**Miniature1-Encoded Cell Wall Invertase Is Essential for Assembly and Function of Wall-in-Growth in the Maize Endosperm Transfer Cell**

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The miniature1 (mn1) seed phenotype in maize (*Zea mays*) is due to a loss-of-function mutation at the *Mn1* locus that encodes a cell wall invertase (INCW2) that localizes exclusively to the basal endosperm transfer cells (BETCs) of developing seeds. A common feature of all transfer cells is the labyrinth-like wall-in-growth (WIG) that increases the plasma membrane area, thereby enhancing transport capacity in these cells. To better understand WIG formation and roles of INCW2 in the BETC development, we examined wild-type and *mn1* mutant developing kernels by cryofixation and electron microscopy. In *Mn1* seeds, WIGs developed uniformly in the BETC layer during 7 to 17 d after pollination, and the secretory/endocytic organelles proliferated in the BETCs. Mitochondria accumulated in the vicinity of WIGs, suggesting a functional link between them. In the *mn1* BETCs, WIGs were stunted and their endoplasmic reticulum was swollen; Golgi density in the mutant BETCs was 51% of the *Mn1* Golgi density. However, the polarized distribution of mitochondria was not affected. INCW2-specific immunogold particles were detected in WIGs, the endoplasmic reticulum, Golgi stacks, and the trans-Golgi network in the *Mn1* BETCs, while immunogold particles were extremely rare in the mutant BETCs. Levels of WIG development in the *empty pericarp4* mutant was heterogeneous among BETCs, and INCW2 immunogold particles were approximately four times more abundant in the larger WIGs than in the stunted WIGs. These results indicate that polarized secretion is activated during WIG formation and that INCW2 is required for normal development of WIGs to which INCW2 is localized.

The maize (*Zea mays*) endosperm is composed of four distinct tissue types: the aleurone layer, the starch endosperm, the embryo-surrounding region, and the basal endosperm transfer layer (BETL; Costa et al., 2004; Olsen, 2004). The functions of cells in the first two tissue types are considered essential to seed germination, whereas the other two, which are spatially distinct in the kernel, are postulated to play roles in seed development. At maturity, the bulk of the endosperm consists of storage cells packed with starch and storage proteins. The aleurone layer is the outermost layer of the endosperm and is often composed of a single cell layer. The embryo-surrounding region cells surround the cavity in which the embryo develops and are predominantly located underneath a developing embryo. The BETL is at the base of the endosperm adjacent to the maternal pedicel tissue, and this is the outermost filial cell layer in direct contact with the maternal pedicel. The BETL is composed of two to three strata of highly specialized transfer cells (Kiesselbach, 1949; Davis et al., 1990). The major morphological feature of the transfer cell is the labyrinth-like proliferation of their cell wall into the cytoplasm, called wall-in-growth (WIG), which serves to increase the plasma membrane area. This unique feature of the BETL cell is postulated to confer a greater solute transport capacity to these cells.

Transfer cells are ubiquitous among plants and are most often located at the aposymplastic junctions between maternal and filial generations of developing seeds (Offler et al., 2003; Vaughn et al., 2007). Vaughn et al. (2007) demonstrated that WIGs in the *Vicia faba* epidermal transfer cells are rich in cellulose, hemicellulose, pectin, callose, and arabinogalactan proteins, similar to the primary cell wall. Although transfer cells in developing seeds are described in a large number of plant species (Thompson et al., 2001), the most elaborate cellular morphology as well as extensive support for their possible roles in the metabolic and developmental biology of endosperm have been reported in...
maize (Felker and Shannon, 1980; Davis et al., 1990; Miller and Chourey, 1992; Cheng et al., 1996; Brugiere et al., 2008; Jain et al., 2008). Additionally, many BETL-specific gene markers (i.e. unique to the BETL) have also been described in maize (Hueros et al., 1995; Maitz et al., 2000; Serna et al., 2001; Cai et al., 2002; Gomez et al., 2002; Costa et al., 2003; Gutierrez-Marcos et al., 2004, 2007). Of these marker genes, though, only a few have been associated with well-defined functions unique to the BETL. These include a family of BETL genes that encode proteins with antimicrobial function (Hueros et al., 1995; Serna et al., 2001; Cai et al., 2002), BETL-specific transcriptional activation (Gomez et al., 2002), and the miniature1 (Mn1)-encoded cell wall invertase (Cheng et al., 1996). There is also mounting evidence that BETL secretes peptides that may have signaling functions (Gutierrez-Marcos et al., 2004) or provide regulatory signals between the dead placento-chalazal cells in the maternal pedicel and filial cells in the endosperm (Kladnik et al., 2004).

It has been demonstrated that the BETL is critical for normal seed development in maize. Charlton et al. (1995) suggested that seed abortion resulting from crosses between diploid maize lines with 4N male parents is attributable to a complete suppression of BETL development at an early stage of seed development in such crosses. In another study, Maitz et al. (2000) reported a reduced grain filling locus that is associated with reduced expression of BETL markers and a loss of 70% seed weight at maturity. Similarly, the globy1 (Costa et al., 2003), empty pericarp4 (emp4; Gutierrez-Marcos et al., 2007), and baseless1 (Gutierrez-Marcos et al., 2006) mutants exhibit abnormal BETL at an early stage of seed development and, ultimately, the aborted seed-lethal phenotypes. The nuclear Emp4 gene codes for a novel type of pentatricopeptide repeat protein that is necessary in the proper regulation of expression of a small subset of mitochondrial transcripts in various parts of the plant, including a developing endosperm. Loss of the EMP4 protein in the emp4 mutant is associated with fewer mitochondria and irregular differentiation of transfer cells in the BETL, consistent with the observations that the normal BETL cells are metabolically active and mitochondrial deficiency can lead to reduced WIG formation in these cells.

