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REVIEW

Wheat waxy proteins: polymorphism, molecular characterization and effects on starch properties

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Abstract The starch fraction, comprising about 70 % of the total dry matter in the wheat grain, can greatly affect the end-use quality of products made from wheat kernels, especially Asian noodles. Starch is associated with the shelf life and nutritional value (glycaemic index) of different wheat products. Starch quality is closely associated with the ratio of amylose to amylopectin, the two main macromolecules forming starch. In this review, we briefly summarise the discovery of waxy proteins—shown to be the sole enzymes responsible for amylose synthesis in wheat. The review particularly focuses on the different variants of these proteins, together with their molecular characterisation and evaluation of their effects on starch composition. There have been 19 different waxy protein variants described using protein electrophoresis; and at a molecular level 19, 15 and seven alleles described for *Wx-A1*, *Wx-B1* and *Wx-D1*, respectively. This large variability, found in modern wheat and genetic resources such as wheat ancestors and wild relatives, is in some cases not properly ordered. The proper ordering of all the data generated is the key to enhancing use in breeding programmes of the current variability described, and thus generating wheat with novel starch properties to satisfy the demand of industry and consumers for novel high-quality processed food.

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Introduction

Wheat is one of the most important crops in the world, with total harvested area of 218.5 million hectares and annual yields of almost 713 million tonnes (FAO 2013). This crop originated in the Fertile Crescent and presents a wide adaptation capacity to different latitudes, which has been key to its success, and is grown in all continents except Antarctica. Although wheat is truly a complex constituted by species with different levels of ploidy (di-, tetra- and hexaploid), there are now two main species cultivated: durum or pasta wheat (*Triticum turgidum* ssp. *durum* Desf. em. Husn.; $2n = 4\times = 28$, AABB) and common wheat (*T. aestivum* L. ssp. *aestivum*; $2n = 6\times = 42$, AABBDD). Both species have the unique ability to produce a broad range of nutritious, appealing foods. Depending on the geographical region, wheat is used to make bread (Europe and America), tortillas (North America), cous-cous (North Africa), chapatti (South Asia), noodles (East Asia) and pasta (Europe and America), among other products (see Faridi and Faubion 1995 for a review). This ability of wheat is based on large variation of the three main traits that determine wheat quality and its end-use: grain hardness, gluten quality and starch (Ram and Mishra 2008).

The grain hardness or texture is the single most important trait that determines end-use and technological utilisation, forming the fundamental basis of differentiating the world trade of wheat grain. According to this trait, wheat is classified in very hard (durum wheat), hard and soft (common wheat). Due to hardness differences, common wheat is used for bread (hard) or cookies and pastries

(soft) production due to the different amounts of damage to starch generated in the milling, which strongly affects the water absorption of dough. The molecular genetic basis of this trait is now clear and two small grain proteins named puroindolines have been shown to be responsible. These proteins are encoded by the *Pina-D1* and *Pinb-D1* genes located in the locus *Ha* (*Hardness*), which is present in the short arm of the chromosome 5D of common wheat (Morris 2002).

Gluten is another major factor affecting wheat quality. The main constituents of this protein viscoelastic network that confer to wheat the ability to form cohesive dough are the endosperm storage proteins (glutenins and gliadins). Glutenins are divided in two groups according to their molecular weight: high molecular weight subunits (HMWGs) encoded at the *Glu-1* loci located on the long arm of chromosome 1-group; and low molecular weight subunits (LMWGs) encoded at the *Glu-3* loci on the short arm of the same chromosomes. Gliadins are divided into ω- and γ-gliadins synthesised by genes of the *Gli-1* loci (short arm of chromosome 1) and α/β-gliadins encoded by the *Gli-2* loci on the sort arm of chromosome 6 (Payne 1987). Although the variation and genetics of all these proteins are not completely understood, there have been great advances in recent decades and several alleles have been associated with good or bad quality for different products in both bread and durum wheat (see Wrigley et al. 2006 for a review).

Finally, the starch fraction, comprising about 70 % of the total dry matter in the wheat grain (Hucl et al. 1996), can greatly affect the products made from wheat kernels, especially Asian noodles (Huang and Lai 2010; Miura and Tanii 1994;). In addition to noodle quality, starch is associated with shelf life (Hayakawa et al. 1997, 2004) and nutritional value (Regina et al. 2006) of the products, and with biofuel yield from wheat (Sosulski and Sosulski 1994; Wu et al. 2006). Starch quality is closely associated with the starch granule structure and the distribution of the two major glucan macromolecules, amylose and amylopectin, that accumulate within the granules. The former is related with the presence/absence or activity of the enzymes involved in starch synthesis (James et al. 2003; Morell et al. 2001).

In this review, we briefly summarise the discovery of waxy proteins—shown to be the sole enzymes responsible for amylose synthesis. We especially focus on the different variants found for these proteins, together with their molecular characterisation and evaluation of their effects on starch composition.

Starch: composition and synthesis

As mentioned above, starch comprises two macromolecules: amylose and amylopectin. Amylose is essentially a linear molecule, in which glucosyl monomers are joined via α (1 → 4)-linkages. Amylopectin contains linear chains of various lengths with α (1 → 4)-linkages, together with approximately 5 % of branched chains generated by α (1 → 6)-linkages. The ratio of both polymers in wheat varies within 22–35 % for amylose and 68–75 % for amylopectin. The physical and chemical properties of starch (gelatinisation, pasting and gelation), and consequently the quality of the end-products are dependent on the relative amounts of amylose and amylopectin (Fredriksson et al. 1998; Zeng et al. 1997).

Starch synthesis occurs within the amyloplast, an organelle derived from the same proplastids as chloroplasts but without photosynthetic apparatus. Starch is stored in granules in which have been detected several proteins, termed starch granule proteins (SGP). In 1987, Schofield and Greenwell described up to ten of these SGPs that they divided in two sets: five 'surface' SGPs with molecular weight of 5–30 kDa; and five 'integral' SGPs of 59–149 kDa. Later studies suggested that the main enzymes of starch synthesis are in the second group: granule-bound starch synthase I (GBSSI or waxy; 59 or 61 kDa), starch synthase I (SSI or SGP-3; 80 kDa), starch synthase II (SSII or SGP-1; 100–115 kDa) and starch branching enzyme I (SBEI or SGP-2; 92 kDa). Data of Yamamori and Endo (1996) indicated that the genes encoding waxy, SGP-1 and SGP-3 proteins are located in the short arm of chromosomes 7A, 7B and 7D, with exception of the *waxy* gene from genome B that appears in 4AL due to a translocation from 7BS (Chao et al. 1989).

Although the enzymes involved in synthesis of both starch polymers (amylose and amylopectin) differ, the initial substrate for both is ADP-glucose. This substrate can be generated inside the amyloplast with glucose-1-phosphate and ATP by ADP-glucose pyrophosphorylase or transferred from the cytoplasm. Amylopectin synthesis is a complex pathway that involves at least three starch synthases (SSI or SGP-3, SSII or SGP-1 and SSIII) and several branching (SBEI or SGP-2, SBEIIa and SBEIIb) and de-branching enzymes. Studies have focussed on all these enzymes and breeding approaches have also been carried out, as in the case of common wheat lines generated by Yamamori et al. (2000) that lacked the three SSII proteins (SGP-1A, SGP-1B and SGP-1D) and showed high amylose content and resistant starch (Yamamori et al. 2006).

In contrast, amylose synthesis in wheat endosperm is only carried out by GBSSI or waxy protein (E.C. 2.4.11.11), which was demonstrated by the development of the first waxy wheats (Nakamura et al. 1995). However, other studies have suggested that this enzyme could also have an indirect effect on the amylopectin synthesis, probably due to a feedback process (Rahman et al. 2000).

