Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.12 No.1, Issue of January 15, 2009

Received May 20, 2008 / Accepted August 7, 2008
DOI: 10.2225/vol12-issue1-fulltext-2
RESEARCH ARTICLE

Genetic variability for *waxy* genes in Argentinean bread wheat germplasm

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Financial support: Grant from the Government of Argentina, FONCyT – INTA PICTO 12948.

Keywords: characterization, molecular markers, *Triticum aestivum* L., starch, *waxy* genes, wheat.

Abbreviations: 1D: one dimensional
2D: two dimensional PCR: polymerase chain reaction
GBSS I: Granule Bound Starch Synthase I
MAS: marker assisted selection
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SNPs: single nucleotide polymorphisms

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Amylose and amylopectin are the two polysaccharides that constitute starch in bread wheat and the enzyme GBSSI (*Granule-bound starch synthase* I), also known as waxy protein, is responsible for amylose synthesis in storage tissues. Decrease of the amylose content in starch has been associated with the lack of waxy protein(s). In this work, different sets of PCR markers were used to characterize the genetic variability of *waxy* loci from 103 Argentinean bread wheat cultivars. For the *Wx-A1* locus, *Wx-A1a* and a novel molecular allele designed *Wx-A1g* were detected. *Wx-B1* locus showed three alleles (*Wx-B1a, Wx-B1b, Wx-B1e*), and *Wx-D1* locus showed only the *Wx-D1a* allele. Novel single-locus allele specific markers for *Wx-A1b, Wx-B1b* and *Wx-D1b* null alleles were also described. To our best knowledge this is the first study focused to characterize the genetic variability for *waxy* genes in bread wheat cultivars from South America.

Starch is a major component of wheat grain, accounting up to 65-70% of the dry matter in mature grain. Wheat starch is a polymer composed of two types of glucose carbohydrates: amylose, a lineal α-1,4 glucan, and amylopectin, a lineal α-1,4 glucan containing α-1,6 branch points. The granule-bound starch synthases (GBSSI or waxy proteins), are the enzymes responsible for amylose synthesis in storage tissues (Yamamori et al. 1994). Because bread wheat (*Triticum aestivum* L.) is an allohexaploid species (2n= 6x= 42, genomic formula AABBDD) it has three different waxy proteins (Wx-A1, Wx-B1, Wx-D1), encoded by three different genes: *Wx-A1*, *Wx-B1*, *Wx-D1*; located on chromosome arms 7AS, 4AL and 7DS, respectively (Nakamura et al. 1993a). Bread wheats with one or two non-functional (null) *waxy* genes produce starch with significant lower levels of amylose (partial waxy starch) (Nakamura et al. 1993b; Vrinten et al. 1999; Wickramasinghe and Miura, 2003). Partial waxy starch is a desirable trait in the development of wheat cultivars suitable for certain types of noodles (Epstein, 2002; Liu et al. 2003). Around the world several wheat collections have been characterized searching for null waxy proteins by 1D or 2D SDS-PAGE (Rodríguez-Quijano et al. 1998; Demeke et al. 2000; Urbano et al. 2002) and PCR markers (Briney et al. 1998; Boggini et al. 2001; Nakamura et al. 2002; Urbano et al. 2002). In wheat, PCR markers can co-amplify two or three *waxy* genes simultaneously making difficult its use in marker-assisted selection (MAS); this is the case of PCR markers developed by Briney et al. (1998), McLauchlan et al. (2001), Nakamura et al. (2002), Urbano et al. (2002). Therefore, the development of single-locus allele specific markers for *waxy* null alleles is a desirable goal for MAS. In this study we report the development of single-locus allele specific markers for *waxy* nulls alleles suitable for MAS programs, and the characterization of the genetic variability of the *Wx-A1*, *Wx-B1* and *Wx-D1* loci in Argentinean bread wheat cultivars using molecular markers as a tool.

