BIOLOGIA PLANTARUM 59 (2): 220-226, 2015 DOI: 10.1007/s10535-015-0507-0

Molecular characterization of several *Wx* alleles in durum wheat

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Abstract

The *Wx* gene, which encodes waxy proteins, is the sole gene responsible for amylose synthesis in the wheat seed endosperm. In this study, we characterized, at the molecular level, several *Wx* alleles in durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.) that had previously been catalogued at the protein level. Our data show a misclassification of the alleles in both *Wx* genes: *Wx-A1* alleles from Blanqueta (*Wx-A1a*) and Astrodur (*Wx-A1b*) have been reclassified as *Wx-A1a'* and *Wx-A1h*, respectively. A sequence comparison of the *Wx-B1e* allele shows that there were up to five different alleles under this denomination, which confirms that the protein analysis by SDS-PAGE separation needs to be combined with the PCR amplification-sequencing analysis in order to obtain a correct classification of the alleles, which will facilitate their use in wheat quality improvement.

Additional key words: PCR amplification; SDS-PAGE; *Triticum turgidum*, waxy proteins.

Introduction

Starch is the main component of wheat seed endosperm and accounts for up to 70 % of its dry mass. This polysaccharide is a mix of two components: amylose (22 - 35 %) and amylopectin (65 - 78 %). Although environmental factors such as temperature can influence this ratio (Sumesh *et al*. 2008), this is a trait governed mainly by a genetic control. Although several enzymes are involved in amylopectin synthesis, only the granule bound starch synthase I (GBSS-I or a waxy protein) has been associated with amylose synthesis (for a review, see Baldwin 2001). The starch physicochemical properties and the end-use quality of different wheat products are conditioned by the amylose/amylopectin ratio which depends on these proteins. This fact has led during the last 20 years to a large number of studies that have mostly focused on searching for variability in the waxy proteins.

In durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.; 2n = 4× = 28, BBA^uA^u), these proteins are synthesised by *Wx* genes (*Wx-A1* and *Wx-B1*) that are located on chromosomes 7AS and 4AL (translocated from 7BS), respectively (Chao *et al*. 1989, Ainsworth *et al*. 1993, Yamamori *et al*. 1994). In general, the studies on durum wheat have shown that there is a very little waxy protein variation, and wild variants are clearly hegemonic (Yamamori *et al*. 1995, Nieto-Taladriz *et al*. 2000, Urbano *et al*. 2002).

Of a particular importance are *null* alleles or those encoding Wx proteins with modified functions because they decrease amylose content, specially the *Wx-B1b* allele (Sharma *et al*. 2002). As the presence of these alleles is low in durum wheat, a search for them has a great relevance.

Although classification of different variants has been mainly carried out by an electrophoretic separation (SDS-PAGE), a method for detecting the null variants using a specific primer set was developed by Nakamura *et al*. (2002). In this study, the *null* alleles were easily distinguished from the others, but later studies have

Submitted 13 August 2014, *last revision* 5 January 2015, *accepted* 7 January 2015.
Abbreviations: PCR - polymerase chain reaction; SDS-PAGE - sodium dodecylsulphate-polymerase chain reaction; Wx - waxy.
Acknowledgements: This research was supported by a grant No. AGL2010-19643-C02-01 of the Spanish Ministry of Economy and Competitiveness and a grant from the European Regional Development Fund (FEDER) of the European Union. The technical assistance of M. Ayala is appreciated. We thank to the Centro de Recursos Fitogenéticos (Alcalá de Henares, Spain), the National Small Grain Collection (Aberdeen, USA), and the Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany) for supplying the analysed material.
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shown that other *null* alleles with different origins can also be detected (Vanzeti *et al*. 2010, Guzmán *et al*. 2011). In the same way, small differences in the size of the protein variants and the limited resolution of SDS-PAGE electrophoresis mean that using this technique to screen for waxy protein polymorphism may mask part of the true variation, *e.g*., intron differences or amino acid changes that could affect protein properties with a minor impact on the size. At this respect, Yamamori and Guzman (2013) proved that changes in just one amino acid may have an important effect on protein activity. In a previous study (Guzmán *et al*. 2012), we found that an allele classified by SDS-PAGE analysis was resolved in up to seven different nucleotide sequences and five amino acid sequences. Another study (Guzman *et al*. 2011) showed that the predominant Wx-B1 protein in durum and emmer wheat, which was considered as the same allele in previous studies, actually vary by 15 different amino acids in their protein sequences after a molecular characterization of the *Wx-B1* genes in both species. Although the entire sequence is necessary for the characterization of any new allele, our own studies have revealed that sequence differences are more pronounced in the central region of the gene, between the fourth and seventh exon (Guzmán *et al.* 2009, 2010, 2011, 2012, Ortega *et al*. 2014). This suggests that the combined use of SDS-PAGE and PCR amplification could be a useful tool for evaluating *Wx* gene variation.

