Nuclear Ribosome Biogenesis Mediated by the DIM1A rRNA Dimethylase Is Required for Organized Root Growth and Epidermal Patterning in Arabidopsis

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Position-dependent patterning of hair and non-hair cells in the Arabidopsis thaliana root epidermis is a powerful system to study the molecular basis of cell fate specification. Here, we report an epidermal patterning mutant affecting the ADENOSINE DIMETHYL TRANSFERASE 1A (DIM1A) rRNA dimethylase gene, predicted to participate in rRNA posttranscriptional processing and base modification. Consistent with a role in ribosome biogenesis, DIM1A is preferentially expressed in regions of rapid growth, and its product is nuclear localized with nucleolus enrichment. Furthermore, DIM1A preferentially accumulates in the developing hair cells, and the dim1A point mutant alters the cell-specific expression of the transcriptional regulators GLABRA2, CAPRICE, and WEREWOLF. Together, these findings suggest that establishment of cell-specific gene expression during root epidermis development is dependent upon proper ribosome biogenesis, possibly due to the sensitivity of the cell fate decision to relatively small differences in gene regulatory activities. Consistent with its effect on the predicted S-adenosyl-l-Met binding site, dim1A plants lack the two 18S rRNA base modifications but exhibit normal pre-rRNA processing. In addition to root epidermal defects, the dim1A mutant exhibits abnormal root meristem division, leaf development, and trichome branching. Together, these findings provide new insights into the importance of rRNA base modifications and translation regulation for plant growth and development.

INTRODUCTION

The proper development of multicellular organisms from a single cell requires precise regulation of cell specification and patterning. In plants, the root epidermis of Arabidopsis thaliana provides a useful model tissue for studying the molecular mechanisms involved in these processes (Schiefelbein et al., 2009). The root epidermis contains two types of cells, hair cells and non-hair cells, which arise in a stereotyped pattern influenced by cell position. Root hair cells are located outside the boundary between two cortical cells (designated the “H” cell position), whereas non-hair cells occur over a single cortical cell (designated the “N” position) (Figure 1A). Root epidermal cells are formed and differentiate continuously in cell files that reflect their history, which means that a complete sequence of developmental events can be observed along the axis of root growth.

Molecular genetic analyses have provided a framework for understanding the regulatory network for root epidermal cell specification in Arabidopsis (Bruex et al., 2012). The five genes, WEREWOLF (WER), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), TRANSPARENT TESTA GLABRA1 (TTG1), and GL2 are required to specify the non-hair fate (Galway et al., 1994; Masucci et al., 1996; Lee and Schiefelbein, 1999; Bernhardt et al., 2003). Current models suggest that WER (an R2R3-MYB protein), GL3/EGL3 (related basic helix-loop-helix proteins), and TTG1 (a WD40 repeat protein) work together in N position cells as a core transcriptional activation complex to promote the non-hair cell fate (Lee and Schiefelbein, 2002; Pesch and Hülskamp, 2004) by directly promoting expression of the homeodomain transcription factor GL2 (Rerie et al., 1994; Di Cristina et al., 1996; Koshino-Kimura et al., 2005) (Figure 1A). GL2 is necessary for non-hair cell specification (Masucci et al., 1996), by negatively regulating root hair-specific genes and positively regulating non-hair-specific genes (Masucci et al., 1996; Lee and Schiefelbein, 1999, 2002; Ohashi et al., 2003). The core activation complex also promotes lateral inhibition by inducing transcription of CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF CPC AND TRY (ECT1), which act semi-redundantly to promote the hair cell fate (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004; Wang et al., 2008) by competing with WER for binding to other activation complex members (Lee and Schiefelbein, 1999, 2002; Tominaga et al., 2007). In an additional feedback loop, GL3 and EGL3 are negatively regulated by the core activation complex in the non-hair cells (Bernhardt et al., 2005). SCRAMBLED (SCM), a leucine-rich repeat receptor-like kinase, is necessary for the position dependence of root epidermal cell fate specification (Kwak et al., 2005) due to its ability to repress WER transcription in H position cells (Kwak and Schiefelbein, 2007) (Figure 1B).

Although the importance of ribosomes in mRNA translation is well known, recent studies have implicated ribosomal proteins and ribosome assembly factors in regulatory aspects of plant development (reviewed in Byrne, 2009). Ribosome biogenesis...
Figure 1. Cell-Type Expression of GL2pro-GUS Is Altered in 45-137/dim1A.
is a complex multistep process requiring coordinated transcription, RNA processing, RNA modification, and the folding and complexing of RNAs and proteins. For nucleus-derived ribosomes in eukaryotes, transcription of the rDNA genes and the majority of rRNA processing events occur in the nucleolus, followed by ribosome assembly in the cytoplasm. The rDNA gene is transcribed as a single 35S/45S pre-rRNA, which is processed and chemically modified (reviewed in Brown and Shaw, 1998). These rRNA modifications primarily occur in functionally significant regions, although their precise role(s) is largely unknown (Decatur and Fournier, 2002). Among these, only three post-transcriptional rRNA modifications identified to date are conserved in all three domains of life (Van Knippenberg et al., 1984; McCloskey and Rozenski, 2005), with two of these involving methylation of adjacent adenosines in the 3'-terminal loop (helix 45) of the small subunit rRNA (Van Knippenberg et al., 1984). Research findings from Saccharomyces cerevisiae suggest that in eukaryotes, these methylation are not required for ribosome function, but the dimethylase enzyme that catalyzes the methylation reaction, Dim1p, is indispensable due to its role in pre-rRNA processing (Lafontaine et al., 1994, 1995; Pulicherla et al., 2009). Interestingly, in prokaryotes, a lack of the methylations in Dim1p, is indispensable due to its role in pre-rRNA processing (Lafontaine et al., 1994, 1995; Pulicherla et al., 2009). Interestingly, in prokaryotes, a lack of the methylations and/or the dimethylase enzyme only modestly affects ribosome function and organism fitness (Heiser et al., 1972; Poldermans et al., 1979; van Buul et al., 1984; O'Connor et al., 1987).

