Regulation of Plasmodesmatal Permeability and Stomatal Patterning by the Glycosyltransferase-Like Protein KOBITO1\textsuperscript{1[W][OA]}

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The differentiation of stomata provides a convenient model for studying pattern formation in plant tissues. Stomata formation is induced by a set of basic helix-loop-helix transcription factors and inhibited by a signal transduction pathway initiated by TOO MANY MOUTHS (TMM) and ERECTA family (ERf) receptors. The formation of a proper stomata pattern is also dependent upon the restriction of symplastic movement of basic helix-loop-helix transcription factors into neighboring cells, especially in the backgrounds where the function of the TMM/ERf signaling pathway is compromised. Here, we describe a novel mutant of KOBITO1 in Arabidopsis (Arabidopsis thaliana). The kob1-3 mutation leads to the formation of stomata clusters in the erl1 erl2 background but not in the wild type. Cell-to-cell mobility assays demonstrated an increase in intercellular protein trafficking in kob1-3, including increased diffusion of SPEECHLESS, suggesting that the formation of stomata clusters is due to an escape of cell fate-specifying factors from stomatal lineage cells. While plasmodesmatal permeability is increased in kob1-3, we did not detect drastic changes in callose accumulation at the neck regions of the plasmodesmata. Previously, KOBITO1 has been proposed to function in cellulose biosynthesis. Our data demonstrate that disruption of cellulose biosynthesis in the erl1 erl2 background does not lead to the formation of stomata clusters, indicating that cellulose biosynthesis is not a major determining factor for regulating plasmodesmatal permeability. Analysis of KOBITO1 structure suggests that it is a glycosyltransferase-like protein. KOBITO1 might be involved in a carbohydrate metabolic pathway that is essential for both cellulose biosynthesis and the regulation of plasmodesmatal permeability.

The majority of neighboring plant cells are connected symplastically by tiny plasma membrane-lined cytoplasmic channels called plasmodesmata. These channels play an essential role in intercellular communications, as they lead to the formation of symplastic domains allowing cell-to-cell trafficking of small molecules, proteins, and mRNA. The symplastic movement of molecules can provide positional information to cells during development and is at the root of the non-cell-autonomous function of multiple transcription factors. For example, while mRNA of the maize (Zea mays) homeobox protein KNOTTED1 is expressed in the L2 and L3 layers of shoot apical meristems, the protein is able to move and functions in the epidermis (Hake and Freeling, 1986; Jackson et al., 1994). In roots, the SHOORTROOT protein moves symplastically from the stele into the adjacent cell layer, where it promotes endodermis cell fate (Nakajima et al., 2001).

Some plasmodesmata form directly in the cell plate during cytokinesis, while others develop de novo by penetrating existing cell walls (Ehlers and Kollmann, 2001). At first, most plasmodesmata are simple linear channels, but later, they may become branched and gain a central cavity. The permeability of plasmodesmata strongly depends on the developmental stage, with cells in meristems and young developing tissues being connected by multiple open plasmodesmata that become closed as tissues mature (Duckett et al., 1994; Kim and Zambryski, 2005). The selectivity of trafficking through plasmodesmata depends on their aperture and is defined by the size exclusion limit that specifies the maximum size of the macromolecules able to pass through. The major factor regulating the size of the aperture is the turnover of callose at the constricted ends (neck regions) of plasmodesmata. Callose deposition by a plasma membrane-localized callose synthase causes a plasmodesma to close, while callose degradation by extracellular glycosylphosphatidylinositol-anchored \( \beta-1,3 \)-glucanases results in a plasmodesma opening (Iglesias and Meins, 2000; Levy et al., 2007; Guseman et al., 2010). In addition, callose accumulation at the
Regulation of Plasmodesmal Permeability by KOB1

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Using map-based cloning, we determined that the mutation is located on the long arm of chromosome 3 and then fine-mapped it to a 138-kb fragment between markers KDY42 and F17O14b (Fig. 1A). Analysis of genes in this fragment revealed the presence of KOBITO1. As descriptions of KOBITO1 mutant alleles such as kob1-1, kob1-2, eld1-1, eld1-2, and ab18 (Cheng et al., 2000; Pagant et al., 2002; Lertpiriyapong and Sung, 2003; Brocard-Gifford et al., 2004) highly resembled the phenotype of our mutant, we sequenced KOBITO1 and found a single C→T substitution at position 2,166 bp that results in a Ser-371→Leu-371 substitution (Fig. 1B). We named the new allele kob1-3.

RESULTS

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This mutation does not change the expression of KOBITO1 mRNA as determined by semiquantitative reverse transcription (RT)-PCR (Supplemental Fig. S1). A genetic cross confirmed that kob1-3 is allelic with eld1-1 (Lertpiriyapong and Sung, 2003).

**Figure 1.** Positional cloning of KOBITO1. A, Fine-mapping of KOBITO1. The kob1-3 mutation was mapped to the long arm of chromosome 3 between molecular markers KDY42 and F17014b. The number of recombinants obtained is indicated in the middle. Markers are positioned to scale. The corresponding bacterial artificial chromosome clones are indicated in the middle. Markers are positioned to scale. The corresponding bacterial artificial chromosome clones and the location of the KOBITO1 locus (At3g08550) are indicated below. B, KOBITO1 gene structure and positions of the mutations in kob1-3 and eld1-1. Boxes indicate exons and thick lines indicate introns. In kob1-3, a C-to-T substitution results in Ser-371→Leu-371. In eld1-1, an A-to-G substitution results in a premature stop codon.