Developing maize seeds are sympatkically isolated from their maternal tissue, and one of the most often postulated functions of the BETL is acquisition of nutrients from maternal postphloem regions in the pedicel (for review, see Thompson et al., 2001; Offler et al., 2003, and refs. therein). The BETL is thus spatially and temporally the first filial cell layer in which the photoassimilates enter from the mother plant. Among the sugars, hexoses may enter during the very early stages, ~4 to +2 d after pollination (DAP; Mclaughlin and Boyer, 2004), but Suc is the predominant sugar (Felker, 1992) that enters the BETL during the major part of subsequent seed development. Not surprisingly, the lack of the Mn1-encoded cell wall invertase (INCW2) in the BETL causes the mn1 seed phenotype (Cheng et al., 1996), which is associated with reduced cell size and cell number in developing endosperm (Vilhar et al., 2002) and a loss of nearly 70% of the seed weight relative to the wild type. The mn1 mutant seed, however, is nonlethal, presumably because of the residual low level (approximately 1% of the wild type) of cell wall invertase activity (Miller and Chourey, 1992) encoded by a nonallelic locus, Incw1 (Chourey et al., 2006). Although the genetic analyses suggest that the INCW2-mediated Suc hydrolysis is a major physiological function of the enzyme in the BETL, exogenously supplied hexose sugars to in vitro kernel cultures of the mn1 seeds failed to restore the Mn1 seed phenotype (Cheng et al., 1999).

Our objectives for this study are 2-fold. (1) To monitor BETL development in Mn1 and mn1 kernels by light and electron microscopy to test whether WIG development in the mn1 mutant is similar to or impaired in comparison with the wild type. Given that several seed-lethal phenotypes in maize are correlated with structural aberrations in the BETL (discussed above) and that mn1 is a well-defined single gene mutant with a known causal biochemical alteration specific to this region of the kernel, characterizing WIG formation in the mn1 mutant is of great interest. (2) To determine subcellular localization of the Mn1-encoded INCW2 by immunoelectron microscopy. Cryofixation and low-temperature embedding techniques provide plant samples in which proteins are immobilized to their native locations and are thus suitable for high-resolution localization by immunogold labeling (Kang and Staehelin, 2008).

RESULTS

WIG Development Is Retarded in mn1 Mutant Kernels

Figure 1 shows the BETL region in semithin sections (500 nm) of the Mn1 (A–C) and mn1-1 (D–F) kernels at 7, 12, and 17 DAP. In both genotypes, basal endosperm transfer cells (BETCs) are aligned side by side, forming a cell layer on top of the nucellar placento-chalazal layer. WIGs are easily observed in 12- and 17-DAP Mn1 BETCs after staining by toluidine blue. Accumulation of cell wall material on the basal cell wall was clearly resolved in the 12-DAP BETCs (Fig. 1B, asterisks), but Mn1 BETCs at 7 DAP were devoid of such basal cell wall thickening (Fig. 1A). The 17-DAP WIGs were long and dense, occupying the basal side of the BETCs (Fig. 1C). The nucleus and vacuoles are too big to be fitted into the space between the WIGs and appeared to have been driven to the apical cytoplasm (Fig. 1C). Toluidine blue staining was weaker in the 17-DAP WIGs than in the 12-DAP WIGs, suggesting a change in the chemical composition of the WIGs. WIGs were most elaborate in the BETCs in contact with the placento-chalazal layer but gradually dimin-
ished in the BETCs farther from the placento-chalazal layer (Fig. 1B). Davis et al. (1990) observed a similar gradient in 23-DAP BETCs in a variety of field corn, TX5855.

WIGs were not observed in 7-DAP mn1-1 BETCs, similar to the Mn1 wild-type BETCs (data not shown). Even at 12 DAP, most mn1-1 BETCs were devoid of WIGs when examined by bright-field light microscopy (Fig. 1D). Only a few cells had thin ingrowths from the basal cell walls at this stage (Fig. 1D, dashed circle). WIGs proliferated in the mn1-1 BETCs during the period from 12 to 17 DAP (Fig. 1E). However, the amount of WIG formation in the mutant was far reduced when compared with the massive growth in the Mn1 BETCs during the same period (Fig. 1, C and F, double arrows). Similarly impaired WIG development was observed in the BETCs of mn1-Neuffer, another loss-of-function allele of mn1 (data not shown).

**Proliferation and Remodeling of Golgi Stacks in the BETC during WIG Development**

To characterize WIG formation and the accompanying reorganization of the BETCs in detail, we examined BETCs from the Mn1 kernels using transmission electron microscopy (Fig. 2). Developing maize kernels were cryopreserved before WIGs were detected (7 DAP), during active WIG growth (12 DAP), and after WIGs had enlarged (17 DAP). At 7 DAP, no regular WIG was detected but short WIG initials were budding from the basal primary cell wall (Fig. 2B, arrows). Only a few Golgi stacks, trans-Golgi network compartments (TGNs), and multivesicular bodies were seen in these immature BETCs lacking WIGs (for Golgi density, see Fig. 6A below).

At 12 DAP, BETCs have convoluted WIGs at the basal cell wall and along the side walls. The WIGs were thickest at the basal wall and decreased in size from the basal to the apical direction along the side wall (Fig. 2C). In the electron micrographs, two regions in the WIGs were distinguished by their differential staining properties. The electron-opaque cell wall containing fibrous substances (Fig. 2D, solid circle) constituted the principal structure, and dark blotches (Fig. 2D, dashed circle) of varying sizes were deposited over the fibrous cell wall. The spotty pattern of staining was obvious from the 7-DAP WIG initials (Fig. 2B) but had faded in the 17-DAP WIGs (Fig. 2I).

