacrylamide

concentrations. The Tris/glycine buffer was used.

Cloning of PCR products and sequencing analysis

PCR products were excised from polyacrylamide gel and cloned into pGEM T-easy vector (Promega, Madison, WI, USA) for sequencing. For the cloning of the genes, the third fragment of the waxy genes was reamplified in two different fragments with minor size in order to clone and sequence this fragment better. The primers designed and used were: Wx-3º1Fw (5´-CGTTCTGCATCCACAACATC-3´); Wx-3º1Rv (5´-CAGCCGCTCAAACTTCTTCT-3´); Wx-3º2Fw (5´-CACCGGGAAGAAGAAGTTTG-3´); and Wx-3º2Rv (5´-ACACCCAGTTCCAAAAGCAC-3´). The conditions of the PCR for these primers were the same than the ones described above for primers WxVT1F/WxVTR. Inserts were sequenced from at least three different clones using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsban, CA, USA). The sequences were compared to the sequences available in the databases (durum wheat cv. Langdon; *Wx-A1,* AB029063; *Wx-B1,*AB029064; and common wheat cv. Chinese Spring: *Wx-B1* AB019622).

Amylose content

A preliminary analysis of the influence of the allelic variants detected on the amylose content was carried out using several lines representative of the variation found in emmer wheat, along with cvs. Langdon and Mexicali. The amylose content was determined in triplicate using Megazyme Amylose/Amylopectin kit. Data were analysed by an ANOVA and the means were compared by the least significant difference (LSD) test.

Results

Polymorphism at GBSSI or waxy proteins.

The electrophoretic analysis of the *waxy* proteins of 87 lines of Spanish emmer wheats revealed no polymorphism of the *Wx-A1* locus. All the materials showed the *Wx-A1a* allele as the standards cvs. Langdon and Mexicali. For the *Wx-B1* locus, three alleles were detected (ESM 1, Fig. 1): *Wx-B1b* (*null* type) that lacks protein (lane 8); *Wx-B1c*,* with the same mobility of *Wx-B1c´* of cv. Mexicali (lanes 5 and 6), and lower electrophoretic mobility than the *Wx-B1a* (lane 3); and a new allele, called *Wx-B1g* (lanes 2

and 4). This new allele present a mobility slightly higher than the *Wx-B1a* allele, but lower than the *Wx-B1d* allele (lane 1) present in the KU 4213D line found by Yamamori et al. (1995). The *Wx-B1g* allele was the most abundant, being detected in 85 accessions. The *null* type (*Wx-B1b* allele) was detected only in one line (emmer-52). Likewise, the *Wx-B1c** allele was solely found in the emmer-49 line.

PCR amplification of Wx genes

In order to confirm our results and reveal further polymorphism in the *waxy* proteins, PCR analyses were carried out. Besides the standards cvs. Langdon (*Wx-B1a*) and Mexicali (*Wx-B1c'*), the emmer-52 (*Wx-B1b*), emmer-49 (*Wx-B1c**) and emmer-71 (*Wx-B1g*) lines were selected for the PCR analyses. Three pairs of specific primers designed by Monari et al. (2005) were used to amplify the *waxy* genes in three different fragments (Table 1). With each pair of primers, two bands (*Wx-A1* and *Wx-B1* genes) were obtained.

Primer combination WxF3/WxVT1R was used to amplify the first region of the *waxy* genes, which corresponds to the signal peptide and the beginning of the mature protein. Some differences between the PCR products were observed. Cv. Langdon was shown to have a larger *Wx-B1* gene than the other samples of emmer wheat and cv. Mexicali (Fig. 2.A, lane 3. The *Wx-A1* gene was of the same size in all samples.

To amplify the central region of the *waxy* genes, the WxBAF/WxBAR pair was used. Once again, the results found (Fig. 2.B) apparently showed that the genes present in the cv. Langdon were different to emmer wheat samples and cv. Mexicali: the two bands co-migrated in cv. Langdon and ran separated in the rest of the samples.

