The Maize Transcription Factor Myb-Related Protein-1 Is a Key Regulator of the Differentiation of Transfer Cells

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Transfer cells are highly modified plant cells specialized in the transport of solutes. They differentiate at many plant exchange surfaces, including phloem loading and unloading zones such as those present in the sink organs and seeds. In maize (Zea mays) seeds, transfer cells are located at the base of the endosperm. It is currently unknown how apical-basal polarity is established or why the peripheral cells at the base of the endosperm differentiate into transfer instead of aleurone cells. Here, we show that in epidermal cells committed to develop into aleurone cells, the ectopic expression of the transfer cell-specific transcriptional activator Myb-Related Protein-1 (MRP-1) is sufficient to temporarily transform them into transfer cells. These transformed cells acquire distinct transfer cell features, such as cell wall ingrowths and an elongated shape. In addition, they express a number of MRP-1 target genes presumably involved in defense. We also show that the expression of MRP-1 is needed to maintain the transfer cell phenotype. Later in development, an observed reduction in the ectopic expression of MRP-1 was followed by the reversion of the transformed cells, which then acquire aleurone cell features.

INTRODUCTION

The regulation of solute and solvent fluxes across the plant body and between source (e.g., leaves) and sink (e.g., seeds or fruits) areas is a crucial process that influences plant growth and development. The entry, branching, and exiting zones of the vascular network are priority points where this regulation is executed. Specialized cells differentiate to facilitate the loading/unloading processes at these areas, and very frequently (albeit not universally) these cells develop deep cell wall ingrowths (CWIs), resulting in a remarkable increase in the membrane surface area available for transport. The term transfer cell (TC) (Pate and Gunning, 1972) has traditionally been used to describe cells involved in transport that show these surface modifications. Histological studies have identified modified TCs strategically positioned at the interfaces where solute exchange takes place (reviewed in Offler et al., 2002), such as the loading areas of minor leaf veins, areas surrounding the vascular bundle at stem nodes, points of glandular secretion, and places of delivery of nutrients at sink organs (i.e., at the base of flowers and fruits). Highly differentiated TCs have also been found at the symplastic discontinuities between individuals belonging to different generations (e.g., at the interface between gametophyte and sporophyte generations [Ligrone and Gambardella, 1988] and at the base of seeds [Kiesselbach, 1949; Cochrane and Duffus, 1980; Offler and Patrick, 1993; Talbot et al., 2001]) and symbiotic species (e.g., at mycorrhizal and rhizobium-root nodule interfaces [Gunning et al., 1974; Allaway et al., 1985]), and even at points of plant-parasite interaction (e.g., in nematode infections [Jones and Northcote, 1972]). This work makes use of the maize (Zea mays) basal endosperm TC layer as a model for investigating the regulatory pathways that govern TC differentiation.

Seeds originate from a double fertilization event that gives rise to the embryo and the endosperm (reviewed in Becraft, 2001; Berger, 2003; Olsen, 2004). The endosperm develops from the fertilized triploid central cell of the ovule, which undergoes nuclear divisions without cytokinesis to produce a coenocyte containing nuclei that are evenly distributed throughout the peripheral cytoplasm. In cereals, periclinal cell division of the first cellularized layer of the endosperm gives rise to two different cell types: the aleurone and starchy endosperm initials (Becraft, 2001; Olsen, 2004). The aleurone layer serves as the epithelium of the filial tissues, surrounding both the endosperm and embryo. At the base of the maize endosperm, however, epithelial cells differentiate to form the basal endosperm transfer cell layer (BETL) instead of an aleurone layer, producing an interface between the filial and maternal tissues of the seed and thus facilitating nutrient uptake from the apoplastic space in the placenta-chalaza area. The differentiation and function of this cell layer therefore critically influence the agronomically key process of grain filling (Kiesselbach, 1949; Thompson et al., 2001; Royo et al., 2007). The embryo-surrounding region (ESR), located around and below the developing embryo, represents a further expression domain within the endosperm (Opsahl-Ferstad et al., 1997; Balandin et al., 2005).
In maize endosperm, the process of TC differentiation is gradual and regulated by the position cells occupy on two axes (Royo et al., 2007). Along the basal-apical axis, the BETL extends inwards into the endosperm as several cell layers. The outer (i.e., most basal) cell layer develops TC features, while the inner cell layers become progressively less elongated and contain fewer CWIs. There is also a TC differentiation gradient along the germinal-abgerminal axis. Whereas the cells located near the embryo pole are morphologically distinct at 8 d after pollination (8 DAP) and subsequently develop a complex network of CWIs, the cells on the abgerminal side of the basal layer morphologically resemble aleurone cells until very late in development (15 to 20 DAP), and never develop extensive CWI, although they express TC markers from the earlier developmental stages. Studies of the maize TC differentiation process have been facilitated by the identification of genes specifically and exclusively expressed in these cells (reviewed in Royo et al., 2007). Among these, MRP-1 was the first TC-specific transcriptional activator identified (Gómez et al., 2002). The protein contains an MYB-related DNA binding domain identified in several DNA binding proteins belonging to the SHAQK(Y/F)F subfamily: for example, MybSt1 (for Myb Solanum tuberosum 1; Baranowskij et al., 1994), Le MYB1 (for Lycopersicum esculentum Myb 1; Rose et al., 1999), LATE ELONGATED HYPOCOTYL (Schaffer et al., 1998), and CIRCADIAN CLOCK ASSOCIATED1 (Wang et al., 1997). The expression of Myb-Related Protein-1 (MRP-1; Gómez et al., 2002) is readily detected in the basal part of the endosperm as early as 3 DAP, when the endosperm coenocyte is still organized into nuclear-cyttoplasmic domains, and continues during the development of the TCs. In addition, MRP-1 regulates the expression of several TC-specific genes, namely, BETL-1 and BETL-2 (Gómez et al., 2002), Meg-1 (for Maternally Expressed Gene 1; Gutiérrez-Marcos et al., 2004), and TCRR-1 (for Transfer Cell Response Regulator 1; Muñiz et al., 2006), through its interaction with a specific sequence in the corresponding promoters (Barrero et al., 2006). These findings suggest that MRP-1 might be a key player in the TC differentiation process.

