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Molecular characterization of new waxy mutants identified in bread and durum wheat

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Abstract Recently, electrophoretic analyses of waxy proteins in several hexaploid and tetraploid wheat accessions from worldwide collections have permitted the identification of new variants at the *waxy* loci, including allelic forms with different mobilities and partial null types. In this paper, the molecular characterization of mutated *waxy* loci in four bread wheat cultivars (two lacking the Wx-B1 and two lacking the Wx-D1 protein, respectively) and in four durum wheat cultivars (one lacking Wx-A1 and the remainder with Wx-B1 proteins showing different electrophoretic mobilities) was conducted by means of PCR, Southern and DNA sequence analyses. Three primer pairs were developed that identified six of the above-mentioned mutations and allowed their molecular description, providing a useful tool for further germplasm screening or marker assisted progeny selection in breeding programs involving the newly identified material. We have found that a complete gene deletion is responsible for a null allele at the *Wx-B1* locus in one bread wheat line, whereas sequencing of the corresponding fragments showed a 724 bp deletion in the *Wx-D1* locus in one line of bread wheat and an insertion of 89 bp in the *Wx-A1* locus in one line of durum wheat, respectively. In addition, nucleotide substitutions and various insertions/deletions ranging from 3 to 30 bp were detected in the PCR fragments of one durum wheat line with a Wx-B1 protein with a different electrophoretic mobility. A fourth primer set, specific for this mutation, was consequently derived.

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Introduction

Starch, which accounts for 65–75% of wheat grain dry weight, is the major constituent of flour and semolina and is composed of two types of polymers, amylose and amylopectin. Amylose is an essentially linear α-1,4 glucan and contributes about 20–30% to the total starch, while amylopectin is a branched α-1,4 glucan containing about 5% α-1,6 branch points and constitutes the remaining 70–80% of the total starch. The physical and chemical properties of starch, and consequently the quality of the end products are dependent on the relative amounts of amylose and amylopectin (Fredriksson et al. 1998). Many enzymes are involved in starch synthesis with at least five isoforms of the starch synthases (SS, as reviewed by James et al. 2003) existing. Four of these are involved only in amylopectin synthesis, with two forms of branching and debranching enzymes. The granule bound starch synthases (GBSSI or waxy proteins) are the sole starch synthases responsible for amylose synthesis in storage tissues (Nakamura et al. 1993). In bread wheat there are three waxy proteins, with a molecular weight ranging from 59 to 60 kDa (Murai et al. 1999), designated Wx-A1, Wx-D1 and Wx-B1. They are encoded by three genes: *Wx-A1* (2,781 bp), *Wx-D1* (2,862 bp) and *Wx-B1* (2,794 bp), each consisting of 11 exons and ten introns (Murai et al. 1999) located on chromosome arms 7AS (*Wx-A1*), 7DS (*Wx-D1*) and 4AL (*Wx-B1*). The latter was originally located on Chromosome 7BS before a translocation occurred between chromosomes 7BS and 4AL during wheat evolution (Miura and Tanii 1994a; Yamamori et al. 1994). In durum wheat, only the Wx-A1 and Wx-B1 proteins are present.

Waxy mutations occur spontaneously in cereals (Eriksson 1970), including maize, rice and barley. In wheat, *waxy* polymorphism has been identified and studied only within the last decade and has immediately gained great relevance. Extensive electrophoretic studies on bread wheat have led to the identification of partial *waxy* mutant lines, characterized by the lack of one or two waxy proteins. Crossing of these materials has permitted the combination of different null alleles detected with the production of the entire set of partial waxy lines along with the total waxy production (Nakamura et al. 1995; Urbano et al. 2002); the different effects of the three isoforms on the amylose content was consequently assessed, with the *Wx-B1* allele showing the highest effect, followed by the *Wx-D1* allele and finally by *Wx-A1* (Miura et al. 1994b, 1999). Null *Wx-A1* and *Wx-B1* alleles have been found in Asian, European and North American wheat cultivars. Null alleles at the *Wx-A1* locus were found to be fairly common in bread wheat from Japan, Korea and Turkey, while null alleles at the *Wx-B1* locus are very common in bread wheat from Australia and India (Yamamori et al. 1994, 1998). In the USA, null alleles have been found in one cultivar, Ike, carrying null alleles both at the *Wx-A1* and *Wx-B1* loci (Graybosch et al. 1998). Several null alleles at the *Wx-B1* locus and one at the *Wx-A1* locus were also found in bread wheat lines bred or grown in Italy (Boggini et al. 2001). Null alleles at the *Wx-D1* locus seem to occur more rarely and at present have been identified only in one Chinese cultivar, BaiHuo (Yamamori et al. 1994), and in one Italian landrace (Boggini et al. 2001). Recent studies carried out on durum wheat have revealed a low degree of polymorphism for the waxy proteins. Yamamori et al. (1995) found two alleles at the *Wx-A1* locus and only one at the *Wx-B1* locus, and Nieto-Taladríz et al. (2000) found two alleles for the *Wx-A1* locus and four for *Wx-B1*.

