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maternally expressed gene1 Is a Novel Maize Endosperm Transfer Cell–Specific Gene with a Maternal Parent-of-Origin Pattern of Expression [W]

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Growth of the maize (*Zea mays*) endosperm is tightly regulated by maternal zygotic and sporophytic genes, some of which are subject to a parent-of-origin effect. We report here a novel gene, *maternally expressed gene1* (*meg1*), which shows a maternal parent-of-origin expression pattern during early stages of endosperm development but biallelic expression at later stages. Interestingly, a stable reporter fusion containing the *meg1* promoter exhibits a similar pattern of expression. *meg1* is exclusively expressed in the basal transfer region of the endosperm. Further, we show that the putatively processed MEG1 protein is glycosylated and subsequently localized to the labyrinthine ingrowths of the transfer cell walls. Hence, the discovery of a parent-of-origin gene expressed solely in the basal transfer region opens the door to epigenetic mechanisms operating in the endosperm to regulate certain aspects of nutrient trafficking from the maternal tissue into the developing seed.

INTRODUCTION

Double fertilization in flowering plants results in the formation of two very different structures—the embryo and endosperm (Nawaschin, 1898; Guignard, 1899; Kiesselbach, 1949). The embryo is diploid, whereas the endosperm is a triploid structure in most angiosperms, containing two maternal genomes and one paternal genome, a combination that is held to be essential for correct seed development (Lin, 1984; Scott et al., 1998).

In maize (*Zea mays*), as in most other species, the endosperm initially develops as a coenocyte and subsequently follows a program of cellularization requiring close coordination between nuclear division and cell wall formation (Olsen, 2001; Sørensen et al., 2002; Dickinson, 2003). After cellularization, the maize endosperm differentiates a range of tissues that assume specialized roles within the developing seed. The basal endosperm transfer layer (BETL) is responsible for the uptake of nutrients from the maternal tissue; the embryo surrounding region acts as a nutritive and protective layer investing the embryo; and the starchy endosperm accumulates storage proteins and carbohydrates, whereas the aleurone is involved in the breakdown and mobilization of storage products upon germination (Lopes and Larkins, 1993; Becraft, 2001; Olsen, 2001). Apart from acting to accumulate and process nutrients for eventual transfer to the developing embryo and seedling, the endosperm is held to assume both a regulatory function during embryogenesis (Lopes and Larkins, 1993) and play a pivotal role in the control of seed size (Lin, 1982; Birchler and Hart, 1987; Kermicle and Alleman, 1990; Scott et al., 1998). The endosperm may also be involved in speciation, for it has been proposed to prevent wide hybridization by acting as a postzygotic barrier to seed development (Cooper and Brink, 1942; Gutiérrez-Marcos et al., 2003).

Both embryo and endosperm develop within the ovule integuments, and although little is known of the interactions that take place between these tissues, it is likely that a coordinated interplay between the sporophytic and gametophytic tissues is a crucial component of seed formation (Lopes and Larkins, 1993). The extent to which maternal tissue is essential for this process is unclear because somatic embryogenesis and endosperm development can occur in vitro in the absence of maternal tissue (Zimmerman et al., 1989; Kranz et al., 1998). There is, however, clear genetic evidence that female sporophytic and gametophytic genes govern early endosperm development (Chaudhury and Berger, 2001; Evans and Kermicle, 2001; Grini et al., 2002; Garcia et al., 2003); for instance, a number of female-gametophytic mutations in *Arabidopsis thaliana* have been shown to severely affect development of the seed, particularly the endosperm (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kohler et al., 2003). The subsequent molecular characterization of these mutations revealed the existence of a set of proteins (MEDEA [Grossniklaus et al., 1998; Luo et al., 1999], FIS2 [Luo et al., 1999], and FIE

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[Ohad et al., 1999]) that are closely related to the *Drosophila melanogaster* Polycomb-group (PcG) proteins. In plants, as in flies, these PcG proteins aggregate into complexes (Kohler et al., 2003) that are required for the establishment of the anterior–posterior axis in the endosperm (Sørensen et al., 2001) and repression of precocious embryo and endosperm development until fertilization (Luo et al., 2000; Spillane et al., 2000). Interestingly, the expression of these genes in the endosperm is restricted to the maternal alleles by a mechanism conventionally termed genomic imprinting (Kinoshita et al., 1999; Luo et al., 2000; Grossniklaus et al., 2001). Although genomic imprinting is well characterized in mammals (Reik and Walter, 2001), it remains poorly understood in plants, with only few imprinted loci reported to date (reviewed in Alleman and Doctor, 2000; Walbot and Evans, 2003).

In maize, the majority of imprinted loci are subject to a parent-of-origin pattern of expression at a particular allele, otherwise known as allele-specific imprinting; examples include α-*tubulin3* and α-*tubulin4* (Lund et al., 1995a, 1995b), the *R* locus controlling aleurone pigmentation (Kermicle, 1970), and *dzr1* that regulates accumulation of 10-kD zeins (Chaudhuri and Messing, 1994). By contrast, only a few maize loci, namely *fie1*, *fie2* (Danilevskaya et al., 2003; Gutiérrez-Marcos et al., 2003), and *nrp1* (Guo et al., 2003), display gene-specific imprinting, as has been reported in Arabidopsis for *MEDEA*, *FIE*, *FIS2* (reviewed in Baroux et al., 2002), and *FWA* (Kinoshita et al., 2004). Whereas allele-specific imprinting occurs in later stages of maize endosperm development (Alleman and Doctor, 2000), gene-specific imprinting usually only occurs during early stages of development (for examples, see Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Danilevskaya et al., 2003; Gutiérrez-Marcos et al., 2003). The only exceptions so far identified are *fie1* (Danilevskaya et al., 2003; Gutiérrez-Marcos et al., 2003) in maize and *FIS2* (Luo et al., 2000) in Arabidopsis, which remain imprinted throughout endosperm development.

To explore the nature and extent of this parent-of-origin gene expression in the maize endosperm, we performed a molecular screen to identify genes that exhibit either a maternal or paternal pattern of expression (Gutiérrez-Marcos et al., 2003). We report here the characterization of a novel gene belonging to a family of sequences predominantly expressed in the endosperm. *maternally expressed gene1* (*meg1*) is preferentially expressed through the maternal allele during early endosperm development but at later stages is expressed from both parental alleles. Interestingly, *meg1* encodes a small, glycosylated, Cys-rich polypeptide exclusively localized within the labyrinthine walls of the BETL. The discovery of *meg1* reveals the existence of a previously unknown class of BETL-specific proteins, while adding significantly to a rapidly increasing group of sequences exhibiting either constitutive or transient parent-of-origin transcriptional regulation during early endosperm development.

RESULTS

Identification and Cloning of the *meg* Gene Family

A genomic screen based on allelic message display (AMD) was designed to identify endosperm transcripts showing parent-of-origin patterns of expression (Gutiérrez-Marcos et al., 2003). For AMD, we used RNA isolated from endosperms that were dissected from four reciprocally crossed parental inbred lines. This analysis resulted in the identification of several fragments exhibiting monoallelic maternal expression, one of which was termed *meg1* (Figure 1). The full-length meg1 cDNA and three other similar but nonidentical cDNAs, consequently termed meg2, meg3, and meg4, were identified using the gel-purified DNA fragment to screen a 7 d after pollination (DAP) endosperm cDNA library. After searching the maize genome database, an additional two ESTs showing partial similarity to the C terminus of the translated MEG1 protein sequence were identified and named meg5 and meg6. We further identified by database search several ESTs from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) with open reading frames displaying strong similarity to the *meg* gene family.

