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Identification of a Promoter Sequence from the *BETL1* Gene Cluster Able to Confer Transfer-Cell-Specific Expression in Transgenic Maize[1]

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The maize (*Zea mays* L.) *betl1* locus, encoding a basal endosperm transfer layer-specific protein, has been mapped and molecularly cloned in its entirety. The locus is shown to consist of three gene copies in the maize inbred line A69Y. To distinguish the three transcription units from the locus name, we have termed them *BETL1a, BETL1b,* and *BETL1c*. Two of the copies are expressed, whereas one is inactive and contains retrotransposon-like insertions in both promoter and intron regions. Based on this information, and a restriction site map covering 17 kb around the *BETL1* locus, a DNA fragment putatively containing an active promoter sequence was identified. This fragment was tested for its ability to confer transfer-cell-specific expression in transient and stably transformed maize tissues. The transgenic maize plants obtained showed the predicted cell-type specificity of expression restricted to the basal endosperm transfer cells, although there were minor deviations in promoter strength and timing and accumulation of the transgene product from the corresponding BETL-1 endogene expression pattern.

The endosperm is the main storage organ in maize seeds, nourishing the embryo while the seed develops and providing nutrients to the seedling on germination. Thus, the uptake of assimilates by the growing endosperm is a critical process in seed development. There are no symplastic connections between maternal and embryonic tissues (Thorne, 1985); instead, phloem unloading releases nutrients into the apoplastic compartment of the pedicel. Uptake of nutrients by the endosperm from the pedicel is facilitated by the conversion of the cells at the base of the endosperm to transfer cells (Davis et al., 1990). Basal endosperm transfer cells possess anatomical modifications such as extensive cell wall ingrowths, which increase the membrane surface area and therefore the solute transport capacity (Pate and Gunning, 1972). The absence of this layer is correlated with reduced rates of grain filling and eventual abortion of the seeds (Brink and Cooper, 1947).

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The endosperm is a triploid tissue, mainly consisting of two cell types, the central endosperm cells, which accumulate starch and proteins, and outer aleurone cells at the periphery of the endosperm. Aleurone cell differentiation takes place between 6 and 10 d after pollination (DAP), producing a single cell layer of small cuboidal cells that accumulate spherosomes and protein bodies. Endosperm transfer cells develop instead of the aleurone cell phenotype in the basal endosperm cell layer bordering the pedicel.

The most basal layer (also termed "modified aleurone") consists of a sheet of elongated cells densely covered on the basal surface by cell wall ingrowths. Two or three adjacent endosperm cell layers also possess cell wall ingrowths, successively decreasing in extent toward the center of the kernel. (Schel et al., 1984; Shannon et al., 1986; Davis et al., 1990). To date, physiological and cytological studies have been carried out on the endosperm transfer cell layer, but little is known about the regulation of development of this cell type. The lack of development of endosperm transfer cells in 4n endosperm has been attributed to a possible regulatory effect of genomic imprinting (Charlton et al., 1995).

A number of genes expressed specifically in different tissues of the seed have been isolated. The corresponding promoter sequences have been functionally analyzed, and sequences conferring tissue specificity in starchy endosperm (Thomas and Flavell, 1990; Thompson et al., 1990; Quayle and Feix, 1992; Muller and Knudsen, 1993) and aleurone (Leah et al., 1994; Kalla et al., 1996) cells have been identified. In contrast, only two transfer-cell-specific cDNAs have been reported, *BET1* in maize (Hueros et al., 1995, synonymous with *BETL1* [basal endosperm transfer layer-specific protein] in this article) and END1 in barley (Doan et al., 1996). The basis for transfer-cell-specific expression is currently unknown.

We report here the identification of functional promoter sequences for the *BETL1* gene. A genomic fragment of 9 kb isolated from the maize line A69Y contained three tandem copies of the gene present at the *betl1* locus. On sequencing, the genes were found to be embedded in a complex array of transposon relic and satellite DNA repeats. A search for transcripts derived from each of the three *BETL1* genes

demonstrated that only two of the three copies were functionally active. Based on these data, we defined a promoter region having a maximum length of 1.8 kb. Particle bombardment experiments showed that a 930-bp promoter fragment proximal to the coding sequence was sufficient to drive β-glucuronidase (GUS) expression in the endosperm transfer cell layer. Finally, maize transgenic plants containing the 930-bp promoter region fused to GUS directed reporter gene expression in a manner resembling that of the *BETL1* transcript, both in timing and cellular localization.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. cv A69Y) shoots, leaves, immature seeds, tassels, and roots were extracted either from greenhouse-grown plants or from seedlings germinated on moist filter paper in Petri dishes.

Plant Transformation

Transgenic maize plants transformed with vector pMON17570 containing the *BETL1* promoter sequence fused to the GUS gene were produced using microprojectile bombardment (Klein et al., 1988; Gordon-Kamm et al., 1990; Walters et al., 1992). Embryogenic callus initiated from immature maize embryos was used as a target tissue. Plasmid DNA at 1 mg mL^{-1} in Tris-EDTA (TE) buffer was precipitated onto M10 tungsten particles using a calcium chloride plus spermidine procedure, essentially as described by Klein et al. (1988). The plasmids also contained the neomycin phosphotransferase II gene (*nptII*) driven by the 35S promoter from cauliflower mosaic virus and fused to the HSP70 intron. The gene of interest and the selectable marker were transcribed in the same direction. Embryogenic callus target tissue was pretreated on culture medium osmotically buffered with 0.2 M mannitol plus 0.2 M sorbitol for approximately 4 h prior to bombardment (Vain et al., 1993). Tissue was bombarded two times with DNA-coated tungsten particles using the gunpowder version of the particle delivery system (PDS 1000 device, Bio-Rad, Hercules, CA). Approximately 16 h after bombardment, tissue was subcultured onto a medium minus mannitol and sorbitol, but containing an appropriate aminoglycoside antibiotic, e.g. G418 to select for cells expressing the *35S/nptII* gene. Actively growing tissue sectors were transferred to fresh selection medium approximately every 3 weeks. About 3 months after bombardment, plants were regenerated from surviving embryogenic callus essentially as described by Duncan and Widholm (1988).