Here, we report the identification of a root epidermal patterning mutant of Arabidopsis that affects a member of the KsgA/Dim1 family of highly conserved nuclear rRNA dimethylases, named DIM1A. We conducted a detailed analysis of the dim1A mutant and its effect on epidermal patterning as well as root meristem cell division, leaf morphogenesis, and trichome branching. Furthermore, we show that the point mutation in the dim1A mutant generates an enzyme that lacks methylase activity but maintains pre-rRNA processing ability, allowing us to study the functional importance of DIM1A-dependent rRNA methylation. Taken together, we propose that the modifications catalyzed by Arabidopsis DIM1A are necessary for generating appropriate patterns of gene expression during root development, including the cell-specific expression of transcriptional regulators in the root epidermis.

### RESULTS

#### Identification of a Mutant of the Arabidopsis DIM1A Gene

A recessive mutant, preliminarily designated 45-137, was identified in a forward genetic screen for root epidermal patterning defects among ethyl methanesulfonate–mutagenized plants expressing the non-hair-cell-specific reporter GL2pro::GUS (for β-glucuronidase). The 45-137 mutant was found to express GL2pro::GUS in a pattern that is not strictly associated with the position of epidermal cells relative to the underlying cortical cells. In wild-type roots, ~95% of developing epidermal cells in the H position lack GL2pro::GUS expression, and 100% of cells in the N position express GL2pro::GUS (Figures 1C and 1D). In 45-137 roots, only 80% of developing epidermal cells in the H position lack GL2pro::GUS, and 94% of cells in the N position express GL2pro::GUS (Figures 1C and 1D). Accordingly, in the mature portion of the root, the 45-137 mutant displays a significant

### Table 1. Specification of Cell Types in the Root Epidermis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>H Cell Position</th>
<th>N Cell Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hair Cells (%)</td>
<td>Non-Hair Cells (%)</td>
</tr>
<tr>
<td>Wild type (Ws)</td>
<td>47.5 ± 3.0</td>
<td>95.0 ± 6.1</td>
</tr>
<tr>
<td>dim1A</td>
<td>40.8 ± 10.1</td>
<td>80.3 ± 15.1a</td>
</tr>
<tr>
<td>wer-1</td>
<td>94.5 ± 5.5</td>
<td>96.5 ± 6.7</td>
</tr>
<tr>
<td>dim1A wer-1</td>
<td>83.5 ± 9.6</td>
<td>94.5 ± 6.9</td>
</tr>
</tbody>
</table>

At least 20 5-d-old seedlings were examined for each line. Values represent mean ± se.

* aDiffers significantly from the wild type (P < 0.01; Student’s t test).

### Figure 1. (continued).

(A) Diagram of a transverse section of an Arabidopsis root meristem. The primary root is organized in concentric rings of cells including the endodermis (En), cortex (C), and the epidermis (which is comprised of non hair cells [N] and hair cells [H]).

(B) Current model of epidermal patterning in Arabidopsis. Note: solid lines represent gene regulation, and dashed lines indicate protein movement.

(C) Analysis of position-dependent expression of GL2 using promoter-reporter transgene GL2pro::GUS in the wild type (WT; Ws), the 45-137/dim1A mutant, two dim1A complementation lines, two DIM1Apro::DIM1A-GFP lines, and the wild type treated with 5 µg/mL cycloheximide. Images are representative roots showing the expression pattern of GL2pro::GUS in the root meristem of 4-d-old seedlings.

(D) Quantification of the epidermal cell-type pattern, showing frequencies of ectopic GL2pro::GUS-expressing cells in the H cell position (gray bars) and ectopic non-GL2pro::GUS–expressing cells in the N cell position (black bars), for each line. The mean and SD are indicated for each line. Complementation lines A- and B- are included as negative controls for complementation lines A and B, respectively. Symbols indicate a value significantly different than the corresponding wild-type (WT) value (using t test): asterisk, P < 0.01.
Figure 2. Mapping and Identification of a Mutant of the Arabidopsis DIM1A Gene.
increase in the frequency of non-hair cells in H cell files (20% compared with 5% in the wild type) (Table 1). The dim1A root phenotype was variable, with some roots appearing nearly normal and others exhibiting severe defects in epidermal pattern, yet the average difference between the dim1A and the wild type was statistically significant (Figure 1D, Table 1).

