

**Molecular characterization of a new *waxy* allele with partial expression in spelt wheat**

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## **Abstract**

Starch composition which is dependent on the waxy protein, the enzyme responsible for amylose synthesis in the grain, is an important aspect of the wheat quality. In this report, we describe the characterization of a novel *Wx-A1g* allele (*Wx-A1g* formerly known as *-Wx-A1a'*) in Spanish spelt wheat lines which is responsible for a remarkable decline in the concentration of Wx-A1 protein found in the endosperm. Comparison of the DNA sequences in the *Wx-A1a* and *Wx-A1g* alleles showed the presence of a 160-bp insertion within the fourth intron in the latter. This insertion had some characteristics of a transposable-like element. RT-PCR analysis showed the presence of normal and aberrant mRNA transcripts in the *Wx-A1g* lines, indicating that the aberrant transcripts are un-spliced and contained the longer fourth intron. This may be related to the low level of Wx-A1 protein in these lines. In addition, a simple and fast PCR assay was designed for differentiating among different *Wx-A1* alleles (*a*, *b*, *f* and *g*). The mutation described here is not related to either of the *Wx-A1* mutations identified previously in common and durum wheats and could help to extend the range of amylose content of wheats.

**Keywords:** Null mutations, Spelt wheat, Starch, Waxy proteins.

**Abbreviations:** GBSSI: granule-bound starch synthase I; ORF: open reading frame; PCR: polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;

## Introduction

One important component of wheat grain is starch, which accounts for between 65 and 75% of its dry weight. This macromolecule is composed of two types of glucose polymer: amylose and amylopectin (James et al. 2003). The ratio of the polymers (usually 22-35% amylose, 68-75% amylopectin) is very important as it affects properties of the starch such as gelatinization, pasting and after gelation which are determinants of the end-uses of the flour or starch (Zeng et al. 1997).

Recently studies have investigated wheat lines with varying ratios of amylose/amylopectin, and these lines have then been used in breeding programmes to develop new wheat lines with a modified ratio of these components (Nakamura et al. 1995; Kiribuchi-Otobe et al. 1997; Yamamori et al. 2000). As amylose is digested more slowly than amylopectine, and slow digestion is beneficial to human health, wheat lines with high amylose content may be used in the manufacture of healthy food (Higgins et al. 2004; Behall and Scholfield 2005). On the other hand, wheat lines containing lower or zero of amylose may have commercial importance since starch containing reduced levels of amylose was reported to produce noodles of better quality (Oda et al. 1980) and to extend the shelf life of various baked products (Lee et al. 2001).

Although several enzymes are involved in starch synthesis in the cereal endosperm, GBSSI or waxy protein is the sole enzyme responsible for amylose synthesis (Denyer et al. 2001). Three waxy proteins are present in common wheat (*Triticum aestivum* L. ssp. *aestivum*) with molecular weights ranging from 59 to 60 kDa, and are encoded by three genes: *Wx-A1* located on chromosome 7AS, *Wx-B1* on chromosome 4AL, and *Wx-D1* on chromosome 7DS (Yamamori et al. 1994). Each gene has eleven exons and ten introns (Murai et al. 1999). Several studies have shown that variation in amylose content is associated with the status of the Wx proteins expression (Yamamori et al. 1994; Rodríguez-Quijano et al. 1998; Urbano et al. 2002), particularly important is the occurrence of null alleles (absence of protein) of any of the three Wx genes.

In a worldwide survey of common wheat, Yamamori et al. (1994) found that the null *Wx-A1* allele frequently occurred in Japanese, Korean and Turkish cultivars,

whereas the null *Wx-B1* allele was present in many cultivars from Australia and India. Other authors also found these null variants in materials from all over the world (Graybosch et al. 1998; Rodríguez-Quijano et al. 1998; Urbano et al. 2002). The null *Wx-D1* allele is however, extremely rare and has only been described in few cultivars (Yamamori et al. 1994; Urbano et al. 2002; Guzman et al. 2010).

These null alleles have been subjected to molecular characterization to identify the nature of their inactivation. Vrinten et al. (1999) analysed the null alleles present in cv. Kanto 107 (null for the *Wx-A1* and *Wx-B1* genes) and cv. Bai Huo (null for the *Wx-D1* gene), both of which had previously been crossed by Nakamura et al. (1995) to develop waxy wheat. They found that the inactivation of these genes is caused by different deletions and insertions in each of the *waxy* genes. However, other types of the null *Wx-A1* allele have been also identified, for example Saito et al. (2004) detected an insertion of 173-bp inside the fourth exon of *Wx-A1f* that changes the ORF and leads to a premature stop codon. Monari et al (2005) described both deletions and insertions in different materials. In addition, Saito and Nakamura (2005) found an insertion and a deletion of one nucleotide that changed the ORF of the gene in wild and cultivated emmer (*T. turgidum* ssp. *dicoccoides* Körn. ex Asch. & Graebner em. Thell. and *T. turgidum* ssp. *dicoccum* Schrank, respectively) that lacked the Wx-A1 protein.