TC differentiation presumably occurs as a response to an increased demand for solute transport. Little is known about the molecular signals that induce TC differentiation, but it is likely that transported solutes are involved (Offler et al., 2002). Certainly, it has been shown that a transient increase in monosaccharide concentration early in development induces the differentiation of TC in the adaxial surface of fava bean (Vicia faba) cotyledons (Offler et al., 1997; Farley et al., 2000). However, the molecular mechanisms underlying this induction are unknown. In addition, our group has recently shown that, in Arabidopsis thaliana and tobacco (Nicotiana tabacum), the promoter of MRP-1 functions specifically in areas of active transport (such as at the base of fruits, branching areas, and nematode-induced galls) while tissues are developing. It was also found that the promoter is modulated by sugars in Arabidopsis and baker’s yeast (Saccharomyces cerevisiae) (Barrero et al., 2009).

These results show that the ectopic expression of MRP-1 in cells committed to differentiate as aleurone cells causes their differentiation into TCs instead. This differentiation is appreciable both at the morphological level, as the transformed cells develop the intricate cell wall architecture that characterizes TCs at the base of the endosperm, and at the molecular level, as they express TC-specific markers. It is also shown that MRP-1 is required for the maintenance of TC. The downregulation of MRP-1 expression observed later in development resulted in the reversion of the transformed cells’ TC phenotype and the acquisition of an aleurone cell morphology.

RESULTS
Expression of MRP-1 at the Abgerminal Side of the Maize Endosperm Aleurone Layer

Initial attempts to ectopically express MRP-1 under the control of ubiquitous promoters (such as those from the cauliflower mosaic virus 35S protein, the maize ubiquitin gene, and the cassava vein mosaic virus) failed to produce stable transgenic lines. In all cases, maize primary transformants were produced, albeit at a low frequency, but they did not transmit the construct to the progeny. Identical results were obtained in attempts to overexpress MRP-1 under the control of the 35S promoter in Arabidopsis and tobacco. Plants containing constructs expressing significant amounts of MRP-1 transcript produced nontransgenic progeny.

We next attempted to express MRP-1 under the control of the aleurone-specific promoter AL-9. AL-9 was originally isolated in a search for compartment-specific molecular markers and encodes a protein of unknown function but related in sequence to the barley (Hordeum vulgare) TC-specific gene END-1 (Doan et al., 1996) and to the END-1 maize putative ortholog (Gruis et al., 2006), termed BETL-9 in our BETL-specific gene collection. The AL-9 transcript can be detected by in situ hybridization exclusively at the maize aleurone layer between 3 and 25 DAP (see Supplemental Figure 1 online). The tissue specificity of the AL-9 promoter was demonstrated using promoter-β-glucuronidase (GUS) maize transgenic lines that produce a reporter gene expression pattern essentially reproducing that of the AL-9 gene (Figure 1A; see Supplemental Figure 1 online for expression at 25 DAP).

Three independent transgenic maize lines containing the ProAL-9:MRP-1 construct were selected for this study. No special phenotypes were observed for these plants besides the features described for the immature kernels below. Adult plants and mature seeds were indistinguishable from nontransgenic material.

Pollination of heterozygous ProAL-9:MRP-1 transgenic maize plants with nontransgenic pollen produced a 1:1 segregation in the sibling kernels. These were collected for analysis at two developmental stages: 12 and 17 DAP. In situ hybridization analyses using a MRP-1 antisense probe and 12-DAP kernels (Figures 1B to 1E) showed that half of the kernels expressed MRP-1 in the BETL (large arrow in Figure 1B), while the other half also expressed the gene in the epithelial layer on the abgerminal side of the endosperm (arrowheads in Figure 1C). Part of the outer cell layer of the endosperm (i.e., the abgerminal side of the aleurone [magnified in Figure 1D]) and the entire BETL [magnified in Figure 1E]) was thus labeled with the MRP-1 antisense probe.
The Expression of MRP-1 Induced the TC Differentiation Process When Expressed in the Abgerminal Aleurone Layer

The expression of MRP-1 promoted a profound change in the morphology of the cells otherwise committed to differentiate into aleurone cells (Figure 2). At 12 DAP, wild-type seeds showed multiple layers of small, cubic cells with no obvious asymmetric features in either the upper or abgerminal endosperm (Figure 2A). In the transgenic kernels, however, the cells on the abgerminal side (Figure 2D) were seen to undergo a dramatic differentiation process. These cells appeared elongated along the axis perpendicular to the endosperm surface and started to accumulate newly deposited cell wall material at their outer side (stained white-blue with the cell wall stain Calcofluor white), resembling the CWIs that densely fill the mature TC at the base of the endosperm (Figure 2J). The inner cell layers immediately adjacent to the transdetermined cells also elongated in the same direction as the external layer, further reinforcing the similarity between this area and the BETL (see Supplemental Figure 2 online for a diagram).

The cell morphology was then examined using semithin (0.5 μm) sections of LR white–embedded material at a ×1000 magnification. The very thin cell walls of the aleurone cells at the abgerminal endosperm side (Figure 2B) contrasted with the thick cell wall (stained pale blue with toluidine blue) developed by the cells expressing MRP-1 (Figure 2E). The cell wall outgrowth completely covered the outer side of the cells and about one-third of their lateral sides. The density of CWI found at these cells was much lower than that observed at the more differentiated BETL cells (Figure 2K) but remarkably similar to the those observed in the less developed TCs that can be observed at the abgerminal side of the BETL (Figure 2L).

The cell wall structure was further studied by examining ultrathin sections of the LR white–embedded material with transmission electron microscopy (TEM). Abgerminal aleurone cells (Figure 2C, equivalent area framed in red in Figure 2B) possessed thin cell walls with no sign of wall outgrowth and very few mitochondria. Cells expressing MRP-1 developed instead a thick, massive cell wall from which small, limitedly branched CWIs protruded into the cytoplasm (composite Figures 2F and 2G, equivalent area framed in 2E). The inner surface of the cell wall thickenings was densely covered by mitochondria; these mitochondria are also evident in the lower magnification image (Figure 2E), but appeared now to be part of a complex synthetic machinery (SM in Figures 2G to 2I) in an organelle-rich cytoplasm also composed of reticulum, Golgi cisternae, and cytoskeletal elements, resembling the situation previously described in models for CWI formation (Offler et al., 2002). TEM analyses of the CWIs developed at the BETL cells (Figures 2M and 2N) revealed a higher structural organization than that observed in the CWIs of transformed cells. Even in the higher-density areas (Figure 2M) it was possible to distinguish each flange of CWI as an individual entity emerging from an otherwise thin intercellular cell wall. The relatively regular pattern of formation of individual CWI was easily observed at the initial stages of cell wall growth (Figure 2N). As compared with the situation in the BETL, the transformed cells’ intercellular cell walls were thicker, probably reflecting an initial disorganized growth preceding the formation of CWIs. Occasionally, a very long, unbranched, flange-like CWI formed in the transgenic abgerminal aleurone (Figure 2E, TEM image in 2H). However, the CWIs generally had very limited extension. Examination of the initial stages of cell wall growth at the lateral sides of the cells (Figure 2I) showed that the massive intercellular cell wall probably formed by coalescence of rather amorphous CWIs.