A map-based cloning approach was taken to identify the gene affected by the dim1A mutation. Using bulk-segregant analysis of F3 pools followed by detailed mapping with simple sequence length polymorphism, cleaved-amplified polymorphic sequence, and derived cleaved-amplified polymorphic sequences primers (see Supplemental Table 1 online), we narrowed the mutation to an 83-kb region (19.408 to 19.490) containing 41 genes (Figure 2). Sequence analysis of genes in this region identified a G-to-A change in locus At2g47420 at position 19,457,770, which causes a predicted Gly to Glu missense at position 66 in the gene product (Figure 2). To verify that the mutation in this locus caused the 45-137 mutant phenotype, a construct containing a full-length genomic fragment of At2g47420 was stably transformed into 45-137 plants. This construct fully complemented the GL2pro::GUS cell-type expression defect of the 45-137 mutant (Figures 1C and 1D), indicating that the phenotypic defects seen in 45-137 result from the single base change identified in locus At2g47420. As the At2g47420 gene was previously designated D1M1A (Richter et al., 2010), we refer to the 45-137 mutant as dim1A.

**DIM1A** encodes a putative 18S rRNA S-adenosyl-L-Met (SAM)-dependent adenosine dimethyltransferase. It exhibits extensive sequence similarity to members of the KsgA/Dim1 family of rRNA dimethylases, including the well-characterized Dim1p in *S. cerevisiae* and KsgA in *Escherichia coli* (45.6 and 21.8% amino acid sequence identity and 59.7 and 31.1% sequence similarity, respectively). These enzymes catalyze the methylation of two adjacent conserved adenosines at the 3’ end of 18S rRNA (in eukaryotes) or 16S rRNA (in prokaryotes and eukaryotic organelles) (Helser et al., 1972; Lafontaine et al., 1995) and are involved in processing of precursor rRNA to generate mature rRNA for subunit assembly (Lafontaine et al., 1995; Pulicherla et al., 2009). The *Arabidopsis* genome encodes a total of three rRNA dimethylase enzymes: DIM1A (At2g47420), described here; PALEFACE1 (At1g01860), a plastid-localized enzyme important for cold tolerance (Tokuhisa et al., 1998); and DIM1B (At5g66360), a mitochondrial-localized enzyme recently shown to methylate mitochondrial rRNA (Richter et al., 2010). Thus, DIM1A appears to be the only 18S rRNA dimethylase in *Arabidopsis* devoted to the biogenesis of nuclear-derived ribosomes.

**The dim1A Mutation Affects rRNA Base Modification but Not Processing**

The dim1A missense mutation alters a conserved Gly residue within the canonical SAM binding sequence, shown in bacterial KsgA to form part of the cofactor binding pocket (Tu et al., 2009) (Figure 2). Considering this, we hypothesized that the dim1A gene may be expressed normally and its product may process pre-rRNA normally, but it may have altered methylation ability due to the change in its predicted SAM binding sequence. As a first test of this hypothesis, we used RT-PCR to analyze **DIM1A** mRNA accumulation, and we found similar transcript levels in **dim1A** and wild-type seedlings (see Supplemental Figure 1 online), indicating that the **DIM1A** gene is transcribed normally in the **dim1A** mutant.

To assess the methylation status of the two adjacent adenosines at the 3’ end of 18S rRNA (A1785 and A1786) in **dim1A** and the wild type, we used a primer extension experiment, which takes advantage of the inability of reverse transcriptase to extend an oligonucleotide past a N-6 dimethylated adenosine (Hagenbüchle et al., 1978) (Figure 3A). Using RNA from wild-type plants, primer extension was inhibited at the rRNA bases A1785 and A1786, indicating that these adenosines possess the N-6 dimethyations (Figure 3B). However, using RNA from **dim1A**, the primer extension reaction proceeded beyond A1785 and A1786, terminating at U1783 as a result of inclusion of ddATP instead of dATP in the nucleotide mixture to force the reaction to stop at a measurable distance (Figure 3). This result indicates that **DIM1A** is necessary for the N-6 dimethylation of the adenosines at positions 1785 and 1786 of the 18S rRNA and that disruption of a single conserved Gly within the SAM binding domain is sufficient to abolish this methylation.

Yeast Dim1p is required not only for 18S rRNA adenosine dimethylation, but also for 35S pre-rRNA processing (Lafontaine et al., 1995), and these two functions are separable (Lafontaine et al., 1998). To determine whether the *Arabidopsis dim1A* mutation affects rRNA processing, we used an RT-PCR–based assay with primers designed to flank the putative processing

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**Figure 2.** (continued).
(A) Fine mapping using individual F2 plants delimited the 45-137 mutation to an 83-kb region on the lower arm of chromosome 2 between markers CER447606 and CER446003. At2g47420 contains two exons (black bars) and one intron (gray bar), and it encodes **DIM1A**, a putative rRNA dimethyladenosine transferase.

