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Molecular characterization of *waxy* mutations in wheat

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Abstract To date, few mutations in wheat have been characterized at the molecular level. In this study, the mutations in the three *waxy* alleles in waxy wheat (*Wx-A1b, Wx-B1b* and *Wx-D1b*) were characterized, and *waxy* gene expression was compared in several wheat lines, including hexaploid and tetraploid waxy lines of wheat. Southern analysis showed that the *Wx-B1b* allele had sustained a deletion which included the entire coding region of the *Wx-B1* gene. DNA homologous to *waxy* gene sequences was still present in the *Wx-A1b* and *Wx-D1b* alleles of the hexaploid *waxy* mutant. Transcripts of *waxy* alleles were also detected in both hexaploid and tetraploid mutants at 10 days post-anthesis, but the transcript level was dramatically reduced compared to that found in non-waxy lines. Isolation of cDNAs showed that transcripts were produced by both the *Wx-A1b* and *Wx-D1b* alleles. A 23-bp deletion sustained by the *Wx-A1b* allele at an exon-intron junction results in the use of a cryptic splice site during mRNA processing. The deduced translation product encoded by the *Wx-A1b* cDNA lacks 39 amino acids, including the putative ADP-glucose binding site and a portion of the transit peptide. The C-terminal region of the deduced protein encoded by the *Wx-D1b* cDNA lacks the last 30 amino acids. Comparison of the genomic sequences of the null and wild-type *Wx-D1* alleles indicated that 588 bp were deleted in the *Wx-D1b* mutation, and that the last 261 bp of the *Wx-D1b* cDNA originated from the normally non-transcribed 3′ flanking region. Like several deletion mutations characterized in other plant species, both *Wx-A1b* and *Wx-D1b* alleles contain small DNA insertions, or filler DNA, between the deletion end-points.

Key words Granule-bound starch synthase · Starch · Waxy · Wheat · Mutation

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Introduction

Spontaneous or induced mutations in plants are useful for investigating the genetic defects that can affect gene expression. In hexaploid wheat, few such mutations are available, since a mutation at one homoeolocus is usually masked by the presence of a wild-type gene product derived from one or both of the remaining loci. However, hexaploid and tetraploid waxy wheat lines which lack amylose in endosperm starch have recently been produced (Nakamura et al. 1995). The *waxy* gene product, waxy protein or granule-bound starch synthase (GBSSI), is responsible for amylose synthesis in storage tissue (Nelson and Rines 1962). Partial *waxy* mutants, lacking one or two of the three waxy proteins, were identified by modified two-dimensional gel electrophoresis (Nakamura et al. 1993a,b; Yamamori et al. 1994). Hexaploid waxy wheat was obtained by crossing Bai Huo, which lacks Wx-D1 protein, and Kanto 107, which lacks Wx-A1 and Wx-B1 proteins, while tetraploid waxy wheat was produced by crossing Kanto 107 with a tetraploid wheat line (Nakamura et al. 1995).

Hexaploid waxy wheat thus carries spontaneous mutations at each of the three homeologous *waxy* loci. Mutations affecting GBSSI have been studied in a number of plant species (Wessler and Varagona 1985; Okagaki and Wessler 1988; Denyer et al. 1995) and several of these mutations have been characterized at the molecular level (Visser et al. 1989; Wessler et al. 1990; Okagaki et al. 1991; Wang et al. 1995). The level at which expression is altered in such mutants varies, and although GBSSI is often completely absent (Sano

1984; Nakamura et al. 1995; Wang et al. 1995), a reduced level of GBSSI (Denyer et al. 1995) or inactive GBSSI (Echt and Schwartz 1981) may be found. In maize *waxy* mutants, transcripts may be of the same size as wild-type transcripts, or be altered due to insertions or incorrect processing, and transcript levels may vary from undetectable to equivalent to wild type (Shure et al. 1983; Wessler et al. 1987; Marillonnet and Wessler 1997). In many rice cultivars, GBSSI and amylose contents are correlated with the post-transcriptional regulation of the *waxy* gene, and vary with the efficiency of excision of the first intron from the pre-mRNA (Wang et al. 1995).

The molecular characterization of the three mutant *waxy* alleles in waxy hexaploid wheat, and the effects of these mutations on *waxy* gene expression, are presented here.

Materials and methods

Plant material

Tetraploid waxy wheat was obtained by crossing the tetraploid wheat Aldura with the partial *waxy* mutant Kanto 107 (K107), and hexaploid waxy wheat was produced by crossing the partial waxy mutants K107 and Bai Huo (Nakamura et al. 1995). The waxy wheat types 1 to 8 (see Table 1) represent all possible homozygous combinations of the *waxy* alleles of K107 and Bai Huo (Yamamori et al. 1994). Several of these wheat types, as well as the non-waxy hexaploid wheat cvs. Chinese Spring and Nanbu, were used in these studies. Table 1 indicates the presence or absence of waxy proteins encoded by each genome in the plants used in this study. The designation of alleles follows that in Yamamori et al. (1994). The wild-type alleles of the A, B and D genomes are designated *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, respectively, whereas null alleles, which produce no detectable GBSSI, are designated *Wx-A1b*, *Wx-B1b* and *Wx-D1b*.

DNA extraction and Southern analysis

Plant DNA was extracted from young leaves using the method of Dellaporta et al. (1983). The purified DNA was digested with *Hin*dIII, fractionated on a 0.8% agarose gel and transferred to GeneScreen Plus membrane (NEN Research Products, Boston, Mass.) by capillary transfer. A 434-bp PCR fragment amplified from a conserved area of the coding region of the barley pcWx27 clone (Rohde et al. 1988) using the primers 5′-GCTACCTCAAGAGCAACT-3′ and 5′-CTCCTTGTTCAGCGCCTT-3′ was employed as a probe. Hybridization was performed in 2 × SSC containing 50% formamide, 1% SDS, 10% dextran sulfate, 0.5 × Denhardt's solution, 250 μg/ml denatured salmon sperm DNA, and ^{32}P-labeled probe. After hybridization, the blots were washed twice for 30 min each in 1 × SSC, 1% SDS at 65° C, then subjected to autoradiography.

