Combining Expression and Comparative Evolutionary Analysis. The COBRA Gene Family

Siobhan M. Brady, Shuang Song, Kanwarpal S. Dhugga, J. Antoni Rafalski, and Philip N. Benfey

Department of Biology and The Institute for Genome Sciences and Policy, Duke University, Durham, North Carolina 27708 (S.M.B., P.N.B.); Syngenta Biotechnology, Research Triangle Park, North Carolina 27709 (S.S.); Genetic Discovery Group, Crop Genetics Research and Development, Pioneer Hi-Bred International, Johnston, Iowa 50131–1004 (K.S.D.); and Genetic Discovery Group, DuPont Crop Genetics Research, Wilmington, Delaware 19880 (J.A.R.)

Plant cell shape is achieved through a combination of oriented cell division and cell expansion and is defined by the cell wall. One of the genes identified to influence cell expansion in the Arabidopsis (Arabidopsis thaliana) root is the COBRA (COB) gene that belongs to a multigene family. Three members of the AtCOB gene family have been shown to play a role in specific types of cell expansion or cell wall biosynthesis. Functional orthologs of one of these genes have been identified in maize (Zea mays) and rice (Oryza sativa; Schindelman et al., 2001; Li et al., 2003; Brown et al., 2005; Persson et al., 2005; Ching et al., 2006; Jones et al., 2006). We present the maize counterpart of the COB gene family and the COB gene superfamily phylogeny. Most of the genes belong to a family with two main clades as previously identified by analysis of the Arabidopsis family alone. Within these clades, however, clear differences between monocot and eudicot family members exist, and these are analyzed in the context of Type I and Type II cell walls in eudicots and monocots. In addition to changes at the sequence level, gene regulation of this family in a eudicot, Arabidopsis, and a monocot, maize, is also characterized. Gene expression is analyzed in a multivariate approach, using data from a number of sources, including massively parallel signature sequencing libraries, transcriptional reporter fusions, and microarray data. This analysis has revealed that the expression of Arabidopsis and maize COB gene family members is highly developmentally and spatially regulated at the tissue and cell type-specific level, that gene superfamily members show overlapping and unique expression patterns, and that only a subset of gene superfam-ly members act in response to environmental stimuli. Regulation of expression of the Arabidopsis COB gene family members has highly diversified in comparison to that of the maize COB gene superfamily members. We also identify BRITTLE STALK 2-LIKE 3 as a putative ortholog of AtCOB.

The shape of a plant cell is achieved through a combination of oriented cell division and cell expansion and is defined by the cell wall. The shape of a plant cell is important to its function, and manipulation of cell shape occurs in response to both biotic and abiotic signals. Understanding how the cell wall grows is of great importance to plant biologists, as the process of cell wall biogenesis and expansion during cell growth is crucial to plant development. Of additional agro-nomic importance is the thick secondary wall that is deposited once cells have attained their final shape and size. Secondary cell wall, by virtue of being rich in cellulose, is the primary determinant of tissue strength and is used as raw material in the generation of textiles, paper, and cellulosic ethanol (Appenzeller et al., 2004).

Two main types of cell walls have been reported to occur in flowering plants, and it is the balance between the types of cell wall components (cellulose, hemicellulose, and pectin) and their chemical structure that defines Type I and Type II cell walls (Carpita and McCann, 2000). The Type I cell wall is found in cells of dicotyledonous plants and noncommelinoid monocotyledonous plants. The Type II wall is found in the cells of commelinoid monocotyledons, which includes grasses like rice (Oryza sativa) and maize (Zea mays; Carpita and McCann, 2000). Cellulose, an unbranched (1,4)-linked β-D-glucan, is common to both types of cell walls. In Type I cell walls, cellulose and the hemicellulosic polysaccharide, xyllo glucan, exist in roughly equal amounts. In Type II cell walls, however, glucoronarabinoxylans or mixed link glucans are the major hemicellulosic polysaccharides. The Type I cellulose-xyl glucan framework is embedded in a pectic gel. Compared to the pectin-abundant Type I wall, the Type II cell wall is pectin poor. The Type II wall is further differentiated from Type I walls by the presence of an extensive network of phenylpropanoids (Carpita and McCann, 2000). A comparative genomic analysis of a set of genes involved in the synthesis, modification, assembly, and disassembly of the cell walls of Arabidopsis (Arabidopsis thaliana) and rice revealed that in some cases the differences between

1 These authors contributed equally to the paper.
* Corresponding author; e-mail benfeyp@duke.edu; fax 919–613–8177.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Philip N. Benfey (benfeyp@duke.edu).

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Type I and Type II cell walls were reflected at the genomic level (Yokoyama and Nishitani, 2004). A number of plant cell types undergo dramatic cell expansion; the elongation of pollen tubes, xylem vessels, fiber cells in the vasculature, trichomes and root hairs, and even the lobing of epidermal pavement cells are all examples (Smith and Oppenheimer, 2005). In response to biotic and abiotic stimuli, cell wall materials are trafficked toward the cell wall, the wall matrix yields, and new cell wall materials are deposited in the expanding cell wall (Ray, 1967; Cosgrove, 2005). Cell wall expansion can occur isotropically (oriented uniformly in all directions) or anisotropically. Under isotropic turgor pressure, perpendicular arrangement of the cellulose microfibrils to the axis of growth determines the direction of cell expansion (Green, 1962). Expansion can occur via tip-directed growth where cell wall expansion is highly polarized at a single site on the tip of the cell or via diffuse growth where new cell wall materials are distributed across the cell surface.

Molecular and genetic studies have identified additional components associated with biosynthesis of the cell wall and in regulating cell expansion. Components of the plant cytoskeleton, including cortical microtubules and F-actin, have been shown to influence cell shape (Green, 1962; Smith and Oppenheimer, 2005). An additional cell expansion component identified through such studies in Arabidopsis is the COBRA (COB) gene (Benfey et al., 1993; Hauser et al., 1995; Schindelman et al., 2001; Roudier et al., 2002, 2005). COB partial loss-of-function and null alleles exhibit abnormal anisotropic cell expansion during root development and display reduced levels and improper orientation of crystalline cellulose microfibrils (Benfey et al., 1993; Hauser et al., 1995; Schindelman et al., 2001; Roudier et al., 2002, 2005). However, root hair expansion and pollen development are normal in these mutants, indicating that COB is required for the regulation of anisotropic expansion only and not tip-directed growth (Roudier et al., 2005).