BETCs at 12 DAP had a large number of Golgi stacks and TGNs with their Golgi density increased seven times from that of the 7-DAP BETCs (see Fig. 6A below). Numerous TGN cisternae on the trans-side of Golgi stacks, free-floating TGN cisternae, and secretory vesicles derived from the TGN were seen in the vicinity of WIGs (Fig. 2, E–G; Supplemental Fig. S1C) as well as in the central cytoplasm (Supplemental Fig. S1D). In addition, 12-DAP BETCs had clusters of multivesicular bodies of diverse sizes in the cytoplasm (Fig. 2F; Supplemental Fig. S1D). Long endoplasmic reticulum (ER) tubules permeated the cytoplasm in both 7- and 12-DAP BETCs, but the ER tubules were excluded from the WIG zone (Supplemental Fig. S1, A and B). The proliferation of secretory/recycling compartments and vesicles provides evidence that membrane trafficking is up-regulated during WIG development.

In 17-DAP BETCs, Golgi stacks, mitochondria, and the ER were tightly packed in the central cytoplasm (Fig. 2, H and J) and interstices of the WIGs (Fig. 2, I and K). The ER in 17-DAP BETCs was thicker than the thin tubular ER seen in the 12-DAP BETCs (Fig. 2K). In earlier stages, nuclei were round and located in the cell.
center (Figs. 1, A and B, and 2, A and C). In 17-DAP BETCs, dark and pleiomorphic nuclei were located at the apical tip (Figs. 1C and 2H). Golgi stack density was higher in the 17-DAP BETCs than in the 12-DAP BETCs (Fig. 6A), and their morphology differed from the earlier stage Golgi stacks. The outer rim of medial and trans-Golgi cisternae in the 17-DAP Golgi stacks was hypertrophied (Fig. 2K; see Fig. 4B below), and their TGN cisternae were associated with vesicles larger than vesicles of the 12-DAP TGNs (Fig. 2, J and K; see Fig. 4, B and C, below). Similar morphological features of Golgi stacks have been reported from electron microscopy investigations of mucilage-secreting plant cells (Craig and Staehelin, 1988; Staehelin et al., 1990; Young et al., 2008). Golgi stacks and TGN cisternae were seen in clusters, and large numbers of vesicles accumulated in their vicinity at this stage (Fig. 2, J and K). While the density of Golgi stacks increased in 17-DAP BETCs, few multivesicular bodies were observed.

Spatial Proximity of Mitochondria and the WIGs

Mitochondria were easily noticed in the BETCs, owing to their abundance and unique spatial distribution. Most of the mitochondria were spherical in all the examined stages. Mitochondria at 17 DAP were larger (0.636 ± 0.169 μm) than mitochondria at 12 DAP (0.335 ± 0.079 μm), and their matrix staining was weaker (Fig. 2, E and I, asterisks). Interestingly, mito-
chondria accumulated at the basal cytoplasm adjacent to the WIG in the BETCs. Mitochondria started concentrating in the basal cytoplasm at 7 DAP even before any WIG was detected (Fig. 2 A, B, and L). This early onset of mitochondrial concentration indicates that the BETC polarity is established prior to WIG formation. In 12-DAP BETCs, mitochondria clearly displayed spatial association with the WIG and the spatial proximity persisted throughout the WIG development (Fig. 2, M and N), suggesting a functional link between them. In 17-DAP BETCs, the spaces between the WIGs were so crowded with mitochondria and the ER that membranes of the organelles were oppressed to each other and to the plasma membrane (Fig. 2I). Unlike the mitochondria, Golgi stacks, TGN cisternae, and multivesicular bodies did not display spatial association with the WIGs (data not shown).

Impaired WIG Development and Swelling of the ER in the mn1 Mutant BETCs

We carried out electron microscopy analyses of mn1-1 mutant BETCs (Fig. 3) to compare their ultrastructural features with those of Mn1 BETCs. The 12-DAP mn1-1 BETL consisted of cells with varying amounts of WIGs, as shown in Figure 1D. The two BETCs in Figure 3, A and B, are from a 12-DAP mn1-1 kernel. The BETC in Figure 3A has WIGs, while no WIG is detected in Figure 3B BETC. In the mn1-1 BETCs at 12 DAP, however, individual WIGs are not fully interconnected and the dark blotchy pattern is less discernible (Fig. 3, A and C). Another abnormality in the mn1-1 BETCs was their swollen ER. Most of the ER lumina were round and dilated (Fig. 3, A, C, and G), in contrast to the thin ER tubules of the same stage Mn1 BETCs (Fig. 2, E and F; Supplemental Fig. S2, A and B).

The mn1-1 WIG elaborated during the period from 12 to 17 DAP (Fig. 3D). The 17-DAP mn1-1 WIGs were analogous to the basal WIGs of 12-DAP Mn1 BETCs in that the WIGs formed a large network and the dark WIG segments in the WIG were easily distinguished (Fig. 3, D and E). The ER swelling became more extreme in the 17-DAP BETCs. In particular, extensive ER cisternae with spherical holes were often noticed in the mutant BETCs (Fig. 3, D, F, and H).