The development of an ectopic endosperm transfer cell layer (EETL) in the aleurone area expressing MRP-1 was also evident
Figure 2. The Ectopic Expression of MRP-1 Induces TC-Like Modifications.

(A) to (C) Images of the abgerminal side of nontransgenic endosperms at 12 (A) or 10 (B) and (C) DAP. 
(D) to (I) Images of the abgerminal side of transgenic endosperms at 12 (D) or 10 DAP (E) to (I). (Enclosed in a common frame.)
(J) to (N) Images of the BETL at 12 (J) or 10 DAP (K) to (N).

(A), (D), and (J) Wax-embedded material sectioned at 8 m and stained with calcofluor white. CWIs appear light blue with calcofluor white staining (some indicated by arrowheads in (D)). Transformed cells (those containing CWIs) appear in (D), elongated along the germinal-abgerminal axis; compare these with the cubic cells at the aleurone layer (Al) or the rounded starchy endosperm cells (SE). The germinal-abgerminal (G-AB) or apico-basal (AP-B) polarity is indicated for figures (A), (D), and (J). The image in (J) has been rotated 90˚ for comparison with the EETL. However, note that the images in (K) and (L) are shown in a natural orientation, with the placento-chalaza at the bottom part of the micrograph.

(B), (E), (K), and (L) Semithin sections (0.5 m) of LR white–embedded material (10 DAP) stained with toluidine blue. Cell wall secondary growth is stained pale blue in the transformed cells (E) and TCs (K) and (L).

(C), (F) to (I), (M), and (N) Ultrathin sections (0.05 m) of LR white–embedded material stained with lead nitrate and uranyl acetate. Images were taken using TEM. The corresponding areas of the cells shown in these images are framed in red in (B), (E), and (L).

P, pericarp side; PCH, placento-chalaza; CW, cell wall; M, mitochondria, SM, synthesis machinery associated with the internal side of the CWI. Bars = 50 m in (A), (D), and (J), 25 m in (B), (E), (K), and (L), and the indicated values in the TEM images. Materials analyzed in (A), (D), and (J) were derived from the transgenic line EER-3b. Materials analyzed in (B), (C), (E) to (I), and (K) to (N) were derived from the transgenic line EER-2a.
from the in situ hybridization and immunolocalization results presented. The EETL images shown in this article were obtained from the analyses of two different transgenic events and two plant generations, and the EETL was always found on the abgerminal side of the endosperm. No other aleurone cells developed an obvious TC phenotype, although isolated cells at different positions were found to express TC markers (see below).

The accumulation of the TC proteins BETL-1 and BETL-2 was investigated in immunolocalization experiments. Both proteins were expressed at high levels in the EETL (Figure 3A to 3C for BETL-2; see Supplemental Figure 3 for BETL-1), in agreement with previous results indicating that MRP-1 directly transactivates the promoters of BETL-1 and BETL-2 (Gómez et al., 2002). No strong cross-reacting signal was detected with these antibodies outside the BETL and the abgerminal EETL, although a weak signal was occasionally detected in isolated cells in other parts of the aleurone layer (Figure 3A, inset).

Both BETL-1 and BETL-2, but especially the latter, were secreted into the maternal placento-chalazal region of the pedicel when expressed in the BETL (see arrowheads at the basal part of the endosperm in Figure 3A [magnified in 3B]); this observation was previously reported for BETL-2 by Serna et al. (2001). Similarly, BETL-1 and BETL-2 expressed by the EETL accumulated on one side of the kernel, this time immediately under the pericarp (arrowheads in the EETL region, Figures 3A and 3C). This pattern of protein accumulation reinforced the polarized appearance of the EETL. No sign of BETL-2 protein secretion was observed in the abgerminal aleurone cells from nontransgenic sibling kernels (Figure 3D).

The EETL Expresses a Complete Set of TC-Specific Markers

Since the EETL is physically separated from the BETL, it was possible to quantitatively compare the expression of domain-specific markers in both tissues by real-time RT-PCR in the upper (Top) and lower (Bottom) halves of the wild-type and transgenic kernels (Figure 4A, left and right diagrams, respectively). TC-specific genes expressed in the EETL (shown in red in Figure 4A, right) should only be detected in the upper halves

![Figure 3. The Ectopic Expression of MRP-1 Induces the Expression of TC-Specific Genes.](image-url)

- **(A)** Immunolocalization of the BETL-2 protein (brown color, greyish in areas that accumulate less protein) in both the BETL and the EETL in a 12-DAP transgenic seed. Inset, higher (5) magnification of cells expressing BETL-2 at the top of the endosperm (framed).
- **(B)** Confocal microscopy immunodetection of BETL-2 in the BETL and pedicel (Pd).
- **(C)** Confocal microscopy immunodetection of BETL-2 in the EETL and pericarp (P).
- **(D)** Negative result for the immunodetection of BETL-2 in the abgerminal aleurone layer (AL) of a nontransgenic kernel at 12 DAP; the rounded cells below the aleurone are starchy endosperm cells (Se). BETL-2 is produced in the TCs and EETL (arrows) but accumulates in the adjacent maternal tissue (arrowheads). P indicates the pericarp side.

