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Identification of glutinous maize landraces and inbred lines with altered transcription of *waxy* gene

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Abstract Waxy maize has little or very low content of amylose (<5 %) in grain starch and carries null mutations of the *waxy* (*Wx*) gene. With important uses as fresh maize or as an ingredient in food, textile, adhesive, and paper industries, two types of *wx* allele, *wx-D7* and *wx-D10*, had been identified. In the present study, 10 accessions carrying neither *wx-D7* nor *wx-D10* allele were identified from a collection of 325 waxy maize accessions. The *Wx* locus of these 10 accessions was sequenced, but no potential causative mutation was detected. Further sequencing of the full-length complementary DNAs revealed that 6 of the 10 accessions had alternative splicing patterns while the other 4 had wild-type *Wx* transcripts. Among the six accessions, one generated transcripts identical to the *wx-D7* allele, two had the same transcripts as the *wx-D10* allele, another two generated transcripts with deletion of the 10th and the 11th exon, yet the other one produced transcripts of various lengths due to deletions from part of the 2nd exon to part of the 12th exon. The *wx* alleles with the above alternative splicing modes are referred to as *wx-tD7*, *wx-tD10*, *wx-tD10-11*, and *wx-tD2-12*, respectively. Real-time quantitative reverse-transcription polymerase chain reaction analysis of two waxy accessions that produced *Wx* transcripts showed significantly decreased expression, having only 47.3 and 3.6 % transcription level compared with B73. Sequence analysis of deletions in the transcripts with comparison with wild one showed short direct repeats at deletion endpoints, similar to reported signatures of DNA deletions. The waxy accessions present unique *wx* alleles for waxy maize breeding as well as for transcriptional regulation studies in plants.

Keywords Granule-bound starch synthase · *Wx* mutation · Alternative splicing · Chinese waxy maize

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Introduction

Waxy maize is a special type of maize that has little or very low content of amylose (<5 %) in grain starch; it is consumed either as fresh maize or as an important ingredient used in food, textile, adhesive, and paper industries. The waxy phenotype mainly results from null mutations of the *Waxy* (*Wx*) locus, which encodes enzyme granule-bound starch synthase I (GBSSI, EC 2.4.1.242) that catalyzes elongation of amylose molecule by transfer of α-D-glucose from adenosine diphosphate (ADP)-glucose. In maize, molecular analyses have shown that various types of spontaneous *wx* mutations exist in maize landraces and inbred lines, namely: (a) stable or unstable insertion of transposable elements into intron, exon, untranslated region (UTR), and intergenic regions of *Wx* locus, hence disrupting the gene structure (Fedoroff et al. 1983; Marillonnet and Wessler 1997; Liu et al. 2007); (b) insertions and deletions in the *Wx* locus, resulting in altered transcripts (Wessler and Varagona 1985; Okagaki et al. 1991) which may generate premature stop codon or loss of amino acids in key domain of GBSSI (Fan et al. 2008, 2009; Tian et al. 2008); and (c) alternative splicing or decreased expression of *Wx* gene, which has no obvious mutations at the DNA level (Varagona et al. 1992; Ding et al. 2009).

Waxy maize was first found in China in 1908 and has since been reported in many other locations in Asia (Collins 1909, 1920; Kuleshov 1954). A wide range of genetic diversity has been observed in Chinese waxy maize collections; at least 767 different accessions have been found in China, and most of them (525) were collected from Yunnan and Guangxi Provinces (Huang and Rong 1998). Some of these lines have been investigated in terms of, e.g., their overall genetic diversity (Yong et al. 2009), and molecular features of mutations at the *Wx* locus (Liu et al. 2007; Fan et al. 2008; Tian et al. 2008) and also in other genes related to starch biosynthesis (Fan et al. 2009; Ding et al. 2009).