The expression patterns of *meg1* and the other *meg* cDNAs were investigated by RT-PCR using gene-specific oligonucleotide pairs (Figure 2A). meg1 transcript was only present in endosperm samples from 4 to 20 DAP, indicating that its expression is endosperm specific. *meg2* and *meg4* also showed a similar pattern of expression. By contrast, *meg3* was expressed in endosperm as well as in anther and pollen samples,

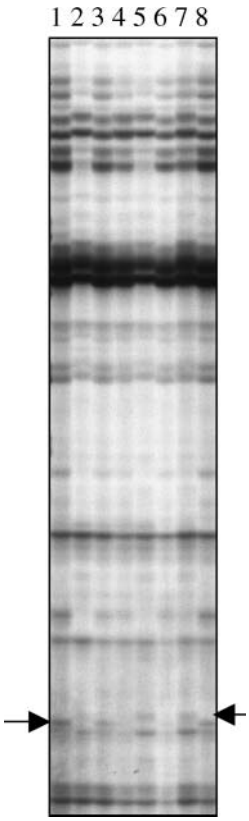

Figure 1. Autoradiograph of an AMD Gel.

Arrows highlight maternal allelic expression of *meg1*. F2 selfed (lane 1); Mo17 selfed (lane 2); F2 × Mo17 (lane 3); Mo17 × F2 (lane 4); A69Y selfed (lane 5); F2 selfed (lane 6); A69Y × F2 (lane 7); F2 × A69Y (lane 8).

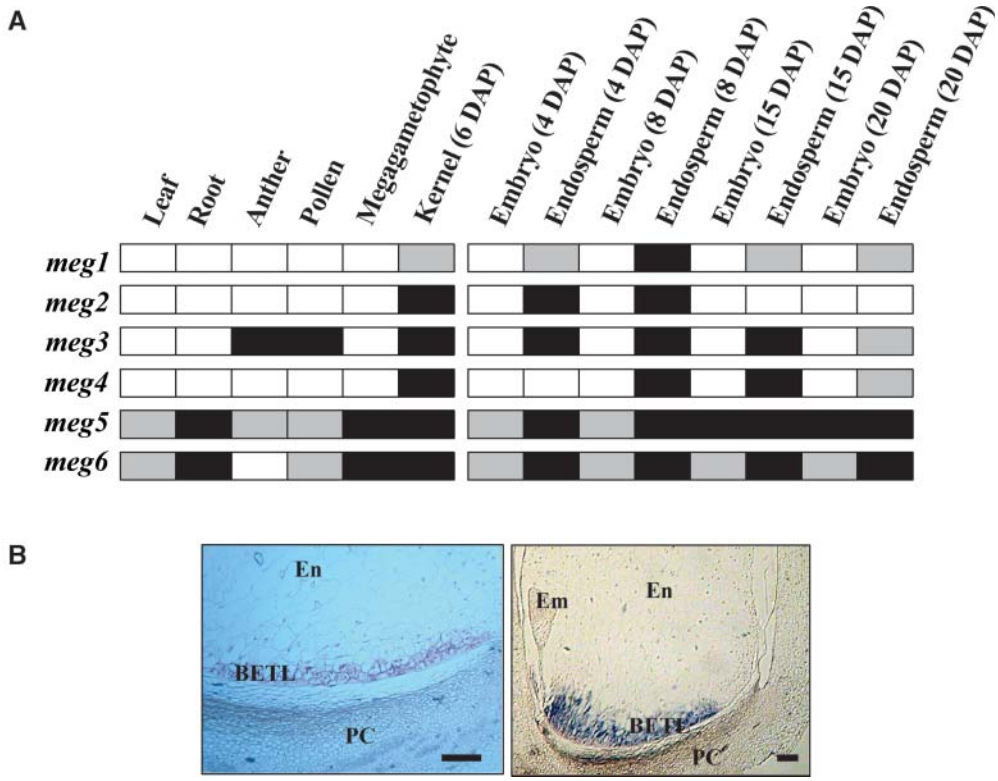

Figure 2. Expression Analysis of *meg1* and Related Sequences.

(A) RT-PCR of *meg1* and five related cDNAs using RNA samples isolated from a range of tissues and amplified with gene-specific primers (see supplemental data online). Dark and light coloration indicate high and intermediate signal, respectively, and open boxes indicate no expression.
(B) mRNA localization of *meg1* in developing seeds. Left, 4 DAP; right, 12 DAP. Em, embryo; En, endosperm; PC, pedicel. Scale bars = 100 μm.

whereas *meg5* and *meg6* were found to be expressed in most tissues tested (Figure 2A).

In situ hybridization of *meg1* was performed on kernel sections at various stages of development with a gene-specific probe. No signal was detected with the sense probe (data not shown), but the antisense probe generated a strong signal found only in the transfer cells from 4 DAP and showing maximum expression at 10 to 12 DAP (Figure 2B). Expression declined thereafter and was absent in >25 DAP kernel sections (data not shown).

DNA gel blot analysis showed that ~4 to 5 copies of *meg1* are present in most maize inbred lines, teosintes, and other grasses (data not shown). Subsequent restriction fragment length polymorphism analysis using a population of immortalized F2 maize lines enabled us to map the *meg1* gene cluster to the short arm of chromosome 7, between markers *csu13* and *bngl1200*.

meg1 Has a Maternal Parent-of-Origin Pattern of Expression

The maternal parent-of-origin expression pattern of *meg1* was further investigated by allele-specific RT-PCR using oligonucleotides designed to detect polymorphisms in the 3′ untranslated region of the gene between inbred lines F2 and A69Y, and W23 and other standard inbred lines. The results confirmed exclusive expression of the *meg1* maternal allele at early stages of endosperm development (i.e., from 4 DAP; Figure 3A). Surprisingly, we found that at later stages (12 DAP) *meg1* expression became biallelic (Figure 3A). To study the effects of altering the maternal to paternal genomic ratio in the endosperm on *meg1* expression, reciprocal crosses between diploid and tetraploid inbred lines were performed. Allele-specific RT-PCR showed that the *meg1* parent-of-origin expression pattern remained unaltered (Figure 3A).

Maternal *meg1* Alleles Are Hypomethylated in the Endosperm

Because *meg1* is subject to a parent-of-origin pattern of expression in the endosperm, we hypothesized that the male and female alleles could be differentially methylated. We tested our hypothesis by examining the methylation status of *meg1* parental alleles. Genomic DNA obtained from embryo and endosperm samples harvested at 6 DAP was digested with a methylation-insensitive restriction enzyme (*Hin*dIII) and a methylation-sensitive enzyme (*Ava*II) in separate reactions. These samples were subsequently analyzed by DNA gel blot hybridization using a gene-specific probe. No parental profiles were observed in the endosperm when samples were digested with

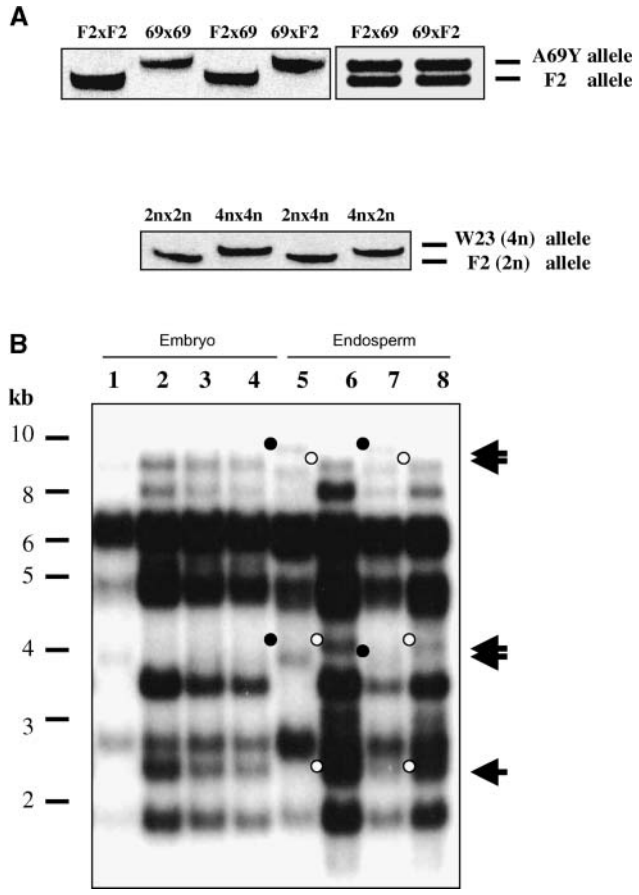

Figure 3. Parent-of-Origin Expression and Methylation Analysis of *meg1*.