The COB gene encodes a plant-specific glycosylphosphatidylinositol (GPI)-anchored protein with an ω-attachment site at the C terminus, a hydrophilic middle, a CCCV domain, a potential N-glycosylation site, an N-terminal secretion signal sequence, and a predicted cellulose binding site (Roudier et al., 2002). GPI anchors are added through an amide bond onto the last amino acid residue remaining after cleavage of the ω-site (Udenfriend and Kodukula, 1995). The COB protein follows a typical GPI secretion path as it is found in vesicles, associated with the Golgi, and finally, at the outer face of the cell wall (Roudier et al., 2005). The distribution of the COB protein is dependent on microtubule organization. AtCOB belongs to a multigene family consisting of 12 members and two subgroups in Arabidopsis, the second of which is distinguished by an additional N-terminal 170 amino acids (Roudier et al., 2002). All members of the AtCOB gene family have been confirmed to be GPI anchored except AtCOBRA-LIKE1 (AtCOBL1), AtCOBL4, and AtCOBL5 (Borner et al., 2003; Elortza et al., 2003; Lalanne et al., 2004; Roudier et al., 2005). Two additional AtCOB gene family members have been ascribed a function through a combination of expression profiling and genetic studies. AtCOBL4 is required for cellulose biosynthesis in the secondary wall and was identified by the similarity of its expression to Arabidopsis cellulose synthase (AtCesA) genes implicated in secondary cell wall synthesis (Brown et al., 2005; Persson et al., 2005). AtCOBL9 is required for tip-directed growth in root hair development and was identified by its enrichment in the root hair morphogenesis transcriptome (Parker et al., 2000; Jones et al., 2006). The functions of these genes demonstrate that multiple members of the AtCOB gene family are involved in diverse types of cell expansion and cell wall biosynthesis. The COB gene family is also found in monocots. The rice mutant brittle culm1 (bc1) and the maize brittle stalk 2 (bk2) mutant were found to have mutations in putative orthologs of AtCOBL4 and affect the mechanical strength of plant tissue (Li et al., 2003; Ching et al., 2006). Nine additional rice BRITTLE CULM-LIKE (OsBC1L) genes were found in the rice genome (Li et al., 2003). The primary function of AtCOB and AtCOB family proteins remains to be fully determined. One theory, based on analysis of AtCOB protein localization, microtubule dependence, and rate of cellulose production in a conditional mutant, suggests that COB is primarily responsible for controlling microfibril orientation in a microtubule-dependent manner. Any effect on cellulose biosynthesis is believed to be secondary (Roudier et al., 2005). Another line of reasoning refutes the role of these proteins in cellulose biosynthesis as being secondary. In this theory, AtCOB and related proteins perform equivalent functions in regulating cellulose microfibril orientation and synthesis (Wasteneys and Fujita, 2006). Previous studies analyzed cellulose production in multiple cell types; however, the AtCOB protein has been shown to be specifically localized to the outer surface of the epidermal cell wall. Further studies on the rate of cellulose production should focus on this particular face of the epidermal cell wall before the role of AtCOB in cellulose biosynthesis is dismissed as being secondary. Also, cellulose-deficient phenotypes of AtCOBL4 and its orthologs in maize and rice suggest that this family of proteins plays a conserved general role in cell wall biosynthesis in multiple types of cell walls (Li et al., 2003; Brown et al., 2005; Persson et al., 2005; Ching et al., 2006).

In this genomic analysis, we present a phylogenetic analysis of the COB gene family phylogeny in Arabidopsis (AtCOB), maize (ZmBk2), and rice (OsBC1). This phylogeny identifies two major subgroups in the family as in Roudier et al. (2005). Within these subgroups, there is a clear difference between monocot and eudicot members. Because expression profiling studies have been successful in assigning function to two AtCOB gene family members (Parker et al., 2000; Brown et al., 2005; Persson et al., 2005; Jones et al.,
2006), we examined the tissue-specific, developmental, and environmental regulation of expression of the COB gene family in a eudicot (Arabidopsis) and commelinoid monocot (maize) using a combination of digital expression profiling, massively parallel signature sequencing (MPSS) analysis, and reporter gene analysis. This expression analysis shows that the expression of COB gene family members in Arabidopsis and maize is highly regulated in many developmental stages, tissues, and cell types in expanding cells and in response to a variety of environmental stimuli. In addition, we describe the similarities and differences between regulation of the Arabidopsis and maize members of this family.

RESULTS
The Maize Bk2 Gene Family

The maize bk2 locus was recently found to contain a mutation in a AtCOBL4 ortholog (Ching et al., 2006). Maize sequences corresponding to the members of a ZmBk2-LIKE (Bk2L) gene family were identified by searching public and DuPont databases (Supplemental Fig. S1). While the rice OsBC1L family contains nine members, the maize ZmBk2L family contains only eight members. These eight members were identified and named based on similarity to the rice OsBC1L gene family (Li et al., 2003). No gene similar to OsBC1L2 was found in the maize genome. All ZmBk2L family members were examined for features characteristic of COB and GPI-anchored proteins. The features include a CCVS (Cys-rich) motif, an N-terminal signal peptide sequence for secretion, a hydrophilic middle portion, a highly hydrophobic C terminus, and specific features around the o-site for GPI processing (Fig. 1C; Supplemental Fig. S2). By these criteria, ZmBk2L1 and ZmBk2L3 to 9 are all COB-LIKE proteins. In addition, ZmBk2L1, ZmBk2L5, and ZmBk2L8 contain an additional 170-amino acid N-terminal sequence as found in the Arabidopsis COBL7 subgroup (Supplemental Fig. S1; Roudier et al., 2002).

Phylogenetic Analysis Identifies Monocot- and Eudicot-Specific COBL Proteins

To determine the evolutionary relationship of maize Bk2, rice BC1, and Arabidopsis COB family members, an unrooted tree was built using heuristic search and random sequence addition over 1,000 replications with bootstrap analysis from the alignment of whole amino acid sequences (Fig. 2). In the phylogenetic tree, AtCOBL1 to 5 and AtCOBL7 to 11 form two major clades with high bootstrap support, similar to a previous analysis examining only Arabidopsis COBL family members (Roudier et al., 2002), with the exception of AtCOBL6 falling out of these two groups. Three genes, ZmBk2L9, OsBC1L p1 (a pseudogene), and AtCOBL6, stand excluded from these two clades. Within this family, AtCOBL1 to 5 and AtCOBL7 to 11 form two subgroups. In the clade of AtCOBL1 to 5 and associated rice and maize genes, OsBC1L1 is most closely related to ZmBk2, which is consistent with the phenotypic similarity of the corresponding mutants (Li et al., 2003; Ching et al., 2006). The rice BC1 protein has been previously shown to be most similar to AtCOBL4; however, our phylogenetic analysis suggests that COBL4 function in monocots may be shared by two co-orthologs: ZmBk2/OsBC1L and ZmBk2L7/OsBC1L7. Co-orthologs are defined as genes that duplicated after a speciation event that are then orthologous to a single gene in the other species (O’Brien et al., 2005). Expression analysis, however, suggests that ZmBk2L7 does not carry out the same function as ZmBk2 (see below).

