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Molecular Characterization of *BET1*, a Gene Expressed in the Endosperm Transfer Cells of Maize

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A cDNA clone, BET1 (for basal endosperm transfer layer), was isolated from a cDNA bank prepared from 10–days after pollination (DAP) maize endosperm mRNA. *BET1* mRNA was shown to encode a 7-kD cell wall polypeptide. Both the mRNA and protein were restricted in their distribution to the basal endosperm transfer layer and were not expressed elsewhere in the plant. *BET1* expression commenced at 9 DAP, reached a maximum between 12 and 16 DAP, and declined after 16 DAP. The initial accumulation of the BET1 polypeptide reached a plateau by 16 DAP and declined thereafter, becoming undetectable by 20 DAP. The antibody raised against the BET1 protein reacted with a number of polypeptides of higher molecular mass than the BET1 monomer. Most of these were present in cytosolic fractions and were found in nonbasal cell endosperm extracts, but three species appeared to be basal cell specific. This result and the reactivity of exhaustively extracted cell wall material with the BET1 antibody suggest that a fraction of the protein is deposited in a covalently bound form in the extracellular matrix. We propose that the BET1 protein plays a role in the structural specialization of the transfer cells. In addition, *BET1* provides a new molecular marker for the development of this endosperm domain.

INTRODUCTION

Grain filling is a critical determinant of yield in many crops. The process depends on the rate and efficiency of two different mechanisms: phloem unloading, and the uptake and utilization of assimilates for seed growth and storage product formation (Gifford et al., 1984). In maize, nutrients are unloaded from the phloem terminals located in the maternal tissue at the base of the seed, the pedicel. The parenchyma of the pedicel provides a symplastic route for assimilates destined for the kernel until they enter the apoplast of the placental–chalazal region, which is the tissue that is directly in contact with the endosperm. No symplastic connection exists between the maternal and embryonic tissues (Thorne, 1985); this situation precludes the entry of infectious agents such as viruses into the seed (Miller and Chourey, 1992). Uptake of assimilates by the endosperm from this apoplastic space is facilitated by the conversion of the cells at the base of the endosperm to transfer cells, which are anatomically and functionally distinct from other endosperm cell types (Schel et al., 1984; Shannon et al., 1986; Davis et al., 1990). These cells have been characterized as having extensive fingerlike wall projections that increase the surface area of the associated plasmalemma up to 20-fold (Pate and Gunning, 1972).

Despite a series of physiological and cytological studies, little is known about the precise function of the basal endosperm transfer layer, and no enzyme to our knowledge has been shown to be exclusively or preferentially present in these cells. The extent to which these cells are actively involved in solute transfer from the endosperm cavity into the endosperm is also unclear. In contrast, metabolic conversions and the postphloem solute route in the pedicel are better documented (Lyznik et al., 1982; Wang and Fisher, 1994a, 1994b). Results from the "empty ovule" technique (Wolswinkel, 1992), in which the discharge of solute from the placental–chalazal region into the endosperm cavity was analyzed after surgical removal of the contents, suggest that an active role for endosperm cells in solute transfer from the phloem terminals may not be required; however, the short-term (~1 day) nature of such experiments may mask an important role for transfer cells. Such issues might be more readily resolved if specific promoters or other molecular markers for this cell type were available. Here, we report the isolation of a cDNA clone, BET1, that is specifically expressed in basal endosperm layer cells and describe features of the encoded protein, thereby suggesting a role for BET1 in endosperm development.

RESULTS

Cloning and Sequencing of BET1

A cDNA library was prepared in the phagemid insertion vector λZAPII. The template was poly(A)$^+$ RNA purified from

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CTCTCTTCGTCACTAGCTCGTGGTAATAAG 30

GGAAGCTTGATCATCTTTCCTTATGGCGGT 60
                          M  A  V 3

GATGAAGAGCAGCACAATCGTCGCGCTTCT 90
  M  K  S  S  T  I  V  A  L  L 13

ATTGGCTGTTGCCATTCTGTCCTCACTGTC 120
  L  A  V  A: I  L  S  S  L  S 23

TCCATGCTACGAAGCCGGCGGCTGCATCGG 150
  P  C  Y  E  A: G  G  C  I  G 33

GAAACCCAAGAAGTCACCGCCACCGCCGAG 180
  K  P  K  K  S  P  P  P  P  R 43

AAGGCCATACTTCTCGTCCTATTCCGAGGA 210
  R  P  Y  F  S  S  Y  S  E  D 53

CCACCAGAATTGCCGCTTGATCTGTAGCTC 240
  H  Q  N  C  R  L  I  C  S  S 63

CAAGGGTTTCAAGGACGGTGGCTGGTGTGA 270
  K  G  F  K  D  G  G  W  C  D 73

CGAAAGCGTGGAGCACAAAGTGTGCTGCTG 300
  E  S  V  E  H  K  V  C  C  C 83

CTCCCATTAGAAATATAATAGCGTATGTTT 330
  S  H  *                      85

GTTGATGCATATATATATCAGCGTCCATCT 360

ACCTCTGTGGTGCAAAAATTCTGAATAAAA 390

ATCTGGCTTTTTTGGT-poly(A) tail
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Figure 1. Nucleotide Sequence of BET1 and Deduced Amino Acid Sequence.

The SPPPP motif is underlined in the amino acid sequence. Two possible signal peptide cleavage sites are indicated by colons, and the stop codon is indicated with an asterisk. The polyadenylation signal is double underlined in the nucleotide sequence. The positions of the oligonucleotide primers used for PCR amplification and cloning of the protein coding sequence are underlined with arrows.

manually dissected endosperms harvested at 10 days after pollination (DAP). A cDNA probe prepared from 10-DAP endosperm mRNA was ^{32}P labeled with α-^{32}P-dCTP by reverse transcription (RT). This probe was subtracted for maturation stage transcripts by hybridization with a 10:1 molar excess of photobiotinylated mRNA from 25-DAP endosperm. The sequences common to both mRNA populations were removed by streptavidin binding and subsequent phenol extraction. The subtraction hybridization procedure was repeated twice as described previously (Sive and St. John, 1988).