The ancient wheats, along with their relatives, have showed to be an important source of variability for the bread- and pasta-quality traits, and could be used in the breeding of the modern wheat (Sharma et al. 1981). Recently, diverse studies on the *waxy* genes have been carried out in three ancient wheat species of Spanish origin: einkorn (*T. monococcum* L. ssp. *monococcum*), emmer and spelt (*T. aestivum* ssp. *spelta* L. em. Thell). These species have been associated with a certain degree of variation including null alleles (Guzman et al. 2009, 2010, 2011). In a previous study on one broad collection of Spanish spelt (Guzman et al. 2010), we detected different null alleles, including *Wx-A1* null (*Wx-A1g* formerly known as *Wx-A1a'*) in 36 out of 420 accessions evaluated.

The aim of the current study was the molecular characterization of this null *Wx-A1g* allele found in spelt in order to determine the cause of its reduced expression.

## **Materials and methods**

### **Plant material**

All spelt (*T. aestivum* ssp. *spelta* L. em. Thell) accessions that presented the novel *Wx-A1g* allele (36), together with a representative sample of accessions carrying the wild allele (*Wx-A1a*), classified in a previous survey by SDS-PAGE means (Guzman et al. 2010), were analysed in this study. The common wheat (*Triticum aestivum* L. ssp. *aestivum*) cultivar Chinese Spring (National Small Grain Collection, Aberdeen, USA) that carries the *Wx-A1a* allele was used as standard as well as a *Wx-A1b* line (9906-155) generated from the cross of cvs. Bai Huo and Kanto 107. Further, the cultivar Kanto 107 (*Wx-A1b* allele) and the Turkey-124 and Turkey-140 accessions (*Wx-A1f* allele) kindly supplied by the National Small Grain Collection (Aberdeen, USA) and NIAS (Tsukuba, Japan), respectively, were included for comparison in DNA analysis. All plants were grown to maturity in a greenhouse.

#### Starch extraction and electrophoretic analysis

Preparation of starch granules and separation of waxy proteins by low-bis acrylamide SDS-PAGE was performed as described by Echt and Schwartz (1981) and Zhao and Sharp (1996).

For two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE), 8.0 mg of starch was swollen at room temperature in 300  $\mu$ l of lysis buffer [8 M urea, 2% Nonidet-P40, 2% ampholine pH 3.5-10 (Pharmacia LKB) and 5% 2-mercaptoethanol]. After centrifugation, the supernatant containing the solubilised proteins was subjected to 2D-PAGE using isoelectric focusing (IEF) for the first dimension and modified SDS-PAGE for the second (Nakamura et al. 1993). IEF gels contained 2.5% (v/v) ampholines (pH 3.5-10/5-8, 1:1). Focusing was begun from the acidic end (0.01 M H<sub>3</sub>PO<sub>4</sub>) and continued at 400 V for 15 h, then 800 V for 60 min at room temperature. Proteins were revealed by silver staining according to stain kit (Wako Pure Chemical Industries, Ltd., Japan).

#### DNA extraction and PCR amplification

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

The primers designed by Monari et al. (2005) were used to amplify the *waxy* genes in three regions. The region spanning the first to the third exon was amplified

using the primers W<sub>x</sub>F3 (5'-TCTGGTCACGTCCCAGCTCGCCACCT-3') and W<sub>x</sub>VT1R (5'-ACCCCGCGCTTGTAGCAGTGGAAAGT-3'); the pair W<sub>x</sub>BAF (5'-ACTTCCACTGCTACAGCGCGGGGT-3') and W<sub>x</sub>BAR (5'-GCTGACGTCCATGCCGTTGACGATG-3') was used to amplify the region spanning the third to the sixth exon; and the pair W<sub>x</sub>VT1F (5'-CATCGTCAACGGCATGGACGTTTCAGC-3') and W<sub>x</sub>VTR (5'-CCAGAAGCACGTCCTCCCAGTTCTTG-3') for the region spanning the sixth to the eleventh exon. Each 15  $\mu$ l reaction included 50 ng DNA, 1.5 mM MgCl<sub>2</sub>, 0.2  $\mu$ M of each primer, 0.2 mM dNTPs, 1.5  $\mu$ l 10 $\times$  PCR Buffer and 0.75U DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 3 min at 94  $^{\circ}$ C followed by 35 cycles as follows: for W<sub>x</sub>F3/W<sub>x</sub>VT1R, 40 s at 94  $^{\circ}$ C, 1 min at 62  $^{\circ}$ C then 1 min at 72  $^{\circ}$ C; for W<sub>x</sub>BAF/W<sub>x</sub>BAR, 45 s at 94  $^{\circ}$ C, 2 min at 62  $^{\circ}$ C then 1 min 5 s at 72  $^{\circ}$ C; and for W<sub>x</sub>VT1F/W<sub>x</sub>VTR, 40 s at 94  $^{\circ}$ C, 1 min at 62  $^{\circ}$ C then 1 min 30s at 72  $^{\circ}$ C. After the 35 cycles all reactions included a final extension of 5 min at 72  $^{\circ}$ C.