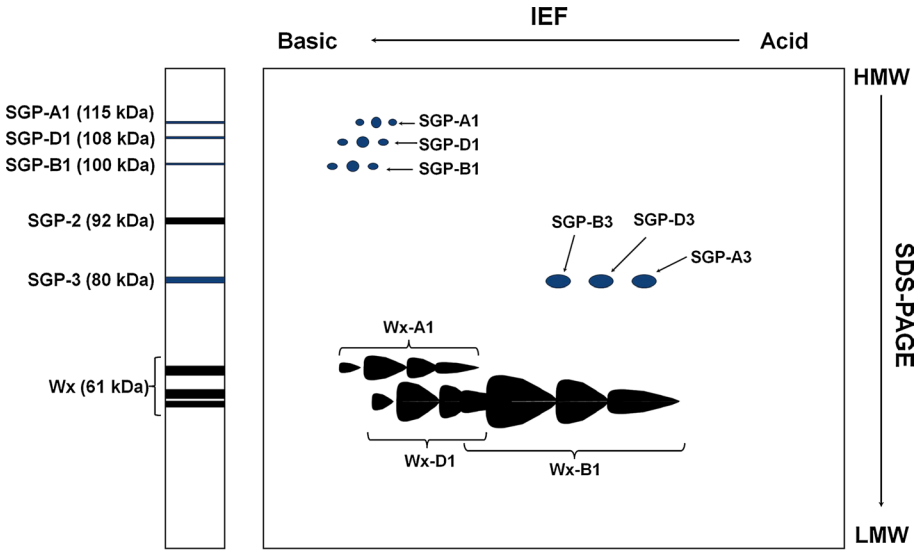

Fig. 1 Diagrammatic representation of the separation of wheat starch proteins by (**a**) SDS-PAGE electrophoresis, and by (**b**) 2D electrophoresis (IEF + SDS-PAGE)

The waxy protein was properly identified in maize (Echt and Schwartz 1981), where its lack was shown to cause the *waxy* phenotype of the endosperm, which was characterised by the waxy appearance of the endosperm due to the absence of amylose in the grain starch. Later, the *waxy* gene was identified and isolated in maize (Shure et al. 1983) as well as rice (Okagaki and Wessler 1988; Sano 1984) and barley (Rohde et al. 1988). In wheat, the discovery or detection–identification of waxy proteins was closely linked with the Japanese udon noodle end-use quality, which is inversely correlated with the amylose content of wheat grain (Oda et al. 1980). This was confirmed in the studies of Nakamura et al. (1992) and Yamamori et al. (1992), because the cultivars preferred for noodles due to their low amylose content, such as cv. Kanto107, showed a reduced expression of the waxy proteins (Yamamori et al. 1992) or even a lack of any of their components (Nakamura et al. 1992).

The first analysis of these proteins by separation in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed low discrimination between the three potential proteins, one for each genome, expected in common wheat (Nakamura et al. 1992; Yamamori et al. 1992), mainly due to the small variation in their molecular weight (Wx-A1: 62.8 kDa, Wx-B1: 56.7 kDa and Wx-D1: 58.7 kDa). One year later, the same group of Japanese scientists developed a 2D electrophoresis methodology (Isoelectrofocusing, IEF × SDS-PAGE; Fig. 1) that enabled them to separate and correctly identify three waxy proteins in common wheat (Nakamura et al. 1993a). Additionally, the lack of one or two waxy proteins (Wx-A1 or Wx-B1) in some Japanese cultivars was confirmed (Nakamura et al. 1993b) and thus the presence of *waxy null* alleles, which would have been indirectly selected and fixed in the breeding process focussed on noodle quality. The identity and chromosomal location of each waxy protein (named Wx-A1, Wx-B1 and Wx-D1) were confirmed using nullisomic-tetrasomic lines of cv. Chinese Spring for chromosomes 7A, 4A and 7D (Ainsworth et al. 1993).

Once the methodology to detect waxy proteins was developed and working, the first studies to determine the effect or role of each waxy protein in amylose synthesis were performed (Miura and Tanii 1994; Miura et al. 1994) in which Wx-B1 protein was shown to be the most important in amylose synthesis. In addition, there were screenings to find cultivars or accessions carrying polymorphic waxy variants, different from that present in cv. Chinese Spring (Wx-A1a, Wx-B1a and Wx-D1a). Until that time, only *Wx-A1* and *Wx-B1 null* alleles had been detected, but not for the *Wx-D1* locus. In a huge and crucial study, Yamamori et al. (1994) analysed 1960 cultivars of different origins by 2D electrophoresis. Several cultivars lacked the Wx-A1 or W-B1 protein (177 and 159, respectively) but only one, Chinese cv. Bai Huo, showed the *null* allele *Wx-D1b*. With this discovery, the first waxy common wheat (carrying starch with 0 % amylose) was developed (Nakamura et al. 1995) by crossing cv. Kanto 107 (*Wx-A1b* and *Wx-B1b*) with cv. Bai Huo (*Wx-D1b*). The same authors also developed waxy durum wheat by crossing durum cv. Aldura with cv. Kanto 107 (Nakamura et al. 1995).

Variability of waxy proteins

The variability detected by electrophoretic separation of proteins is very limited, because of insufficient resolution to detect minor changes in the protein size/sequence. In fact, only 16 protein variants for the three genomes have been described by this procedure, mainly when 2D electrophoresis was carried out (Table 1).

Table 1 Waxy protein variants identified by protein electrophoresis

Allelic variant	Difference to wild variant in electrophoresis[a]	Cv. or accession of reference	References
Wx-A1b	Null	cv. Kanto107 (common wheat)	1
Wx-A1c	Greater mobility and more basic isoelectric point	QT105 (common wheat)	2
Wx-A1d	Greater mobility	KU8937B (wild emmer)	3
Wx-A1e	More basic isoelectric point	KU3655 (durum wheat)	3
Wx-A1g	Reduced amount of protein	PI-348476 (spelt)	4
Wx-A1i	Reduced amount of protein	KU9259 (durum wheat)	3
Wx-A1j	More basic isoelectric point than Wx-A1c. Same mobility.	PI-242428 (common wheat)	5
Wx-B1b	Null	cv. Kanto107 (common wheat)	1
Wx-B1c	More basic isoelectric point	cv. Cikotaba (common wheat)	2
Wx-B1d	Greater mobility and more basic isoelectric point	KU4213D (durum wheat)	3
Wx-B1e [Wx-B1c´]	Less mobility	cv. Cartaya (common wheat)	6
Wx-B1f	Less mobility than Wx-B1a, similar to Wx-D1a	BGE-012413 (durum wheat)	7
Wx-B1g	Slightly less mobility than Wx-B1a	BGE-012302 (emmer)	8
Wx-D1b	Null	cv Bai Huo (common wheat)	2
Wx-D1c	More basic isoelectric point	cv. Scoutland (common wheat)	2
Wx-D1g	Less mobility	PI-348701 (spelt)	9

Ref.: *1* Nakamura et al. (1993a, b;) *2* Yamamori et al. (1994); *3* Yamamori et al. (1995); *4* Caballero et al. (2008v; *5* Yamamori and Yamamoto (2011;) *6* Rodriguez-Quijano et al. (1998); *7* Nieto-Taladriz et al. (2000); *8* Guzmán et al. (2011); and *9* Guzmán et al. (2010)

[a] Allelic variants are Wx-A1a, Wx-B1a and Wx-D1a detected in cv. Chinese Spring (common wheat) by Ainsworth et al. (1993)