(A) Allele-specific RT-PCR analysis of *meg1* in the endosperm. Top left, *meg1* sequence polymorphism detected in endosperms from selfed and reciprocally crossed F2 and A69Y (69) lines. Note that endosperms resulting from reciprocal crosses show expression of the maternal *meg1* allele at 4 DAP. Top right, *meg1* expression is biparental in 12 DAP endosperms resulting from F2 and A69Y reciprocal crosses. Bottom, *meg1* sequence polymorphism in F2 diploid (2n) and W23 tetraploid (4n) endosperms and showing monoallelic maternal expression in 4 DAP endosperms resulting from reciprocal interploidy crosses.

(B) Methylation analysis of *meg1* in 6 DAP embryos and endosperms. Embryo samples (lanes 1 to 4): W22 selfed (lane 1); A69Y selfed (lane2); W22 × A69Y (lane 3); A69Y × W22 (lane 4). Endosperm samples (lanes 5 to 8): W22 selfed (lane 5); A69Y selfed (lane 6); A69Y × W22 (lane 7); W22 × A69Y (lane 8). Closed circles, W22-specific fragment. Open circles, A69Y-specific fragment.

*Hin*dIII (data not shown), whereas differences were observed between parental *meg1* polymorphic alleles when these samples were digested with *Ava*II (Figure 3B). Two W22-specific fragments (~4.0 and 8.5 kb) and two A69Y-specific fragments (~2.7 and 4.5 kb) were present in endosperm samples only when W22 and A69Y lines were used as a pollen source, respectively, but not when used as females, indicating that *meg1* maternal alleles are hypomethylated. These data point to a correlation between methylation status of a given *meg1* allele and its expression in the endosperm.

MEG Proteins Contain Conserved Cys-Rich Motifs

The full-length meg1 cDNA contained an open reading frame of 88 amino acids (Figure 4A), encoding a predicted 9794-D protein with a pI of 7.5. The MEG1 polypeptide contains a hydrophobic N-terminal region with characteristics of a 27–amino acid signal peptide (von Heijne, 1986). Detailed analysis identified a putative cleavage site between His-26 and Glu-27, thus producing a polypeptide containing 61 amino acids, with a predicted molecular mass of 6730 D and pI of 6.2. A protein alignment of MEG1 and the other MEG proteins (i.e., MEG2 to MEG6) revealed the presence of a highly conserved Cys-rich domain in the C-terminal portion (Figure 4A). Further, a conserved amino acid motif comprising eight Cys and two Tyr residues were present in most MEG proteins as well as in the related proteins from *T. aestivum* and *H. vulgare* (Figure 4A). A further interesting feature common to most maize MEG proteins is a conserved group of amino acids with high homology to a glycosylation sequon (Mellquist et al., 1998; Wormald and Dwek, 1999), located in the predicted cleaved polypeptide (Figure 4A).

To better understand the function of the conserved Cys and Tyr residues in the native MEG1 protein, we generated a sequence homology–based model. In developing this model, the pattern of disulfide bonds in MEG1 was assumed to be similar to that described for other proteins containing eight conserved Cys residues, such as SP11 and plant defensins (Thomma et al., 2002; Mishima et al., 2003). The molecular architecture of this group of proteins consists of a small α/β structure with extensive loop regions, held together by four disulfide bonds (Thomma et al., 2002). When the sequence of MEG1 was aligned with that of SP11, deletions were revealed in the α-helical region between Cys 2 and 3, in addition to an almost complete deletion of the region containing the hypervariable loop between Cys 3 and 4 and a significant insertion between Cys 6 and 7 (Figure 4B). It is interesting to note that despite these disparities, the extended loop between Cys 6 and 7 in MEG1 occupies a similar structural domain to that of the loop between Cys 3 and 4 in SP11 and plant defensins (Figure 4B). Importantly, in our predicted structure for MEG1, the conserved Tyr residues are located in accessible positions at the surface of the protein (Figure 4B).

The MEG1 Protein Is Localized to the Wall Ingrowths of the Basal Endosperm Transfer Cells

To determine the localization of MEG1 protein in maize endosperm, we raised a polyclonal antiserum using a synthetic peptide for the N terminus of the putatively processed MEG1 polypeptide. Immunolocalization was performed using the purified antiserum, which detected MEG1 protein adjacent to the cell wall ingrowths of basal endosperm transfer cells (Figure 5A). To obtain biochemical evidence of protein localization to the cell wall, protein extracts from 10 DAP endosperms were fractionated as described by Serna et al. (2001) and analyzed by immunoblotting (Figure 5B). We found that a number of