The number of members in most of the cell wall-related gene families identified in Yokoyama and Nishitani (2004) in rice and Arabidopsis was similar, and the same holds for the COB gene family with 12 members in Arabidopsis, 11 in rice, and nine in maize. It cannot be excluded, however, that the differences in maize COB family sizes may be due to incomplete sequence information. Contrary to the findings of the Yokoyama study (2004) where putative orthologs with highly similar amino acid sequences were found in both plant genomes, no clear predictions for direct orthology were found between Arabidopsis and monocot genes. Instead, a rice BC1L gene was primarily paired with a presumably orthologous maize counterpart. This is particularly striking in the COBL7 subgroup where ZmBk2L8/OsBC1L8 and ZmBk2L1/OsBC1L1 form a monocot-specific clade.

Arabidopsis and Maize COB Gene Family Members Are Expressed in Multiple Organs

Members of the Arabidopsis COB family have been demonstrated to be expressed in specific organs using reverse transcription-PCR (Roudier et al., 2002). Publicly available expression data from the AtGenExpress organ series further support the organ-specific expression patterns of AtCOB family members (Fig. 3A; Zimmermann et al., 2004; Schmid et al., 2005). Indeed, the majority of COB family members are expressed at varying levels in most organs examined. The exceptions to this are AtCOBL10 and AtCOBL11, which are expressed only in inflorescence or floral tissue. Hierarchical clustering of these expression profiles support AtCOBL10 and AtCOBL11 expression being the most similar to each other and suggests the possibility of subfunctionalization or neofunctionalization of this gene pair. AtCOB also exists as an outgroup from all other AtCOB family members, presumably due to its high expression in nearly all tissues. Transcripts of all maize ZmBk2L gene family members were found in MPSS libraries (data not shown). Organ-specific expression profiling of the ZmBk2L gene family was performed using libraries representing specific developmental stages (Fig. 3B; Brenner et al., 2000). While AtCOBL family members were expressed in most organs examined, only four ZmBk2L family members were
expressed in the majority of organs examined. *ZmBk2L1* and *ZmBk2L3* are expressed in all organs at varying levels, while *ZmBk2L4* expression is excluded from the root, ear, and embryo, and *ZmBk2* expression is excluded from floral and embryonic tissue. *ZmBk2L7* and *ZmBk2L9* are expressed in a subset of these tissues.

The Expression of Arabidopsis and Maize COB Gene Family Members Is Regulated in a Tissue-, Cell-Type, and Developmental Stage-Specific Manner

*AtCOB1L9*, *AtCOB1L4*, and *AtCOB1* function in a cell type-specific manner in cell expansion and/or cell wall biosynthesis. Cell expansion can occur both spatially and temporally in the development of an organism. For example, during leaf development, cell expansion and cell cycling occur in specific tissues, at specific stages in developmental time, and in response to environmental stimuli (Kang and Dengler, 2002; Tsukaya, 2006). Cell expansion in roots also occurs in multiple dimensions in a zone-specific manner across multiple tissues, in specific cell types outside of this zone of elongation, such as xylem cells, columella cells, or in root hair cells, and in response to environmental stimuli like gravity. Expression profiling of specific organs could not therefore reflect the cell-type specific expression of COB gene family members. The tissue, cell type, and developmental stage specificity of *AtCOB1* and *ZmBk2L* genes was therefore examined.

Arabidopsis COB family member expression was analyzed using a β-glucuronidase (GUS)-green fluorescent protein (GFP) fusion reporter under control of expression and cell cycling occur in specific tissues, at specific stages in developmental time, and in response to environmental stimuli. Cell expansion in roots also occurs in multiple dimensions in a zone-specific manner across multiple tissues, in specific cell types outside of this zone of elongation, such as xylem cells, columella cells, or in root hair cells, and in response to environmental stimuli like gravity. Expression profiling of specific organs could not therefore reflect the cell-type specific expression of COB gene family members. The tissue, cell type, and developmental stage specificity of *AtCOB1* and *ZmBk2L* genes was therefore examined. Arabidopsis COB family member expression was analyzed using a β-glucuronidase (GUS)-green fluorescent protein (GFP) fusion reporter under control of
each COBL upstream regulatory region. In cases where GFP expression was not high enough to visualize using confocal imaging, the increased sensitivity of GUS was exploited. A series of MPSS libraries covering a diverse number of tissues and developmental stages was examined for spatial and temporal expression patterns of members of the ZmBk2L gene family.

Expression in the Root

Expression analysis in the root revealed distinct expression patterns for each AtCOBL reporter. (Fig. 4). AtCOBL10 and AtCOBL11 were not expressed in the root using these reporter fusions, consistent with the organ-level microarray analysis. The expression patterns conferred in combination by AtCOBL1, AtCOBL2, AtCOBL5, AtCOBL6, AtCOBL8, and AtCOBL9 cover all cells in the root tip (Fig. 4A). In most tissues, a number of AtCOBL genes show overlapping expression. This is true for the columella (AtCOBL1, AtCOBL2, and AtCOBL5), the quiescent center (AtCOBL2 and AtCOBL8), and the lateral root cap (AtCOBL1, AtCOBL5, AtCOBL6, and AtCOBL9). AtCOBL1 is weakly and uniquely expressed in the stele, and AtCOBL8 is uniquely expressed in the newly differentiating cortex and endodermal cell lineages. Although many AtCOBL genes are expressed in the lateral root cap, only AtCOBL6 is expressed very precisely and bilaterally in four cells of the lateral root cap. AtCOBL5 is expressed in a zone-specific manner in the zone of

![Figure 2. The COB gene family phylogeny. Unrooted cladogram of the encoded proteins of the COB gene superfamily in Arabidopsis, rice, and maize determined by the heuristic algorithm. Bootstrap values are indicated for only those branches where they exceeded 50%. AtCOB represents the Arabidopsis COB family members. OsBC represents the rice COB family members. ZmBk2 represents the maize COB family members. The monocot-specific clade is marked with a bracket. AtCOBL4 and its probable functional orthologs ZmBk2 and OsBC1 are indicated in shading.](http://www.plantphysiol.org)
elongation. Expression was also analyzed in more mature regions of the root (Fig. 4B) and in lateral root primordia (Fig. 4C). AtCOBL4, AtCOBL6, AtCOBL7, and AtCOBL9 are all expressed in the mature root. AtCOBL4 expression is highly localized to the xylem, AtCOBL6 to the epidermis and weakly in the stele of mature cells, and AtCOBL9 to trichoblasts. A number of AtCOBL genes were found to be expressed in lateral root primordia (AtCOBL1, AtCOBL7, and AtCOBL8) or expressed in the cells through which the primordia emerge (AtCOBL2). AtCOBL7 and AtCOBL8 are also expressed in the pericycle cells and vasculature associated with the developing primordia.

Maize MPSS libraries do not cover cell types in the root at as high a resolution as the Arabidopsis reporter lines. However, expression in root libraries covering developmental time and seminal, nodal, and lateral root tips do reveal changes in ZmBk2L expression (Fig. 5A). In various root tissues, ZmBk2, ZmBk2L1, and ZmBk2L3 were expressed, with the ZmBk2L3 transcript being the most abundant. Levels of ZmBk2 transcript increase over time in the primary root. Lateral root tip and nodal root tip meristems also displayed similar transcript abundance of ZmBk2L family members compared to the primary root with high levels of ZmBk2L3 and low levels of ZmBk2L1 and ZmBk2. Expression of ZmBk2L members in seminal lateral roots differs from the primary root by the absence of ZmBk2L1 expression.