Together with the above-mentioned *null* alleles, Yamamori et al. (1994) detected three of these variants: Wx-A1c (seven Pakistani cultivars, slightly lower weight and more basic isoelectric point), Wx-B1c (11 cultivars from six countries, more basic isoelectric point) and Wx-D1c (American cv. Scoutland, more basic isoelectric point). In a study with 303 accessions of tetraploid wheat, Yamamori et al. (1995) detected novel variants with different mobility to the standard ones such as Wx-A1e (more basic isoelectric point) and Wx-B1d (greater mobility and more basic isoelectric point). In this study were analysed both durum wheat and its ancestors: emmer (*T. turgidum* spp. *dicoccum* Schrank em. Thell.) and wild emmer (*T. turgidum* ssp. *dicoccoides* Korn. ex Asch. & Graebner em. Thell.). In these species, the null proteins were very infrequent (Wx-A1b: 2.64 % and Wx-B1b: 0 %), as confirmed by Rodríguez-Quijano et al. (2003). However, Demeke et al. (1997) identified several Wx-A1 and Wx-B1 null variants in Japanese, Australian and Canadian cultivars of common wheat. Yamamori et al. (1995) also found one durum wheat accession with reduced expression of Wx-A1, later named Wx-A1i (Yamamori and Yamamoto 2011). In spelt wheat (*T. aestivum* ssp. *spelta* L. em. Thell.), Caballero et al. (2008) also found a novel variant for Wx-A1 with reduced expression that was named Wx-A1a′ and later catalogued by McIntosh et al. (2013) as Wx-A1g.

The methodology to separate waxy proteins was improved by different means (Rodríguez-Quijano et al. 1998; Zhao and Sharp 1998), and the resolution of SDS-PAGE electrophoresis was sufficient to separate the three waxy proteins of hexaploid wheat. Because of this, Rodriguez-Quijano et al. (1998) detected in several accessions of a collection of spelt and common wheat a novel variant for Wx-B1 with slightly reduced mobility compared to Wx-B1a, which they named Wx-B1c′ and was later catalogued as Wx-B1e by Yamamori and Quynh (2000). Nieto-Taladriz et al. (2000) described in durum wheat another novel variant named Wx-B1f with similar mobility to Wx-D1a from common wheat. More recently, Yamamori and Yamamoto (2011) added one more Wx-A1 variant with a more basic isoelectric point than Wx-A1a and even Wx-A1c, named Wx-A1j.

In addition to the mentioned studies on ancient wheat species, the search for the variability of waxy proteins was progressively applied to other *Triticum* species and wheat relatives, mainly einkorn (*T. monococcum* L. ssp. *monococcum*), *T. urartu* Thum. ex Gandil and the diploid species of *Aegilops*, which have been related with the A, B and D genomes of wheat (Salamini et al. 2002). In this way, Taira et al. (1995) and Fujita et al. (1996) showed the SDS-PAGE mobility differences and N-terminal sequence of waxy proteins in these species. Later, Rodríguez-Quijano et al. (2004) did not show intraspecific polymorphism for waxy proteins in einkorn and *Aegilops*, but their mobility differed from their homologs in common wheat, Wx-A1a and Wx-B1a.

Caballero et al. (2008) also identified and catalogued the waxy protein variants found in relatives of wheat (einkorn and *Aegilops*), although their nomenclature has not been

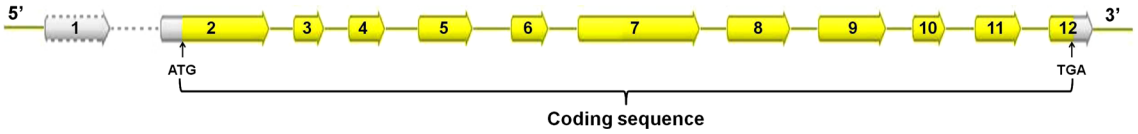

Fig. 2 Diagrammatic representation of the waxy gene structure, composed by 12 exons and 11 introns

used in further studies, probably because of the absence of molecular data that would better illustrate their results and allow good comparisons with other alleles.

Guzmán et al. (2009) found one einkorn accession with a waxy protein, named Wx-A^{m}1a′, with slightly less mobility than the common one of this species, described previously by Rodriguez-Quijano et al. (2004). Finally, Guzmán et al. (2010) found apparently null variants for Wx-A1, Wx-B1 and Wx-D1 proteins in a Spanish spelt collection. Additionally, they also found a novel variant for Wx-D1, with less mobility than Wx-D1a, named Wx-D1g.

Although the above-mentioned alleles were the end results obtained, the procedure to find this variability involved the analysis of multiple materials of different geographical origins. For many of these studies, the main result was the detection of null variants that permitted their use in the development of novel cultivars with low amylose content. In this respect, Graysbosch et al. (1998) found null proteins for Wx-A1 and Wx-B1 in USA common wheat, Zhao et al. (1998) confirmed the abundance of null Wx-B1 protein in Australian varieties and Demeke et al. (2000) only detected Wx-B1 null mutants in Canadian materials. In European common wheat, Marcoz-Ragot et al. (2000) identified Wx-A1 and Wx-B1 nulls and Wx-B1e variants, and Boggini et al. (2001) found null proteins for the three *waxy* loci in Italian common and durum wheat accessions, including the extremely rare Wx-D1b, although they presented no information to properly identify the accessions carrying those alleles. In another study, Urbano et al. (2002) identified, among others, two common wheat accessions lacking the Wx-D1 protein (one Iranian and one Italian). They detected more polymorphism in other tetraploid wheat species, notably one Wx-B1 allele from durum wheat with higher mobility, but those alleles were neither classified by the authors nor appear in the Wheat Gene Catalogue (McIntosh et al. 2013).

Structure of the *Wx* gene

The first study at molecular level on the *Wx* gene was carried out in maize, in which were detected *null* alleles due to the insertion of transposable elements inside *Wx* (Shure et al. 1983). In maize and rice (Olsen and Purugganan 2002), this gene has an internal structure fragmented in 13 exons and 12 introns. Later, other *Wx* genes from different species such as pea (Dry et al. 1992), barley (Rohde et al. 1988) and potato (Visser et al. 1989) were obtained, in all cases as a single-copy gene (Mason-Gamer et al. 1998), with exception of the Rosaceae where the gene appears duplicated (Evans et al. 2000).

The first report in wheat was by Clark et al. (1991), who obtained a *waxy* cDNA sequence of 2186 bp, with an open reading frame (ORF) of 1845 bp, although the association of each *waxy* gene with its corresponding genome in common wheat could not be established. Based on the sequence reported in Clark et al. (1991), Briney et al. (1998) developed a PCR marker that distinguished between *Wx-A1* wild and null variants in Australian wheat.

Murai et al. (1999) carried out the complete isolation and characterisation of the three *waxy* genes in cv. Chinese Spring. They showed that the size of *Wx-A1*, *Wx-B1* and *Wx-D1* was 2781, 2794 and 2862 bp, respectively, from the start to stop codon, with the mature protein region very similar between them (homology of 95.6–96.3 %). Each of these *Wx* genes is formed by 12 exons and 11 introns (Yan et al. 2000), similar to the barley genomic sequence (Rohde et al. 1988), although in the first studies, that data differed (11 exons and ten introns) because only the coding sequence was considered (Fig. 2).