Lastly, the third region of the *waxy* genes, amplified with the WxVT1F/WxVTR pair, showed two bands with the expected size of 1168 bp for the *Wx-A1* gene and 1154 bp for the *Wx-B1* gene. The separation of the amplification products in polyacrylamide gels did not detect clear differences in size between the bands of the different samples (Fig. 2.C).

The *null* allele for the *Wx-B1* gene present in the emmer-52 line showed amplification products for the three fragments of the gene.

Restriction analysis of the PCR products

On the basis of the published sequences of durum wheat, endonucleases that cut the *Wx-B1* gene but not the *Wx-A1* were chosen, so as to visualise more clearly polymorphism

in this gene. The amplification product of the first region of the gene was digested with *Stu*I, which cut once inside the first exon (Fig. 3.A), and separated in polyacrylamide gels. The result revealed that only the *Wx-B1* gene of cv. Langdon was digested and displayed the expected bands of 346 and 294 bp. No emmer wheat sample was affected by the enzymatic digestion.

The second fragment of *Wx-B1 gene* was digested with *Rse*I. This was expected to cut in the fifth exon. Once again, only the gene present in cv. Langdon was digested, whereas the other samples appeared unaltered (Fig. 3.B).

As in the other two regions of the *waxy* genes, the amplification products of the third fragment were digested with a restriction enzyme that was expected to cut only the *Wx-B1* gene. In this case, the *Bgl*II enzyme was used which cut at 307 bp inside the seventh exon of the *Wx-B1* gene. In this instance, the enzyme digested the *Wx-B1* gene of all the samples (Fig. 3.C) and two fragments of 306 bp and 848 bp were detected. Digestion fragments of *Wx-B1* gene from cv. Langdon were different to the rest of the samples, displaying a larger slow band and a smaller fast band than the others.

Sequence analysis of Wx-B1 genes

The amplification products of the *Wx-B1* gene of the three emmer wheat lines, together with the gene of cv. Mexicali were cloned for sequencing, and their nucleotide sequence are available in the GenBank Nucleotide Sequence Databases (emmer-49, GQ205418; emmer-52, GQ205419; emmer-71, GQ205417; and cv. Mexicali, GQ205420). Although no variability was found in SDS-PAGE and PCR analysis for the *Wx-A1* gene, its sequence was also obtained and analysed in two emmer lines (HM751941) for confirming the variation or not in this gene.

With these new data of the sequences of the *waxy* genes, the exact size and the real identity of the fragments amplified previously (Table 1) could be determined. For the second fragment of the *waxy* genes, the *Wx-B1* fragment from the three emmer wheat lines was the faster band and the *Wx-A1* the slower one, contrary to cv. Langdon. Similarly, with the third fragment of the gene, the *Wx-B1* gene fragment from cv. Langdon was faster than *Wx-A1* one, whereas in emmer wheat the contrary was the case (Table 1). A homology of 99.9 % and two SNPs in the eleventh exon were found between emmer *Wx-A1* gene and

durum one, which did not modify the predicted protein sequence. The two sequences of emmer showed a homology of 100 % between them.

The sequences of the *Wx-B1* gene were highly homologous among emmer wheat lines (99.5%), and very similar to the cv. Mexicali sequence (99%). DNA sequences were compared with the corresponding gene sequence of cv. Langdon available from the databases (AB029064). The pairwise identity between the *Wx-B1* genes present in cv. Langdon and emmer-71 line was 91.2%, showing diverse differences between them (ESM 2).