Isolation of RNA and DNA and Analysis by Filter Hybridization

Standard methods of DNA and RNA manipulation were carried out as described previously (Hueros et al., 1995). For the filter hybridizations presented in Figure 5, digoxigenin PCR-generated probes were labeled with digoxigenin, as recommended by the supplier (Boehringer Mannheim, Mannheim, Germany), CPD-Star was used as the substrate for the alkaline phosphatase (Boehringer Mannheim), and the filters were exposed for 30 min unless otherwise stated.

Inverse-PCR Reactions

To clone the genomic sequences immediately upstream of the coding region contained in the cDNA clone, a protocol based on that designed for genomic sequencing (Pfeifer et al., 1989) was used. Genomic DNA was digested with *Xba*I, denatured, and annealed to an 18-mer oligonucleotide derived from the cDNA sequence. The oligonucleotide was used in a primer extension reaction using Sequenase (Amersham-Pharmacia, Buckinghamshire, UK). The resulting blunt ends were ligated to an unphosphorylated synthetic double-stranded linker. The genomic sequences located between the restriction sites and the 5′ end of the cDNA were subsequently amplified by PCR using primers from the cDNA and synthetic linkers.

Transient Expression Analysis

Maize kernels harvested 10 DAP were surface-sterilized and hand-dissected to isolate the endosperm. Endosperms were maintained in the solid medium described below until being bombarded with DNA-coated gold particles. The coating and bombardment was performed according to the method of Knudsen and Müller (1991). After bombardment, tissue samples were incubated at 25°C for 24 h in the dark, in solid (0.5% [w/v] agarose) Murashige and Skoog medium containing 100 mg L^{-1} myo-inositol, 2 g L^{-1} Asn, 2 g L^{-1} Gln, 30 g L^{-1} Suc, and Murashige and Skoog vitamins (Sigma, St. Louis).

GUS Assay

Expression of the GUS gene was detected by histochemical staining according to the method of Jefferson et al. (1987). In vitro-cultured endosperms and fresh tissues were stained for GUS in a medium containing: 0.5 mg mL^{-1} X-glucuronide (CLONTECH, Palo Alto, CA), 0.5 mM K^+-ferrocyanide, 0.5 mM K^+-ferricyanide, 10 mM Na_2EDTA, 50 mM phosphate buffer (pH 7.0), and 0.1% (w/v) Triton X-100. A blue background, caused by endogenous glucuronidase activity observed in the pedicel, was eliminated by including 20% (v/v) methanol in the staining solution.

Immunological Quantification of Proteins in Transgenic Kernel Extracts

Immature kernels were removed, cut longitudinally, and half-kernels were stained for GUS activity as above. The remaining half-kernels from GUS-positive samples were ground in 100 μL of 3× SDS-PAGE loading buffer (Rotiload, Roth, Karlsruhe, Germany) and centrifuged in an Eppendorf minifuge at 13,000 rpm for 5 min. The supernatant (30 μL) was fractionated by 15% (w/v) SDS-PAGE, electroblotted onto polyvinylidenefluoride (PVDF, Milli-

pore, Bedford, MA) membrane, and proteins detected by enhanced chemiluminescence (ECL, Amersham, Little Chalfont, UK) using antibodies raised to BETL1 (Hueros et al., 1995), immunophilin mzFKBP66 (Hueros et al., 1998), and GUS (A-5790, Molecular Probes, Eugene, OR). Images were quantitated using a CCD recording camera (Lumimager, Boehringer Mannheim)

RESULTS

BETL1 Is Present in Three Copies in the Maize Genome

Analysis of the organization of *BETL1* coding sequences by Southern hybridizations indicated that the locus probably consisted of a short array of three copies of the gene (Hueros et al., 1995). To confirm this hypothesis, a gene copy number reconstruction was carried out by serially diluting *BETL1* plasmid DNA with maize genomic DNA (Fig. 1). Aliquots (8 μg) of *Hin*dIII-digested genomic DNA (corresponding to approximately 1 million maize nuclei) were mixed with increasing amounts of linearized plasmid containing the *BETL1* cDNA. After Southern blotting, the filter was hybridized to a *BETL1* probe. The genomic band intensity seen was equal to the intensity of 8.9-pg plasmid DNA (Fig. 1, arrowhead), which is equivalent to 2.4 million copies. Therefore, each *Hin*dIII genomic band contains at least one copy of *BETL1* per haploid genome. The existence of more than one copy was further confirmed by physical mapping of the restriction sites upstream of the coding sequence. For this purpose, double digests including *Nsi*I (which cuts at the 3′ end of the cDNA) and various other restriction enzymes were blotted and hybridized to a 5′-specific cDNA probe (data not shown).

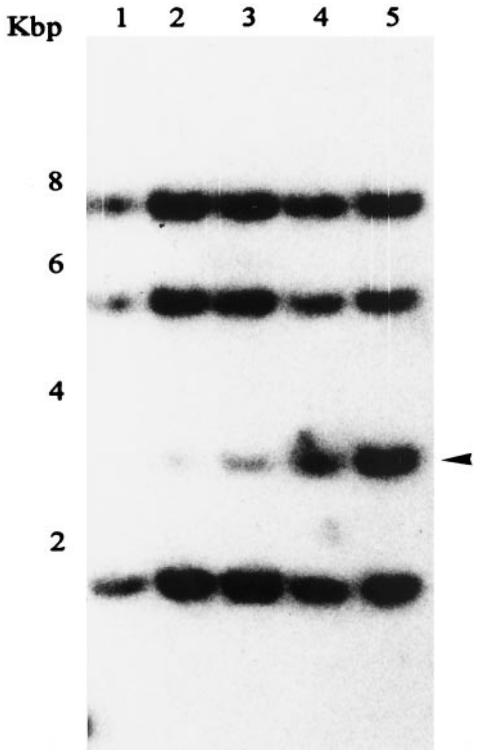

Figure 1. Three copies of the *BETL1* gene are present in maize variety A69Y. *Hin*dII-digested genomic DNA (5 μg [lane 1] or 8 μg [lanes 2–5]) was run along with increasing amounts of plasmid DNA containing the *BETL1* cDNA. Lane 1, 0 pg of plasmid DNA; lane 2, 2.22 pg; lane 3, 4.44 pg; lane 4, 8.88 pg; lane 5, 17.76 pg. The resulting Southern blot was hybridized with a *BETL1* cDNA probe. Genomic fragments are located just below the 8-, 6-, and 2-kb markers, with the *BETL1* plasmid DNA control located at 3.5 kb (arrowhead).