Natural mutations are very useful for the production of waxy wheat lines with a low amylose content such as durum wheat and those used for bread (Nakamura et al. 1995, 2002; Miura et al. 1999). The most common natural variants used to produce waxy wheat lines are the Japanese bread wheat cultivar Kanto 107 (*Wx-A1*/*-B1* null) and the Chinese cultivar BaiHuo (*Wx-D1* null). Their mutations have been characterized at the molecular level by Vrinten et al. (1999), who found a 19 bp deletion in the gene at an exon-intron junction, a deletion which included the entire coding region of the *Wx-B1* gene, and a 588 bp deletion in the C-terminal region of the *Wx-D1* gene. In a subsequent study, a new null *Wx-A1* allele was described by Saito et al. (2004), who demonstrated a 173-bp insertion responsible for gene inactivation in six Turkish cultivars.

Recently, Urbano et al. (2002) have analyzed several cultivars and accessions of bread and durum wheat from worldwide collections and identified new waxy variants, including null forms and waxy proteins showing different electrophoretic mobilities in SDS-PAGE. In this study we report the molecular characterization of some of these mutations (four null lines in bread wheat, two of these at the *Wx-B1* locus and two at *Wx-D1*, and a further four in durum wheat, three of these being polymorphic at the *Wx-B1* locus and one null at *Wx-A1*) and report molecular markers for their identification and progeny selection for the development of new waxy wheat lines.

Materials and methods

Plant material

Seed accessions of four hexaploid wheats, two from Italy (Glutinoso, *Wx-B1* null; MG 20506, *Wx-D1* null), one from Nepal (MG 27124, *Wx-B1* null), and one from Iran (Iran 689, *Wx-D1* null) and four tetraploid wheats, three from Iran (MG 689, MG 787, MG 3072) and one (MG 826, *Wx-A1* null) from Turkey, were obtained from the seed collection of the Institute of Plant Genetics, Bari, Italy. The bread wheat cultivar Chinese Spring, the durum wheat cultivar Langdon and the partial waxy mutant cultivar Kanto 107 (*Wx-A1*/*-B1* null) were used as references for protein and DNA pattern comparisons. All plants were grown to maturity in a greenhouse.

Protein extraction and analysis

The preparation of starch granules from half seeds and the separation of waxy proteins by SDS-PAGE followed the method reported by Zhao and Sharp (1996) with some modifications, as in Mohammadkhani et al. (1999). Protein bands were visualized by silver staining.

DNA extraction

Plant genomic DNAs were extracted from 3 g of young leaves following the method of Dvorak et al. (1988) with modifications described by D'Ovidio et al. (1992).

Primers and PCR conditions

Primers were designed on the basis of the published sequences of *waxy* genes in *Triticum aestivum* (Murai et al. 1999) and *T. durum* (GenBank accessions AB029063 and AB029064). The following primer combinations were used to characterize the homologous *waxy* genes in specific regions: WxBAF (5′-ACTTCCACTGCTACAAGCGCGGGGT-3′) and WxBAR (5′-GCTGACGTCCATGCCGTTGACGATG-3′) for the region spanning the third to the sixth exon; WxF3 (5′-TCTGGTCACGTCCCAGCTCGCCACCT-3′) and WxVT1R (5′-ACCCCGCGCTTGTAGCAGTGGAAGT-3′) for the region spanning the first to the third exon; WxVT1F (5′-CATCGTCAACGGCATGGACGTCAGC-3′) and

WxVTR (5′-CCAGAAGCACGTCCTCCCAGTTC-TTG-3′) for the region spanning the sixth to the eleventh exon; and WxAM7 (5′-CTTGAGGCACCCAG-GATCCT-3′) and WxAM6 (5′-GTTGAGCTGCGC-GAAGTCGT-3′) spanning the fourth to the sixth exon. PCR amplifications were carried out in 50 µl reaction volumes consisting of 50 ng genomic DNA, 1.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM dNTPs, 1× PCR reaction buffer and 2.5 U of Go *Taq* polymerase (Promega, Madison, Wisc., USA). The PCR conditions included an initial denaturation step of 3 min at 94°C followed by 35 cycles as follows: for WxBAF/WxBAR, 45 s at 94°C, 2 min at 62°C then 1 min at 72°C; for WxF3/WxVT1R, 45 s at 94°C, 1 min at 62°C, then 1 min at 72°C; for WxVT1F/WxVTR, 45 s at 94°C, 2 min at 64°C, than 90 s at 72°C, and for WxAM7/WxAM6, 45 s at 94°C, 45 s at 61°C, then 1 min at 72°C. After the 35 amplification cycles, all reactions included a final extension of 5 min at 72°C.