The main differences were presented in intron regions although several changes were detected inside exons that change the amino acid sequence of the *Wx-B1* protein. The deduced sequences of all sequencing genes were analysed and compared (Figure 4). The similarity of the amino acid sequence between the emmer-71 line and cv. Langdon was of 97.4% (15 amino acids changes out of a total of 578), while that between the emmer-71 line and cv. Mexicali was 99.5%. Several amino acids changes were present in the transit peptide of the protein (the first 70 amino acids) and only a few in the region corresponding to the mature protein. The amino acids changes found between cv. Langdon and emmer wheat were: Ala 34 → Ser, Ala 39 → Pro, Val 41 → Gly, Ile 46 → Thr, - 55 → Gln, Ser 62 → Thr, Gly 76 → Ala, Asn 246 → Ser, Met 250 → Arg, Thr 358 → Ala, Val 365 → Ala, Asn 451 → Ser, Val 510 → Met, Arg 520 → His, and Gly 554 → Glu.

The *Wx-B1* gene sequence of the emmer-52 line did not show any variation with respect to the rest of samples analysed that could justify the null *Wx-B1* protein detected in this accession. Neither deletion nor stop codon in the open reading frame were detected. The sequence of the emmer-49 line showed few differences with respect to emmer-71 and emmer-52. Specifically, four SNPs and two amino acids changes were detected in contrast to the other two sequences of emmer wheat: Met 510 → Val, His 520 → Arg. These two changes in emmer-49 are also present in the gene found in cvs. Langdon and Mexicali. Cv. Mexicali also showed one amino acid different from the rest: Asn 24 → Ser.

Because of these results, it can be asserted that the *Wx-B1c'* allele from emmer wheat is different to the *Wx-B1c** from durum wheat. Likewise, data demonstrated the differences between the *Wx-B1g* allele present in emmer wheat and the *Wx-B1a* allele of durum wheat. The comparison between emmer *Wx-B1* alleles and common wheat one described by Murai et al. (1999) showed high homology between both species (99.7% for *Wx-B1g* and 99.6%

for *Wx-B1c**). However, some amino acid changes were detected in the predicted sequence of the respective proteins (Arg 520 → His in *Wx-B1g* and Met 510 → Val in *Wx-B1c**), which confirm that the emmer *Wx-B1* alleles must be catalogued as novel.

Amylose content

The same lines evaluated by PCR and sequencing analysis (cv. Langdon, cv. Mexicali, emmer-49, emmer-52 and emmer-71) were analysed for amylose content. The mean amylose content of each sample is shown in Table 2. Although these data must be considerate as preliminary, cv. Langdon (*Wx-B1a*) showed higher value than emmer wheat ones and than cv. Mexicali. The emmer-52 line, which lacked the *Wx-B1* protein, showed the lowest amylose content, remarkable lower than the rest of the samples. Similarly, the emmer-49 line (*Wx-B1c**) presented slightly higher amylose content than the emmer-71 line (*Wx-B1g*), although both lines were not significantly different of cv. Mexicali.

Discussion

In the last twenty years, wheat starch associated proteins have been the topic of several studies due to their importance in the starch properties. Specifically, *waxy* proteins (Granule-bound starch synthase) that are the key enzymes in the amylose synthesis, have been the main subject of multiple studies, mainly focused on the search of null forms of these proteins or mutants with less activity (Yamamori et al. 1994; Rodríguez-Quijano et al. 1998; Yanagisawa et al. 2001). These null proteins can be used in breeding programs to produce partial (with less amylose) or total (free of amylose) *waxy* wheats. Starches with different amylose contents are still demanded and this makes it necessary to evaluate all possible alternatives of starch proteins in those species that can serve as a source of variability to increase the genetic basis of these components in modern wheat. Previous studies have suggested that emmer wheat could be one of these species (Sharma et al. 1981; Srivastava and Damania 1989).