The Golgi stacks in the 17-DAP Mn1 BETCs were characterized by the bulbous periphery of trans-Golgi

Figure 3. Electron microscopy analysis of mn1-1 BETCs. A to C, mn1-1 BETCs at 12 DAP. A, mn1-1 BETC with WIGs are less convoluted than wild-type WIGs. B, mn1-1 BETC devoid of WIG. C, Higher magnification image of WIGs and peripheral cytoplasm of a mn1-1 BETC. Mitochondria (arrowheads) and swollen ER (asterisks) are marked in A to C. D, mn1-1 BETC at 17 DAP. The large ER cisternae are denoted with dashed ovals. E, Higher magnification image of the peripheral cytoplasm in a 17-DAP mn1-1 BETC. Mitochondria are marked with arrowheads. F, Higher magnification image of the abnormal ER cisternae. Multivesicular bodies are marked with arrows. G and H, Golgi stacks in 12-DAP (G) and 17-DAP (H) mn1-1 BETCs. G, Golgi stack; N, nucleus; PCW, primary cell wall. Bars = 2 μm in A and B, 500 nm in C and E to H, and 5 μm in D.
cisternae and large TGN vesicles. Golgi stacks in 17-DAP *mnl-1* BETCs did not display such morphological characteristics (Fig. 3H), suggesting that cell wall polysaccharide secretion is affected in the mutant BETCs. In both 12- and 17-DAP *mnl-1* BETCs, Golgi cisternae were thin and few vesicles were associated with Golgi stacks and TGN compartments (Fig. 3G and H). Golgi densities in the *mnl-1* BETCs were also lower (approximately 50%) than in the *Mnl* BETCs at both 12 and 17 DAP, although the density in the *mnl-1* BETCs increased during that period (see Fig. 6A below).

 Despite the retarded WIG development and the abnormal ER in the *mnl-1* mutant, polar distribution of mitochondria adjacent to the WIG was not disrupted (Fig. 3, A, B, and E, arrowheads), indicating that the cell polarity establishment is functional in the mutant BETCs. We have examined BETCs in the *mnl-Neuffer* mutant allele by electron microscopy and observed cellular abnormalities consistent with the results from *mnl-1* mutant BETCs (data not shown).

### Localization of INCW2 in the *Mnl* and *emp4* BETCs

To determine the cellular localization of INCW2, we performed immunogold labeling experiments with an INCW2 antibody (Figs. 4 and 5). This antibody is INCW2 specific by several criteria; most notably, it showed no INCW2 protein in the *mnl-1* mutant by both western-blot analysis and light microscopy immunolocalization in developing kernels. Additionally, this absence of INCW2 epitope agrees with the fact that the *mnl-1* mutant lacks the *Mnl* RNA throughout endosperm development (Cheng et al., 1996; Chourey et al., 2006). In the cryofixed *Mnl* BETC samples, INCW2-specific gold particles were primarily located in the WIGs (Fig. 4A). The immunogold particles were also found in the ER, Golgi stacks, and TGNs (Fig. 4, B and C). The localization of INCW2 in the WIG is consistent with the previous biochemical result that the protein is associated with the cell wall (Lauriere et al., 1988). INCW2 has an N-terminal signal peptide (Taliertico et al., 1999), and the immunogold labeling of the ER and the secretory organelles is consistent with the idea that INCW2 is synthesized by ER-bound ribosomes and delivered to the WIG via the Golgi and TGN compartments. The WIG association of INCW2 was observed in earlier stages (10 DAP, Fig. 4D; 12 DAP, data not shown). When we carried out immunogold labeling experiments with *mnl-1* BETCs, virtually no immunogold particles were detected, confirming that *mnl-1* is a null mutant for *Mnl* expression (Cheng et al., 1996) and validating the specificity of the INCW2 antibody.

The BETC cell wall consists of the outer primary cell wall and inner WIGs. The primary cell wall is constructed during the early stage of endosperm development and constitutes the original cell boundary. The WIGs are built as a secondary cell wall during BETC maturation. In electron micrographs, the primary cell wall can be distinguished from WIGs based on its position, staining property, and layered pattern, which WIGs do not have (Figs. 2 and 5). The localization of INCW2 gold particles was specifically confined to the WIGs, indicating that the primary cell wall is devoid of INCW2 (Fig. 5, A and B).

To further investigate the relation between INCW2 and WIG development, we analyzed INCW2 localization in a previously described seed-lethal *emp4* mutant, in which WIG development is aberrant (Gutierrez-Marcos et al., 2007). It is impossible to investigate whether WIG formation is linked to proper localization of INCW2 using the *mnl* mutants, because INCW2 is absent in the mutant. In cryofixed *emp4*...
BETL samples, the WIGs were smaller than Mn1 WIGs and WIG density was heterogeneous among BETCs (Fig. 5C). INCW2-specific immunogold particles were detected in the WIGs in the emp4 mutant BETCs as in the Mn1 BETCs, but the immunogold particles were fewer in the mutant WIGs than in the Mn1 WIGs (Fig. 6B). Interestingly, the immunogold particles were more abundant in BETCs with elaborate WIGs than in the BETCs with stunted WIGs (Fig. 5D, W and W'). The elaborate WIGs have up to four times more immunogold particles than the stunted WIGs do in the emp4 BETCs (Fig. 6B).

DISCUSSION

In this study, we demonstrated that Golgi number increases, that Golgi remodeling indicative of massive polysaccharide secretion occurs during WIG development, and that INCW2, a BETC-specific cell wall invertase, is delivered to the WIG by Golgi-derived vesicles. We also showed that WIG formation is impaired in the mn1 mutant BETCs where INCW2 is absent. In the mutant, Golgi stacks are fewer and do not display the morphological changes observed in the wild-type BETCs. Given that the INCW2 deficiency of the mn1-1 mutant leads to reduced hexose levels (S. LeClere, E.A. Schmelz, and P.S. Chourey, unpublished data), we suggest that the defective WIG formation in the mn1 mutant may result from rate-limiting levels of monosaccharides that are essential for cell wall polysaccharide synthesis and glycosylation reactions.