Expression in Aboveground Tissue (Leaves, Stalk, and Vasculature)

Loss-of-function alleles of AtCOBL4 are cellulose deficient and display a collapsed secondary xylem phenotype (Brown et al., 2005; Persson et al., 2005). Consistent with these findings, AtCOBL4 was expressed in a localized manner in all degrees of vasculature...
in cotyledons, rosette leaves, and cauline leaves (Fig. 6, A–C). However, AtCOBL family members in addition to AtCOBL4 are expressed in leaf vascular tissue (AtCOBL1, AtCOBL4, AtCOBL5, AtCOBL7, and AtCOBL8). A number of AtCOBL family members are expressed in patterns dependent on leaf type and/or in a longitudinal gradient of the leaf. AtCOBL1, AtCOBL7, and AtCOBL8 are expressed in all leaf types (cotyledon, rosette, and cauline), while AtCOBL1 and AtCOBL5 are expressed only in cauline leaves. A distal-to-proximal gradient of expression was also seen with AtCOBL7 (cauline leaves) and AtCOBL8 (rosette and cauline leaves). Finally, AtCOBL9 was not expressed in leaf vasculature, but instead was expressed at the base of trichomes overlying the primary vasculature, reminiscent of its expression in root hairs.

In maize leaf tissue, expression of ZmBk2L members varies along the longitudinal axis; however, a proximal-to-distal gradient is not observed as in Arabidopsis (Fig. 5B). Instead, extremely low levels of ZmBk2L transcripts were followed by a peak of ZmBk2 and ZmBk2L3 in the transition zone (30–35 mm from the base of the leaf). In the available MPSS libraries, leaf vascular tissue is not represented. The stalk and elongating internode tissues were therefore examined for the expression of genes associated with vascular bundles, as the stalk provides the majority of mechanical support for the maize plant (Fig. 5C). ZmBk2 shows its highest transcript abundance in the transition zone of the elongating internodes, as would be expected of a gene involved in secondary wall formation. ZmBk2L3 is most highly expressed in the elongating zone of the internode, and its expression declines as the internode tissues enter the developmental stages where secondary wall is deposited, i.e. transition and mature zones. We next examined MPSS libraries generated from elongating internodes that were further dissected into the component cell types: the ground tissue, pulvinus (the tissue that connects the leaf sheath to the node), rind, and vascular bundles. The ground tissue, pulvinus, and rind show similar transcript abundance patterns characterized by high levels of ZmBk2L3. In vascular bundles, however, ZmBk2 expression predominates, with lower level expression of ZmBk2L1, ZmBk2L3, and ZmBk2L4. This strong ZmBk2 expression in vascular tissue further supports the orthology of ZmBk2 with AtCOBL4 and OsBc1.

Reproductive Stage Expression

All AtCOBL family members except AtCOBL1 are expressed in a variety of floral organs in male, female,
and supporting tissue (Fig. 7A). *AtCOBL4* is expressed in all floral organs. All other *AtCOBL* family members are expressed in a subset of tissues. *AtCOBL2* is expressed in the vasculature of the sepals, on the papillary surface of the stigma, and in ovules. *AtCOBL5* is expressed on the vasculature of the pedicel and stigma and below the papillary surface of the receptacle. *AtCOBL6* is expressed in the anthers and in ovules. *AtCOBL7* is expressed in the filaments of the stamen and in the vasculature of the sepals. *AtCOBL8* is expressed in the pedicel and in the transmitting tube of the stigma. *AtCOBL9* is expressed in the receptacle of the flower. *AtCOBL10* is expressed in the pedicel, anther filaments, pollen grains, and germinating pollen tubes. *AtCOBL11* is expressed in pollen grains.

Maize MPSS libraries representing male and female reproductive tissue types were examined (Fig. 5, D–F). *ZmBk2L1* and *ZmBk2L3* are expressed in tassel, ear, anther, and silk tissues, although at lower levels compared to root, leaf, and stalk tissues. *ZmBk2L7* is expressed in the tassel only when the tassel is undergoing meiosis. The *ZmBk2L4* transcript is only found postpollination in silk and ear libraries, and the *ZmBk2L5* transcript, which is expressed only in the pollen grain and pollen tubes (data not shown), is weakly expressed in the pollinated silk (Fig. 5F).

Expression during Embryogenesis

Expression of *AtCOBL* family members was also examined in siliques at the shattering stage (approximately 15–25 d after pollination [DAP]; Fig. 7B). *AtCOBL2* is expressed in the funiculus and along the outer surface of the silique pod upon dehiscence. *AtCOBL8* is expressed in the pedicel and in the replum. *AtCOBL10* were found to be expressed in mature, bending cotyledon-stage embryos (Fig. 7C). *AtCOBL2* is expressed strongly only at the tip of the embryonic root. *AtCOBL8* and *AtCOBL10* are expressed throughout the embryo, with *AtCOBL10* being expressed more
strongly than AtCOBL8. AtCOBL8 is expressed more strongly at the tips of the embryonic root and at cotyledon tips.

MPSS libraries representing the products of fertilization, the kernel and its associated tissues, embryo, endosperm, and pericarp, were also examined for ZmBk2L family abundance. There is very low transcript abundance in the embryo from 15 DAP to approximately 21 DAP (Fig. 5G). After this point, ZmBk2L3 and ZmBk2L4 transcript abundance increases. The endosperm also has variable levels of expression of some of the ZmBk2L genes throughout its development (Fig. 5H). Two peaks of transcript abundance can be seen; the first is of ZmBk2L3 from 6 DAP to 21 DAP, and this peak is followed by an increase in ZmBk2L4 transcript abundance. That ZmBk2L3 peaks in the developing endosperm at approximately 8 to 10 DAP again supports its role in cell expansion. ZmBk2L6 and ZmBk2L8 were also found to be expressed in cultured or dissected endosperm (data not shown). The pericarp has a small peak of ZmBk2L4 transcript abundance at 15 DAP and a massive increase of ZmBk2L3 transcript abundance at 27 DAP (Fig. 5I).

Figure 6. Unique GUS expression patterns conferred by AtCOBL family member upstream regulatory regions in the cotyledon (A), vegetative rosette leaf (B), and cauline leaf (C). Images in A are at 16 × magnification. B and C, Bar = 250 μm. A, COBL4 is expressed in localized areas of cotyledon vasculature, while COBL7 and COBL8 are expressed throughout cotyledon vasculature. COBL7 is also expressed in stomata overlying cotyledon vasculature (inset). B, COBL4 is expressed in localized areas of rosette leaf vasculature and in the petiole, while COBL7 and COBL8 are expressed throughout the leaf vasculature, although COBL8 is expressed in an acropetal manner. COBL9 is expressed at the base of trichomes overlying leaf primary vasculature. C, COBL1 is expressed in hydathodes and in a localized manner throughout the leaf vasculature. COBL4 is expressed in localized areas of the leaf vasculature. COBL5 is expressed strongly at the apex of the leaf in the vasculature. COBL7 is expressed in a basipetal manner throughout the leaf. COBL8 is expressed strongly at the base of the leaf in the vasculature.