In the current study, three different alleles for the *Wx-B1* gene were found by SDS-PAGE in emmer wheat; results were confirmed by PCR-RFLP and DNA sequence analyses. Several differences were found between the durum wheat cultivars with well-known alleles used as standards and emmer wheat, mainly in the *Wx-B1* gene. The comparison of the DNA sequences made it possible to confirm two new alleles (*Wx-B1c**

and *Wx-B1g*) in emmer wheat. The electrophoretic mobility in SDS-PAGE of *Wx-B1g* allele from emmer wheat is very similar to the *Wx-B1a* from durum wheat, but its internal structure show to be clearly different by DNA sequence analysis, showing 15 amino acid changes between the two proteins, nine of these changes appeared in the mature protein. Given that this allele is the most abundant in the current emmer collection, it could have been previously confused with the *Wx-B1a* allele due to its similar electrophoretic mobility (Yamamori et al. 1995; Rodríguez-Quijano et al. 2003). Besides, the *Wx-B1 null* allele was detected, although no mutation was detected in the coding sequence that could explain the absence of the protein. This suggests that this gene could be inactivated due to mutations in the gene regulatory regions.

In previous studies (Vrinten at al. 1999, Saito et al. 2004), the inactivation of the *Wx-B1b null* alleles in common wheat were explained by a sustained deletion in the *Wx-B1* gene. However, Monari et al. (2005) did not found any mutation in the sequence of a *Wx-B1null* durum line, as in our case. On the other hand, similarities between the *Wx-B1c´* and *Wx-B1c** sequences were greater than with respect to *Wx-B1g*. Given that the electrophoretic mobility of *Wx-B1c´* and *Wx-B1c** proteins is clearly different from *Wx-B1g* one and their DNA sequences are very similar, it is possible that part of these differences is the consequence of post-traductional mechanisms. In addition to this, *Wx-B1* emmer alleles showed a high homology to *Wx-B1a* allele from common wheat. This data has phylogenetic relevance and supports the hypothesis for the origin of hexaploid wheats as derived from the cross of *Aegilops tauschii* Cosss. with emmer and not with durum wheat (Salamini et al. 2002). The sequence data presented in this study could be used in phylogenetic analysis due to the high variability found in introns.

As a first approach to study the effect of the new alleles discovered, amylose content was measured in order to know if the observed differences for genes and proteins could affect the amylose content of these species. The highest value of amylose content was found in durum cv. Langdon, while that emmer wheat and cv. Mexicali showed similar contents. The same results were found by Rodríguez-Quijano et al. (2003). The exception was emmer-52 line that presented the lowest amylose content due to carry the *Wx-B1 null* protein. Other authors have demonstrated that the lack of *Wx-B1* causes a remarkable effect in amylose content, having a greater effect than the lack of *Wx-A1* or *Wx-D1* protein (Yamamori and Quynh, 2000; Araki et al. 2000). Although further works should be carried

out to study the effects of the described alleles on the amylose content and starch properties, the differences detected in gene and protein predicted sequences could help to explain better some of these differences in amylose content between durum and emmer wheat. It seems that the *Wx-B1* alleles described at a molecular level in this study tend to reduce the amylose content respect the common allele *Wx-B1a* of durum cv. Langdon. Therefore, although the introgression of any character between different species need additional information about the differential expression of these genes in a different background, this information could be useful for evaluating the possible use of this ancient crop in the quality breeding programmes of durum wheat.

On the other hand, because of the revival of the ancient wheat crops as emmer wheat in the context of the current Agriculture, the use of emmer lines without *Wx-B1* protein could be useful for breeding programmes focused on the development of partial-*waxy* emmer wheat with lower amylose content.

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Table 1.- Region amplified and expected fragment sizes (bp) from waxy alleles in durum and emmer wheat.

Primer pair	Region amplified	Expected length cv. Langdon	Length found in emmer wheat
WxF3 / WxVT1R	First to the third exon (I)	Wx-A1: 620 bp Wx-B1: 640 bp	Wx-A1: 620 bp Wx-B1: 648 bp
WxBAF / WxBAR	Third to the sixth exon (II)	Wx-A1: 953 bp Wx-B1: 969 bp	Wx-A1: 953 bp Wx-B1: 935 bp
WxVT1F / WxVTR	Sixth to the eleventh exon (III)	Wx-A1: 1168 bp Wx-B1: 1154 bp	Wx-A1: 1168 bp Wx-B1: 1174 bp

Table 2.- Amylose content of the lines evaluated.