Aberrant WIGs in the mn1 BETL

Bright-field imaging of semithin sections revealed that the WIGs in the mn1 BETL kernels are stunted and
display nonuniform growth along the BETC layer (Fig. 1D). The retarded growth of mn1 WIGs is similar to that reported from studies of 2N × 4N hybrids (Charlton et al., 1995), globby1 (Costa et al., 2003), baseless1 (Gutierrez-Marcos et al., 2006), and emp4 (Gutierrez-Marcos et al., 2007) kernels, but the causal basis for aberrant WIGs in these mutants is not straightforward. The defective WIG assembly seen in the mn1 mutants can be better understood because biochemical activity of INCW2 is well known. Indeed, the loss of approximately 99% of the total cell wall invertase activity in the mn1 mutant relative to the Mn1 kernels (Cheng et al., 1996; Chourey et al., 2006) leads to a severe sugar imbalance in the basal part of the kernel that includes the BETL. Specifically, at 12 DAP, the mutant basal region shows approximately 5-fold less hexose (Glc and Fru) and a significant increase in Suc relative to the Mn1 kernels (S. LeClere, E.A. Schmelz, and P.S. Chourey, unpublished data). Although no biochemical data are available on the composition of maize WIGs, histochemical and biochemical analyses of transfer cells in V. faba cotyledons demonstrated that they are rich in cell wall polysaccharides and glycoproteins that are also found in the primary cell wall (Vaughn et al., 2007). Assuming that WIG in the maize BETC consists mainly of polysaccharides and glycoproteins, the stunted growth of mn1 WIGs may result from the lack of monosaccharides for polysaccharide biosynthesis, glycosylation, and numerous other metabolic and signaling functions. The lack of Suc hydrolysis in the mutant could also contribute to a loss of osmotic pull essential to drive water uptake in the developing seed. The possibility of such sugar-mediated cellular abnormality suggested here is in complete agreement with the recent molecular data (Barrero et al., 2009) that showed a significant up-regulation of the ZmMRP-1 promoter activity by monosaccharide hexoses relative to disaccharide Suc in transgenic Arabidopsis (Arabidopsis thaliana) seedlings and in yeast. The ZmMRP-1 encodes a transfer cell-specific transcription activator that induces transfer cell differentiation in the maize BETL (Gomez et al., 2002). Several other in vitro experiments showing an increase in transfer cell differentiation by hexose monosaccharides, but not by Suc, are also described in V. faba cotyledons (Offler et al., 2003).

Maize BETL as a Model System for Plant Polarized Secretion

Multiple types of model systems have been utilized for cellular and molecular characterizations of the plant secretory pathway. They include Arabidopsis root tip cells, protoplasts, root hair tips, and pollen tube tips as well as tobacco (Nicotiana tabacum) suspension cultured BY-2 cells and leaf epidermal cells (Brandizzi et al., 2004; Lee et al., 2008; Nielsen et al., 2008; Van Damme et al., 2008). Recently, Young et al. (2008) investigated polarized deposition of mucilage in the Arabidopsis seed coat cells and Golgi stacks in the mucilage-secreting cells. The WIG formation requires massive amounts of cell wall proteins and polysaccharides delivered from Golgi stacks to the site of WIG assembly. Here, we report proliferation of and morphological changes in Golgi stacks that accompany WIG development in the BETC.

The main advantage of the BETL system over other model systems is that the maize BETL consists of a uniform cell population in which transfer cells develop synchronously. This cell arrangement facilitates isolating RNA transcripts and proteins specific to BETCs at certain developmental stages for transcriptomic or proteomic analysis. Although the genome of Arabidopsis is the best-annotated among plant species, the amount of RNA or protein that can be isolated from a particular cell type is limited, due to the small size of Arabidopsis plants. We have been able to isolate micrograms of high-quality RNA from the maize BETL consistently by cryosectioning and microdissection. We are currently listing genes highly expressed in maize BETCs by means of parallel sequencing technology (Margulies et al., 2005) to identify which maize secretory pathway genes are activated during WIG development and to find novel plant-specific factors involved in polysaccharide secretion. Tissue-specific transcriptome analyses were carried out in micellar projection and endosperm transfer cells of barley kernels, revealing their gene expression profiles (Thiel et al., 2008).

The Abnormal Secretory Pathway in the mn1 Mutant BETC

The density and morphological features of Golgi stacks in the mn1 mutant BETCs indicate that Golgi activity is restrained in the mn1 mutant. This observation is not unexpected, given that hexose activity producing hexoses from Suc is limited in the mn1 mutant BETCs. The Golgi is the primary site for protein glycosylation, oligosaccharide addition to glycolipids, and synthesis of noncellulosic cell wall polysaccharides (Staehelin and Newcomb, 2000). It would be impossible for Golgi stacks to carry out these carbohydrate biochemical reactions without a sustained supply of monosaccharide substrates.

It is puzzling, however, why low levels of hexoses led to the ER swelling that resulted in enormous ER cisternae in 17-DAP BETCs. One possible explanation is that protein-folding quality control might be affected in the mutant ER. Oligosaccharide residues are added to Asn residues of nascent polypeptides in the ER lumen, and this N-glycosylation is involved in the quality control system that blocks misfolded proteins from leaving the ER (Pattison and Amtmann, 2009). It is possible that the N-glycosylation for ensuring proper protein folding is malfunctioning in the mn1 BETCs due to a shortage of monosaccharide supply. Aberrant N-glycosylation in the ER can disturb the quality control mechanism, inhibiting export of nascent proteins from the ER. This general block in ER
export can lead to a large-scale accumulation of secretory proteins in the ER, swelling the compartment.