Regulation of Arabidopsis and Maize COB Gene Family Members in Response to Stress and Hormones

Plants are able to respond dynamically to environmental stimuli by cell expansion or cell division. The association of the COB gene family with cell expansion suggests that some family members may be involved in the response to such stimuli. Publicly available AtGenExpress stress and hormone series were therefore assayed for AtCOBL gene family expression (Fig. 8, A and B; Zimmermann et al., 2004; Schmid et al., 2005). Three main patterns were observed: (1) a subset of genes showed no response to different stresses or hormone treatments; (2) stress and hormone treatments elicit the response of specific AtCOBL family members; and (3) no repression of COBL family member expression in response to a treatment was observed. For example, AtCOBL7, AtCOBL9, and AtCOBL5 do not show responses to the stresses analyzed, and the response to heat versus cold elicits a different combination of AtCOBL genes. In the case of responses to hormones, even fewer AtCOBL family members showed a response. AtCOBL11 and, to a lesser extent, AtCOBL10, both of whom showed very little expression in any organs, demonstrated a striking response to a number of hormones.

In maize MPSS libraries of leaf or root tissue exposed to different stress or hormone treatments, generally similar patterns of expression were seen as with the response of AtCOBL family members to stress/hormone treatments. Only a subset of genes show response to different treatments: ZmBk2, ZmBk2L1, ZmBk2L3, ZmBk2L4, and ZmBk2L8. Stress and hormone treatments elicit the response of specific family members. As an example, the wounding treatment elicits a change in expression of ZmBk2L8, and an abscisic acid (ABA) treatment elicits a change in expression of ZmBk2, ZmBk2L1, ZmBk2L3, ZmBk2L4, and ZmBk2L8. In opposition to the regulation of Arabidopsis family members, however, repression of ZmBk2L family members in response to stress was observed, particularly in the case of ZmBk2L4.
ZmBk2L3 expression is highly correlated with cellulose synthase genes involved in primary cell wall formation

Analysis of these MPSS libraries has revealed ZmBk2L3 to be expressed in high abundance in the majority of the vegetative tissues. In Arabidopsis, COBL4 is also expressed abundantly in vegetative organs actively undergoing secondary cell wall deposition. AtCOBL4’s expression is coregulated with AtCesA4, 7, and 8, three cellulose biosynthesis genes that are involved in secondary cell wall cellulose biosynthesis (Persson et al., 2005). Ching et al. (2006) have shown that the expression of ZmBk2 is coregulated with ZmCesA10, ZmCesA11, and ZmCesA12, which are known to be involved in secondary cell wall formation (Appenzeller et al., 2004). In agreement with previous reports, the expression of ZmBk2 is strongly correlated with ZmCesA10 to 12. Upon correlation analysis of the tag abundance among all the ZmBk2L genes and the CesA genes across 130 tissue libraries, the expression of only one of the ZmBk2L genes, ZmBk2L3, is found to be highly correlated with four maize CesA genes (ZmCesA2, ZmCesA1, ZmCesA7, and ZmCesA8), which had previously been reported to be involved in primary wall formation (Dhugga, 2001; Appenzeller et al., 2004; Table I). The observations that ZmBk2L3 is expressed in more libraries than any of the other genes in this family and that its expression pattern is similar to that of the primary wall-forming ZmCesA genes suggests that ZmBk2L3 is most likely a functional ortholog of AtCOB.

DISCUSSION

The ZmBk2L gene family contains nine members

All nine members of the ZmBk2L gene family in maize contain an omega (ω-site, required for attachment of a GPI moiety, and other characteristics associated with members of the COB gene family: a CCVS motif, an N-terminal signal peptide sequence for secretion, highly hydrophobic N and C termini, and a hydrophilic middle portion. ZmBk2L1, 5, and 8 also contain an extra N-terminal amino acid stretch similar to that found in the derived proteins of the AtCOBL7 subgroup of the AtCOB gene family. Phylogenetic analysis of the Arabidopsis COB family, the rice BC1 family, and the maize Bk2L gene family groups the encoded proteins into two main clades as previously identified, although AtCOBL6 and ZmBk2L9 do fall out of these two groups, indicating significant sequence divergence (Roudier et al., 2002). This COB gene family phylogeny also indicates that the maize and rice gene families are more similar to each other than to the Arabidopsis family, suggestive of common COBL ancestors, one with the additional N-terminal region and one without, followed by monocot and eudicot-specific
The monocot-specific clade of $\text{ZmBk2L8/OsBC1L8}$ and $\text{ZmBk2L1/OsBcL1}$ stands out in this regard in that $\text{ZmBk2L1}$ is expressed in the majority of organs and tissue types examined, while $\text{ZmBk2L8}$ is expressed in only a subset of tissues. Several possibilities exist to explain this evolutionary bias. One such possibility is that these genes may function in Type II cell wall expansion. The primary function of $\text{AtCOB}$ is hypothesized to be in orienting cellulose microfibrils, presumably through some sort of interaction with the $\text{AtCesA}$ complex and microtubules. One hypothesized way of achieving this is through the putative cellulose binding domains present on the $\text{AtCOB}$ protein. However, the homology to a cellulose binding domain is indeed weak, and this domain has not been shown to be functional (Roudier et al., 2002). There is a 6-amino acid insertion in one of these potential cellulose binding domains of $\text{Bk2L1/Bk2L8}$. This suggests the possibility of these proteins binding to an alternative cell wall matrix carbohydrate, potentially a Type II cell wall-specific carbohydrate.

**The Expression of Arabidopsis and Maize COB Gene Family Members Is Highly Developmentally and Spatially Regulated**

The vast increase in digital expression data, using microarray and MPSS technology (Schmid et al., 2005), allows for facile extraction of expression information for large gene families. Analysis of $\text{COB}$ gene family spatiotemporal expression has revealed multiple levels of regulation during plant development at the organ, tissue, cell type, and temporal levels. Organ-specific expression profiling revealed a complex set of expression patterns in both Arabidopsis and maize. A further level of complexity was revealed by examining...
expression over time. Expression during lateral root development, root hair development, in the silk, and in the endosperm postpollination are all examples of changing both expression levels and which COB family members are expressed over developmental time. All of these expression pattern changes over time can be correlated with multiple types of cell expansion, such as pollen tube elongation and lateral root primordium expansion through multiple layers of tissue in the primary root.