In our previous studies, we analyzed the *Wx* locus by sequencing of 55 waxy maize accessions; however, only two *wx* alleles, *wx-D7* and *wx-D10*, were identified (Fan et al. 2008, 2009). The *wx-D7* allele involves a 30-bp deletion at the junction of the 7th exon–intron and consequently produces an abnormal transcript retaining the 7th intron, which introduces an immature stop codon and inactivates GBSSI. The 30-bp deletion is similar to the *wx1240* and *wx-BL2* mutations identified in a spontaneous and a chemically induced mutant (Okagaki et al. 1991). The *wx-D10* allele involves a 15-bp deletion in the 10th exon and hence causes loss of five amino acids in the glucosyl transferase domain 1 (GTD1) of GBSSI (Fan et al. 2008). The *wx-D10* mutation has been independently detected in another 12 waxy maize accessions (Tian et al. 2008). In this study, a total of 325 Chinese waxy maize accessions of both inbreds and landraces were characterized for the *Wx* gene in an attempt to further disclose the molecular features of Chinese waxy maize germplasm.

Materials and methods

Germplasm accessions and amylose content analysis

The 325 investigated accessions of waxy maize including 90 landraces and 157 inbred lines, some of which had been used for analyses in our previous studies (Fan et al. 2008, 2009). They were provided by Institute of Crop Science, Chinese Academy of Agricultural Sciences and Institute of Crop Science, Zhejiang Academy of Agricultural Sciences. Amylose content was measured using seeds provided according to the National Standards of the People's Republic of China, GB-T17891 (China Standards Press 1999). The 10 accessions that were identified to be of neither *wx-D7* nor *wx-D10* genotype were grown into mature plants at the Experiment Farm of Zhejiang University in Hangzhou, China for further studies including gene expression (see below) and amylose content measurement.

DNA extraction, genotyping, and sequencing

Genomic DNA was extracted from fresh leaves of 14-day-old seedlings using the protocol described by Sambrook and Russell (1989) with minor modifications. DNA quality was confirmed by gel electrophoresis and quantified using a NanoDrop® ND-1000 spectrophotometer.

The waxy maize collections were genotyped using functional DNA markers for the known *wx* mutations *wx-D7* (with a 30-bp deletion at the junction of exon 7–intron 7) and *wx-D10* (with a 15-bp deletion in the

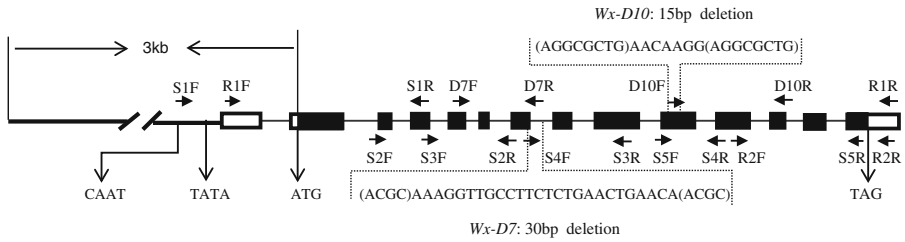

Fig. 1 Schematic illustration of *Wx* gene and primers used for DNA and cDNA sequencing and marker development in maize: S1F/R-S5F/R for DNA sequencing, R1F/R and R2F/R for cDNA sequencing, and D7F/R and D10F/R for *Wx* genotyping, with arrows indicating their approximate locations and directions. Important cassettes in the promoter region and the start and stop codons are also indicated

Table 1 Primers and sequences used for PCR

Gene	Primer code	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)
Wx	R1	CAGGGCTGCTCATCTCGTCG	AGCACAAGCAAGCAGCTACA	1,965
	R2	ACCATCATCGAAGGCAAGAC	CACACGCAAGCAATTAGTTCA	486
	S1	AGTACCAGCACAGCACGTTG	GTACCCGTCTCCCATCTTGA	1,672
	S2	GTCTTCTTCGTGCTCTTGCC	GATGCCGTGGGACTGGTAG	907
	S3	GTTGACCACCCACTGTTCCT	ATGAGCTCCTCGGCGTAGTA	880
	S4	AACTACCAGTCCCACGGCATCT	CACGTCCTCCACCATCTCCAT	902
	S5	GACAAGTACATCGCCGTGAA	AGCACAAGCAAGCAGCTACA	1,398
	D7	CTGCTATGCCAGGTCAGGAT	AGCAGAGAAGGCAACCTTTG	394
	D10	CAAGGCGCTGAACAAGGA	GTCTTGCCTTCGATGATGGT	632
Actin	R3	GCATGAAGGTGAAGGTGGTT	ACCAAATGCGAAACCTGTTC	532