**Stomatal Patterning Defects Conferring by kob1-3**

The kob1-3 erl1-2 erl2-1 plants contain a dramatically increased number of stomata clusters in cotyledons (Fig. 3A). In kob1-3 erl1-2 erl2-1, 38% ± 5% and 35% ± 1% of stomata are in clusters versus 3% ± 3% and 2% ± 4% in erl1 erl2 on the abaxial and adaxial sides of cotyledons, respectively (Fig. 4, B and D). In other organs of kob1-3 erl1-2 erl2-1 plants, we also detected stomata clusters, but the phenotype was weaker compared with cotyledons. When kob1-3 is in the background of functional ERL1 and ERL2, the stomata clustering phenotype is absent (Figs. 3C and 4, B and D). Outcrossing of the KOB1 allele eld1-1 into an erl1-2 erl2-1 background produced seedlings with stomata clusters in cotyledons, confirming that the observed phenotype is due to a mutation in the KOBITO1 gene (Fig. 3, I and J). Next, we analyzed the genetic interaction of kob1-3 with two other components of the stomatal patterning pathways: ERECTA and TMM. In the erecta background, the kob1-3 mutation increased the number of cells dividing asymmetrically but did not lead to the formation of stomata clusters (Fig. 3E). We observed a very strong stomata clustering phenotype in the background of the tmm-1 mutation (Fig. 3, G and H) (Nadeau and Sack, 2002), with 99% ± 1% of stomata in clusters of more than three on the abaxial side of cotyledons in kob1-3 tmm-1 versus 36% ± 7% in tmm-1 (Fig. 4F). In addition to stomata clustering, the kob1-3 mutation also had an effect on the stomatal index (the ratio of stomata to total epidermal cell count). In cotyledons, kob1-3 increases the fraction of cells that are stomata on the adaxial epidermis on its own (Fig. 4C) and on both the abaxial and adaxial epidermis in the erl1-2 erl2-1 and tmm-1 backgrounds (Fig. 4, A, C, and E).

These results suggest that KOBITO1 is necessary for proper stomatal patterning and for the inhibition of excessive stomata differentiation when the TMM/ERECTA gene family pathway is compromised. This interaction between KOBITO1 and the ERECTA family genes does not happen at the level of gene expression, as expression of ERECTA is not changed in either kob1-3 erl1 erl2 or kob1-3, and KOB1 expression is not changed in the erl1 erl2 mutant (Supplemental Fig. S1).
The KOBITO1 Gene Is Necessary for the Regulation of Plasmodesma Permeability

Published data suggest that a primary function of KOBITO1 is in cell wall metabolism (Cheng et al., 2000; Pagant et al., 2002). Because alterations in plasmodesmal permeability can lead to misspecification of epidermal cell fate (Guseman et al., 2010), we asked whether KOBITO1 is important for the establishment of the proper size exclusion limit of plasmodesmata.

To examine cell-to-cell protein mobility, we bombarded a plasmid carrying cauliflower mosaic virus (CaMV) 35S:GFP into the abaxial epidermis of kob1-3 erl1 erl2 and erl1 erl2 10-d-old seedlings and 18 h later analyzed the movement of GFP from the point of transformation. The particle gun always transforms an individual cell during bombardment, and surrounding cells receive GFP due to diffusion through plasmodesmata. This technique has previously been used to assess changes in plasmodesmata permeability due to the deregulation of callose biosynthesis or degradation (Levy et al., 2007; Guseman et al., 2010). In erl1 erl2 seedlings, GFP was mostly expressed in a single cell (34%) or in small clusters of cells (38% in clusters of two to four). In kob1-3 erl1 erl2 seedlings, GFP was more frequently expressed in big groups of cells (78% in clusters of more than four, 32% in clusters of more than 10; Fig. 5, A and D). The average cluster sizes were 3.8 for erl1-2 erl2-1 plants and 8.4 for kob1-3 erl1-2 erl2-1 mutants. To confirm our findings, we repeated the experiment with two plasmids; one carrying CaMV 35S:GFP and another carrying CaMV 35S:red fluorescent protein (RFP) were cobombarded into the adaxial epidermis of 17-d-old seedlings. In our experiments, RFP was always observed only in an individual transformed cell due to the presence of an endoplasmic reticulum retention signal and a resulting inability to move through plasmodesmata. While GFP had the ability to move, it was still confined to mostly single cells (88%) in erl1 erl2 seedlings. However, in kob1-3 erl1 erl2 seedlings, GFP had moved out of one cell to neighboring cells and was observed in clusters (72%; Fig. 5, B and E). The average cluster sizes were 1.1 for erl1-2 erl2-1 plants and 3.7 for kob1-3 erl1-2 erl2-1 mutants, confirming the increased plasmodesmatal permeability in kob1-3 erl1-2 erl2-1 mutants.

As the number of stomatal lineage cells in the kob1-3 erl1-2 erl2-1 epidermis is much higher than in erl1-2 erl2-1 (Figs. 3, A and B, and 4, A–D), it is possible that our observed variation in plasmodesma permeability is related to a distinct plasmodesmata structure in different cell types. To analyze whether kob1-3 alters plasmodesmata conductivity in seedlings with a comparable epidermal cell differentiation pattern, we bombarded the CaMV 35S-GFP construct into kob1-3 and the wild type. Analysis of the adaxial epidermis of 10-d-old seedlings demonstrated increased GFP diffusion in kob1-3, with 78% of GFP expressed in clusters versus 47% in the wild type (Fig. 5, C and F). The average cluster size of cells expressing GFP was 2.1 in the wild type and 3.2 in kob1-3.