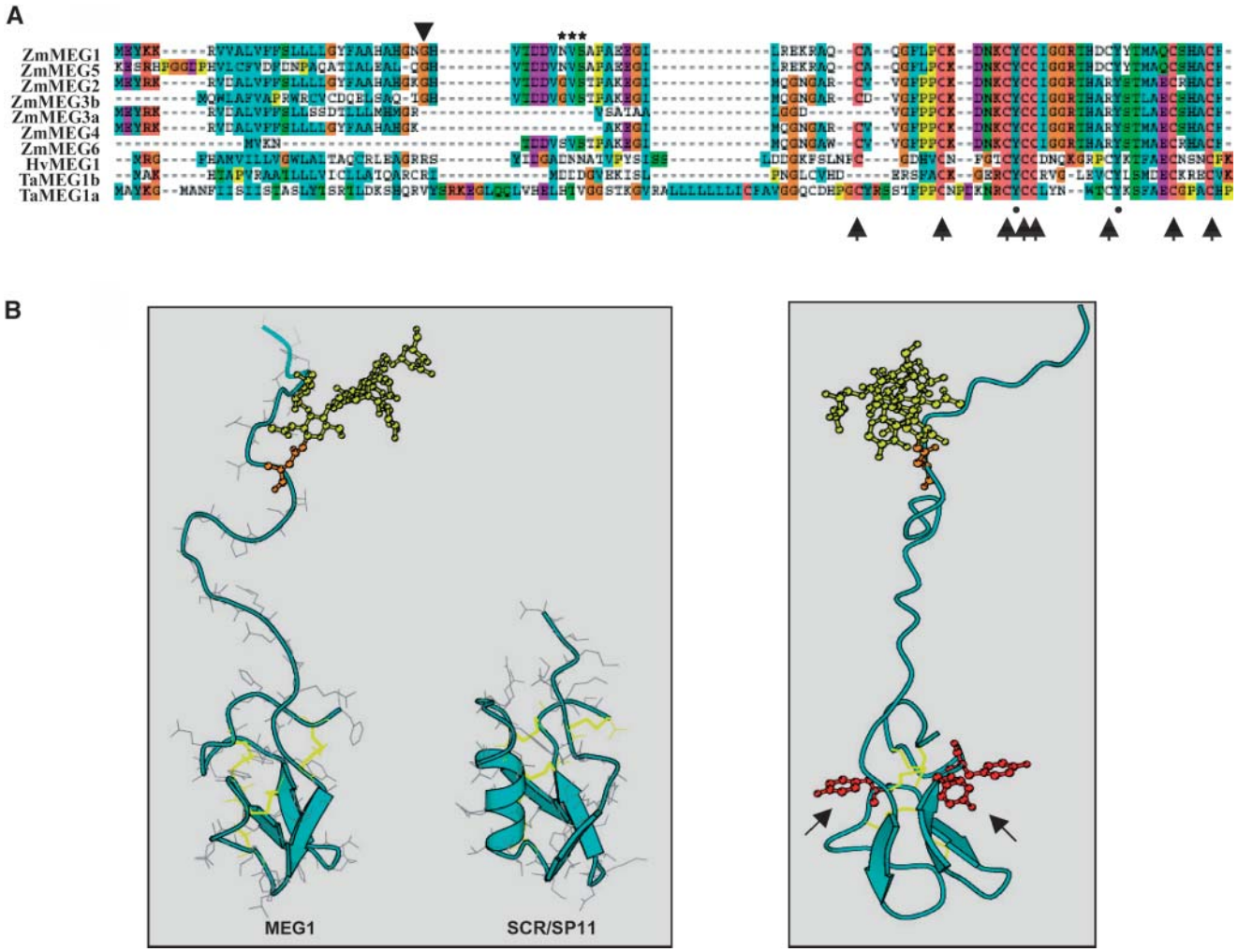

Figure 4. MEG1 Protein Analysis.

(A) Amino acid conservation of predicted MEG polypeptides in maize (Zm), barley (Hv), and wheat (Ta). Arrowhead indicates putative cleavage site of transit peptides. Stars denote the putative glycosylation sequon. Arrows mark the positions of conserved Cys, whereas closed circles highlight the conserved Tyr residues. Note that MEG3 has two predicted polypeptides (MEG3a and MEG3b).

(B) Homology-based model of MEG1 structure. Left, comparison of the predicted model of MEG1 and the crystal structure of SCR/SP11. Disulfide bonds are shown in yellow; single *N*-glycan is shown as a yellow ball-and-stick model. β-Strands are represented by broad arrows and the α helix by a helical ribbon. Right, spatial distribution of conserved Tyr residues on MEG1 predicted structure, shown as red balls and sticks (arrows).

proteins recognized by the antibody with predicted molecular masses ranging from ~20 to 50 kD were present in the cytoplasmic fractions. In cell wall preparations, three proteins of ~4 to 10 kD gave the greatest signal intensity, suggesting that the putative cleaved MEG1 polypeptide is located in the cell wall (Figure 5B). Unexpectedly, an ~25-kD protein was also detected in the same preparation (Figure 5B). To test whether this protein corresponded to a glycosylated form of MEG1, we treated isolated proteins obtained from cell wall fractions of 10 DAP endosperms with exoglycosidases and subsequently detected MEG1 proteins by immunoblotting. After exoglycosidase treatment of the cell wall protein fraction, we found a clear reduction in the amount of this 25-kD protein, accompanied by an increase in the amount of 4- to 10-kD protein detected by the antibody (Figure 5B), thus demonstrating that MEG1 proteins are present in a glycosylated form in the transfer cell walls.

The *meg1* Promoter Is Transcriptionally Activated by ZmMRP1 in Vitro

The *meg1* promoter sequence was obtained from a maize genomic BAC library (O'Sullivan et al., 2001). Interestingly, through a sequence comparison analysis, we noticed that the full-length promoter region of the *meg1* (Figure 6A) shared conserved regions among promoters belonging to known genes that are expressed in maize basal endosperm transfer cells (Yang

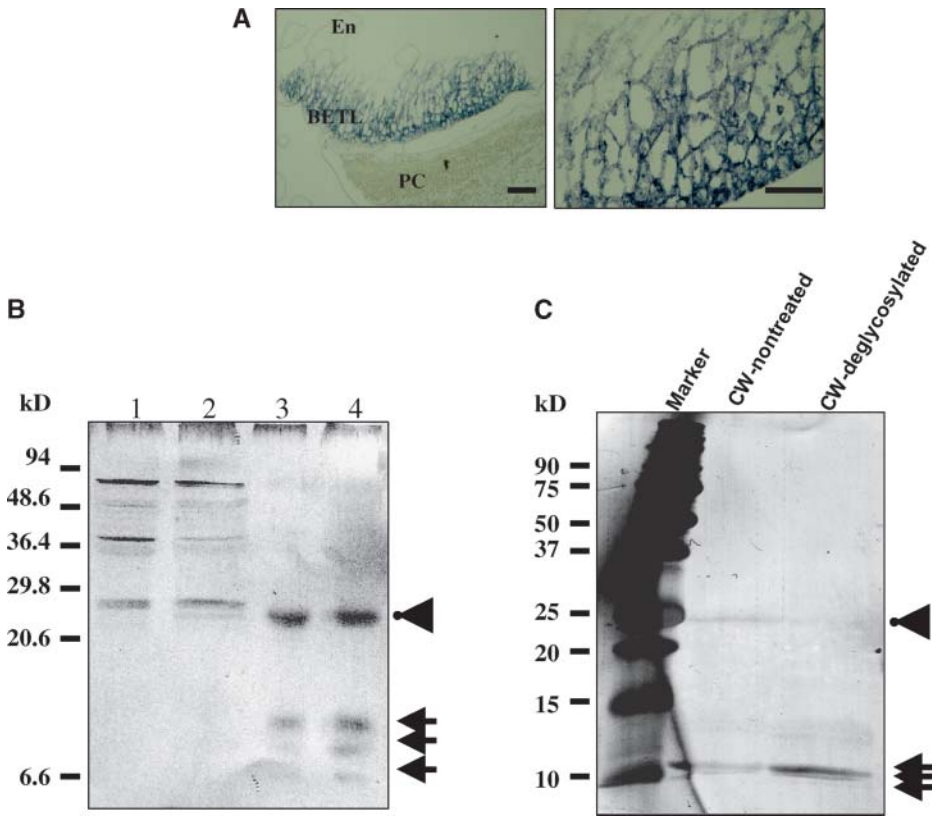

Figure 5. MEG1 Protein Localization.

(A) Immunolocalization of MEG1 proteins. Left, 12 DAP basal endosperm; right, magnification of basal transfer cells showing cell wall localization of MEG1. En, endosperm; PC, pedicel. Scale bars = 100 μm.

(B) Protein gel blot of proteins isolated from 10 DAP endosperms after subcellular fractionation in a 20% SDS-PAGE. Cytoplasmic fraction I (lane 1); cytoplasmic fraction II (lane 2); cell wall fraction I (lane 3); cell wall fraction II (lane 4). Arrows, proteins detected with anti-MEG1 antibody. Arrowhead denotes the presence of an ~25-kD protein(s).