Analysis of PCR products

Aliquots of the PCR products were visualized by electrophoresis on 2% agarose gels. Amplification products resulting from the use of primers WxF3/WxVT1R, WxVT1F/WxVTR and WxAM7/WxAM6 were restricted with the endonucleases *Bam*HI, *Cla*I, *Hind*III and *Pst*I (Invitrogen, UK) following the supplier's instructions.

DNA sequencing and analysis

DNA sequence analyses were performed on PCR fragments amplified using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). Polymorphic bands were excised from the gels and purified with the GFX kit (Amersham Biosciences) and directly sequenced in both directions with the amplification primers. Sequencing was performed using an ABI Prism 373A automated gel reader. The resulting nucleotide sequences were adjusted visually with the software Chromas 1.43 (http://trishul.gu.edu.au/~conor/chromas) and uploaded to DNAMAN-1999 (Lynnon Biosoft, Quebec, Canada) for sequence alignment and translation. Confirmation of the sequence overlaps was obtained by use of inner primer pairs, developed from the sequence data.

Southern analysis

Plant genomic DNAs (10 µg) were digested with *Hind*III, fractionated on a 1% agarose gel and then transferred to nylon membrane (Roche Diagnostics) after HCl depurination. Pre-hybridization (3 h) and hybridization (18 h) reactions were performed at 65°C in a solution containing 5×SSC, 0.02% SDS, 0.1% N-lauroylsarcosine and 0.7% blocking reagent (Roche Diagnostics). A PCR fragment was amplified from the durum wheat cultivar Langdon with the primer pair WxBAF/WxBAR, PCR-labeled with digoxigenin and used as the probe. Post-hybridization washes were performed twice in 2×SSC, 0.1% SDS for 5 min each change at room temperature, and twice in 0.1×SSC and 0.1% SDS for 15 min each change at 65°C. Detection was performed by chemiluminescence.

Results and discussion

Figure 1 shows an SDS-PAGE analysis of the waxy proteins present in the eight mutants identified in bread and durum wheat. The bread wheat cultivars Chinese Spring and Kanto 107 and the durum wheat cultivar Langdon were used as the references. Chinese Spring shows the presence of three waxy proteins: Wx-A1, Wx-D1 and Wx-B1 (in order of increasing mobility); the two bread wheat accessions Glutinoso (lane 1) and MG 27124 (lane 2) show the absence of the Wx-B1 protein (*Wx-B1* null allele), the other two, MG 20506 (lane 3) and Iran 689 (lane 4), lack the Wx-D1 protein (*Wx-D1* null allele). Langdon has two waxy proteins, Wx-A1 and Wx-B1. Three of the durum wheat accessions, MG 689 (lane 7), MG 787 (lane 8) and MG 3072 (lane 9), have a polymorphic Wx-B1 protein with a higher mobility than the control, whereas the accession MG 826 (lane 10) does not produce the Wx-A1 protein (*Wx-A1* null allele).

Primer combination WxBAF/WxBAR was used in a first attempt to characterize these mutations (Fig.2). These primers allow the simultaneous amplification of the central regions of the three *Wx-1* genes. The expected sizes of the three fragments, as deduced from the relative primer positions on the published sequences of wheat *waxy* genes, were 1,017 bp (*Wx-D1*), 953 bp (*Wx-A1*), and 935 bp (*Wx-B1*) in bread wheat, and 969 bp (*Wx-B1*) and 953 bp (*Wx-A1*) in durum wheat. The resulting amplification products displayed fragments of the expected size in all the controls and the null mutants, with the exception of MG 27124 (Fig.2a, lane 3) and Kanto 107 (Fig.2a, lane 6), where the PCR band from the *Wx-B1* gene was absent. Moreover, a fragment of smaller size, associated with the *Wx-B1* locus in the three durum wheat accessions from Iran (Fig.2b, lanes 8–10), and a fragment of larger size than the *Wx-A1* allele (Fig.2b, lane 11) was detected in the null line.

Analysis of the remaining regions of the *waxy* genes was conducted with the primer pairs WxF3/WxVT1R and WxVT1F/WxVTR. In these cases the primers were designed to simultaneously amplify the three homologous *waxy* genes in bread and durum wheat. Since the PCR products were of very similar sizes, the assignation of each band to the corresponding gene region was facilitated by digestion of the PCR products with appropriate restriction enzymes.

Amplification with WxF3/WxVT1R produced the expected fragment of 620 bp (*Wx-A1*) and the co-migrating fragments of 639 and 644 bp (*Wx-D1* and

Wx-B1, respectively) in all mutants and controls, with the exception of Kanto 107 and MG 471053, where the *Wx-B1* products were absent. Digestion with *Bam*HI produced two fragments of 80 and 559 bp originating from the *Wx-D1* PCR product, providing a clearer resolution of the pattern. No size polymorphism could be detected in the mutant lines, with the known exception of the shorter *Wx-A1* fragment of Kanto 107 (data not shown).