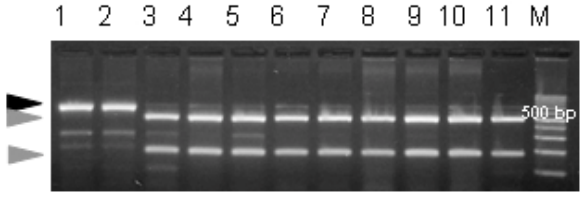

Figure 1. PCR products obtained using Wx-A1L/Wx-A1R primers after *Hind* III digestion. Lanes 1 to 11 are breeding lines/cultivars 1-DHWx12, 2-Komugi Norin, 3-Mariñar, 4-Gamenya, 5-Baguette 10, 6-Buck Brasil, 7-Prointa Molinero, 8-Prointa Puntal, 9-Cronox, 10-Klein Martillo, 11-Prointa Granar, M: 100-pb ladder (Promega), 500-bp fragment is indicated. The black arrowhead indicates the 652-bp fragment from *Wx-A1b* allele, the grey arrowheads indicate 495-bp and 176-bp fragments from *Wx-A1a* allele.

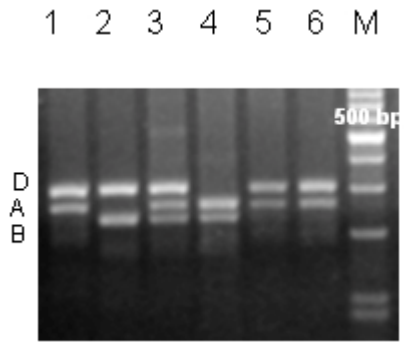

Figure 2a. PCR products obtained using #4F/#4R primers. Lanes 1 to 6 are breeding lines/cultivars 1-N4AT4B, 2-N7AT7B, 3-N7BT7D, 4-N7DT7B, 5-Reeves, 6-Eradu and M: 100-pb ladder (Promega), 500-bp fragment is indicated. Capital letters "D", "A" and "B" indicate PCR fragments from *Wx-D1a*, *Wx-A1a* and *Wx-B1a* alleles.

MATERIALS AND METHODS

Plant materials

A set of 103 bread wheat cultivars (*T. aestivum* L.) from Argentina (Table 1) was screened using a set of molecular markers to assess the frequency of different *Wx-A1, Wx-B1* and *Wx-D1* alleles. Seed stocks were obtained from INTA Marcos Juárez and/or INTA Castelar Wheat Germplasm Collections (Argentina). Australian wheat cultivars Bodallin, Cadoux, Eradu, Gamenya, Halberd and Reeves were used as controls carrying *Wx-B1b* allele (Briney et al. 1998).The Spanish wheat landrace Mariñar (accession BG-018258) was used as control carrying *Wx-B1e* allele (Rodríguez-Quijano et al. 1998). The Chinese wheat cultivar Komugi Norin was used as control carrying *Wx-A1b* allele and the Australian breeding line DHWx12 was used as triple-null control (*Wx-A1b* / *Wx-B1b* / *Wx-D1b*) (Shariflou and Sharp, 1999). Nullitetrasomic lines of cv. Chinese Spring, N7AT7B, N4AT4B, N7BT7D and N7DT7B were used to validate the genome specificity of the designed primers.

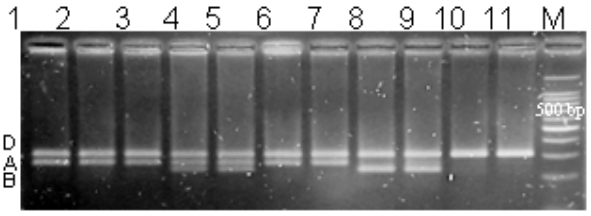

Figure 2b. PCR products obtained using #4F/#4R primers. Lanes 1 to 11 are breeding lines/cultivars 1-Gamenya, 2-DHWx12, 3-Mariñar, 4-Baguette 10, 5-Buck Brasil, 6-Prointa Molinero, 7-Prointa Puntal, 8-Cronox, 9-Klein Martillo, 10-Prointa Granar, 11-ACA 801 and M: 100-pb ladder (Promega), 500-bp fragment is indicated. Capital letters "D", "A" and "B" indicate PCR fragments from *Wx-D1a*, *Wx-A1a* and *Wx-B1a* alleles.