Bars = 1 mm in (A), 100 m in (B), and 50 m in (C) and (D).
of the transgenic kernels. In addition to the expression of MRP-1 itself, the expression of another six TC-specific genes, BETL-1 (Hueros et al., 1995), BETL-2 (Hueros et al., 1999), BETL-9 (a TC-specific homolog of End-1 [Doan et al., 1996; Gruis et al., 2006]), BETL-10 (a defensin-related gene [G. Hueros, unpublished data]), the response regulator TCRR-1 (Muñiz et al., 2006), and the cell wall invertase INCW-2 (Cheng et al., 1996), was examined. As expected, MRP-1 expression (Figure 4B) was detected in the lower half of all the kernels (containing the BETL) and also in the upper half of the transgenic kernels (although the expression level was nearly two orders of magnitude lower than in the BETL). The five TC-specific markers examined in this case were found to be expressed in the upper part of the transgenic endosperm (Figure 4B, red bars), although at a lower level than in the lower, BETL-containing, halves (Figure 4B, blue bars). The expression level in the upper halves of the nontransgenic kernels (Figure 4B, striped red bars) was, for four of the markers, between one and two orders of magnitude lower than in the transgenic kernels. This weak signal might be due to contamination of the top samples with basal endosperm TC during the kernel dissection procedure, and this possibility was strongly supported by examination of the expression of the ESR marker ESR-6 (Balandín et al., 2005). ESR-6 expression was weakly detectable in the upper halves of nontransgenic kernels, at a comparable level to that of the four TC markers detected in this material, indicating that these signals might be derived from contaminating material introduced during the dissection procedure. The expression of ESR-6 in the upper part of the transgenic material was, however, below the limit of detection, suggesting that this material lacked BETL contamination and that the expression of TC markers detected in this tissue was completely derived from the EETL.

Consistent with the in situ hybridization results indicating that the EETL is not very large, all samples contained similar levels of the aleurone-specific marker AL-9. Similar results were obtained using a different transgenic line (Figure 4C). In this case, the comparison of kernels derived from the self-cross of a heterozygous plant, which expressed different levels of MRP-1 in the upper part of the seed, showed a positive correlation between the MRP-1 transcript level and that of the six BETL markers in the upper halves of the kernels. Samples with a lower ectopic MRP-1 expression (presumably heterozygous, HET in Figure 4C) showed, for all markers examined, a similar expression level to that of the transgenic kernels of the line tested above (Figure 4B). Samples with a higher ectopic MRP-1 expression (presumably homozygous, HOM in Figure 4C) showed a much higher expression of TC-specific markers than the heterozygous kernels; in fact, the expression levels were within the same order of magnitude as found in the BETL.

Analysis of the ESR marker, which showed an expression value four orders of magnitude lower than that of the TC markers in the homozygous samples, ruled out the possibility of contamination of the upper halves of the transgenic kernels with material from their lower halves. The relative expression levels found in the upper and lower seed halves for the aleurone marker AL-9 (Figures 4B and 4C) probably reflect the position of the plane through which the seeds were cut.

MRP-1 Is Only Transiently Expressed at the Aleurone Layer and the EETL Reverts to Normal Aleurone Morphology during Development

The transformation of the abgerminal aleurone layer into a TC layer through the expression of the ProAL-9:MRP-1 construct requires a mechanistic explanation. Since ProAL-9 is specifically expressed in the aleurone layer, cells assuming a TC role ought to progressively reduce the expression of the MRP-1 transcript derived from the transgene. To test this hypothesis, the expression of AL-9 and BETL-9 (a TC-specific, AL-9–related sequence) was examined by in situ hybridization using kernels at 12 DAP with and without an EETL (Figure 5). The results showed that while the EETL expressed high levels of the TC marker BETL-9 (Figure 5A) in a pattern similar to that observed in the BETL (see higher magnifications of the EETL and BETL in Figures 5B and 5C, respectively), AL-9 was almost excluded from both areas (Figure 5D). AL-9 homogeneously labeled the aleurone layer in the developing kernels of nontransgenic siblings (Figure 5E).

The data shown in Figure 5 suggest that the MRP-1 expression level detected in the abgerminal aleurone layer during the early developmental stages (in the context of this article 10 to 12 DAP; 11 DAP in Figure 1) is the result of the equilibrium between forces promoting aleurone and TC differentiation. The examination of kernels at later developmental stages indicated that this equilibrium is always broken in favor of the aleurone cell fate. At 17 DAP, although the MRP-1 transcript was detectable by in situ hybridization (see Supplemental Figure 4 online) in the abgerminal part of transgenic kernels, the level of transcript accumulation was greatly reduced. Furthermore, the morphology of the cells on the abgerminal side of the endosperm took on an aleurone or starchy endosperm appearance. In situ hybridization analysis using TC markers and an aleurone marker (Figure 6) confirmed this transformation. The BETL-9 TC marker was never detected at the abgerminal side of the aleurone in kernels at 17 DAP (Figure 6A), while it was readily detected in the BETL. However, the aleurone marker AL-9 became detectable in all aleurone cells (Figure 6B). Nonetheless, immunolocalization studies detected the presence of BETL-2 peptides at 17 DAP in the apoplastic space between the pericarp and the aleurone layer (Figure 6C) at the position where the EETL was normally detected during earlier stages (Figure 3). The endosperm cells positioned beside the apoplastic deposits (labeled as aleurone-like [AL-L] in Figure 6D) had the same size and morphological features as those located at equivalent positions in nontransgenic kernels of the same age (Figure 6D). In some sections, however, patches of four to five cells with no aleurone features (enlarged cells [EC] in Figure 6C) were identified beside the BETL-2 deposits. No comparable cells were observed in preparations from nontransgenic maize kernels. Compared with the aleurone cells (Figure 6E), the enlarged cells contained a less dense cytoplasm and a much larger cell volume. Compared with the EETL cells, the enlarged cells extended in an axis parallel to the endosperm surface, while the characteristic elongation along a surface-orthogonal axis observed in the EETL was not evident. Cell wall modifications were also not observed in the EETL in azure-B stained sections produced from wax-embedded material (Figure 6E). To obtain a better morphological characterization, we analyzed LR white–embedded, 16-DAP
Figure 4. Expression Analyses of BETL Markers at the EETL

(A) Diagram showing a sagittal section of a maize kernel at 15 DAP. En, starchy endosperm; Em, embryo; Al, aleurone; ESR, embryo surrounding region; Pd, pedicel (maternal tissue).