In durum wheat, using cv. Langdon as a reference, the same team of Japanese researchers sequenced the *waxy* genes (*Wx-A1a* and *Wx-B1a*) that despite being different (especially the *Wx-B1a*) from those from cv. Chinese spring were also named *Wx-A1a* and *Wx-B1a.* This sometimes complicates the identification of alleles, especially in studies in which both species (common and durum wheat) or other relatives are included, and indicates that these allele names should be changed in the future with the necessary consensus. To distinguish between them, we provisionally named the durum wheat alleles in this review *Wx-A^{Td}1a* and *Wx-B^{Td}1a.*

Molecular characterisation of the allelic variation

With the information of the waxy sequences available, Vrinten et al. (1999) characterised at a molecular level the *null* alleles of the cultivars used by Nakamura et al. (1995) to create the first waxy common wheat (cv. Kanto 107:

Wx-A1 and *Wx-B1* null; and cv. Bai Huo: *Wx-D1* null). Southern blot analyses revealed the presence of the *Wx-A1* and *Wx-D1* genes, but not *Wx-B1*, which sustained a deletion that included its entire coding region. The size of this deletion was estimated at around 60 kb and could include other genes related to quality (Saito et al. 2009). The comparison among the *Wx-A1b* and *Wx-D1b* alleles and wild alleles showed two different partial gene deletions in different regions of each gene. *Wx-A1b* had a 23-bp deletion at the first exon–intron junction that resulted in the lack of 39 amino acids in the deduced protein, including part of the signal peptide, while *Wx-D1b* showed a 588-bp deletion in the 3′-region of the coding sequence that produced the lack of the last 30 amino acids.

Molecular markers to detect these *waxy* null mutations were developed in several studies. McLauchlan et al. (2001) applied *waxy* markers to detect *null* alleles in Australian breeding programme. Shan et al. (2007) and Liu et al. (2008) successfully used these markers to screen their materials for the presence of *null* alleles. Later Nakamura et al. (2002) developed a single multiplex PCR to simultaneously detect the three *null* alleles (*Wx-A1b*, *Wx-B1b* and *Wx-D1b*) present in cvs. Kanto107 and Bai Huo. Saito et al. (2009) designed an improved codominant marker to detect the *Wx-B1b* allele.

The development of these types of molecular markers has aided screening for the presence of *null* alleles, together with the detection of novel mimetic alleles at protein level in each of the wheat genomes, which have been identified and characterised. The characteristics of the alleles detected in these three genomes are shown in the next sections of this review.

Variants of *Wx-A1* gene

Up to now, the *Wx-A1* gene has shown the highest number of alleles (Table 2). Using the primers designed by Nakamura et al. (2002) for *Wx-A1*, Saito et al. (2004) discovered that some accessions of Turkish common wheat, not showing Wx-A1 protein using 2D electrophoresis, did not produce the expected 370-bp band product of *Wx-A1b* but a 200-bp longer one. The sequence of this PCR product revealed the presence of an extra 173-bp in the fourth exon of *Wx-A1* gene from those accessions. This insertion generates a premature stop codon and likely leads to the formation of a truncated protein. This allele is known as *Wx-A1f*. The insertion had the characteristics of a transposable-like element (TLE).

The TLE presence inside the *Wx* gene was widely described in rice by Nagano et al. (2002), which could affect the posttranscriptional maturation of these genes and thus also their expression (Cai et al. 1998). In spelt, Caballero et al. (2008) detected a *Wx-A1* allele with reduced expression that was characterised by Guzmán et al. (2012a). This allele (catalogued as *Wx-A1g*) presented a 160-bp insertion (TLE) in the fourth intron that could affect the maturation of primary transcripts due to aberrant splicing, which probably reduced the final amount of the Wx-A1 protein in the grain.

More recently, Yamamori and Guzmán (2013) characterised five *Wx-A1* alleles previously identified by means of SDS-PAGE and 2D electrophoresis (Yamamori et al. 1994, 1995). The analysis of the nucleotide sequence of the two alleles (*Wx-A1c* and *Wx-A1i*) detected in common wheat showed that the differences can be both in the coding region and in the untranslated region (UTR). The *Wx-A1c* allele presented two SNPs, one due to a transition (A → G) within the eighth exon generated a change in amino acid sequence (Glu405 → Gly), whereas the *Wx-Ai* allele also presented a 376-bp TLE in the 3′-UTR. These changes could be associated with reduced enzymatic activity in the case of *Wx-A1c*, or reduced gene expression for the *Wx-A1i* allele. In our recent study (Guzmán et al. 2015), a novel null *Wx-A1* allele was characterised in Mexican common wheat landraces, which had a deletion spanning the last three-and-a-half exons of the gene. This allele was catalogued with the tentative name *Wx-A1o*.

More variability for this gene was detected in tetraploid wheat. In the study of Yamamori and Guzmán (2013), the other three alleles characterised (*Wx-A1d*, *Wx-A1e* and *Wx-A1j*) were from tetraploid wheat. Compared with the wild allele (*Wx-A1a*), *Wx-A1d* had only a SNP (G → A) in position 1848 of the eighth exon that led to Val → Met. In contrast, the other two alleles had up to four SNPs compared with the reference allele. In the *Wx-A1e* allele, only one of these SNPs (G → A in position 2123 within the ninth exon) affected the amino acid sequence, producing the change Glu480 → Lys inside a highly conserved area of waxy proteins. For the *Wx-A1j* allele, two of these SNPs caused amino acid changes: one common with *Wx-A1c* (position 2042; Trp453 → Arg) and the other in position 482 (Gln134 → Lys).

Using a partial sequence of *Wx-A1* from cv. Buck Topacio, Vanzetti et al. (2010) found that the absence of the Wx-A1 protein in that durum cultivar was associated with a 1-bp deletion in the sixth exon, resulting in a frameshift generating a stop codon. This allelic variant was catalogued as *Wx-A1h*. Recently, Ortega et al. (2015) obtained the complete sequence of this allele in another durum wheat (cv. Astrodur), whose *null* allele was previously classified as *Wx-A1b* (Nieto-Taladriz et al. 2000).

Although other wheat genes have been included in the Wheat Gene Catalogue (McIntosh et al. 2013), some *Wx* alleles described in the literature have not. Monari et al. (2005) described a durum wheat line (MG 826) without Wx-A1 protein. The evaluation of its nucleotide sequence

Table 2 *Waxy* alleles of the *Wx-A1* gene

Allele	NCBI ID	Species. Standard	Remarkable changes	References
Wx-A1a	AB019622	Bread wheat. cv. Chinese Spring	DNA: Wild sequence of this species Protein: Wild protein	1
Wx-A^{Td}1a	AB029063	Durum wheat. cv. Langdon	DNA: Wild sequence of this species Protein: Wild protein. 3 amino acid changes respect to Wx-A1a	1
Wx-A1^{Tdi}a	AB029061	Wild emmer	DNA: Wild sequence of this species Protein: Wild protein. 2 amino acid changes respect to *Wx-A1a*	1
Wx-A^{m}1a	KF612977	Einkorn. PI 191094	DNA: Wild sequence of this species Protein: Wild protein. 26 amino acid changes respect to Wx-A1a	2
Wx-A1b	AF113843	Bread wheat. cv. Kanto107	DNA: 23 bp deleted at the first exon–intron junction [pseudogene]	3
Wx-A1c	AB737981	Bread wheat. QT105	DNA: 2 SNPS. One novel: position 1804, 8th exon, A → G. Protein: Glu405 → Gly	4
Wx-A1d	AB737982	Wild emmer. KU8937B	DNA: 1 SNP. Position 1848, 8th exon, G → A Protein: Val420 → Met	4
Wx-A1e	AB737983	Durum wheat. KU3655	DNA: 4 SNPs. Only G → A in position 2123, 9th exon, with effect Protein: Glu480 → Lys	4
Wx-A1f	AY376310	Bread wheat. cv. Turkey-124	DNA: 173-bp insertion in 4th exon. Change of ORF [pseudogene]	5
Wx-A1g	HQ625382	Spelt. BGE-012911	DNA: 160-bp insertion in 4th intron. Protein: Reduced expression	6
Wx-A1h	GQ120523	Durum wheat. cv. Buck Topacio	DNA: 1-bp deletion in 6th exon. Change of ORF [pseudogene]	7
Wx-A1i	AB737984	Durum wheat. KU9259	DNA: 376-bp insertion in the 3´ untranslated region Protein: Reduced expression	4
Wx-A1j	AB737985	Bread wheat. PI-242428	DNA: 4 SNPS Protein: Two amino acid changes not affecting activity	4
Wx-A1k	–	Durum wheat. MG-826	DNA: 89-bp insertion in 6th exon. Change of ORF [pseudogene]	8
Wx-A1l	–	Wild emmer. KU13454	DNA: 1-bp insertion in 10th exon. Change of ORF [pseudogene]	9
Wx-A1m	–	Emmer. KU14294	DNA: 1-bp deletion in 4th exon. Change of ORF [pseudogene]	9
Wx-A1n	JN935600	Emmer. CGN161104	DNA: Some SNPs in exons Protein: 5 amino acid changes respect to Wx-A1a	10
Wx-A1o	KF861807	Bread wheat. cv. CWI61656	DNA: 738 bp deleted affecting 9th, 10th, 11th, and 12th exon [pseudogene]	11
Wx-A^{m}1b	AF110373	Einkorn. AUS 22986	DNA: Several SNPs in exons and introns respect to *Wx-A^{m}1a* Protein: Wild protein. 2 amino acid changes respect to Wx-A^{m}1a	12