Many COB Gene Family Members Show Unique Cell- and Tissue-Specific Expression

The complex patterns of COB family expression in organs were further resolved by analyzing cell- and tissue-specific expression patterns. The unique and overlapping cell type-specific expression of each COB family member provides useful information and a platform for understanding the function of each COB family member and putative orthology between family members. Indeed, when looking at the full complement of tissues in which members of the COB gene family are expressed in Arabidopsis, it appears that within almost every tissue and cell type, at least one AtCOBL gene is expressed. To be defined as orthologs, gene pairs must meet several criteria. Such criteria include sequence identity, similarity in its genome context (when available), and conservation of function at the level of expression and activity.

One particularly interesting example of overlapping COB family expression is in the vascular tissue of both Arabidopsis and maize. A loss-of-function mutant in AtCOBL4 displays a very subtle collapsed xylem phenotype (Brown et al., 2005), and expression of AtCOBL4 indicates extremely localized patterns of expression within the vasculature, possibly coincident with isolated instances of secondary cell wall deposition. Mutations in its putative orthologs in maize and rice also resulted in reductions of mechanical strength (Li et al., 2003; Ching et al., 2006). The strong ZmBk2 expression in vascular tissue further supports Bk2’s orthology with AtCOBL4 and OsBc1. Overlapping expression of many family members during vascular development was seen in both Arabidopsis (AtCOBL4, AtCOBL5, AtCOBL7, and AtCOBL8) and maize (ZmBk2, ZmBk2L1, ZmBk2L3, and ZmBk2L4). This expression suggests that multiple COB family members are involved in secondary cell wall deposition. The involvement of AtCOBL4 in secondary cell wall deposition in one study was identified through its close correlation with AtCesA genes associated with secondary cell wall formation (Persson et al., 2005). Although other AtCOBL genes were not identified as being highly correlated with these AtCesA genes, it is still possible that they are loosely correlated with them and that additional AtCOBL members influence secondary cell wall formation. Many additional instances of overlapping expression were found in both Arabidopsis and maize in many tissues undergoing cell expansion.

| Bk2 | Bk2L1 | Bk2L3 | Bk2L4 | Bk2L5 | Bk2L6 | Bk2L7 | Bk2L8 | Bk2L9 | CesA1 | CesA2 | CesA3 | CesA4 | CesA5 | CesA7 | CesA8 | CesA9 | CesA10 | CesA11 | CesA12 |
|-----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|     | 1.00  | -0.04 | 0.52  | -0.05 | -0.04 | -0.08 | -0.04 | -0.04 | 0.36  | 0.44  | 0.01  | 0.51  | -0.17 | 0.28  | 0.63  | 0.30  | 0.02  | 0.89  | 0.75  | 0.80  |
| Bk2L1 |       | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L3 | 0.52  |       | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L4 | -0.05 | 0.52  |       | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L5 | -0.04 | 0.05  | -0.09 | 0.07  | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L6 | -0.08 | -0.06 | -0.08 | 0.02  | 0.07  | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L7 | -0.04 | -0.05 | -0.08 | 0.04  | -0.01 | -0.02 | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L8 | -0.04 | -0.10 | -0.06 | 0.06  | -0.07 | 0.05  | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |
| Bk2L9 | -0.03 | 0.13  | -0.05 | 0.02  | -0.04 | -0.02 | 0.08  | 1.00  |       |       |       |       |       |       |       |       |       |       |       |
| CesA1| 0.36  | 0.06  | 0.73  | -0.16 | -0.08 | -0.10 | -0.01 | -0.04 | 1.00  |       |       |       |       |       |       |       |       |       |       |
| CesA2| 0.44  | 0.05  | 0.81  | -0.11 | -0.08 | -0.13 | -0.13 | 0.01  | 0.62  | 1.00  |       |       |       |       |       |       |       |       |       |
| CesA3| 0.01  | 0.11  | 0.20  | -0.06 | 0.12  | 0.96  | 0.10  | 0.22  | 0.06  | 0.23  | 1.00  |       |       |       |       |       |       |       |       |
| CesA4| 0.51  | 0.02  | 0.59  | -0.02 | -0.07 | 0.07  | -0.09 | 0.05  | 0.47  | 0.48  | -0.14 | 1.00  |       |       |       |       |       |       |       |
| CesA5| -0.17 | 0.21  | 0.20  | -0.05 | 0.08  | -0.05 | 0.06  | 0.09  | 0.05  | 0.20  | -0.20 | 0.00  | 1.00  |       |       |       |       |       |       |
| CesA6| 0.28  | 0.14  | 0.18  | 0.04  | -0.04 | 0.03  | 0.07  | 0.47  | 0.18  | -0.04 | 0.23  | 0.09  | 1.00  |       |       |       |       |       |       |
| CesA7| 0.63  | -0.06 | 0.77  | -0.09 | -0.07 | 0.14  | -0.07 | 0.08  | 0.86  | 0.71  | -0.71 | -0.11 | 0.58  | 0.19  | 0.22  | 1.00  |       |       |       |
| CesA8| 0.30  | 0.09  | 0.73  | -0.19 | -0.06 | 0.13  | 0.07  | 0.04  | 0.67  | 0.73  | -0.13 | 0.42  | -0.10 | 0.25  | 0.56  | 1.00  |       |       |       |
| CesA9| 0.02  | 0.13  | -0.09 | -0.03 | -0.07 | 0.03  | 0.23  | 0.03  | 0.28  | 0.12  | -0.04 | 0.33  | 0.18  | 0.12  | 0.24  | 0.26  | 1.00  |       |       |
| CesA10| 0.89 | 0.00  | 0.58  | -0.04 | 0.08  | 0.06  | 0.04  | 0.51  | 0.46  | 0.61  | -0.10 | 0.42  | 0.63  | 0.38  | 0.05  | 1.00  |       |       |       |
| CesA11| 0.75 | 0.05  | 0.48  | -0.04 | 0.08  | -0.03 | 0.01  | 0.52  | 0.37  | -0.02 | 0.48  | -0.02 | 0.62  | 0.47  | 0.37  | 0.07  | 0.92  | 1.00  |       |
| CesA12| 0.80 | 0.01  | 0.50  | -0.09 | 0.04  | -0.09 | 0.02  | 0.56  | 0.42  | -0.01 | 0.52  | -0.05 | 0.57  | 0.56  | 0.38  | 0.05  | 0.94  | 0.96  | 1.00  |

Highly correlated CesA-Bk2L pairs are indicated in green. Highly correlated CesA pairs are indicated in peach.
suggesting that overlapping expression of AtCOB gene family members is an important mechanism in regulating cell expansion. Unique instances of AtCOB gene family expression were also found. An example of this expression is AtCOBL9 during trichoblast development. Genetic analysis has implicated AtCOBL9 in tip-directed expansion of root hair cells, and the expression of AtCOBL9 supports this role (Jones et al., 2006). AtCOBL9 expression in the base of trichoblasts underlying the vasculature and the lack of AtCOBL9 expression in pollen provides clues as to the function of AtCOBL9. Root hairs and pollen tubes are two classic examples of cells that undergo tip-directed expansion, and one conclusion from this expression analysis is that AtCOBL9 cannot be generally required for all types of tip-directed expansion. However, pollen tubes differ from root hairs by decreased cellulose at the tip of the cell and a corresponding decrease in microtubule arrays. This suggests that cellulose deposition is not a part of the extension process per se (Heslop-Harrison, 1987; Peterson and Farquhar, 1996; Hepler et al., 2001). The expression of AtCOBL9 in the base of trichomes is also interesting in this context. The outgrowth of unbranched trichomes occurs by a highly polarized form of diffuse growth (Quader et al., 1987; Tiwari and Wilkins, 1995). Perhaps AtCOBL9 function is therefore in linking cellulose microfibril and microtubule orientation at sites of polarized cellulose deposition.