Material	Allelic composition	Amylose content (%)
cv. Langdon	*Wx-A1a*, *Wx-B1a*	29.8 ± 0.41 a
cv. Mexicali	*Wx-A1a*, *Wx-B1c'*	26.5 ± 0.56 bc
Emmer-49	*Wx-A1a*, *Wx-B1c**	27.2 ± 0.15 b
Emmer-52	*Wx-A1a*, *Wx-B1b*	20.9 ± 0.65 d
Emmer-71	*Wx-A1a*, *Wx-B1g*	26.2 ± 0.25 c

l.s.d.: 0.81

Means follow of the same letter are not significant different.

Caption figures

Figure 1.- SDS-PAGE gel electrophoresis patterns of *waxy* proteins. Lanes are as follow: 1, KU 4213D (*Wx-A1a, Wx-B1d*); 2, Emmer-71 (*Wx-A1a*, *Wx-B1g*); 3, cv. Langdon (*Wx-A1a*, *Wx-B1a*); 4, Emmer-71 (*Wx-A1a*, *Wx-B1g*); 5, cv. Mexicali (*Wx-A1a*, *Wx-B1c'*); 6, Emmer-49 (*Wx-A1a*, *Wx-B1c**); 7, cv. Langdon (*Wx-A1a*, *Wx-B1a*); and 8, Emmer-52 (*Wx-A1a*, *Wx-B1b*).

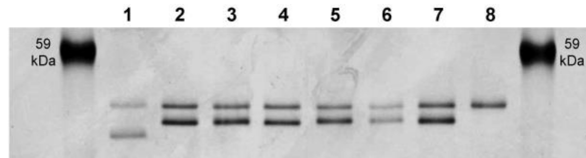

Figure 2.- Amplification products of the *Wx* genes. **A**, first fragment; **B**, second fragment; and **C**, third fragment (see text and Table 1). Lanes are as follow: 1, Emmer-71 (*Wx-A1a, Wx-B1g*); 2, Emmer-49 (*Wx-A1a, Wx-B1c**); 3, cv. Langdon (*Wx-A1a, Wx-B1a*); 4, cv. Mexicali (*Wx-A1a, Wx-B1c'*); and 5, Emmer-52 (*Wx-A1a, Wx-B1b*).

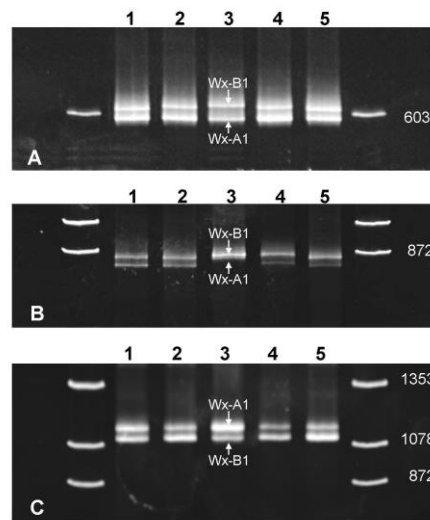

Figure 3.- Diagrammatic representation of the Wx-B1 gene and digestions of the three amplified fragments. **A**, first part of genes digested with *Stu*I; **B**, second part of genes digested with *Rse*I; and **C**, third part of genes digested with *Bgl*II. Lanes are as follow: 1, Emmer-71 (*Wx-A1a, Wx-B1a*); 2, Emmer-49 (*Wx-A1a, Wx-B1c**); 3, cv. Langdon (*Wx-A1a, Wx-B1a*); 4, cv. Mexicali (*Wx-A1a, Wx-B1c'*); and 5, Emmer-52 (*Wx-A1a, Wx-B1b*). Restriction enzymes digestion products are indicated (arrowheads).