Another possibility is that the observed increase in the diffusion of GFP is related to higher GFP accumulation in the smaller epidermal cells of kob1-3. While on average cells are smaller in kob1-3, we often detected GFP expressed in cells of similar size in kob1-3 and the wild type (Fig. 5C) and sometimes in cells with the reversed proportions: small wild-type cells and big kob1-3 cells (Supplemental Fig. S3). We did not detect any correlation between the size of cells or the level of GFP expression and the amount of GFP diffusion. These results suggest that the kob1-3 mutation increases plasmodesma permeability independently of changes in epidermal cell composition or modification of cell size.
Increased Diffusion of SPCH-Yellow Fluorescent Protein in kob1-3 erl1-2 erl2-1

We hypothesized that excessive stomata differentiation in kob1-3 erl1-2 erl2-1 is due to increased diffusion of stomata promoting bHLH transcription factors. To test this hypothesis, we analyzed the diffusion of SPCH-yellow fluorescent protein (YFP), a bHLH transcription factor essential for meristemoid mother cell differentiation (MacAlister et al., 2007). SPCH-YFP and cyan fluorescent protein (CFP) were cobombarded into the adaxial epidermis of 12-d-old cotyledons. Analogous to GFP, diffusion of CFP was increased in kob1-3 erl1-2 erl2-1 versus erl1-2 erl2-1 (Fig. 6, A and B), again suggesting increased plasmodesmata permeability. More importantly, diffusion of SPCH-YFP was also increased in kob1-3 erl1-2 erl2-1 epidermis (Fig. 6, A and C). While in erl1-2 erl2-1 we detected SPCH-YFP only in single cells, in kob1-3 erl1-2 erl2-1 SPCH-YFP was able to diffuse in the neighboring cells in 23% of transformation events (Fig. 6C). This result suggests that the diffusion of SPCH and possibly other stomata-promoting transcription factors from stomatal lineage cells into neighboring cells is likely to occur in kob1-3 mutants.

Callose Accumulation in kob1-3 erl1-2 erl2-1

Plasmodesma permeability strongly depends on callose accumulation at the neck region, with a decrease in callose deposition leading to plasmodesma opening (Iglesias and Meins, 2000; Levy et al., 2007; Guseman et al., 2010). However, previously it was reported that the kob1-1 mutant shows increased, not decreased, callose accumulation (Pagant et al., 2002). Our analysis of kob1-3 by aniline blue staining suggests a similar pattern and level of callose deposition in the cotyledons of kob1-3 erl1 erl2 and erl1 erl2 seedlings (Fig. 7, A and B). In both cases, we detected high aniline blue staining in the vasculature, at the tips of root hairs, at the cell plates of the newly divided guard mother cells, and in the root tip. In some kob1-3 erl1 erl2 seedlings, the levels of aniline blue staining was somewhat higher compared with erl1 erl2, but as the difference was not dramatic it is difficult to conclude significance. The weaker accumulation of callose in kob1-3 compared with kob1-1 is likely related to the considerably weaker phenotype of kob1-3 mutants. More significantly, while observing the abaxial leaf epidermis, we detected callose accumulation at plasmodesmata in the erl1-2 erl2-1 and kob1-3 erl1-2 erl2-1 (Fig. 7, C and D). In the erl1-2 erl2-1 epidermis, aniline blue staining appeared as two narrow parallel rectangles in the cell wall area. These rectangles are most likely two plasmodesmata neck regions. In kob1-3 erl1-2 erl2-1, some plasmodesmata had a similar pattern of staining, but accumulation of callose at others seemed to be reduced and looked patchy (Fig. 7C). This different pattern of callose accumulation could be one of the contributing factors to increased plasmodesma permeability in kob1-3. Alternatively, the difference in observed aniline blue staining could be due to the difficulty in focusing on callose rectangles, as the surface of kob1-3 leaves is very bumpy. While a more precise analysis is needed to measure the exact amount of callose accumulation at plasmodesmata, at this point we can conclude that if there is a change in callose accumulation in kob1-3 it is rather subtle.
Disruption of Cellulose Biosynthesis Does Not Change Stomata Differentiation in the \textit{erl1 erl2} Background

The most obvious defects of \textit{kobito1} mutants are decreased cellulose biosynthesis and the abnormal orientation of cellulose microfibrils (Pagant et al., 2002). Based upon those data, KOBITO1 was proposed to be a part of the cellulose synthase machinery in elongating cells. To check whether a disruption of cellulose biosynthesis could cause an increase in plasmodesma permeability and subsequent formation of stomata clusters, we used two different methods. In the first experiment, we grew wild-type and \textit{erl1 erl2} seedlings on plates with 1 \textmu m 2,6-dichlorobenzonitrile (DCB), a cellulose synthesis inhibitor. The presence of the inhibitor produced dwarf seedlings as cells became smaller, but it did not induce the formation of stomata clusters in either the wild type or in \textit{erl1 erl2} (Fig. 8, A and B). In the second experiment, we analyzed stomata formation in the \textit{rsw1-1} mutant (Arioli et al., 1998), a temperature-sensitive allele of cellulose synthase A1. To examine stomata formation in a background where the TMM/ERECTA family signaling pathway is not fully functional, we outcrossed \textit{rsw1-1} into the \textit{erl1 erl2} background. Again, we observed that the \textit{rsw1-1} mutation caused reduced cell elongation and led to dwarfism, but it did not change the stomatal index or induce the formation of stomata clusters in either background (Fig. 8, D and E).