Table 1 Presence (+) or absence (−) of *waxy* gene products in wheat lines

Cultivar	Type[a]	Waxy proteins		
		Wx-A1	Wx-B1	Wx-D1
Aldura	–	+	+	No D genome
Tetraploid Waxy	–	−	−	No D genome
Chinese Spring, Nanbu	1	+	+	+
Bai Huo	4	+	+	−
	5	+	−	−
	6	−	+	−
Kanto 107	7	−	−	+
Hexaploid Waxy	8	−	−	−

[a] See text for details

RNA extraction and Northern analysis

Total RNA was extracted from developing seeds by the method of Ainsworth et al. (1993). Poly(A)$^+$ RNA was isolated using the Poly(A) Quik mRNA Isolation kit (Stratagene, La Jolla, Calif.). RNA was fractionated on formaldehyde-containing 1% agarose gels and transferred to GeneScreenPlus membrane (NEN) by capillary transfer. The entire insert of the barley *waxy* cDNA clone pcWx27 (Rohde et al. 1988) was used as a probe. Hybridization was performed in buffer containing 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll, 50 mM TRIS-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA and ^{32}P-labeled probe. After hybridization, blots were subjected to two 30-min washes in 2 × SSC, 1% SDS at 65° C, followed by a 10-min wash in 0.1 × SSC at 65° C, then autoradiographed.

cDNA library construction and screening

Poly (A)$^+$ RNA prepared from seed of hexaploid waxy wheat 10 days post-anthesis (DPA) was used to construct a cDNA library using a λZap cDNA synthesis kit (Stratagene). To screen the library, plaques were lifted onto NitroME membranes (MSI, Westboro, Mass.) and hybridized in a solution containing 50% formamide, 5 × Denhardt's solution, 5 × SPPE, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA and ^{32}P-labeled probe. The insert of the barley *waxy* cDNA clone pcWx27 (Rohde et al. 1988) was used as a probe. Sequences of positive clones were determined with an ABI Prism Genetic Analyzer (Perkin Elmer, Foster City, Calif.).

PCR amplification and cloning of amplification products

To obtain the 5′ ends of the cDNAs, adapter-ligated cDNAs were constructed using a Marathon kit (Clontech, Palo Alto, Calif.). The coding region of the cDNA for the *Wx-A1b* allele was amplified using a specific primer based on the 3′ non-translated region of this cDNA (5′-GCCCTTCTTCACTACAACAAGCGGC-3′) and the adapter primer AP1 from the Marathon kit. The coding region of the cDNA for the *Wx-D1b* allele was amplified using a specific primer deduced from the 3′ non-translated region of the cDNA for this allele (5′-CCTTGCGTCATTTGTTGTGTGG-3′) and a primer (5′-TTGCTGCAGGTAGCCACACCCTG-3′) designed using sequences from the 5′ non-translated regions of the barley (Rohde et al. 1988) and wheat (Clark et al. 1991) *waxy* clones.

To obtain genomic sequence information, genomic DNA was isolated from Chinese Spring and hexaploid waxy wheat as described above. Primers flanking the region of the mutations in the *Wx-A1* gene (5′-TTGCTGCAGGTAGCCACACCCTG-3′ and 5′-AGTTGCTCTTGAGGTAGC-3′) and the *Wx-D1* gene (5′-TAGTGCGTCCAGACTCACAG-3′ and 5′-GAGATGGTCAAGAACTGCAT-3′) were used to amplify the corresponding genomic regions from the wild-type (Chinese Spring) and null (waxy wheat) alleles.

The cDNA and genomic amplification products were cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, Calif.), and sequences were determined as described above. Since the primers used for genomic amplification of the *Wx-A1* gene were capable of amplifying all three genes, the correct clones were identified based on homology to the cDNA sequences.

Results

Detection of *waxy* genes

In preliminary PCR and Southern hybridization experiments, no polymorphisms were detected between the alleles *Wx-A1b* and *Wx-B1b*. Therefore, a Southern analysis including DNA from lines carrying only one of these two null alleles was performed (Fig. 1). Lines with the GBSSI genotypes of the hexaploid waxy wheat progenitors K107 (type 7) and Bai Huo (type 4), as well as tetraploid waxy wheat, were included in the analysis.

Digestion of DNA from waxy hexaploid wheat with *Hin*dIII produced two fragments (18 kb and 15 kb) which hybridized to the PCR-generated *waxy* probe (Fig. 1). The 15-kb band is the same size as the single band found in tetraploid waxy wheat and was therefore generated from the *Wx-A1b* and/or *Wx-B1b* alleles. Thus the 18-kb fragment must have originated from the *Wx-D1b* allele; this is corroborated by the presence of a fragment of identical size in the type 4 line. Type 4 has the same GBSSI genotype as Bai Huo (*Wx-A1a*/*Wx-B1a*/*Wx-D1b*), the donor of the *Wx-D1b* allele in waxy wheat. Type 4 also carried 12-kb and 4.2-kb bands. The 12-kb band was absent in lines bearing the *Wx-B1b* allele and present in lines carrying the *Wx-B1a* allele, and thus was generated from *Wx-B1a*, whereas the 4.2-kb band originated from the *Wx-A1a* allele. Fragments generated from the wild-type and null alleles of the D genome were very similar in size, as the *Wx-D1a* allele present in type 7 also generated a band of approximately 18 kb. To determine if the *Wx-A1b* and *Wx-B1b* alleles of hexaploid waxy wheat generate identically sized fragments, or if one gene is missing, hybridization patterns of type 6 (*Wx-A1b*/*Wx-B1a*/*Wx-D1b*) and type 5 (*Wx-A1a*/*Wx-B1b*/*Wx-D1b*) wheat lines were compared. While the 15-kb band is present in type 6 DNA this band was absent from type 5 DNA, and no additional band was present, indicating the *Wx-B1b* allele has sustained a deletion of the *waxy* gene. The deletion appears to include the entire transcription unit, since an additional Southern analysis using the whole insert from the barley *waxy* cDNA as a probe did not detect homologous DNA from the B genome of waxy wheat (data not shown).