Thirty-three plaques giving a strong hybridization signal with the subtracted probe were plaque purified. The clone inserts were isolated by phagemid excision and rescreened with two unsubtracted cDNA probes, which were prepared from endosperm mRNA from plants harvested at 10 and 25 DAP. BET1 represents one of a group of clones that showed a strong hybridization signal with the probe from 10-DAP plants and a much weaker signal with the probe made from mRNA from 25-DAP plants.

The BET1 clone contained a 400-bp insert. Fifteen homologous clones were screened from the nonamplified library using the BET1 insert as a probe, indicating an abundance of *BET1* mRNA of between 0.5 and 1% in the mRNA from 10-DAP endosperm. No significant differences in lengths were found between the 15 homologous inserts, suggesting that BET1 is probably close to a full-length cDNA. The three clones that were sequenced differed only in the length of their poly(A) tails.

The BET1 sequence (Figure 1) includes a single continuous open reading frame (ORF) with a coding capacity for an 85–amino acid protein. This ORF is preceded by two in-frame stop codons. The sequence also contains 52 bp of 5′ untranslated sequence and a 99-bp 3′ untranslated region. The sequence, which has been submitted to the EMBL data base, has been given accession number Z49203. The deduced amino acid sequence does not show any significant homology with sequences in the EMBL data base, although it shares overall features, such as size and high asymmetric charge distribution, with certain other putative structural proteins, such as the threonine- and lysine-rich cell wall protein (Domingo et al., 1994). BET1 is also cysteine rich, like the phloem-specific protein PP2 (Bostwick et al., 1992). However, neither PP2 nor BET1 possesses the hevein-type lectin domain (Lucas et al., 1985), which has been identified in many cell wall proteins.

BET1 has two interesting features. First, there is a single pentapeptide motif, SPPPP, which is characteristic of the extensin cell wall protein family (Showalter, 1993; Kieliszewski and Lamport, 1994). This motif may be present in 20 or more copies per protein molecule. The presence of this motif, which is substituted by hydroxyproline and O-glycosylated in extensins, would be consistent with an extracellular location for BET1. In addition to the SPPPP motif, the sequence contains six lysine, three tyrosine, and eight cysteine residues; any of these could potentially cross-link the polypeptide to other extracellular proteins. Second, BET1 has a hydrophobic region with the characteristics of a signal peptide at the N terminus (Figure 1). This predicted signal peptide is homologous to the corresponding sequences from different extensins (Bown et al., 1993) and to a number of other proteins that are targeted to the endoplasmic reticulum.

The predicted signal peptide cleavage site for BET1 could be at either of two possible positions (Figure 1; von Heijne, 1985). The first site, which is a better fit with the consensus sequence, would result in a 68–amino acid mature protein; use of the second site would result in a 57–amino acid product.

Expression of *BET1* in the Developing Endosperm

To investigate the expression pattern of *BET1* during endosperm development, the ^{32}P-labeled BET1 insert was hybridized with filter-bound mRNA isolated from kernels at various developmental stages (Figure 2). A single hybridizing mRNA species was detected, and it corresponded in size to the 450 to 500 bases predicted from the cDNA sequence. The mRNA was first seen at 9 DAP and reached a maximum at 10

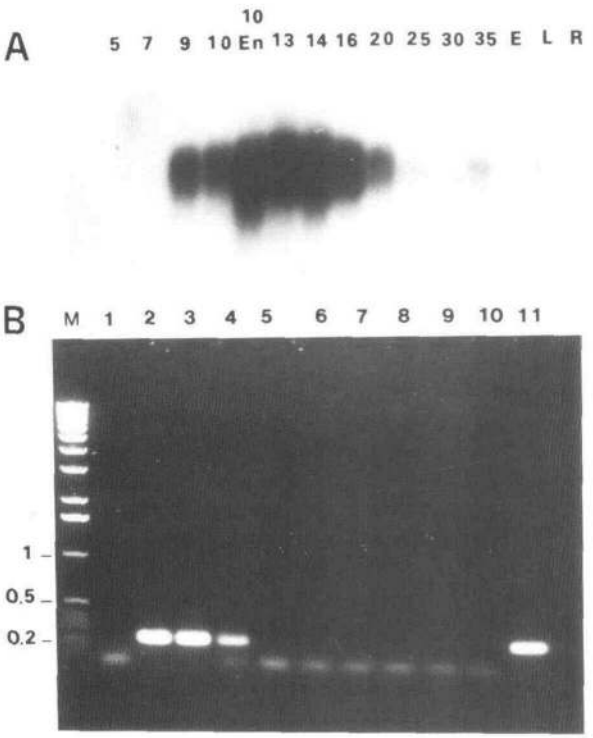

Figure 2. RNA Gel Blot Analysis of *BET1* mRNA and RT-PCR Amplification of the BET1 Coding Sequence.

(A) Each lane contains 1 μg of poly(A)$^+$ RNA from dissected endosperm (En), embryos (E), leaves (L), roots (R), and whole seed (all other lanes) collected at different days after pollination (indicated at the top of each lane). The filter was hybridized with the ^{32}P-labeled cDNA insert of BET1 and exposed for 16 hr. mRNA concentration was controlled by using a ubiquitin probe (Kawalleck et al., 1993) in a subsequent hybridization of the same filter (data not shown).

(B) mRNAs from unpollinated caryopses harvested at anthesis (lane 1), endosperms 15 DAP (lane 2), entire seed 15 DAP (lane 3), dissected embryos 15 to 20 DAP (lane 4), coleoptiles (lane 5), roots (lane 6), root tips (lane 7), and pollen (lane 8) were converted to cDNA using Moloney murine leukemia virus RT and the BET1 sequence amplified with specific primers as described in Methods. In lanes 9 and 10, mRNAs from endosperm and root tips, respectively, were first treated with RNase A and then amplified as described in Methods. These samples served as controls for DNA contamination in RNA preparations. The plasmid containing the BET1 cDNA insert (3 ng) was amplified under the same conditions as the positive control and is in lane 11. Lane M contains the length markers (in kilobases; 1-kb ladder from Bethesda Research Laboratories).

to 14 DAP, decreasing after that to become almost undetectable by 30 DAP. The small increase in signal at 35 DAP may have resulted from the drop in steady state levels of the abundant storage protein mRNAs at 35 DAP, increasing the relative proportion of BET1 in the sample. No signal was detected in mRNA samples from embryo, leaf, or roots (Figure 2). This result was confirmed by RT–polymerase chain reaction (PCR) (Figure 2B). Using PCR primers derived from the BET1 cDNA sequence, no PCR product was detected when cDNA from unfertilized caryopses, coleoptiles, roots, root tips, or pollen was used as a template. A single band of the same length as the plasmid control insert was amplified from endosperm or seed samples. We attributed a faint PCR product derived from embryo cDNA to trace contamination of the embryo mRNA preparation with endosperm mRNA because no mRNA was detectable in the embryo by in situ hybridization (Figure 3).