The main objectives of this study were the molecular characterization of several *Wx* alleles in durum wheat that had previously been described at the protein level, and the reclassification of some of these alleles.

Materials and methods

Plants: Several cultivars of durum wheat that were previously analysed for waxy proteins composition were used (Table 1). These materials were kindly provided by the Centro de Recursos Fitogenéticos (Alcalá de Henares, Spain), the National Small Grain Collection (Aberdeen, USA), and the Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany).

The cultivar Chinese Spring, together with the waxy line WQL6K107-BHWX14-7 (PI 612546) generated by Morris and Konzak (2001), were used as controls. Furthermore, two bread wheat cultivars (Turkey Red and Eureka) that present a putative *Wx-B1e* allele (McInstosh *et al*. 2013) were also used to compare *Wx-B1e* sequences in the materials assigned to this allele.

DNA extraction and PCR amplification: For DNA extraction, about 100 mg of young leaf tissue was excised and immediately frozen in liquid nitrogen. Total genomic DNA was isolated by the cetyltrimethyl ammonium bromide (CTAB) method as described by Stacey and Isaac (1994).

Primer sequences together with PCR conditions for each primer pair are listed in Table 1 Suppl. Primers designed by Nakamura *et al*. (2002) were used to evaluate the presence of the *null* alleles *Wx-A1b* (AFC/AR2) and *Wx-B1b* (BDFL/BRD). To amplify the final region together with the initial part of the 3′-UTR of *Wx* gene (approximately 200 bp), Wx3º2Fw was combined with the primer SUN1R designed by Shariflou and Sharp (1999). The central region of the gene (Wx2Fw/Wx2Rv, from the fourth to seventh exon) as well as its complete translated sequence (Wx1Fw/Wx3Rv) were amplified using primers designed by Guzman and Alvarez (2012). All amplifications were performed in 0.02 cm^3 of final volume containing 50 ng of genomic DNA, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 0.004 cm^3 of 10× PCR buffer, and 0.75 U *Taq* polymerase (*Promega*, Madison, WI, USA). The primer concentrations were 0.3 μM for the central fragment (Wx2Fw/Wx2Rv) and 0.2 μM for the rest.

Amplification products were fractionated in vertical PAGE gels with 8 % (m/v) polyacrylamide and the bands were visualized by ethidium bromide staining. PCR products were purified and ligated using *pGEM T-easy* vector (*Promega*) for sequencing. Inserts were sequenced from at least three different clones using an *ABI Prism 310* genetic analyzer (*Applied Biosystems*, Carlsbad, CA, USA). DNA sequences together with the traits of their deduced proteins, including relative molecular mass (M_r) and isoelectric point (pI), were analysed by the *Geneious Pro v. 5.0.4* software (*Biomatters Ltd*, New Zealand).

Results

In previous studies based on SDS-PAGE protein electrophoresis analysis (Nieto-Taladriz *et al*. 2000), Wx-A1 proteins from durum wheat cultivars Blanqueta and Astrodur were identified as the wild (*Wx-A1a* allele) and *null* (*Wx-A1b* allele) types, respectively (Table 1). However, the analysis of these alleles using the AFC and AR2 primers showed clear differences between the *null* allele in Astrodur and the reference *null* allele (WQL6K107-BHWX14-7). In contrast, no difference could be detected between the apparent wild *Wx-A1* allele in Blanqueta and Chinese Spring (Fig. 1*A*). Nevertheless, when the Wx3º2Fw/SUN1R primers were used, the amplicon obtained from Blanqueta was larger than that obtained from Chinese Spring (Fig. 1*B*).

