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Characterization of *dull1*, a Maize Gene Coding for a Novel Starch Synthase

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The maize *dull1* (*du1*) gene is a determinant of the structure of endosperm starch, and *du1*– mutations affect the activity of two enzymes involved in starch biosynthesis, starch synthase II (SSII) and starch branching enzyme IIa (SBEIIa). Six novel *du1*– mutations generated in *Mutator*-active plants were identified. A portion of the *du1* locus was cloned by transposon tagging, and a nearly full-length Du1 cDNA sequence was determined. *Du1* codes for a predicted 1674-residue protein, comprising one portion that is similar to SSIII of potato, as well as a large unique region. Du1 transcripts are present in the endosperm during the time of starch biosynthesis, but the mRNA was undetectable in leaf or root tissue. The predicted size of the *Du1* gene product and its expression pattern are consistent with those of maize SSII. The *Du1* gene product contains two repeated regions in its unique N terminus. One of these contains a sequence identical to a conserved segment of SBEs. We conclude that *Du1* codes for a starch synthase, most likely SSII, and that secondary effects of *du1*– mutations, such as reduction of SBEIIa, result from the primary deficiency in this starch synthase.

INTRODUCTION

Starch is the most significant carbohydrate reserve in plant storage tissues and comprises the glucose polymers amylose and amylopectin. Amylose is predominantly linear chains of $\alpha(1\rightarrow4)$–linked glucose residues, whereas amylopectin is a highly branched glucan with a specific "clustered" distribution of $\alpha(1\rightarrow6)$ glycosidic bonds (i.e., branch linkages) connecting linear chains (reviewed in French, 1984; Manners, 1989). Despite the relatively simple chemical structure of amylopectin, the enzymatic processes responsible for the formation of the highly specific and complex branching patterns in this polysaccharide are not clearly understood. Biosynthesis of amylose and amylopectin involves the activities of four groups of enzymes, of which each comprises multiple isozymes. These enzymes are ADPglucose pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE) (reviewed in Preiss, 1991; Hannah et al., 1993; Martin and Smith, 1995; Nelson and Pan, 1995; Ball et al., 1996; Preiss and Sivak, 1996; Smith et al., 1997). These enzymatic steps can account for all chemical linkages in starch; however, the specific roles of individual isozymes in the formation of specific branching patterns in amylopectin and determination of starch granule structure and properties remain unknown.

Analysis of maize mutants with abnormal endosperm phenotypes has contributed greatly to the understanding of starch synthesis (reviewed in Shannon and Garwood, 1984; Nelson and Pan, 1995) and facilitated the identification of many genes coding for starch biosynthetic enzymes. Cloned maize genes involved in starch biosynthesis are *waxy* (*wx*), coding for granule-bound starch synthase I (GBSSI) (Shure et al., 1983; Klösgen et al., 1986), *amylose extender* (*ae*), coding for SBEIIb (Fisher et al., 1993; Stinard et al., 1993), *shrunken2* (*sh2*) and *brittle2* (*bt2*), coding for the large and small subunits of AGPase, respectively (Bae et al., 1990; Bhave et al., 1990), *brittle1* (*bt1*), thought to code for an adenylate translocator (Sullivan et al., 1991; Shannon et al., 1996; Cao and Shannon, 1997), and *sugary1* (*su1*), coding for the SDBE SU1 (James et al., 1995) (maize genetic nomenclature is according to Beavis et al., 1995). The transposon-tagging strategy was used to determine that the abnormal endosperm phenotype of *wx*–, *ae*–, *su1*–, or *bt1*– mutants results from primary defects in GBSSI, SBEIIb, SU1, or the adenylate transporter, respectively, and this approach remains the most effective way of characterizing genes, such as *dull1* (*du1*), in which the primary defect cannot be associated with a particular enzyme deficiency.

The *du1*– mutations define a gene with an important function in starch synthesis, as indicated by extensive structural analyses of starch from *du1*– mutant endosperms and by

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the effects of these mutations when combined with other genetic deficiencies in starch biosynthetic enzymes (Shannon and Garwood, 1984; Nelson and Pan, 1995). The reference mutation *du1-Ref* was first identified as a recessive modifier of *su1-Ref* and *su1-amylaceous* (*su1-am*) (Mangelsdorf, 1947). Mutations of *du1*, when homozygous in otherwise nonmutant backgrounds, result in mature kernels with a tarnished, glassy, and somewhat dull appearance, which is referred to as the "dull phenotype." Expression of this phenotype, however, depends on the particular genetic background (Mangelsdorf, 1947; Davis et al., 1955).

Total carbohydrate content in mature *du1*– mutant kernels is slightly lower than normal (Creech, 1965; Creech and McArdle, 1966). The apparent amylose content in starch from *du1*– mutants is slightly or greatly elevated compared with normal, depending on the genetic background (Shannon and Garwood, 1984), although the properties of polysaccharides in the apparent amylose fraction essentially are not altered (Dvonch et al., 1951). Approximately 15% of the starch in *du1*– mutant endosperms is in a form known as "intermediate material," which is distinguished from amylose and amylopectin by the properties of its starch–iodine complex (Wang et al., 1993a, 1993b). Analysis of the combined amylopectin/intermediate material fractions indicated that starch from *du1*– mutants has the highest degree of branching among a wide variety of normal and mutant kernels analyzed (Inouchi et al., 1987; Wang et al., 1993a, 1993b). Starch granules from *du1*– mutants seem to have normal structural and physical properties, although some abnormally shaped granules are found in the mutant endosperm (Shannon and Garwood, 1984).

Mutant *du1*– alleles, when combined with other mutations affecting starch synthesis, result in a broad range of alterations more severe than those in the single mutants (reviewed in Shannon and Garwood, 1984; Nelson and Pan, 1995). Double mutants containing *du1*– together with a *wx*–, *ae*–, *su1*–, or *su2*– mutation have increased soluble sugars and decreased total starch compared with any of the single mutants. The double mutants sometimes produce polysaccharides distinct from the starch found in any single mutant kernels. These pleiotropic effects suggest that the product of *Du1* may have broad effects on starch biosynthesis in maize endosperm; however, without knowing the identity of this protein, it is difficult to assess its specific functions.

Consistent with their pleiotropic effects, *du1*– mutations cause reduced activity in the endosperm of two seemingly unrelated starch biosynthetic enzymes, SSII and SBEIIa (Boyer and Preiss, 1981). SSII is one of two starch synthases identified in the soluble fraction of maize endosperm. SSII activity in vitro requires an exogenous glucan primer, and its native molecular mass has been determined in different studies as being either 95 or 180 kD (Boyer and Preiss, 1981; Mu et al., 1994). SBEIIa is one of the three known SBE isozymes in endosperm (Boyer and Preiss, 1978; Fisher et al., 1993, 1995; Gao et al., 1997). Thus, *Du1* may code for a protein regulating the expression or activity of both SSII and SBEIIa. Another possibility is that *Du1* codes for one of these two enzymes, and the primary deficiency in that enzyme might in some way affect the activity of the second.

To understand the role of the *du1* locus in starch biosynthesis, a transposon-tagging strategy was used to isolate the gene and characterize its polypeptide product. This study reports the tagging of the *du1* locus with a *Mutator* (*Mu*) transposon and the cloning and characterization of a portion of the gene and the sequence of a nearly full-length cDNA. The amino acid sequence deduced from this cDNA indicates that *Du1* codes for a 188-kD polypeptide that is similar to SSIII, a starch synthase from potato tubers (Abel et al., 1996; Marshall et al., 1996). The expression pattern of *Du1* also was characterized. Taken together, these characterizations indicate that *Du1* codes for a starch synthase of maize endosperm, most likely SSII. In addition, the product of *Du1* contains unique sequence features at its N terminus that may mediate direct interactions with other starch biosynthetic enzymes.

RESULTS

Identification of *du1*– Mutations Potentially Induced by *Mu* Transposons

Novel *du1*– mutations were identified in plants derived from parental lines containing an active *Mu* transposable element system by the strategy outlined in Figure 1. Standard non-*Mu* lines were pollinated by *Mu*-active plants, and the resultant F_1 progeny were self-pollinated. Six F_1 ears were found that contained kernels with the dull phenotype at a frequency of ~25%, as shown in Figure 2. Plants grown from the dull kernels were crossed to standard lines to generate presumed *Du1*/*du1* heterozygous kernels. These were grown to maturity and crossed to *du1-Ref*/*du1-Ref* tester plants, resulting in a 1:1 segregating population of dull and normal sibling kernels for each of the six putative *Mu*-induced *du1*– alleles. Thus, in all instances, the dull phenotype is a single gene trait conditioned by a mutation allelic to *du1-Ref.* The mutations are designated *du1-R2370::Mu1*, *du1-R2339*, *du1-R2649*, *du1-R4059*, *du1-R2197*, and *du1-R1178.*

Cloning and Characterization of a *Mu1*-Containing Genomic Fragment That Cosegregates with *du1-R2370::Mu1*

A specific *Mu1* transposon was found to cosegregate with the dull phenotype among progeny of a *du1-R2370::Mu1*/*Du1* heterozygote. The heterozygous parent was crossed to a *du1-Ref* homozygote, generating ears containing ~50% dull kernels (*du1-R2370::Mu1*/*du1-Ref*) and 50% normal kernels (*Du1*/*du1-Ref*). Genomic DNA from 35 kernels of each type was digested with BamHI and subjected to gel

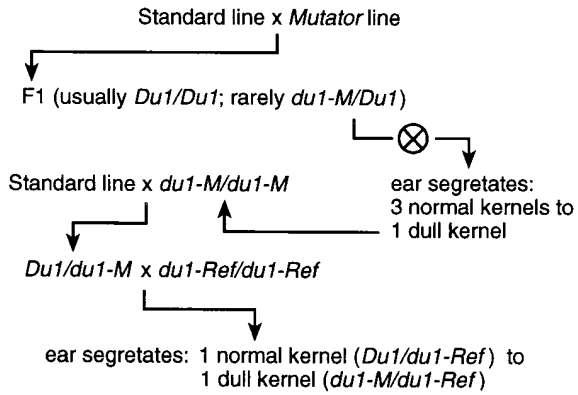

Figure 1. Isolation of *du1*– Mutations and Generation of Segregating Populations.