Similarly, the expected fragments ranging in size from 1,167 to 1,176 bp were produced with primers WxVT1F/WxVTR and their assignation to the corresponding *waxy* genes was obtained through restriction analysis with *Cla*I and *Hind*III (Fig.3). These enzymes cut the products of the *Wx-A1* and *Wx-B1* genes at 836 and 405 bp, respectively. No size heterogeneity was observed in these gene regions, except in Iran 689 (Fig.3, lane 5), where a shorter fragment (approximately 400 bp) derived from the *Wx-D1* gene was identified. Moreover, the bread wheat accession MG 20506 contained a new *Wx-D1* null allele, different from that described in Bai Huo by Vrinten et al. (1999), since the primer WxVTR was developed within the 588-bp deletion responsible for gene inactivation.

No fragment that could be assigned to the *Wx-B1* gene was produced from line MG 27124 when using any of the above listed primers, nor with other primer sets developed for this purpose (data not shown).

Southern analysis of all genomic DNAs digested with *Hind*III (data not shown) was in agreement with the results of Vrinten et al. (1999), who provided the locus assignation of the hybridizing bands using a cDNA probe (~4.2 and 12 kb to *Wx-A1* and *Wx-B1* respectively in bread and durum wheat, and 18 kb to *Wx-D1*).

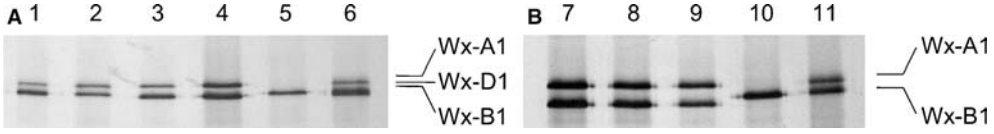

Fig. 1a,b SDS-PAGE of waxy proteins in eight wheat mutant lines, Kanto 107, and wild-type bread and durum wheats. **a** Bread wheats: *lane 1* Glutinoso, *Wx-B1* null; *lane 2* MG 27124, *Wx-B1* null; *lane 3* MG 20506, *Wx-D1* null; *lane 4* Iran 689, *Wx-D1* null; *lane 5* Kanto 107, *Wx-A1*/*-B1* null; *lane 6* Chinese Spring. **b** Durum wheats: *lanes 7*/*9* MG 689, MG 787 and MG 3072, polymorphic at the *Wx-B1* locus; *lane 10* MG 826, *Wx-A1* null; *lane 11* Langdon

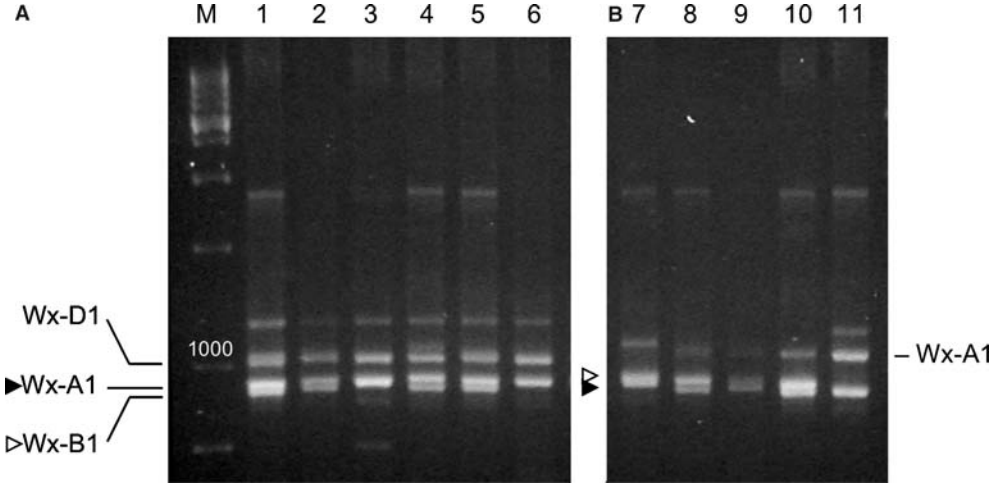

Fig. 2a,b PCR analysis. **a** Bread wheats: *lane 1* Chinese Spring, *lane 2* Glutinoso, *lane 3* MG 27124, *lane 4* MG 20506, *lane 5* Iran 689, *lane 6* Kanto 107. **b** Durum wheats: *lane 7* Langdon, *lane 8* MG 689, *lane 9*, MG 787, *lane 10* MG 3072, *lane 11* MG 826