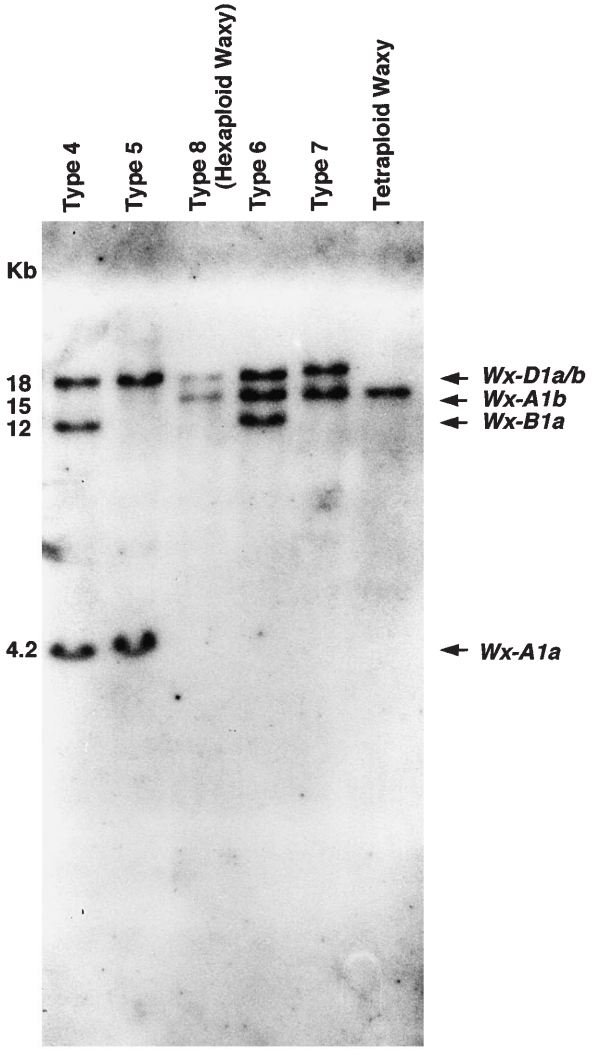

Fig. 1 Southern analysis of *waxy* genes in several wheat genotypes. Genomic DNA was digested with *Hin*dIII and 15 μg of DNA was loaded in each lane. A 434-bp PCR product amplified from the barley *waxy* clone pcWx27 (Rohde et al. 1988) was used as a probe. The approximate sizes of the fragments in kb are shown to the right of the blot, and the *waxy* alleles from which the fragments were generated from are indicated on the left

Accumulation of transcript in waxy and non-waxy lines

RNA blots were used to compare the levels of *waxy* transcript in tetra- and hexaploid waxy wheat, the progenitors of these lines, and wild-type wheat lines (Fig. 2). A very faint hybridizing band was detected in RNA extracted from 10-DPA seed of both hexaploid and tetraploid waxy wheat lines (Fig. 2). This band was somewhat more intense in hexaploid (lane 4) than in tetraploid (lane 2) waxy wheat. Transcript levels in Kanto 107, which carries the null *Wx-A1b* and *Wx-B1b* alleles but a wild-type *Wx-D1a* allele, were lower than those found in Chinese Spring, while transcript levels in Bai Huo, which carries the null *Wx-D1b* allele, appeared comparable with those in Chinese Spring.

To determine whether the transcripts present in waxy wheat are mature, polyadenylated transcripts, poly(A)$^+$ RNA was purified from waxy hexaploid wheat and examined by Northern analysis (Fig. 3). At least a portion of the transcripts present in hexaploid waxy wheat are polyadenylated, and transcripts appear to have a slightly lower molecular weight than those found in non-waxy wheat.

Sequences of cDNAs from waxy wheat

Attempts to isolate full-length cDNA clones from a λZAPII library were unsuccessful. However, partial

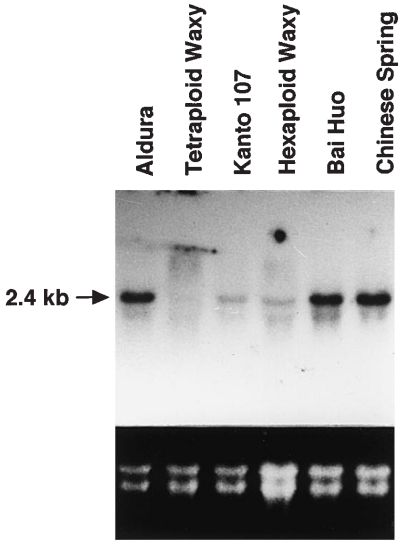

Fig. 2 Northern analysis of total RNA isolated from seeds harvested at 10 DPA. Each lane contains approximately 10 μg of total RNA. The insert from the barley *waxy* cDNA pcWx27 (Rohde et al. 1988) was used as a probe. The size of the *GBSSI* transcript is approximately 2.4 kb. The ethidium bromide-stained gel is shown below the autoradiograph