(C) Protein gel blot of MEG1 proteins before and after treatment with exoglycosidases. Proteins were separated on a 12% SDS-PAGE. Arrowhead, putative glycosylated form of MEG1 detected in a partially purified cell wall fraction. Arrows, nonglycosylated MEG1 protein(s).

et al., 1999; Sevilla-Lecoq et al., 2003). It has recently been shown that an endosperm transfer cell–specific transcription factor, ZmMRP1, is able to transactivate expression of several of these BETL-specific promoters (Gomez et al., 2002). Therefore, to investigate whether ZmMRP1 was also capable of transactivating the *meg1* promoter, we transformed tobacco (*Nicotiana tabacum*) protoplasts with a transcriptional fusion construct containing the full-length *meg1* promoter fused to the *uidA* reporter gene and a nopaline synthase (NOS) terminator (denominated pMEG1-GUS), together with a 35S-driven *ZmMRP1* transcriptional fusion construct (termed pMON-MRP1). Preliminary data revealed that pMEG1-GUS was strongly transactivated by pMON-MRP1 (data not shown). To define the promoter sequence recognized by *ZmMRP1*, we generated a deletion series of the *meg1* promoter. These fragments were individually fused to *uidA*, and each construct was used to cobombard etiolated maize leaves with pMON-MRP1. Transactivation was confirmed by the presence of β-glucuronidase (GUS) staining. We found that by removing 120 bp of the distal-most portion of the promoter (−370 to −250 region), transactivation by pMON-MRP1 was disabled (Figure 6B). To determine whether the presence of this 120-bp minimal promoter region permitted transactivation by pMON-MRP1, either alone or in combination with other regions of the *meg1* promoter, deletions were performed in the reverse orientation. From this analysis, the 120-bp minimal promoter region (−370 to −250) in combination with the putative TATA box region (−127 to 1) emerged as sufficient to confer transactivation of the *meg1* promoter by pMON-MRP1 (Figure 6B). It therefore follows that ZmMRP1 may activate *meg1* expression by direct interaction with the −370 to −250 domain of the promoter, a sequence that is conserved among promoters of other genes expressed in the BETL.

Expression of pMEG1-GUS in BETL Cells Is Dependent on Parental Inheritance

To determine whether the full-length promoter region of *meg1* fused to the *uidA* reporter could also drive transgene expression in the endosperm transfer cells, we stably transformed maize

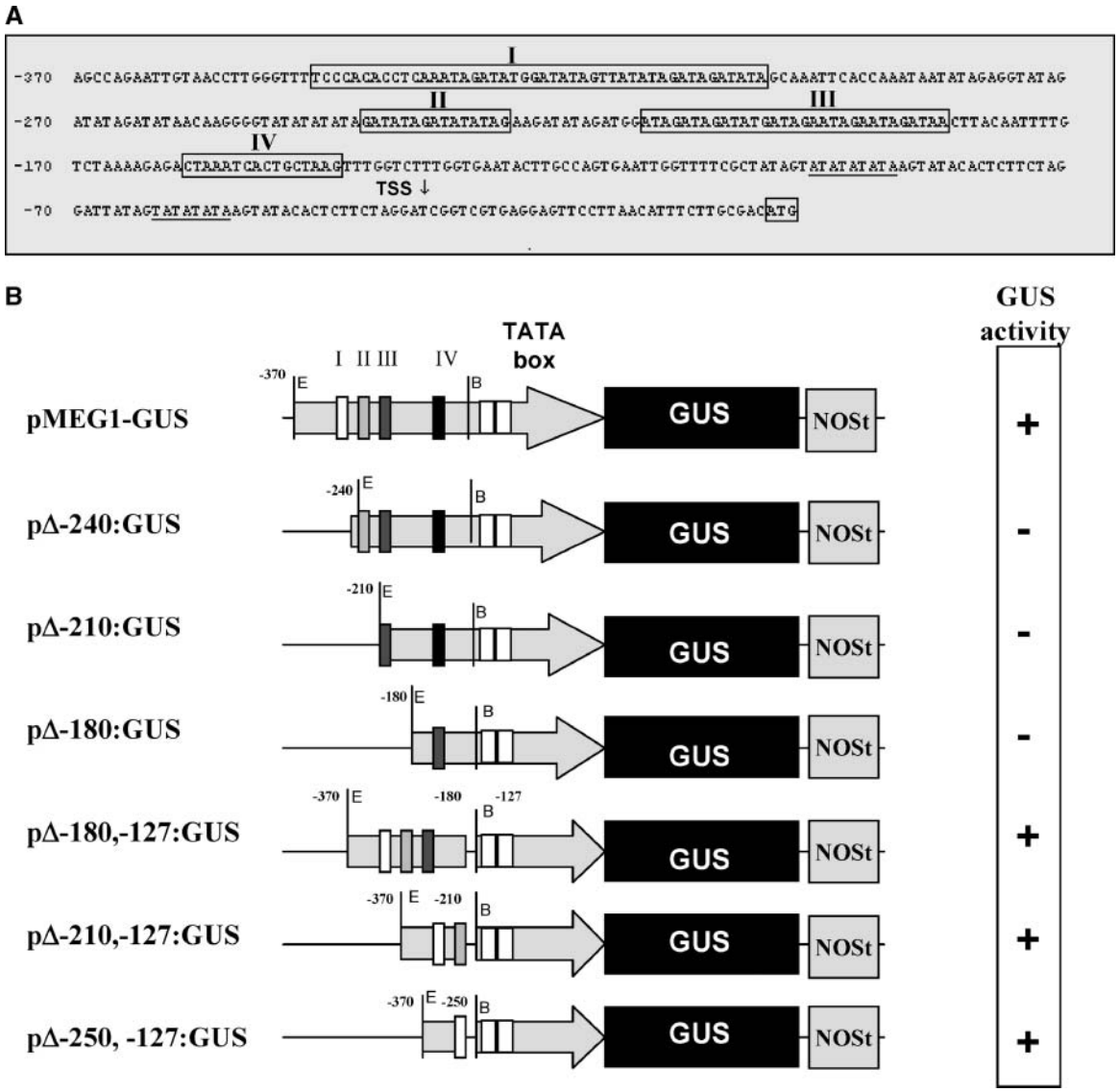

Figure 6. Analysis of the *meg1* Promoter.

(A) Promoter sequence of *meg1* showing four conserved regions also present in the promoters of BETL1, BETL4, and AE1. Putative TATA motifs are underlined. Transcription start site (TSS) is labeled with an arrow, and the first codon (ATG) is boxed.

(B) Deletion analysis of the *meg1* promoter. Left, schematic representations of each promoter-deletion construct used for cobombardment with pMON-MRP1. Right, results obtained from the cobombardment assay; plus sign indicates the presence of GUS staining (transactivation); minus sign denotes absence of GUS staining (lack of transactivation).

plants with the pMEG1-GUS transcriptional fusion construct. Five independent transgenic lines (0757-1C, 0757-2E, 0757-2F, 0757-2D, and 0757-2L) were selected on the basis of high levels of transgene expression, as determined by histochemical staining of GUS. GUS staining was only ever observed in BETL endosperm tissue and absent in all other plant tissues tested (data not shown). To confirm the stable pattern of transgene expression in the endosperm, these lines were backcrossed as females with pollen from A188 standard inbreds for four consecutive generations.

Two of these independent transgenic lines (0757-2E and 0757-2D) were subsequently selected at random and reciprocally outcrossed (i.e., used as females and males) with wild-type A188 plants to investigate any possible changes in transgene expression associated with parental origin. In both instances, 200 to 280 kernels were isolated at different developmental time points and histochemically stained for GUS. Strikingly, the timing at which GUS staining became apparent differed significantly depending on whether the transgene was transmitted maternally or paternally; when plants carrying the pMEG1-GUS transgene

were either self-pollinated or outcrossed as females, GUS staining was present in transfer cells from 4 DAP (i.e., at a time when the endosperm becomes fully cellular), whereas when plants were outcrossed as males (i.e., with the transgene transmitted paternally through pollen), GUS accumulation was evident only after 10 DAP (Figure 7). The apparent delay in expression of the paternally transmitted transgene was confirmed at the transcriptional level by RNA gel blot analysis (data not shown). In all cases a maximum level of GUS staining was attained at 10 to 12 DAP (Figure 7), thereafter declining until 25 DAP, when it was no longer detectable.