Amplification products were fractionated in vertical PAGE gels with 8% polyacrylamide concentration (w/v, C: 1.28%), and the bands were visualized by ethidium bromide staining.

#### RNA extraction, cDNA synthesis and analysis

Developing seeds at 10 days post-anthesis (10-DPA) for RNA extractions were collected, immediately frozen by immersion in liquid nitrogen and stored at -80 $^{\circ}$ C. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and treated with TURBO DNase (RNase-Free; Ambion, Warrington, UK) to eliminate any DNA contamination. The cDNA was generated from total RNA and random nonamers using Superscript II Reverse Transcriptase (Invitrogen), according to the company's instructions. One control PCR amplification was routinely performed to ensure that the specific PCR products were from RNA and not from DNA contamination.

Four pairs of specific primers were designed to amplify different regions of the cDNA surrounding the fourth intron in *Wx-A1* gene (Table 1). Each 15  $\mu$ l reaction included 50 ng cDNA, 1.5 mM MgCl<sub>2</sub>, 0.4  $\mu$ M of each primer, 0.2 mM dNTPs, 1.5  $\mu$ l 10 $\times$  PCR Buffer and 0.75U DNA polymerase. The PCR conditions for each pair are

describing in Table 1. Amplifications products were fractionated as described before.

#### Cloning of PCR products and sequencing analysis

PCR products were excised from polyacrylamide gel and cloned into pGEM T-easy vector (Promega) for sequencing. Inserts were sequenced using an ABI Prism 310 Genetic Analyzer (Perkin Elmer). The sequences were compared to the sequences of spelt (*Wx-A1*; HQ338723) and cv. Chinese Spring (*Wx-A1a*; AB019622) available in the NCBI databases.

#### PCR marker for differentiation among null and partial-null *Wx-A1* alleles

For detection and differentiation of different *Wx-A1* alleles (*a*, *b*, *f* and *g*), a pair of primers that flanked the region where these alleles showed differences was designed: *Wx-A1* Diag Forward (5'-GTAAGCTTGCGCCACTGCCT-3') and *Wx-A1* Diag Reverse (5'-TGTGCCAGTCGTTGCACACA-3'). Each 15  $\mu$ l reaction included 50 ng DNA, 1.5 mM MgCl<sub>2</sub>, 0.2  $\mu$ M of each primer, 0.2 mM dNTPs, 1.5  $\mu$ l 10 $\times$  PCR buffer and 0.75U DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 3 min at 94 °C followed by 35 cycles as follows: 40 s at 94 °C, 30s 66 °C then 1 min 55 s at 72 °C. After the 35 cycles a final extension of 5 min at 72 °C. Amplifications products were fractionated as described above. Additionally, the PCR products were restricted with the *ApoI* endonuclease (Fermentas) following the supplier's instructions.

Amplification and digestion products were fractionated in vertical PAGE gels with 8% polyacrylamide concentration (w/v, C: 1.28%), and the bands were visualized by ethidium bromide staining.

## Results

#### SDS-PAGE and 2D-PAGE electrophoresis

The separation of the waxy proteins in several representative lines of the variation detected is shown in Fig. 1. In a previous study (Guzman et al. 2010) these lines were classified as null for *Wx-A1* protein (*Wx-A1b* allele). However, in the current study, when the SDS-PAGE gels were stained for an extended period, a faint band was detected in the *Wx-A1* position (lanes 1, 3, 4, 5, 7, and 8), although it was much less intense than in the lines carrying the *Wx-A1a* allele (lanes 2 and 6).

One similar result was indicated by Caballero et al. (2008) that provisionally named the partially null allele found as *Wx-A1a'*, although following this discovery, this new allele was designated *Wx-A1g* by McIntosh et al. (2009). The comparison between the three Spanish spelt lines used by Caballero et al. (2008) and the used in the current study suggested that both alleles could be the same. Consequently, these lines were reassigned as partially null for the Wx-A1 protein (*Wx-A1g* allele). This result was confirmed with 2D-PAGE, which showed three faint spots in the expected position of the Wx-A1 protein (Fig. 1b) but with less intensity than that shown in the spelt reference sample (Fig. 1a).

#### Molecular characterization of *Wx-A1g* allele

The PCR amplification of the *Wx-A1* gene was carried out in genomic DNA with the specific primers designed by Monari et al. (2005). This permitted the simultaneous amplification of the three *waxy* genes (A, B, and D) as three fragments. In the first and third fragment of the *Wx-A1* gene no differences were detected between the amplicons of the lines carrying the *Wx-A1g* and *Wx-A1a* alleles (data not shown). However, amplification of the central region of these genes revealed a remarkable difference between the spelt and common wheat lines (Fig. 2, lanes 2 and 6, respectively) which were used as standards. Spelt showed two bands due to the Wx-A1 and Wx-B1 which co-migrated, while the common wheat presented three conspicuous bands. When the amplicons were sequenced, three different sequences for each species were identified. The size of the Wx-A1 (953-bp) and Wx-D1 (1017-bp) was similar in both species, while that of the Wx-B1 bands was different: 973-bp for spelt and 935-bp for common wheat.