**KOBITO1 Is a Glycosyltransferase-Like Protein**

\textit{KOBITO1} encodes a protein of 533 amino acids. While earlier publications have stated that KOBITO1 has no known domains, our search for conserved domains using the Conserved Domain Database (Marchler-Bauer et al., 2011) identified a glycosyltransferase family A domain between amino acids 238 and 382 with an E-value of 2.14e-03 and one between amino acids 146 and 292 with an E-value of 0.01. Ser-371 is a conserved residue in this domain that is sometimes substituted to Ala, Thr, or Pro in putative KOBITO1 orthologs and paralogs. Another search using the Pfam database identified a domain of unknown function (DUF23) with an E-value of 2.1e-03 between amino acids 140 and 373. This domain also belongs to the glycosyltransferase A clan. The TMHMM2 program predicted a transmembrane domain spanning amino acids 27 to 49 and a type II transmembrane topology typical of glycosyltransferases, with a short N-terminal segment within the cytosol and a larger C-terminal domain on the other side. Orthologs of KOBITO1 exist in many different plant species and in green algae (Supplemental Fig. S4). The putative glycosyltransferase domain (amino acids 146–382) of KOBITO1 from the green alga \textit{Chlorella variabilis} is 61% identical and 76% similar to that of \textit{Arabidopsis} KOBITO1.

**DISCUSSION**

In a genetic screen designed to find genes involved in the regulation of stomata development, we found a novel mutant allele of \textit{KOBITO1}. The localization of the \textit{kob1-3} mutation was determined by map-based positional cloning and confirmed by allelic analysis with the \textit{eld1-1} mutant. Several mutant alleles of \textit{KOBITO1}, such as \textit{eld1-1}, \textit{eld1-2}, \textit{kob1-1}, and \textit{kob1-2}, have previously been isolated in genetic screens for growth-defective mutants (Cheng et al., 2000; Pagant et al., 2002), and another allele, \textit{abi8}, has been isolated in a screen for reduced sensitivity to abscisic acid during seed germination (Brocard-Gifford et al., 2004). All of these previous mutants are sterile severe dwarfs.
with abnormal vasculature differentiation. Cell elongation is impaired in all organs; however, the polarized growth of root hair and pollen tubes is not changed (Pagant et al., 2002). The reduced cell elongation in kobito1 mutants cannot be rescued by treatment with growth regulators (Cheng et al., 2000). The phenotype of our novel allele kob1-3 is very similar but slightly less severe. The kob1-3 plants are dwarfs with reduced cell elongation; however, they are partially fertile and do not have the incomplete cell walls that were observed in some other alleles (Pagant et al., 2002). The weaker phenotype of kob1-3 is likely due to the presence of a partially functional KOBITO1 protein with a Ser-371 → Leu-371 substitution.

Our experiments uncovered that, in addition to growth defects, the kob1-3 mutants have increased plasmodesmatal permeability. At first, we noticed that in backgrounds with reduced function of TMM/ERECTA family (ERf), the kob1-3 mutants had increased stomatal index and multiple stomata clusters. The dependence of stomata clustering in kob1-3 on mutations in the signaling pathway, which suppresses stomata differentiation, is strikingly similar to the phenotypes observed for chorus (chor; Guseman et al., 2010). For example, chor has very few stomata clusters when the TMM/ERf pathway is functional, but stomata clusters form in the background of the erecta erl1 and erecta erl2 mutations. In the background of erecta, both kob1-3 and chor produce an increased number of small cells that form through asymmetric divisions, and in the background of tmm, they both generate massive stomata clusters. The increased stomata clustering in chor has been attributed to an increased permeability of plasmodesmata that allows symplastic movement of transcription factors promoting stomata development. The chor mutation is in the GSL8 gene, which encodes a callose synthase (Töller et al., 2008). Reduced deposition of callose at plasmodesmata in chor is the most likely cause of their increased conductivity (Guseman et al., 2010). In the kob1-3 plants, cell-to-cell mobility assays demonstrated an increase in intercellular protein trafficking, including a rise in the symplastic movement of SPCH. In analogy to chor, we speculate that in kob1-3, increased plasmodesmatal permeability leads to an escape of stomata fate-promoting transcription factors such as SPCH, MUTE, FAMA, ICE1/SCREAM, and SCREAM2 from meristems and guard mother cells to neighboring cells. When the TMM/ERf signaling pathway is functional, it inhibits these transcription factors, preventing the formation of stomata clusters. In the backgrounds where this pathway is compromised, such as in erl1 erl2 or tmm, the escaped transcription factors are able to switch the development of cells in the stomata neighborhood to stomata cell fate.

Our findings again illustrate that closure of plasmodesmata is essential for the differentiation of stomata, as...
it prevents the diffusion of cell fate determinants. In that respect, stomata development is unlike the differentiation of the endodermis, root hairs, or trichomes, in which symplastic transport plays a positive role in the establishment of correct cell fate. In each of these three cases, cells communicate with each other through the exchange of transcription factors such as SHORTROOT, TRIPTYCHON, and CAPRICE (Nakajima et al., 2001; Digiuni et al., 2008). On the other hand, stomata formation is correlated with plasmodesmata closure, with mature guard cells being virtually completely symplastically isolated. This isolation is essential for efficient opening and closing of stomata by maintaining the independence of guard cell ionic and electric balance. It appears that the differentiation of stomata and their spacing evolved to rely solely on extracellular communications and not on symplastic transport. This peculiarity of stomata development could be exploited further in the future to find other factors that are important for the regulation of plasmodesma permeability. For example, the G-protein signaling pathway has been shown to regulate stomatal density (Zhang et al., 2008) and cell wall metabolism (Klopfleisch et al., 2011). It would be interesting for future studies to investigate if stomatal density changes in mutants of G-protein α-subunit and G-protein β-subunit are related to changes in plasmodesmal permeability.