Inverse-PCR Cloning of *BETL-1* Genes

To clone the sequences upstream of the coding regions, inverse-PCR (I-PCR) was used. Genomic DNA was digested with *Xba*I, denatured, and allowed to anneal to a reverse primer derived from the cDNA sequence; after primer extension, a blunt-ended adaptor was ligated and PCR was performed using a nested reverse primer derived from the cDNA and a forward primer derived from the adaptor sequence. The *Xba*I-digested I-PCR reaction produced two distinct bands of 1,475 and 942 bp. After cloning and sequencing, it was shown that the 942-bp band contained a single species (*BETL1b*), while the 1,475-bp band was a mixture of two DNA fragments, *BETL1a* and *BETL1c*, having slightly different sequences.

The nested primers derived from the cDNA were designed to amplify a large portion of the coding sequence along with the promoter. In this way, we were able to identify three distinct copies of the gene, all of which contained an intron sequence inserted after T-80, taking the A of the translation start codon as nucleotide no. 1. Two features distinguished *BETL1b*: first, the acceptor splice site was TG instead of AG; second, the intron sequence was larger than that present in *BETL1a* or *BETL1c* at 463 bp instead of 123 bp. As a result, the promoter sequence contained in the I-PCR fragment of *BETL1b* was only 177 bp long.

Isolation of Intergenic Regions and Structure of the *BETL1* Cluster

From the information obtained from the physical mapping of the upstream sequences and the restriction sites identified after sequencing of the I-PCR products, a physical map was constructed that was confirmed by further Southern-blot analysis (data not shown). The three copies of *BETL1* were found to be located on a single *Eco*RV restriction fragment (Fig. 2A). All of the copies of the gene are orientated in the same direction. The map was confirmed by amplifying and cloning the intergenic regions using the primers indicated in Figure 2A. A contig of 9,167 bp containing the three genes was assembled, which is summarized in Figure 2B. The cluster showed a rather complex organization with the three copies of the gene interspersed with a number of distinct repetitive sequences.

The 135 bp of the *BETL1b* promoter obtained from the I-PCR product did not possess any homology with the promoter sequences of *BETL1a* or *BETL1c*. However, it did match (95% identity) a repeated sequence found in the

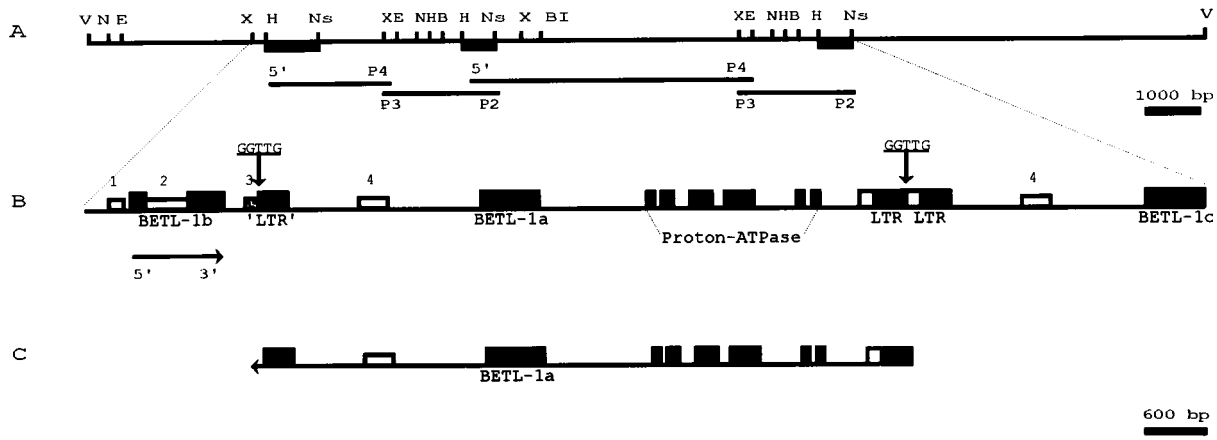

Figure 2. Structure of the *BETL1* cluster. A, Physical map of the 17-kb genomic fragment containing BETL-1a, BETL-1b, and BETL-1c. V, *Eco*R5; N, *Nco*I; E, *Eco*RI; X, *Xba*I; H, *Hin*dII; Ns, Nsi1; B, Bgl2; BI, *Bam*HI. Lines shown under the map represent the PCR fragments used to sequence the 9-kb region outlined in B. Elements identified are shown as numbered boxes. 1, *Spm*-like transposon sequence; 2, *Tourist*-like transposable element; 3, 27 × (TTA) microsatellite repeat; 4, *sleepy*-like transposable element; black boxes, *BETL1* and proton ATPase coding regions (transcription direction is indicated below the map); gray boxes and attached empty boxes, putative retrotransposon LTRs. C, Sequences possibly mobilized through a retrotransposition-like event (direct 5-bp repeats generated after insertion are shown in B).

promoter of the *ZEMa* gene and shown to be related to the Spm/En element (Montag et al., 1996). The sequence contained in the *BETL1* contains two repeats of 36 bp, each formed by a palindromic sequence. Southern-blot analysis confirmed that the promoter region of *BETL1b* contains highly repeated DNA (not shown). The intron sequence present in *BETL1b* was also modified by the presence of a second transposable element insertion. The transposon was 346 bp long and possessed 15-bp inverted repeats at both ends, flanked by 3-bp direct repeats as follows: TAAgggcatgtacagtgg. . . . . .ccactatacatgccc TAA (capital letters = direct repeat; lowercase letters = inverted repeat). The sequences identify this element as a miniature inverted repeat transposable element (MITEs, Wessler et al., 1995), in the same subfamily as Tourist (Bureau and Wessler, 1992). Hybridization of the transposon sequence to maize genomic DNA showed that related sequences are dispersed throughout the maize genome (not shown). The *BETL-1b* gene is also modified by the exchange of the splicing acceptor site of the intron to TG instead of the standard AG found in *BETL1a* and *BETL1c*.