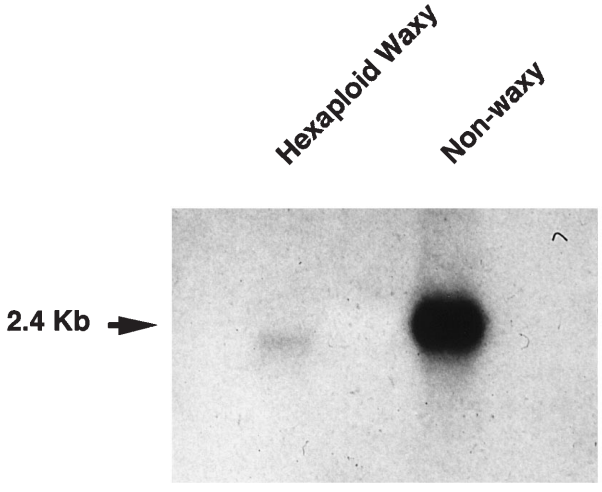

Fig. 3 Northern analysis of poly(A)$^+$ RNA from wild-type and hexaploid waxy wheats. RNA was isolated from seeds harvested at 10 DPA. Approximately 5 μg of poly(A)$^+$ RNA was loaded in each lane. The insert from the barley *waxy* cDNA pcWx27 (Rohde et al. 1988) was used as a probe. The transcript size is approximately 2.4 kb

clones of two types of *waxy* cDNAs were isolated; the longest clones for each type of cDNA being about 1500 bp in length. The use of primers which were specific to each of the two cDNAs allowed the isolation of clones that included the entire coding region. The sequences of both cDNAs were compared to that of the wheat *waxy* clone pcSS22 (Clark et al. 1991; Ainsworth et al. 1993) (Fig. 4). By comparing the amino acid sequences deduced from the cDNAs with partial amino acid sequences of the three waxy proteins (Fujita et al. 1996), it was determined that the cDNAs from waxy wheat originated from the *Wx-A1* and *Wx-D1* genes, and that the Chinese Spring *waxy* cDNA clone isolated by Clark et al. (1991) originated from the *Wx-A1* gene.

The sequence derived for the transcript encoded by the *Wx-A1b* allele was missing 117 nucleotides found in pcSS22, and coded for a translation product that lacks 39 amino acids, including the cleavage site of the transit peptide and the putative substrate (ADP-Glc) binding site (KTGGL) (Fig. 4). The sequence deduced for the transcript encoded by the *Wx-D1b* allele had a completely different 3′ region from that found in the clone reported by Ainsworth et al (1993). This change would result in production of a prematurely terminated translation product which lacks 30 amino acids at the C-terminal end, and has five amino acid substitutions prior to the stop codon (Fig. 4). Both clones also lacked an insertion found in pcSS22 (Ainsworth et al. 1993) that codes for 11 amino acids. This insertion is not found in any other cereal *waxy* cDNAs, and Ainsworth et al. (1993) suggested it was specific to wheat. However, we also failed to find this region in a *Wx-A1a* cDNA isolated from Chinese Spring wheat (data not shown).

Several single or double amino acid substitutions were also found in the deduced translation products of both *Wx-A1b* and *Wx-D1b* (Fig. 4). The substitutions coded for by the *Wx-D1b* cDNA were identical to those found in the deduced translation product of a *Wx-DIa* cDNA from Chinese Spring (data not shown), and thus appear to be specific to the D genome. However, none of the three substitutions encoded by the *Wx-A1b* cDNA were found in a *Wx-A1* clone isolated from Chinese Spring (Ainsworth et al. 1993). The genomic sequence of the *Wx-A1b* allele was identical to the cDNA clone in the regions around these substitutions, indicating the amino acid substitutions are likely to be specific to this genotype, and do not result from errors in sequencing or PCR.

Comparison of genomic DNA and cDNA sequences in the region of the mutation in the *Wx-A1b* allele

Although the *Wx-A1b* cDNA sequence was missing 117 nucleotides, the genomic clone contained most of this sequence (Fig. 5). Only 23 bp of wild-type genomic sequence was deleted, and an insertion of 4 bp was present. The deletion included an exon-intron junction, such that the 5′ end of an intron was missing. A cryptic splice site 117 bp upstream of the normal splice site was used in the cDNA. This site is not the first GT position available upstream of the normal 5′ intron splice site. However, several nucleotides upstream of this cryptic splice site are identical to those found at the normal splice site.

The DNA insertion, or filler DNA, present between the deletion endpoints in this allele is only 4 bp in length, so it was not possible to determine its origin unequivocally. However, it may have originated from a sequence

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TAWAXYSS    MAALVTSQLATSGTVLSVTDRFRRPGFQGLRPRNPADAALGMRTVGASAAPKQSRKPHRFDRRCLSMVVRATGSGGMNLVFVGAE
Wx-A1b      MAALVTSQLATSGTVLSVTDRFRRPGFQGLRPRNPADAALGIRTVGASAAPKQSRKPHRGNRRCLSMV==================
Wx-D1b      MAALVTSQLATSGTVLGITDRFRRAGFQGVRPRSPADAALGMRTVGASAAPTQSRKAHRGTRRCLSMVVRATGSGGMNLVFVGAE

TAWAXYSS    MAPWSKTGGLGDVLGGLPAAMAANGHRVMVISPRYDQYKDAWDTSVISEIKVVDRYERVRYFHCYKRGVDRVFVDHPCFLEKVRG
Wx-A1b      ====================ANGHRVMVISPRYDQYKDAWDTSVISEIKVVDRYERVRYFHCYKRGVDRVFVDHPCFLEKVRG
Wx-D1b      MAPWSKTGGLGDVLGGLPPAMAANGHRVMVISPRYDQYKDAWDTSVVSEIKVVDKYERVRYFHCYKRGVDRVFVDHPCFLEKVRG