(B) Multiple alignment of the amino acid sequences from eukaryotic **DIM1A** homologs. The highly conserved β sheets (β1 and β2) and α helix (α1) important for SAM binding are indicated. Binding “motif 1” (shown as a black bar) contains the canonical GXGXG SAM binding sequence. The asterisk within motif 1 indicates the position of the mutation in **dim1A**. **Arabidopsis** (At), *Zea mays* (Zm), *Chlamydomonas reinhardtii* (Chl), *Physcomitrella patens* (Pp), *Dictyostelium discoideum* (Dd), *S. cerevisiae* (Sc), *Cryptococcus neoformans* (Cn), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), and *Giardia lamblia* (Gl). Black shading indicates 90% consensus, gray shading indicates 50% consensus, the exclamation point is any one of IV, the dollar sign is any one of LM, the percent symbol is any one of FY, and the hash tag is any one of NDQEBZ.
sites (Shi et al., 2005; Huang et al., 2010) (Figure 4A). The results show that similar levels of unprocessed 5' external transcribed spacer (ETS) are detected from wild-type and \textit{dim1A} seedling tissue (Figure 4B), suggesting that the \textit{dim1A} mutation affects 18S rRNA dimethylation, but not pre-rRNA processing.

The results described above suggest that aberrant ribosome formation may be responsible for the root development defects in the \textit{dim1A} mutant. To explore this possibility further, we analyzed the effect of cycloheximide, a specific inhibitor of eukaryotic cytoplasmic ribosome function, on root epidermal patterning in wild-type seedlings. Using a concentration of cycloheximide (5 µg/mL) that affected root growth to the same extent as the \textit{dim1A} mutant on unsupplemented media, we observed a significant change in the specification of the hair and non-hair cell types (Figures 1C and 1D). This suggests that the abnormal patterning in \textit{dim1A} is due, at least in part, to abnormal cytoplasmic ribosome function.

Expression and Localization of \textit{DIM1A} in Developing Tissues

To investigate the transcriptional control of the \textit{DIM1A} gene, a \textit{DIM1A} promoter GUS fusion construct, \textit{DIM1A}_{pro:GUS}, was
generated and characterized. This construct was made with the same promotor region shown to complement the dim1A mutation when driving expression of the DIM1A coding region (Figures 1C and 1D). Examination of 12 independent transgenic lines showed that DIM1Apro:GUS expression was detectable at the tips of emerging 2-d-old roots, with the strongest expression in the epidermis and vasculature (Figure 5A). The level of epidermal expression was greatest in the meristematic region, with cell-to-cell variability within each individual root that did not appear to be related to cell type or position (Figure 5A). In older roots, the level of DIM1Apro:GUS expression was diminished and restricted to the vasculature and lateral root primordia when roots were stained for shorter times, but expression was detected in all root meristematic cells upon longer incubation times (Figures 5B and 5F). In developing cotyledons and leaves, DIM1Apro:GUS expression was observed at the tip of the leaves as well as in the vascular tissue (Figures 5E and 5G). DIM1Apro:GUS was expressed throughout floral tissue, particularly in the vasculature of petals, anther, and pollen, and was enriched in stem vasculature and the siliques abscission zone (Figures 5C and 5D).

As an independent assessment of DIM1A expression, we analyzed DIM1A transcript accumulation data from available Arabidopsis microarray datasets, including Genevestigator (Zimmermann et al., 2004; www.genevestigator.ethz.ch) and the eFP database (Winter et al., 2007; www.bar.utoronto.ca/efp). These data indicate that DIM1A is expressed in rapidly dividing regions of the plant, such as embryonic tissue, leaf primordia, and root meristems. Within the root meristem, DIM1A RNA accumulates in all tissues, with its abundance diminishing as the tissues mature (Brady et al., 2007; www.bar.utoronto.ca/efp). These findings largely confirm our reporter expression data.

To examine the subcellular and organ-wide distribution of the DIM1A protein, we generated a C-terminal green fluorescent protein (GFP) translational fusion construct of the DIM1A coding region, driven by the DIM1A promoter. When introduced into the dim1A mutant, this DIM1Apro::DIM1A-GFP construct fully complemented the dim1A mutant phenotypes (Figures 1C and 1D), suggesting that the DIM1A-GFP fusion protein is functional. Confocal imaging revealed that DIM1A-GFP was localized primarily in root cell nuclei, most strongly in subnuclear regions presumed to be the nucleolus (Figures 6A to 6C). This result is consistent with recent work showing nucleolar localization of DIM1A-GFP transiently expressed in Arabidopsis protoplasts (Richter et al., 2010) and predominantly nucleolar localization of Dim1p in yeast (Lafontaine et al., 1998). At an organ-wide level, DIM1A-GFP was observed in all tissue types in the developing root (Figure 6F). A comparison of DIM1Apro::DIM1A-GFP (Figure 6) to DIM1Apro::GUS results (Figure 5) indicates considerable overlap in root cells expressing the DIM1A promoter and accumulating DIM1A protein, although DIM1A protein appears to persist in differentiated (older) root cells that lack detectable DIM1A promoter activity. Interestingly, in the root epidermis, DIM1A-GFP appeared to accumulate and persist to a greater extent in the H position cells than in the N position cells (Figures 6D and 6E; 14/15 H cell files examined exhibited preferential GFP accumulation relative to N cell files). This is likely a post-transcriptional effect, since differential DIM1Apro::GUS expression was not observed (Figure 5), and it may reflect a preferential requirement for DIM1A and/or ribosome function in the differentiation of root hair cells.