(B) and (C) Real-time RT-PCR expression analyses of MRP-1 and six TC-specific genes (BETL-1, BETL-2, BETL-9, BETL-10, TCRR-1, and INCW-2), an embryo surrounding region marker (ESR-6), and the aleurone marker AL-9. Solid red and blue bars, upper (Top) and lower (Bottom) halves of a transgenic seed, respectively (dissected as shown in [A]); striped red and blue bars, upper (Top) or lower (Bottom) halves of a seed not expressing (B) or expressing low levels (C) of MRP-1. Materials analyzed were derived from the transgenic lines EER-3b and EER-2a, respectively. Values are means + SD of three technical replicates. Asterisks denote that the transcript level was below the technique sensitivity.
endosperms from transgenic and nontransgenic kernels. At this developmental stage, aleurone cells (Figure 6F) have a dense cytoplasm containing numerous storage vesicles. TEM analysis (Figure 6G) showed that the aleurone cell walls are thin and lack any sign of secondary growth. The cytoplasm of the enlarged cells found at the abgerminal aleurone (Figure 6H) appeared more dense than that of the starchy endosperm cells but was much more vacuolated than that of the aleurone cells (cf. starchy endosperm and aleurone cells in Figure 6F). The massive cell wall outgrowth that characterizes the EETL cells was only found in a single case (Figure 6H, arrow). More common was the presence in these enlarged cells (Figure 6I) of a moderately thicker cell wall at the pericarp-facing side, and TEM analysis revealed the presence of minute CWIs protruding from this cell wall (Figure 6J, arrows). The internal cell layers showed almost no evidence of cell wall secondary outgrowth (Figure 6K). The lateral sides of the enlarged cells showed scarcely discrete masses of cell wall material (Figure 6I, arrows) and short stretches of slightly thicker cell wall (Figures 6I, CWT, and 6L).

The comparison of the abgerminal region of the endosperm between 10 to 12 DAP and 16 to 17 DAP showed a dramatic reduction in the number of cells displaying nonaleurone features as well as in the TC-like morphology of these cells. To further confirm this apparent reversion process, we studied the cell morphology in mature kernels. Sixteen segregating mature kernels were imbibed for 72 h, the embryo axis was used for genotyping, and the endosperm and scutellum were fixed and embedded in wax. The central part of the seeds (~3 mm thick) was studied by taking 8-μm-thick sections every 150 μm. Analyses of the BETL, abgerminal aleurone, and upper aleurone regions (see Supplemental Figure 5 online) indicated that although cells in the abgerminal aleurone were slightly longer than cells in the aleurone at the upper part of the seed, their appearance was identical in the seeds containing (10 cases) or lacking (six cases) the ProAL-9:MRP-1 construct.

**DISCUSSION**

This work presents the results of a long effort to achieve in planta overexpression of the TC-specific transcriptional regulator MRP-1. Attempts to ectopically express MRP-1 under the control of various ubiquitous promoters (35s, maize ubiquitin, and Cassava vein mosaic virus) failed to produce stable transgenic lines in maize, tobacco, and Arabidopsis.

An expression study using a functional MRP-1 promoter fragment (Barrero et al., 2009) identified endosperm TC in maize and barley and the vascular tissue–associated parenchyma at the solute exchange surfaces in tobacco and Arabidopsis as the only sites where a presumptive MRP-1 ortholog would be expressed in these species.

Our results suggest the existence of a regulatory mechanism in the kernel that restricts the expression of MRP-1 to the BETL. Consequently, the strong AL-9 promoter only achieved a limited expression of MRP-1: the transgene was neither expressed in all of the aleurone cells nor was it expressed at the expected intensity in those cells in which expression did occur (Figure 1). Although not a primary aim of this work, we have tested this hypothesis (see Supplemental Figure 6 online) using transient expression experiments in immature maize aleurones and V. faba abgerminal cotyledon epidermis, which is completely covered in TCs (Offer et al., 1997; Weber et al., 1997). These experiments suggest that limited ProAL-9:MRP-1 expression in the aleurone cells is not caused by a technical problem in the construct design, but by a regulatory mechanism specifically downregulating the MRP-1 transcript in the aleurone layer. Unfortunately, demonstrating the existence of such a mechanism is not easy, since the AL-9 promoter does not function at all in the maize endosperm TCs, in which the hypothesized MRP-1 downregulating mechanism does not operate.

While the reasons for the restricted expression of MRP-1 can only by hypothesized at this stage, the results of this expression...
Figure 6. The TC-Like Features Observed in Epithelial Cells at the EETL by 12 DAP Are Almost Absent in 16- to 17-DAP Kernels.

(A) and (B) In situ hybridization analyses of a 17-DAP transgenic kernel, using probes against a TC marker (BETL-9; [A]) or an aleurone marker (AL-9; [B]). Cells at the abgermal (AB) side of the endosperm do not express the TC marker (cf. [A] to Figure 5A), while they express the aleurone marker normally.

(C) Immunolocalization of the BETL-2 protein (brown color) in the same transgenic kernel as in (A) and (B). Al-L, aleurone-like cells; EC, enlarged cells.

(D) Immunolocalization of the BETL-2 protein (negative result) in a nontransgenic kernel. Al, aleurone; P, pericarp.

(E) Lower-magnification image for comparison of Aleurone-like cells (Al-L) and a patch of enlarged cells (EC).

(F), (H), and (I) Semithin sections (0.5 m) of LR white–embedded material (16 DAP) stained with toluidine blue.

(F) A normal aleurone layer (Al) and several starchy endosperm cells (SE) in a nontransgenic kernel.

(G), (J), (K), and (L) Ultrathin sections (0.05 m) of LR white–embedded material stained with lead nitrate and uranyl acetate. Images were taken using TEM. The corresponding areas of the cells shown in these images are framed in red in (F) and (I). Arrows in (J) and (K) indicate the position of small CWIs. CW, cell walls.