The *BET1* Gene Is Expressed Only in the Basal Area of the Endosperm

A truncated version of the BET1 cDNA, lacking the poly(A) tail, was used to produce ^{35}S-labeled sense and antisense RNA probes by in vitro transcription with T3 and T7 RNA polymerases, and the probes were used for in situ hybridization of 8-μm-thick sagittal sections of maize kernels at different developmental stages (Figures 3A to 3E). The hybridization signal, which was first detected in the 12-DAP section (Figure 3B), was not evenly distributed over the kernel section but was restricted to a zone of the endosperm that is adjacent to the pedicel. Within this region, only the cells known as endosperm transfer cells appeared to express *BET1*. In particular, no transcript was detected in the placental–chalazal or other maternal cells, nor in the pericarp, aleurone, embryo, or central endosperm cells.

The cells expressing *BET1* are characterized by extensive wall ingrowths that are thought to facilitate the uptake of nutrients from the maternal tissue (Thorne, 1985). As seen in Figures 3B to 3E, only the basal cells directly opposite the maternal vascular tissue contain *BET1* transcripts. The cells transcribing *BET1* at the 12- and 16-DAP stages are apparently not restricted to the basal monolayer, which acquires transfer cell morphology, but a gradation in signal strength from the placental–chalazal region toward the central endosperm is evident, as is a gradient away from the position of the embryo, which can be seen at a higher magnification (Figure 3C). The possibility that the presence of signal over cells adjacent to the basal single-cell layer could be due to scatter from the ^{35}S-labeled probe was eliminated by using digoxigenin-labeled probes (data not shown). Both types of probe gave the same distribution pattern. The signal strength decreased markedly after 16 DAP and was more uniform and confined to the most basal cells. At no stage was signal seen in cells directly bordering the embryo. No hybridization signal was obtained with the sense probe (Figure 3A).

Immunological Characterization of the BET1 Protein

To raise an antiserum against the *BET1*-encoded protein, the coding sequence corresponding to the mature protein was used to express the 68–amino acid ORF as a fusion to an N-terminal hexahistidine tag in *Escherichia coli*. The expression vector pQE60 was used (Hochuli et al., 1988). The protein

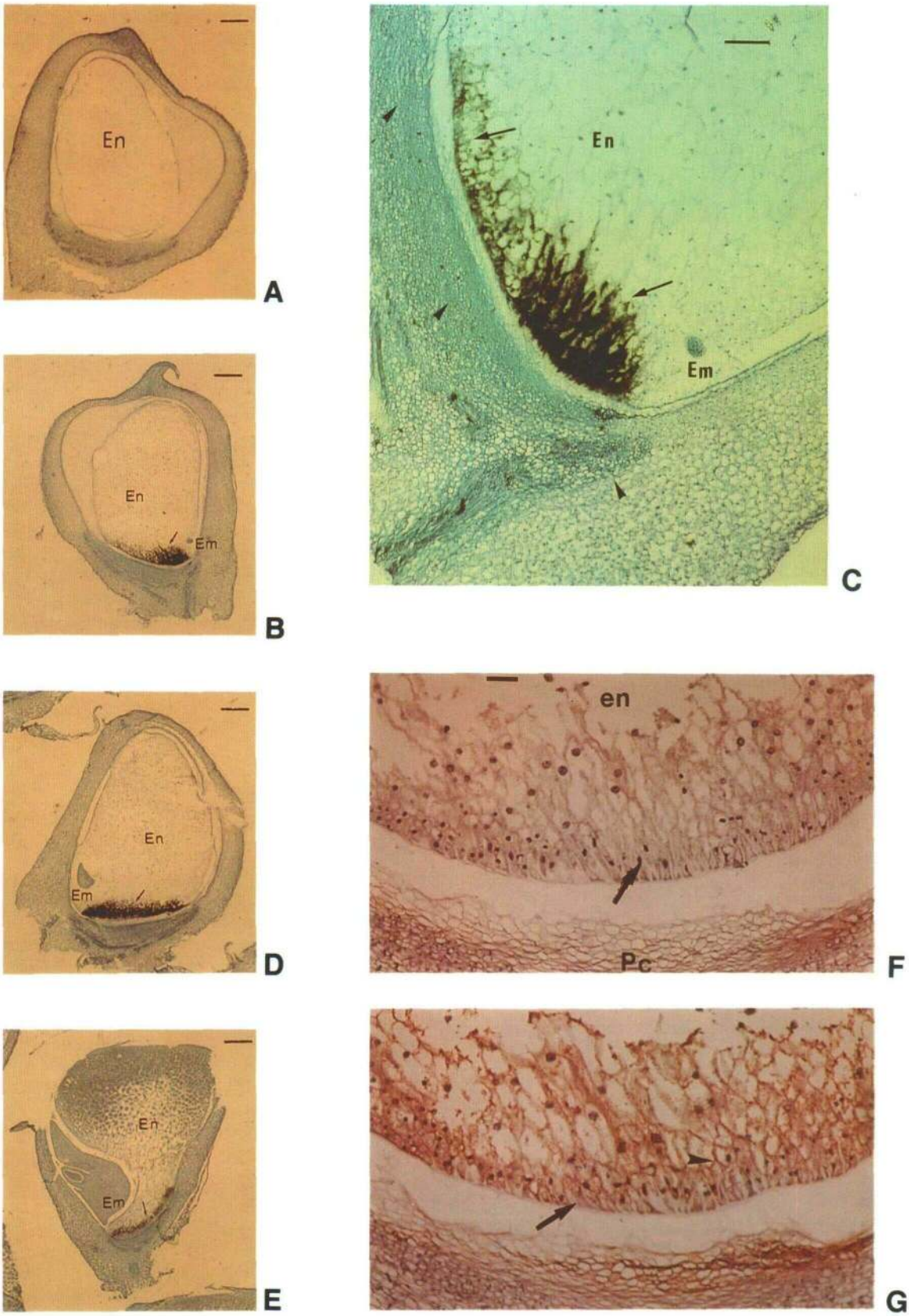

Figure 3. Localization of *BET1* mRNA by in Situ Hybridization and the BET1 Protein by Immunoreactions to Tissue Sections.