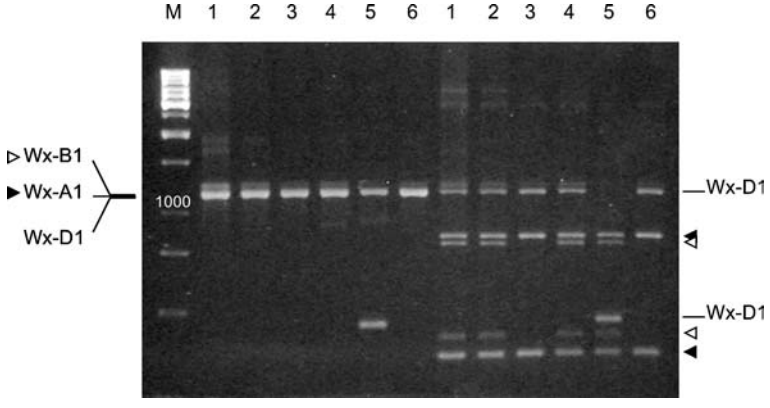

Fig. 3 The PCR (*lanes 1–6*) and PCR-RFLP analysis (*lanes 7–12*) of the bread wheats. *Lanes 1* and *7* Chinese Spring, *lanes 2* and *8* Glutinoso, *lanes 3* and *9* MG 27124, *lanes 4* and *10* MG 20506, *lanes 5* and *11* Iran 689, *lanes 6* and *12* Kanto 107

Southern patterns of the mutant accessions showed the absence of the fragment corresponding to the *Wx-B1* gene in MG 27124, as was also the case in Kanto 107, indicating that the same gene deletion was probably responsible for the absence of the corresponding waxy protein in both genotypes. In contrast, the *Wx-B1* null accession Glutinoso displayed the hybridizing *Wx-B1* band.

The remaining lines investigated showed no polymorphism, even when they were probed with another gene fragment obtained with the same primers as in Vrinten et al. (1999).

Molecular description of the mutations detected by the PCR analyses was obtained by means of DNA sequencing of the polymorphic bands and comparison with the corresponding gene sequences available from the databases. Alignment of the *Wx-D1* fragment amplified from Iran 689 (*Wx-D1* null) with primers WxVT1F/WxVTR and the corresponding gene region in Chinese Spring (Vrinten et al. 1999) showed the occurrence of a 724-bp deletion in this gene region, spanning from the seventh to the tenth exon (Fig.4). Even though we cannot exclude other minor mutations in the flanking regions, this event may be the principal cause for the lack of the corresponding protein, as in the case of the deletion found in *Wx-D1* in the cultivar Bai Huo described by Vrinten et al. (1999). The fragment was 432 bp long and shared 99.54% sequence identity (two nucleotide substitutions, neighboring the deleted region) with both complete *Wx-D1* gene sequences from *T. aestivum* (GenBank accession AB019624) and *T. tauschii* (AF110375).

Sequence analysis of the *Wx-A1* PCR product of durum wheat MG 826 (*Wx-A1* null) with primers WxBAF/WxBAR showed the existence of an insertion of 89 bp in the sixth exon caused by a duplication event that involved the neighboring sequence (Fig.5). The 992 bp fragment displayed one nucleotide substitution (99.9% sequence identity) when compared with the complete *Wx-A1* gene sequence from *T. durum* (AB029063) and two nucleotide changes, located in the fourth exon, with respect to the partial sequence of *Wx-Td2* from the same species (Yan and Bhave 2000). The deduced amino acid analysis revealed that the insertion caused a frameshift with the insertion of a stop codon and thus the gene inactivation observed on SDS-PAGE analysis.

Finally, the nucleotide sequence (886 bp) of the *Wx-B1* PCR band obtained with primers WxBAF/WxBAR in the polymorphic durum accession MG 689 displayed a discrete variability when compared to the complete sequence of the corresponding gene in *T. durum* (AB029064). In addition to nucleotide substitutions, various insertions and deletions ranging from 3 to 30 bp were found (Fig.6). Noteworthy, all the insertions/deletions are located within the intron regions but without affecting their junctions with the exons. The variation detected in intron IV is identical to that described by Yan and Bhave (2000) for the sequences *Wx-Td1* and *Wx1*, corresponding to *Wx-B1* partial sequences (373 bp) from *T. durum* and *T. aestivum*, respectively. In addition, we found another long deletion of 30 bp in the fifth intron. In order to analyze the effect on protein synthesis, the fragment sequence, excluding the intron