The differences observed were confirmed when the complete *Wx-A1* allele sequences (exons + introns) for the two durum wheat cultivars were obtained (Table 2 Suppl.). The *Wx-A1b* allele (NCBI ID: AF113843) had a

Table 1. Current composition for *Wx-A1* and *Wx-B1* genes of the accessions used in this study, and proposed changes in the nomenclature. [a] - BGE: Centro de Recursos Fitogenéticos, Alcalá de Henares, Spain; CItr and PI: National Small Grain Collection, Aberdeen, USA; TRI: Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany. [b] according to Wheat Gene Catalog (McInstosh *et al*. 2013).

Cultivar/line	Accession[a]	Classification current[b]		new tentative	
		Wx-A1	*Wx-B1*	*Wx-A1*	*Wx-B1*
Astrodur	TRI 27109	*b*	*e*	*h*	*e*
Blanqueta	BGE 13702	*a*	*b*	*a′*	*b*
Lobeiro ruivo	BGE 12408	*a*	*e*	*a*	*e′*
Lobeiro oscuro	BGE 20909	*a*	*e*	*a*	*e′*
Mourisco	BGE 12413	*a*	*f*	*a*	*f*
Bread wheat					
Chinese Spring	-	*a*	*a*	*a*	*a*
WQL6K107-BHWX14-7	PI 612546	*b*	*b*	*b*	*b*
Turkey Red	PI 565351	*a*	*e*	*a*	
Eureka	CItr 12812	*a*	*e*	*a*	

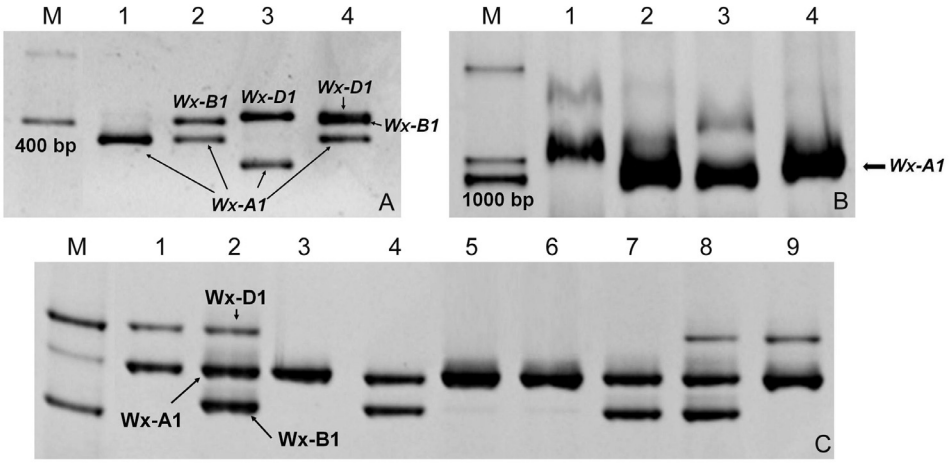

Fig. 1. Polyacrylamide electrophoresis of PCR products obtained from genomic DNA using AFC/AR2 primers (*A*), Wx3º2Fw/SUN1R primers (*B*), and BDFL/BRD primers (*C*). *Lanes* in *A* and *B*: 1 - Blanqueta, 2 - Astrodur, 3 - WQL6K107-BHWX14-7, and 4 - Chinese Spring. *Lanes* in *C*: 1 - WQL6K107-BHWX14-7, 2 - Chinese Spring, 3 - Blanqueta, 4 - Astrodur, 5 - Lobeiro oscuro, 6 - Lobeiro ruivo, 7 - Mourisco, 8 - Turkey Red, and 9 - Eureka. M - a DNA molecular marker.

deletion of 19 bp, which included the end of exon-2 and the beginning of intron-2. However, Astrodur did not show this deletion, but the null character is conferred by a single nucleotide deletion in exon-7, which produced a frameshift that generated a premature stop codon in the gene sequence. This mutation is the same as the one described by Vanzetti *et al*. (2010) in the partial sequence of the *Wx-A1h* allele since the alignment of this partial sequence (458 bp; NCBI ID: GQ120523) shows a 100 % similarity with the *Wx-A1* sequence in Astrodur (Fig. 2*B*), therefore, we classified this allele as putative *Wx-A1h*.