Ref.: *1* Murai et al. (1999); *2* Ortega et al. (2014a, b); *3* Vrinten et al. (1999); *4* Yamamori and Guzmán (2013); *5* Saito et al. (2004); *6* Guzmán et al. (2012a); *7* Vanzetti et al. (2010); *8* Monari et al. (2005); *9* Saito and Nakamura (2005); *10* Guzman et al. (2012b); *11* Guzman et al. (2015); and *12* Yan et al. (2000)

indicated that this allele had an 89-bp insertion in the sixth exon, which leads to a frameshift change with a premature stop codon and the consequent absence of the protein. As the information given is complete, we propose naming this allele *Wx-A1k*. Saito and Nakamura (2005) also reported two different *Wx-A1 null* alleles, one in emmer and another in wild emmer. Both consisted of a single nucleotide insertion and deletion in the tenth and fourth exons, respectively, which changed the ORF and led to the absence of Wx-A1 protein. We propose identifying and cataloguing these alleles not done previously as *Wx-A1l* and *Wx-A1m*. Guzmán et al. (2012b) also described in spelt (and emmer) one new allele that has five amino acid changes with respect to *Wx-A1a* that could be catalogued as *Wx-A1n*.

The variation detected in the cultivated diploid wheat (einkorn) is notably lower. In fact, only two alleles have been sequenced, with small differences between them. The *Wx-A^{m}1b* allele obtained by Yan et al. (2000) has two amino acid changes (Lys360 → Asn; and Asp367 → Asn) with respect to the *Wx-A^{m}1a* allele sequenced by Ortega et al. (2014a).

Table 3 *Waxy* alleles of the *Wx-B1* gene

Allele	NCBI ID	Standard	Remarkable changes	References
Wx-B1a	AB019623	Bread wheat. cv. Chinese Spring	DNA: Wild sequence of this species Protein: Wild protein	1
Wx-B^{Td}1a	AB029064	Durum wheat. cv. Langdon	DNA: Wild sequence of this species Protein: Wild protein. 14 amino acid changes respect to Wx-B1a	1
Wx-B1^{Tdi}a	AB029062	Wild emmer	DNA: Wild sequence of this species Protein: Wild protein. 2 amino acid changes respect to Wx-B1a	1
Wx-B1b	–	Bread wheat. cv. Kanto107	DNA: Entire coding region deleted	2
Wx-B1c	–	Bread wheat. cv. Cikotaba	DNA: At least 4 SNPs in 3rd, 9th and 10th exons. Protein: 4 amino acid changes Gly → Arg in exon 3, Asp → Asn in exon 9, Glu → Met and Asp → Asn in exon 10.	3
Wx-B1d	–	Durum wheat. KU4213D	DNA: Three nucleotides deleted in 8th exon. Protein: Glu deleted.	3
Wx-B1e [*Wx-B1c′*]	GQ205418	Durum wheat. cv. Mexicali	DNA: Several SNPs in exons and introns Protein: 14 amino acid changes respect to Wx-B^{Td}1a and 1 to Wx-B1a.	4
Wx-B1f	–	Durum wheat. BGE-12413	DNA: Not sequence available Protein: Less mobility than Wx-B1a, similar to W-D1a	5
Wx-B1g	GQ205417	Emmer. BGE-275999	DNA: Wild sequence on this species Protein: Wild protein. 15 amino acid changes respect to Wx-B^{Td}1a and 1 to Wx-B1a	4
Wx-B1h [*Wx-B1c**]	GQ205418	Emmer. BGE-275996	DNA: Several SNPs in exons and introns Protein: 14 amino acid changes respect to Wx-BTd1a and 1 to Wx-B1a.	4
Wx-B1i	HQ338721	Spelt. CGN8384	DNA: Some SNPs in exons and introns Protein: 2 amino acid changes respect to Wx-B1a	6
Wx-B1j	JN935595	Spelt. CGN11460	DNA: Some SNPs in exons and introns Protein: 1 amino acid changes respect to Wx-B1a	6
Wx-B1k	KP726909	Indian dwarf wheat. PI 272580	DNA: Insertion of 4 nucleotides in 7th exon (position 1437 from the start codon). Change of ORF [pseudogene]	7
Wx-B1l	KF861808	Bread wheat. CWI60507	DNA: Deletion nucleotide 311 in 2nd exon. Change of ORF [pseudogene]	8
Wx-B1m	KP726910	Club wheat. PI 442911	DNA: Deletion of four nucleotides in 2nd exon (position 157 from the start codon). Change of ORF [pseudogene]	7

Ref.: *1* Murai et al. (1999); *2* Vrinten et al. (1999); *3*, Yamamori et al. (2013); *4* Guzmán et al. (2011); *5* Nieto-Taladriz et al. (2000); *6* Guzman et al. (2012b); *7* Ayala et al. (2015); and *8* Guzmán et al. (2015)

Variants of the *Wx-B1* gene

Although some authors consider that the *Wx-B1* gene has a greater effect on amylose content than the other two *Wx* genes (Araki et al. 2000; Yamamori and Quynh 2000), few alleles of this gene have yet been characterised (Table 3). No sequence of alleles *Wx-B1c* or *Wx-B1d* is included in GenBank, although molecular characterisation has been carried out (Yamamori et al. 2013).

Analysis of the nucleotide sequence indicated that the alleles considered as wild (*Wx-B1a*) in common and durum wheat are clearly different. Therefore, the wild allele of durum wheat present in cv. Langdon, named *Wx-B^{Td}1a*, showed 14 changes in the deduced amino acid sequence (NCI ID: AB029064.1). This circumstance was observed in other alleles exclusively classified by protein separation. Rodriguez-Quijano et al. (1998) named as *Wx-B1c′* a new allele detected in common wheat (cv. Mariñar), although they suggested that this probably corresponded to *Wx-B1c* found in durum (cvs. Junbuk 12 and Cikotaba) by Yamamori et al. (1994). This same Spanish group identified as *Wx-B1c′* the *Wx-B1* alleles present in durum wheat cvs. Astrodur and Loberio ruivo (Nieto-Taladriz et al. 2000). However, Yamamori and Quynh (2000) reclassified this new allele as *Wx-B1e* in common wheat (cv. Bai Huo). Rodriguez-Quijano et al. (2003) also assumed this reclassification for cvs. Astrodur and Mexicali. The nucleotide sequences of this allele were partially obtained by Vanzetti et al. (2009) in cv. Buck Poncho (NCBI ID: AY954026), and completely by Klimushina et al. (2013) in cv. Korotyshka (NCBI ID: KF305522). Our own data obtained with the *Wx-B1* sequence of cv. Mexicali (NCBI ID: GQ205420) suggested that these sequences differ (Guzmán et al. 2011).