TAWAXYSS    KTKEKIYGPDAGTDYEDNQQRFSLLCQAALEVPRILDLNNNPHFSGPYAMLCRAVPRRAGEDVVFVCNDWHTGLLACYLKSNYQS
Wx-A1b      KTKEKIYGPDAGTDYEDNQQRFSLLCQAALEVPRILDLNNNPHFSGPY----------GEDVVFVCNDWHTGLLACYLKSNYQS
Wx-D1b      KTKEKIYGPDAGTDYEDNQQRFSLLCQAALEVPRILNLDNNPYFSGPY----------GEDVVFVCNDWHTGLLACYLKSNYQS

TAWAXYSS    NGIYRTAKVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFDFIDGYDKPVEGRKINWMKAGILQADKVLTVSPYYAEELISGEAR
Wx-A1b      NGIYRTAKVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFDFIDGYDKPVEGRKINWMKAGILQADKVLTVSPYYAEELISGEAR
Wx-D1b      NGIYRAAKVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFDFIDGYDKPVEGRKINWMKAGILQADKVLTVSPYYAEELISGEAR

TAWAXYSS    GCELDNIMRLTGITGIVNGMDVSEWDPIKDKFLTVNYDVTTALEGKALNKEALQAEVGLPVDRKVPLVAFIGRLEEQKGPDVMIA
Wx-A1b      GCELDNIMRLTGITGIVNGMDVSEWDPIKDKFLTVNYDVTTALEGKALNKEALQAEVGLPVDRKVPLVAFIGRLEEQKGPDVMIA
Wx-D1b      GCELDNIMRLTGITGIVNGMDVSEWDPTKDKFLAVNYDITTALEGKALNKEALQAEVGLPVDRKVPLVAFIGRLEEQKGPDVMIA

TAWAXYSS    AIPEIVKEEDVQIVLLGTGKKKFERLLKSVEEKFPTKVRAVVRFNAPLAHQMMAGADVLAVTSRFEPCGLIQLQGMRYGTPCACA
Wx-A1b      AIPEIVKEEDVQIVLLGTGKKKFERLLKSVEEKFPTKVRAVVRFNAPLAHQMMAGADVLAVTSRFEPCGLIQLQGMRYGTPCACA
Wx-D1b      AIPEILKEEDVQIVLLGTGKKKFERLLKSIEEKFPSKVRAVVRFNAPLAHQMMAGADVLAVTSRFEPCGLIQLQGMRYGTPCACA

TAWAXYSS    STGGLVDTIVEGKTGFHMGRLSVDCNVVEPADVKKVVTTLKRAVKVVGTPAYHEMVKNCMIQDLSWKGPAKNWEDVLLELGVEGS
Wx-A1b      STGGLVDTIVEGKTGFHMGRLSVDCNVVEPADVKKVVTTLKRAVKVVGTPAYHEMVKNCMIQDLSWKGPAKNWEDVLLELGVEGS
Wx-D1b      STGGLVDTIVEGKTGFHMGRLSVDCNVVEPADVKKVVTTLKRAVKVVGTPAYHEMVKNCMIQDLSWKGPAWKVSR*==========

TAWAXYSS    EPGIVGEEIAPLALENVAAP*
Wx-A1b      EPGIVGEEIAPLALENVAAP*
Wx-D1b      ====================
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Fig. 4 Deduced amino acid sequences encoded by the *Wx-A1b* and *Wx-D1b* cDNAs from waxy wheat. The *Wx-A1b* and *Wx-D1b* sequences were aligned with the amino acid sequence deduced from the waxy cDNA clone pcSS22 from Chinese Spring wheat (TAWAXYSS; Clark et al. 1991). Sequence alterations with respect to TAWAXYSS are *outlined*; gaps (-) were introduced to maximize the alignment. Gaps in the area of the deletions in Wx-A1b and Wx-D1b are shown by *outlined dashes*, and the positions at which stop codons are encountered in the cDNAs are indicated by *asterisks*. The sequences of the *Wx-A1b* and *Wx-D1b* cDNAs have been deposited in the EMBL database under Accession Nos. AF113843 and AF113844, respectively

located 120 bp downstream, which also contains the 4 nucleotides (GCCT) found at the 3′ deletion endpoint.

Comparison of genomic DNA and cDNA sequences in the region of the mutation in the *Wx-D1b* allele

The *Wx-D1b* allele carries a deletion of 588 bp, and an insertion of 12 bp (filler DNA) (Fig. 6). The normal polyadenylation site found in the *Wx-D1a* allele occurs 369 bp into the deletion, and the deletion extends an additional 219 bp beyond this site. The mutated *Wx-D1* gene is transcribed for 261 bp into the normally non-transcribed region before the addition of a poly(A) tail. Since the *Wx-D1b* transcript is missing 369 bp that are normally transcribed, the overall length of this transcript is 96 bp shorter than the *Wx-D1a* transcript. However, a stop codon occurs closely following the filler DNA.

The 12 bp of filler DNA found in this allele originate from the previous intron-exon junction, which also contains 2 bp of the 5′ deletion endpoint, and 1 bp of the 3′ deletion endpoint.

Discussion

Extensive investigations of mutations at the *waxy* locus have been carried out in maize (Wessler and Varagona 1985; Wessler et al. 1990; White et al. 1994; Marillonnet and Wessler 1997) and rice (Wang et al. 1995; Cai et al. 1998; Isshiki et al. 1998). The presence of three spontaneous mutations in hexaploid waxy wheat allowed us to investigate the level at which these mutations affect gene expression in wheat, and to compare the types of mutations present with those detected in other cereals.