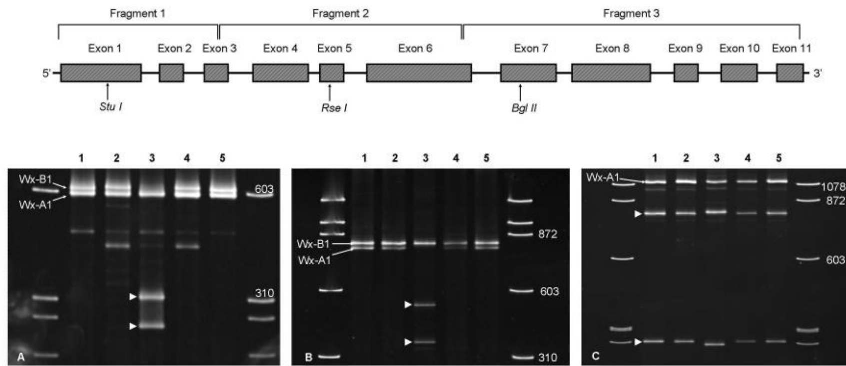

Figure 4.- Alignment of deduced protein sequences of the *Wx-B1* genes from cvs. Langdon and Mexicali (durum wheat) and three emmer lines (emmer-49, emmer 52 and emmer-71).

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                                                               Transit peptid <-┐┌-> Mature waxy protein
Langdon     3 LVTSQLATSGTVLGITDRFRRAGFQGVRPRNPADAALVMRTIGASAAPKQ-SRKAHRGSRRCLSMVVRATGSGGMNLVFVGAEMAPWSKTGGLGDVL
Emmer-49    3 ..............................S....P.G...T........Q.......T.............A........................
Emmer-52    3 ..............................S....P.G...T........Q.......T.............A........................
Emmer-71    3 ..............................S....P.G...T........Q.......T.............A........................
Mexicali    3 ....................H.........S....P.G...T........Q.......T.............A........................

Langdon   100 GGLPPAMAANGHRVMVISPRYDQYKDAWDTSVVSEIKVADEYERVRYFHCYKRGVDRVFVDHPCFLEKVRGKTKEKIYGPDAGTDYEDNQLRFSLLC
Emmer-49  101 .................................................................................................
Emmer-52  101 .................................................................................................
Emmer-71  101 .................................................................................................
Mexicali  101 .................................................................................................

Langdon   197 QAALEAPRILDLNNNPYFSGPYGEDVVFVCNDWHTGLLACYLKSNYQSNGIYMTAKVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFDFIDGYDKP
Emmer-49  198 ................................................S...R............................................
Emmer-52  198 ................................................S...R............................................
Emmer-71  198 ................................................S...R............................................
Mexicali  198 ................................................S...R............................................

Langdon   294 VEGRKINWMKAGILQADKVLTVSPYYAEELISGEARGCELDNIMRLTGITGIVNGMDVSEWDPTKDKFLAVNYDVTTALEGKALNKEALQAEVGLPV
Emmer-49  295 ...............................................................A......A..........................
Emmer-52  295 ...............................................................A......A..........................
Emmer-71  295 ...............................................................A......A..........................
Mexicali  295 ...............................................................A......A..........................

Langdon   391 DRKVPLVAFIGRLEEQKGPDVMIAAIPEILKEEDVQIVLLGTGKKKFERLLKSVEEKFPNKVRAVVRFNAPLAHQMMAGADVLAVTSRFEPCGLIQL
Emmer-49  392 ...........................................................S.....................................
Emmer-52  392 ...........................................................S.....................................
Emmer-71  392 ...........................................................S.....................................
Mexicali  392 ...........................................................S.....................................

Langdon   488 QGMRYGTPCACASTGGLVDTIVEGKTGFHMGRLSVDCNVVEPADVKKVVTTLKRAVKVVGTPAYHGMVKNCMIQDLSWKGPAKNWEDVLLELGV
Emmer-49  489 .................................................................E............................
Emmer-52  489 .....................M.........H.................................E............................
Emmer-71  489 .....................M.........H.................................E............................
Mexicali  489 .................................................................E............................
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