DNA extraction, primer design, PCR reactions and sequencing

Genomic DNA from leaves of single plants was isolated as described before (Weining and Langridge, 1991). Different primer combinations based on previously published sequences were used to amplify preferentially different alleles from *Wx-A1*, *Wx-B1* and *Wx-D1* genes (Murai et al. 1999; Vrinten et al. 1999). Details about primer sequences, amplified loci and cycling conditions are in Table 2. PRIMER3 program (Rozen and Skaletsky, 2000) was used for primer design. Primers #4F/#4R were developed by McLauchlan et al. (2001). The PCR reactions were performed in a MJ Research thermocycler model PTC 100 in a 25 µl reaction mixture. Each reaction consisted of 1X *Taq* polymerase buffer (Promega Corp. Madison WI), 1.0 U *Taq* DNA polymerase (Promega), 200 µM of each dNTP (Promega), 0.2 µM of each primer, and 100-150 ng of wheat genomic DNA as template, Magnesium Chloride concentrations are detailed in Table 2. Following amplification with primers Wx-A1L/Wx-A1R, 10 µl of PCR products were directly digested with restriction enzymes *Hind* III (New England Biolabs Inc. Beverly MA), by adding 5 units of enzyme to the PCR products and incubating for 90 min at 37ºC. Direct PCR fragments and digested products were separated by electrophoresis on 2% agarose gels in 1X SB Buffer (Brody and Kern, 2004), stained with Ethidium bromide [0.5 g/L] and visualized by UV exposure. DNA sequencing was performed directly from PCR fragments purified using Wizard SV Gel and PCR clean-Up System Kit (Promega) using the amplification primers in both directions. Detected mutations were confirmed by sequencing of PCR fragments from at least two independent PCR reactions.

Waxy protein extraction and electrophoresis

In a subset of 53 bread wheat cultivars (underlined in Table 1) starch granule-bound proteins (including waxy protein) were extracted from embryoless grains, and later separated by SDS-PAGE as described by Rodríguez-Quijano et al. (1998). Protein patterns were visualized by silver stain using the Silver Express kit (Invitrogen Corp. Carlsbad CA).

RESULTS

Wx-A1 locus

Three sets of PCR primers considering proximal and central portions of *Wx-A1* gene were used. Primers Wx-A1F/Wx-A1R amplify a 671-bp fragment from the proximal region of the *Wx-A1* locus in plants carrying the *Wx-A1a* allele and a 652-bp fragment in plants with the *Wx-A1b* allele described by Vrinten et al. (1999). The amplification products from *Wx-A1a* (671-bp) and *Wx-A1b* (652-bp) alleles can not be easily separated in agarose gels, therefore, we found a polymorphic *Hind* III restriction site in the sequence from the *Wx-A1a* allele that divides the 671-bp fragment into two fragments of 495-bp and 176-bp (Figure 1, lanes 4 to 11), this restriction site is not present into the *Wx-A1b* sequence (Figure 1, lanes 1 and 2). The described markers are an effective tool to select *Wx-A1b* allele in marker-assisted selection breeding. When primers Wx-A1F/Wx-A1R were used to evaluate the genetic variability in the selected 103 Argentinean bread wheats, all the samples showed the *Wx-A1a* allele.

Primers #4F/#4R were initially designed to detect the null *Wx-B1b* allele (McLauchlan et al. 2001). These primers co-amplify exons 5 and 6 of the three homoeoalleles of *Wx-A1*, *Wx-B1* and *Wx-D1* genes and were used to detect genetic variability in this portion of the *Wx-A1* locus. In Figure 2a, the larger fragment (299-bp) belongs to the D genome, the intermediate (257-bp) to the A genome and the smaller one (227-bp) to the B genome. When this marker was used to evaluate the genetic variability in Argentinean germplasm, 78 cultivars (76%) showed the 257-bp fragment associated with the *Wx-A1* locus (*Wx-A1a* allele) (Figure 2b lines 4, 5, 6 and 7), and 25 (24%) showed absence of the 257-bp fragment (Figure 2b lines 8, 9, 10 and 11), which is an unexpected high proportion of putative *Wx-A1* null alleles. We also found that DHWx12 (*Wx-A1b* control) amplified

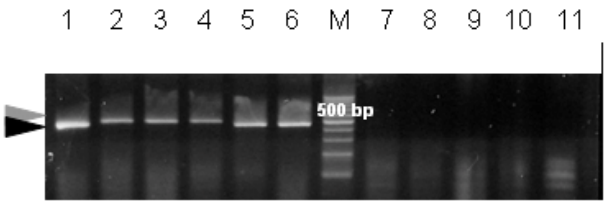

Figure 3. PCR amplification products obtained using Wx-B1F/Wx-B1R primers. Lanes 1 to 11 are breeding lines/cultivars 1-Biointa 3000, 2-Mariñar, 3-Buck. Pingo, 4-Buck Poncho, 5-Triguero 100, 6-Prointa Bon. Hurón, 7-Prointa. Milenium, 8-Prointa Granar, 9-Gamenya, 10-Cadoux, 11-DHWx12 and lane M is a DNA size standard (100-bp ladder, Biodynamics Corp.). The black arrowhead indicates the 461-bp fragment from *Wx-B1a* allele, the grey arrowhead indicate 495-bp fragment from *Wx-B1e* allele and lack of amplification is *Wx-B1b* allele.