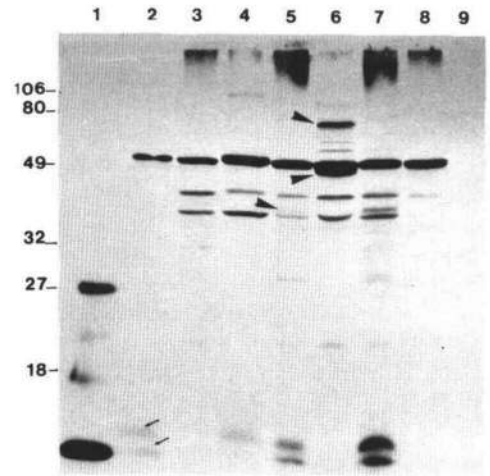

Figure 4. Protein Gel Blotting Using the Anti-BET1 Antiserum.

Equivalent amounts (50 μg of protein) of embryoless seed and endosperm extracts (in 50 mM $CaCl_2$, pH 3.2) at different developmental stages were electrophoresed on a 12% SDS–polyacrylamide gel. Lane 1 contains 100 ng of *E. coli*–expressed BET1 protein; lane 2, 10 μg of protein eluted from intact endosperms by diffusion in 50 mM $CaCl_2$, pH 3.2; lane 3, 50 μg of whole 8-DAP seed; lane 4, 10-DAP seed; lane 5, 15-DAP seed; lane 6, 22-DAP seed; lane 7, basal part of the seed at 15 DAP; lane 8, upper part of the seed at 15 DAP; lane 9, negative control, 50 μg of maize leaf protein extract. After electroblotting the gel onto a nitrocellulose membrane, the filter was immunoreacted as given in Methods. Arrows show the positions of the two polypeptides with electrophoretic mobilities similar to that of the *E. coli*–expressed protein. Arrowheads show BET1-related polypeptides specific to the basal endosperm that appear later during seed development. The apparent protein sizes by reference to size markers are indicated at left in kilodaltons.

was purified to apparent homogeneity by Ni^{2+}–affinity chromatography and used to raise a rabbit antiserum. In protein extracts from maize seeds harvested between 8 and 22 DAP, the antiserum recognized two proteins on immunoblots. They had electrophoretic mobilities similar to that of the *E. coli*–synthesized protein (Figures 4 and 5, indicated by arrows). The presence of two cross-reacting polypeptides might have been due to the use of alternative signal peptide cleavage sites, to the existence of more than one related gene (see also Figure 7), or to post-translational modifications, such as O-glycosylation of the endosperm BET1 protein, which would be consistent with their slightly lower mobility compared with the *E. coli*–synthesized product.

A number of polypeptides with higher apparent molecular masses also cross-reacted with the antiserum. However, we did not believe that they were derived from BET1, because they also appeared in extracts from the upper part of the seed (Figure

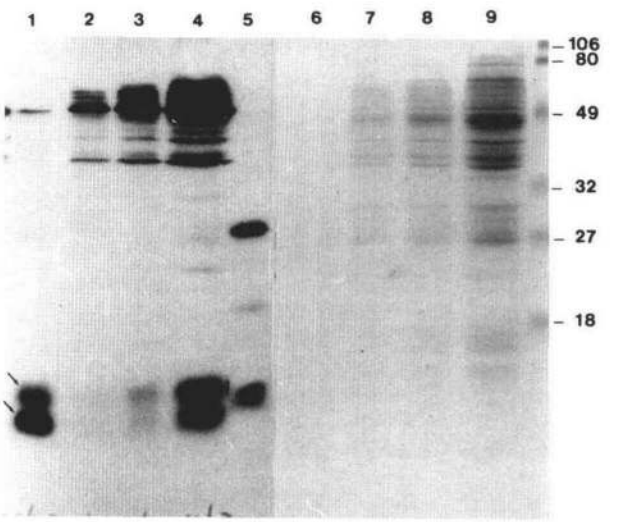

Figure 5. Immunodetection of the BET1 Protein in Cell Wall Eluates from Intact Cells.

Twelve micrograms of protein from intact endosperms (10 DAP) eluted in 50 mM $CaCl_2$, pH 3.2 (lane 1), was electrophoresed on a 15% SDS–polyacrylamide gel along with different amounts of protein extracted from endosperms crushed in the same buffer. Lane 2 contains 12.5 μg of protein; lane 3, 25 μg; lane 4, 75 μg; lane 5, 100 ng of BET1 protein expressed in *E. coli*. Lanes 6 to 9 have the same contents as lanes 1 to 4 but were stained with Coomassie Brilliant Blue R 250. Arrows show the positions of the two polypeptides with electrophoretic mobilities similar to that of the *E. coli*–expressed protein. Protein size markers are indicated at right in kilodaltons.

Figure 3. (continued).

(A) to **(C)** Sagittal sections (8 μm thick) from a maize kernel at 12 DAP. Hybridization with the *BET1* sense probe is shown in **(A)**, and hybridization with antisense probe is shown in **(B)** and **(C)**. **(C)** is a higher magnification of **(B)** in which the signal is seen to extend over at least three cell layers in the basal endosperm. Hybridization conditions are as given in Methods. Arrows in **(B)** and **(C)** indicate hybridization signals in the endosperm transfer cells, and arrowheads in **(C)** designate the vascular tissue and placental–chalazal cells. Bars in **(A)** and **(B)** = 200 μm; bar in **(C)** = 10 μm.

(D) Sagittal sections (8 μm thick) from a maize kernel at 16 DAP. Bar = 200 μm.

(E) Sagittal section (8 μm thick) from a maize kernel at 22 DAP. Bar = 200 μm.