Taken together, these data strongly suggest that the full-length promoter of *meg1* is not only able to confer transgene expression in the BETL but that it is also able to regulate expression of the transgene differentially, depending upon whether it is maternally or paternally transmitted.

DISCUSSION

meg1 Shows a Transient Parent-of-Origin Pattern of Expression in the Endosperm

Gene expression in the triploid endosperm is unusual in that, for some sequences, inherited alleles are expressed according to their parental origin (reviewed in Baroux et al., 2002; Walbot and Evans, 2003). Dosage is certainly responsible for much of this differential expression (Birchler, 1993), and it has recently been shown that allelic dosage accounts for the expression pattern of the majority of endosperm genes in maize (Guo et al., 2003). Evidence is, however, accumulating that a variety of other mechanisms can regulate allele-specific gene activity in the endosperm. These range from temporal asymmetry in expression of parental alleles (Springer et al., 2000; Vielle-Calzada et al., 2000) to a complete silencing of the paternal allele (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kinoshita et al., 1999). To determine the extent to which postfertilization gene expression in the maize endosperm is regulated by these mechanisms, a screen was developed to identify endosperm genes expressed from either maternal or paternal alleles (Gutiérrez-Marcos et al., 2003). We report here that *meg1* is expressed maternally only during early endosperm development but is expressed from both parental alleles at later stages.

The mechanism for which the maternal alleles of *meg1* are transcribed while the paternal allele is transiently silenced is not fully understood. It is possible that a general suppression of the paternal genome, which has been reported for Arabidopsis by Vielle-Calzada et al. (2000), may be responsible. However, evidence is now accumulating that some maize, and indeed some Arabidopsis genes, are transcribed shortly after transmission through the pollen (Weijers et al., 2001; Scholten et al., 2002).

More feasible, perhaps, is the preferential expression of the maternal *meg1* allele simply resulting from a dosage effect reflecting the 2:1 maternal to paternal allelic balance in the endosperm. However, in this case *meg1* monoallelic expression would be expected to be constitutively and not transiently regulated, as reported for other dosage-dependent sequences (Guo et al., 2003). Deviations in timing and level of

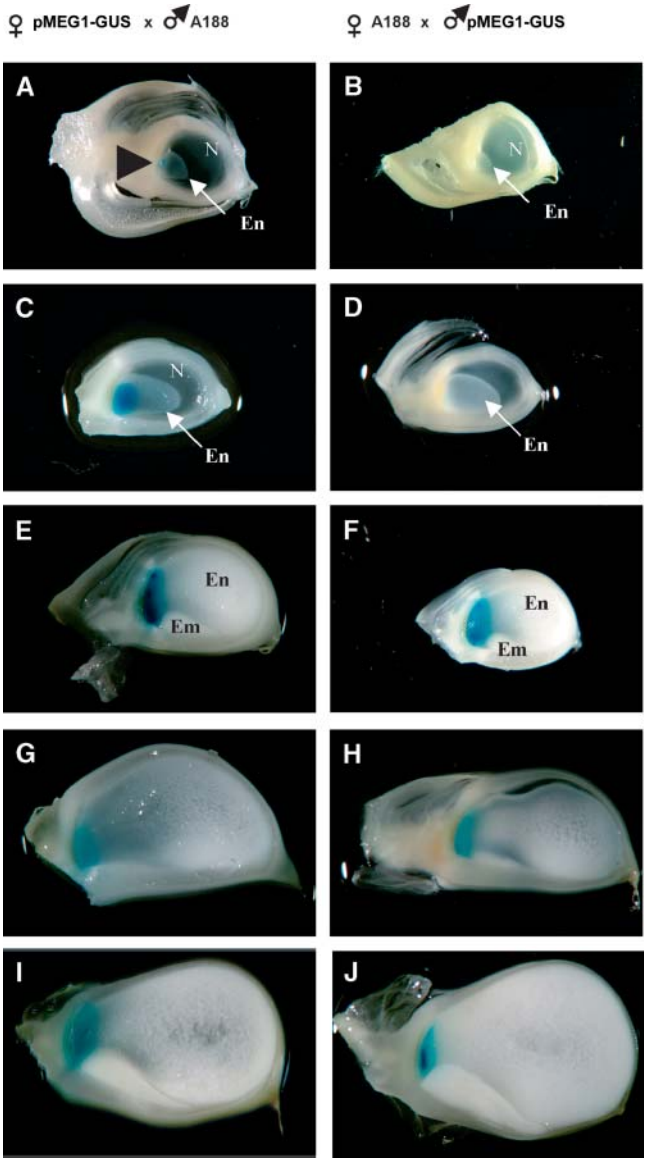

Figure 7. Pattern of Maternal and Paternal pMEG1-GUS Expression during Endosperm Development.

Saggital section of kernels resulting from reciprocal crosses between A188 and transgenic plants carrying a transgene with the promoter sequence of *meg1* fused to *uidA* and analyzed by GUS staining. Arrowhead marks region of GUS staining. Em, embryo; En, endosperm; N, nucellus.

(A), **(C)**, **(E)**, **(G)**, and **(I)** Seeds from plants carrying the pMEG1-GUS transgene after crossing with A188 pollen.

(B), **(D)**, **(F)**, **(H)**, and **(J)** Seeds from A188 plants crossed with pollen from transgenic plants bearing the pMEG1-GUS transgene.

(A) and **(B)** 4 DAP.

(C) and **(D)** 6 DAP.

(E) and **(F)** 10 DAP.

(G) and **(H)** 15 DAP.

(I) and **(J)** 20 DAP.

dosage-dependent allelic expression do, however, occur frequently in maize endosperm among different inbred lines and have been attributed to heterochronic allelic variation (Guo et al., 2003). However, for the parental inbred lines tested, we did not find evidence of variation in *meg1* parent-of-origin allelic expression (Figure 3A).

Alternatively, the paternally inherited allele may be silenced by an epigenetic mechanism similar to that already reported for both Arabidopsis and maize (reviewed in Alleman and Doctor, 2000; Baroux et al., 2002). The monoallelic maternal expression of *meg1* is gene specific, and, thus, similar to that reported for several developmentally important genes, including *MEDEA*, *FIS2*, *FIE*, and *FWA* in Arabidopsis (Ohad et al., 1996; Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Kinoshita et al., 2004) and *fie2* and *nrp1* in maize (Danilevskaya et al., 2003; Guo et al., 2003; Gutiérrez-Marcos et al., 2003).

The molecular basis of this epigenetic control of gene expression in the endosperm has yet to be fully established. In Arabidopsis, maternal allelic expression of *MEDEA* and *FWA* is induced by *DEMETER* (Choi et al., 2002; Kinoshita et al., 2004), but little is known of the mechanism(s) responsible for parent-of-origin expression of genes in maize and, indeed, other sequences in Arabidopsis. In mammals, most genes showing this parent-of-origin imprinting exhibit a parental asymmetry in methylation (Reik and Walter, 2001). Similar studies in maize revealed that some imprinted loci displayed a strong correlation between hypomethylation of maternally inherited alleles and maternal allelic expression in the endosperm (Lund et al., 1995a, 1995b; Alleman and Doctor, 2000). Our data also suggest that *meg1* maternal alleles may be hypomethylated in the endosperm during early development. A strong correlation thus seems to exist between methylation status and allelic gene expression; whether this methylation asymmetry involves only a few genes or is the result of a more global, genome-wide mechanism operating in the maize endosperm remains to be determined. Interestingly, Kinoshita et al. (2004) and Xiao et al. (2003) have demonstrated that the expression of imprinted *FWA* and *MEDEA* from maternal alleles in the Arabidopsis endosperm is dependent on the maintenance of a CpG DNA methyltransferase activity, which maintains a silenced, methylated state of the paternal allele.