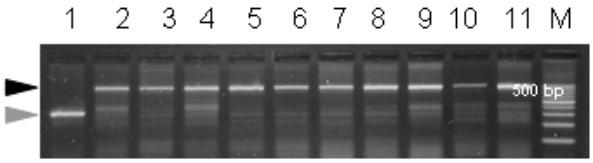

Figure 4. PCR amplification products obtained using Wx-D1L/Wx-D1R primers. Lanes 1 to 11 are breeding lines/cultivars 1-DHWx12, 2-Buck Arriero, 3-Biointa 2001, 4-Klein Gavilán, 5-Baguette 10, 6-Buck Brasil, 7-Prointa Molinero, 8-Prointa Puntal, 9-Cronox, 10-Klein Martillo, 11-Prointa Granar, and lane M: 100-pb ladder (Promega), 500-bp fragment is indicated. The black arrowhead indicates the 930-bp fragment from *Wx-D1a* allele and the grey arrowhead indicates the 342-bp fragment from *Wx-D1b* allele.

the 257-bp fragment of the *Wx-A1a* allele (Figure 2b lane 2).

To confirm that result, local cultivars were tested with a third set of primers *Wx-A1*-specific that included #4F/#4R primers region (Wx-A2L/Wx-A2R), and surprisingly, all of them amplified a 491-bp fragment. The specificity of Wx-A2L/Wx-A2R primers for *Wx-A1* locus was confirmed by the lack of amplification of the 491-bp fragment in N7AT4B Chinese Spring nullitetrasomic line. Sequence comparison of PCR products amplified with Wx-A2L/Wx-A2R primers from local cultivars previously scored as "nulls" for *Wx-A1* locus using #4F/#4R primers, showed two silent single nucleotide polymorphisms (SNPs), one of them located in the annealing site of the primer #4R (Figure 5). The primer #4R has an additional mismatch at position 17 (starting from the 3' end), as #4F/#4R primers amplify simultaneously *Wx-A1*, *Wx-B1* and *Wx-D1* genes, these two mismatches (one in the *Wx-A1g* sequence, one in the #4R primer) would favour *Wx-B1* and *Wx-D1* amplification against *Wx-A1g*. This is not a real "null" allele, so we propose to designate it as *Wx-A1g* (GeneBank accession DQ431232).

Wx-B1 locus

When using #4F/#4R primers, 81 tested cultivars (79%) showed the 227-bp PCR fragment for the *Wx-B1*a allele (Figure 2b lanes 4, 5, 8 and 9) and 22 (22%) showed absence to the 227-bp PCR fragment (*Wx-B1b* allele, Figure 2b lanes 6, 7, 10 and 11). Wheat cultivars Gamenya, Cadoux, Reeves and Bodallin (*Wx-B1b* controls), and unexpectedly, the landrace Mariñar (control *Wx-B1e*, Rodríguez-Quijano et al. 1998) (Figure 2b lane 3) showed absence to the 227-bp PCR fragment.

To confirm that result a second combination of *Wx-B1*-specific primers (Wx-B1L/Wx-B1R) was included in the analysis. This marker amplifies a 461-bp fragment in bread wheats with *Wx-B1a* allele and no PCR fragments are observed in wheats carrying *Wx-B1b* allele (nulls). With this marker, 81 of 103 cultivars (78%) showed the 461-bp PCR fragment and were scored as *Wx-B1a* as before (Figure 3 lanes 1, 5 and 6), but only 17 cultivars (16%) showed absence of the 461-bp fragment and were scored as *Wx-B1b* (Figure 3 lanes 7 and 8). The 5 remaining cultivars (5%), including the control *Wx-B1e* landrace Mariñar, amplified a slightly larger fragment 495-bp long (Figure 3 lanes 2, 3 and 4).