10th exon) (Fan et al. 2009) using primer pairs D7F/R and D10F/R, respectively (Fig. 1; Table 1). Another primer pair S4F/R was designed for amplification of the exon 7–exon 10 region for sequencing (Fig. 1; Table 1).

Five overlapping pairs of PCR primers (S1F/R, S2F/R, S3F/F, S4F/R, and S5F/R; Fig. 1 for position; Table 1 for sequence information) were designed according to the *Wx* genomic sequence of B73 (GenBank acc. no. X03935) and used for amplification of the *Wx* locus of the 10 accessions that carried neither *wx-D7* nor *wx-D10* allele. For PCR with S1F/R primer, LA Taq^{TM} polymerase was used with a GC buffer kit (Takara, Japan) and the following program: 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C, and final for 8 min at 72 °C. For all PCRs of other primers, the following program was used: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 54 °C, and 90 s at 72 °C, and final for 8 min at 72 °C. PCR products were sequenced directly by Invitrogen Inc. (Shanghai, China).

RNA extraction, RT-PCR, and FL-cDNA sequencing

Self-pollination of the accessions was achieved by bagging the cob before flowering and pollinating with pollens from the tassel of the same plant. Immature cobs were sampled 21 days after pollination and flash-frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated from the immature cobs using the RNA extraction kit TRIzol (Invitrogen, USA) according to the manufacturer's guideline. DNase-treated total RNA (~2 μg) was reverse-transcribed to cDNA using an oligo-dT primer at 42 °C in volume of 20 μl with a Revert Aid^{TM} first-strand cDNA synthesis kit (MBI Fermentas, #K1622). The full length (FL)-cDNA of *Wx* locus was amplified through PCR of cDNA using the primer pair R1F/R (Fig. 1; Table 1). One microliter of synthesized cDNA was used as DNA template for PCR with total volume of 20 μl, with the following program: 5 min at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at

72 °C; and 8 min at 72 °C. Since the 5′ end region of the *Wx* gene has high GC content, LA Taq™ with a GC buffer kit (Takara, Japan) was used.

PCR products of cDNA (040429, 622157-CN-159, 230525, 232001, 231989, 20V-18, 20V-19, 622081-CN-82, 041057) were sequenced directly, except cDNA sequence of 613177-CN-9762 which was sequenced after TA clone (pMD19-T kit; Takara, Japan). Primer synthesis and sequencing services were provided by Invitrogen Inc. (Shanghai, China).

Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using Maxima® SYBR Green qPCR master mix (MBI Fermentas) on an Applied Biosystems 7300 real-time PCR system. The maize housekeeping gene *Actin* was analyzed as internal control together with the *Wx* gene. Forty cycles of quantitative real-time PCR were performed with primers R2 for *Wx* and R3 for *Actin*. Mean values of *Ct* were used to estimate the relative quantification (RQ) values in waxy gene expression by the $2(-\Delta\Delta Ct)$ method ($RQ = 2^{-\Delta\Delta Ct}$). The experiment was replicated twice.

Sequence analysis

Primers were designed online using Primer3 (Rozen and Skaletsky 2000). Sequence quality was manually checked using Chromas software (http://www.technelysium.com.au/chromas.html), and sequences were assembled using DNAMAN 6.0 (Lynnon Biosoft, Los Angeles, USA). Sequence similarity analysis used GeneDoc 3.2 (http://www.psc.edu/biomed/genedoc). All sequence data including FL-cDNA sequences were deposited in GenBank under accession numbers HQ423233–HQ423257 and JN854287–JN854289.