(F) and **(G)** Sagittal sections (8 μm thick) from maize kernels at 20 DAP used to immunolocalize the BET1 protein. The section in **(F)** was incubated with preimmune serum, and the section in **(G)** was incubated with the anti-BET1 antiserum. Arrows indicate the position of the basal cell layer; the arrowhead indicates the presence of signal in cells adjacent to the basal monolayer. Bar in **(F)** = 10 μm.

Em, embryo; en, endosperm; Pc, placental–chalazal region.

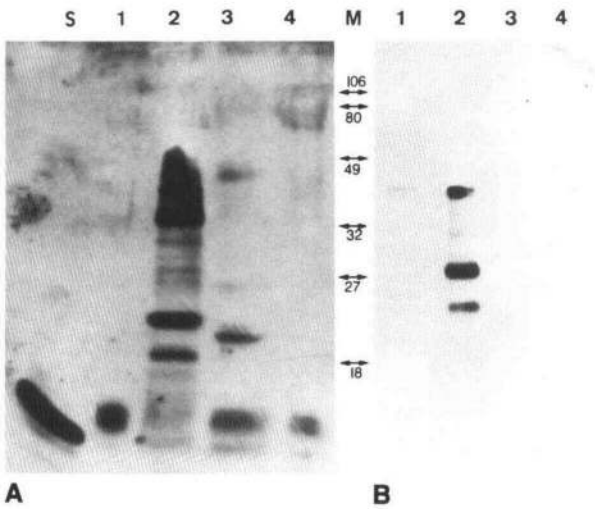

Figure 6. Immunodetection of BET1 in Cell Wall Extracts.

Fifty micrograms of protein extracts from cell wall fractions of maize endosperm at 10 DAP was prepared as given in Methods, fractionated on a 15% SDS–polyacrylamide gel, and electroblotted onto nitrocellulose. M indicates molecular length markers that are given in kilodaltons.

(A) Lane S contains the *E. coli*–derived BET1 protein as the control (1 μg); lane 1, 13,000*g* pellet from crude filtrate; lane 2, 13,000*g* supernatant from crude filtrate; lane 3, 0.2 M $CaCl_2$ cell wall extract; lane 4, 0.2 M $CaCl_2$–2% SDS cell wall extract. The filter was incubated with anti-BET1 antiserum.

(B) Lanes 1 to 4 are as given for **(A)**; they were incubated with antiserum raised against cytosolic glyceraldehyde 3-phosphate dehydrogenase from white mustard (Cerff and Kloppstech, 1982).

4, lane 8), whereas the two bands comigrating with *E. coli*–expressed BET1 only appeared in the lower part of the seed (Figure 4, lane 7), which contains the endosperm transfer cell layer. However, the high molecular mass cross-reacting polypeptides were also expressed tissue specifically in that they were not detectable in the leaf extract. The bands corresponding in mobility to the *E. coli BET1* gene product accumulated during endosperm development, reaching a maximum by 15 DAP, but decreased again to become almost undetectable in these extracts by 22 DAP. This loss of a readily extractable 7-kD BET1 polypeptide coincided with the accumulation of three new immunoreacting polypeptides with higher apparent molecular masses (Figure 4, lanes 5 and 6, arrowheads). The immunoblot analysis shown in Figure 5 also demonstrates that BET1 polypeptides are secreted from the cell because they were extractable from intact endosperms (Figure 5, lane 2) with a calcium-containing buffer that has been reported not to plasmolyse cells (Brownleader and Dey, 1993). The relative enrichment of BET1 in washed as opposed to ground endosperms was assayed by immunoblotting (Figure 5). A comparison of the samples confirmed that most of this protein was selectively leached out without cell lysis, because 12 μg of protein eluted from washed endosperms in extraction buffer gave the same signal strength as 75 μg of proteins extracted by crushing the endosperms in the same buffer. The latter sample also contained additional nonspecific cross-reacting polypeptides. We assumed that the selective enrichment obtained by eluting the proteins without crushing the tissue was due to secretion of the BET1 protein from the cell into the extracellular matrix.

In addition, purification of cell wall proteins according to Stafstrom and Staehelin (1986) showed that the BET1 monomer bands were restricted to this subcellular fraction (Figure 6A). Elution of cell wall fractions with 0.2 M $CaCl_2$ either with 2% SDS (Figure 6A, lane 4) or without SDS (lane 3) released very similar amounts of BET1. The same polypeptides were found in the pellet obtained by centrifuging the crude endosperm extract in 20 mM potassium phosphate, pH 6.0, 10 mM DTT extraction buffer at 13,500*g* (lane 1). However, polypeptides of higher molecular masses that were detected by the anti-BET1 antibody were restricted to the supernatants from the crude endosperm filtrate (lane 2). Slot blot immunoreaction of the samples of the residues from cell wall preparations after $CaCl_2$ elution (data not shown) confirmed that a large proportion of the BET1 protein remains tightly, probably covalently, attached to cell walls, as has also been inferred for the threonine- and lysine-rich cell wall protein (Domingo et al., 1994); therefore, it was not detected by electrophoresis. As a control for the efficiency of fractionation of cytosolic from cell wall–associated proteins, corresponding samples were also probed with an antibody raised against cytosolic glyceraldehyde 3-phosphate dehydrogenase. The resulting signal was essentially restricted to low-speed supernatants (Figure 6B).

The localization of the BET1 protein at the base of the

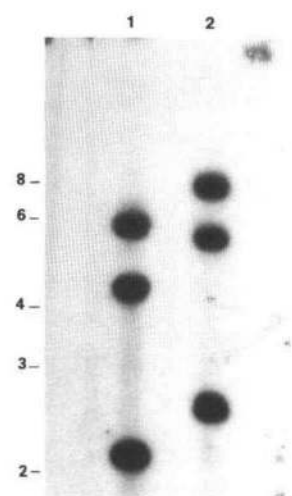

Figure 7. Genomic DNA Gel Blot of Maize DNA Probed with the ^{32}P-Labeled BET1 cDNA.

Lane 1 contains DNA (10 μg) digested with EcoRI; lane 2, DNA (10 μg) digested with HindIII. The filter was processed as described in Methods and exposed for 4 days. Markers are indicated at left in kilobases.

endosperm was demonstrated by in situ immunolocalization (Figures 3F and 3G). The signal (a reddish brown precipitate) obtained with the anti-BET1 antiserum is visible in the endosperm region directly adjacent to the maternal vascular tissues, as shown in Figure 3G, but also extends up three cell layers toward the central endosperm (arrowhead). As was observed with the RNA probe, at no stage is the BET1 protein detectable in cells bordering the embryo, and no signal could be detected with the preimmune serum (Figure 3F).