The specific maize lines used in this procedure are listed in Methods. The allele designation *du1-M* indicates a putative recessive mutation in the *du1* locus caused by the insertion of a *Mu* transposon.

blot analysis, using the 960-bp internal MluI fragment of *Mu1* as a probe. Figure 3A illustrates that a 2.0-kb *Mu1*-containing fragment was detected in all analyzed plants bearing *du1-R2370::Mu1* but not in any plants lacking this allele.

This 2.0-kb *Mu1*-containing genomic DNA fragment was cloned by screening a size-fractionated genomic library prepared from a *du1-R2370::Mu1*/*du1-Ref* heterozygote in vector λZAPII express, using an internal fragment of *Mu1* as a probe. Figure 3B shows the structure of the cloned fragment. As expected, this fragment contains two 9-bp direct repeats (5′-GTGAGAATG-3′) flanking a *Mu1* element. Figure 3C illustrates DNA gel blot analysis confirming that the cloned *Mu1*-containing fragment was derived from the genomic interval that cosegregates with *du1-R2370::Mu1.* The probe F500, which is adjacent to the *Mu1* element (Figure 3B), detected a fragment of ~0.6 kb in all plants of the segregating population. It also detected a fragment of ~2.0 kb in plants derived from dull kernels (*du1-R2370::Mu1*/*du1-Ref*). In all, 27 kernels of each type were characterized. The size difference of 1.4 kb indicates that the longer 2.0-kb fragment most likely arose from insertion of a 1.4-kb *Mu1* element within the 0.6-kb region delineated by these two BamHI sites. These data indicate that the cloned *Mu1*-containing fragment is either located within or closely linked to the *du1* locus.

Additional support for this conclusion is shown in Figure 4A, which illustrates DNA gel blot analyses of other restriction fragments using fragment F500 as a probe. The difference of 1.4 kb, indicating a *Mu1* insertion, was observed in the 6.0-kb EcoRI fragment detected in both genotypes, with the 7.4-kb fragment being found only in the plants containing *du1-R2370::Mu1.* Two different XbaI fragments were detected in the *Du1*/*du1-Ref* plants of the segregating population. In sibling plants containing *du1-R2370::Mu1*, the shorter of these two fragments, ~3.0 kb, was invariably replaced by a 4.4-kb fragment. These genomic DNAs were derived from eight dull kernels and eight normal kernels. The difference of 1.4 kb between the longer fragment detected only in plants bearing the mutant allele *du1-R2370::Mu1* and the shorter fragment associated with the wild-type *Du1* allele is consistent with the *Mu1* insertion causing the *du1*– mutation.

Characterization of the cDNA Product of the Cloned Locus

A longer genomic fragment overlapping the cloned 2.0-kb BamHI fragment was isolated from wild-type genomic DNA. A 6.0-kb EcoRI fragment from wild-type genomic DNA contains sequences flanking the cloned *Mu1* (Figure 4A). A 1.3-kb portion of this EcoRI fragment, designated BE1300, was cloned by one-sided, nested-primer polymerase chain reaction (PCR) amplification. Figure 4B illustrates that fragment BE1300 extends from within the shorter *Mu1*-flanking region of the original cloned 2.0-kb BamHI fragment to one of the termini of the 6.0-kb EcoRI fragment. The nucleotide sequence of fragment BE1300 confirmed its overlap with the 2.0-kb BamHI fragment. Fragment BE1300 was then used as a probe to screen a maize endosperm λgt11 cDNA library.

A nearly full-length cDNA sequence of 6027 bp was obtained from three overlapping cDNA clones (Figure 4B). These clones were isolated from three consecutive rounds of screening of ~3×10^6 plaque-forming units (pfu). Plasmid pMg6Aa contains a 4.3-kb cDNA insert internal to the nearly full-length cDNA (nucleotides 1002 to 5367), and the cDNA inserts in plasmids pMgf10 (nucleotides 1 to 1657) and pMgt6-2M (nucleotides 4433 to 6027) overlap and extend the cDNA sequence in this central cDNA fragment at the 5′ and 3′ ends, respectively (Figure 4B). The resulting continuous sequence revealed an ATG-initiated coding sequence of 1674 codons (Figure 4B). Multiple stop codons in

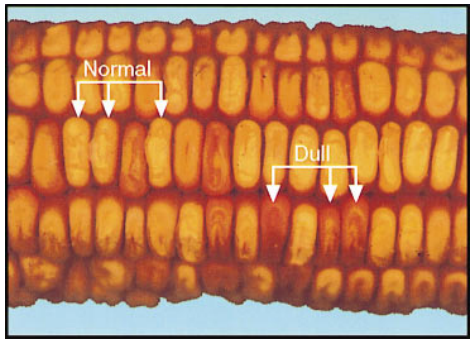

Figure 2. The Dull Mutant Phenotype.

The ear shown was obtained by self-pollination of a *du1-R2370::Mu1*/*Du1* heterozygote. Dull kernels and wild-type kernels are present at approximately the Mendelian frequency of 1:3, respectively.

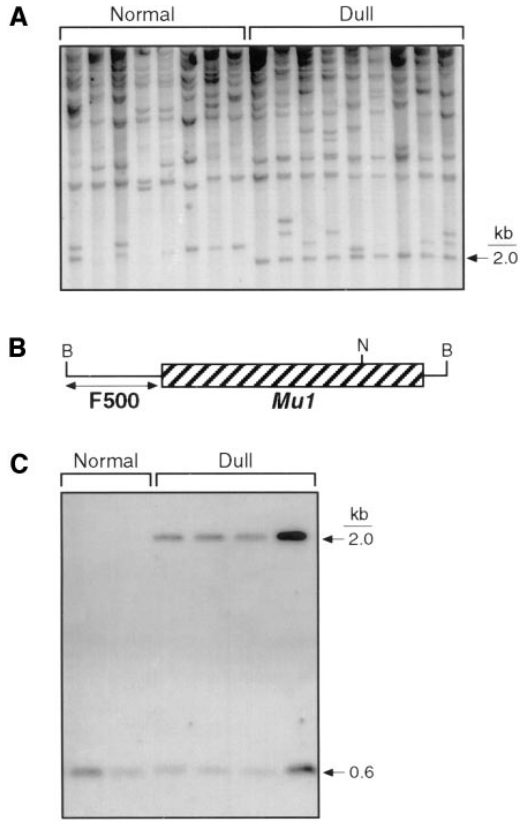

Figure 3. A *Mu1*-Containing BamHI Genomic DNA Fragment Co-segregates with *du1-R2370::Mu1.*

(A) Detection of *Mu1*-containing genomic DNA fragments. BamHI-digested genomic DNA of seedlings grown from segregating (1:1) nonmutant and dull sibling kernels was separated on a 1% agarose gel, blotted, and probed with the 960-bp internal MluI fragment of *Mu1* excised from plasmid pMJ9 (Barker et al., 1984). The length of the fragment indicated by the arrow was estimated by comparison with fragments of known sequence run in the same gel.

(B) Structure of the cloned 2.0-kb BamHI fragment. The hatched bar indicates the position of *Mu1* as determined by the nucleotide sequence of the cloned fragment. The position of the 500-bp probe fragment F500 is indicated. The diagram is drawn to scale. Restriction sites are indicated for BamHI (B) and NotI (N).

(C) Detection in genomic DNA of restriction fragments homologous to the cloned fragment. The analysis was conducted as given in **(A)**, except that the blot was hybridized with a single-stranded probe generated by PCR, using fragment F500 shown in **(B)** as the template. The lengths of the fragments indicated by arrows were estimated by comparison with fragments of known sequence run in the same gel.

all three reading frames at the 5′ end of the cDNA insert of pMgf10 indicate that the coding sequence begins within this fragment. The size of a DNA fragment amplified from total endosperm RNA by 3′ rapid amplification of cDNA ends indicated that the 3′ end of the cloned cDNA is very close to the polyadenylation site(s) of the corresponding transcript (data not shown). The cloned cDNA, therefore, is nearly full length and contains the entire coding sequence. This conclusion was supported further by the detection of a 6-kb transcript in nonmutant endosperm RNA using the cDNA insert of pMg6Aa as a probe, as described in a following section.