Transient parent-of-origin expression such as we describe here for *meg1* is reminiscent of that reported for some imprinted genes in Arabidopsis (*MEDEA* and *FIE*) and maize (*fie2*). Importantly, the full-length promoter of *meg1* is capable of driving transgene expression in an identical manner to that of the endogenous *meg1* gene, both temporally and according to its parental origin. Similarly in Arabidopsis, it has been reported that the expression of transgenic reporters fused to the promoters of imprinted *MEDEA* and *FIE* genes display a parent-of-origin expression pattern during early endosperm development, followed by a reactivation of the paternally inherited transgene before cellularization of the endosperm (Luo et al., 2000; Yadegari et al., 2000). Why some genes should commence biallelic transcription at midstage in endosperm development is not yet known. In maize, however, it may not be a coincidence that the point at which both parental alleles of *meg1* (and *fie2*) are expressed coincides with the onset of endoreduplication (Schweizer et al., 1995; Larkins et al., 2001; Dilkes et al., 2002). Endoreduplication has been associated with a decrease in histone 1 and posttranscriptional changes in high mobility group I/Y proteins (Zhao and Grafi, 2000), events that may lead to alterations in chromatin structure and/or changes in DNA methylation and perhaps to transcriptional activation of previously silenced paternal sequences.

MEG1 Is Specific of the Basal Endosperm Transfer Region

Several endosperm transfer cell–specific genes have been identified in maize, many of which belong to large gene families such as the *BETLs* and *BAPs* (Hueros et al., 1995; Serna et al., 2001). Whereas mRNA in situ and promoter-GUS fusion expression data revealed that *meg1* is specifically located in the basal transfer region of the endosperm from 4 to 20 DAP, sequence analysis at the nucleotide and amino acid level revealed no homology to other known proteins, thus identifying MEG1 as a novel transfer cell–specific protein. MEG1 is a small polypeptide that is either localized in or adjacent to the massive, labyrinthine cell wall ingrowths of the transfer cells. The majority of transfer cell–specific proteins reported to date are small and either secreted into the pedicel region or localized to the BETL cell walls (Hueros et al., 1995; Serna et al., 2001). An unusual feature of MEG1 proteins is the presence of a conserved Cys-rich motif in the C-terminal region and a putative N-terminal glycosylation signal (Mellquist et al., 1998; Wormald and Dwek, 1999). This Cys-rich motif, which consists of eight Cys residues and two Tyr residues, is conserved in all the MEG proteins and bears strong resemblance to Cys-rich domains identified in a number of other plant proteins (Domingo et al., 1999; Schopfer et al., 1999). Cys-rich domains are believed to generate protein conformations that can expose side chain residues (Berg and Shi, 1996), such as the Tyr residues found in MEG1. Although yet to be confirmed for MEG1, the exposure of Tyr residues in synthetic proteins has been shown to facilitate binding to cell wall components (reviewed in Cassab, 1998).

Unlike other maize transfer cell–specific proteins, such as the BETL family, MEG1-like proteins are present also in other grasses, suggesting that aspects of MEG1 function are conserved among the cereals. The molecular configuration and location of MEG1 protein in maize suggests that it either plays a structural role in the basal endosperm transfer region, given its tight association with the cell wall ingrowths, or a defensive function against pathogens, which has been hypothesized for the BETL1 and BETL3 proteins (Hueros et al., 1995, 1999a). Alternatively, because a number of Cys-rich proteins such as the pollen determinant of sporophytic incompatibility in Brassica, SCR/SP11 (Schopfer et al., 1999), function as signaling molecules, MEG1 may play a role in signaling, perhaps operating as part of a cross talk between the endosperm and the maternal tissue. The posttranslational modification of MEG1 by *N*-linked glycosylation may further support this inference because in plants, protein glycosylation is required for a variety of biological functions, including cell–cell communication and signaling (Cheung et al., 1996; Wilson, 2002).

Although there is clear evidence that endosperm development is under strong maternal genetic control (Felker et al., 1985; Ohad

et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kohler et al., 2003), the interactions between the developing seed and the surrounding maternal tissues remain enigmatic (reviewed in Lopes and Larkins, 1993). The BETL region is in direct contact with the maternal pedicel tissue and is established early in endosperm development (Costa et al., 2003). Further, the transfer cells that comprise this tissue are sites of nutrient translocation (reviewed in Becraft, 2001; Olsen, 2001; Thompson et al., 2001), and it is reasonable to expect that evolutionary pressure has resulted in significant maternal control over their activity. In this context, it is interesting that only the maternal *meg1* allele is transcribed during BETL differentiation (Becker et al., 1999). Recently, another gene has been identified, which is both expressed in the basal region of the maize endosperm and exhibits a similar parent-of-origin pattern of expression (Magnard et al., 2003). This discovery of mechanisms regulating maternal expression of transfer cell–specific genes increases the likelihood that resource allocation to the endosperm is maternally modulated, which has implications for seed size and, ultimately, yield. This evidence that the female parent can regulate nutrient supply to the seed strengthens the view that paternally and maternally derived genes compete over resources in the progeny (Haig and Westoby, 1989), an hypothesis that has also been supported by experiments involving intraspecific interploidy crosses (Lin, 1984; Haig and Westoby, 1991; Scott et al., 1998). After such crosses in maize, both gene expression in the BETL and its cellular structure are greatly disrupted (Charlton et al., 1995; Gutiérrez-Marcos et al., 2003).

meg1 represents a novel gene that is specifically expressed in the endosperm transfer cell region and is subject to a transient parent-of-origin effect. Although the mechanism responsible for this pattern of allelic expression is not yet well understood, our findings contribute to an increasing body of data that point to the existence of a group of transfer cell–specific genes whose expression is under maternal control. This level of control would, of course, provide the maternal parent with the opportunity to distribute resources effectively, taking into account overall nutrient levels within the plant and other local and environmental factors.

METHODS

Plant Material and Growth Conditions

Maize (*Zea mays*) diploid inbred lines W22, F2, B73, and Mo17 and tetraploid inbred W23 (Maize Genetics Cooperation Stock Center, Urbana, IL) were glasshouse grown at Oxford University and in Jealott's Hill (Syngenta, Berkshire, UK) between 1996 and 2002, under the following regime: 16-h daylength (supplemented with metal halide lamps at 250 mmols, when required) at 22°C to 28°C during the day, and at 16°C to 20°C at night. Humidity levels were set at ~40% to 50% daytime and 60% to 70% at night. Kernels were harvested at 4 to 25 DAP. Embryos and endosperms were isolated and pooled, then frozen in liquid nitrogen and stored at −80°C.

Allelic Message Display

Total endosperm RNA was extracted from plants that were either selfed or reciprocally crossed among W22, F2, B73, and Mo17 inbred lines and used for AMD-PCR according to a modified protocol of Hagiwara et al. (1997). Briefly, reverse-transcribed RNA was used as source material for PCR (HIEROGLYPH kit; Genomix-Beckman, Fullerton, CA), with labeled [α-^{33}P]dATP. A combination of 24 random oligonucleotides and 10 degenerated poly(T) primers (240 combinations) was used to carry out PCR reactions on endosperm samples, and products were analyzed in a semiautomatic Genomix LR DNA sequencing system. After exposure to film (Biomax MR; Kodak, London, UK), candidate bands were excised from the gel, PCR amplified, and subcloned into a suitable plasmid vector.

Identification of meg1 cDNA and Related Sequences

Full-length cDNAs were obtained after screening a 7 DAP maize endosperm cDNA library (Hueros et al., 1999b). Among the 500,000 plaques screened, five cDNA clones were identified and sequenced at the biochemistry sequencing unit (Oxford University). After screening an F2 BAC library (O'Sullivan et al., 2001), six genomic fragments that hybridized with a meg1 probe were subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) and sequenced.