It was shown that Golgi stacks in the Arabidopsis seed coat cells proliferate and display morphological features indicative of active polysaccharide synthesis and secretion during mucilage export (Young et al., 2008). Mum4 is an Arabidopsis enzyme converting UDP-Glc into UDP-rhamnose (Oka et al., 2007), and inactivation of Mum4 leads to a decrease in mucilage secretion. In the mum4 mutant seed coat cells, the Golgi remodeling associated with mucilage synthesis is not observed. Interestingly, the increase in Golgi density was not affected in the mum4 cells, suggesting that Golgi proliferation is part of a developmental program that takes place irrespective of mucilage synthesis (Young et al., 2008). This observation contrasts with the decreased Golgi stack density in the mn1-1 BETCs. This discrepancy can be understood if we consider that Glc is the raw material for all carbon compounds in the developing kernel and that Glc also serves as an important signal molecule influencing transcription, translation, and protein degradation in plant cells (Rolland et al., 2006). In the mum4 mutant, an enzyme involved in a specific UDP- monosaccharide conversion is defective. On the contrary, an insufficient Glc supply is a fundamental problem that can cause multiple cellular processes in the mn1-1 BETCs to deviate from the normal developmental program.

Despite the abnormalities in the secretory pathway, cell polarity is established normally in the mn1 BETCs. This conclusion is supported by the polarized distribution of mitochondria and WIG assembly on the basal cell wall in the mutant, suggesting that cell polarity establishment is not affected by the low hexose levels in the mn1 mutant BETC.

INCW2 Localizes Specifically to the WIG in the Cell Wall

Plants have three types of invertases, which are located in the cell wall, in the cytoplasm, and in the vacuole (Roitsch and Gonzalez, 2004). Subcellular localization of cell wall invertases by electron microscopy has not previously been reported in plant species, although the proteins have been considered to be apoplastic, containing sink-source relationships in various parts of the plant (Chourey et al., 2006, and refs. therein). INCW2 has the characteristics of secreted soluble proteins, including an N-terminal signal peptide, three predicted glycosylation sites, and lack of transmembrane domains (Taliercio et al., 1999). The distribution of INCW2 in the WIG and secretory compartments is consistent with the structural features of INCW2.

Construction of the primary cell wall cellularizes BETCs during the syncytial stage, and the WIG is a secondary cell wall built on the original primary cell wall later in endosperm development (Scanlon and Takacs, 2009). The WIG is positioned immediately outside of the BETC plasma membrane, and the spatial association of INCW2 with the plasma membrane is likely to accelerate absorption of hexoses instantly after the hydrolysis and to enhance transport efficiency by minimizing possible loss from diffusion of newly produced hexose. Furthermore, dynamic interaction between INCW2 and hexose transporters could facilitate nutrient acquisition through the mechanism of substrate channeling (Winkel, 2004).

WIG development is impaired in the emp4 BETCs, and INCW2 immunogold particle density in the emp4 WIG was generally lower than in the Mn1 WIGs. Interestingly, BETCs with larger WIGs had as many as four times more immunogold particles than BETCs with smaller WIGs in the emp4 mutant kernel. This positive correlation between INCW2 accumulation in the WIG and WIG proliferation suggests that they are interdependent, reinforcing each other. The monosaccharides produced by the INCW2 are essential building blocks for constructing the WIG. For INCW2 to function properly, BETCs have to up-regulate polysaccharide synthesis to build WIGs that accommodate INCW2.

MATERIALS AND METHODS

Plant Materials

Immature maize (Zea mays) kernels of homozygous Mn1 and the mn1 reference alleles in the W22 inbred line were harvested at various stages of development marked by DAP. At the time of harvest, kernels were excised from the ear with care to include the pedicel region, to ensure preservation of the basal part of the endosperm. Heterozygous Emp4-1 plants were selfed, and the F2 ears showing 3:1 segregation for normal and defective kernels were marked. Homozygous emp4-1 defective kernels were readily identifiable at as early as the 12-DAP stage; mutant kernels were processed for semithin sections and immunogold labeling as described below.

Bright-Field Microscopy and Transmission Electron Microscopy

Developing maize kernels were dissected with a biopsy punch (2 mm in diameter) to isolate the basal area of the kernels. The dissected kernel specimens containing the BETL were transferred to B-type aluminum planchettes (Technontrade International). A Suc solution (150 mM) was added to fill the planchettes, and the kernel samples were frozen with a HPM 100 high-pressure freezer (Leica). The whole process from dissection to freezing was completed within 1 min. The frozen BETL samples were freeze substituted in acetone containing 2% OsO4 at −80°C for 4 d. After substitution, the samples were slowly warmed up to −20°C over 24 h, from −20°C to 4°C over 12 h, and from 4°C to room temperature over 4 h in the AFS2 automatic freeze substitution system (Leica). The samples were washed three times with anhydrous acetone and embedded in Epon resin (Ted Pella). After polymerization, samples were sliced into 50- and 80-nm sections for bright-field imaging and transmission electron microscopy imaging, respectively. The 500-nm sections were stained with toluidine blue and examined with an Olympus BH2 compound microscope using a Retiga 200R digital camera. The 80-nm sections were stained with an aqueous uranyl acetate solution (2%, w/v) and subsequently with a lead citrate solution (26 g L−1 lead nitrate and 35 g L−1 sodium citrate). Electron micrographs were captured with a Hitachi H-7000 transmission electron microscope operated at 80 kV.

Immunogold Labeling

The high-pressure-frozen BETC samples were freeze substituted in 0.1% uranyl acetate and 0.25% glutaraldehyde in acetone at −80°C for 3 d. After substitution, the samples were warmed up to −50°C over 30 h and washed.
four times with dry acetone at −50°C. The BETC samples were then embedded in HM20 acrylic resin (Ted Pella) at −50°C, and the resin was polymerized under UV light at −50°C for 36 h. All of the freeze substitution, temperature transition, resin embedding, and UV polymerization were carried out in the AFS2 automatic freeze substitution system (Leica). The polymerized samples were sliced into 100-nm-thick sections and immunogold labeled with an anti-INCW2 antibody (Cheng et al., 1996; 1.50 [v/v] dilution) as described by Kang and Staehelin (2008). After poststaining as explained above, the BETC sections were examined with a Hitachi H-7000 transmission electron microscope operated at 80 kV.