In other species, excessive production of a normal or catalytically inactive member of the KsgA/Dim1 protein family can affect growth or viability (Connolly et al., 2008; Pulicherla et al., 2009). To assess the effect of excessive DIM1A or mutant dim1A protein in Arabidopsis, we overexpressed C-terminal GFP fusion constructs of the DIM1A or dim1A alleles using the strong cauliflower mosaic virus 35S promoter. Each of these constructs, 3SS::DIM1A-GFP and 3SS::dim1A-GFP, were introduced into wild-type and dim1A mutant plants, and multiple homozygous transgenic lines were analyzed for each combination. In the dim1A mutant background, overexpressing the G66E dim1A mutant did not have any impact on epidermal patterning, while overexpressing the wild-type DIM1A essentially complemented the dim1A mutant phenotype (see Supplemental Figure 2 online). In the wild-type background, overexpressing either wild-type DIM1A or mutant G66E dim1A had little or no effect on epidermal patterning (see Supplemental Figure 2 online) or overall plant development. Overall, the results from these overexpression experiments suggest that, in plants, an excessive amount of the normal DIM1A or methylase-deficient version of DIM1A protein does not affect epidermal patterning or meristem development.

Figure 5. DIM1A is Expressed in Developing Tissues.

Histochemical GUS staining pattern of transgenic plants carrying the DIM1Apro::GUS transgene. T3 plants identified as homozygous for DIM1Apro::GUS were observed: (A) 2-d-old root tip; (B) 4-d-old root tips stained for moderate (left) and extended (right) times; (C) floral tissue (stigma and petals); (D) anther, pollen grains, and sepal vasculature; (E) developing leaves and cotyledon of 10-d-old plant; (F) lateral root emergence site and vascular tissue staining in two different 4-d-old seedlings; and (G) 2-d-old cotyledon.
Our initial analyses showed that the dim1A mutant affects the position-dependent pattern of GL2 promoter activity in the root epidermis (Figure 1). To define the role of DIM1A further, we analyzed the effect of dim1A on the expression of the hair cell reporter EGL3::GUS and the non-hair cell reporters CPC::GUS and WER::GFP, which were independently introduced into the dim1A mutant by crossing.

In the dim1A EGL3::GUS plants, we did not observe a significant change in the amount or pattern of GUS activity in the root epidermis (Figure 7B). This suggests that the cell position-dependent regulation and expression level of EGL3 in the root epidermis does not require wild-type DIM1A.

In the dim1A CPC::GUS plants, we observed a severe reduction in GUS activity in the root epidermis compared with wild-type plants, although the characteristic expression of this GUS reporter in the developing vascular tissue was still present (Figure 7A). After a long staining period, we did observe weak CPC promoter activity in the root epidermis. These results show that DIM1A is necessary for proper epidermal cell expression of CPC.

In the dim1A WER::GFP line, we observed the usual non-hair-specific pattern of the WER::GFP reporter, but we noticed a difference in the relative abundance of GFP accumulation in cells located in the H and N files, relative to the wild-type WER::GFP line (Figures 7C and 7D). Quantification of the level of WER::GFP expression, performed by measuring pixel content in individual cells in the upper one-third of the root meristem, revealed a significant reduction in the N/H cell ratio of GFP in dim1A compared with the wild type (1.5 versus 3.2) (Figure 7E), due to an almost twofold increase in WER::GFP expression in the H file cells of dim1A. These results suggest that DIM1A is required to establish or maintain the high N/H ratio of WER expression during root epidermis cell specification.

**Effect of dim1A on the GL2, CPC, and WER Patterning Genes**

The results described above suggest that DIM1A is required for proper specification of the hair cell fate through its effects on CPC and WER expression. To analyze further the effect of DIM1A on epidermal cell fate specification, we introduced the dim1A mutation into the wer, scm, gl2, and ttg1 single mutant and egl3/gl3 double mutant backgrounds, in the presence of the GL2::GUS reporter, to investigate possible genetic interactions.

WER is necessary for specification of the non-hair cell fate and for GL2 expression, as illustrated by the excess root hairs and reduced GL2::GUS expression in the wer-1 mutant (Lee and Schiefelbein, 1999, 2002). Interestingly, we found that the wer-1 dim1A double mutant contained dramatically more GL2::GUS-expressing epidermal cells than the wer-1 single mutant (Figure 7F). Specifically, we found that only 4% of wer-1 dim1A roots (n = 83) completely lacked GL2::GUS-expressing meristematic epidermal cells, compared with 75% of wer-1 roots (n = 44). Furthermore, while only 9% of wer-1 mutant roots had more than five GL2::GUS-expressing epidermal cells, 72% of wer-1 dim1A roots showed more than five GL2::GUS-expressing cells. Consistent with its increased GL2 promoter activity, the wer-1 dim1A double mutant had significantly fewer root hair cells than the wer-1 mutant (83.5 and 94.5%, respectively),
Figure 7. Genetic Interaction of dim1A and Epidermal Patterning Genes.

(A) to (D) Expression pattern of the following transcription factor reporter genes in roots of wild-type (WT) and dim1A 4-d-old seedlings: CPCpro:GUS (A), EGL3pro:GUS (B), and WERpro:GFP (C) and (D). Images in (D) represent magnification of region within boxes diagrammed in (C). Bars = 50 μm in (A), (B), and (D) and 25 μm in (C).