Gene Expression Analysis

Tissue-specific expression of *meg1* and other *meg* sequences was assessed by RT-PCR (see supplemental data online for primer details). For mRNA localization, in situ hybridization was performed on developing kernels according to a published method (Jackson, 1991), with minor modifications (Costa et al., 2003).

For allele-specific RT-PCR, we sequenced *meg1* alleles from several inbred lines (diploid A188, B73, Mo17, F2, W22, and tetraploid W23), which exhibited 99.5% identity (data not shown). To differentiate between *meg1* alleles, an amplified and cleaved polymorphic sequences technique was used (Neff et al., 1998). RT-PCR analysis was performed with primers (MEG1 [5′-TGCTGCTCATGCGCATGGGGCTG-3′] and MEG1HpaI [5′-TTGTATATAAAAACAGTGATGTTAA-3′]), and PCR products were subsequently digested with *Hpa*I to generate the following fragments: 177 bp in F2 and 198 bp in A69Y or W23 standard inbred lines. The *glutathione synthase1* gene was amplified as a control (see supplemental data online).

Immunolocalization

Polyclonal antiserum was raised in rabbit against a synthetic peptide (N-APAEEGILREKRAQC-C) and affinity purified with an immobilized peptide using a Sulpholink coupling gel system (Pierce, Rockford, IL). Maize kernels were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 12 to 24 h depending on the tissue volume. Samples were dehydrated in an ethanol series and wax embedded. Sections were deparaffinized and blocked in 1% BSA in PBS (10 mM sodium phosphate and 150mM NaCl, pH 7.4) for 30 min at room temperature and incubated overnight with anti-MEG1 antiserum or preimmune serum (both diluted 1:500). The immunoreactions were detected using an alkaline phosphatase-coupled secondary antibody (Sigma, St. Louis, MO; diluted 1:1000) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

Subcellular Fractionation of Proteins

Fractionation of subcellular components of endosperms (obtained from a standard F2 inbred) was performed as described by Serna et al. (2001). Proteins were separated by 20% SDS-PAGE according to Laemmli (1970) and electroblotted onto a polyvinylidine difluoride membrane. Proteins were immunodetected using an enhanced chemiluminescence method (Amersham, Buckinghamshire, UK).

Glycosylation Analysis

A partially purified 10 DAP endosperm cell wall fraction was incubated in the presence of 1 unit of β-*N*-acetylhexosaminidase (New England Biolabs, Hitchin, UK) and 4 units of α-mannosidase (New England Biolabs) in 40 μL of 100 mM sodium acetate, pH 5.0, for 48 h at 37°C. Proteins were fractionated by 12% SDS-PAGE and immunodetected as above.

Protein Modeling

Molecular modeling was performed on a Silicon Graphics Fuel workstation using the programs InsightII and Discover (Accelrys, San Diego, CA). The C-terminal region of MEG1 (residues 47 to 88) was modeled based on the crystal structure of SP11 (Mishima et al., 2003). The sequence similarity between this region of MEG1 and SP11 is not high, except for the conserved presence of eight Cys residues. The N-terminal region of MEG1 (residues 27 to 46) was modeled as a random chain in the absence of any structural data or sequence similarity with proteins of known structure. In the absence of any sequence data for the glycan, a typical plant glycan, Xylβ1-2(Manα1-3)(Manα1-6)Manβ1-4GlcNacβ1-4(Fucα1-3)GlcNAc (Wilson, 2002), was added to Asn-36. The *N*-linked glycan structure was built using average crystallographic torsion angles for the glycosidic linkages (Wormald et al., 2002; Petrescu et al., 2004), and the conformation of the Asn-GlcNAc linkage was based on average crystallographic values (Petrescu et al., 2004).

Generation and Analysis of Transgenic Lines

The promoter region of *meg1* was isolated by PCR using two specific oligonucleotides (pMEG1-GUS primers; see supplemental data online) and subcloned into pGEM-T easy vector (Promega, Madison, WI). After digestion with *Eco*RI and *Cla*I, the 371-bp fragment was fused to the *uidA* sequence and the NOS terminator of the pSLJ4K1 vector (Jones et al., 1992) to generate the $PRO_{ZmMEG1\text{-}1}$-*uidA*-NOS transcriptional fusion, denoted pMEG1-GUS. Embryogenic type II calli were transformed with the construct and regenerated as described previously (Bonello et al., 2000). Plants were genotyped for the presence of the transgene via PCR using specific oligonucleotides (GUS.FOR and GUS.REV; see supplemental data online), backcrossed to wild-type A188 plants for four successive generations, and then bulked by selfing. Histochemical analysis of transgenic lines was performed as described previously (Costa et al., 2003). Briefly, stained kernels were hand-sectioned, fixed then dehydrated to 70% ethanol, and digitally imaged.

Promoter Deletion Analysis

The serial deletion analysis was performed by PCR amplification of different regions of the *meg1* promoter, using multiple oligonucleotide pairs (see supplemental data online), then subcloned into pGEM-T easy vector (Promega). Constructs were digested with *Eco*RI and *Bst*XI, and fragments were subcloned into the pMEG1-GUS construct predigested with *Eco*RI and *Bst*XI. By following this approach, each deletion was subcloned immediately upstream of the putative TATA boxes identified in the *meg1* promoter.

The *ZmMRP-1* coding region was isolated by PCR using oligonucleotides MRP.FOR (5′-GGATCCATGAATCCCAACTTCAACAGTG-3′) and MRP.REV (5′-GAATTCTTATCGGTTATATATCTGGCTCTCC-3′). PCR fragments were subcloned in pGEM-T easy (Promega), digested with *Bam*HI/*Eco*RI, and the 327-bp fragment was subcloned into pMON30049 (Pang et al., 1996) to generate the construct pMON-MRP1.

Plasmid DNA was isolated by the QIAprep midi kit (Qiagen, Hilden, Germany) and coated onto tungsten (M10) particles according to Klein et al. (1992). For transient transformation, Hi-II maize seeds were surface-sterilized and germinated in the dark. Etiolated leaves (2 cm wide) were sectioned into 1 to 2 cm–long pieces and cobombarded with each *meg1* promoter deletion construct and pMON-MRP1 using a Bio-Rad Biolistic PDS-1000/He device (Hertfordshire, UK). Gold particles (0.6 nm; Bio-Rad) were coated with the DNA plasmid mixture, including 2.5 g of pMON-MRP1 derived plasmid and 2.5 g of each *meg1* promoter deletion construct. Tissues were positioned 6 cm from the microcarrier stopping screen, itself located 5 cm below the 6.2 MPa rupture disc. After bombardment, samples were incubated in the dark on MS solid media containing 100 mg/L of *myo*-inositol, 2 g/L of Gln, 30 g/L of sucrose, and MS vitamins (Sigma, Poole, UK) for 24 h at 26°C. A minimum of three independent experiments were conducted for each promoter deletion made. Transcriptional activation was confirmed by GUS staining the leaf discs according to Jefferson (1989), with modifications. Leaf tissue was stained in a solution containing 0.5 mg/mL of X-glucuronide (Clontech, Palo Alto, CA), 0.5 mM phosphate buffer, pH 7.0, 0.1% Triton X-100, and 20% (v/v) methanol.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY536120 (*meg1* genomic locus), AY536121 (meg1 cDNA), AY536122 (meg2 cDNA), AY536123 (meg3 cDNA), AY536124 (meg4 cDNA), AY536125 (meg5 cDNA), and AY536126 (meg6 cDNA).

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