Increased plasmodesmal permeability is most often associated with decreased callose deposition at the neck regions (Bucher et al., 2001; Guseman et al., 2010). Previously, it has been reported that callose deposition in kob1-1 is increased (Pagant et al., 2002). Likewise, we observed equal or higher levels of callose accumulation in kob1-3 mutants compared with controls. These data indicate that callose biosynthesis is not impaired in kobito1 mutants. There is still a possibility that while callose is synthesized, it is inappropriately deposited at the neck regions. While we were able to detect callose accumulation at plasmodesmata, we saw some evidence that in kob1-3 there was a higher density of plasmodesma with slightly less callose deposition at individual plasmodesma (Fig. 7C). Whether this result reflects the real situation or is due to the difficulty with focusing a microscope on plasmodesmata in kob1-3 leaves, as they are fairly undulating, is still an open question. The structure of kob1-3 plasmodesmata and the level of callose deposition at individual plasmodesmata shall be addressed in the future.

Multiple previous lines of evidence suggest an involvement of KOBITO1 in cellulose biosynthesis. The dwarf phenotype of kobito1 mutants with radically swelled cells is reminiscent of cellulose-deficient mutants and seedlings treated with cellulose inhibitors. Analysis of the kob1-1 mutant demonstrated a 33% decrease in cellulose content and a random orientation of cellulose microfibrils in the root cell elongation zone (Pagant et al., 2002). In addition, classification of Arabidopsis cell wall mutants using Fourier transform infrared microspectroscopy showed that kobito1 mutants appear in the same cluster as cellulose-deficient mutants (Mouille et al., 2003). Could cellulose biosynthesis be essential for the regulation of plasmodesmal permeability? Recently, high-resolution scanning electron microscopy of plasmodesmata suggested that the plasmodesmal plasma membrane and the cell wall are connected by spokes that are either composed of or stabilized by cellulose or pectin (Brecknock et al., 2011); hypothetically, disruption of the formation of those spokes might change plasmodesmata structure. Therefore, we analyzed whether the disruption of cellulose biosynthesis might increase the permeability of plasmodesmata and thus induce the formation of stomata clusters. Two independent experiments suggested that it does not. Analysis of the cellulose biosynthesis mutant...
rsow-1 and seedlings grown on the cellulose biosynthesis inhibitor DCB demonstrated that disruption of cellulose biosynthesis does not result in the formation of stomata clusters; thus, is not likely to increase plasmodesmatal permeability. These data suggest that kobito1 mutants are deficient in some other aspect besides cellulose biosynthesis and that decreased biosynthesis of cellulose might be an indirect effect of kobito1 mutation.

An analysis of KOBITO1 structure suggests that it has a DUF23 domain that belongs to the glycosyltransferase family A clan. This domain is highly conserved between KOBITO1 homologs in land plants and green algae but is not present in other organisms. While KOBITO1 is not part of CAZy, a database of carbohydrate active enzymes, a bioinformatics approach aimed at finding additional plant glycosyltransferase genes has identified KOBITO1 as one of 27 non-CAZy-classified putative glycosyltransferases (Egelund et al., 2004). Two genes out of this group of 27 have been proven to encode glycosyltransferases that function in pectin biosynthesis (Egelund et al., 2006). The overall structure of the KOBITO1 protein is also consistent with its suspected function as a glycosyltransferase, as it is predicted to be a type II transmembrane protein with four functional domains: a short cytoplasmic tail, a membrane-anchoring domain, a stem region, and an approximately 350-amino-acid-long putative catalytic domain. Several different subcellular localizations have previously been reported for KOBITO1. When overexpressed under the control of a 35S promoter, KOBITO1 was detected in the plasma membrane and in distinct punctate patches (Pagant et al., 2002) as well as in the cell wall (Lertpiriyapong and Sung, 2003). However, when expressed under the control of its own promoter, KOBITO1 was observed in punctate structures within the cytoplasm (Brocard-Gifford et al., 2004), which hypothetically could be Golgi complexes. This expression in punctate structures is sufficient to complement the abi8 mutant. The possibility of KOBITO1 localization in the Golgi is suggested by Golgi Predictor (Yuan and Teasdale, 2002). Based on the transmembrane domain, this program determines whether a membrane protein would stay within the Golgi or move beyond it to a post-Golgi compartment. The transmembrane domain of KOBITO1 is surprisingly evolutionarily conserved, with 64% similarity between Arabidopsis and Physcomitrella patens. Finally, a recent proteomic analysis of Golgi membranes purified by free-flow electrophoresis from Arabidopsis cell cultures has reproducibly identified KOBITO1 as a
component of this organelle (J.L. Heazlewood, personal communication). In contrast, KOBITO1 has not been identified in any proteomic survey of the Arabidopsis plasma membrane, which have collectively identified over 3,000 proteins from this structure (Heazlewood et al., 2007). While it is possible that KOBITO1 is a transient member of the secretory system, its absence in plasma membrane preparations would support the notion that it is in fact a resident and functional component of the Golgi apparatus.