Results

Identification of accessions with novel *wx* genotype

Two types of *wx* allele, *wx-D7* and *wx-D10*, had been previously identified with a 30-bp deletion and 15-bp deletion in the 7th exon–intron region and in the 10th exon region, respectively (Fig. 1). The three types of *Wx* allele, i.e., *Wx*, *wx-D7*, and *wx-D10*, could be differentiated by analyzing fragments amplified using two sets of PCR primers, i.e., D7F/R and D10F/R. Two amplicons, of 394 and 632 bp in length, could be amplified for *Wx* allele (cv. B73, Fig. 2a) using these two sets of primers; however, only one of them could be amplified for *wx-D7* (the 632-bp fragment) and *wx-D10* (the 394-bp fragment) (Fig. 2a) because D7R and D10F are located in the deletion regions (Fig. 1).

Among the 325 fresh waxy maize accessions, 10 accessions (5 inbred lines and 5 landraces) were identified to be carrying neither *wx-D7* nor *wx-D10* allele because both fragments were amplified like B73 (Fig. 2); hereafter, they are referred to as *non-wx-D7/10* lines. Analysis of amylose content of the 10 accessions showed that they were not significantly different from *wx-D7* and *wx-D10* lines (Table 2).

Wx sequences of non-*wx-D7/10* lines

To identify potential causative mutations in the *Wx* locus, overlapped PCR fragments were sequenced using five sets of PCR primers, and the *Wx* DNA sequences were assembled for the 10 accessions (see Table 2 for GenBank accession numbers of the sequences). Alignment analysis, however, revealed no unique mutations in exon, intron, splicing site, and 5′ upstream (~3 kb) that could be potentially linked with the waxy phenotype of the 10 accessions, compared with nonwaxy maize (data not shown).

Identification of alternatively spliced *Wx* transcripts

To test whether the waxy phenotype of the 10 accessions resulted from abnormal transcription of the *Wx* locus, their FL-cDNAs were cloned and sequenced. Length polymorphisms of *Wx* transcripts were observed among these 10 accessions (Fig. 2b). The FL-cDNA of three lines, 232001, 231989, and 613177-CN-9762 (lane 8, 9, and 10, respectively, in Fig. 2b), seemed much shorter than other lines (Fig. 2b).

Sequencing of the transcripts indicated that four accessions (inbred lines 20V-18, 20V-19, and 622081-CN-82, and landrace 041057) produced *Wx* transcripts highly identical to the wild type (nonglutinous maize), although some synonymous mutations were observed, while the other six accessions generated alternatively spliced transcripts (Fig. 3).