The wheat *Wx-B1b* allele has sustained a deletion that appears to remove the entire GBSSI transcription unit. In maize, one of seven deletion mutants characterized by Wessler and Vargona (1985) involved the deletion of the entire *waxy* transcription unit. However, the maize mutation was induced by gamma irradiation. Spontaneous mutations involving large deletions are likely to be maintained more easily in self-pollinated, hexaploid species such as wheat. Such major mutations may therefore be more common in wheat. The *Wx-B1* gene has been mapped to the distal end of chromosome arm 4AL in Chinese Spring wheat (Yamamori et al. 1994). The *Wx-D1* and *Wx-A1* genes are located on group 7 chromosomes, and the non-homoeologous location of the *Wx-B1* gene is due to a translocation between chromosomes 4A and 7B (Naranjo et al. 1987;

A

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Wx-A1b cDNA       CGGCGGTGCCTCTCCATGGTG
Wx-A1b genomic    CGGCGGTGCCTCTCCATGGTGGTGCGCGCCACGGGCTGCGGCGGCATGAACCTCGTGTTCGTCGGCGCCGAGATGGCGCCCTGGAGC
Wx-A1a genomic    CGGCGGTGCCTCTCCATGGTGGTGCGCGCCACGGGCAGCGGCGGCATGAACCTCGTGTTCGTCGGCGCCGAGATGGCGCCCTGGAGC

Wx-A1b cDNA
Wx-A1b genomic    AAGACTGGCGGCCTCGGCGACGTCCTCGGGGGCCTCCCCGCCGC GGAC                    gccttcttataaatgtttc
Wx-A1a genomic    AAGACTGGCGGCCTCGGCGACGTCCTCGGGGGCCTCCCCGCCGC CATGGCCgtaagcttgcgccact gccttcttataaatgtttc

Wx-A1b cDNA                                                          GCCAACGGTCACCGGGTCATGGTCATCTCCCCGCGCTACG
Wx-A1b Genomic    ttcctgcagccatgcctgccgttacaacgggtgccgtgtccgtgcagGCCAACGGTCACCGGGTCATGGTCATCTCCCCGCGCTACG
Wx-A1a Genomic    ttcctgcagccatgcctgccgttacaacgggtgccgtgtccgtgcagGCCAACGGTCACCGGGTCATGGTCATCTCCCCGCGCTACG

Wx-A1b cDNA       ACCAGTACAAGGACGCCTGGGACA
Wx-A1b Genomic    ACCAGTACAAGGACGCCTGGGACA
Wx-A1a Genomic    ACCAGTACAAGGACGCCTGGGACA
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B

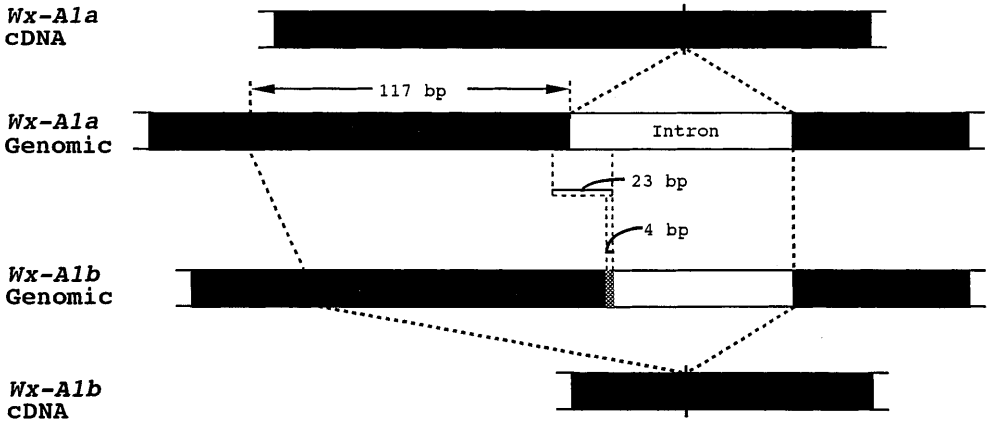

Fig. 5A,B The deletion in the *Wx-A1b* allele of waxy wheat. **A** Genomic and cDNA sequences in the region of the mutation in the *Wx-A1* gene. PCR amplification was used to obtain partial genomic clones from waxy wheat (*Wx-A1b*) and Chinese Spring wheat (*Wx-A1a*). The intron of the *Wx-A1* gene is shown in *lower case letters* and the nucleotides within the deletion are shown in *italics*. Filler DNA is shown in *bold* and is *underlined*, and the sequence from which the filler DNA may have originated is *underlined*. **B** Schematic diagram of the mutation in the *Wx-A1b* allele. The *stippled box* represents filler DNA, and *black boxes* represent exons

Lui et al. 1992). It is not yet known if the entire end of chromosome 4AL is missing in waxy wheat.

In the *Wx-D1* and *Wx-A1* null alleles, DNA homologous to the *waxy* gene is present (Fig. 1). Although these mutations also involve deletions, transcripts are produced from both alleles (Fig. 4). The deletion in the *Wx-A1b* allele includes an intron-exon junction, which affects splicing of the transcript (Fig. 5). Plant introns are flanked by highly conserved /GU and /AG dinucleotides at the 5′ and 3′ splice sites, although other nucleotides at the splice sites are less conserved than in other eukaryotic systems (reviewed in Brown 1996). Mutations at 5′ splice sites are known to result in the activation of cryptic splice sites in plants (reviewed in Brown 1996). McCullough et al. (1996) suggest that 5′ splice sites in plant nuclei are selected on the basis of their upstream position relative to the intron elements, and by their complementary to the U1 small nuclear RNA. Like the *Wx-A1b* mutation, a 5′-splice site mutation in the *aux1-22* allele in Arabidopsis results in cryptic splicing and the deletion of a portion of the previous exon (Marchant and Bennett 1998). In rice, a single G-to-T mutation at the 5′ splice site of the first intron in the *waxy* gene results in splicing at a number of cryptic sites (Cai et al. 1998; Isshiki et al. 1998). Since the rice mutation is caused by a single base pair mutation, a low level of correctly spliced product is produced, and the mutation has been maintained in many cultivars with intermediate amylose levels. In these rice cultivars, unspliced transcripts containing intron 1 also accumulate (Cai et al. 1998; Isshiki et al. 1998); this is not observed in waxy wheat (Figs. 2, 3).