Verification of the Cloned cDNA as a Product of the *du1* Locus

Physical characterization of another independently isolated *du1* allele, *du1-R2649*, indicated that the cloned cDNA is coded for by the *du1* locus rather than by a different gene closely linked to *du1.* Genomic restriction fragments from sibling *du1-R2649*/*du1-Ref* and *Du1*/*du1-Ref* plants were probed with the cDNA insert of plasmid pMgf10. Figure 5 shows that a 6.6-kb SalI fragment was detected in all plants bearing *du1-R2649*, whereas a 5.2-kb fragment was the only one detected in the *Du1*/*du1-Ref* plants. The shift of 1.4-kb in the SalI fragment associated with *du1-R2649* most likely resulted from insertion of a *Mu1* element. This alteration is distinct from the one associated with *du1-R2370::Mu1*, because the probe that detects that polymorphism does not identify any abnormal fragment in *du1-R2649* mutants (data not shown). That two independent genomic rearrangements in the same locus coincide with appearance of the dull phenotype most likely is explained by *Mu1* insertions being the causative agents of the *du1*– mutations. The structure of *du1-R2370::Mu1* is also consistent with this conclusion. Figure 4B shows the intron–exon structure deduced by comparing the sequences of the cloned cDNA and genomic fragments. The *Mu1* insertion in the cloned 2.0-kb BamHI fragment is within an exon; thus, it is expected to generate a mutant *du1*– allele.

Characterization of Du1 mRNA Levels

Figure 6A shows that wild-type endosperm RNA contains a transcript of ~6.0 kb that hybridizes with the Du1 cDNA. The transcripts detected by this probe in *du1-Ref*, *du1-R2370::Mu1*, *du1-R2339*, or *du1-R2197* mutants were greatly reduced in abundance in endosperm harvested 20 days after pollination (DAP) (Figure 6A and data not shown). The residual transcripts in the *du1-R2370::Mu1* or *du1-R2339* endosperms were ~1.4 kb longer than normal (Figure 6A and data not shown), possibly resulting from transcriptional readthrough of a *Mu1* insertion. The residual transcripts in the *du1-Ref* and *du1-R2197* endosperms were the same size as the wild-type mRNA (Figure 6A and data not shown). That four independently isolated *du1*– mutant alleles, including *du1-Ref*, are associated with severely reduced expression of the transcript detected by the cDNA probe provides further evidence that the cloned gene is in fact *du1.*

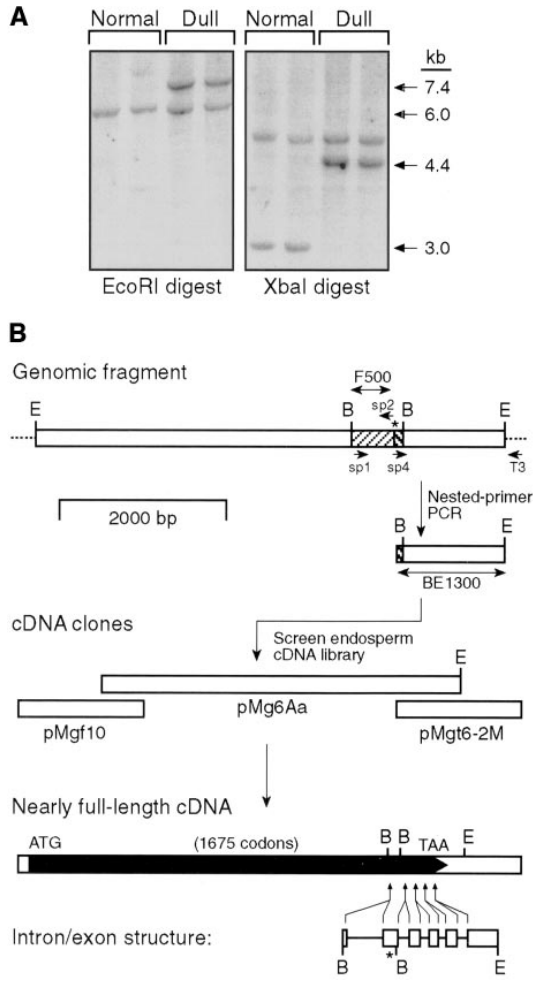

Figure 4. Isolation of a Nearly Full-Length Du1 cDNA Clone.

(A) Identification of genomic fragments containing regions flanking the *Mu1* element in the cloned 2.0-kb BamHI fragment. EcoRI- and XbaI-digested genomic DNA from *du1-R2370::Mu1*/*du1-Ref* mutants and sibling *Du1*/*du1-Ref* nonmutant seedlings was probed with fragment F500. The lengths of the fragments indicated by the arrows were estimated by comparison with fragments of known sequence run in the same gel.

(B) Illustration of the procedure for cloning the nearly full-length Du1 cDNA. Genomic fragment BE1300 was cloned using nested-primer PCR, as described in Methods. The wild-type counterpart of the original cloned BamHI fragment (indicated by hatched boxes) was shown to be part of a 6.0-kb EcoRI fragment that is indicated in **(A)**. A population of EcoRI genomic fragments of ~6.0 kb was ligated to pBluescript SK+ (dashed lines). The ligation mixture was used to amplify a 2.0-kb fragment with primers du1-sp1 and T3. Fragment BE1300 was then amplified from the 2.0-kb fragment by using primers du1-sp4 and T3. The position of the *Mu1* insertion in *du1- R2370::Mu1* is indicated by the asterisks. The positions of PCR primers used for fragment amplification are indicated. Restriction sites are indicated for EcoRI (E) and BamHI (B). The nearly full-length cDNA diagram represents the continuous sequence from the three overlapping cDNA fragments. The solid bar and arrow indicate the location and the 5′ to 3′ direction of the *Du1* coding sequence. The partial intron–exon structure was deduced by comparing the available genomic sequence with the cDNA sequence.

The 6.0-kb transcript present in wild-type endosperm appears to be completely absent in certain *du1*– mutants, for example, *du1-R2370::Mu1* (Figure 6A). It is unlikely, therefore, that a gene other than *Du1* produces an endosperm transcript of 6.0 kb that is detected by the cDNA probe, at least at the 20-DAP point of development. Accordingly, the 6.0-kb transcripts detected at relatively low levels in the *du1-Ref* and *du1-R2197* mutants must result from residual activity of the mutant *du1*– gene. The longer transcripts of 7.4 kb present in *du1-R2370::Mu1* or *du1-R2339* endosperms also are derived from residual transcription of the *du1* locus, because they are not detected in wild-type endosperm or other *du1*– mutants. That transcription of a mutant gene is reduced but not completely eliminated is typical of many maize mutations affecting endosperm starch biosynthesis (Giroux et al., 1994; James et al., 1995; Fisher et al., 1996).

The developmental period of Du1 mRNA accumulation coincides with that during which starch biosynthesis occurs (Figure 6A). Du1 transcripts were not detected in endosperm collected at 7 DAP. The Du1 mRNA level was maximal in endosperm at the early developmental age of ~12 DAP. The steady state level of the Du1 transcripts declined gradually over time, reaching a minimum for the starch biosynthetic period ~22 to 26 DAP. The Du1 transcript level increased once again in more mature endosperm of 32-DAP kernels. Figure 6B illustrates these transcript levels quantified relative to the amount of 26S rRNA present in each sample.

Figure 6C presents the results of a qualitative reverse transcriptase–PCR (RT-PCR) analysis designed to detect the presence of Du1 transcripts in various plant tissues. The sensitivity of this assay was demonstrated by the fact that Du1 mRNA present at very low levels in two *du1*– mutants (data not shown) was detected clearly by RT-PCR (Figure 6C). The expected 940-bp cDNA fragment was amplified from total endosperm RNA; this fragment was not amplified from RNase-digested total RNA from 22-DAP endosperm (Figure 6C), indicating that it was derived from mRNA rather than from contaminating genomic DNA. In DNA gel blot analysis, the 940-bp fragment hybridized with a Du1 cDNA probe under high-stringency conditions (data not shown). The additional fragment of ~500 bp did not hybridize with the Du1 cDNA probe (data not shown) and thus is a nonspecific amplification product. Complete absence of the 940-bp fragment after amplification of total leaf or root RNA (Figure 6C) indicated that *Du1* is not expressed in these tissues. Du1 mRNA appears to be present in embryos and tassels, however, as indicated by a positive RT-PCR signal (Figure 6C). Transcripts that hybridize with the Du1 cDNA also were detected in total tassel and embryo RNAs by using gel blot

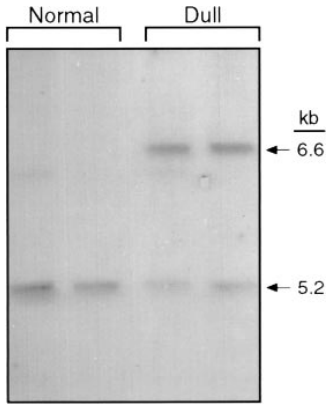

Figure 5. Physical Alteration of the Cloned Locus in Plants Bearing *du1-R4059.*

SalI-digested genomic DNA of seedlings grown from *du1-R2649*/*du1-Ref* mutant kernels and sibling *Du1*/*du1-Ref* nonmutant kernels was blotted and probed with the cDNA insert of pMgf10. The lengths of the fragments indicated by the arrows were estimated by comparison with fragments of known sequence run in the same gel.

analysis, although at low levels compared with endosperm (data not shown). The amplified fragments most likely are derived from transcription of *Du1*, as opposed to a heterologous gene, because the cDNA probe containing the amplified region detects only a single fragment in high-stringency DNA gel blots (data not shown).

Du1 Codes for a Putative Starch Synthase with Conserved and Unique Features

The amino acid sequence deduced from the cloned cDNA indicates that *Du1* codes for a starch synthase. The longest open reading frame of the continuous Du1 cDNA sequence codes for a polypeptide, designated DU1, with a predicted molecular mass of 188 kD and including a potential amyloplast transit peptide. Sequence similarity searches found that the deduced amino acid sequence of DU1 is most similar to that of the potato starch synthase SSIII (Abel et al., 1996; Marshall et al., 1996) among all proteins in the databases.