The alignment between the *Wx-A1* allele in Blanqueta and the *Wx-A1a* allele (Chinese Spring) shows that there were nine single nucleotide polymorphisms (SNPs) inside the translated region, so it has been reclassified as *Wx-A1a'*. Only five of these differences had an effect on amino acid sequence and were responsible for three changes that affected predicted Mr and pI (Table 2 Suppl.). In exon-2, changes appeared between position 177 and position 181, which is A-TTG-G in the wild sequence (*Wx-A1a*) and T-GGG-A in the *Wx-A1a′* allele. This generated two amino acid changes: Phe60 → Gly and Asp61 → Asn. Another amino acid change was detected in exon-9 where Trp453 → Arg was due to a transversion (T → A) in nucleotide 2042. Additional differences were found in the 3′-UTR located inside a microsatellite described by Shariflou and Sharp (1999). The differences were an $(AT)_6A(AT)_2$ repeat in the *Wx-A1a* allele and $(AT)_8A(AT)_2$ in *Wx-A1a′*. The allele present in Astrodur had a perfect $(AT)_7$ repeat (Fig. 3).

According to Vrinten *et al*. (1999), *null* allele *Wx-B1b* is characterized by the deletion of the entire *Wx* gene, so the absence of amplicon corresponding to the B genome in cv. Blanqueta when the AFC/AR2 primers were used