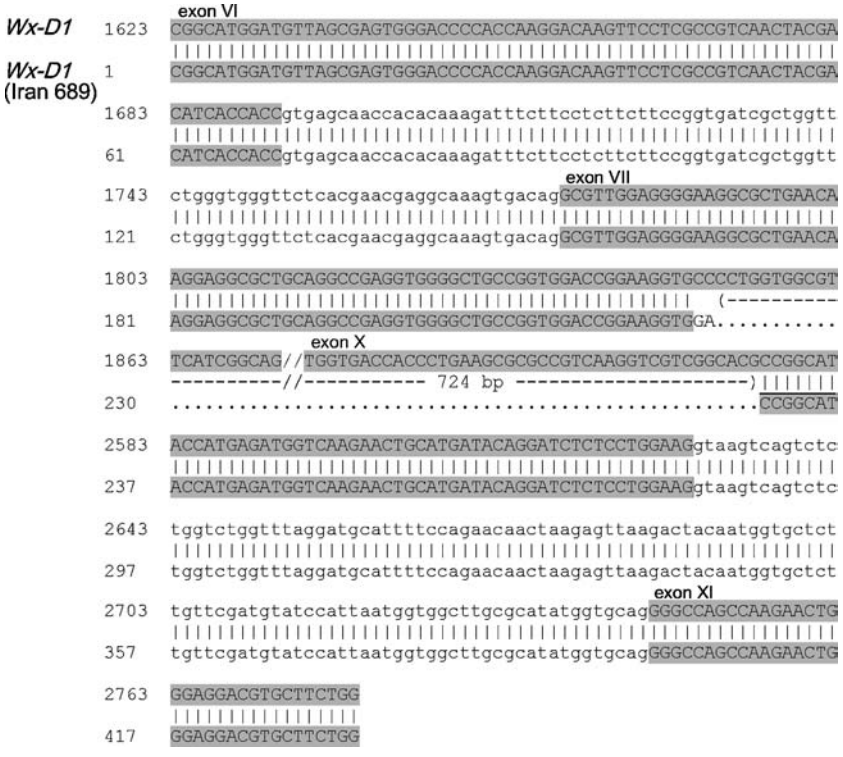

Fig. 4 Comparison of genomic DNA sequence of the *Wx-D1* gene region within the fragment amplified from Iran 689 using primers WxVT1F/WxVTR and the corresponding region in Chinese Spring. A 724-bp deletion is present in the mutated wheat line, spanning from exons 7 to 10. Exon and intron regions are indicated in *grey-shaded*, *capital letters* and *lower case letters*, respectively