The promoter sequences of *BETL1a* and *BETL1c* are nearly identical up to position −1,609, numbering from the A of the start codon. At −1,609, *BETL1c* contains two tandem repeats of 365 bp, while *BETL1a* contains a microsatellite repeat of $(ATT)_{27}$ (labeled "3" in Fig. 2B). A 230-bp fragment homologous to the 365-bp repeats is found downstream of the microsatellite in *BETL1a*. These findings suggest that a retrotransposition event may have been responsible for the integration of *BETL1a* between *BETL1b* and *BETL1c*. A fragment of about 5.5 kb, delimited by the 365-bp elements described above, functioning as the long terminal repeats (LTR) of a retrotransposon element (Fig. 2C), would have moved into the tandem duplication previously formed by *BETL1b* and *BETL1c* to give the present structure (Fig. 2B).

In support of this hypothesis, it is known that retrotransposons possess a primer binding site at the 5′ end, whose sequence resembles that of a tRNA. A 15-bp sequence, partially homologous to a primer binding site, is present in the 365-bp repeated units at 6,494-TGGTCCTCGC-CGAAGG-6,479. Further evidence for a transposon relic is the presence of fragments of an unrelated gene between the two LTRs. A sequence downstream of *BETL1a* shows homology to plasma membrane proton ATPases. The most similar sequence was that of a maize H^+ ATPase, with a stretch displaying 75% identity extending over 238 residues. Intriguingly, this sequence was also found to be part of the maize retrotransposon-like sequence Bs1 (Young-Kwan and Bennetzen, 1994). However, the sequence in the *BETL1* cluster is fragmented into six segments and contains two stop codons and seven frameshifts, indicating an accumulation of mutations.

An additional feature found in the *BETL1a* and *BETL1c* promoters is the presence of a 242-bp element showing 62% identity with the transposable element "sleepy" (labeled "4" in Fig. 2B, Winkler and Helentjaris, 1995).

A *BETL1* Gene Cluster Is Present in Different Maize Varieties

To investigate the degree of conservation of the *BETL1* gene cluster, we compared the organization of the locus in different maize lines by filter hybridizations. DNA was prepared from maize lines of diverse origin and from teosinte (*Zea diploperennis*). The DNA was digested with *Hin*dIII, which cuts once at the 5′ end of the *BETL1* transcribed sequence, and thus gives an estimate of the number of copies of *BETL1* present in each line (Fig. 3). Several of the samples, including the teosinte DNA, contain more than one *BETL1* gene copy. Interestingly, the highest number of copies (three) was found in lines that had been

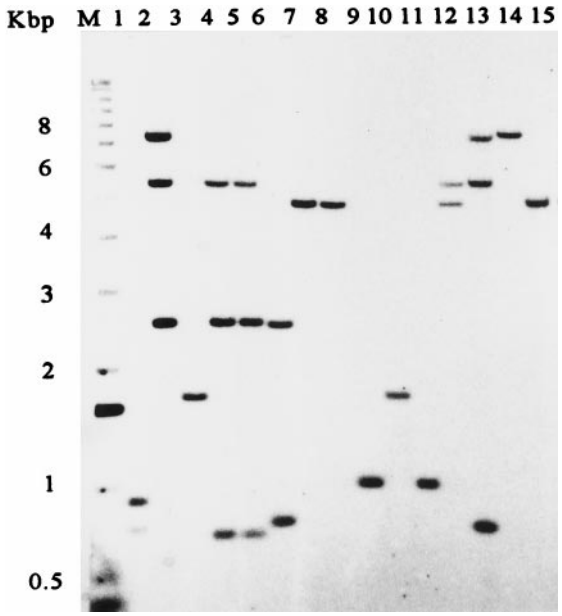

Figure 3. *BETL1* gene copy number in different maize varieties. Genomic DNAs (8 μg per line) were digested with *Hin*dII and the resulting Southern blot was hybridized with a *BETL1* cDNA probe. Lane M, 1-kb DNA ladder; lane 1, teosinte (*Zea diploperennis*); lane 2, maize variety A69Y; lane 3, A239; lane 4, A632; lane 5, B73; lane 6, H99; lane 7, Pa91; lane 8, W64A; lane 9, FR16; lane 10, F2; lane 11, F252; lane 12, F1110; lane 13, F1444; lane 14, DBTS; lane 15, Y204.

produced after intensive breeding programs (lanes 2, 4, 5, and 12) as compared with those considered as primary lines or first derivatives (lanes 1, 3, 7–11). The process of gene amplification is illustrated by the line F2 (Fig. 3, lane 11, one copy) and its derivatives, F252 (lane 12, one copy), F1110 (lane 13, two copies), and F1444 (lane 14, three copies).

Expression Analysis of *BETL1* Genes

The determination of copy number and mapping of each copy at the *betl1* locus enabled us to approach the issue of which copies of the gene were transcribed. The alignment of the three *BETL1* coding sequences, which was deduced from the genomic sequence after removing the introns, showed up to 23 base substitutions between genes. However, they are evenly distributed, which precluded the design of gene-specific probes for analyzing the expression of *BETL1a*, *BETL1b*, and *BETL1c* separately by RNA filter hybridizations. Therefore, two alternative approaches were used. First, RT-PCR analysis was performed using either a *BETL1b* or a *BETL1a/BETLc* specific primer (Fig. 4). Two primers annealing to all BETL1 copies amplified a single band from endosperm cDNA (lane 1), two bands from genomic DNA (due to the different sizes of the *BETL1b* and *BETL1a/BETLc* introns, lane 2) and one band from each genomic clone containing either *BETL1a* or *BETL1b* (lanes 3 and 4, respectively). When a *BETL1b* gene-specific primer was used, only a faint band was amplified from endosperm cDNA (lane 5), while the *BETL1a* gene specific primer produced an intense band from the same cDNA preparation (lane 8).

Gene specificity of the primers was confirmed by the fact that PCR from genomic clones containing either *BETL1b* (Fig. 4, lanes 7 and 10) or *BETL1a* (lanes 6 and 9) rendered a PCR product only when the corresponding specific primers were used in the reaction. This experiment suggests that *BETL1b* is only weakly expressed, which is consistent with numerous modifications found in this gene, including transposon insertions in promoter and intron sequences. RT-PCR cannot be easily used to distinguish between *BETL1a* and *BETL1c*. To know if they are expressed at the same level, 16 independent *BETL1* cDNA clones were sequenced and classified into three groups, a, b, or c. No clone was found that contained the sequence derived from *BETL1b*, 10 clones contained the *BETL1a* sequence, and six clones, in addition to the original *BETL1* clone, contained the *BETL1c* sequence. We conclude that both *BETL1a* and *BETL1c* promoters are functional. Comparison of the 5′ end sequences of the cDNA clones indicates a probable transcription start site at −53 or −51 upstream of the translation start codon.