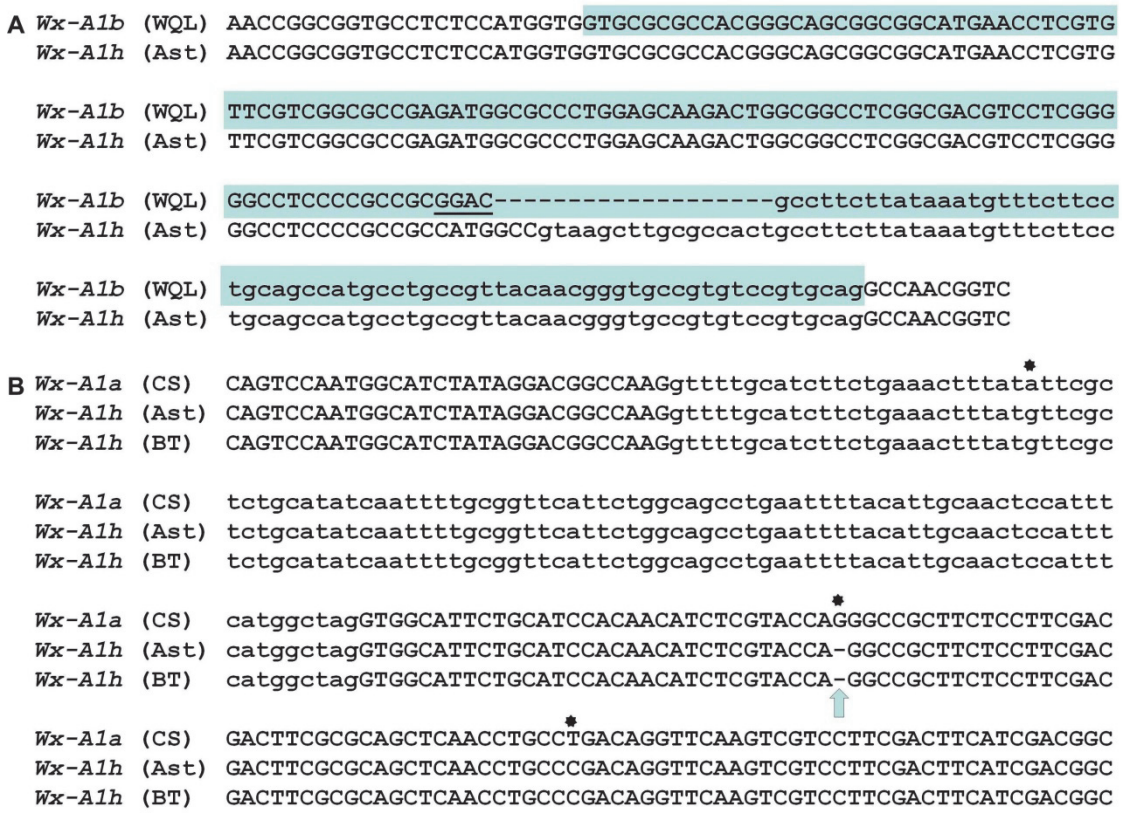

Fig. 2. The alignment of DNA sequences. *A* - comparison between the second and third exons (*upper case letters*), and the second intron (*lower case letters*) from the *null* alleles of Astrodur (Ast, *Wx-A1h*) and WQL6K107-BHWX14-7 (WQL, *Wx-A1b*). The *shaded region* indicates alternative processing in *Wx-A1b* as consequence of one cryptic splice site located 117 bp upstream of normal splice site. The filler DNA appears underlined. *B* - the comparison of central region between the *Wx-A1* alleles of Chinese Spring (CS), Astrodur (Ast), and Buck Topacio (BT). The *asterisks* show SNPs found and the *arrow* indicates the nucleotide deletion responsible for a change in the frameshift.

Fig. 3. The alignment of the ending of exon 11 (*upper case letters*) and the beginning of the 3'-UTR (*lower case letters*) of the *Wx-A1* alleles of Chinese Spring (CS), Blanqueta (Blq), and Astrodur (Ast). The *asterisks* indicate SNPs found in the 3'-UTR region and the *arrow* a non-synonymous substitution. The *shaded region* corresponds with a microsatellite described by Shariflou and Sharp (2002).

suggests that it possessed this allele (Fig. 1*A*). This was confirmed using a primer pair designed by Nakamura *et al*. (2002) for the specific detection of *Wx* null (Fig. 1*C*). In addition, two different patterns were detected among the five alleles classified as *Wx-B1e* (Table 1) by SDS-PAGE (Nieto-Taladriz *et al*. 2000). *Wx-B1e* alleles from Astrodur and Turkey Red showed an expected amplicon with a similar mobility to those from *Wx-B1a* and *Wx-B1f* (Fig. 1*C*). Conversely, in *Wx-B1e* alleles from both Lobeiro and Eureka, this amplicon was not present

(Fig. 1*C*), showing a pattern similar to those with a *null* allele; although a higher intensity of the band corresponding to the *Wx-A1* gene in these accessions led us to think of overlapping *Wx-A1* and *Wx-B1* amplicons due to a similar size. To molecularly characterize these differences, complete *Wx-B1* gene was sequenced from Astrodur and both the Lobeiro accessions, as well as the central fragment (the fourth to seventh exon) from Turkey Red and Eureka. *Wx-B1f* allele was also included for complete sequencing as it has never been characterized at nucleotide level.

The comparison of the sequences shows that Lobeiro oscuro and Lobeiro ruivo had the same allele (a 100 % similarity, so we refer to this allele as Lobeiro), but all other sequences were different. Astrodur and Turkey red, although very similar, were not the same and were very different from Lobeiro and Eureka which were also slightly different from each other (Fig. S1) and very close (a 99.4 and 99.6 % homology, respectively) to the *Wx-B1e* allele (NCBI ID: KF305522) described by Klimushina *et al* (2013). Therefore, these cultivars have four different alleles that should be classified separately. In this study, the *Wx-B1* allele detected in Astrodur has been named *Wx-B1e*, whereas the Lobeiro allele was named *Wx-B1e′*.

Genomic sequences were also compared to the *Wx-A1a* sequence of Chinese Spring (Table 2 Suppl, Fig. 2 Suppl.). Large differences, particularly in the case of *Wx-B1e′*, were found between these alleles. To check whether they led to changes in protein, the deduced proteins of the novel alleles together with those from *Wx-B1a* (Chinese Spring) and *Wx-B1c′* (Mexicali), which were described in a previous study (Guzman *et al*. 2011), were compared (Table 2). Proteins from *Wx-B1e* and *Wx-B1f* were similar, and only one amino acid change was found between them and that from *Wx-B1a* (Met510 → Val) or *Wx-B1c′* (His24 → Arg). Differences were higher for *Wx-B1e′* (Table 2) which showed 13 amino acid changes and one deletion (Gln54) compared to *Wx-B1a*, and one more compared to *Wx-B1c′*.

Table 2. Comparison of amino acid composition among deduced proteins from alleles *Wx-B1a* of bread wheat cv. Chinese Spring (reference), *Wx-B1c′* of cv. Mexicali, and novel alleles detected in the current study: *Wx-B1e* allele of cv. Astrodur, *Wx-B1e'* allele of cvs. Lobeiro oscuro and L. ruivo, and *Wx-B1f* of cv. Mourisco.