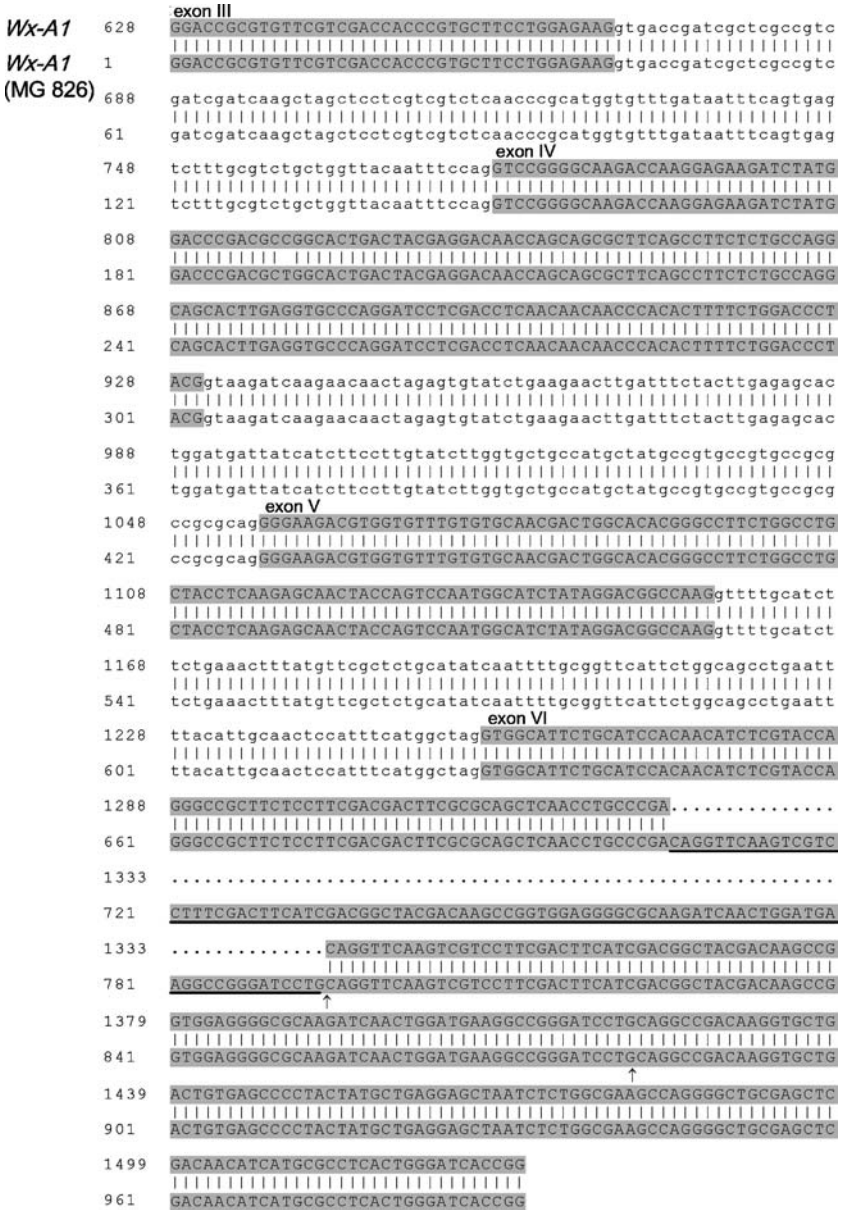

Fig. 5 Comparison of the genomic DNA sequence of the *Wx-A1* gene region within the fragment amplified from MG 826 using primers WxBAF/WxBAR and the corresponding region in a wild-type durum wheat (GenBank accession AB029063). An 89 bp insertion (caused by a sequence duplication) is present in exon 6 of the mutated wheat line. Exon and intron regions are indicated in *grey-shaded*, *capital letters* and *lower case letters*, respectively; the insertion is indicated with *arrows* and the duplicated sequence is *underlined*

regions, was joined to the *Wx-B1* coding sequence obtained from Genbank. The deduced amino acid sequence revealed that most of nucleotide variations do not result in amino acid substitutions with the exception of an Asn → Ser substition at position 175 and a Met → Arg at position 179 of the mature protein. The substitution at position 179 is shared with *T. monococcum* (Yan et al. 2000), *T. urartu*, *T. longissima* and *T. bicornis* (Yan and Bhave 2000). Interestingly, sequence *Wx-Td1* (Yan and Bhave 2000) shared both the amino acid substitutions detected in the present study, and differed by only one nucleotide in exon IV. However, these authors did not provide a description of the *T. durum* line they studied, as being either mutant or wild type.

These two amino acid substitutions may explain the different mobility seen on SDS-PAGE, even considering that other substitutions that may have accumulated in the remaining parts of the gene cannot be excluded. Yanagisawa et al. (2001) showed that a single amino acid change (Ala → Thr) at position 258 in the mature WX-D1 protein of Tanikei A6599-4 is responsible for amylose reduction, even though no difference in mobility on SDS-PAGE was seen.

These findings for MG 689 may be extended to MG 787 and MG 3072, which all share the same geographical origin (Turkey) and show identical patterns on SDS-PAGE and PCR analysis. All *Wx-B1* polymorphic durum wheat accessions were analyzed with the primers WxAM7/WxAM6, developed after sequencing, and further digested with the restriction enzyme *Pst*I that cuts only within the PCR product of the polymorphic region. They all displayed fragments with the same mobility (data not shown).