On the other hand, the main difference between spelt lines containing the *Wx-A1a* and *Wx-A1g* alleles was the presence of an extra band larger than the *Wx-D1a* band (Fig. 2, lanes 1, 3, 4, 5, 7 and 8) in *Wx-A1g* lines. This larger band was purified and sequenced. It showed 100% homology with respect to the *Wx-A1a* allele from spelt reference line and cv. Chinese Spring, with the exception of one insertion of 160-bp located in the fourth intron (Fig. 3). This fragment had the characteristics of a transposable-like element, with target site duplications and inverted repeats, although the inverted repeats were not located in the terminal regions of the insertion and did not share 100% homology.



The rest of the *Wx-A1* gene (first and third fragment) was also sequenced in two *Wx-A1g* lines (PI-348437, BGE-012911) in order to identify other differences that had not been detected by PCR analysis. In this case no variation was found with respect to the *Wx-A1a* allele however there was 100% homology with the spelt reference sample. The entire *Wx-A1g* sequences of both lines are available in GenBank (HQ625382, HQ625383).

#### RT-PCR analysis of the *Wx-A1* gene

Because low levels of *Wx-A1* protein were detected in lines containing the *Wx-A1g* allele in comparison with the lines containing *Wx-A1a* allele, it was important to determine whether the insertion found in the fourth intron of the *Wx-A1g* allele affected mRNA processing and, consequently, the expression of the protein. Total RNA was extracted from the 10-DPA endosperm for use in the synthesis of cDNA and RT-PCR analysis. Four pairs of specific primers were designed on the exons flanking the fourth intron which was expected to be removed during the splicing as was the remainder of the introns (Fig. 4a). Both the *Wx-A1a* and *Wx-A1g* alleles showed transcripts containing only the exons, all the introns having been removed (Fig. 4b; and Fig. 4d lanes 1-4.694 bp bands). In addition to this, lines carrying the *Wx-A1g* allele showed larger transcripts that were not present in the *Wx-A1a* lines with each pair of primers used (Fig. 4c; and Fig. 4d lanes 1,2 975 bp bands). Sequencing revealed these products consisted of *Wx-A1* sequences carrying the fourth intron which had not been spliced. This gave a clear indication that splicing of part of the mRNA had been unsuccessful in the *Wx-A1g* genotypes. In addition, it was found with one pair of primers that part of the first exon was also deleted in some fragments of mRNA of *Wx-A1g* lines during the splicing, resulting in an incomplete *Wx-A1* coding sequence (Fig. 4c).

#### Development of a molecular marker to differentiate *Wx-A1* alleles

Two null *Wx-A1* alleles (*Wx-A1b* and *Wx-A1f*) together with the wild allele (*Wx-A1a*) were compared with the *Wx-A1g* alleles by the amplification of the gene region between the first and fifth intron (Fig. 5a). This area include the In/Del sequences that differentiate the three mutant alleles and the wild ones. In addition to this, the primers were designed in areas of the *Wx-A1* gene that have several mismatches with respect to *Wx-B1* and *Wx-D1* ones, which avoid the simultaneous amplification of the *Wx-B1* and

*Wx-D1* homoeologous genes when the annealing temperature increases.

The *Wx-A1a* allele showed a 770-bp amplicon (Fig. 5b, lanes 1 and 2). However, as it was expected, the *Wx-A1b* allele present in cv. Kanto 107 did not show any PCR product, since the forward primer was designed on a region of the *Wx-A1* sequence that is deleted in this allele, exactly at the first exon-intron junction. The other two alleles (*Wx-A1f* and *Wx-A1g*) showed amplicons with a higher size of the *Wx-A1a* allele (Fig. 5b, lanes 4-7), but with slightly differences between them (943 and 930 bp, respectively). To make a more clear differentiation between *Wx-A1g* and *Wx-A1f* alleles, the PCR product was digested with *ApoI* endonuclease that has a sequence target in *Wx-A1f* allele (Fig. 5c). *Wx-A1a* and *Wx-A1g* products were not affected by this enzyme. Consequently, the use of this PCR assay permitted to discriminate among all alleles evaluated here, with special interest between both null allele type (*Wx-A1b* and *Wx-A1f*) and the new *Wx-A1g* allele that shows a partial expression.

## Discussion

Waxy protein (GBSSI) is the key enzyme in amylose synthesis, which variation has a considerable influence on the amylose content, mainly due to the presence of null alleles (Yamamori et al. 1994; Rodríguez-Quijano et al. 1998). Numerous studies have been carried out to characterize the mutations that cause these null *Wx* variants (Vrinten et al. 1999; Saito et al. 2004; Monari et al. 2005).