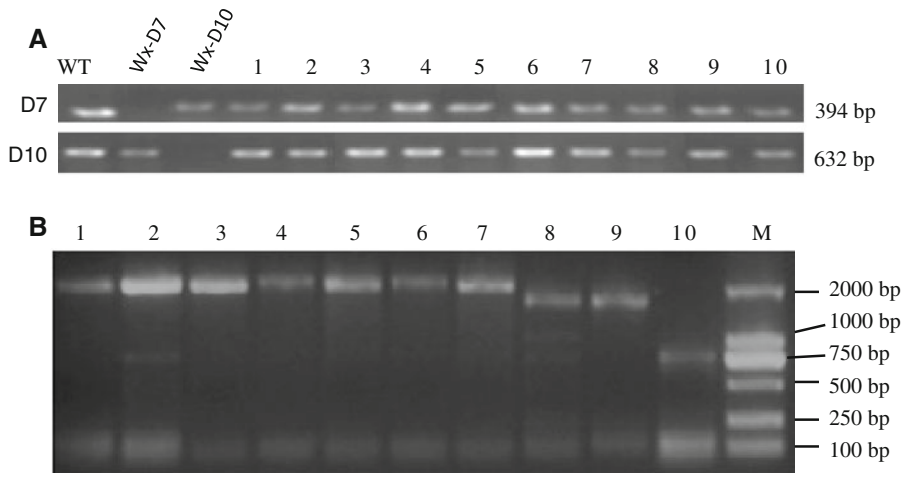

Fig. 2 Molecular characterization of different alleles of the maize *Wx* gene. **a** Amplification of *Wx* gene fragments using primer pairs of D7F/R (*upper*) and D10F/R (*bottom*); Mutant allele *wx-D7* and *wx-D10* only produced one fragment while other alleles could generate two fragments of 632 and 394 bp. **b** FL-cDNA amplified by RT-PCR with primer pair R1F/R with expected length of 1,965 bp for the WT allele. M: DNA marker (DL2000); WT stands for cultivar B73 and *lanes 1–10* for *non-wx-D7/10* lines: 040429, 622157-CN-159, 230525, 20V-18, 20V-19, 041057, 622081-CN-82, 232001, 231989, and 613177-CN-9762

Table 2 New *Wx* genotypes identified with altered transcription of fresh waxy maize with DNA and cDNA sequence GenBank accession number

Accession	Type	Amylose content (%)[a]	Designed *Wx* genotype	Novel splicing	DNA	cDNA
040429	Landrace	1.80	*wx-tD7*	Exon 7	HQ423237	HQ423250
622157-CN-159	Inbred line	2.23	*wx-tD10*	Exon 10	HQ423235	HQ423248
230525	Landrace	3.28			HQ423236	HQ423249
232001	Landrace	1.74	*wx-tD10-11*	Exon 10–11	HQ423234	HQ423247
231989	Landrace	1.56			HQ423233	HQ423246
613177-CN-9762	Inbred line	2.79	*wx-tD2-12*	Exon 2–12	HQ423242	HQ42325–7
						JN854287–9
20V-18	Inbred line	2.66	*wx-tN1*	Not found	HQ423240	HQ423253
20V-19	Inbred line	4.20			HQ423241	HQ423254
622081-CN-82	Inbred line	0.94			HQ423238	HQ423251
041057	Landrace	3.03			HQ423239	HQ423252

[a] Amylose content in other type of maize: *wx-D7*, average 2.18 % (0–5.73 %); *wx-D10*, average 2.65 % (0–9.66 %); wild type, average ~ 25.00 %

Further analysis of these *Wx* transcript sequences showed that landrace accession 040429 had *Wx* transcript identical to those of *wx-D7* genotype, while landrace 230525 and inbred line 622157-CN-159 generated the same *Wx* transcript as those of *wx-D10* genotype. Furthermore, the *Wx* transcript of landrace accessions 232001 and 231989 was trimmed due to the absence of exon 10 and 11 as compared with wild-type (WT) transcript (hence being 375 bp shorter). The transcription of the inbred line 613177-CN-9762 seemed to be the most complex, and its PCR fragments could not be sequenced directly. By clone-sequencing, 6 types of *Wx* transcript in 12 clones were identified, all having a large fragment deletion between exon 2 to exon 11 or exon 12. The results were confirmed by several independent samplings and sequencings (Fig. 3).