(E) Graphical representation of the ratio of the amount of GFP (expressed from the WER promoter) measured in cells in the N position to the amount measured in cells in the H position in wild-type (gray bars) and dim1A roots (black bars). GFP pixel quantification was done in cells in the uppermost (shootward) one-third of the meristem. Measurements are expressed in terms of total pixels (left) and pixels/area (right). The mean and SD are indicated for each line.

(F) and (G) The genetic interaction of dim1A and epidermal patterning mutants using expression level and pattern of GL2pro:GUS as a readout.
due to a reduction in the fraction of N-position cells that differentiate into hair cells (Table 1). These additive genetic interactions suggest that *wer* and *dim1A* mutations affect root epidermal cell specification in opposing ways, with *wer* promoting the hair pathway and *dim1A* the non-hair pathway.

The SCM leucine-rich repeat receptor-like kinase is required for epidermal cells to interpret their position relative to underlying cortical cells, and mutations in *scm*, like *dim1A*, result in a disorganized pattern of specified cell fates (Kwak et al., 2005; Kwak and Schiefelbein, 2007). Following the introduction of *dim1A* into the *scm-2* loss-of-function mutant, we discovered a partial rescue of the *scm-2* epidermal cell-type patterning phenotype by *dim1A* (Figures 7F and 7G). The double mutant showed a slight reduction in *GL2pro:GUS*-expressing cells in the H position, from 40.6% in *scm-2* to 32.2% in *scm-2 dim1A*, and a greater reduction in *GL2pro:GUS* nonexpressing cells in the N position, from 30.6% in *scm-2* to 9.2% in *scm-2 dim1A* (Figure 7G).

The inclusion of the *dim1A* mutation in the *ttg1*, *gl3 egl3*, or *gl2* mutant backgrounds did not alter their *GL2pro:GUS* root expression phenotypes compared with the respective single mutant phenotypes (Figure 7F), which suggests a lack of genetic interaction between *dim1A* and these patterning genes.

**Effect of *dim1A* On Root and Shoot Vegetative Development**

Primary roots of *dim1A* seedlings are shorter than wild-type seedlings due to an ∼50% reduction in root growth rate (Figures 8A and 8B). Quantitative analysis of root epidermal cell length showed that *dim1A* does not differ significantly from the wild type (Figure 8C), implicating reduced cell production rather than reduced cell size as the primary cause for its decreased root length and growth rate. Examination of root meristems (defined as the region of the root tip containing actively dividing cells; Figure 8D) revealed a reduction in cell number in *dim1A*. Compared with wild-type roots, the *dim1A* mutant meristematic region contained 60.5% of the H position epidermal cells, 67.9% of the N-position epidermal cells, and 49.9% of the cortical cells (Figure 8E). Interestingly, the cells observed in the *dim1A* meristematic region were longer than their wild-type counterparts (Figure 8E), suggesting diminished cell division activity.

To assess the relative rate of cell division within the meristem, we examined epidermal clones in wild-type and *dim1A* roots. These clones arise from rare longitudinal-oriented divisions of epidermal cells within the meristem that produce a recognizable pair of short cell files within a single cell file, with the final cell number in the clone dictated by the number of rounds of cell division that occur (Figure 9A) (Berger et al., 1998). Examination of more than 100 clones each from wild-type and from *dim1A* roots suggest a reduced cell division rate in the *dim1A* clones (Figure 9B). The most frequent type of clone in wild-type root meristems was derived from five rounds of division (class five clones), whereas the most frequent *dim1A* clone was due to three rounds of division (class three clones). Additionally, while 18.9% of wild-type clones were class six, we only observed one class six clone in *dim1A* roots (Figure 9B). Together, these results indicate that cell division is reduced in the *dim1A* roots.

To determine whether the reduced cell division rate in the *dim1A* mutant might be responsible for its epidermal cell patterning defect, we analyzed the pattern of *GL2pro:GUS* reporter expression in wild-type roots treated to inhibit cell division. For this purpose, we used hygromycin, streptomycin, puromycin, and tetracycline, which were all effective in reducing cell number in the root epidermis (see Supplemental Figure 3A online). However, none of these treatments significantly affected the *GL2pro:GUS* expression pattern (see Supplemental Figures 3B and 3C online), suggesting that the epidermal cell-type patterning defect in the *dim1A* mutant cannot be mimicked by merely inhibiting meristem cell division.

We observed other (non-root) morphological defects in the *dim1A* mutant, including pointed leaves, reduced leaf size, and disrupted leaf venation (Figure 10). These phenotypes are similar to ones observed in other *Arabidopsis* ribosome assembly factor mutants, including nuc-11/par1 (Kojima et al., 2007; Petricka and Nelson, 2007; Pontvianne et al., 2007), oligocellula2 (Fujikura et al., 2009), and apum23-1 (Abbasi et al., 2010), as well as the phenotypes of *Arabidopsis* ribosomal protein mutants, including rpl24b/stv1, rpl4a, and rps23a (Nishimura et al., 2005; Degennhardt and Bonham-Smith, 2008; Rosado et al., 2010). Interestingly, we did not detect a difference in leaf cell shape or stomatal development (Figure 10E) as observed in other ribosomal mutants (Fujikura et al., 2009; Rosado et al., 2010). However, we did detect a reduction in trichome branching in the *dim1A* mutant (Figure 10F), suggesting that functional *DIM1A* may be needed for normal trichome development.