In the case of the *Wx-A1* locus, several In/Del mutations that caused a change in the ORF which led to a premature stop codon have been found. Vrinten et al. (1999) described a 23-bp deletion at an exon-intron junction in cv. Kanto 107 that cause the lack of *Wx-A1* protein. In the same way, Saito et al. (2004) described one mutation in Turkish common wheat accessions lacking *Wx-A1* protein that consisted of a 173-bp insertion in the fourth exon of the *Wx-A1* locus (*Wx-A1f* allele). They also found that the insertion had the characteristics of a transposable element of class II which affected RNA maturation; this element was designated *Hikkoshi* (Saito et al. 2004). In fact, Wessler and Varagona (1985) indicated that some spontaneous waxy mutations in maize were associated with the presence of these transposable elements. Monari et al. (2005) also identified a Turkish durum wheat accession that lacked the *Wx-A1* protein as consequence of an insertion of 89-bp in the sixth exon.

Although in a recent study (Debiton et al. 2010), a non-functional truncated

protein has been found for one null *Wx* allele (*Wx-D1b*), which presented a smaller size than the wild protein, the possible proteins of the abovementioned null *Wx-A1* alleles have not been detected yet. On the contrary, the corresponding protein in the *Wx-A1g* allele detected in spelt by Caballero et al. (2008) and in the current study was found at very low levels. The analysis of its sequence indicated the presence of a 160-bp insertion in the fourth intron which showed some characteristics typical of transposable-like elements such as target site duplications and inverted repeats. Due to the similarity of the remaining sequence in the *Wx-A1a* and *Wx-A1g* alleles, it is likely that this insertion is related to the reduced expression of the *Wx-A1* protein. The RT-PCR analysis of developing seeds showed that although the correct processed form of *Wx-A1* transcript was present in the *Wx-A1a* and *Wx-A1g* lines, transcripts with the un-spliced fourth intron were also found in all *Wx-A1g* lines. Saito et al. (2005), who also found un-spliced transcripts in null mutant lines, suggested that these aberrant mRNA products were rapidly degraded by a process referred to as nonsense-mediated mRNA decay (NMD), or formed a truncated protein that would be promptly eliminated because of its lack of function. In the current case, the 160-bp insertion found in this intron appeared to prevent the complete splicing of all the RNA molecules, thus reducing the yield of correctly processed RNA leading to a remarkable decrease in the final concentration of *Wx-A1* protein.

The presence of three *Wx* loci in hexaploid wheat has made it difficult to develop wheats with low or null amylose content. Yamamori et al. (1994) classified these wheats in eight types by combining wild and null alleles at the *Wx-A1*, *Wx-B1* and *Wx-D1* loci. The wild type was named as type 1, waxy wheat as type 8 and the partial waxy lines as types 2-6. In general, the effect of any active *Wx* gene makes that the mutations of any of the other *Wx* genes have scarce impact on the amylose content due to one dosage effect, as it was showed again later by Yamamori and Quynh (2000). Consequently, the partial waxy lines show amylose contents almost always over 20%, and only the triple null mutant (type 8) present values less than 1 % (Nakamura et al. 1995). Although further studies, similar to that made by Yamamori (2009) and Yamamori and Yamamoto (2011) with other *Wx* alleles, should be carried out, data suggest that the allele describe in the current survey (*Wx-A1g*) could have one different behaviour in combination with the null alleles for the *Wx-B1* and *Wx-D1* loci, which open the possibility to obtain quasi-waxy wheats with amylose content above 1% but

less than 20%.

The main difficulty of these materials is the correct and unequivocal identification of the *Wx-A1* alleles (*a*, *b*, *f* and *k*) that they present. The evaluation by SDS-PAGE (uni- or bi-dimensional) is low efficient for its use in breeding programmes. Consequently, the development of one easy and fast PCR marker associated with these mutations, as the designed in the current study, is an important key for the successful development of new waxy, partial waxy or quasi-waxy wheats.

In conclusion, a new *waxy* allele (*Wx-A1g*) has been characterized at a molecular level. The mutation described here is not related to either of the *Wx-A1* mutations identified previously in common and durum wheats and could help to extend the range of amylose content of wheats or to breed wheats with very low amylose content.

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**Table 1** Description of PCR primers pairs for amplifying of the different regions of the cDNA surrounding the fourth intron in *Wx-A1* gene

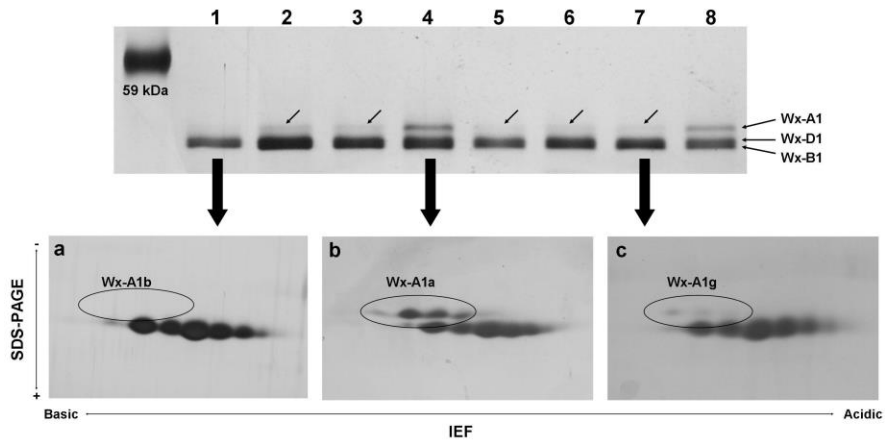
Primer	Sequence (5' → 3')		
Wx-A1AFw:	AGATCTATGGACCCGACGCC		
Wx-A1A3Rv:	TGCTCCTCCAGCCTGCCGAT		
Wx-A1BFw:	CAGCACTTGAGGTGCCCAGG		
Wx-A1B1Rv:	CGGCGATCATCACGTCGGGG		
Wx-A1B2Rv:	CAGCGCCTTCCCCTCCAACG		
Wx-A1DFw:	GGCGCCCTGGAGCAAGACTG		