A Subset of COB Gene Family Members Are Responsive to Environmental Stimuli

Although not all possible situations were examined, the response of COB gene family members to environmental stimuli is quite different than its expression during development. While most genes are highly spatially and temporally regulated during development, only a subset of genes is regulated in stress situations or in response to hormone treatments. In Arabidopsis, AtCOBL10 and AtCOBL11 are the only members that respond to hormone treatments. Also, in opposition to the developmental expression patterns, AtCOBL11 is more strongly responsive to hormone application. In response to heat, AtCOBL2 shows a response of high magnitude. In response to other stresses, however, a different subset of AtCOBL genes are weakly up-regulated in response to different stress situations. In the stress and hormone libraries examined for ZmBk2L2 members, a similar result was obtained. Stress and hormone responses elicit the response of specific family members, although the magnitude of the response is much decreased in comparison to Arabidopsis and in comparison to regulation of expression during development. In some cases, ZmBk2L members responded with a decrease in expression. The cellular response of the plant in response to hormone treatment has often been analyzed in reference to changes in cell expansion or division (indole-3-acetic acid, ethylene). However, the same cannot be said for many stress treatments. The lower magnitude of response of COB family genes to stress treatments suggests that cell expansion may not be the primary response to these different treatments.

ZmBk2L3 Appears to Be a Functional Ortholog of AtCOB

Any orthology of AtCOB with ZmBk2L3 was not obvious from its position in the COBL gene family phylogeny. AtCOB appeared in a solitary clade, while the amino acid sequence of ZmBk2L3 is most similar to ZmBk2L4 with high bootstrap support. Correlation analysis of the gene expression patterns of the ZmBk2L and ZmCesA gene families indicates that ZmBk2L3 may be a functional ortholog of AtCOB, as demonstrated by its close correlation in expression with four of the primary wall-forming maize CesA genes (Table I). Expression profiling analysis further supports this orthology with ZmBk2L3 being most highly expressed in the elongating zone of the internode and its expression declining as the internode tissues enter the transition and mature zones where secondary wall is deposited. However, the fact that ZmBk2L3 was also expressed at a moderate level in mature cells of both the leaf and stalk tissues suggests that it may play a wider role in cell development in monocots than just in cell expansion. Three of the four genes, ZmCesA1, ZmCesA7, and ZmCesA8, that the expression of ZmBk2L3 is most highly correlated with, have also been shown to be expressed, albeit at a lower level, in the tissues where a transition from primary to secondary wall formation occurs (Dhugga, 2001; Appenzeller et al., 2004).

Regulation of Arabidopsis COB Gene Family Members Is Highly Diversified Compared to Maize Members

Analysis of expression of COB gene family members has revealed both similarities and differences between monocot and eudicot members. The correlation in expression to At/ZmCesA enzymes of AtCOBL4/ZmBk2 and AtCOB/ZmBk2L3 (Persson et al., 2005; Ching et al., 2006) demonstrates a close connection of these genes to cellulose biosynthesis for both primary and secondary cell walls. The expression of family members in expanding tissues, the overlapping expression of family members in a number of tissues, the unique expression of some family members in specific tissues, and a subset of genes being weakly responsive to environmental stimuli are additional examples of commonalities between the monocot and eudicot members. One main difference between monocot and eudicot family expression, however, is that the majority of AtCOBL family members are expressed in most tissues, while only some ZmBk2L members are expressed in most tissues (ZmBk2, ZmBk2L1, ZmBk2L3, and ZmBk2L4), suggesting that regulation of Arabidopsis members is highly diversified compared to maize members.

Two possible explanations for this diversification can be suggested. The phylogeny of the Arabidopsis
members provides some clues as to the mechanism behind this diversification of regulation. *AtCOBL10* and *AtCOBL11* are similar to each other with high bootstrap support in the COB gene family phylogeny, providing evidence of gene duplication. An analysis of gene duplication in Arabidopsis provides further support to this hypothesis, as *AtCOBL10* and *AtCOBL11* reside on a block of three duplicated genes covering approximately 50 kb on chromosomes 3 and 4, respectively (Blanc et al., 2003). These genes also cluster close to each other in organ expression profiling. Tissue-specific expression and expression in response to hormones suggests subfunctionalization of these two members or neofunctionalization of one member. *AtCOBL10* is primarily hormone responsive, while *AtCOBL10* is primarily expressed in stamen tissue. Using this phylogeny and the available expression profiling data to examine similar types of relationships for maize family members was less informative, particularly in the case of the monocot-specific *ZmBkL8*/ZmBk2L clade. A second possible suggestion for this diversification lies within the regulatory sequences of this gene family. Acquisition of maize upstream regulatory sequences would be useful in this context to find cis-regulatory modules that could explain these expression similarities and differences. An additional approach to determine the biological significance of the differences in regulation of monocot and eudicot members could include correlation of expression of these genes to enzymes associated solely with Type II cell wall biosynthesis.

In conclusion, this genomic analysis described the COB gene family phylogeny using sequence data from Arabidopsis, maize, and rice members. The phylogeny reflects differences between monocot and eudicot members, and multivariate expression analysis further supports these differences, primarily through diversification of expression regulation. Members are expressed in a diverse number of developmental stages, expanding tissues and cell types, and in response to environmental stimuli. This comprehensive analysis suggests that COB gene family members are expressed in a diverse number of ways to facilitate plant cell expansion.

**MATERIALS AND METHODS**

**GPI and COB Predictions of the Maize Bk2L Family**

The maize (*Zea mays*) sequences were assembled from public and DuPont databases. Clustal analysis was carried out using ClustalW (Thompson et al., 1994). The signal peptide was predicted with SignalP Version 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Dyrløv Bendtsen et al., 2004), and the hydrophobic profile using the Kyte-Doolittle method according to Roudier et al. (2002). GPI modification was predicted using Big-PI (http://mendel.imp.ac.at/gpi/gpi_server.html; Eisenhaber et al., 1998).