A 985-bp Promoter Sequence from *BETL1a* Directs the Expression of the GUS Reporter Gene in the Endosperm Transfer Cell Layer

The promoter sequences of *BETL1a* are delineated at −1.8 kb by the insertion of *BETL1b* sequences. Because we have evidence that *BETL1a* is expressed, it is likely that a 1.8-kb promoter fragment will be sufficient to confer this expression pattern. Furthermore, the presence of a transposon-like sequence inserted at position −761 sug-

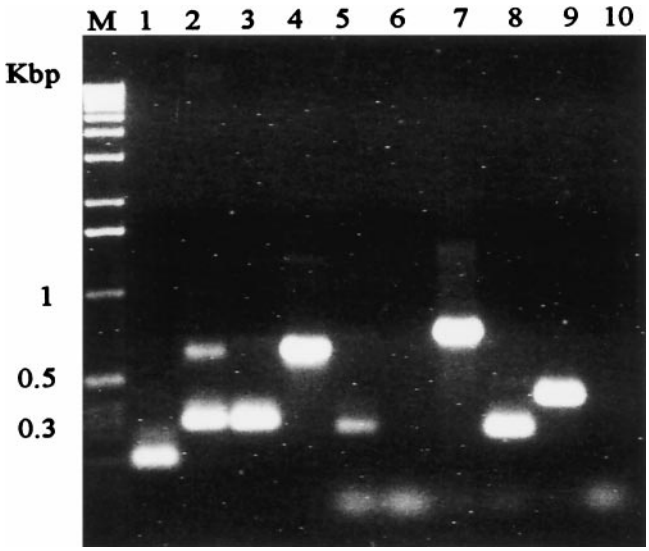

Figure 4. RT-PCR analysis of the expression of the *BETL1* genes. Endosperm cDNA samples (lanes 1, 5, and 8), genomic DNA (lane 2), and genomic clones containing either *BETL1a* (lanes 3, 6, and 9) or *BETL1b* (lanes 4, 7, and 10) were amplified using unspecific forward and reverse primers (lanes 1–4), *BETL1b* specific primers (lanes 5–7), or *BETL1a/BETL1c* specific primers (lanes 8–10). Lane M, 1-kb DNA ladder.

gests that only the sequences downstream of this element might be needed.

A −985 (*Kpn*I–*Hin*dIII) *BETL1a* promoter fragment was fused to GUS, and the cassette was used in transient expression experiments by particle bombardment of immature dissected endosperms (not shown). Immature (12 DAP) endosperms expressed GUS at the sites of bombardment on the outer endosperm layer when a positive control, pAHC25 (Christensen et al., 1992), containing a maize polyubiquitin promoter fused to GUS was used. No signal was observed after bombardment with a promoterless GUS construct. Finally, blue spots were restricted to the basal area of the endosperm, when a −985-bp-*BETL1a* promoter-GUS construct was used. The GUS expression obtained in basal endosperm cells was weak and subject to large variation between individual experiments, thus preventing any quantitative conclusions.

Generation and Analysis of Transgenic Maize Plants Containing the *BETL1* Promoter-GUS Construct

The 985-bp *BETL1* promoter fragment was active specifically in the transfer cell layer of the kernel. The ability of this promoter fragment to confer transfer-cell-specific expression in transgenic maize was tested by maize transformation with the GUS reporter gene. Three independent transgenic lines, numbered 1, 11, and 17, were randomly chosen to be analyzed in detail.

Plants containing the GUS gene were identified by Southern filter analysis (Fig. 5, top). Since the plants analyzed derived from the crosses between the primary transformants and non-transgenic plants, the segregation observed (1:1, transformant:non-transformant) was consistent with a single-copy integration. Nevertheless, the additional hybridizing bands present in line 17 (lanes 15 and 16) and the complex hybridizing patterns observed in all the lines when other restriction enzymes were used (Fig. 5, bottom) indicate the presence of multiple copy insertions, presumably linked or catenated.

The transgenic lines were further analyzed using *Dra*I, an enzyme that does not cleave within the reporter gene. Probing *Dra*I-digested DNA with the GUS coding sequence gave different patterns of hybridization for all three transgenic lines, confirming their independent origin (Fig. 5, bottom, lanes 2–4). Additionally, the comparison between blots A and B of Figure 5, which were probed with a fragment of the *BETL1* promoter and the GUS coding sequence, respectively, demonstrates that the integration