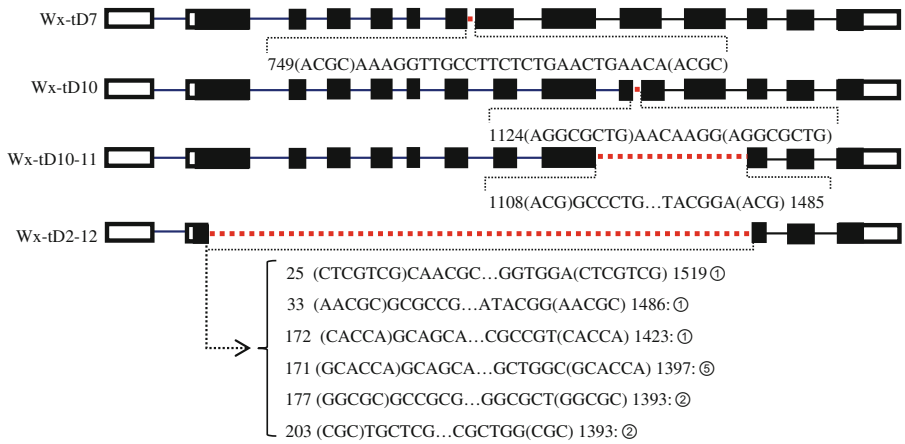

Fig. 3 Types and positions of deletion in novel transcripts identified in four new waxy genotypes. *Dark* and *empty boxes* refer to coding sequences and UTR regions, respectively. *Dark solid lines* refer to intron, while *dotted lines* refer to deleted sequences. The short direct repeats are highlighted in *parentheses*, and the *numbers* indicate the start and end nucleotide of each deleted sequence. For *wx-tD2-12* genotype, the number of clones identified is given in a *circle*. The position information refers to *Wx* cDNA sequence X03935

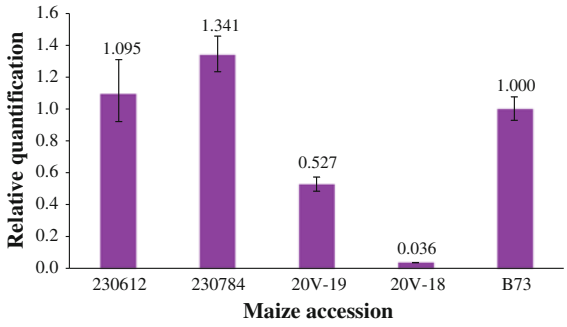

Fig. 4 Real-time quantitative RT-PCR analysis of *Wx* gene in fresh waxy maize. Relative quantification ($RQ = 2^{-\Delta\Delta Ct}$) calculated using the housekeeping gene *Actin* as internal control

For convenience and simplicity, waxy genotypes that have wild-type gene (*Wx*) sequence but with transcripts identical to *wx-D7* or *wx-D10* allele are hereafter referred to as *wx-tD7* and *wx-tD10*, respectively (Table 2). Accordingly, these produce transcripts with deletions either in exon 10 and exon 11, or with deletions across exon 2 and exon 12, being designated as *wx-tD10-11* and *wx-tD2-12*, respectively.

Quantitative analysis of *Wx* expression

Two accessions, namely 20V-18 and 20V-19, of the four accessions that produced *Wx* transcripts with identical sequence to nonwaxy maize were subjected to quantitative real-time RT-PCR analysis of the *Wx* locus, together with nonwaxy maize B73 and two *wx-D10*-type waxy lines (230612 and 230784). Results indicated that 20V-18 and 20V-19 had significantly lower transcription than B73, being only 47.3 and 3.6 % of the level of B73 (Fig. 4). No significant differences were observed among the two *wx-D10* lines 230612 and 230784, which had slightly higher, but insignificant, expression level compared with nonwaxy maize cultivar B73 (Fig. 4). Waxy maize with wild-type DNA and cDNA sequences (*Wx*) but with reduced transcription level is hereafter referred to as *wx-tN* line.

Discussion

Stable spontaneous and induced *wx* mutations had been characterized previously in several collections of waxy maize; both abnormal *Wx* transcripts (longer or shorter than WT locus) and reduced expression of the *Wx* locus had also been observed. In this study, a large Chinese collection of waxy maize was genotyped for the *Wx* locus and consequently 10 accessions were identified to be carrying neither *wx-D7* nor *wx-D10* allele, all having altered transcriptions quantitatively or qualitatively. This is, to the best of our knowledge, the first comprehensive and detailed analysis of waxy maize with abnormal transcriptions of the *Wx* locus. The 10 waxy accessions present a novel type of *wx* resource not only for waxy maize breeding but also for gene transcriptional regulation studies in plants.