**Phylogenetic Analysis**

The rice (*Oryza sativa*) accessions are as in Li et al. (2003). Multiple amino acid sequence alignment was carried out using ClustalW (Thompson et al., 1994). Phylogenetic analysis was performed with the PAUP 4.0 program, as previously described, using the heuristic algorithm over 1,000 replications with random sequence additions (Dhugga, 2004). Bootstrap analysis to determine support for each branch of the tree was performed over 10,000 replications with random sequence addition (Felsenstein, 1985). The bootstrap values are presented as percentages, and the cutoff for monophyletic branch was at least 50% support.

**Arabidopsis Gene Expression Profiling**

The stress, hormone, and organ expression profiles of the COB1 gene family were extracted from the Genevestigator meta-analyzer program (Zimmermann et al., 2004; Schmid et al., 2003) using the AtGenExpress plant organ expression series. For stress and hormone treatments, expression is indicated as fold-change relative to a control treatment. For the organ dataset, the raw Affymetrix values were log2 transformed. Data was clustered using hierarchical clustering, Euclidean distance, and average linkage with the Institute for Genomic Research MeV software package (Eisen et al., 1998). For all stress treatments, green tissue and root experiments were combined relative to controls. For the heat, cold, osmotic, oxidative, drought, and wounding treatments, experiments at 6, 12, and 24 h of treatment were combined relative to controls. Further details about the AtGenExpress stress treatments can be found at http://www.weigelworld.org/resources/microarray/AtGenExpress and the sesnence experiments in Schmid et al. (2005). For AtGenExpress hormone treatments, experiments at 1 and 3 h on wild-type seedlings were combined relative to controls. Hormone concentrations were as follows: 10 μM ABA, 10 μM 1-aminoacyclopropane-1-carboxylic acid, 10 mM brassinolide, 1 μM GA3, 1 μM indole-3-acetic acid, 10 μM methyl jasmonate, and 1 μM zeatin.

**Gene Expression Analysis in Maize through MPSS**

Maize Bk2 and Bk2L transcript expression levels were measured using MPSS from Solexa (Brenner et al., 2000). A total of 120 MPSS libraries covering 17 nonoverlapping tissues, diverse developmental stages, and stress and hormone treatments were assayed. For the stress and hormone treatments, expression categories were chosen according to Meyers et al. (2004). Stress treatments are outlined as follows: wounding, leaves and roots are from a V5 plant collected 24 h after wounding; nitrate starvation, leaves and roots were collected from plants grown in hydroponics in the absence of nitrate or treated with 5 mM nitrate for 2 h after starvation; drought stress, plants grown for 4 weeks with an ample supply of water and water then withheld for approximately 1 week or until leaves start rolling; ABA treatment, V5-stage plants were removed from the soil and the roots were placed in a 0.1 mM ABA solution for 24 h, then the third leaf from the base was collected; benzyladenine treatment, leaf discs from across the ear leaf at V10 stage were treated for 6 h. Tags were a 17-base sequence read beginning with “GATC.” Tag-to-gene associations were done by direct sequence matching. Data were normalized and filtered according to the Solexa protocol. Relationships among the expression patterns of the Bk2L and CesA genes were quantified by measuring the pairwise Pearson correlation coefficients of the tag abundances in 130 libraries from the B73 genotype, which were obtained with the improved MPSS protocol (Appenzeller et al., 2004). The overall pattern of correlations among different pairs for the CesA genes is in general agreement with the previous reports (Dhugga, 2001; Appenzeller et al., 2004; Ching et al., 2006). Minor differences in the magnitude of the correlation coefficients resulted from a larger set of libraries, many of them not represented in the previous analyses, obtained with the improved MPSS protocol. The varoius libraries were derived from the following tissues: anther, three; young ear, six; embryo, three; endosperm, 15; kernel, four; leaf, 33; meristematic zone, 14; pericarp, three; pollen, one; root, 31; seedling, two; silk, two; stalk, seven; and whorl, six.

**Construction of Arabidopsis Promoter:GFP-GUS Reporter Lines**

 Primers were designed to amplify the noncoding region of COBL1 genes from the end of the upstream gene to the translational start site. This upstream noncoding region was first amplified by PCR from genomic DNA and cloned into the Gateway pENTR vector. The promoter of each COBL gene was subsequently introduced into the pBastaGWFS7 binary vector that carries an enhanced GFP-GUS fusion and a glufosinate (Basta) selectable marker (Karimi et al., 2002) through recombination. The promoter lengths and primer sequences are as follows: COBL1: 687 bp, COBL1F 5′-caccctgcaagactgtttattcctgttg-3′
COBL1R 5’-tttttgtaagaaaaagagagag-3’; COBL2 2.5/8 hp, COBL2F 5’-cac- 
cctcctaattaaattgcttc-3’; COBL2R 5’-atctctatgcgttatgcta-3’; COBL4 832 bp, 5’-caccttcatttttttctgcaacctagtac-3’; COBL4F 5’-atctctatgcgttatgcta-
tca-3’; COBL4R 5’-cttttgagtaaaggtaaggatggt-3’; COBL5 416 bp, COBL5F 5’-cacctcgtagttaactaccgta-3’; COBL5R 5’-tttctgctctgccagaa-3’; COBL7: 1,752 bp, COBL7F 5’-caccgatgccaaacctaatcatt-
tcg-3’; COBL7R 5’-ggacacacaatactgctgttgac-3’; COBL6: 1,549 bp, COBL6F 5’-cacctgctctcaaatctgcttgac-3’; COBL8R 5’-gattactaatctactctcact-3’; COBL9 2,400 bp, COBL9F 5’-ccacattagctgccagctgatagtct-3’; COBL9R 5’-tgctg-
ttttctctagagaaataag-3’. Each construct was introduced into the Arabidopsis (Arabidopsis thaliana) Columbia ecotype genome through Agrobacterium-mediated trans-
formation and individuals selected in the T1 for Basta resistance. For each 
construct, at least eight T2 lines were examined for GFP or GUS expression 
with a minimum of five plants per line, and results shown are representative 
of these lines.

Plant Material and Growth Conditions
Arabidopsis seedlings were grown on vertically oriented 0.15% Phytagel 
plugs with 1 × Murashige and Skoog salt mixture containing 1% Suc and 
0.5 g/L MES, pH 5.7. Seedlings and roots were examined for expression at 5 d. 
All other developmental stages were from plants that were transferred to soil 
after approximately 7 d. All plants were grown under a 16-h-light/8-h-dark 
day length. Developmental stages sampled are as follows: root, 5 d after germina-
tion; cotyledon, 5 d after germination; rosette leaf, adult leaves 8, 9, or 10; 
cauline leaf, cauline leaves along the main inflorescence stem; flower, mature 
flowers or just-pollinated flowers (Beyes stage 6.50); siliqua, shattering siliqua 
age (Smyth stage 13–14); and embryo, mature bending cotyledon stage 
embryos at approximately 20 to 25 DAP were excised from the seed coat.

Imaging
In cases where GFP fluorescence was detected, it was often at a lower 
intensity (or not present) compared to the GUS reporter. GFP fluorescence was 
monitored in the roots only. In lines where no GFP was detected in the roots,
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