Our analysis of KOBITO1 structure and localization suggests that it could be involved in some sort of glycosylation process in the Golgi that is essential for both cellulose biosynthesis and for the closing of plasmodesmata. Cellulose biosynthesis has been shown to be dependent on at least one other glycosylation pathway: N-glycosylation of proteins (Lukowitz et al., 2001). But considering that the biosynthetic steps and enzymes involved in N-glycosylation are conserved between yeast, mammals, and plants (Lehle et al., 2006) and that KOBITO1 is a plant-specific gene, its function in N-glycosylation is unlikely. The kob1-1 seedlings have an increased accumulation of pectin, callose, and lignin (Pagant et al., 2002); therefore, KOBITO1 is not likely to function in those pathways either. Analysis of gene coexpression data can be useful for finding genes belonging to the same metabolic pathway. For example, it has been used successfully to identify gene clusters associated with cellulose biosynthesis during primary and secondary cell wall formation (Persson et al., 2005) and to find glycosyltransferases responsible for arabinosylation of cell wall Hyp-rich glycoproteins (Velazquez et al., 2011). In agreement with our idea that its involvement in cellulose biosynthesis is indirect, KOBITO1 is not coexpressed with genes involved in cellulose biosynthesis, based on data in the ATTED-II database. However, KOBITO1 does coexpress with several suspected glycosyltransferases of unknown function (At1g52420, At1g34270, and At3g10630) and with FUCOSYLTRANSFERASE1, an enzyme responsible for the addition of the terminal fucosyl residue on xyloglucan side chains (Vanzin et al., 2002). Further analysis of the KOBITO1 coexpression network might be helpful in the future to identify its metabolic pathway.

CONCLUSION

Our analysis of kobito1 mutants in backgrounds having deficient signaling of the TMM/ERF signaling pathway shows that KOBITO1 function is required for proper stomatal patterning and for the suppression of excessive stomata differentiation. We have further demonstrated that KOBITO1 function is essential for the regulation of symplastic trafficking, a factor influencing stomata development. Interestingly, the increased plasmodesmatal permeability we observed in the kobito1 mutant was not due to decreased accumulation of callose, as has typically been ascribed. Based
on the structure of KOBITO1 and previously published localization data, we propose that KOBITO1 is a Golgi-localized glycosyltransferase-like protein functioning in a carbohydrate metabolic pathway that is essential for proper plasmodesma closure and for cellulose biosynthesis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was used as the wild type. The kob1-3 mutant was obtained from an ethyl methanesulfonic acid-mutagenized (0.3% for 14 h) erl2-2 erl2-1 population (Shpak et al., 2004). Individual M2 seed lines were grown on modified Murashige and Skoog medium plates supplemented with 1× Gamborg B5 vitamins and 1% (w/v) Suc and screened for stomata patterning defects. dst-1 (CS6557), tmn-1 (CS6140), and rsu1-1 (CS6554) mutants were received from the Arabidopsis Biological Resource Center. The erecta-105 mutant was described previously (Torii et al., 1996). Plants were grown on a soil mixture (Promix PGX [Premier Horticulture]:vermiculite [Palmetto Vermiculite Co.], 1:1) supplemented with Miracle-Gro (Scotts) and approximately 3.5 mg cm⁻³ Osmocote 15-9-12 (Scotts). In the experiment in which cellulose biosynthesis was disrupted with DCB, seedlings were grown for 10 d on plates containing modified Murashige and Skoog medium as above with 1 µM DCB (Sigma; catalog no. D97558) + 0.05% dimethyl sulfoxide or with only 0.05% dimethyl sulfoxide. Both seedlings on plates and plants in soil were grown at 20°C under long-day conditions (18-h-light/6-h-dark cycle). For the analysis of rsu1-1 stomata development, we grew seedlings at 28°C.

Map-Based Cloning of kob1-3

A mapping population of kob1-3 was generated by crossing kob1-3 erl2 erl2 to the Landsberg erecta (Ler) ecotype. A bulked segregant analysis (Lukowitz et al., 2000) with a combined pool of DNA from 55 kob1-3-like seedlings revealed a strong linkage of kob1-3 to the long arm of chromosome 3 between simple sequence length polymorphism (SSLP) markers NGA172 and NGA162. Fine-mapping performed within this region using 3,172 F2 plants localized the marker kob1-3 to chromosome 3 Osmocoat 15-9-12 (Scotts). In the experiment in which cellulose biosynthesis was disrupted with DCB, seedlings were grown for 10 d on plates containing modified Murashige and Skoog medium as above with 1 µM DCB (Sigma; catalog no. D97558) + 0.05% dimethyl sulfoxide or with only 0.05% dimethyl sulfoxide. Both seedlings on plates and plants in soil were grown at 20°C under long-day conditions (18-h-light/6-h-dark cycle). For the analysis of rsu1-1 stomata development, we grew seedlings at 28°C.

Crosses and Genotyping

To generate kob1-3/dst-1/dst-1 plants, kob1-3/+;+ was crossed to dst-1/dst-1. The kob1-3/dst-1/dst-1 plants were identified in F1 based on their phenotype, and then their genotype was confirmed by DNA analysis. We distinguished wild-type, kob1-3/+;+ and kob1-3−/−;− genotypes by amplifying genomic DNA with the primers kdy 550–1100 (5′-GCCTAATCGTCTGTGCTGGTC-3′) and kdy 550–1770 (5′-GCCATCTAATGACATCAC-3′) and then digesting the PCR product with the restriction enzyme DraI. As the restriction site for DraI is present only in plants that contain kob1-3, after digestion we detected the 1.4-kb band in wild-type plants, the 1.4-kb, 1.2-kb, and 200-bp bands in kob1-3−/−;− and the 1.2-kb and 200-bp bands in kob1-3−/−/+ plants. We distinguished wild-type and dst-1/dst-1−;− plants by amplifying genomic fragments with kdy 550–1100 and kdy 550–1770-rc primers and sequencing the product to check for a G-to-A substitution at position 2,566 bp.