Molecular characterization of waxy maize, of spontaneous or induced origin, was started as early as in the mid-1980s (Wessler and Varagona 1985; Klosgen et al. 1986). However, concrete sequence data of DNA and transcripts of the *Wx* locus were not available until recently when Fan et al. (2008) and Tian et al. (2008) independently identified the *wx-D10* mutant allele, and Fan et al. (2008) and Ding et al. (2009) detected the *wx-D7* allele in Chinese maize collections. In the present study, we further identified 10 waxy maize accessions with altered transcription while having DNA sequences of the *Wx* locus highly similar to wild-type nonwaxy maize; they either generated altered transcripts with deletions (*wx-tD7*, *wx-tD10*, *wx-tD10-11*, and *wx-tD2-12*) or had significantly low level of expression (*wx-tN*).

For the *wx-tN* waxy maize accessions with reduced transcription levels, the promoter region ($\sim$3 kb) of the *Wx* locus was sequenced but no mutations could be considered to be responsible for the low expression level. Therefore, there might be other regulatory element(s) responsible for its low transcription level of *Wx* locus, similar to what was observed in waxy sorghum (McIntyre et al. 2008). Several other studies have also shown that causative mutations are sometimes located at remote promoter regions; for example, a miniature transposon element (MITE) positioned 70 kb upstream of an *AP2*-like transcription factor is strongly associated with flowering time in maize (Salvi et al. 2007; Buckler et al. 2009) and a single nucleotide polymorphism (SNP) at a noncoding region located 12 kb upstream of *qsh1* significantly reduces shattering in rice (Konishi et al. 2006). Therefore, it may also involve mutations of the regulatory element for *Wx* locus in these waxy maize accessions.

For waxy maize carrying alleles of *wx-tD7*, *wx-tD10*, *wx-tD10-11*, and *wx-tD2-12*, the waxy phenotype could be explained by the abnormal transcripts, because all these transcripts are expected to encode nonfunctional GBSSI. Although alternative splicing is not an uncommon phenomenon for many genes (see review Reddy 2007), there is no publication to the authors' knowledge that reports such deletions in transcripts as observed in the present study.

At the DNA level, Wessler et al. (1990) indicated that there is a GC-rich region of $\sim$1,000 bp that is capable of forming stable secondary structures, which are prone to spontaneous deletions in the *Wx* locus. Furthermore, they found that all of the *wx* alleles have DNA insertions (filler DNA) of 1–131 bp between the deletion endpoints. Such filler DNA sequences were also observed in the *wx-D7* and *wx-D10* alleles in the present study, with the AGGCGCTG and ACGC direct repeat at the deletion endpoints, respectively (Fig. 1). Similar finding of filler DNA was also reported in *wx* mutants of wheat (Nakamura et al. 1995) and sorghum (McIntyre et al. 2008). The importance of such direct repeats in DNA deletion is explained by the following: Pairing of one direct repeat copy with its complement at the second repeat would initiate deletion formation. Replication or repair of these "slipped mispaired" structures may result in deletion of the intervening DNA and one copy of the repeat (Wessler et al. 1990).

In the present study, deletions were observed in transcripts of several *wx* accessions (Fig. 3), while no differences were observed at the DNA level. Interestingly, we notice that there are short direct repeats at the deletion termini of all cDNAs including different transcripts of the *wx-tD2-12* alleles (Fig. 3); For example, an ACG repeat was observed at the deletion endpoint of *wx-tD10-11* allele, and a CTCGTCG repeat for one of the *wx-tD2-12* cDNAs (Fig. 3). Although mutations at the *Wx* locus have been characterized in several other cereals, including rice (Hirano and Sano 1991), sorghum (McIntyre et al. 2008), barley (Domon et al. 2002; Patron et al. 2002), and wheat (Nakamura et al. 1995), none of these studies reported similar deletions in *Wx* transcripts. Review of literature indicated that transcript deletion is often related to diseases and disorders in humans (see, for example, Lemos and Thakker 2008; Polke et al. 2011). However, our present study is the first detailed analysis of transcript deletion in plants to the authors' knowledge; hence, further research should be performed to clarify whether such genetic systems exist in plants or not.

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