**Morphological Measurements from Transmission Electron Micrographs**

To calculate Golgi stack densities (numbers of Golgi stacks μm−2 cytoplasm), images from six BETCs at each stage were taken and numbers of Golgi stacks in the electron micrographs were counted. Surface areas of the cytoplasm were measured with ImageJ 1.38X (National Institutes of Health; http://rsb.info.nih.gov/ij/). The cytoplasmic areas were measured after excluding nuclei and WIGs in the cells. A total of 19 Golgi stacks at 7 DAP, 93 Golgi stacks at 12 DAP, and 104 Golgi stacks at 17 DAP were identified in the wild-type BETCs. For mn1-1 mutant BETCs, 49 Golgi stacks at 12 DAP and 57 Golgi stacks at 17 DAP were counted. Golgi density in 7-DAP mn1-1 BETCs was not determined because Golgi stacks are extremely rare (<10) in the images. Golgi stacks in which stack architecture was clearly distinguished were counted for measuring Golgi density.

Immunogold particle density (numbers of immunogold particles μm−2 WIG) was calculated in a similar way. We enumerated 927, 772, and 163 INCW2-specific gold particles in the wild-type WIGs, thick WIGs of the wild-type BETCs. For emp4–/emp4– specific transcriptional activator, ZmMRP-1. Plant Cell 14: 599–610

**Supplemental Data**

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

Supplemental Figure S1. Organization of the ER in 7-DAP and 12-DAP BETCs.

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**LITERATURE CITED**

Barrero C, Royo J, Grijota-Martínez C, Faye C, Paul W, Sanz S, Steinbiss HH, Hueros G (2009) The promoter of ZmMRP-1, a maize transfer cell-specific transcriptional activator, is induced at solute exchange surfaces and responds to transport demands. Planta 229: 235–247

Brandizzi E, Irons SL, Johansen J, Kotzer A, Neumann U (2004) GFP is the way to glow: bioimaging of the plant endomembrane system. J Microsc 138: 154–158

Brugiere N, Humbert S, Rizzo N, Bohn J, Habben JE (2008) A member of the maize isopentenyl transferase gene family, Zea mays isopentenyl transferase 2 (ZmIPT2), encodes a cytokinin biosynthetic enzyme expressed during kernel development. Plant Mol Biol 67: 215–229

Cai G, Faleri C, Del Casino C, Hueros G, Thompson RD, Cresti M (2002) Subcellular localisation of BETL-1, -2 and -4 in Zea mays L. endosperm. Sex Plant Reprod 15: 85–98

Charlton WL, Keen CL, Merriman C, Lynch P, Greenlan AJ, Dickinson HG (1995) Endosperm development in Zea mays: implication of genetic imprinting and paternal excess in regulation of transfer layer development. Development 121: 3089–3097

Cheng WH, Taliercio EW, Chourey PS (1996) The Miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell 8: 971–983

Cheng WH, Taliercio EW, Chourey PS (1999) Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3’ untranslated region in a cell suspension culture of maize. Proc Natl Acad Sci USA 96: 10512–10517

Chourey P, Jain M, Li QB, Carlson S (2006) Genetic control of cell wall invertases in developing endosperm of maize. Planta 223: 159–167

Costa LM, Gutierrez-Marcos JF, Brutnell TP, Greenland AJ, Dickinson HG (2003) The globby1-1 (glo1-1) mutation disrupts nuclear and cell division in the developing maize seed causing alterations in endosperm cell fate and tissue differentiation. Development 130: 5009–5017

Costa LM, Gutierrez-Marcos JF, Dickinson HG (2004) More than a yolk: the short life and complex life of the plant endosperm. Trends Plant Sci 9: 507–514

Craig S, Staehelin LA (1988) High pressure freezing of intact plant tissues: evaluation and characterization of novel features of the endoplasmic reticulum and associated membrane systems. Eur J Cell Biol 46: 81–93

Davis RW, Smith JD, Cobb BG (1990) A light and electron-microscope investigation of the transfer cell region of maize caryopses. Can J Bot 68: 471–479

Felker FC (1992) Participation of cob tissue in the uptake of medium components by maize kernels cultured in vitro. J Plant Physiol 139: 647–652

Felker FC, Shannon JC (1980) Movement of C-14-labeled assimilates into kernels of Zea mays L. 3. An anatomical examination and micro-auto-radiographic study of assimilate transfer. Plant Physiol 65: 864–870

Gomez E, Royo J, Guo Y, Thompson R, Hueros G (2002) Establishment of cereal endosperm expression domains: identification and properties of a maize transfer cell-specific transcription factor, ZmMRP-1. Plant Cell 14: 599–610

Gutierrez-Marcos JF, Costa LM, Biderre-Petit C, Khabba Y, O’Sullivan DM, Wormald M, Perez P, Dickinson HG (2004) maternally expressed gene is a novel maize endosperm transfer cell-specific gene with a maternal parental-origin pattern of expression. Plant Cell 16: 1298–1301

Gutierrez-Marcos JF, Costa LM, Evans MMS (2006) Maternal gameto- phytic baseless1 is required for development of the central cell and early endosperm patterning in maize (Zea mays). Genetics 174: 317–329

Gutierrez-Marcos JF, Dal Pra M, Giulini A, Costa LM, Gavazzi G, Cordelier S, Sellam O, Tatout C, Paul W, Perez P, et al (2007) empty pericarp4 encodes a mitochondrion-targeted pentatricopeptide repeat protein necessary for seed development and plant growth in maize. Plant Cell 19: 196–210