Table 4 *Waxy* alleles of the *Wx-D1* gene

Allele	NCBI ID	Standard	Remarkable changes	References
Wx-D1a	AB019624	Bread wheat. cv. Chinese Spring	DNA: Wild sequence of this species Protein: Wild protein	1
Wx-D1b	AF113844	Bread wheat. cv. BaiHuo	DNA: 588 bp deleted in 3′ coding region [pseudogene]	2
Wx-D1c	–	Bread wheat. cv. Scoutland	DNA: SNP in 2nd exon. Protein: Asn → Lys in exon 2	3
Wx-D1d	–	Bread wheat. K107Wx1 and K107Wx2	DNA: Not sequence available Protein: No protein detected	4
Wx-D1e	–	Bread wheat. NP-150	DNA: No amplification with primers in the 3′end of waxy genes. [pseudogene]	5
Wx-D1f	–	Bread wheat. Tanikei A6599-4	DNA: G → C in exon Protein: Ala258 → Thr.	6
Wx-D1h	–	Bread wheat. Iran-689	DNA: 724-bp deletion spanning from 7th to 10th exon [pseudogene]	7

Ref.: *1* Murai et al. (1999); *2* Vrinten et al. (1999); *3* Yamamori et al. (2013); *4* Yasuie et al. (1998), Shariflou and Sharp (1999), 6; 5 Yanagisawa et al. (2001); and *7* Monari et al. (2005)

In this study by our group, one *Wx* gene of emmer, whose Wx-B1 protein looked slightly lighter than that of durum in SDS-PAGE electrophoresis, was sequenced. Molecular data confirmed the result, and 15 amino acids were found to differ from durum allele *Wx-B*^{Td}*1a* and one from common wheat *Wx-B1a*. This allele was named *Wx-B1g*. Another emmer allele with the same electrophoretic mobility as protein variant Wx-B1e (Wx-B1c′) was also sequenced, and had one amino acid different from that of cv. Mexicali. For this reason, this emmer allele was considered novel and named *Wx-B1c**. Following a more logical nomenclature according to the official gene catalogue (McIntosh et al. 2013), we propose reassigning *Wx-B1c** as *Wx-B1h*. A recent study by our group found that under the *Wx-B1e* denomination were several alleles with similar mobility in protein separation but different nucleotide sequence (Ortega et al. 2015).

Two additional novel *Wx-B1* alleles were found in spelt wheat (Guzmán et al. 2012b), which showed changes of two and one amino acid compared to the reference alleles and we propose naming these alleles *Wx-B1i* and *Wx-B1j*, respectively. A novel *null* allele (*Wx-B1l*) was characterised in common wheat Mexican landraces (Guzmán et al. 2015). This allele had a deletion of only one nucleotide, and not all the gene as for *Wx-B1b*. This is particularly interesting because in the region deleted with the *Wx-B1b* allele, other genes related to quality could be included (Saito et al. 2009), and these would not be removed in the *Wx-B1 l* allele. Two additional novel *Wx-B1 null* alleles (*Wx-B1k* and *Wx-B1m*) had been recently discovered (Ayala et al. 2015), which also do not involve the deletion of the full gene and other adjacent regions.

Variants of the *Wx-D1* gene

Little variation has been detected in the *Wx-D1* gene, with only six alleles described in the Wheat Gene Catalogue (McIntosh et al. 2013). Two of them, wild (*Wx-D1a*) and *null* (*Wx-D1b*) alleles, have been used as references for the evaluation of the other four (Table 4). The *Wx-D1c* allele, identified in cv. Scoutland (Yamamori et al. 1994), presented two SNPs: one transversion (C/G) inside the second exon that led to Asn → Lys; and one transition (A/G) within the third intron (Dr. Yamamori pers. commun.).

Shariflou and Sharp (1999) detected a microsatellite marker in the 3′ *waxy* gene associated with *null* Wx-A1 and Wx-D1 variants. In their study, line NP-150 carrying a null *Wx-D1* allele, and not related to cv. Bai Huo, showed no amplified fragment for chromosome 7D. This novel *null Wx-D1* allele was catalogued as *Wx-D1e*. Previously, the *null Wx-D1* allele generated in cv. Kanto 107 seeds treated with ethyl methanesulphonate (EMS) that resulted in waxy lines K107Wx1 and K107Wx2 was named *Wx-D1d*, although no sequence data were presented supporting this nomenclature (Yasui et al. 1998).

The use of different primers and molecular markers constructed based on the waxy sequences allowed the identification of novel alleles not distinguishable at protein level. Yanagisawa et al. (2001) found a point mutation (G/C) that resulted in Ala → Thr in the Tanikei A6599-4 line; this allele was initially named *Wx-D1e*, but was finally catalogued as *Wx-D1f* (McIntosh et al. 2013). Supplementing this first study, a derived cleaved amplified polymorphic sequence (dCAPS) marker was developed to detect this allele (Yanagisawa et al. 2003).

Other novel mutations were identified in subsequent years. Monari et al. (2005) characterised at a molecular

level some of the *null* alleles previously described by Urbano et al. (2002) at a protein level. Two wheat lines showing the infrequent lack of Wx-D1 protein, Iran-689 and MG-20506, were shown to carry a different allele from the *Wx-D1b* previously described by Vrinten et al. (1999), as amplification was produced with primers designed using the deleted area of *Wx-D1b* allele. Iran-689 had a deletion of 724-bp spanning exons 7–10 that caused the absence of the protein. This allele was not catalogued at that time. As the information presented by the authors is complete (protein + DNA sequence data), we propose naming this allele *Wx-D1h* and including it in the gene catalogue. The MG-20506 *Wx-D1* allele was not completely sequenced and no mutation was detected that explained the absence of the protein. The authors suggested that a point mutation may be present in regions of the gene that were not sequenced.

Wx gene in wheat relatives

The interest in wheat relatives as sources of variation for *Wx* genes has been parallel to the search of the variation in wheat, as described above. However, here we comment on the data obtained with wild species donors of the genomes present in wheat, such as *T. urartu* and *Aegilops* spp.

Yan et al. (2000) reported the *waxy* genes from two of the putative genome (B and D) donor species of common wheat (*Ae. speltoides* and *Ae. tauschii*). In Table 5, we propose considering these sequences as wild alleles in these species (*Wx-B*^{Ast}*1a* and *Wx-D*^{At}*1a*, respectively). The nucleotide sequence of *Ae. speltoides* showed great polymorphism compared to the *Wx-B1a* allele of common wheat, which was traduced in 13 amino acid changes in the deduced sequence. However, the similarity was clearly greater in the *Ae. tauschii* sequence, and was almost equal to that of *Wx-D1a*.

Other *Aegilops* species are indirectly related to the wheat genomes, mainly diverse species of the *Sitopsis* section, such as *Ae. longissima*, *Ae. searsii*, *Ae. sharonensis* and *Ae. speltoides* that some authors have associated with the B genome (see Tsunewaki and Ogihara 1983 for a review). In a recent study, Ortega et al. (2014b) analysed *Wx* gene variation in accessions of these species, together with other diploid *Aegilops* species (*Ae. comosa*, *Ae. markgrafii* and *Ae. umbellulata*), whose genomes have been related with the D genome. There were 19 new alleles detected in this study, suggesting great potential of these species as sources of variation in *Wx* genes that could be used in wheat breeding. We propose a nomenclature for these alleles that could be included in the official gene catalogue (Table 5).