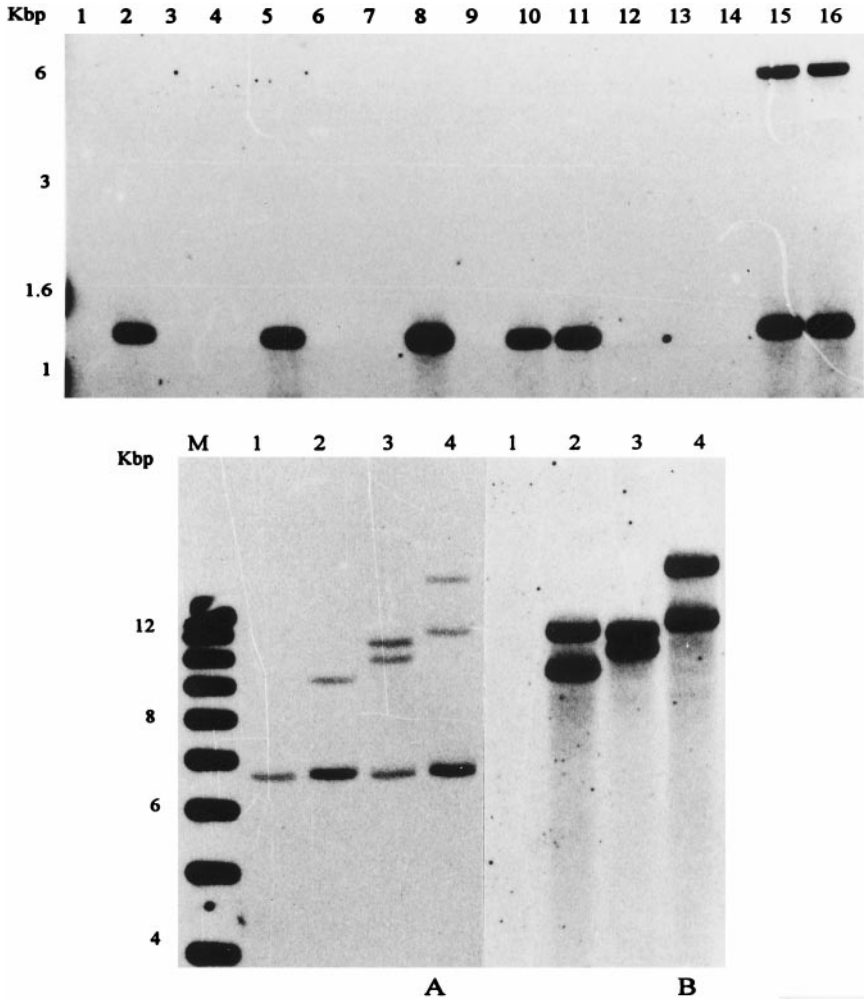

Figure 5. Top, Southern-blot analysis of putative transgenic plants. Genomic DNA from a negative control plant (10 μg, lane 1, A69Y) and five plants segregating the transgene locus from each transgenic line (line 1, lanes 2–6; line 11, lanes 7–11; and line 17, lanes 12–16) were digested with *Eco*R5, blotted onto a positively charged nylon filter, and hybridized with a probe derived from the GUS gene coding sequence. Size markers are shown on the left. Bottom, Southern-blot analysis of the transgenic loci present in three transgenic lines. Genomic DNA from a negative control plant (10 μg; lane 1, A69Y) and one plant from each transgenic line (line 1, lane 2; line 11, lane 3; and line 17, lane 4) were digested with *Dra*I, blotted, and hybridized with either a 300-bp fragment from the proximal promoter of *BETL1* (A) or a fragment derived from the GUS gene coding sequence (B). Lane M, DNA size marker (1-kb ladder).

sites detected contain both GUS gene and *BETL1* promoter sequences.

Transgenic plants from each line were grown to maturity under greenhouse conditions. Histochemical staining of leaves, roots, adventitious roots, anthers, silks, and female flowers for GUS did not give signals for any transgenic line analyzed (not shown). The transgenic plants were either self-pollinated or crossed in both directions with a non-transgenic maize line (A69Y), and the developing kernels were stained for GUS enzyme activity at various stages during development. The position of the immature embryo, endosperm with transfer cells, and phloem terminals in the pedicel are indicated schematically in Figure 6F.

Some of the seeds stained were GUS negative (Fig. 6A), as would be expected for plants segregating for the transgene. In other seeds, however, a pale blue staining corresponding to weak GUS activity appeared after 11 DAP, which was highly specific for the transfer cell layer (Fig. 6B). At later stages of seed development, the GUS signal remained confined to the basal transfer cells and the intensity peaked by 16 DAP (Fig. 6D), with no decrease in intensity observed until 27 DAP (Fig. 6C). Figure 6E shows a higher magnification of the 16 DAP staining pattern photographed under phase contrast microscopy; the presence of cell wall ingrowths in the transfer cell layer is evident, and the concentration of GUS activity in these cells can be seen. The comparison between the staining intensity obtained from seeds resulting from reciprocal crosses indicated staining proportional to gene endosperm dosage (not shown). A very weak endogenous GUS activity in the placentochalazal region of the pedicel appeared in both transgenic and non-transgenic kernels at approximately 16 DAP, and could be largely eliminated by the inclusion of 20% (v/v) methanol in the staining solution, a technique that did not affect staining in the transgenic basal layer.

Comparison of the Accumulation of *BETL1* and GUS Gene Products in Transgenic Maize

GUS protein has been reported to be very stable in the plant cell, and consequently might not accurately reflect either the rate of transcription or the steady-state mRNA