The 495-bp PCR fragment was amplified from the local cultivar Buck Poncho and sequenced (GeneBank AY954026). Sequence comparison of that fragment and the *Wx-B1a* allele showed nine SNPs in intron 5; six SNPs in exons 5 and 6, including four silent mutations and two amino acid changes in exon 5 (Ser to Asn and Arg to Met) and a 34-bp insertion in intron 5 that explain the different size (Figure 6). In addition, SDS-PAGE analysis in cultivars carrying Buck Poncho allele showed a *Wx-B1* protein that migrate slightly slower than *Wx-B1a*, having the same size of the *Wx-B1e* allele in Mariñar (Figure 7). Therefore, PCR fragment size and waxy protein band size were used as arguments to assign the 495-bp PCR fragment amplified with primers Wx-B1L/Wx-B1R from the local

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                                  ---- #4 F ------
                                  AAGAGCAACTACCAGT
Wx-A1a GGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAATGGCATCTATAGGACGGCCAAGgttttgcatcttctga 80
Wx-A1g GGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAATGGCATCTATAGGACGGCCAAGgttttgcatcttctga 80

Wx-A1a aactttatattcgctctgcatatcaattttgcggttcattctggcagcctgaattttacattgcaactccatttcatggc 160
Wx-A1g aactttatattcgctctgcatatcaattttgcggttcattctggcagcctgaattttacattgcaactccatttcatggc 160

Wx-A1a tagGTGGCATTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCTGA 240
Wx-A1g tagGTGGCATTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCGA 240