Figure 7A shows the alignment of the DU1 and SSIII deduced amino acid sequences, and Figure 7B indicates three discrete regions with varying degrees of similarity between the two proteins. The C-terminal regions, over a span of 645 amino acids (DU1 residues 1029 to 1674), share the highest degree of similarity in the alignment; 73% of the aligned residues are identical in these sequences, with only a single gap of one amino acid. In the central regions of DU1 and SSIII, corresponding to DU1 residues 770 to 1028, 51% of the 259 aligned residues are identical, with no gaps in the alignment. The remaining N-terminal region of DU1 (residues 1 to 769) has no significant similarity to that of potato SSIII or to any polypeptide sequence currently available in the databases. A 440-residue extension relative to SSIII is present in the DU1 N terminus.

Sequence comparisons with various cloned starch synthases and glycogen synthases indicate that the C-terminal region of DU1 beyond residue 1226 is likely to provide α(1,4)-glycosyltransferase activity. This stretch of $\sim$450 residues is similar to the corresponding amino acid sequence near the C termini of many distinct types of α(1,4)-glycosyltransferase, including fungal, mammalian, and bacterial glycogen synthases, pea GBSSI and GBSSII, and maize GBSSI. As an example, 28% of 438 aligned C-terminal residues are the same in both DU1 and *Escherichia coli* glycogen synthase (GenBank accession number P08323). Local similarity is much higher, for example, 67% of the 48 aligned residues of DU1 from positions 1550 to 1597 are identical in the corresponding region of the *E. coli* enzyme, with no gaps in the alignment (data not shown). Three short spans of amino acids within the C-terminal portion of DU1 fit the consensus sequence of the conserved regions identified by comparison of *E. coli* glycogen synthase with GBSSI from a wide variety of plant species (Figure 7A) (Preiss and Sivak, 1996). Thus, the 450 C-terminal residues of DU1 are highly likely to constitute the complete catalytic domain for a starch synthase. This speculation is further supported by the observation that the central regions of DU1 and SSIII, in which 51% of the amino acids are the same, have no significant similarity to any of the other cloned glycogen synthases or starch synthases. This exclusive sequence conservation, therefore, is expected to define functions belonging solely to a subgroup of plant starch synthases represented by SSIII and DU1. The unique 769-residue sequence at the N terminus of DU1 is expected to contain an amyloplast-targeting peptide and to define functions unique to this enzyme.

Two Groups of Repeats Are Present in the Unique N-Terminal Region of DU1

Figure 7A indicates two distinct groups of repeats comprising a total of 180 and 85 amino acids, respectively, that were identified in the unique N-terminal region of DU1 by intrasequence dot-plot analysis. Figure 8A shows that the group of 180 residues (positions 418 to 597) is a hierarchical repeat. This sequence contains three tandem repeats of 60 residues designated the “SBE superrepeat.” Each of these in turn is composed of six tandem repeats of 10 residues designated the “SBE repeat” (Figure 8A). The designation “SBE” in these names reflects the fact that the repeating unit is similar to a sequence found in all SBEs, as described in a following paragraph. This two-level repeating structure was deduced from the pattern of sequence conservation among the 18 SBE repeats. Each individual SBE repeat is most similar to the two repeats positioned either 60 or 120 residues distant (Figure 8A). Moreover, within single SBE

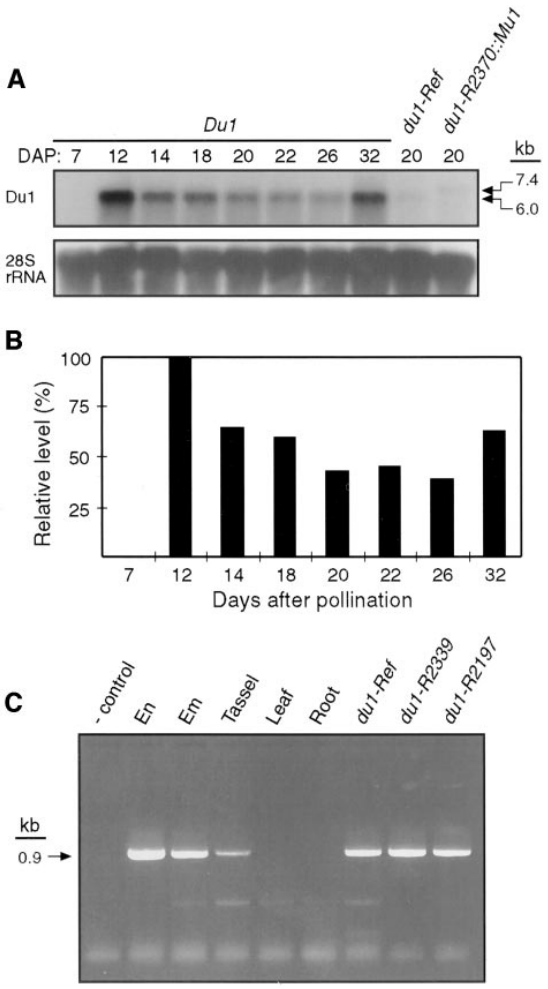

Figure 6. Du1 Transcript Levels.

(A) Gel blot analysis of total RNAs from developing endosperm. Total RNAs were extracted from endosperm of W64A kernels harvested at various developmental ages and from *du1-Ref* and *du1-R2370::Mu1* mutant kernels harvested at 20 DAP. The RNAs were fractionated on a formaldehyde–agarose gel, blotted, and probed with the cDNA insert in pMg6Aa (top). The lengths of the transcripts indicated by the arrows were estimated by comparison with RNA fragments of known sequence run in the same gel. Minor loading differences were calibrated by hybridization of the 28S rRNA on the same blot (stripped of the cDNA probe) with a tomato rRNA cDNA probe (bottom).

(B) Relative steady state level of the Du1 transcript in developing endosperm. Radioactivity of the cDNA probe hybridized with Du1 transcripts was measured by using a PhosphorImager, which was quantified using the program ImageQuant (Molecular Dynamics), and expressed as the percentage of the maximal signal strength on the same blot (relative level) after calibration of minor loading differences. The data represent the average of three repeats of the analysis with standard error $<10\%$.

(C) RT-PCR analysis. DNA fragments amplified from total RNAs by RT-PCR, using primers du1-F3 and du1-R1, were separated in an agarose gel and visualized by ethidium bromide staining. Endosperm (En) and embryo (Em) RNAs were from tissue collected 22 DAP. The lane designated -control is from the same sample as the En lane, except that the RNA was pretreated with RNase A before amplification. RNAs from the indicated *du1*– mutants were obtained from endosperm collected 22 DAP. The length of the fragment indicated by the arrows was estimated by comparison with fragments of known sequence run in the same gel.

superrepeats, each individual SBE repeat is always more similar to the repeat that precedes it in the N-terminal direction than to the one that follows it.

These patterns of sequence similarity strongly indicate a hierarchical repeating process involving duplication of the SBE superrepeat as a unit rather than as 18 individual repeating events. Each SBE repeat consists of two "half-repeats" of six and four residues, respectively, as deduced from (1) the different degrees of sequence conservation exhibited by the first and second half-repeats among all SBE repeats and (2) the presence of four residues between two complete SBE repeats (Figure 7A; residues 414 to 417), possibly resulting from an unequal crossover mechanism (Smith, 1976; Lewin, 1997).

The nature of the 180-residue repeat suggests that it is involved in a specific function of DU1. The SBE repeats that begin each SBE superrepeat are more similar to each other than to the SBE repeats at any of the other five positions in the superrepeat (Figure 8A). This suggests that these three SBE repeats were subjected to the highest selection pressure and thus may represent a functional domain. The consensus sequence among these three conserved SBE repeats is DQSIVG in the first half-repeat, designated as the "M-box," and SHKQ in the second half-repeat. The M-box sequence was found to match exactly with a sequence located in SBEI family members. Figure 8B shows that the M-box sequence is invariant in maize SBEI, pea SBEII, and wheat SBEI; the sequence also is conserved precisely in the rice branching enzyme (RBEI) and potato SBEI. The M-box is conserved, with substitutions of two residues of similar properties yielding the sequence DQALVG, in the corresponding region of SBEII family members, including maize SBEIIa and SBEIIb and pea SBEI (Figure 8B); the same sequence is found in rice RBEIII, wheat SBEII, and Arabidopsis SBE2.1 and SBE2.2. The DQALVG sequence also is present in glycogen branching enzymes from yeast and humans (Figure 8B).