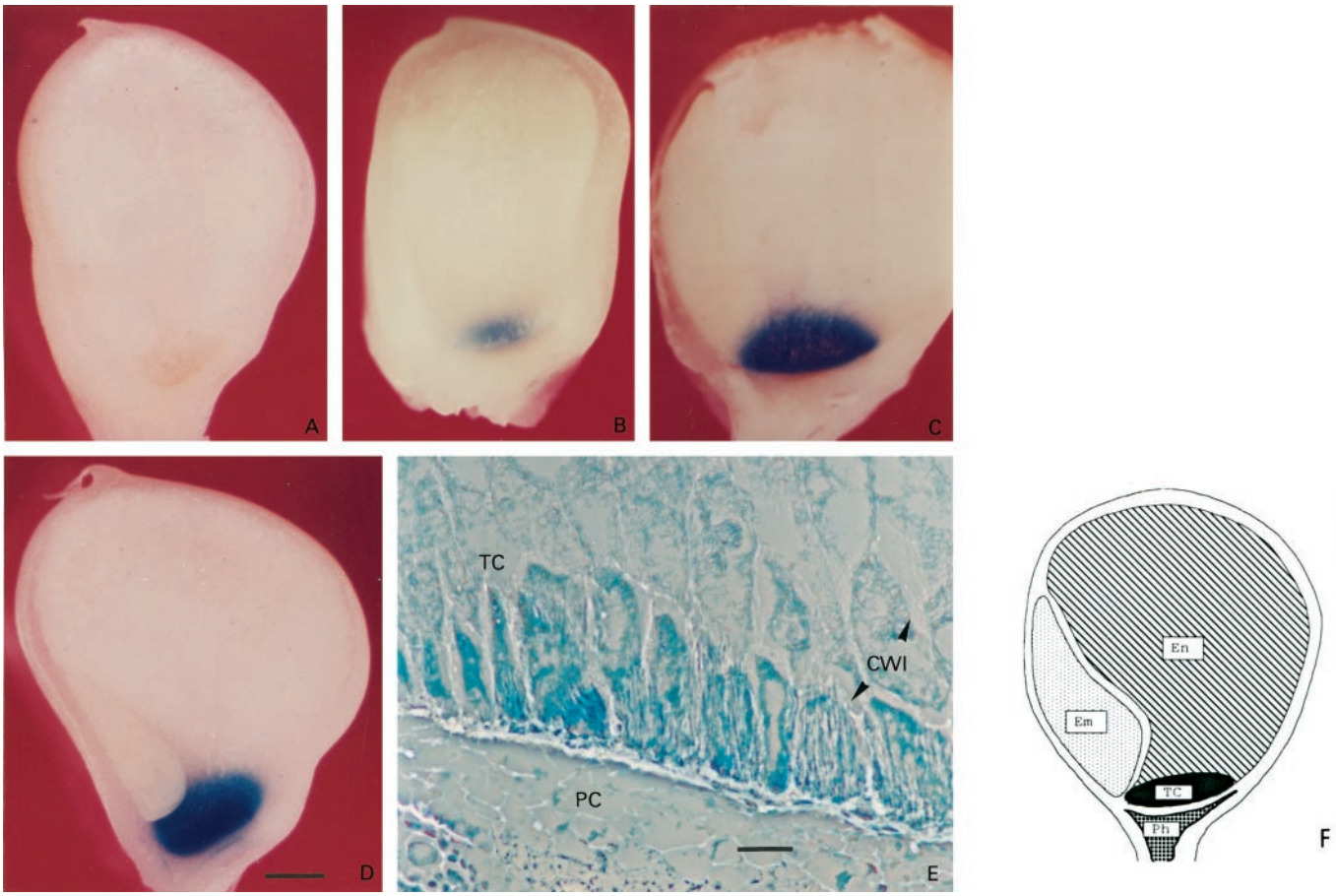

Figure 6. GUS staining of immature seeds from transgenic plants. Immature kernels at various stages of development were hand-dissected and stained for 24 h as described in "Materials and Methods." A, Negative control at 16 DAP; B, 11-DAP kernel; C, 27-DAP kernel; D, 16-DAP kernel; E, phase-contrast image at higher magnification of transfer cell region shown in D; F, a schematic representation of a longitudinal section of the kernel. GUS activity is seen as a blue precipitate of dichloro-dibromoindigo. Magnification: B, ×16; A, C, and D, ×6.4; and E, ×200. Bars: D, 1 mm; E, 50 μm. F, Schematic representation of the component tissues of the developing kernel. En, Endosperm; Em, embryo; TC, transfer cell layer of endosperm; Ph, placentochalaza.

concentration derived from the transgene. Therefore, the steady-state GUS mRNA concentration was estimated by northern filter hybridization of poly(A^+) RNA (Fig. 7, top). Seeds from the cross between the transgenic line 11 and a wild-type parent (A69Y) were collected at five different developmental stages. Half of each kernel was stained for GUS activity, and the remainder stored at −80°C for RNA extraction if GUS activity was seen. A comparison between GUS mRNA (Fig. 7A) and that of the endogenous BETL1 (Fig. 7B) shows that the kinetics of accumulation of GUS mRNA differs from that of the endogenous *BETL1* transcript. GUS mRNA is first detected at 13 DAP, later than that for BETL1, while the dramatic decrease in BETL1 mRNA after 17 DAP contrasts with a much more modest decline in GUS mRNA concentration.

A comparison of the accumulation of BETL1 and GUS protein during endosperm development was made by immunoblotting with specific antisera. As control for a constitutively expressed intracellular protein, an immunophilin antibody was used (Fig. 7). It is evident that in contrast to the quantitative turnover of BETL1 after 20 DAP, GUS continues to accumulate and is present in mature kernels.

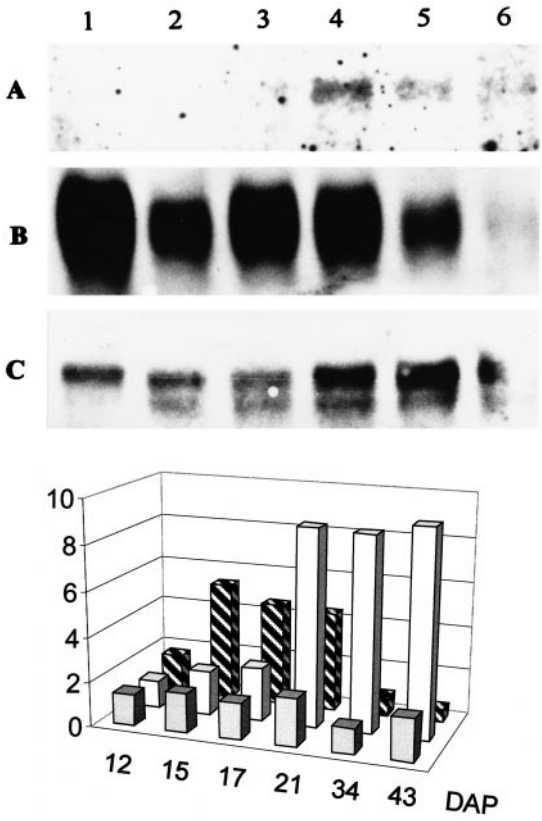

Figure 7. Top, Northern-blot analysis of the GUS gene expression. mRNA from non-transgenic (A69Y) seeds at 10 DAP (lane 1, 1 μg) or transgenic seeds (line 11 crossed by A69Y) at various stages of development (0.7 μg per lane) were electrophoresed in a formaldehyde gel, blotted, and sequentially hybridized with a GUS probe (A), a *BETL1* probe (B), and a ubiquitin probe (C). Lane 2, 9 DAP; lane 3, 13 DAP; lane 4, 17 DAP; lane 5, 21 DAP; lane 6, 27 DAP. Filters were exposed for 2 h (A), 1 min (B), or 15 min (C). Bottom, Accumulation of *BETL1* (hatched bars), GUS (white bars), and immunophilin (shaded bars) in *BETL1*/GUS transgenic maize kernels. Total protein extracts (15% of one kernel per track) were prepared from kernels harvested at different days after fertilization as indicated, loaded onto 15% SDS-polyacrylamide gels, and electroblotted onto PVDF membranes. Immunoblots were probed successively with antisera against *BETL1* (Hueros et al., 1995), GUS (A-5790, Molecular Probes), and mzFKBP-66 (Hueros et al., 1998), detected by enhanced chemiluminescence, and changes in protein concentration (expressed in arbitrary units) derived by image quantitation.