Materials analyzed in (A) to (E) were derived from the transgenic line EER-3b. Materials analyzed in (F) to (L) were derived from the transgenic line EER-2a. Bars = 1 mm in (A) and (B), 100 m in (E), 50 m in (C) and (D), 25 m in (F), 20 m in (H) and (I), and the indicated value in the TEM images.
on the differentiation of the abgerminal endosperm epidermal cells were very clear. Aleurone cells expressing MRP-1 acquired the features of endosperm TCs (Royo et al., 2007), forming what has here been called an EETL (Figure 2). The development of an extensive network of CWIs is among the best characterized of TC features (for an ultrastructural characterization of maize TC CWIs, see Davis et al., 1990). These ingrowths provide the increase in plasma membrane surface area needed to enhance the transport function of the TC (reviewed in Pate and Gunning, 1972; Offler et al., 2002) and are clearly observed in the EETL (Figure 2; see diagram in Supplemental Figure 2 online). Their reactivity to cell wall stains was indistinguishable from that observed for the BETL CWI, although the extension of the intracellular network of CWIs in the EETL never reached the density observed in the BETL. TEM analyses (Figure 2) revealed the presence in the EETL of the cellular elements previously associated with the synthesis of CWIs (Offler et al., 2002) but also indicated structural differences between the CWIs of the BETL and EETL, which appear to develop in a less organized pattern in the latter, where the CWIs acquired a floccular structure. This suggests that additional factors, other than MRP-1, might be required for the complete development of the CWI network found in the BETL. These additional factors might implicate the expression of genes not regulated by MRP-1 and/or the requirement of an energy supply that cannot be obtained in cells physically separated from the nutrient unloading zone at the pedicel. Cell elongation is another remarkable feature of the endosperm TCs compared with the round starchy endosperm cells and the cubic aleurone cells (Becraft and Asuncion-Crabb, 2000; Olsen, 2004). Several layers of TCs elongate along an axis orthogonal to the endosperm surface, the elongation rate being a function of TC maturity (Royo et al., 2007). This gives a domed appearance to the BETL, with the longest cells positioned at the center of the layer and slightly closer to the germinal pole (as shown in Figures 3A, 5A, and 6A). The same cell elongation and general appearance was observed for the EETL (Figures 3A, 5A, and 5B). Interestingly, since the elongation axis of the EETL cells was again orthogonal to the endosperm surface, it was also orthogonal to the elongation axis of the BETL. This, together with the observed secretion of TC-specific peptides toward the surrounding apoplast, provided a strongly polarized appearance to both layers. While the achievement of cell polarity is a complex process that probably involves the reorganization of the cytoskeleton and contacts with neighboring cells (as has been found for animal epithelia [Gibson and Perrimon, 2003; Nelson, 2003]), it is clear that the preexisting polar information in aleurone cells is efficiently passed on via MRP-1 in the formation of highly polarized TCs.

The availability of MRP-1–regulated TC-specific genes (i.e., BETL-1 and BETL-2 [Gómez et al., 2002], TCRR-1 [Muñiz et al., 2006], and BETL-9 and BETL-10 [this work]) allowed the transcriptional regulatory activity of MRP-1 to be correlated with morphological observations (Figure 3 for BETL-2; Figure 5 for BETL-9). Furthermore, the induction of these genes by MRP-1 could be quantified (Figure 4). The real-time RT-PCR data suggest that all TC markers respond in a quantitative mode to the expression of MRP-1. The induction of TC markers in the transgenic heterozygous kernel shown in Figure 4B resembles that seen for a presumably heterozygous kernel of a different line (Top and HET in Figure 4C), whereas a presumably homozygous sample (Top and HOM in Figure 4C) expressed much higher levels of both MRP-1 and the TC markers. Although sample-to-sample variation might reflect the concurrent influence of other factors regulating the expression of the transgene, for instance, nutrient availability or small differences in maturity, it is clear from the data shown in Figure 4 that the expression levels of the TC markers follow that of MRP-1. Interestingly, the analysis of the expression of a TC-specific gene not previously related with the MRP-1–regulated gene set, the TC-specific cell wall bound invertase INCW-2 (for Cell Wall Invertase 2; Cheng et al., 1996), produced results comparable to those found for the BETL genes. The expression of INCW-2 at the EETL suggests that this gene is part of the gene complement of TCs and is thus expressed as the result of the partial TC differentiation process induced by MRP-1. Alternatively, INCW-2 might be another direct target of the MRP-1 transcriptional regulator. In this context, we have identified a sequence motif in the INCW-2 promoter (5′-GAGATAAGAAT-3′) that strongly resembles the sequence identified as the target of MRP-1 in the BETL promoters (5′-GAGATAAGATA-3′; Barrero et al., 2006), although in the case of INCW-2, this motif would be positioned 227 bp upstream of the position reported for the other TC promoters (as measured from the start codon of the protein). Consistent with these results, we have found that the expression level of MRP-1 in the miniature-1 mutant endosperms (not expressing INCW-2; Cheng et al., 1996) is at least as strong as in the wild-type endosperm (see Supplemental Figure 7 online), whereas the TCs develop their characteristic features (Cheng et al., 1996), suggesting that INCW-2 acts downstream of MRP-1.

The quantitative RT-PCR results indicate that the extension of the EETL is limited to a discrete patch of cells right at the abgerminal pole of the endosperm; hence, the almost normal expression levels detected for AL-9 in the upper halves of the kernels (Figures 4B and 4C) while the EETL cells showed reduced AL-9 expression in situ hybridization experiments (Figure 5D).