Figure 8C shows that the smaller group of repeats of 85 residues in the N terminus of DU1 (amino acids 150 to 233) is composed of three tandem repeats of 28 residues. The basic repeating unit also consists of two halves, 12 and 16 residues each, which again are likely to have evolved via imperfect tandem duplications through an unequal crossover mechanism. This conclusion is supported by the distinct degree of sequence conservation of the two half-repeats among the three tandem repeats. The first half-repeat is

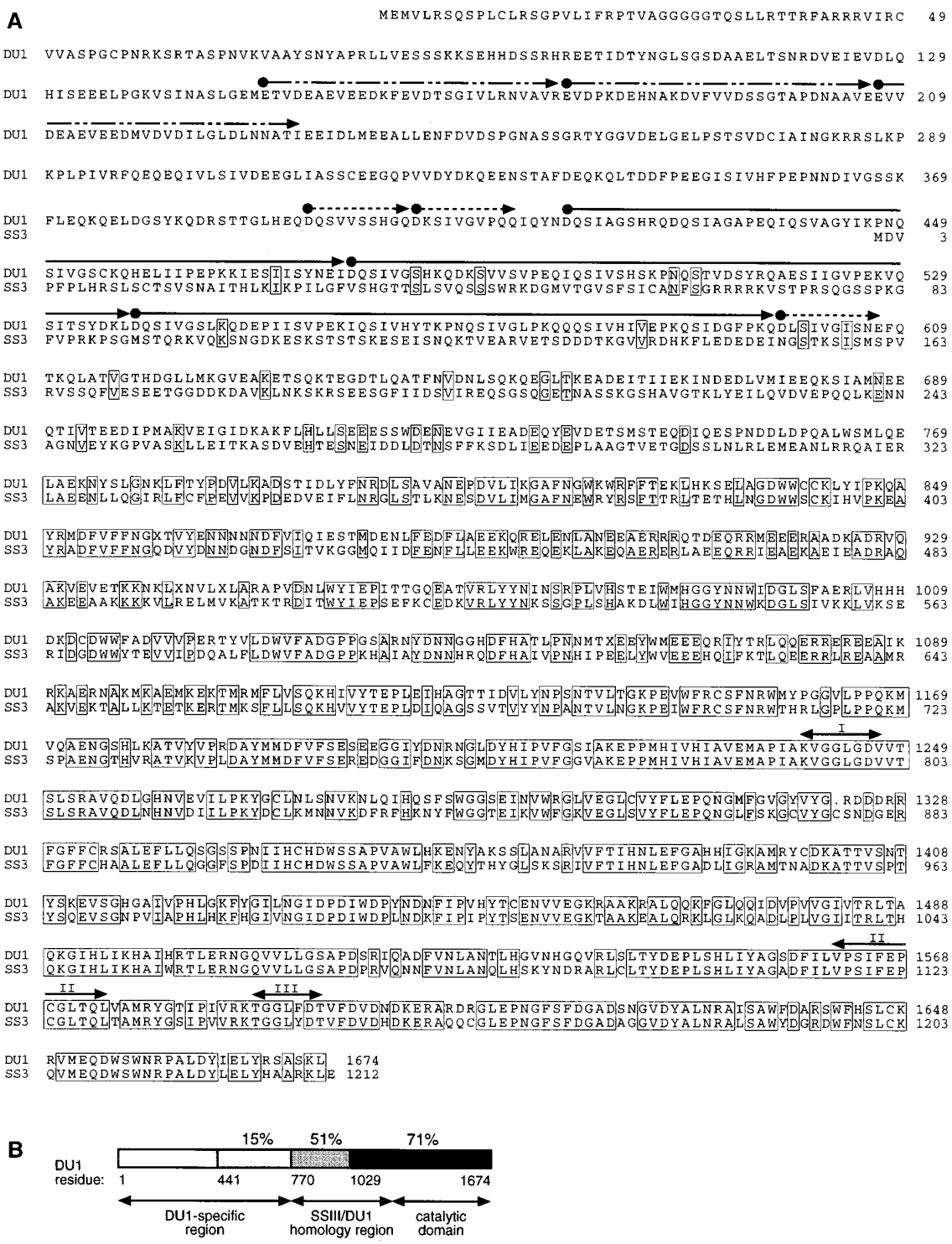

Figure 7. Comparison of the DU1 and Potato SSIII Amino Acid Sequences.

(A) Primary sequence alignment. The deduced amino acid sequences of DU1 (GenBank accession number AF023159) and potato SSIII (SS3; GenBank accession number X95759) are aligned. Boxed residues are the same in both polypeptides. Solid directional arrows indicate the positions of the three 60–amino acid SBE superrepeats. Dotted arrows denote individual copies of the SBE repeat. Dashed arrows indicate the posi-

highly conserved in the first and the third copies of the 28-residue repeat, whereas the second half is more conserved in the first and second copies of the repeats (Figure 8C).

DISCUSSION

The following four lines of evidence support the conclusion that the genomic locus cloned in this study is a portion of the *du1* gene. First, the cloned genomic interval is either within or tightly linked to the *du1* locus, because it cosegregated with the dull phenotype in 70 progeny plants. Second, two independent mutations of *du1* arose coincidentally with 1.4-kb insertions at distinct positions in the cloned transcription unit. One insertion is known to be a *Mu1* element located within an exon. Third, the transcript hybridizing with the cloned cDNA is reduced drastically to the same extent in endosperm of *du1-Ref* and three independently isolated *du1*– mutants. In two of these mutants, including the one with a characterized *Mu1* insertion, the residual transcript is 1.4 kb longer than the wild-type mRNA, which is consistent with insertion of a *Mu1* element in an exon. Fourth, the cloned gene codes for a putative starch synthase, which is consistent with the fact that *du1*– mutants have greatly reduced SSII activity.

The fact that DU1 possesses starch synthase activity is indicated by the extensive similarity of its predicted amino acid sequence to potato SSIII and by the similarity between the C-terminal residues of DU1 and a large group of phylogenetically diverse starch and glycogen synthases. Particularly striking are two regions that together comprise more than half of the deduced DU1 sequence of 1674 residues, which are 51 and 73% similar, respectively, with the corresponding regions of potato SSIII. Within a stretch of 450 amino acids at the C terminus of DU1, ~30% of the best-aligned residues are identical in comparisons with a wide variety of starch and glycogen synthases, suggesting the location of a domain within DU1 that provides α(1,4)-glycosyltransferase activity. Thus, five distinct starch synthase homologs have been identified in maize, including DU1, GBSSI (Shure et al., 1983; Klösgen et al., 1986), and the products of three recently identified cDNA clones (Harn et al., 1998; Knight et al., 1998).

The starch synthase coded for by *Du1* is likely to account for the soluble isozyme identified biochemically as SSII (Ozbun et al., 1971; Boyer and Preiss, 1981). The deduced molecular mass of DU1, including a potential transit peptide, is 188 kD, which is consistent with that of 180 kD reported for native SSII (Mu et al., 1994). The tissue-specific expression pattern of the Du1 mRNA also matches that of SSII. Du1 transcripts were undetectable in leaves either by RNA gel blot (data not shown) or RT-PCR analyses (Figure 6C). This expression pattern corresponds with the fact that no detectable SSII activity was present in leaf extracts (Dang and Boyer, 1988). Moreover, the activity of SSII, along with that of SBEIIa, was greatly reduced in *du1*– endosperm (Boyer and Preiss, 1981). The simplest explanation for these results is that the *du1* locus codes for SSII. Direct demonstration of the identity between DU1 and SSII, however, will require purification of the enzyme to homogeneity and determination of its polypeptide composition and partial amino acid sequence. Based on amino acid sequence similarity, DU1 is the counterpart of potato SSIII. The enzymes differ, however, in the structure of their N-terminal regions. Also, DU1 is expressed in specific tissues, whereas SSIII is expressed throughout the plant (Abel et al., 1996).

Two apparent discrepancies are known regarding the potential identity of DU1 and SSII. First, the size of the enzyme was reported initially as 95 kD (Boyer and Preiss, 1981), but subsequently an activity with the same enzymatic properties was ascribed to a 180-kD protein (Mu et al., 1994). Second, the total starch synthase activity in crude endosperm extracts was not decreased in *du1*– mutants (Singletary et al., 1997), even though there was a major reduction of the SSII activity peak in fractionated mutant extracts (Boyer and Preiss, 1981). Even though the explanation for these discrepancies is not yet obvious, the fact that *du1*– mutants produce endosperm starch with abnormal structure provides definitive evidence that the affected protein, presumably SSII, plays a significant role in starch biosynthesis.

Du1 transcripts accumulate in endosperm during the time of starch biosynthesis, as expected for an mRNA that codes for a starch biosynthetic enzyme. That the highest level of accumulation was observed relatively early in the starch biosynthetic period suggests the possibility that DU1 provides a specialized starch synthase function during specific periods of kernel development. Further developmental analysis

Figure 7. (continued).

tions of the three repeat units that make up the 85-residue repeat. Double-headed arrows labeled with roman numerals indicate the positions of correspondingly designated conserved sequence blocks identified in the glucan synthase family (Preiss and Sivak, 1996).

(B) Domains of DU1. Similarity scores between each segment of DU1 and SSIII are shown above each region. “Catalytic domain” indicates the region of DU1 similar in amino acid sequence to $\alpha(1\rightarrow4)$–glycosyltransferases in general. The SSIII/DU1 homology domain indicates the region shared specifically by DU1 and SSIII among known proteins. “DU1-specific region” indicates the portion of DU1 that is unique in amino acid sequence among known proteins.