**DISCUSSION**

The Effect of *dim1A* On Epidermal Cell Fate Specification

The position-dependent patterning of root hair and non-hair cells of the *Arabidopsis* root epidermis serves as a useful experimental model to investigate the molecular basis of cell fate specification in plants. Here, we discovered a previously unrecognized requirement for a ribosome assembly factor for the appropriate regulation of this cell-type patterning process.

Figure 7. (continued).

(F) Expression of the *GL2pro:GUS* transcriptional reporter in the following lines: the wild-type and *dim1A*; *ttg1* and *dim1A ttg1*; *gi3 egl3* and *dim1A gi3 egl3*; *wer* and *dim1A wer*; *scm-2* and *dim1A scm-2*; and *gl2* and *dim1A gl2*. Bars = 50 µm.

(G) Quantification of the epidermal cell-type pattern, showing frequencies of ectopic *GL2pro:GUS*-expressing cells in the H cell position (gray bars) and ectopic non-*GL2pro:GUS*–expressing cells in the N cell position (black bars) in wild-type, *dim1A*, *scm-2*, and *dim1A scm-2* lines. The mean and SD are indicated for each line.
Using a cell-specific reporter screen, we identified a mutant with abnormal root epidermal patterning and showed that the corresponding gene encodes the rRNA dimethyltransferase DIM1A. Our findings suggest that the dim1A mutation alters root epidermal patterning by disrupting the establishment of distinct gene expression patterns required for root hair and non-hair cell specification. Several lines of evidence support this conclusion. First, the functional DIM1A-GFP fusion protein exhibits differential accumulation in the two root epidermal cell types, preferentially persisting in developing cells in the H position, which implies that DIM1A is associated with the differentiation of these cell types. Second, the major effect of the dim1A mutation is a decrease in the frequency of root hair cells, together with a corresponding increase in non-hair cells and GL2pro:GUS expression, in the H position of the root epidermis. Given that ectopic GL2 expression can cause epidermal cells to differentiate as non-hair cells (Lee and Schiefelbein, 2002; Wada et al., 2002), it is likely that the misspecification of the H position cells is due to ectopic GL2 gene expression. Third, the dim1A mutation diminishes the differential expression of WER in the developing N and H cells and reduces CPC expression in the N cells. According to current models, epidermal cell specification relies on the balance between non-hair cell–promoting R2R3 MYB domain proteins (WER and MYB23) and hair cell–promoting R3 MYB domain proteins (CPC, TRY, and ETC1) (Lee and Schiefelbein, 1999, 2002; Wada et al., 2002; Simon et al., 2007; Tominaga et al., 2007). Given this, the increase in GL2pro:GUS-expressing cells in the H position of dim1A may result from a relatively high level of WER in the H position, which reduces the ability of CPC to compete successfully with WER for binding to the TTG-GL3/EGL3 factors. Furthermore, the abnormal accumulation of the core transcriptional activation complex in H position cells may direct CPC-dependent lateral inhibition against the N cells, which may be responsible for the small number of ectopic hair cells in the N position observed in the dim1A mutant. Consistent with this suggestion, expression of WER equally in the N and H cells in a wer mutant background has been shown to cause a largely random pattern of GL2pro:GUS expression and cell fates (Lee and Schiefelbein, 2002).

In addition to increased GL2pro:GUS expression in the dim1A single mutant, we observed excessive GL2pro:GUS expression in the dim1A wer and dim1A scm-2 double mutants (compared with the respective single mutants), which further supports a role for DIM1A in restricting GL2 expression during wild-type root development. It is notable that an increase in GL2pro:GUS-expressing cells and non-hair cell specification is also observed in the wer cpc double mutant (relative to wer single mutant) (Lee and Schiefelbein, 2002; Simon et al., 2007). As mentioned...
previously, CPC negatively affects GL2 expression by competing with WER for binding to TTG-GL3/EGL3 (Lee and Schiefelbein, 2002). Considering this, and given the severe effect of dim1A on CPC reporter expression, it appears that CPC transcription is most sensitive to the dim1A defect, and this may be responsible for the other observed changes in this highly integrated regulatory network.

Several lines of evidence indicate that abnormal ribosome function in the dim1A mutant is most likely to be responsible for its altered root epidermal patterning. First, DIM1A is a member of a universal family of ribosome biogenesis factors, known to participate in the modification of the small subunit rRNA during ribosome assembly. This, by itself, does not preclude the possibility that DIM1A may have other functions and may therefore affect root epidermis development in a ribosome-independent manner. Second, we demonstrated a defect in methylated residues in the dim1A mutant, consistent with the predicted role of DIM1A and the point mutation within the SAM binding motif of the DIM1A protein. Third, the dim1A mutant exhibits non-root phenotypes that are similar to those found in other ribosome mutants, including RPL24b/STV1, RPS6a, and RPS6b, also result in reduced root length despite mature cell lengths similar to the wild type (Nishimura et al., 2005; Creff et al., 2010). Additionally, the rps6a and rps6b mutants, like the dim1A mutant, show about a 50% reduction in meristem cell content (Creff et al., 2010). These findings suggest a common requirement for ribosome biogenesis to maintain the root meristem cell division rate, and they indicate that reduced meristem cell production may be responsible for the short root phenotype observed in other ribosomal gene mutants as well. Interestingly, ribosome protein accumulation has been linked to cell cycle progression in animals. Depletion of the RPS6 homolog in mice liver revealed that the rate of ribosome production is sensed and regulates cell cycle progression (Volarevic et al., 2000). Thus, it is tempting to speculate that the reduced root length phenotype may result from root meristem cells slowing cell cycle progression after sensing a defect in the rate of ribosome biogenesis.