Studies on the *Wx* gene in *T. urartu*, putative donor of the A genome, are limited and, in general, with partial sequences (Mason-Gamer et al. 1998; Yan and Bhave 2000). The first complete genomic sequence of this gene (*Wx-A1*^{u}*a*) in this species was reported in 2012, and had 27 amino acids different from those of *Wx-A1a* (Guzmán and Alvarez 2012). More recently, four new alleles were found in this species (Ortega et al. 2014a), which could also be added to the official gene catalogue.

Effect of *Wx* gene mutations on amylose content and end-use quality

The effect of the variation previously described on starch properties has been widely studied, although many of these studies used the *null* alleles of each *Wx* gene (Araki et al. 2000; Kim et al. 2003; Miura and Sugawara 1996, Miura et al. 1999, 2002; Wickramasinghe et al. 2003). In this respect, in common wheat, Yamamori et al. (1994) described eight types by combining wild and *null* alleles at the *Wx-A1*, *Wx-B1* and *Wx-D1* loci. The wild type (*Wx-A1a*, *Wx-B1a*, *Wx-D1a*) was named type 1, whereas type 8 corresponded with waxy wheat (*Wx-A1b*, *Wx-B1b*, *Wx-D1b*). The remaining types (2–7) were classified as partial waxy lines. In general, the presence of three *Wx* loci in hexaploid wheat has made it difficult to develop wheat with low or null amylose content, due to the dosage effect of any active *Wx* gene on amylose content (Yamamori and Quynh 2000). Consequently, the partial waxy lines usually show amylose contents over 16 %, and only the triple null mutant (type 8) has very low values (less than 3 %) (Kim et al. 2003; Miura et al. 2002; Nakamura et al. 1995).

Several studies have reached similar conclusions concerning the effect of *null Wx* alleles on starch properties (Araki et al. 2000; Kim et al. 2003; Miura and Sugawara 1996, Miura et al. 1999, 2002; Yamamori and Quynh 2000; Wickramasinghe et al. 2003)—the Wx-B1 protein has the most striking effect on amylose synthesis in all studies. However, the lack of Wx-A1 or Wx-D1 protein does not always lead to a significant decrease in amylose content (Kim et al. 2003); while the absence of Wx-D1 was reported to have a greater effect than that of Wx-A1 (Yamamori and Quynh 2000), this difference was not significant in other studies.

A different level of expression that confers different amounts of each waxy protein is the likely reason for the different effects of the *null* alleles (Yamamori and Quynh 2000), with the Wx-B1 protein more abundant than the others. The three *null* alleles combined lead to the waxy phenotype (0 % amylose) as mentioned above. The properties of waxy wheats were well reviewed in Graybosch (1998) and Yasui (2006).

Although in initial analyses they were considered as *null*, some of the above-mentioned variants showed truly low or reduced expression. Demeke et al. (1997) found

Table 5 Waxy alleles in wheat relatives

Species	Allele	NCBI ID	Standard	Remarkable changes	References
T. urartu	*Wx-A1^{u}a*	JN857937	MG-26992	DNA: Wild sequence of this species Protein: Wild protein. 27 amino acid changes respect to Wx-A1a	1
	Wx-A1^{u}b	KF612973	PI 427328	DNA: Several SNPs in exons and introns respect to *Wx-A1^{u}a* Protein: 4 amino acid changes respect to Wx-A1^{u}a	2
	Wx-A1^{u}c	KF612974	PI 428217	DNA: Several SNPs in exons and introns respect to *Wx-A1^{u}a* Protein: 8 amino acid changes respect to Wx-A1^{u}a	2
	Wx-A1^{u}d	KF612975	TRI 6734	DNA: Several SNPs in exons and introns respect to *Wx-A1^{u}a* Protein: 3 amino acid changes respect to Wx-A1^{u}a	2
	Wx-A1^{u}e	KF612976	PI 538733	DNA: Several SNPs in exons and introns respect to *Wx-A1^{u}a* Protein: 6 amino acid changes respect to Wx-A1^{u}a	2
Ae. comosa	*Wx-M1a*	JX402790	PI 542172	DNA: Wild sequence of this species Protein: Wild protein. 9 amino acid changes respect to Wx-D1a	3
	Wx-M1b	JX625135	PI 551022	DNA: Three SNPs respect to *Wx-M1a* Protein: No change respect to Wx-M1a	3
	Wx-M1c	JX679003	PI 551038	DNA: Several SNPs in exons and introns respect to *Wx-M1a* Protein: 2 amino acid changes respect to Wx-M1a	3
	Wx-M1d	JX679004	PI 551048	DNA: Several SNPs in exons and introns respect to *Wx-M1a* Protein: 6 amino acid changes respect to Wx-M1a	3
	Wx-M1e	JX679005	PI 551064	DNA: Several SNPs in exons and introns respect to *Wx-M1a* Protein: No change respect to Wx-M1a	3
Ae. longissima	*Wx-S^{l}1a*	JX679018	PI 604106	DNA: Wild sequence of this species Protein: Wild protein. 16 amino acid changes respect to Wx-B1a	3
	Wx-S^{l}1b	JX679019	PI 604130	DNA: Several SNPs in exons and introns respect to *Wx-S^{l}1a* Protein: 1 amino acid changes respect to Wx-S^{l}1a	3
Ae. markgrafii	*Wx-C1a*	JX679009	PI 263554	DNA: Wild sequence of this species Protein: Wild protein. 8 amino acid changes respect to Wx-D1a	3
	Wx-C1b	JX679010	PI 551129	DNA: Several SNPs in exons and introns respect to *Wx-C1a* Protein: 6 amino acid changes respect to Wx-M1a	3
Ae. searsii	*Wx-S^{s}1a*	JX679011	PI 599124	DNA: Wild sequence of this species Protein: Wild protein. 16 amino acid changes respect to Wx-B1a	3
	Wx-S^{s}1b	JX679012	PI 599157	DNA: Several SNPs in exons and introns respect to *Wx-S^{s}1a* Protein: 3 amino acid changes respect to Wx-S^{s}1a	3
Ae. sharonensis	*Wx-S^{sh}1a*	JX679016	PI 584372	DNA: Wild sequence of this species Protein: Wild protein. 16 amino acid changes respect to Wx-B1a	3
	Wx-S^{sh}1b	JX679017	PI 584396	DNA: Several SNPs in exons and introns respect to *Wx-S^{sh}1a* Protein: 3 amino acid changes respect to Wx-S^{sh}1a	3
Ae. speltoides	*Wx-B^{Ast}1a*	AF110374	AUS 21638	DNA: Wild sequence of this species Protein: Wild protein. 13 amino acid changes respect to Wx-B1a	4
	Wx-S1a	JX679013	PI 219867	DNA: Several SNPs in exons and introns respect to *Wx-B^{Ast}1a* Protein: 12 amino acid changes respect to Wx-B^{Ast}1a	3
	Wx-S1b	JX679014	PI 554296	DNA: Several SNPs in exons and introns respect to *Wx-B^{Ast}1a* Protein: 9 amino acid changes respect to Wx-B^{Ast}1a	3
	Wx-S1c	JX679015	PI 487232	DNA: Several SNPs in exons and introns respect to *Wx-B^{Ast}1a* Protein: 10 amino acid changes respect to *Wx-B^{Ast}1a*	3
Ae. tauschii	*Wx-D^{At}1a*	AF110375	CPI110799	DNA: Wild sequence of this species Protein: Wild protein. No differences with bread wheat	4
Ae. umbelullata	*Wx-U1a*	JX679006	PI 298906	DNA: Wild sequence of this species Protein: Wild protein. 6 amino acid changes respect to Wx-D1a	3
	Wx-U1b	JX679007	PI 478219	DNA: Two SNPs respect to *Wx-U1a* Protein: No change respect to Wx-U1a	3
	Wx-U1c	JX679008	PI 542372	DNA: Several SNPs in exons and introns respect to *Wx-U1a* Protein: Protein: 2 amino acid changes respect to Wx-U1a	3

Ref.: *1* Guzmán and Alvarez (2012); *2* Ortega et al. (2014a); *3*, Ortega et al. (2014b); and *4*, Yan et al. (2000)

that the Canadian cv. Reward had a reduced amount of the Wx-B1 protein, probably associated with the contribution of one parent (cv. Prelude). However, the molecular reason for this reduced expression has not been evaluated. A similar process was observed by Caballero et al. (2008) for the Wx-A1 protein in some Spanish spelt accessions. The molecular characterisation of this allele (*Wx-A1g*) showed the presence of a TLE inside the fourth intron that modified the normal splicing of the mRNA (Guzmán et al. 2012a). This implies that only a small part of this mRNA is correctly processed and the Wx-A1 protein appears in reduced amounts, which could reduce amylose synthesis. This opens the way for development of new waxy wheat types where these allelic variants are associated with double *null* alleles for the other two *Wx* genes, which could be termed 'quasi-waxy'.