Hueros G, Varotto S, Salamini F, Thompson RD (1995) Molecular characterization of Bet1, a gene expressed in the endosperm transfer cells of maize plants. Plant Cell 7: 747–757

Jain M, Li QB, Chourey PS (2008) Cloning and expression analyses of sucrose non-fermenting-1-related protein kinase 1 (SnRK1b) gene during development of sorghum and maize endosperm and its implicated role in sugar-to-starch metabolic transition. Physiol Plant 134: 161–173

Kang BH, Staehelin LA (2008) ER-to-Golgi transport by COPII vesicles in Arabidopsis involves a ribosome-excluding scaffold that is transferred with the vesicles to the Golgi matrix. Proteoplasma 234: 51–64

Kieselbach T (1949) The structure and reproduction of corn. Univ Nebr Agric Exp Stn Res Bull 61: 1–96

Kladnik A, Chamusko K, Dermastia M, Choury P (2004) Evidence of programmed cell death in post-phloem transport cells of the maternal pedicel tissue in developing caryopsis of maize. Plant Physiol 136: 3572–3581
Lauriere C, Lauriere M, Sturm A, Faye L, Chrispeels MJ (1988) Characterization of beta-fructosidase, an extracellular glycoprotein of carrot cells. Biochimie 70: 1483–1491

Lee YJ, Szumalnski A, Nielsen E, Yang ZB (2008) Rho-GTPase-dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth. J Cell Biol 181: 1153–1168

Maitz M, Santandrea G, Zhang ZY, Lai S, Hannah LC, Salamini F, Thompson RD (2003) cgt1, a mutation reducing grain filling in maize through effects on basal endosperm and pedicel development. Plant J 23: 29–42

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Maitz M, Santandrea G, Zhang ZY, Lal S, Hannah LC, Salamini F, Margulies E, Cheung AY, Ueda T (2008) The regulatory RAB and ARF GTPases for vesicular trafficking. Plant Physiol 147: 1516–1526

Miller ME, Chourey PS (1992) The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4: 297–305

Nielsen E, Cheung AY, Ueda T (2008) The regulatory RAB and ARF GTases for vesicular trafficking. Plant Physiol 147: 1516–1526

Offler CE, McCurdy DW, Patrick JW, Talbot MJ (2003) Transfer cells: cells specialized for a special purpose. Annu Rev Plant Biol 54: 431–454

Oka T, Nemoto T, Jigami Y (2007) Functional analysis of Arabidopsis thelana RHM2/MUM4, a multidomain protein involved in UDP-D-glucose to UDP-L-rhamnose conversion. J Biol Chem 282: 5389–5403

Olsen OA (2004) Nuclear endosperm development in cereals and Arabidopsis thaliana. Plant Cell (Suppl) 16: S214–S227

Pattison RJ, Amtmann A (2009) N-Glycan production in the endoplasmic reticulum of plants. Trends Plant Sci 14: 92–99

Roitsch T, Amtmann MC (2004) Function and regulation of plant invertases: sweet sensations. Trends Plant Sci 9: 606–613

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 57: 675–709

Scanlon MJ, Takacs EM (2009) Kernel biology. In J Bennetzen, S Hake, eds, Handbook of Maize, Its Biology. Springer, New York, pp 121–129

Serna A, Maitz M, O’Connell T, Santandrea G, Thevissen K, Tienens K, Hueros G, Faleri C, Cai G, Lottspeich F, et al (2001) Maize endosperm secretes a novel antifungal protein into adjacent maternal tissue. Plant J 25: 687–698

Staelin LA, Giddings TH Jr, Kiss JZ, Sack FD (1990) Macromolecular differentiation of Golgi stacks in root tips of Arabidopsis and Nicotiana seedlings as visualized in high pressure frozen and freeze-substituted samples. Protoplasma 157: 75–91

Staelin LA, Newcomb EH (2000) Membrane structure and membrane organelles. In B Buchanan, W Gruissem, RJ Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, MD, pp 19–22

Taliercio EW, Kim JY, Mahe A, Shander S, Choi J, Cheng WH, Prioul JL, Chourey PS (1999) Isolation, characterization and expression analyses of two cell wall invertase genes in maize. J Plant Physiol 155: 197–204

Thiel J, Weier D, Sreenivasulu N, Strickert M, Weichert N, Melzer M, Czauderna T, Wobus U, Weber H, Heschke W (2008) Different hormonal regulation of cellular differentiation and function in nucellar projection and endosperm transfer cells: a microdissection-based transcriptome study of young barley grains. Plant Physiol 148: 1436–1452

Thompson RD, Hueros G, Becker HA, Maitz M (2001) Development and functions of seed transfer cells. Plant Sci 160: 775–783

Van Damme D, Inze D, Russinova E (2008) Vesicle trafficking during somatic cytokinesis. Plant Physiol 147: 1544–1552

Vaughn KC, Talbot MJ, Offler CE, McCurdy DW (2007) Wall ingrowths in epidermal transfer cells of Vicia faba cotyledons are modified primary walls marked by localized accumulations of arabinoxylan proteins. Plant Cell Physiol 48: 159–168

Vilhar B, Kladinik A, Blejer A, Chourey PS, Dermastia M (2002) Cytochemical evidence that the loss of seed weight in the miniature1 seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. Plant Physiol 129: 23–30

Winkel BS (2004) Metabolic channeling in plants. Annu Rev Plant Biol 55: 85–107

Young RE, McFarlane HE, Hahn MG, Western TL, Haughn GW, Samuels AL (2008) Analysis of the Golgi apparatus in Arabidopsis seed coat cells during polarized secretion of pectin-rich mucilage. Plant Cell 20: 1623–1638