                         --------- #4 R -------
                         TCGACTTCATCGACGGGTACGA
Wx-A1a CAGGTTCAAGTCGTCCTTCGACTTCATCGACGGCTACGA 279
Wx-A1g CAGGTTCAAGTCGTCCTTCGACTTCATTGACGGCTACGA 279
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Figure 5. Best-fit alignment of partial nucleotide sequences (middle region of the *Wx-A1* gene) including *Wx-A1a* (AB019622) and *Wx-A1g* (DQ431232) alleles from bread wheat. Mismatches are gray-shaded, and exons 5 and 6 regions are in capitals. Locations of #4 F and #4 R primers are also indicated.

```
                                ----- #4 L -----
                                AAGAGCAACTACCAGT
Wx-B1a   GGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAGTGGCATCTATAGGACGGCCAAGgttttgcatcttctca 80
Wx-B1e   GGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAATGGCATCTATATGACGGCCAAGgttttgcaacttctga 80

Wx-B1a   aactttatattctctctgca----------------------------------gaattttacattgcaacttcatttca 126
Wx-B1e   aacttcatattctctccgcatatcaattcttttgcggttcattcattctggcctggattttacattgcaactccatttca 160

Wx-B1a   tgtccagGTAGCGTTCTGCATCCACAACATCTCGTATCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGC 206
Wx-B1e   tggatagGTGGCGTTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGC 240

                               -------- #4 R --------
                               TCGACTTCATCGACGGGTACGA
Wx-B1a   CCGACAGGTTCAAGTCGTCCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAG 286
Wx-B1e   CCGACAGGTTCAAGTCGTCCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAG 320

Wx-B1a   GCCCGGGATCCTGCAGGCCGACAAGGTGCTCACGGTGAGCCCCTACTACGCGG 338
Wx-B1e   GCCCGGGATCCTGCAGGCCGACAAGGTGCTGACGGTGAGCCCCTACTACGCTG 372
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Figure 6. Best-fit alignment of partial nucleotide sequences (middle region of the *Wx-B1* gene) including *Wx-B1a* and *Wx-B1e* alleles from bread wheat (GenBank accessions AB019623 and AY954026). Gaps were introduced to maximize nucleotide alignment and are indicated with dashes, mismatches are gray-shaded, exons are in capital. Annealing sites of #4 L and #4 R primers (McLauchlan et al. 2001) are also indicated.

cultivar Buck Poncho to the *Wx-B1e* allele.

Wx-D1 locus

For the molecular characterization of the *Wx-D1* locus two set of primers were used. The set of primers Wx-D1L/Wx-D1R was developed to detect the *Wx-D1b* allele described by Vrinten et al. (1999) in the cultivar Bai Huo. These primers amplified a 930-bp fragment from the distal region of the wild type *Wx-D1a* allele and also included a 588-bp deletion present in the *Wx-D1b* allele. The *Wx-D1b* control breeding line DHWx12 showed the mutation described by Vrinten et al. (1999) in Bai Huo (Figure 4 lane 1). When this molecular marker was used to evaluate the genetic variability in 103 Argentinean bread wheat cultivars, all tested samples showed the *Wx-D1a* allele. (Figure 4 lanes 2 to 11).

Primers #4F/#4R were also used to characterize the genetic variability of the *Wx-D1* locus and no molecular variability was detected. SDS-PAGE protein analysis showed the GBSSI subunit corresponding to the *Wx-D1* locus in all tested cultivars, confirming previous data observed with PCR markers (data not shown). The lack of variability observed in the *Wx-D1* locus in comparison with *Wx-A1* and *Wx-B1* loci agreed with previous data (Graybosh et al. 1998).

DISCUSSION

Urbano et al. (2002) expressed that waxy protein polymorphism in wheat is not very high, especially when compared with other group of proteins, such as storage proteins of wheat kernels. A possible explanation could be that most of the initial characterization studies of waxy proteins in wheat were performed by SDS PAGE of GBSSI proteins from starch granules, and with this technology small differences between proteins might be underestimated. In this context, molecular markers can be a valuable tool to characterize the genetic variability of *waxy* genes at DNA level, as sequences from *Wx-A1, Wx-B1, Wx-D1* genes are available (Murai et al. 1999). Moreover, the polyploid nature of bread wheat and the high homology between A, B and D genomes, are difficult issues to overcome in the development of single-locus allele specific markers because of the always latent possibility of coamplifying homologous alleles. This is the case of markers for *waxy* genes developed by Briney et al. (1998), McLauchlan et al. (2001), Nakamura et al. (2002) and Urbano et al. (2002).