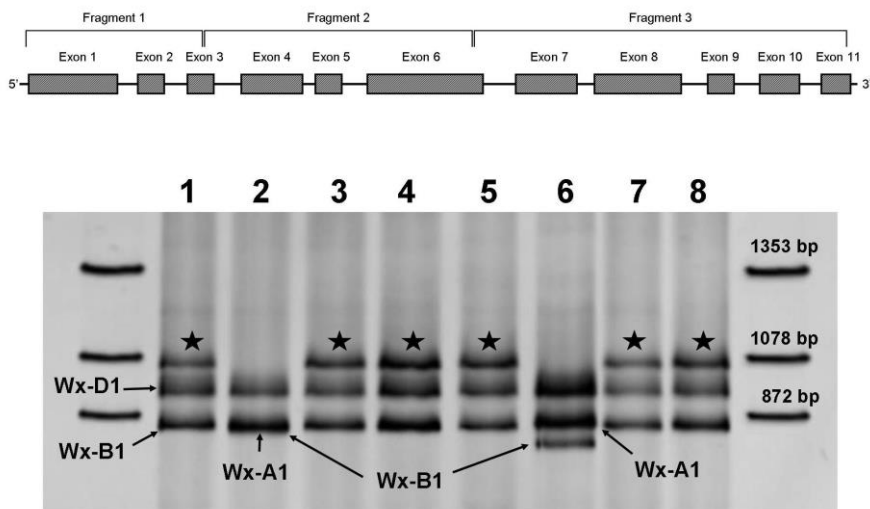
Pair	PCR conditions		
	Initial denaturation	35 cycles	Final extension
Wx-A1DFw/Wx-A1B2Rv	3 min at 94 °C	45 s at 94 °C	5 min at 72 °C
		30 s at 64 °C	
Wx-A1AFw/Wx-A1A3Rv	3 min at 94 °C	1 min 40s at 72 °C	5 min at 72 °C
		40 s at 94 °C	
Wx-A1BFw/Wx-A1B1Rv	3 min at 94 °C	30 s at 66 °C	5 min at 72 °C
		1 min 10 s at 72 °C	
Wx-A1BFw/Wx-A1B1Rv	3 min at 94 °C	45 s at 94 °C	5 min at 72 °C
		45 s at 64 °C	
Wx-A1BFw/Wx-A1B2Rv	3 min at 94 °C	1 min 10 s at 72 °C	5 min at 72 °C
		45 s at 94 °C	
Wx-A1BFw/Wx-A1B2Rv	3 min at 94 °C	45 s at 64 °C	5 min at 72 °C
		50 s at 72 °C	

**Caption figures**

**Fig. 1** SDS-PAGE and 2D-PAGE electrophoresis patterns of waxy proteins. Lanes are as follow: 1, Line 9906-155 (W<sub>x</sub>-A1b); 2, PI-348437 (W<sub>x</sub>-A1g); 3, BGE-012911 (W<sub>x</sub>-A1g); 4, PI-469029 (W<sub>x</sub>-A1a); 5, PI-348518 (W<sub>x</sub>-A1g); 6, PI-348741 (W<sub>x</sub>-A1g); 7, PI-348728 (W<sub>x</sub>-A1g); and 8, cv. Chinese Spring (W<sub>x</sub>-A1a). All lines present the W<sub>x</sub>-B1a and W<sub>x</sub>-D1a proteins. Faint W<sub>x</sub>-A1g proteins are indicated by arrows. In 2D-PAGE electrophoresis **a** is W<sub>x</sub>-A1b, **b** is W<sub>x</sub>-A1a and **c** is W<sub>x</sub>-A1g.