Position	24	34	39	41	45	54	62	76	246	250	358	365	451	510	554
Wx-A1a	Arg	Ser	Pro	Gly	Thr	Gln	Thr	Ala	Ser	Arg	Ala	Ala	Ser	Met	Glu
Wx-B1e						Gln								Val	
Wx-B1e′		Asn	Ala	Val	Ile	-	Ser	Gly	Asn	Met	Thr	Val	Asn	Val	Gly
Wx-B1f						Gln								Val	
Wx-B1c′	His					Gln								Val	

Discussion

According to data in the Wheat Gene Catalogue (McInstosh *et al*. 2013), waxy protein polymorphism in wheat is limited. However, it is important to determine if this limited variation is true or is a consequence of analysis techniques used. In the case of waxy proteins, a similar size among the products of the three genes, together with the possibility of amino acid changes that have no effect on the protein size, could easily generate incorrect classifications when variations in these genes are exclusively determined by the electrophoretic separation of their proteins as suggested for other wheat endosperm proteins (Goldsbrough *et al*. 1989).

In this study, several primer pairs have been used in the PCR analysis of the *Wx* alleles from durum wheat cultivars previously classified by protein analysis (Nieto-Taladriz *et al*. 2000). Our data show that the nucleotide sequences of the alleles present in these materials did not correspond with putative *Wx* alleles assigned by SDS-PAGE protein separation. For the *Wx-A1* gene, this misclassification of two *null* alleles has been caused by two factors that are impossible to differentiate by protein analysis. The *null Wx-A1* allele of Astrodur did not correspond with the *Wx-A1b* allele, according to its DNA sequence, but with the *Wx-A1h* allele. The null character of this last allele is due to one single nucleotide deletion in exon-7, which produces a frameshift that leads to the appearance of a premature stop codon. In the *Wx-A1b* allele, the null character is the result of aberrant splicing derived from a 23-bp deletion, which cause the loss of 39 amino acids including part of the transit peptide and the ADP-glucose binding site (Vrinten *et al*. 1999). Even though the presence of one or other allele would have identical effects on amylose content, a correct classification of the *Wx* alleles is very important if they are to be used in wheat breeding programs. This study correctly detected and properly identified both alleles using specific molecular markers. However, it is important to note that wheat lines carrying the *Wx-A1b* allele would lead to a negative result for the markers designed by Vanzetti *et al*. (2010) because they were designed to detect the *Wx-A1h* allele and *vice versa* for *Wx-A1h* allele detection using the markers designed

by Nakamura *et al*. (2002) for *Wx-B1b* detection.

Other possible causes of the misclassification of *waxy* alleles are that the differences inside the coding sequence cannot be detected using SDS-PAGE because these changes have no effect on the apparent protein size. This was the case of the *Wx-A1* allele in Blanqueta which was classified as the wild-type allele (*Wx-A1a*). However, our data show differences in the deduced translated sequence which was different from the Wx-A1 protein of cv. Chinese Spring and led to the reclassification of this allele as *Wx-A1a'*.

A paradigmatic case is the *Wx-B1e* allele analysed in this study. Diverse authors have identified this allele in durum and bread wheat cultivars (Nieto-Taladriz *et al*. 2000, Yamamori and Quynh 2000) and we have used some of these cultivars as controls during its evaluation. A previous work suggested that at least two different allelic variants could have been classified as *Wx-B1e* (Divashuk *et al.* 2011), but the comparison of the complete or partial sequence produced in this study together with sequences obtained previously (Guzman *et al*. 2011, Klimushina *et al*. 2013) shows that up to six different nucleotide sequences could have been misclassified, as Mr and pI of these deduced proteins were very similar. Consequently, it is very difficult in this context to establish clear differences among the different sequences and the error is highly understood.

In conclusion, the data presented in this survey confirm the necessity of combining protein analysis by SDS-PAGE separation with PCR amplification-sequencing analysis in order to obtain a correct classification of the alleles, which will facilitate their future use in wheat quality improvement. Obviously, any allele whose protein shows a clear difference in mobility should be accepted as being different. However, any proteins that have the same mobility should only be classified as being the same if DNA analysis also shows no differences between the protein sequences.

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