In this work we used allele-specific mutations to develop single-locus allele specific markers for *Wx-A1, Wx-B1, Wx-D1* genes. As expected, the most frequent alleles in *waxy*

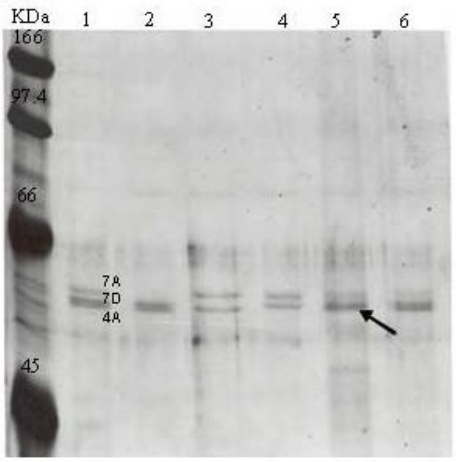

Figure 7. SDS-PAGE of waxy proteins from bread wheat. Lanes 1 to 6 are: 1-Chinese Spring, 2-N7AT7B, 3-N7DT7B, 4-N4AT4B, 5-BuckPoncho (*Wx-A1a, Wx-D1a, Wx-B1e*) and 6-Buck Arriero (*Wx-A1a, Wx-D1a, Wx-B1a*). The arrow (lane 5) indicate the *Wx-B1e* allele. The chromosomal locations of the isoproteins are indicated. Protein size standards are included in the left (lane M).

Table 1. Genetic variability of *Wx-A1*, *Wx-B1* and *Wx-D1* loci in Argentinean bread wheat cultivars obtained using PCR markers. In underlined, cultivars in which waxy proteins were also characterized by SDS-PAGE.

Locus and allele *Wx-A1* *Wx-B1* *Wx-D1*			Cultivar Name
a	a	a	Acienda, Baguette 10, Baguette 19, Baguette 20, Baguette Premium 13, Biointa 3000, Biointa 3003, Bonaerense Cauquén, Bonaerense Pasuco, Bordenave Puán Sag., Buck Arriero, Buck Brasil, Buck Chacarero, Buck Chambergo, Buck Charrúa, Buck Farol, Buck Guapo, Buck Guatimozín, Buck Manantial, Buck Mataco, Buck Mejorpan, Buck Namuncurá, Buck Ñandú, Buck Palenque, Buck Pampero, Buck Panadero, Buck Patacón, Buck Sureño, Buck Yapeyú, Buck Yatasto, Caudillo, Cooperación Liquen, Cooperación Millán, Cooperación Nahuel, Diamante INTA, Furlani Accidio, General Roca INTA, Inia Churrinche, Inia Condor, Inia Tijetera, Inia Torcaza, Klein Cobre, Klein Don Enrique, Klein Escudo, Klein Flecha, Klein Gavilán, Klein Jabalí, Klein Proteo, Klein Tauro, Leones INTA, Lona, Malambo, Marcos Juárez INTA, Pergamino Gaboto, Prointa Bon. Hurón, Prointa Bon. Redomón, Prointa Cauquén, Prointa Colibrí, Prointa Elite, Prointa Gaucho, Prointa Guazú, Prointa Hurón, Prointa Imperial, Prointa Oasis, Prointa Súper, Triguero 100
a	b	a	Buck Mataco, Granero INTA, Klein Chajá, Klein Escorpión, Klein Rendidor, Prointa Molinero, Prointa Puntal
a	e	a	ACA 302, ACA 303, Biointa 2001, Buck Pingo, Buck Poncho
g	a	a	ACA 223, Buck Biguá, Buck Pronto, Cronox, Greina, Klein Martillo, Klein Pegaso, Klein Salado, Klein Volcán, Las Rosas INTA, Pampa INTA, Prointa 5 Cerros, Prointa Bon. Alazán, Prointa Huen Pan, Zorzal
g	b	a	ACA 801, Agrovic 2000, Buck Guaraní, Cooperación Nahuel, Klein Sagitario, Prointa Amanecer, Prointa Don Humberto, Prointa Federal, Prointa Granar, Prointa Milenium

genes from tested wheat cultivars were the wild types (Table 1). Moreover, additional genetic variability in *Wx-A1* and *Wx-B1* loci was detected.

Wx-A1g is a new allele whose origin is probably CIMMYT, as the most ancient reference stocks in this work are Pampa INTA and Las Rosas INTA (year of release 1984) which are selections from CIMMYT germplasm and Klein Salado (year of release 1985) which is a selection from local and CIMMYT germplasm. As previously expressed, mutations associated with changes in aminoacid composition were not detected in partial *Wx-A1g* sequence, suggesting that protein function has not been affected in this allele. In line with these arguments, waxy protein patterns in cultivars scored as *Wx-A1a* and *Wx-A1g* were identical, different from *Wx-A1c* allele with a slightly altered isoelectric point and from *Wx-A1b* and *Wx-A1f* which showed real null protein alleles (Yamamori et al. 1994; Saito et al. 2004). *Wx-A1d* and *e* alleles were described in the bread wheat relatives *T. dicoccoides* (*Wx-A1d*) and *T. durum* (*Wx-A1*e), respectively (Yamamori et al. 1995) as protein bands with sizes different from *Wx-A1a*, which is not the case of *Wx-A1g*.

The origin of *Wx-B1b* allele in local germplasm is also unclear, as it was detected in cultivars developed using CIMMYT germplasm (Prointa Puntal, Klein Escorpión, Klein Chajá) and old local germplasm (Klein Rendidor, year of release 1954). The cultivars scored in this study as *Wx-B1b* showed lack of PCR products with two different sets of primers (#4F/#4R, Wx-B1L/Wx-B1R) and lack of the diagnostic *Wx-B1a* protein fragment by SDS-PAGE. This data suggest the presence of a large mutation similar to the null *Wx-B1b* allele described by Vrinten et al. (1999) in the cultivar Kanto 107. This mutation is the most frequent null mutation for *waxy* genes in wheat, having been detected in germplasm from Asia, Europe and North America (Saito et al. 2004) but not from South America, being this study the first report. Unfortunately the extend of the *Wx-B1b* deletion has not been established yet; therefore, it is uncertain if the size of the putative deletion observed in local cultivars carrying *Wx-B1b* allele is the