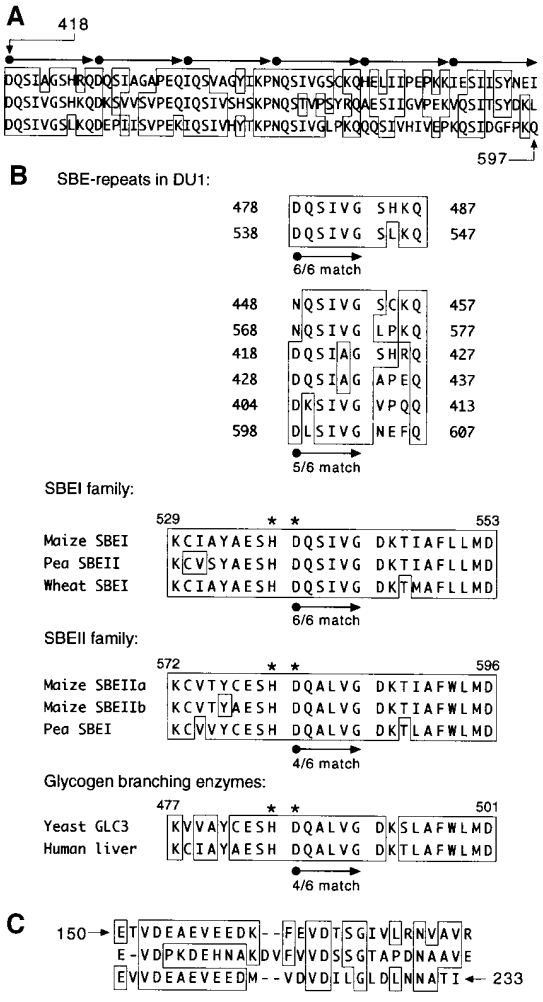

Figure 8. Repeats in the N Terminus of DU1.

(A) Alignment of the SBE superrepeats. Numbers refer to positions of residues within the DU1 coding sequence. Each row represents one complete copy of the SBE superrepeat. Boxed residues are the same in two or three copies of the SBE superrepeat. Each 60-residue SBE superrepeat comprises six copies of the 10–amino acid SBE repeat (indicated by arrows). The degree of sequence conservation between each SBE repeat descends toward the C terminus of each SBE superrepeat.

(B) Alignment of selected copies of the SBE repeat and conservation of the M-box within branching enzymes. In the first grouping, numbers refer to positions within the DU1 coding sequence. Boxed residues are identical to the consensus sequence of the SBE repeat. Arrows indicate the M-box sequence (DQSIVG). The M-box sequence is almost completely conserved in members of the SBEI family, including maize SBEI (GenBank accession number D11081), pea SBEII (GenBank accession number X80010), and wheat SBEI (GenBank accession number Y12320). The M-box sequence is also well conserved in members of the SBEII family and glycogen branching enzymes, including maize SBEIIa (Gao et al., 1997), maize SBEIIb (GenBank accession number L08065), pea SBEI (GenBank accession number X80009), glycogen branching enzyme from human liver (GenBank accession number D29685), and the yeast glycogen branching enzyme (the *GLC3* product; GenBank accession number M76739). In these instances, there is substitution of two residues of similar properties. Residue numbers refer to the first enzyme in each group. Arrows indicate the occurrence of M-box sequences or related sequences. Asterisks indicate conserved residues that in amylolytic enzymes of determined structure are thought to be part of the active site.

(C) Sequence conservation of the 28–amino acid repeat. The three repeats within the 85-residue repeat region are aligned to show the pattern of sequence conservation among the two portions of the 28-residue basic repeat unit. Numbers refer to positions within the DU1 coding sequence. Dashes indicate gaps in the alignment.

in different genetic backgrounds and environmental conditions is required, however, to assess the significance of the expression pattern reported here. That Du1 mRNA is detected in endosperm but not leaf tissue indicates a specific function of DU1 in storage starch synthesis that is not utilized for production of transient starch.

This characterization of DU1 implies that the phenotypic effects of *du1*– mutations, including changes in starch structure, deficiencies of both SSII and SBEIIa, and genetic interactions with *ae*–, *su1*–, *su2*–, and *wx*– mutations, all result either directly or indirectly from alteration of a starch synthase. The reduction of SBEIIa activity in *du1*– mutant endosperm could result secondarily from the SSII deficiency because of physical interaction between the two proteins. Loss of such an interaction due to a *du1*– mutation might result in abnormally rapid proteolytic turnover of SBEIIa or prevent branching enzyme activity by some other means. This general explanation is consistent with the fact that peak activities of both SSII and SBEIIa coincide in the same anion exchange column fractions (Boyer and Preiss, 1978, 1981; Dang and Boyer, 1988). Another possible explanation is that expression of the *Sbe2a* gene in *du1*– mutant endosperm is inhibited as a more indirect consequence of the deficiency in SSII, for example, through a transcriptional regulatory mechanism.

Mutations of the *ST-3* gene of the unicellular chlorophyte Chlamydomonas cause deficiency in a starch synthase with enzymatic properties generally similar to maize SSII (Fontaine et al., 1993). Starch from *st-3*– mutants is similar to that of maize *du1*– mutants in that the apparent amylose percentage is increased and amylopectin structure is altered in part by increased branch frequency. Amylopectin from *du1*– kernels, however, does not exhibit the marked decrease in intermediate-size glucan chains characteristic of the *st-3*– mutants (Inouchi et al., 1987; Fontaine et al., 1993; Wang et al., 1993a, 1993b). More detailed comparisons of the structures of *du1*– maize starch to *st-3*– Chlamydomonas starch (Buléon et al., 1997) and of the chlorophyte SSII to the anal-

ogous maize enzyme are likely to provide further insights into the roles of SSII in amylopectin synthesis.

Conservation of the M-box sequence that is repeated in DU1 specifically in starch and glycogen branching enzymes from phylogentically divergent species is particularly striking, considering that SBEs and starch synthases act in a concerted biosynthetic pathway. Molecular modeling based on the known structures of several members of the α-amylase enzyme family, which includes the SBEs and glycogen branching enzymes, indicates that the M-box sequence constitutes the loop between β strand 7 and α helix 7 in the $(\alpha/\beta)_8$ structural motif (Jespersen et al., 1993). Parts of this loop interact with residues distant in the primary sequence to form the active site, and the aspartic acid residue of the M-box and the strictly conserved histidine residue preceeding it (Figure 8B) are thought to be directly involved in catalysis and substrate binding, respectively, in these amylolytic enzymes (Jespersen et al., 1993; Kuriki et al., 1996). The M-box sequence in SBEs, therefore, must be accessible from the exterior of the enzyme. This suggests a possible mechanism by which the M-boxes might mediate direct interaction of DU1 with one or more SBE. An interaction could occur if the M-box region within the SBE were displaced from the active site and replaced by the same or similar sequence from the N terminus of DU1.

Identification of the primary lesion in *du1*– mutants is expected to facilitate assessment of the contributions of SSII and SBEIIa to amylopectin synthesis. Any structural abnormality of starch in *du1*– mutants, however, may not result solely from the deficiency of DU1, because both SSII and SBEIIa are affected. The fact that the dull mutant phenotype arises from a primary lesion in a starch synthase, most likely SSII, and resultant reduction of SBEIIa raises the question of whether the same mutant phenotype might result from a primary effect on SBEIIa and resultant reduction of SSII. Thus, the contributions of both SSII and SBEIIa to amylopectin synthesis can be assessed accurately by future studies that focus not only on SSII or SBEIIa but also on their interactions during the production of starch.

METHODS

Nomenclature, Plant Materials, and Isolation of *Mutator*-Induced *du1*– Mutations

The nomenclature follows the standard maize genetics format (Beavis et al., 1995). Alleles beginning with a capital letter indicate a functional non-mutant form of the gene (e.g., *Du1*). Unspecified mutant alleles are indicated by dashes with no following designation (e.g., *du1*–). Gene products are indicated by unitalicized uppercase letters (e.g., DU1). Transcripts and cDNAs are indicated by the unitalicized gene symbol (e.g., Du1).

Standard lines used in this study were the F_1 hybrids B77/B79 or Q66/Q67. These products of four inbred lines have no history of *Mutator* (*Mu*) activity. The *Mu*-active parents used in the mutant isolation scheme were those described by Robertson (1978). Maize inbred line W64A was used for detection of the Du1 transcript in kernels and other tissues.

Mutant alleles *du1-R2197*, *du1-R2339*, *du1-R2649*, *du1-R2370::Mu1*, *du1-R4059*, and *du1R-1178* were identified from the ears of self-pollinated F_1 plants 87-2197-9, 87-2339-2, 87-88-2649-11, 87-2370-20, 82-4059-23, and 89-1178-3, respectively (Figure 1). Inclusion of the letter *R* in the allele names indicates that the stocks originally are from the laboratory of Donald S. Robertson, and inclusion of the term Mu1 in allele name *du1-R2370::Mu1* indicates that this transposon has been identified definitively within the mutant gene. Stock number X10A from the Maize Genetics Cooperation Stock Center (Urbana, IL) was used for complementation tests and to generate segregating populations (Figure 1). It is homozygous for the reference allele *du1-Ref.*

Cloning of the 2.0-kb BamHI Fragment That Cosegregates with the Dull Phenotype

The methods used for genomic DNA extraction and DNA gel blot analysis were described previously (James et al., 1995). Most probes were ^{32}P-labeled by the standard random-primed method (Sambrook et al., 1989). The 2.0-kb BamHI fragment that contains *Mu1* and cosegregates with *du1-R2370::Mu1* was isolated from a size-selected λZAPII express (Stratagene, La Jolla, CA) library constructed from BamHI-digested genomic DNA from a *du1-R2370::Mu1*/*du1-Ref* plant, essentially as described previously (James et al., 1995), and subcloned into pBluescript SK+ (Stratagene) to form plasmid pJW3. Fragment F500 (Figure 3B) was amplified for use as a probe by polymerase chain reaction (PCR) from pJW3, using primers du1-sp1 (5′-GTACAATGACAACTTTATCCC-3′) and du1-sp2 (5′-CATTCTCAC-AAGTGTAGTGGACC-3′). The single-stranded ^{32}P-labeled F500 probe was generated by PCR, using primer du-sp1 and the gel-purified BamHI fragment from pJW3 as a template, according to Konat et al. (1994).