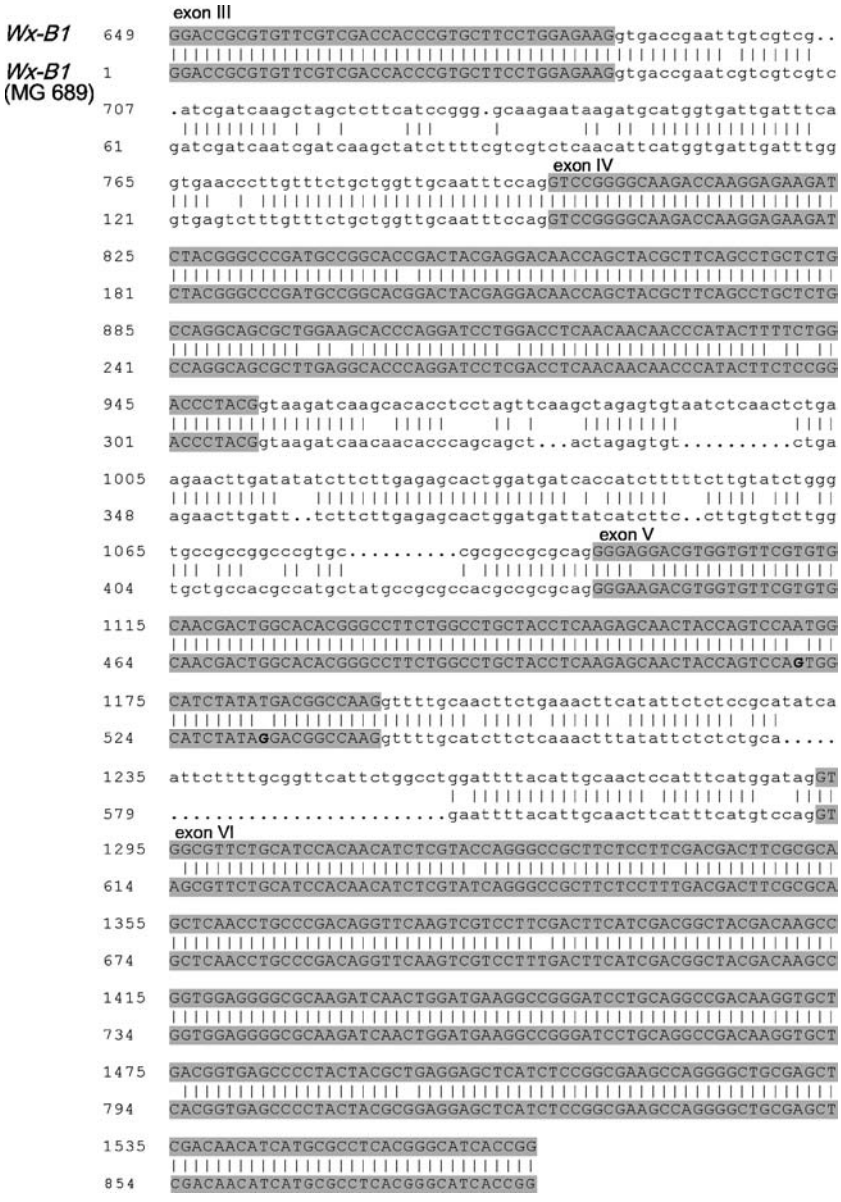

Fig. 6 Comparison of the genomic DNA sequence of the *Wx-B1* gene region amplified with primers WxBAF/WxBAR from accession MG 689 and the corresponding region in a wild-type durum wheat (GenBank accession AB029064). Non-synonymous substitutions in the exon regions are in *bold*. Exon and intron regions are indicated in *grey-shaded*, *capital letters* and *lower case letters*, respectively

In maize, many spontaneous *waxy* mutations are caused by transposable elements (Fedoroff et al. 1983; Wessler and Varagona 1985; Wessler et al. 1987), whereas a single nucleotide substitution leads to aberrant splicing of *waxy* mRNA in rice (Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). In barley, a deletion in the promoter region and in the first intron, as well as a single nucleotide polymorphism have been reported in amylose-free cultivars (Patron et al. 2002; Domon et al. 2002). In wheat, a 23-bp deletion at an exon-intron junction of the *Wx-A1* gene, or a 173-bp insertion in the same gene, a deletion which included the entire coding region of *Wx-B1* gene, and a 588-bp deletion in the C-terminal region of the *Wx-D1* gene have been described (Vrinten et al. 1999; Saito et al. 2004).

In this paper we present four primer pairs that allowed us to identify and describe at the molecular level three new *waxy* alleles in bread wheat and two in durum wheat. Very probably, the bread wheat accession MG 471053 contains the same mutation as Kanto 107 at the *Wx-B1* locus. The bread wheat line Iran 689 revealed a new deletion in the *Wx-A1* gene as the possible cause for its inactivation. Both the *Wx-B1* and *Wx-D1* null alleles in genotypes Glutinoso and MG 20506 are different from those described in previous works and we assume the causes for the inactivation of their *Wx-B1* and *Wx-D1* genes are probably related to minor mutations that will necessitate inspection of the full gene at the nucleotide level. Three durum wheat lines displayed sequence polymorphisms in the exons at the *Wx-B1* locus, whereas the fourth durum line showed an insertion in the *Wx-A1* gene.

The technological properties of novel starches have revealed many possible uses for wheat breeders and food

processors. In fact, flour with a reduced amylose content produces higher quality Asian noodles (Miura et al. 1994b) and extends the shelf life of various baked products (Lee et al. 2001; Graybosch 1998). Moreover, waxy wheat starch could be used as a substitute for waxy maize in the same type of food and non-food industrial applications (Reddy and Seib 2000). The potential use for wheat quality improvement has stimulated worldwide germplasm screening and the molecular characterization of the mutations identified, necessary for a better understanding and management of the existing variability. With reference to this, the mutations we report here enlarge the range of known *waxy* mutants and present new alternatives for bread and durum wheat breeding; the three polymorphic durum lines especially may constitute interesting candidates as soon as their amylose synthesis capacity is tested.

Molecular analysis has revealed that different mechanisms have operated on the *waxy* genes during evolution: deletion of the complete gene, sequence insertions, deletions, duplications, amino acid substitutions, and it is reasonable to expect the identification of new variations as more germplasm is screened. At the same time, correlation with the biochemical data and the technological properties of the mutants are needed. All primer pairs developed will be useful as molecular markers in future breeding programs involving the recently identified material and the traditionally used cultivar Kanto 107.

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