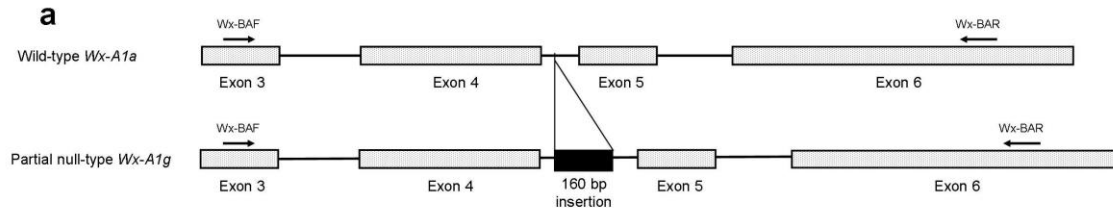


**Fig. 2** Diagrammatic representation of the waxy gene structure and amplification of the central region (Fragment 2) in several representative lines. Lanes are as follow: 1, PI-348437 (W<sub>x</sub>-A1g); 2, PI-469029 (W<sub>x</sub>-A1a); 3, PI-348518 (W<sub>x</sub>-A1g); 4, PI-348741 (W<sub>x</sub>-A1g); 5, PI-348728 (W<sub>x</sub>-A1g); 6, cv. Chinese Spring (W<sub>x</sub>-A1a); 7, PI-469029 (W<sub>x</sub>-A1g); and 8, BGE-012911 (W<sub>x</sub>-A1g). W<sub>x</sub>-A1g bands are indicated with stars.





**Fig. 3** The insertion in the partial null *Wx-A1g* allele of spelt wheat. **a**, Diagrammatic representation of the central region in the *Wx-A1a* and *Wx-A1g* alleles. **b**, Comparison of genomic DNA sequences of *Wx-A1* alleles between the primers Wx-BAF and Wx-BAR. The exons are showed in capital letter. Target site duplications are indicated by arrows and terminal inverted repeats like sequences are showed in grey boxes.



**b**

*Wx-A1a* ACTTCCACTGCTACAAGCGCGGGTGGACCGCGTGTTCGTGACACCACCGTGCTTCCCTGGAGAAGgtgaccgatcgctcgccgtgagatcgatcaagctag  
*Wx-A1g* ACTTCCACTGCTACAAGCGCGGGTGGACCGCGTGTTCGTGACACCACCGTGCTTCCCTGGAGAAGgtgaccgatcgctcgccgtgagatcgatcaagctag

*Wx-A1a* ctccctcgctgctcacaaccgcatggtgttgataattcagtgagctcttgcgctgctggttacaatttccagGTCCGGGCAAGACCAAGGAGAAGAT  
*Wx-A1g* ctccctcgctgctcacaaccgcatggtgttgataattcagtgagctcttgcgctgctggttacaatttccagGTCCGGGCAAGACCAAGGAGAAGAT

*Wx-A1a* CTATGGACCCGACGCGGCACCGACTACGAGGACAACCAGCAGCGCTTCAGCCTTCTCTGCCAGGCAGCACTTGAGGTGCCAGGATCCTCGACCTCAAC  
*Wx-A1g* CTATGGACCCGACGCGGCACCGACTACGAGGACAACCAGCAGCGCTTCAGCCTTCTCTGCCAGGCAGCACTTGAGGTGCCAGGATCCTCGACCTCAAC

*Wx-A1a* AACAAACCACACTTTTCTGGACCTACGgtaagatcaagaacaactagagtgtatctgaagaacttgattttctacttgagagca-----  
*Wx-A1g* AACAAACCACACTTTTCTGGACCTACGgtaagatcaagaacaactagagtgtatctgaagaacttgattttctacttgagagcaactggatgaggaact

-----  
*Wx-A1a* -----  
*Wx-A1g* aetteaeggaogatcttttgctgaacgatatacgaagatagctaaatcaagggttagggctgcccaggttgccaaaggatgggtccctgaagtagcgt

*Wx-A1a* -----  
*Wx-A1g* cgtgcacatctcgctagcaagtgtcgtctgctgtagcactgctcctggatgattatactctccttgatcttgggtgctgccatgctatgccgtgcccgtg

*Wx-A1a* cgcgcgcgcgcagGGGAAGACGTGGTGTGTTGTGTGCAACGACTGGCACACGGGCCTTCTGGCCTGTACTCAAGAGCAACTACCAGTCCAATGGCATCT  
*Wx-A1g* cgcgcgcgcgcagGGGAAGACGTGGTGTGTTGTGTGCAACGACTGGCACACGGGCCTTCTGGCCTGTACTCAAGAGCAACTACCAGTCCAATGGCATCT

*Wx-A1a* ATAGGACGGCCAAGgttttgcatctctgaaactttaattogctctgcaatcaatcttgggttcaattctggcagcctgaattttacattgcaactcc  
*Wx-A1g* ATAGGACGGCCAAGgttttgcatctctgaaactttaattogctctgcaatcaatcttgggttcaattctggcagcctgaattttacattgcaactcc