Cloning of Genomic Fragment BE1300 by Nested Primer PCR

For PCR amplification of a longer genomic fragment overlapping the sequence flanking the *Mu1* element in the 2.0-kb BamHI fragment, size-selected fragments were prepared from 80 μg of EcoRI-digested genomic DNA of sibling wild-type plants (*Du1*/*du1-Ref*; see Figure 1) fractionated on a 0.5% preparative agarose gel. Five fractions of EcoRI fragments were isolated by electroelution (Sambrook et al., 1989) from consecutive gel slices bracketing the 6.0-kb marker. These fractions were checked for presence of the *Mu1*-flanking sequences in the original cloned BamHI fragment by PCR, using primers du1-sp1 and du1-sp2. The DNA in the two fractions containing the highest amounts of the fragment were joined in a standard 20-μL ligation reaction to EcoRI-linearized pBluescript SK+. A 1-μL portion of each ligation mixture was used directly for PCR amplification of the region overlapping the cloned BamHI fragment with primer du1-sp1 or du1-sp2 in combination with primer T3 or T7 from pBluescript SK+. A fragment of ~2.0 kb amplified by the primer pair du1-sp1 and T3 was confirmed as containing the BamHI fragment by subsequent PCR amplification, using primers du1-sp1 and du1-sp2. This fragment was used as the template for PCR

amplification, using the nested primer du1-sp4 (Figure 4B) (5′-GTCGTAGGAATCGTCACTCG-3′) and primer T3. The specifically amplified 1.3-kb fragment was polished with T4 DNA polymerase, digested with EcoRI to remove the remaining vector sequence, and cloned into the EcoRV and EcoRI sites of pBluescript SK+ to form plasmid pMg1A.

cDNA Library Screen

Random-primed maize endosperm cDNA libraries in λgt11 were kindly provided by K. Cone (University of Missouri, Columbia). Standard procedures were followed for preparation of phage lifts, phage amplification, and single-plaque purification (Ausubel et al., 1989; Sambrook et al., 1989). Phage lifts were hybridized at 65°C for 16 to 18 hr with probes labeled with ^{32}P-dCTP by the random-primed method and washed under high-stringency conditions, as described by Church and Gilbert (1984). cDNA inserts in phage clones were subcloned in pBluescript SK+ or pBluescript KS+ from phage DNAs prepared by the Wizard DNA purification kit (Promega).

cDNA inserts in purified phage were characterized regarding their length by direct PCR amplification from disrupted phage, using primers λ1030 (5′-ATTGGTGGCGACGACTCCTG-3′) and λ1356 (5′-GTGTGGGGGTGATGGCTTCC-3′). Their sequences are located 19 bp proximal to the EcoRI cloning site in the left arm and 281 bp distal to EcoRI site in the *LacZ′* region of the right arm in λgt11 phage DNA, respectively. An aliquot of homogeneous purified phage (1 μL of 10^{10} plaque-forming units [pfu] per μL suspension) was disrupted for 15 to 20 min at 96°C in 20 μL of optimal PCR buffer (10 mM Tris-HCl, pH 9.2, 1.5 mM $MgCl_2$, 25 mM KCl) containing 0.2 μM each of the two primers and 0.2 mM each of four deoxynucleotide triphosphates. PCR amplification of the cDNA inserts typically was as follows: 94°C for 4 min for one cycle (with 1 unit of Taq DNA polymerase added at the end); 10 cycles of 58°C for 45 sec, 72°C for 0.5 to 3 min (depending on the insert size), and 94°C for 45 sec; 20 cycles of 61°C for 1 min, 72°C for 0.5 to 3 min (depending on the insert size), and 94°C for 1 min; and one cycle of 61°C for 5 min and 72°C for 7 min. Lengths of cDNA inserts were determined by gel electrophoresis of 5 to 10 μL of the PCR products.

Details of the cDNA library screen are as follows. In the first round, ~340 positive signals were obtained in primary screening of ~0.5×10^6 pfu, using fragment BE1300 as a probe. The longest cDNA insert among 15 further purified and characterized clones was 3.2 kb (nucleotides 2577 to 5782 in the nearly full-length sequence). This insert was subcloned as two EcoRI fragments in plasmids pMg271L and pMg271S containing the 2.7-kb cDNA at the 5′ end and the 0.5-kb cDNA at the 3′ end, respectively. In the second round, the 0.5-kb EcoRI-ScaI fragment at the extreme 5′ end of the 2.7-kb cDNA insert in pMg271L and the 0.5-kb EcoRI fragment of pMg271S were used separately as probes in the primary screening of an additional 1.5×10^6 pfu of phage.

The longest insert identified by the 5′ end probe in one of 24 purified and characterized phage clones, 4.3 kb, was subcloned in plasmid pMg6Aa. The probe from pMg271S identified an ~4.0-kb cDNA insert containing a 3′ end EcoRI fragment of 0.67 kb that overlapped with and extended the original cloned 3′ end fragment. The 1.4-kb portion from the 3′ end of this 4.0-kb cDNA insert was amplified by PCR directly from purified phage and cloned as a BamHI-HindIII fragment in pMgt6-2M. The original terminal EcoRI site was mutated to a HindIII site during PCR amplification to facilitate subsequent reconstruction of the complete cDNA. The BamHI fragment of 240 bp at the 5′ end of the cDNA in pMg6Aa was then used as a probe for the primary screening of another 10^6 pfu in the third round. Among 19 purified and characterized phage clones, the cDNA insert that overlapped with the insert in pMg6Aa and containing the longest extension, of ~1.5 kb at the 5′ end, was subcloned in plasmid pMgf10. The continuous sequence of three overlapping cDNA fragments in plasmids pMgf10, pMg6Aa, and pMgt6-2M represents the nearly full-length cDNA sequence (Figure 4B).

RNA Gel Blot Analyses and Reverse Transcriptase–PCR

Extraction of total RNA from various tissues of maize inbred W64A and RNA gel blot analysis was essentially as previously described (Gao et al., 1996). Radioactivity of the cDNA probe hybridized with Du1 transcripts was detected and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and expressed as the percentage of the maximal signal strength on the same blot. Minor loading differences among samples on each blot were calibrated using a tomato cDNA probe hybridizing to the 26S rRNA in the appropriate lane to normalize the Du1 mRNA signal strength.

The Titan reverse transcriptase (RT)–PCR system (Boehringer Mannheim) was used for the RT-PCR assay. The primers were du1-F3 (5′-ATAAATGTGTGGCGTGGACT-3′) and du-R1 (5′-CGTTCCTTGTCATTGTCCAC-3′). Thus, the amplified fragment comprised the 934-bp cDNA region from nucleotide 3997 to nucleotide 4930. Total RNA (1 μg) from various samples was used as the template. To distinguish RT-PCR amplification of mRNA from PCR amplification of potential residual genomic DNA, total RNA from one of the samples (22 days after pollination [DAP] endosperm) was treated with RNase A (100 ng/mL) for 10 min at 37°C before its use as a template. The RT-PCR products were analyzed on a 1% agarose gel and then blotted and hybridized using the cDNA insert of pMg6Aa as the probe to confirm the identity of the product.

Nucleic Acid Sequences and Computational Analysis

Nucleotide sequences were obtained using the ABI Prism automated sequencing system (Perkin-Elmer, Foster City, CA) at the Iowa State University Nucleic Acid Sequencing and Synthesis Facility (Ames, IA). Double-stranded plasmids were used as templates. All nucleotide sequences were confirmed by analysis of both strands. The available genomic sequence from within the *du1* locus (Figure 4B) has GenBank accession number AF023160. The nucleotide sequence of the nearly full-length Du1 cDNA has GenBank accession number AF023159. Computational analyses were performed using the Wisconsin Package (Genetics Computer Group, Madison, WI) and the Lasergene software package (DNASTAR Inc., Madison, WI).

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REFERENCES

Abel, G.J.W., Springer, F., Willmitzer, L., and Kossmann, J. (1996). Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). Plant J. **10,** 981–991.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K., eds (1989). Current Protocols in Molecular Biology. (New York: John Wiley and Sons).

Bae, J.M., Giroux, M., and Hannah, L. (1990). Cloning and characterization of the *brittle-2* gene of maize. Maydica **35,** 317–322.

Ball, S., Guan, H.-P., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P., and Preiss, J. (1996). From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. Cell **86,** 349–352.

Barker, R.F., Thompson, D.V., Talbot, D.R., Swanson, J., and Bennetzen, J.L. (1984). Nucleotide sequence of the maize transposable element *Mu1.* Nucleic Acids Res. **12,** 5955–5967.

Beavis, W., Berlyn, M., Burr, B., Chandler, V., Coe, E., Fauron, C., Nelson, O., Polacco, M., Rodermel, S., Sachs, M., and Wetzel, C. (1995). A standard for maize genetics nomenclature. Maize Genet. Coop. Newsl. **69,** 182–184.

Bhave, M.R., Lawrence, S., Barton, C., and Hannah, L.C. (1990). Identification and molecular characterization of *Shrunken-2* cDNA clones of maize. Plant Cell **2,** 581–588.

Boyer, C.D., and Preiss, J. (1978). Multiple forms of (1,4)-α-D-glucan-6-glucosyl transferase from developing *Zea mays* L. kernels. Carbohydr. Res. **61,** 321–334.