BET1 Is a Low Copy Number Locus That Maps to Maize Chromosome 2

Figure 7 shows DNA filter hybridization of maize genomic DNA probed with the BET1 cDNA. A similar hybridization pattern appears in digests using two different enzymes (Figure 7). The pattern of three hybridizing bands having similar size increments in both digests could reflect a locus organization of a short repeated array of three copies of the *BET1* gene. The *BET1* locus has been mapped using a segregating population from the cross CM×T232. It mapped to a single location on chromosome 2, which is closely linked to the white pollen (*Wp*) locus (Franken et al., 1991), and in the same region in which three different defective kernel loci, *dek4* (1024 A), *dek16* (1414), and *dek23* (1428), have been mapped (Neuffer and Sheridan, 1980; numbers within parentheses refer to the nomenclature used in their article).

DISCUSSION

BET1 Expression Is Spatially and Temporally Distinct from That of Previously Characterized Endosperm Genes

RNA gel blot analysis and RT-PCR (Figure 2) demonstrated that the *BET1* gene is expressed exclusively in the developing endosperm of maize, because no signal or PCR product was detected in any other plant tissue tested. The faint PCR product observed in the embryo sample (Figure 2B, lane 4) is most likely due to contamination of the RNA preparation with endosperm RNA, because no signal was detected in embryo samples by RNA gel blot analysis or in situ hybridization (see the following discussion). However, the site and timing of *BET1* expression were quite distinct from that seen for most other previously characterized endosperm transcripts. The mRNA was first detectable at 9 DAP, reached a maximum at 12 to 15 DAP, and decreased rapidly after 20 DAP, in contrast to a typical maturation phase transcript in maize. Typically, such an mRNA is first expressed at 11 to 12 DAP, reaches a maximum at 25 DAP, and decreases markedly only after the onset of kernel drying at 35 to 40 DAP. Because the subtractive hybridization procedure was designed to select for genes more highly expressed at 10 than at 25 DAP, this result was anticipated. However, most of the other clone sequences that were preferentially expressed at 10 DAP continued to be expressed throughout the maturation phase (G. Hueros, unpublished data), suggesting distinct functions for the two classes of genes.

BET1 Is Expressed Only in the Basal Region of the Endosperm

In situ hybridization analysis (Figure 3) confirmed the results shown by RNA gel blot hybridization. A *BET1* transcript was not detectable in 5-DAP seed sections (data not shown), was highly expressed at 12 to 16 DAP, and decreased markedly after 21 DAP. The tissue specificity of *BET1* expression was also confirmed by in situ hybridization; the signal was absent from pedicel, seed coat, and embryo. Furthermore, it was not evenly distributed over the entire area of the endosperm but was concentrated in a thin layer at the basal part of the endosperm. Thus, *BET1* expression is confined to the cells known as endosperm transfer cells (Thorne, 1985) and at a lower level in neighboring endosperm cells. The protein encoded by *BET1* was localized using in situ immunolocalization to the same region (Figures 3F and 3G). The protein did not appear to be mobilized to any other region of the seed, and the kinetics of its accumulation followed that of the appearance of the mRNA up to 17 DAP. Possible turnover later in endosperm development has not yet been studied because it is difficult to extract the majority of the protein from the cell wall.

BET1 is presumably very highly expressed in the transfer cells because it represents between 0.5 and 1% of the clones in the unamplified library, although this layer constitutes only a small fraction of the endosperm. The existence of a small multigene family for *BET1* (Figure 7), which has been confirmed by cloning distinct gene copies from an inbred line (G. Hueros, unpublished data), may represent an adaptation to fulfill the *BET1* expression requirements.

The transfer cells are highly modified endosperm cells specialized to facilitate nutrient uptake from the maternal tissues. They are located at the base of the endosperm, directly adjacent to the vascular network formed at the phloem terminus, and contain extensive wall ingrowths, which amplify the absorptive surface of the associated plasmalemma (Pate and Gunning, 1972; Schel et al., 1984; Thorne, 1985). The same wall ingrowths are found at lower density in adjacent endosperm cells up to seven cell layers toward the crown (Davis et al., 1990). Both the time of appearance and the cellular distribution of the wall ingrowths and *BET1* expression coincide, strongly implying a functional relationship. Currently, a role for *BET1* in active solute transfer can only be surmised. Solute transfer from phloem terminals to seed has been studied mainly in systems that only measure the maternal tissue contribution. From the results obtained using the empty corn pedicel system (Porter et al., 1985) and seed coats (Wolswinkel, 1992), it has been concluded that the rate of solute import into the endosperm is controlled by the maternal tissues. Diffusion

and bulk flow appear to be the main forces that affect the symplastic movement of nutrients from phloem termini to the endosperm cavity in wheat (Wang and Fisher, 1994a, 1994b). Although active transport processes may also be involved, little evidence for this has been thus far obtained. On the other hand, the metabolic events that take place in this region can have a major influence on the processes of protein and starch biosynthesis and, thus, on the final endosperm composition (Miller and Chourey, 1992; Lopes and Larkins, 1993). The isolation of the *BET1* clone presents the opportunity to analyze the role played by this specialized region, independent of the specific function of BET1 protein. For this purpose, the regulatory sequences controlling *BET1* expression in transfer cells are of particular interest.

The BET1 Protein Is a Component of the Extracellular Matrix

The deduced BET1 amino acid sequence (Figure 1) contains two features indicative of a cell wall protein. First, the predicted coding sequence begins with a putative 17-residue signal peptide that displays sequence homology with signal peptides encoded by the extensin genes (Bown et al., 1993). In the absence of further targeting domains, this feature would result in secretion of the protein. Second, a Ser-$(Pro)_4$ motif, which is characteristic of the extensins and hydroxyproline-rich structural cell wall proteins (Cassab and Varner, 1988; Showalter, 1993; Kieliszewski and Lamport, 1994), is present. This motif is the site of extensive post-translational modifications by hydroxylation and glycosylation in extensins, where it may be present in more than 20 copies per molecule (Kieliszewski and Lamport, 1994).