Further investigations are required to understand why cells at the abgerminal pole of the endosperm can be induced to differentiate into an EETL. This is very likely connected to the fact (discussed above) that these cells are the only endosperm epidermal cells that expressed detectable levels of MRP-1 in the transgenic material (Figure 1). Certainly, these cells are the last to acquire aleurone morphological and molecular features. Studies using transposon-induced gain- and loss-of-function sectors of the maize Deficient kernel1 (Dek-1; Becraft and Asuncion-Crabb, 2000), a gene required for aleurone cell fate determination (Lid et al., 2002), revealed that aleurone cell fate is not fixed until late in development. Rather, aleurone and starchy endosperm fates are interchangeable for a long period. Furthermore, positional cues are required to specify and maintain the identity of an aleurone cell; the differentiation gradient along the germinal-abgerminal axis seems to dictate that the abgerminal cells are less surely destined to acquire an aleurone cell identity. In addition, in situ hybridization experiments using an aleurone marker in very young material (AL-9 in Supplemental Figure 8 online) showed that the abgerminal epithelial cells are the last to acquire aleurone cell molecular features. We propose that the cells on the
abgerminal side of the endosperm remain in an undifferentiated, MRP-1–responsive stage because they are exposed to maternal signals for a longer period than cells in other regions of the aleurone (see Supplemental Figure 9 online for a picture of a kernel section at 3 DAP, showing possible routes for the input of maternal signals into the endosperm space). The strict dependence on maternally derived signals for the expression of TC-specific genes has been elegantly demonstrated by Gruis et al. (2006); the expression of a fluorescent reporter under the control of a BETL-specific promoter occurred normally in planta, but the reporter switched off in in vitro–grown endosperms. As in in planta endosperms, the in vitro–cultured endosperms expressed aleurone- and starchy endosperm-specific reporter constructs, indicating that these cell fates are correctly specified in the absence of maternally derived signals. These maternal signals, possibly derived from vascular tissues, are thus responsible for the induction of the BETL at the base of the endosperm and might participate in the formation of the EETL in a process triggered by the expression of MRP-1. In this context, analyses of the MRP-1 promoter (Barrero et al., 2009) have shown that its expression is upregulated by phloem components (mainly glucose and sucrose). The role of sugars as signaling molecules in plants is well established (reviewed in Smeeekens, 2000; Rolland et al., 2002). Changes in sugar concentration cause induction or repression of gene transcription, providing a mechanism for interaction between sink and source tissues for carbohydrate metabolism (Koch, 1996). In addition, sugar sensing also affects gene expression posttranscriptionally by changing mRNA stability, translation, or protein stability (Chan and Yu, 1998; Rook et al., 1998; Cheng et al., 1999; Yanagisawa et al., 2003; Wiese et al., 2004). Interestingly, the regulation of the maize cell wall–bound invertase Incw-1 is included among these examples (Cheng et al., 1999). Incw-1 is expressed in maize TCs, and we reported the association between metabolizable sugars and the increase in the steady state abundance of functional Incw-1 RNA. Regardless of the identity of the signals that maintain MRP-1 expression in the EETL area, it is clear that the BETL cells cease to express MRP-1 and TC markers later in development (from 17 DAP to maturity) and go on to acquire aleurone morphological features, the accumulation of TC peptides at the apoplasm being the only remnant of the transient acquisition of TC identity (Figure 6C). We have shown that this process is gradual; very few cells with modified cell walls remained at 16 to 17 DAP, and these cell wall modifications were less evident than those observed at earlier developmental stages (Figure 6), and no sign of modified cells was found in an extensive analysis of the abgerminal aleurones of mature plants. Within the model explained above, this observation suggests that the exposure of the endosperm abgerminal cells to the vascular tissue–derived substances ceases in older kernels, which results in the cessation of MRP-1 expression. This also implies that continuous expression of MRP-1 is required to maintain the TC differentiated stage, which agrees with previous observations of MRP-1 expression in the BETL as late as 29 DAP, when the TCs are completely differentiated (Gómez et al., 2002). Furthermore, the results imply that the TC phenotype is reversible, presenting a remarkable illustration of cell plasticity. In the absence of adequate levels of MRP-1, cells rapidly (in <5 d) change their shape and, more strikingly, their cell wall structure. As discussed above, dynamic interconversions between the aleurone and starchy endosperm cell fate have also been demonstrated using unstable alleles of the aleurone developmental regulator _dek-1_ (Becraft and Asuncion-Grabb, 2000). EETL reversion notably implies the remobilization of the cell wall modifications and ingrowths that characterize TCs (Figure 3). Although the in vitro transformation of garden zinnia (_Zinnia elegans_) mesophyll cells into highly differentiated vascular elements is a well-known process (Fukuda, 1997; McCann, 1997), no such remobilization of cell wall modifications has previously been reported. The reversible modifications observed here (cf. Figures 2 and 6; see Supplemental Figure 5 online) might cast light on the biochemical components involved in cell wall plasticity. Many of the TC-specific genes identified so far encode small hydrophilic proteins of unknown function, although there is increasing evidence that these proteins might be involved in the defense of the developing seed against mother plant–borne pathogens (Serna et al., 2001). In addition, it has been shown that MRP-1 can transactivate various TC-specific promoters, including those of _BETL-1_ and _BETL-2_ (Gómez et al., 2002), _Meg-1_ (Gutiérrez-Marcos et al., 2004), and the TC-specific response regulator _TCRR-1_ (Muñiz et al., 2006). This transactivation occurs through the interaction of MRP-1 with a TC-box found in the promoters of these genes (Barrero et al., 2006) and also in the promoters of _BETL-9_ and _BETL-10_ used in this work. The results presented here leave open the possibility that these peptides have a role in cell–to-cell communication and signaling, in addition to or alternative to the role in defense proposed above, as has been suggested for other TC-specific peptides (Gutiérrez-Marcos et al., 2004). Indeed, a role in cell–to-cell communication and signaling has already been demonstrated for the barley nucellar TC-specific gene Jekyll, which plays a decisive role in the differentiation of nucellar projection TCs (Radtchuk et al., 2006). METHODS Plant Material Transgenic maize (_Zea mays_ ) plants were produced from the variety A188. All plants were grown at 24°C under a 16-h-light/8-h-dark regimen. Quantitative Real-Time RT-PCR The Brilliant SYBR green quantitative RT-PCR kit (Stratagene) was used for real time RT-PCR. Reactions contained 100 ng DNase-treated total RNA from 12-DAP kernels and were performed in an ABI 7000 real-time thermocycler. CT values were normalized against those obtained for a ubiquitously expressed gene, _FKBP-66_ (Hueros et al., 1998), thus obtaining the ΔCT value. Relative quantification was determined by subtracting the ΔCT value of one of the lower kernel halves in the experiment from all the normalized CT values. This provided the ΔΔCT value. Finally, since the efficiency of the PCR reactions was −1 for all genes, the expression 2ΔΔCT was considered to be a function of the starting transcript concentration. The mean (and SD) 2ΔΔCT value of three experiments (technical replicates) was recorded. The oligonucleotides used are listed in Supplemental Table 1 online. In all cases, one of
the primers used spanned an intron to avoid amplification from contaminating DNA.

Cell Wall Staining

The sections in Figure 2 were stained for cellulose using 0.1% calcofluor Fluorescent Brightener 28 ( Sigma-Aldrich). Under UV light, cellulose-containing primary walls appear dark blue, whereas newly deposited wall thickenings appear light blue.

In Situ Hybridization and Immunolocalization

Maize seeds at 10 to 12 or 16 to 17 DAP were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 12 to 24 h, depending on the tissue volume. Samples were dehydrated, embedded in Fibrowax (Plano), and cut into 8-μm sections. For in situ hybridization, slides were probed as previously described (Hueros et al., 1995) with sense and antisense 35S-labeled riboprobes, synthesized from cDNA clones in a pBluescript vector. Slides were exposed to LM-1 silver grain emulsion for 2 to 4 d at 4°C (Amersham). After developing, sections were stained with calcofluor (0.01%).

In situ hybridization results were photographed under bright-field (hybridization signals appear as black spots) or dark-field (hybridization signals appear as white spots) illumination. In some cases, combined dark-field and epifluorescence images were produced to reveal the calcofluor white counterstaining.