Although several /GT dinucleotides are present in the area upstream of the mutation in the *Wx-A1b* allele (Fig. 5), the splice site selected in the *Wx-A1b* allele shows greater homology to the 5′ splice site consensus sequence reported in Arabidopsis (Brown 1996) than do sequences around /GT dinucleotides closer to the normal splice site.

The deletion in the *Wx-D1b* allele includes the 3′ untranslated region and the polyadenylation signal (Fig. 6). Normally AATAAA-like sequences upstream of the poly(A) tail function as polyadenylation signals,

A

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Wx-D1b cDNA       CTCTCCTGGAAG
Wx-D1b genomic    CTCTCC TGGAAGgtaagt cagtctctggtctggtttaggatgcactttccagaacaactaagagttaagactacaatggtgctcttg
Wx-D1a genomic    CTCTCC TGGAAGgtaagtcagtctctggtctggtttaggatgcattttccagaacaactaagagttaagactacaatggtgctcttg

Wx-D1b cDNA                                                GGGCCAGCCTGGAAGGTAAGT                    CGAT
Wx-D1b genomic    ttcgatgtatccattaatggtggcttgcgcatatggtgcagGGGCCAGCC TGGAAGGTAAGT                    CGAT
Wx-D1a genomic    ttcgatgtatccattaatggtggcttgcgcatatggtgcagGGGCCAGCC AAGAACTGGGAG...GTGTTTTTG ..... CGAT
                                                                    {-----369 bp------}*
                                                                    {---------------588 bp-----------}

Wx-D1b cDNA       GACCGCCAAGATCAATGCGACACAACCAGGAAATGACAGATGACCGCCAAGATCAACGCACACAACAAATGACGCAAGGGGAGCGAT
Wx-D1b genomic    GACCGCCAAGATCAATGCGACACAACCAGGAAATGACAGATGACCGCCAAGATCAACGCACACAACAAATGACGCAAGGGGAGCGAT
Wx-D1a genomic    GACCGCCAAGATCAATGCGACACAACCAGGAAATGACAGATGACCGCCAAGATCAACGCACACAACAAATGACGCAAGGGGAGCGAT

Wx-D1b cDNA       CATGGCTGAAACAGCTTCACTATTTTCCTTGCTAGTACAGTACTACTTGCTCAGTTTGCTGTTAAACTGTGAGTCTGTGACGCACTA
Wx-D1b genomic    CATGGCTGAAACAGCTTCACTATTTTCCTTGCTAGTACAGTACTACTTGCTCAGTTTGCTGTTAAACTGTGAGTCT
Wx-D1a genomic    CATGGCTGAAACAGCTTCACTATTTTCCTTGCTAGTACAGTACTACTTGCTCAGTTTGCTGTTAAACTGTGAGTCT