To generate kob1-3, dst-1; dst-1; dst-2 plants were crossed with the wild type (ecotype Columbia). To generate kob1-3, erct-105, kob1-3, erct-105 erl1, and kob1-3 erct-105 erl1 mutants, kob1-3 dst-1 dst-2 plants were crossed with erct-105 erl1. To generate kob1-3, tmn-1; tmn-1 mutant, kob1-3 dst-1 dst-2 plants were crossed with tmn-1 plants. The presence of the erl1-2 and erl1-2 mutations was determined by PCR using gene-specific primer pairs and a combination of T-DNA annealing primer (JL-202) with gene-specific primers as described previously (Shpak et al., 2004). The presence of the ercita-105 and tmn-1 mutations was determined by phenotypic analysis in kob1-3/+;+ plants. Plants of the correct genotype were isolated from the F2 or F3 populations.

To generate rsu1-1, erlt-2, erlt-2, rsu1-1 plants were crossed with erlt-2 erlt-2. The presence of the erlt-2 and erlt-2 mutations was determined as above, and the presence of the rsu1-1 mutation was determined based on the dwarf phenotype of seedlings grown at 28°C.

RNA Isolation and Expression Analysis by Semiquantitative RT-PCR

Total RNA was isolated from 15-d-old seedlings using the Spectrum Plant Total RNA Kit (Sigma). The first-strand cDNA was synthesized from 1 µg of total RNAs with the ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs) according to the manufacturer’s instructions. For the primers used, see Supplemental Table S2. The PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel with ethidium bromide and captured using a CCD camera.

Analysis of Mutant Phenotypes

Images of embryos were made and measurements of stomata index and clustering were done on 17- or 10-d-old seedlings as described using differential interference contrast (DIC) microscopy. For DIC, seedlings were incubated in a solution of 9:1 ethanol:acetic acid overnight and then cleared in a mixture of 8:1:1 chloral hydrate:distilled water:glycerol. For the stomata measurements, we looked at the cotedyledon epidermis of six seedlings per genotype and analyzed an area in the center of the cotedyledon excluding the midvein region. The size of pavement cells was measured on the abaxial side of 15-d-old cotedyledons (n = 60; 10 cells per cotedyledon) using the ImageJ program.

Transient Transformation of Seedlings and Cell-to-Cell Mobility Assay

In the first experiment, the abaxial epidermis of kob1-3 erl2 erl2 and erl1 erl1 10-d-old Arabidopsis seedlings was transiently transformed with the vector pAVA 321 (CaMV 35S:egFP657; von Aim, et al., 1998). In the second experiment, the adaxial epidermis of kob1-3 erl2 erl2 and erl1 erl1 17-d-old Arabidopsis seedlings was transiently transformed with the vectors pAVA 321 and pAN456 (CaMV 35S:PF with endoplasmic reticulum retention signal; Nelson et al., 2007). In the third experiment, the adaxial epidermis of kob1-3 and wild-type 10-d-old Arabidopsis seedlings was transiently transformed with the vector pAVA 321. And in the fourth experiment, the adaxial epidermis of kob1-3 erl2 erl2 and erl1 erl1 12-d-old Arabidopsis seedlings was transiently transformed with the vectors pAVA 574 (CaMV 35S:CFP) and pLJP 170 (CaMV 35S:SPCH-YFP). For transient transformation, we used 1.3-µm tungsten microcarriers (Bio-Rad) and a PDS-1000/He particle bombardment system (Bio-Rad). To determine the number of cells into which fluorescent proteins diffused, we analyzed fluorescent signal at 18 h post bombardment using a Nikon Eclipse 80i epifluorescence microscope or a Zeiss Axio Observer.Z1 and obtained images with a 12-megapixel cooled color DCM-1200i (Nikon) camera or with a 1.3-megapixel cooled black-and-white ORCA-AG (Hamamatsu) camera. We analyzed RFP expression to confirm that only one cell was transformed during bombardment.

Aniline Blue Staining

The 7-d-old seedlings were fixed in 95% ethanol overnight. The next day, they were incubated first for 30 min in 0.09 µ sodium phosphate buffer (pH 9) and then for 1 h in 0.01% aniline blue dissolved in the same buffer. The seedlings were observed with a Nikon Eclipse 80i epifluorescence microscope equipped with a 12-megapixel cooled color camera and a UV-2A filter (Nikon).

The GenBank accession number for the KOBITO1 (At1S968550) mRNA sequence is NM111689.

Supplemental Data

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

Supplemental Figure S1. Semiquantitative RT-PCR analysis of KOBITO1, ERECTA, ER2, and actin transcripts in 15-d-old seedlings.
Supplemental Figure S2. The effect of kob1-3 on the elongation of hypocotyls and roots.

Supplemental Figure S3. Increased plasmodemata conductivity in kob1-3 does not correlate with a decrease in cell size.