For the immunolocalization experiments, endogenous peroxidase was inhibited by incubating the sections in 0.3% v/v H2O2 in methanol for 10 min. The sections were then washed in PBS and blocked with 10% normal donkey serum (Chemicon International) for 20 min before being exposed to anti-BETL-1 or anti-BETL-2 rabbit sera (diluted 1:400) for 1 h at room temperature. The immunoreaction was performed using a biotinylated anti-rabbit antibody (Sigma-Aldrich) and a streptavidin-peroxidase conjugate (Zymed). The peroxidase was detected using 3,3′-diaminobenzidine tetrahydrochloride with a metal enhancer (Sigma-Aldrich). PBS was used as the washing buffer. Finally, the sections were stained with Azure B, pH 4.0, for 3 min.

For confocal microscopy, the sections were rehydrated in an ethanol series, exposed to Alexa enhancer (Invitrogen) for 30 min, and then preincubated in 10% normal donkey serum diluted in 1% (w/v) BSA (fraction V) in phosphate buffer (0.1 M, pH 7.5). After five washes in phosphate buffer (0.1 M, pH 7.5), the sections were reacted with the primary antibody (polyclonal rabbit anti-BETL-2.1:400) for 2 h. The antigen-antibody binding reaction was revealed by applying goat anti-rabbit Alexa Fluor 488 antibodies (Invitrogen) diluted 1:1000 in 1% (w/v) BSA (fraction V) in phosphate buffer (0.1 M, pH 7.5). After several washes in phosphate buffer (0.1 M, pH 7.5), the sections were stained with toluidine blue (0.01%; Sigma-Aldrich) to quench cell wall autofluorescence. A Leica TCS-SL confocal microscope equipped with an argon laser was used for the localization of Alexa 488 green fluorescence. Red cell wall autofluorescence was also recorded to visualize tissues.

For LR-White embedding, fixed tissue was infiltrated with benzoyl peroxide-activated LR white embedding media (Sigma-Aldrich). The infiltration began with a graded infiltration series for 24 h at room temperature: 1:3, 1:1, and 3:1 (LR white:100% ethanol), followed by two changes of pure LR white at 4°C for 1 week each. Samples were positioned into aluminum weight boats, covered, and polymerized at 60°C for at least 24 h. Samples were first sectioned at 1-μm thickness using tungsten-carbide knives. These whole-kernel sections were stained with toluidine blue and used to explore the aleurone, and blocks were then carved and sectioned with glass knives. Semithin sections (0.5 μm) were stained with toluidine blue, and ultrathin (0.05 μm) sections in nickel grids were stained with Reynolds’ lead nitrate and uranyl acetate as described by Hayat (2000). Ultrathin sections were examined using a Zeiss EM-10 electron microscope.

Transgenes

To generate pAL-9-GUS, a 2.2-kbp AL-9 promoter fragment was amplified by PCR from a maize BAC clone hybridizing to AL-9, such that the fragment contained the entire 5′ untranslated region up to the ATG. The PCR fragment was cloned in front of the GUS reporter gene and a cauliflower mosaic virus 3SS polyadenylation signal, in pSK, forming plasmid AL-9GUS-sk(PmeI). Embryogenic type II maize calluses were transformed with the AL-9GUS-sk(PmeI) construct by biolistic bombardment and regenerated as described by Komari et al. (1996).

To generate pAL-9-MRP-1, a full-length MRP-1 cDNA was first cloned into a GATEWAY entry vector (Invitrogen) and then transferred via an LR clonase reaction into the plant binary destination vector pBIOS873, forming a pAL-9-MRP-1 chimeric gene. The vector pBIOS873 is a derivative of pSB12 (Komari et al., 1996) that contains a selectable marker gene for maize transformation and the 2.2-kb AL-9 promoter in front of a GATEWAY cassette, which is followed by an Arabidopsis thaliana polyadenylation sequence. The resulting plasmid, pBIOS880, was transferred into Agrobacterium tumefaciens strain LBA4404 (pSB1) as described by Komari et al. (1996). The maize cultivar A188 was transformed with this clone essentially as described by Ishida et al. (1996).

Transient Expression Assays

Plant material was surface sterilized, sectioned, and maintained on the solid medium described below until bombardment with DNA-coated gold particles. Coating and bombardment was performed according to Knudson and Müller (1991). Five micrometers of each plasmid to be transformed was used to prepare a gold particle batch that was then used to bombard seven tissue samples. After bombardment, samples were incubated at 25°C for 24 h in the dark on solid (0.5% agarose) Murashige and Skoog medium containing 100 mg/L myo-inositol, 2 g/L Asn, 2 g/L Gln, 30 g/L sucrose, and Murashige and Skoog vitamins (Sigma-Aldrich).

Histochemical detection of GUS expression was performed by staining, according to Jefferson et al. (1987). Samples were stained for GUS in a medium containing 0.5 mg/mL X-glucuronide (Clontech), 0.5 mM K+−ferrocyanide, 0.5 mM K+−ferricyanide, 10 mM Na2EDTA, 50 mM phosphate buffer, pH 7, 0.1% Triton X-100, and 20% (v/v) methanol.

Accession Numbers

Supplemental data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: BETL-10, FN400765; BETL-9, FN400766; AL-9, FN400767; MRP-1, AJ318518; BETL-1, Z49203; BETL-2, NM_001112109; TCRR-1, AM085299; INCW-2, AF050128; and ESR-6, AJ849917.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Expression Analyses of the AL-9 Gene.

Supplemental Figure 2. Interpretation of the Transformation of Endosperm Epithelial Cells into Transfer Cells, Induced by the Expression of MRP-1.

Supplemental Figure 3. The Ectopic Expression of MRP-1 Induces the Expression of BETL-1.

Supplemental Figure 4. The Expression of MRP-1 in the Abgerminal Aleurone Decreases as the Kernel Matures.
Supplemental Figure 5. Morphological Analyses of the Mature Endosperm Epidermis.

Supplemental Figure 6. The Presence of the MRP-1 Coding Sequence Decreases the Expression Efficiency of Reporter Constructs in Transient Expression Experiments.

Supplemental Figure 7. Expression Analyses in miniature-1 and Wild-Type Kernels.

Supplemental Figure 8. Expression Pattern of the Aleurone Marker Gene AL-9 in Young Kernels.

Supplemental Figure 9. A Model Explaining How Maternal Signals Might Be Perceived by the Abgerminal Side of the Endosperm at Its Initial Developmental Stages.

Supplemental Table 1. Oligonucleotides Used for Quantitative RT-PCR.

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