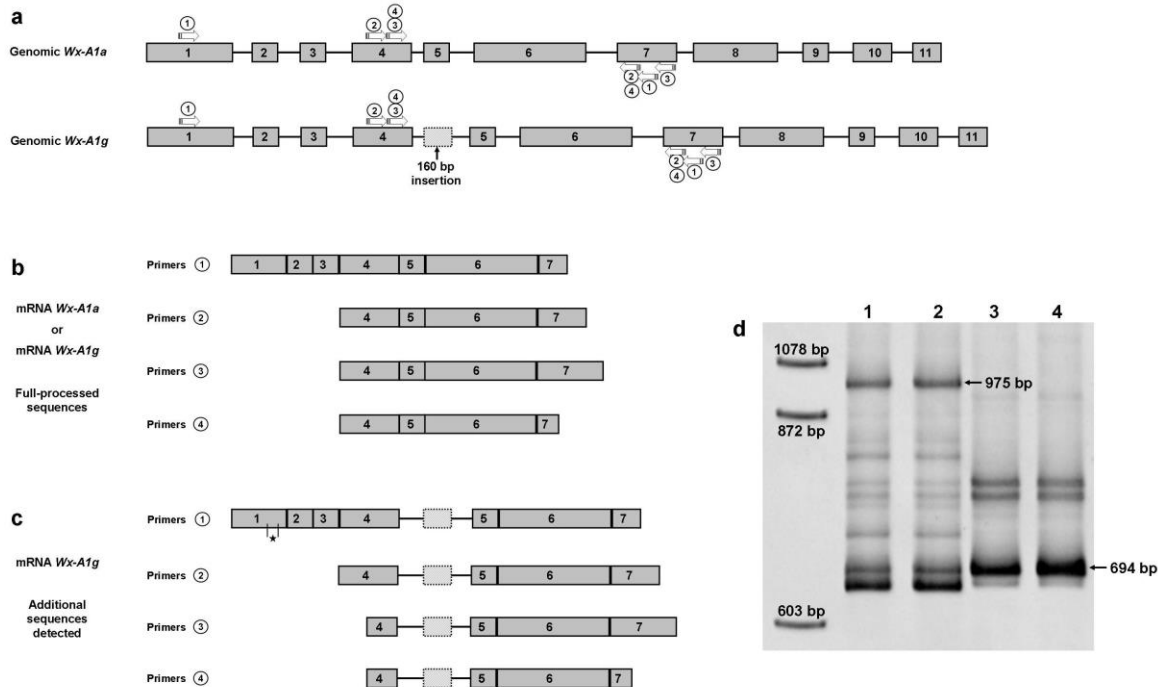
*Wx-A1a* atttcatggctagGTGGCATTCTGCATCCACAACATCTGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCTGACAGGTTCAAG  
*Wx-A1g* atttcatggctagGTGGCATTCTGCATCCACAACATCTGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCTGACAGGTTCAAG

*Wx-A1a* TCGTCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGGCCGACAAGGTGCTGACTG  
*Wx-A1g* TCGTCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGGCCGACAAGGTGCTGACTG

*Wx-A1a* TGAGCCCCTACTATGCTGAGGAGCTAATCTCTGGCGAAGCCAGGGCTGCGAGCTCGACAACATCATGCGCCTCACTGGGATCACCGGCATCGTCAACGG  
*Wx-A1g* TGAGCCCCTACTATGCTGAGGAGCTAATCTCTGGCGAAGCCAGGGCTGCGAGCTCGACAACATCATGCGCCTCACTGGGATCACCGGCATCGTCAACGG

*Wx-A1a* CATGGACGT CAGC  
*Wx-A1g* CATGGACGT CAGC

**Fig. 4** **a**, Schematic representation of the *Wx-A1a* and *Wx-A1g* alleles. **b**, fragments amplified from total RNA of *Wx-A1a* and *Wx-A1g* lines using RT-PCR. **c**, fragments amplified exclusively from total RNA of *Wx-A1g* lines. Four pairs of primers were used: 1, *Wx*A1DFw/*Wx*A1B2Rv; 2, *Wx*A1AFw/*Wx*A1A3Rv; 3, *Wx*A1BFw/*Wx*A1B1Rv; and 4, *Wx*A1BFw/*Wx*A1B2Rv. Small deletion in first exon was detected (star). **d**, representative gel of amplicons obtained from cDNA with primers *Wx*A1AFw/*Wx*A1A3Rv. Bands of 974 bp contained fourth intron unspliced with insertion. Lanes are as follow: 1, PI-348437 (*Wx-A1g*); 2, BGE-012911 (*Wx-A1g*); 3, PI-469029 (*Wx-A1a*); and 4, Pi-348515 (*Wx-A1a*)



**Fig. 5** Molecular marker to differentiate *Wx-A1* alleles. **a**, schematic representation of the *Wx-A1a*, *Wx-A1b*, *Wx-A1f* and *Wx-A1g* alleles. Primers Wx-A1 Diag forward and reverse are indicated in their annealing area. **b**, PCR products with Wx-A1 Diag primers fractionated in polyacrylamide gels. **c**, Digestion with *ApoI* of PCR products. Lanes are as follow: 1, cv. Chinese Spring (*Wx-A1a*); 2, spelt PI-469029 (*Wx-A1a*); 3, cv. Kanto 107 (*Wx-A1b*); 4, Turkey-124 (*Wx-A1f*); 5, Turkey-140 (*Wx-A1f*); and 6, spelt PI-348437 (*Wx-A1g*).