Table 2. Primer names, sequences, amplified loci and cycling conditions.

Name	Sequence	Amplified loci	Cycling Conditions
Wx-A1L	CCCCAAAGCAAAGCAGGAAAC	Wx-A1	39 cycles of 94ºC 45 sec, 55ºC 30 sec, 72ºC 1 min.
Wx-A1R	CGGCGTCGGGTCCATAGATC		
Wx-A2L	CGCAGGGGAAGACGTGGT	Wx-A1	39 cycles of 94ºC 45 sec, 65ºC 40 sec, 72ºC 50 sec.
Wx-A2R	CGTTGACGATGCCGGTGATC		
Wx-B1L	CGCAGGGGAAGACGTGGT	Wx-B1	39 cycles of 94ºC 45 sec, 65ºC 40 sec, 72ºC 50 sec
Wx-B1R	CGTTGACGATGCCGGTGATG		
Wx-D1L	GCCGACGTGAAGAAGGTGGTG	Wx-D1	39 cycles of 94ºC 45 sec, 55ºC 30 sec, 72ºC 1 min.
Wx-D1R	CCCCTTGCGTCATTTGTTGTGT		
#4F *	AAGAGCAACTACCAGT	*Wx-A1*, *Wx-B1* and *Wx-D1*	(1) Touch down step of 94ºC 1 min, 64ºC to 58ºC (-1ºC/2 cycles) 1 min, 72ºC 1 min. (2) 35 cycles of 94ºC 1 min, 58ºC 1 min, 72ºC 30 sec.
#4R *	TCGTACCCGTCGATGAAGTCGA		

*[$MgCl_2$] 3 mM. Other primer combinations [$MgCl_2$] 1.5 mM.

same that the *Wx-B1b* allele described by Vrinten et al. (1999) in the cultivar Kanto 107. Further fine mapping studies focused on *WxB1b* allele can answer this question.

In the case of the *Wx-B1e* allele, the most probable origin is Buck Poncho, a selection from local germplasm released in 1986, because all the other cultivars carrying *Wx-B1e* allele share Buck Poncho in their pedigree. The *Wx-B1e* allele is difficult to detect with markers #4L/#4R developed by McLauchlan et al. (2001) and by GBSS pattern detection in SDS-PAGE because of fragment overlapping. Cultivars with the *Wx-B1e* allele using #4L/#4R primers will generate fragments of 261-bp, which is very close to the 257-bp of *Wx-A1a* allele, and they will be probably scored as "nulls" for *Wx-B1* locus in agarose gels. The *Wx-B1e* allele is also difficult to be detected by SDS-PAGE because its mobility is similar to the *Wx-D1a* allele (lower mobility than *Wx-B1a*) (Demeke et al. 2000; Marcoz-Ragot et al. 2000; Yamamori and Quynh, 2000). In this work we describe a novel single-locus allele specific marker that accurately detects the *Wx-B1e* allele in agarose gels.This marker is a valuable tool to develop isogenic lines for *Wx-B1e* allele to evaluate the effect of amino acid changes detected in exon 5 of the *Wx-B1e* allele in amylose/amylopectin ratio.

The importance of the identification of new forms of waxy protein is related to a reduction in the amylose content found in genotypes carrying these mutations (null mutations) (Nakamura et al. 1993b). In this work we have detected one allele (*Wx-B1b*) with a deleterious effect in protein function, and a second allele (*Wx-B1e*) carrying several mutations whose effect in protein function still have to be elucidated. The *Wx-B1b* allele was detected in 16% of wheat cultivars. These cultivars carrying partial waxy starch can be an attractive target in the development of local adapted cultivars, suitable for certain specialties like dry white chinese noodles (Liu et al. 2003). No null alleles for *Wx-A1* or *Wx-D1* loci were detected in our bread wheat collection, which agree with data observed in European germplasm (Marcoz-Ragot et al. 2000). The vast majority of germplasm carrying *Wx-A1b* or *Wx-D1b* alleles is from Turkey, Korea, Japan (Yamamori et al. 1994) and probably, China. The molecular markers single-loci allele specific for nulls *Wx-A1b*, and *Wx-D1b* alleles are being used to introgress foreign null *Wx-A1b* and *Wx-D1b* alleles in local germplasm by marker assisted selection programs in order to develop adapted wheats with partial and total waxy starchs with different levels of amylose content.

ACKNOWLEDGMENTS

L. Vanzetti expresses his gratitude to FONCYT-INTA for a PICTO fellowship during this work which is part of his PhD Thesis. The authors want to express their gratitude to Paola Romina Aurelia for her excellent technical assistance, to Beatriz Formica for providing the wheat seeds used in this study and to Jorge Dubcovsky for his valuable suggestions in the writing of this paper.

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