To test whether BET1 is indeed secreted from the cells into the cell wall matrix, endosperm protein extracts were prepared using a technique selective for cell wall proteins (Brownleader and Dey, 1993). The selective enrichment for BET1-related polypeptides obtained by noncytolytic elution, as compared with the extract obtained by grinding the endosperms in the same buffer, indicated that the protein is indeed present extracellularly (Figure 5). The extractability of protein monomers by ionic desorption has been reported to be a cell wall protein characteristic (Miller and Fry, 1992). Subcellular fractionation also showed that BET1 is restricted to the cell wall (Figure 6), whereas the majority of the larger cross-reacting polypeptides, which are not restricted to the basal endosperm (see Figure 4, lanes 6 to 8), are in the cytosol. Only trace amounts of higher molecular mass forms were extractable from cell walls (Figure 6), suggesting the possibility of BET1 insolubilization by cross-linking to other cell wall components, as has been reported for other proteins during cell wall maturation (Bradley et al., 1992). This possibility was supported by a positive immunoblot reaction of insoluble cell wall residues remaining after elution with $CaCl_2$ (0.2 M) and SDS (data not shown).

Possible Roles for BET1 Protein

There are three possible roles for BET1 to be considered. First, BET1 could be secreted to protect the seed from the entry of infectious agents. The defensive role of cell wall proteins has been documented both for extensins (Cassab and Varner, 1988) and for arabinogalactan proteins and gums (Kieliszewski and Lamport, 1994). However, the ability of these proteins to interfere with the pathogen attack is thought to be due to the production of structural barriers via network formation. Because BET1 is much smaller than previously described extensins and possesses only one Ser-$(Pro)_4$ motif, it seems more probable that it could limit the formation of such networks by chain termination.

A second possible role for BET1 would be mechanical. Cell wall characteristics, such as elasticity or tensile strength, are thought to be determined by the complement of cell wall proteins (Cassab and Varner, 1988). The maize mutant *miniature-1* (*mn1*; Lowe and Nelson, 1946) has been characterized by the breakage of the cell walls at the placental–chalazal region. *mn1* mutant seed develop normally up to 9 DAP (when *BET1* is first expressed); the maternal cells of the placenta then degenerate, and a gap appears between the pedicel and the endosperm basal region, resulting in the near-arrest of seed development at 14 DAP. Lowe and Nelson (1946) suggested that the wild-type endosperm tissue produces some substance that promotes longevity of the chalazal layer. This hypothesis is supported by genetic analysis of different maternal/zygote *mn1*/*Mn1* combinations (Miller and Chourey, 1992) in which a role for reduced endosperm and pedicel invertase levels in causing the phenotype was proposed.

To test whether *BET1* expression is affected in *mn1*, *BET1* mRNA levels in wild-type and *mn1* seeds were compared by gel blot hybridization. Although the *BET1* mRNA level was reduced by 50% in *mn1* (data not shown), this may represent a secondary effect of the mutation and cannot be attributed to direct genetic control or cause of the *mn1* lesion. *Mn1* maps on the short arm of chromosome 2, whereas *BET1* maps on the long arm of the same chromosome and is therefore nonallelic to *mn1*.

A third possible role for BET1 protein would be to promote the deposition of cell wall components in the extensive wall ingrowths that characterize transfer cells. The expression of *BET1* (Figures 2 and 3) started at the same time point (7 to 9 DAP) as the formation of cell wall modifications in transfer cells. Furthermore, the location of the *BET1* mRNA was restricted to those cells possessing characteristic ingrowths in the cell walls (Schel et al., 1984; Davis et al., 1990), which is consistent with a role in this structural modification.

The extensin-like Ser-$(Pro)_4$ motif present in BET1 appears to confer local molecular rigidity on the extensin protein by adopting a polyproline II conformation, to facilitate the interaction with other cell wall components, and to assist incorporation of extensin into the wall matrix. All these features implicate proteins containing extensin motifs in the process of wall self-

assembly. Other extensin-related proteins and arabinogalactan proteins, which are hydroxyproline rich and located in the cell wall but lack the Ser-$(Pro)_4$ motif, also have structures and cell-type-specific localization, suggesting a role in directing cell wall assembly, possibly by acting as matrices for the orderly addition of nascent wall precursors (Kieliszewski and Lamport, 1994). A small (25 kD) arabinogalactan-like protein has also been correlated with maize pollen coat patterning (Chay et al., 1992). Both the structural features and location of BET1 would therefore be consistent with a role in the formation of wall ingrowths in transfer cells.

METHODS

Plant Material

Mature leaf, pollen, and seed samples were extracted from greenhouse grown *Zea mays* cv A69Y. Seedlings were germinated aseptically on moist filter paper in Petri dishes. A DNA filter bearing digests of isogenic lines from F_2 plants from the cross CM×T232 (supplied by B. Burr, Brookhaven National Laboratories, Upton, NY) was a gift from G. Theissen (Max-Planck-Institut für Züchtungsforschung).

RNA Isolation and Analysis by Filter Hybridization

Poly(A)$^+$ RNA from different sources was isolated by the phenol–proteinase K method as described in Bartels and Thompson (1983). Poly(A)$^+$ RNA was directly purified from the deproteinized aqueous phase by batch chromatography on oligo(dT) cellulose and stored at −70°C under ethanol. Capillary transfer to nylon membranes was performed as described previously (Maniatis et al., 1982). mRNA was electrophoresed through 1.5% formaldehyde agarose gels and transferred in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to Hybond-N membranes (Amersham). Blots were hybridized at 68°C in 7% SDS, 0.5 M Na_2HPO_4, pH 7, with 3 × 10^6 cpm/mL and washed at 65°C in 0.1 × SSC, 0.1% SDS.

DNA Isolation and Filter Hybridizations

Genomic DNA was isolated from the aqueous phase of the RNA extraction mentioned previously. After oligo(dT) cellulose chromatography, the DNA was precipitated from the supernatant with two volumes of ethanol, dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0, and incubated with RNase A (100 μg/mL, 37°C, 30 min) and proteinase K (100 μg/mL, 50°C, 1 hr). The incubation was terminated by phenol–chloroform extraction, and the DNA was recovered by ethanol precipitation.