DISCUSSION

The *betl1* locus, encoding an abundant, endosperm transfer-cell-specific transcript (Hueros et al., 1995), has been found to consist of a cluster of three tandemly arranged gene copies. In the maize line A69Y, the three gene copies *BETL1a*, *BETL1b*, and *BETL1c*, (Figs. 1 and 2) are located on a 9-kb DNA fragment. The distribution of these sequences allowed us to define a region of maximum 1.8 kb in which an active promoter sequence resides; 985 bp of this promoter is sufficient to confer expression specifically in basal endosperm transfer cells. The structure of the gene cluster (Fig. 2) and the sequence comparisons suggest a model in which *BETL1b* and *BETL1c* have arisen by spontaneous duplication (Ohno, 1979), followed by the insertion of the *BETL1a* copy by a transposition event (see below). Interestingly, the different maize inbred lines examined all show evidence of gene duplication, having two to three copies (Fig. 3).

The *BETL1a* gene is located on a DNA fragment (from position 1,259–6,745, Fig. 2B) with similarities to TY3/gypsy retrotransposons, such as "reina" (SanMiguel et al., 1996). Both reina and the element described here are flanked by LTRs of around 0.3 kb. The total length of both elements is 5.5 kb and both apparently are flanked by 5-bp repeats of GGTTG (only detected at the 3′ end in the case of reina) at the integration site. The potential transcription direction of the retro-element, as deduced from the position of the putative primer-binding site, is opposite to that of *BETL1a*. This has two consequences. First, this orientation would explain why the 3′ flanking LTR adjoins a microsatellite repeat (27× AAT, if read on the noncoding strand).

The A-rich microsatellites found at the 3′ end of LTR-like repeats are thought to be derived from the poly-dA tail of retrotransposons, as suggested for Alu repeats (Nadir et al., 1996) or Artiodactyl retroposons (Kaukinen and Varvio, 1992). This would also explain why the 3′ LTR found in the *BETL1* cluster is shorter than the elements found at the 5′ end. Retrotransposon LTRs have a tripartite structure, i.e. are formed by three different elements, when integrated into the host genome. After transcription, however, the 3′ LTR lacks the 3′ element and the 5′ LTR lacks the 5′ element; complete LTRs are regenerated only after successful integration. A second consequence of the transcription polarity is the presence of introns in the genes contained in the retrotransposon. Had *BETL1a* been inserted in the sense orientation within the retrotransposon, the *BETL1* intron would have been spliced out after transcription, as re-

ported for the H-ATPase fragment found in Bs1 (Young-Kwan and Bennetzen, 1994).

These considerations strongly suggest that the 365 bp repeats, and the truncated repeat associated with the $(AAT)_{27}$ microsatellite, are the LTRs of a maize retrotransposon, although this sequence configuration could also derive from homologous recombination between Solo-LTR elements. In this case, LTRs would have provided recombination sites used in generating multiple copies of *BETL1*.

In addition to the putative retrotransposon described above, the 9-kb *BETL1* cluster harbors a tourist-like transposon element (Bureau and Wessler, 1992; Rio et al., 1996), a sleepy-like transposon fragment (Winkler and Helentjaris, 1995), and a sequence highly homologous to a member of the suppressor/mutator family (Montag et al., 1996). Such a high level of interspersion between repeated mobile elements and genes seems to be a characteristic of the maize genome. SanMiguel et al. (1996) found that retrotransposons accounted for more than 60% of a 280-kb region containing the *ADH1-F* maize gene. Furthermore, the comparison of the region containing the genes *Sh2* and *a1* from maize, sorghum, and rice showed that, despite the conserved order of genes, the intergenic regions had accumulated extensive differences due to the integration of unrelated, repeated sequences (Chen et al., 1997).

Once those regions of the cluster belonging to repeated/mobile DNA families had been identified, a promoter sequence was selected for testing specificity of expression in transgenic plants. As *BETL1b* was not expressed (Fig. 4 and cDNA sequencing results), a −983-bp fragment of *BETL1a* was selected for functional analysis. Preliminary experiments using particle bombardment with promoter-GUS constructs (data not shown) showed specific expression in the transfer cells of immature maize kernels. Nevertheless, the GUS expression seen was too weak to be quantitated. This may be due to the technical difficulty of exposing the basal cells of immature endosperms to particle bombardment without extensive cell damage. Another possibility is that when the transfer cell layer is removed from the influence of solute flux through the pedicel, it may be altered in its expression characteristics. Interestingly, a similar finding was made for expression of an aleurone-specific promoter in transient assays (Kalla et al., 1996).

The functionality of the *BETL1* promoter was demonstrated by generation of transgenic maize plants containing the *BETL1* promoter-GUS gene construct. Inspection of transgene organization indicates that multiple copies are clustered in a few discrete regions of the maize genome (Fig. 5, top). The transgenes were integrated in different restriction fragments in the three lines analyzed, indicating that they are of independent origin (Fig. 5, bottom). Finally, histochemical GUS staining demonstrated that the −983 *BETL1* promoter-GUS construct introduced in transgenic plants can direct reporter gene activity (Fig. 6) in a way that resembles the spatial and temporal pattern of *BETL1* expression (Hueros et al., 1995).

Comparison of transgene-derived GUS mRNA with the endogene-derived *BETL1* transcript (Fig. 7, top) shows that the behavior of the *BETL1* promoter fragment in the GUS fusion differs in two respects from that of the native promoter. First, the reporter gene mRNA was present at a much lower concentration than BETL1 mRNA. Second, the pattern of accumulation of GUS mRNA showed a delay compared with that from the *BETL1* gene. These effects could be due to the lack of enhancer sequences, for example, located upstream of −983 or in the *BETL1* intron, which were not in the region used for transformation, but alternative explanations such as the influence of position effects or differential mRNA stability are possible. Despite these minor differences in expression profiles, GUS protein accumulates in seeds approaching seed maturity, in contrast to the BETL1 protein (Fig. 7, bottom). It may be that BETL1 and other secreted proteins of the transfer cell layer are selectively degraded by extracellular proteases. An alternative might be their quantitative incorporation in insoluble cell wall material, which would render them nonextractable.

The presence of transfer cells in the basal endosperm region suggests that this layer may promote solute transfer into the kernel, but the relative inaccessibility of the tissue makes this contribution difficult to assess by physiological techniques. Furthermore, to date, no mutant has been unequivocally identified whose primary site of action is in the transfer cells, although this may very well be the case for *mn1*, which affects one enzyme located in the transfer layer, cell wall-bound invertase (Cheng et al., 1996). We have shown that a 983-bp *BETL1* promoter fragment directs expression exclusively in basal endosperm cells of maize. The *BETL1* promoter may become a valuable tool for the identification of components influencing solute transfer into the endosperm, and could potentially be used to manipulate grain filling.

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