Yamamori (2009) and Yamamori and Yamamoto (2011) started the process, with the transfer of different *waxy* alleles described previously, to a complete waxy line (*null* for the three waxy genes). This allowed individual analysis of the effect of each allele or protein variant, without the interaction of the homologous proteins. Common wheat lines carrying a waxy protein produced by one variant (e.g. Wx-A1c) and one control (e.g. Wx-A1a) allele were bred and their amylose contents compared (Yamamori 2009). It was concluded that the *Wx-A1e* allele did not have amylose synthesis activity, while *Wx-A1c*, *Wx-B1c* and *Wx-B1d* reduced amylose content compared to *Wx-A1a* and *Wx-B1e* (around 6.5, 4.0 and 3.0 %, respectively) and therefore had reduced enzymatic activity. The *Wx-D1c* allele encoded a variant that led to slightly higher amylose content, whereas the *Wx-A1i* allele, with reduced protein expression, decreased amylose content by around 14 % compared to *Wx-A1a*, enabling the generation of the above-mentioned quasi-waxy lines with amylose content of 6–8 % (Yamamori and Yamamoto 2011). However, *Wx-A1j* did not show significantly different activity from *Wx-A1a.* Yamamori and Guzman (2013) confirmed part of these results.

In many of the studies mentioned in this section, in addition to amylose content, starch properties, such as pasting, swelling power or paste clarity, were also analysed, with differences generally found based on amylose content changes. These differences have significant effects on different processing and end-use quality traits of diverse products. Although this is a vast topic and a full discussion is outside the scope of this document, some of the main conclusions reached by several studies are commented below.

Most of the research done about the effect of starch properties on end-use quality has been carried out in oriental noodles, because the direct impact of starch properties on their characteristics is well known. High peak viscosity, high breakdown and swelling power were identified as factors responsible for superior Japanese udon noodles quality (Crosbie 1991; Oda et al. 1980), and to some extent in Cantone and instant noodles (Baik et al. 1994). Those starch properties are related to less amylose content (Miura and Tanii 1994; Zeng et al. 1997). However, although showing higher breakdown and higher swelling power than conventional starches, flours made from waxy wheat (no waxy protein, 0 % amylose) were found not suitable for making noodles, being sticky, extremely soft upon cooking, and did not maintain the integral structure of the noodle strands (Baik and Lee 2003; Hayakawa et al. 2004). The use of partial waxy lines (flour with reduced amylose content, around 15–19 %) or flours blend with waxy flour (30–50 %) seems to be more suitable for that product as showed by Baik et al. (2003). This could be also used to prepare more increased staling- and freezing-tolerant grain-based foods (Hayakawa et al. 2004).

Other studies have analysed the bread-making quality of partial and full waxy lines. With full waxy flour, Morita et al. (2002) developed bread with slightly higher initial volume than those done with non-waxy flour, but with breadcrumbs soft, viscous and glutinous, which could not keep the original form after baking. Similar results were found by Jonnala et al. (2010): the initial loaf volume was high with full waxy flour, but the structure becomes unstable and collapses within the first day of baking. Besides the internal crumb showed dark brown colour and poor appearance with large gas cells. Park and Baik (2007) reached the same conclusions. For pasta-making, durum waxy wheat was not found suitable because of its softening effect (Vignaux et al. 2005). Martin et al. (2008) showed that the smaller reductions in amylose content (*Wx-B1 null)* did not cause an important effect on bread quality. Using flours with ≈16 % amylose content (double null waxy), Park and Baik (2007) obtained bread of comparable loaf volume to that of non-waxy flours but with greater crumb moisture content and softer crumb texture, which could recommend its use for bread-making.

Future trends and conclusions

The knowledge on the effect of the *Wx* alleles mentioned in the previous sections has opened the way for the direct use of this variation to develop new wheat cultivars with starch modification. As mentioned above, Nakamura et al. (1995) were the first to develop a waxy wheat by a cross between two partial waxy cultivars. Afterward, several groups developed new waxy lines by classical or modern breeding procedures (Kiribuchi-Otobe et al. 1997; Morris and King 2007; Morris and Konzak 2001; Urbano et al. 2002; Yasui et al. 1997; Zhao et al. 1998).

More studies are required to determine more about the specific effects on starch properties of the other non-null alleles described in this document. The studies of Yamamori (2009) and Yamamori and Yamamoto (2011) are good examples of this kind of research that should be expanded. In addition, effects on processing and end-use quality traits of the novel starches generated need deeper study. The transfer of alleles found in genetic resources (neglected wheats and wild relatives) to modern wheat is also required to give value to all the previous characterisation work on variability.

Parallel studies on other starch enzymes, mainly SGP-1, have permitted the alternative development of wheat lines with high amylose content (Yamamori et al. 2006), which has been associated with a higher content of resistant starch, for which health benefits are well-established (Asp et al. 1996; Topping 2003). The combined use of the null variants for both enzymes (GBSS-I and SGP-1) permitted development of a wheat line without amylose and with reduced starch content, but high contents of sucrose and maltose (Nakamura et al. 2006).

Although part of the natural allelic variation described in the previous sections has been used, other research groups have opted for the generation of new variation using mutagenic procedures such as TILLING technology (Targeting Induced Local Lesions in Genomes). The TILLING technology that combines the mutagenic chemical agents (sodium azide or EMS) and PCR-based screening has been successfully used for the generation of mutant *SSIIa* and *SBEIIa/b* variants (Botticella et al. 2011; Hazard et al. 2012; Sestili et al. 2010a; Slade et al. 2012; Uauy et al. 2009;). However, its utility in *Wx* genes has been more limited because these genes have higher natural variation, including *null* variants for the three wheat genomes.

Despite the low social acceptability of genetically modified organisms, transgenic approaches have been used for starch modification in wheat. Sestili et al. (2010b) used RNA interference technology to generate durum wheat with higher amylose content by the suppression of *SBEIIa* genes. In this case, similar to the above-mentioned TILLING approach, its use in *Wx* genes has generated little interest due to the possibility of using natural variation.

This review has shown that there has been much research in the last three decades concerning these important proteins that control in great part the amylose/amylopectin ratio of wheat starch. This trait is important to determine the processing, end-use and nutritional quality of wheat, and is becoming increasingly important with the growing demand for processed novel food of high quality.

Tens of papers focussed in modern wheat, landraces, wheat ancestors and wild relatives have described large variability for these proteins and their respective genes. The proper ordering of all the data generated is the key to enhancing use in breeding programmes of the current variability described, and thus generating wheat with novel starch properties to satisfy the demands of industry and consumers.

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Compliance with ethical standards

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

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