Capillary transfer to nylon membranes (Hybond-N; Amersham) was performed according to Maniatis et al. (1982). The filters were hybridized and washed as described for RNA filters.

Radioactive DNA Probe Preparation

A BET1 (for basal endosperm transfer layer) clone insert was amplified by polymerase chain reaction (PCR) using flanking universal forward and reverse primers from pBluescript SK− (Stratagene). The PCR was phenol–chloroform extracted and ethanol precipitated. Labeling was performed by random primer labeling using ^{32}P-dCTP as the radioactive nucleotide, to a specific activity of 10^9 cpm/μg.

Expression of BET1 cDNA in *Escherichia coli*

To raise an antiserum against the *BET1*-encoded protein, the coding sequence corresponding to the mature protein was amplified from the BET1 clone by PCR. The PCR product was used to express the 68-amino acid open reading frame (ORF) as a fusion to an N-terminal hexahistidine tag in *E. coli*, using expression vector pQE60 (Hochuli et al., 1988; supplied by Quiagen, Hilden, Germany). The protein was deposited predominantly in inclusion bodies. The inclusion bodies were isolated by differential centrifugation and redissolved in 6 M guanidine hydrochloride according to the manufacturer's protocol. BET1 was purified from this extract to apparent homogeneity by Ni^{2+}–agarose chromatography and used to raise a rabbit antiserum by repeated subcutaneous injections of 50 μg of protein samples at 4-week intervals.

In Situ Hybridization

Maize seeds were collected at 12, 16, 21, 25, and 30 days after pollination (DAP) and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 12 to 24 hr at 4°C depending on the kernel size. Seeds were dehydrated in a graded ethanol–xylene series and embedded in Fibrowax (Plano GmbH, Marburg, Germany). Sections (8 to 10 μm) were prepared using a microtome (Reichert-Jung, Nussloch, Germany).

Before hybridization, the sections were deparaffinized and treated with 5 μg/mL proteinase K (Merck). In situ hybridization was performed essentially as described by Cox and Goldberg (1988). The BET1 insert minus the poly(A) tail in pBluescript SK− was used as the template for synthesis of ^{35}S-labeled riboprobes with T3 and T7 RNA polymerases. Mounted slides were incubated with 50 to 60 ng of RNA per slide for 14 hr at 50°C. After hybridization, the sections were incubated with 40 μg/mL RNase A, washed several times with 2 × SSC, coated with LM-1 silver grain emulsion (Amersham), and exposed for 4 days at 4°C. After developing, sections were stained with 0.025% azure B (Sigma) and mounted with DPX mounting medium (Agar Scientific, Stansted, UK).

For immunolocalization, sections were deparaffinized and blocked with 1% BSA in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) for 20 min at room temperature and incubated overnight with anti-BET1 antiserum or preimmune serum (both diluted 1:300). The immunoreaction was detected using horseradish peroxidase–coupled second antibody (Sigma A-6154; diluted 1:800) and 3-amino-9-ethylcarbazole staining (Sigma AEC-101 staining kit).

Selective Elution of Cell Wall Proteins from Intact Cells

Proteins were extracted either by grinding tissue in 50 mM $CaCl_2$, pH 3.2 (Brownleader and Dey, 1993), with a pestle and mortar to obtain a total cell extract or using a selective elution for extraction of cell wall proteins. In this case, endosperms (10) were isolated from the kernels by manual dissection, washed in a large volume (500 mL) of chilled water, and then floated in 500 μL of 50 mM $CaCl_2$, pH 3.2. After 5 min,

the liquid was removed with a pipette. Protein concentrations were determined by the Bradford assay (Bio-Rad). After separation by SDS–PAGE, proteins were electroblotted onto a nitrocellulose membrane (BA85; Schleicher & Schuell) in Tris-glycine buffer, pH 8.3 (Towbin et al., 1979). The membrane was treated with a 1:1000 dilution of BET1 serum in TTBS (0.05% Tween 20 in Tris-buffered saline) containing 5% nonfat dried milk powder for 1 to 3 hr. Detection was performed with horseradish peroxidase–coupled second antibody (goat anti-rabbit; Sigma A-6154) at a 1:5000 dilution for 45 min. The reaction was detected using enhanced chemiluminescence (Amersham).

Analysis of Proteins in Cell Wall Extracts

Cell wall fractions were isolated essentially as previously described (Stafstrom and Staehelin, 1986). Ten DAP PA91 maize kernels (9.5 g) were ground in 45 mL of extract buffer (20 mM potassium phosphate, pH 6.0, 10 mM DTT) on ice and filtered through two layers of Miracloth (Calbiochem). The filtrate was centrifuged for 10 min at 13,000*g* in a microcentrifuge to give pellet 1 and supernatant 1 (Figure 6, lanes 1 and 2, respectively). The residue retained on the Miracloth filter was reextracted in extract buffer twice by grinding each time in 25 mL of extract buffer and recovering by centrifugation at 5000*g* for 10 min. The resulting pellet was split in two. Half was extracted by grinding in 0.2 M $CaCl_2$ (Figure 6, lane 3), and the remainder was ground in 0.2 M $CaCl_2$, 2% (w/v) SDS (Figure 6, lane 4). Fifty micrograms of protein from each sample was loaded on duplicate 15% SDS–polyacrylamide gels and processed by immunoblotting either for BET1 detection or for detection using antiserum raised against maize glyceraldehyde 3-phosphate dehydrogenase (Cerff and Kloppstech, 1982; kindly provided by R. Cerff, Biozentrum, Technische Universität, Braunschweig).

Polymerase Chain Reactions

For the reverse transcription–PCR amplification, cDNA was synthesized using the Moloney murine leukemia virus reverse transcriptase, oligo(dT) primer, and 200 ng of mRNA template. Eight nanograms of cDNA (out of a 200 ng yield) was taken for PCR amplification using the oligonucleotides 5′-GAGACCATGGCCATTCTGTCCTCACTGTC-3′ and 5′-TGT-GAGATCTATGGGAGCAGCAGCACAC-3′ (locations indicated in the BET1 sequence in Figure 1). The reactions were run in a Biometra Trio block (Biometra, Göttingen, Germany) at 93°C for 30 sec, 68°C for 1 min, and 72°C for 1 min for 35 cycles.

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