Supplemental Figure S4. ClustalW alignment of the predicted amino acid sequences for KOBITO1 from Arabidopsis, Pupulus trichocarpa, Zea mays, Oryza sativa, Selaginella moellendorffii, Physcomitrella patens, Chlorella variabilis, and Micronema strain RCC299.

Supplemental Table S1. Primer sequences used in map-based cloning of kob1-3.

Supplemental Table S2. Primer sequences used in RT-PCR.

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LITERATURE CITED

Abrashe EB, Bergmann DC (2010) Regional specification of stomatal production by the putative ligand CHALLAH. Development 137: 447–455

Arioli T, Peng L, Betznser AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R, et al (1998) Molecular analysis of cellulose biosynthesis in Arabidopsis. Science 279: 717–720

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D (2009) Control of Arabidopsis meristem development by thioredoxin-dependent regulation of intercellular transport. Proc Natl Acad Sci USA 106: 3615–3620

Bergmann DC, Dibbayawan TP, Vesk M, Vesk PA, Faulkner C, Barton DA, Jackson D (2001) Local expression of enzymatically active class I β-glucan synthase-like 8. Development 128: 166–175

Bergmann DC, Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL, Somerville CR (2007) A plasmodesmata-mediated complex formation mechanism underlies trichome patterning on Arabidopsis leaves. Mol Syst Biol 3: 217

Buchert CM, Opara KJ, Prior DAM, Dolan L, Roberts K (1994) Dye-coupling in the root epidermis of Arabidopsis is progressively reduced during development. Development 120: 3247–3255

Clausen H, Ulvskov P, Geshi N, Ulvskov P, Petersen BL (2004) A competitive complex formation mechanism mediates crosstalk between cell-to-cell communication and innate immunity in Arabidopsis. Plant Cell 23: 3533–3537

Lehle I, Strahl S, Tanner W (2006) Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases. Angew Chem Int Ed Engl 45: 6802–6818

MacAlister CA, Bergmann DC (2008) Arabidopsis stomatal initation is controlled by MAPK-mediated regulation of the bHLH transcription factor SHR in root patterning. Nature 457: 581–585

Levy A, Elander M, Rosenthal M, Epel BL (2007) A plasmodesmata-associated β-1,3-glucanase in Arabidopsis. Plant J 49: 669–682

Lukowitz W, Gillmor CS, Scheible WR (2000) Positional cloning in Arabidopsis: why it feels good to have a genome initiative working for you. Plant Physiol 123: 795–805

Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL, Somerville CR (2001) Arabidopsis cytoplasmic β1,3-glucanase-deficient mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. Proc Natl Acad Sci USA 98: 2262–2267

MacAlister CA, Ohashi-Ito K, Bergmann DC (2007) Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature 445: 537–540

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Feng JH, Geer LY, Geer RC, Gonzales NR, et al (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39: D225–D229

Mouille GG, Robin SP, Lecomte MG, Pagant SR, Höfte H (2003) Classification and identification of Arabidopsis cell wall mutants using Fourier-transform infrared (FT-IR) microscopy. Plant J 38: 393–404

Nadeau JA, Sack FD (2002) Control of stomatal distribution on the Arabidopsis leaf surface. Science 296: 1697–1700

Nakajima K, Sena G, Nawy T, Beneyf PN (2001) Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413: 307–311

Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J 51: 1126–1136

Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T (2007) The secretory peptide gene EPF11 drives the stomatal one-cell-spacing rule. Genes Dev 21: 1720–1725

Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T (2009) Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis leaves. Plant Cell Physiol 50: 1019–1031
Ohashi-Ito K, Bergmann DC (2006) Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell 18: 2493–2505

Pagant S, Bichet A, Sugimoto K, Lerouxel O, Desprez T, McCann M, Lerouge P, Vernhettes S, Höfte H (2002) KOBITO1 encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in Arabidopsis. Plant Cell 14: 2001–2013

Persson S, Wei H, Milne J, Page GP, Somerville CR (2005) Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. Proc Natl Acad Sci USA 102: 8633–8638

Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU (2007) Termination of asymmetric cell division and differentiation of stomata. Nature 445: 501–505

Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Harashimura I (2010) Stomagen positively regulates stomatal density in Arabidopsis. Nature 463: 241–244

Thomas CL, Bayer EM, Ritzenhailer C, Fernandez-Calvino L, Maule AJ (2008) Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. PLoS Biol 6: e7

Töller A, Brownfield L, Neu C, Twell D, Schulze-Lefert P (2008) Dual function of Arabidopsis glucan synthase-like genes GSL8 and GSL10 in male gametophyte development and plant growth. Plant J 54: 911–923

Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell 8: 735–746

Vanzin GF, Madson M, Carpita NC, Raikhel NV, Keegstra K, Reiter WD (2002) The mur2 mutant of Arabidopsis thaliana lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. Proc Natl Acad Sci USA 99: 3340–3345

Velasquez SM, Ricardi MM, Dorosz JG, Fernandez PV, Nadra AD, Pol-Fachin L, Egelund J, Gille S, Harholt J, Ciancia M, et al (2011) O-Glycosylated cell wall proteins are essential in root hair growth. Science 332: 1401–1403

von Arnim AG, Deng X-W, Stacey MG (1998) Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene 221: 35–43

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 19: 63–73

Yuan Z, Teasdale RD (2002) Prediction of Golgi type II membrane proteins based on their transmembrane domains. Bioinformatics 18: 1109–1115

Zhang L, Hu G, Cheng Y, Huang J (2008) HeterotrimERIC G protein alpha and beta subunits antagonistically modulate stomatal density in Arabidopsis thaliana. Dev Biol 324: 68–75