Boyer, C.D., and Preiss, J. (1981). Evidence for independent genetic control of the multiple forms of maize endosperm branching enzymes and starch synthases. Plant Physiol. **67,** 1141–1145.

Buléon, A., Gallant, D.J., Bouchet, B., Mouille, G., D'Hulst, C., Kossman, J., and Ball, S. (1997). Starches from A to C. *Chlamydomonas reinhardtii* as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal. Plant Physiol. **115,** 949–957.

Cao, H., and Shannon, J.C. (1997). BT1, a possible adenylate translocator, is developmentally expressed in maize endosperm but not detected in starchy tissues from several other species. Physiol. Plant. **100,** 400–406.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Creech, R.G. (1965). Genetic control of carbohydrate synthesis in maize endosperm. Genetics **52,** 1175–1186.

Creech, R.G., and McArdle, F.J. (1966). Gene interaction for quantitative changes in carbohydrates in maize kernels. Crop Sci. **6,** 192–194.

Dang, P.L., and Boyer, C.D. (1988). Maize leaf and kernel starch synthases and starch branching enzymes. Phytochemistry **27,** 1255–1259.

Davis, J.H., Kramer, H.H., and Whistler, R.L. (1955). Expression of the gene *du* in the endosperm of maize. Agron. J. **47,** 232–235.

Dvonch, W., Kramer, H.H., and Whistler, R.L. (1951). Polysaccharides of high amylose corn. Cereal Chem. **28,** 270–280.

Fisher, D.K., Boyer, C.D., and Hannah, L.C. (1993). Starch branching enzyme II from maize endosperm. Plant Physiol. **102,** 1045–1046.

Fisher, D.K., Kim, K.-N., Gao, M., Boyer, C.D., and Guiltinan, M.J. (1995). A cDNA encoding starch branching enzyme I from maize endosperm. Plant Physiol. **108,** 1313–1314.

Fisher, D.K., Gao, M., Kim, K.-N., Boyer, C.D., and Guiltinan, M.J. (1996). Allelic analysis of the maize (*Zea mays* L.) amylose-extender locus suggests that independent genes encode starch branching enzymes IIa and IIb. Plant Physiol. **110,** 611–619.

Fontaine, T., D'Hulst, C., Maddelein, M.-L., Routier, F., Pépin, T.M., Decq, A., Wieruszeski, J.-M., Delrue, B., Van den Koornhuyse, N., Bossu, J.-P., Fournet, B., and Ball, S. (1993). Toward an understanding of the biogenesis of the starch granule: Evidence that *Chlamydomonas* soluble starch synthase II controls the synthesis of intermediate size glucans of amylopectin. J. Biol. Chem. **268,** 16223–16230.

French, D. (1984). Organization of starch granules. In Starch: Chemistry and Technology, R.L. Whistler, J.N. BeMiller, and E.F. Paschall, eds (Orlando, FL: Academic Press), pp. 183–247.

Gao, M., Fisher, D.K., Kim, K.-N., Shannon, J.C., and Guiltinan, M.J. (1996). Evolutionary conservation and expression patterns of maize starch branching enzyme I and IIb genes suggests isoform specialization. Plant Mol. Biol. **30,** 1223–1232.

Gao, M., Fisher, D.K., Kim, K.-N., Shannon, J.C., and Guiltinan, M.J. (1997). Independent genetic control of maize starch-branching enzymes IIa and IIb. Plant Physiol. **114,** 69–78.

Giroux, M.J., Boyer, C., Feix, G., and Hannah, L.C. (1994). Coordinated transcriptional regulation of storage product genes in the maize endosperm. Plant Physiol. **106,** 713–722.

Hannah, L.C., Giroux, M., and Boyer, C.D. (1993). Biotechnological modification for sweet corn and maize improvement. Sci. Hortic. **55,** 177–197.

Harn, C., Knight, M.E., Ramakrishnan, A., Guan, H.P., Keeling, P.L., and Wasserman, B.P. (1998). Isolation and characterization of the SS2 starch synthase cDNA clones from maize endosperm. Plant Mol. Biol., in press.

Inouchi, N., Glover, D.V., and Fuwa, H. (1987). Chain length distribution of amylopectins of several single mutants and the normal counterpart, and sugary-1 phytoglycogen in maize. Starch/ Staerke **39,** 259–265.

James, M.G., Robertson, D.S., and Myers, A.M. (1995). Characterization of the maize gene *sugary1*, a determinant of starch composition in kernels. Plant Cell **7,** 417–429.

Jespersen, H.M., MacGregor, E.A., Henrissat, B., Sierks, M.R., and Svensson, B. (1993). Starch- and glycogen-debranching and branching enzymes: Prediction of structural features of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. J. Prot. Chem. **12,** 791–805.

Klösgen, R.B., Gierl, A., Schwarz-Sommer, Z., and Saedler, H. (1986). Molecular analysis of the *waxy* locus of *Zea mays.* Mol. Gen. Genet. **203,** 237–244.

Knight, M.E., Harn, C., Lilley, C.E.R., Guan, H.P., Singletary, G., Mu-Forster, C., Wasserman, B.P., and Keeling, P.L. (1998). Molecular cloning of starch synthase I from maize (W64A) endosperm and expression in *E. coli.* Plant J., in press.

Konat, G.W., Laszkiewicz, I., Grubinska, B., and Wiggins, R. (1994). Generation of labeled DNA probe by PCR. In PCR Technology:

Current Innovations, H.G.G. Griffin and M. Annette, eds (Boca Raton, FL: CRC Press), pp. 37–42.

Kuriki, T., Guan, H., Sivak, M., and Preiss, J. (1996). Analysis of the active center of branching enzyme II from maize endosperm. J. Prot. Chem. **15,** 305–313.

Lewin, B. (1997). Genes VI. (Oxford, UK: Oxford University Press).

Mangelsdorf, P.C. (1947). The inheritance of amylaceous sugary endosperm and its derivatives in maize. Genetics **32,** 448–458.

Manners, D.J. (1989). Recent developments in our understanding of amylopectin structure. Carbohydr. Polym. **11,** 87–112.

Marshall, J., Sidebottom, C., Debet, M., Martin, C., Smith, A.M., and Edwards, A. (1996). Identification of the major starch synthase in the soluble fraction of potato tubers. Plant Cell **8,** 1121–1135.

Martin, C., and Smith, A.M. (1995). Starch biosynthesis. Plant Cell **7,** 971–985.

Mu, C., Harn, C., Ko, Y.-T., Singletary, G.W., Keeling, P.L., and Wasserman, B.P. (1994). Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. Plant J. **6,** 151–159.

Nelson, O.E., and Pan, D. (1995). Starch synthesis in maize endosperms. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 475–496.

Ozbun, J.L., Hawker, J.S., and Preiss, J. (1971). Adenosine diphosphoglucose–starch glucosyl transferases from developing kernels of *waxy* maize. Plant Physiol. **78,** 765–769.

Preiss, J. (1991). Biology and molecular biology of starch synthesis and its regulation. Oxf. Surv. Plant Mol. Cell Biol. **7,** 59–114.

Preiss, J., and Sivak, M. (1996). Starch synthesis in sinks and sources. In Photoassimilate Distribution in Plants and Crops, E. Zamski and A.A. Schaffer, eds (New York: Marcell Dekker), pp. 63–96.

Robertson, D.S. (1978). Characterization of a mutator system in maize. Mutat. Res. **51,** 21–28.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Shannon, J.C., and Garwood, D.L. (1984). Genetics and physiology of starch development. In Starch: Chemistry and Technology, R.L. Whistler, J.N. BeMiller, and E.F. Paschall, eds (Orlando, FL: Academic Press), pp. 25–86.

Shannon, J.C., Pien, F.-M., and Liu, K.-C. (1996). Nucleotides and nucleotide sugars in developing maize (*Zea mays* L.) endosperms: Synthesis of ADP-glucose in *brittle-1.* Plant Physiol. **110,** 835–843.

Shure, M., Wessler, S., and Fedoroff, N. (1983). Molecular identification and isolation of the *waxy* locus in maize. Cell **35,** 225–233.

Singletary, G.W., Banisadr, R., and Keeling, P.L. (1997). Influence of gene dosage on carbohydrate synthesis and enzymatic activities in endosperm of starch-deficient mutants of maize. Plant Physiol. **113,** 293–304.

Smith, A.M., Denyer, K., and Martin, C. (1997). The synthesis of the starch granule. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 67–87.

Smith, G.P. (1976). Evolution of repeated DNA sequences by unequal crossing over. Science **191,** 525–535.

Stinard, P.S., Robertson, D.S., and Schnable, P.S. (1993). Genetic isolation, cloning, and analysis of a *Mutator*-induced, dominant antimorph of the maize *amylose extender1* locus. Plant Cell **5,** 1555–1566.

Sullivan, T.D., Strelow, L.I., Illingworth, C.A., Phillips, R.L., and Nelson, O.E., Jr. (1991). Analysis of maize *Brittle-1* alleles and a defective *Suppressor-mutator*–induced mutable allele. Plant Cell **3,** 1337–1348.

Wang, Y.-J., White, P., Pollak, L., and Jane, J.-L. (1993a). Amylopectin and intermediate materials in starches from mutant genotypes of the Oh43 inbred line. Cereal Chem. **70,** 521–525.

Wang, Y.-J., White, P., Pollak, L., and Jane, J.-L. (1993b). Characterization of starch structures of 17 maize endosperm mutant genotypes with Oh43 inbred line background. Cereal Chem. **70,** 171–179.