Wx-D1b cDNA       AACTTATTTAATAAGTTGTGCAGCAGCAACTTATTTAATGTAAGTCATGCAAAGAGCCAGCTTCTAAATACTTCCTAAAATACAAAAAAA
```

B

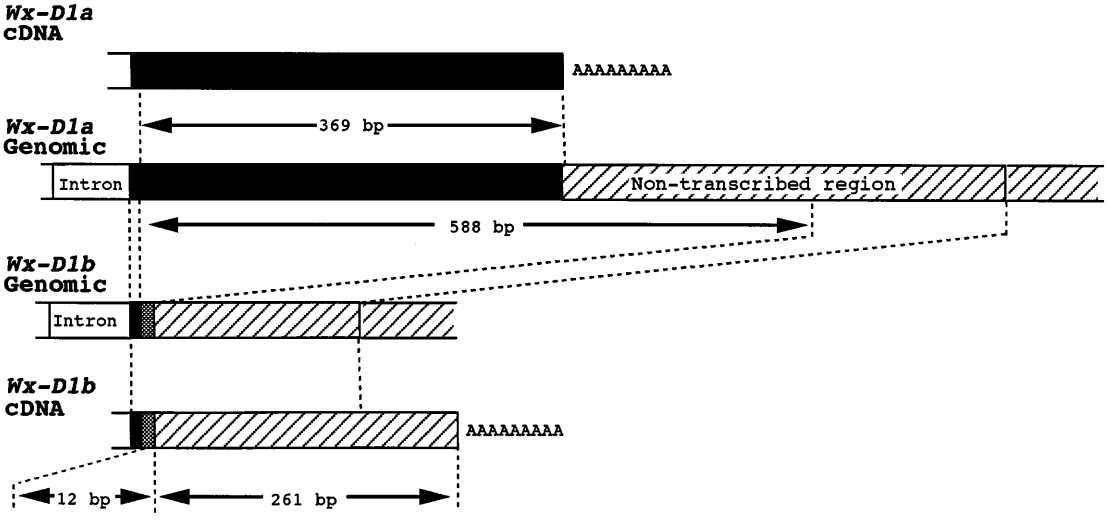

Fig. 6A,B The deletion in the *Wx-D1b* allele of waxy wheat. **A** Genomic and cDNA sequences in the region of the mutation in the *Wx-D1* gene. PCR amplification was used to obtain partial genomic clones from waxy wheat (*Wx-D1b*) and Chinese Spring wheat (*Wx-D1a*). The last intron of the *Wx-D1* gene is shown in *lower case letters* and the position of the 588-bp deletion is indicated. A portion of the sequence within the deletion is shown in *italics*, and the polyadenylation site in *Wx-D1a* is indicated by an *asterisk*. Filler DNA is shown in *bold* and is underlined, and the sequence from which the filler DNA may have originated is underlined. **B** Schematic diagram of the mutation in the *Wx-D1b* allele. The *stippled box* represents filler DNA, and *black boxes* represent the last exon of the *Wx-D1* gene

although a high level of sequence variation may be tolerated in plants (Rothnie et al. 1994). Luehrsen and Walbot (1994) have suggested that the presence of AU-rich regions in a transcript leads to polyadenylation as a default pathway in the absence of a 5′ splice site. Although a strong polyadenylation signal was not found in the *Wx-D1b* allele, the region preceding the poly(A) tail is AT-rich (Fig. 6).

Filler DNA was present in both *Wx-A1b* and *Wx-D1b* alleles (Figs. 5, 6). In maize, filler DNA was associated with five spontaneous deletions at the *waxy* locus and one at the *Bronze* locus (Wessler et al. 1990). In four of the maize deletions the filler DNA was derived from a nearby sequence that also contained the 3′ and 5′ deletion endpoints. In the wheat deletions, the sequences from which filler DNA originated contained both deletion endpoints in the *Wx-D1b* allele and only the 5′ endpoint in the *Wx-A1b* allele (Figs. 5, 6). The model put forward to explain the maize deletions involves two slipped mispairing events during lagging-strand DNA replication (Wessler et al. 1990), but it is also possible that the wheat deletions resulted from the repair of double-strand DNA breaks. Gorbunova and Levy (1997) have shown that when a linearized plasmid was transferred into tobacco cells and products of non-homologous end-joining were recovered, 30% of analyzed clones contained filler DNA.

Northern analysis indicated that the *waxy* mRNA found in waxy wheat had a slightly lower molecular weight than the transcripts found in wild-type wheat (Fig. 3). This is in agreement with sequence data showing that the *Wx-A1b* cDNA is missing 117 bp and the

Wx-D1b cDNA is missing 96 bp of wild-type sequence. Although two types of transcript were produced in waxy wheat, the transcript level was much lower than in Chinese Spring wheat (Fig. 2). Mutations leading to premature stop codons in the 5′ region of transcripts often result in mRNAs with greatly reduced half-lives (Jofuku et al. 1989; Marillonnet et al. 1997; Marchant and Bennett 1998); however, the splice site used in the *Wx-A1b* transcript does not create a stop codon. It is possible that other splicing events which result in stop codons occur, but the resulting transcripts are rapidly degraded, and thus were not detected in this study. However, a 5′ splice site mutation in the human lysyl hydroxylase gene, which, like the *Wx-A1b* deletion, leads to an in-frame deletion, also results in a marked reduction in the steady-state level of mRNA (Pajunen et al. 1998). Thus, the reduction in *Wx-A1b* mRNA may result from a decrease in formation of mature transcript in the nucleus due to inefficient splicing, rather than a reduction in mRNA stability. In the *Wx-D1b* allele, the wild-type 3′-untranslated region is deleted. Both the polyadenylation signal and upstream sequences in the 3′-untranslated region may affect 3′ end processing (Wu et al. 1993; Rothnie 1996). Therefore, the *Wx-D1b* deletion may result in inefficient 3′-end formation, causing a reduction in the level of transcript accumulation.

Although GBSSI transcripts are present in waxy wheat, granule-bound translation products of either normal or altered molecular weight are not detectable during endosperm development (Nakamura et al. 1995; Vrinten and Nakamura, unpublished data). In the case of the *Wx-A1b* mutation, the putative translation product will lack the transit peptide at the 5′ end of GBSSI, which is responsible for correct localization to the amyloplast (Klösgen et al. 1989). The mutation carried by the *Wx-D1b* allele affects the C-terminal region of the GBSSI enzyme. GBSSI is thought to be tightly bound to the amylopectin matrix (van de Wal et al. 1998), and the C-terminal region may be involved in this attachment. If translation products are produced but not bound to the starch granule, these proteins should be present in the soluble fraction. However, proteins antigenically related to potato GBSSI could not be detected in the soluble fraction from 15-DPA waxy wheat endosperm (data not shown), although this antibody clearly recognized wheat GBSSI in the granule-bound fraction from Chinese Spring endosperm (Nakamura et al. 1998). Therefore, it appears that the transcripts in waxy wheat may not be translated. Alternatively, the low amount of transcript available for translation, possibly combined with the rapid degradation of inactive translation product, may result in protein levels that are too low to be detected.

The characterization of the *waxy* null alleles presented here provides information on how mutations may affect gene expression in wheat, and suggests some avenues that may be pursued in efforts to gain further understanding of GBSSI function, such as detailed investigations on the role of the C-terminus of GBSSI in enzyme function or localization. Also, the design of PCR primers to identify individuals carrying the *Wx-A1b*, *Wx-B1b* or *Wx-D1b* alleles will be facilitated by the sequence information presented here. Such primers may be useful in breeding programs and in evolutionary studies. It is not yet known how or when the *waxy* mutations arose during the development of the lines used to produce waxy wheat. However, the pedigree of K107 is known (Yamamori et al. 1997); therefore, PCR studies could be used to elucidate the origin of the *Wx-A1b* and *Wx-B1b* alleles.

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