In addition to defects in root epidermal cell patterning and development, we also detected changes in development of shoot tissue. To date, 12 of the 17 observed ribosomal protein mutants display altered leaf shape (the pointed first leaf phenotype) (Creff et al., 2010) and many also display defects in vascular patterning (Byrne, 2009). Both of these phenotypes have often been attributed to a reduction in auxin-mediated cell divisions at the leaf margin (Petricka and Nelson, 2007;
Figure 10. dim1A Affects Leaf Morphology and Higher-Order Trichome Branching, but Not Leaf Epidermal Cell Shape or Stomata.

(A) Relative sizes of wild-type (top) and dim1A mutant (bottom) 14-d-old seedlings (left panel). Leaf shape characteristics of the first pair of true leaves emerging in 6-d-old seedlings (right panel).

(B) Quantification of the surface area of the first pair of true leaves (left) and second pair of true leaves (right) in wild-type (WT; gray bars) and dim1A (black bars) seedlings.
Degenhardt and Bonham-Smith, 2008; Rosado et al., 2010). In support of this, Weijers et al. (2001) observed that the leaf vascular patterning defect in the rps5a mutant resulted from a defect in cell division rather than differentiation of the vascular tissue. While further analysis is needed to determine the precise mechanism of ribosome protein and biogenesis factor involvement, it is clear that leaf development is sensitive to a disruption in these processes.

**Arabidopsis Ribosome Biogenesis and DIM1A**

The assembly of ribosomes from RNA and proteins is a complex process facilitated by a large number of ribosome biogenesis factors, including the KsgA/Dim1 rRNA dimethylase proteins (Karbstein, 2011). Based on findings in other systems, the KsgA/Dim1 proteins are important for rRNA methylation and pre-rRNA processing, as well as other potential aspects of ribosome assembly or cellular metabolism (Lafontaine et al., 1995; Inoue et al., 2007; Connolly et al., 2008; Strunk et al., 2011). For example, recent structural analyses indicate a role for KsgA/Dim1 proteins in regulating ribosome formation to prevent premature translation, by obstructing the binding of initiation factors and/or the large subunit (Strunk et al., 2011; Boehringer et al., 2012). Although members of the KsgA/Dim1 family appear to share some biochemical activities, it is important to note that significant differences are likely to exist between proteins from different species. As evidence of this sort of functional diversification, Dim1 from yeast and from Methanothermobacter arboracensi (an archaeal species) are able to complement the E. coli KsgA mutant, but neither the E. coli nor the archael gene could substitute for the yeast Dim1 (O’Farrell et al., 2006; Pulicherla et al., 2009).

Our characterization of the *Arabidopsis dim1A* mutant revealed a defect in rRNA methylation, but not pre-rRNA processing activity (Figures 3 and 4, respectively). The fact that this mutant is viable indicates that the methylated adenesines are not essential for ribosome function in plants. Interestingly, a similar catalytically inactive protein in *E. coli* generates severe growth defects when expressed in otherwise wild-type cells (Connolly et al., 2008), even though the ksgA null mutant lacking the 16S dimethyladenosines has only mild growth defects (Helser et al., 1972; Poldermans et al., 1979; O’Connor et al., 1997). These and other results led Connolly et al. (2008) to hypothesize that methylation is required for KsgA to be released from the small subunit rRNA (Connolly et al., 2008). Interestingly, this result differs from observations in yeast, where the growth of the catalytically inactive E85A dim1 mutant cells does not differ from the wild type (Pulicherla et al., 2009). Our results here show that expressing the catalytically inactive dim1A in wild-type *Arabidopsis* plants does not affect growth or epidermal patterning (see Supplemental Figure 2 online), suggesting that, unlike *E. coli* and similar to yeast, rRNA methylation may not be required for ribosome biogenesis. Additionally, our inability to isolate a *DIM1A* null mutant suggests that, unlike bacteria (Helser et al., 1972) but similar to yeast (Lafontaine et al., 1994, 1995), the *DIM1A* gene may be essential for *Arabidopsis* viability.

The precise effect of the methylase-deficient dim1A protein on ribosome formation is unclear. One possibility is that the lack of the adenosine dimethylations causes a conformational change in the ribosomal subunit that alters its function. Another possibility is that catalytically inactive dim1A may remain bound to the small subunit and block ribosome biogenesis. Structural analyses have indicated that interaction of *E. coli* KsgA and yeast Dim1 with their small subunits prevents binding of translation initiation factors, which opens the possibility that KsgA/Dim1 release may regulate ribosome biogenesis (Xu et al., 2008; Strunk et al., 2011; Boehringer et al., 2012). However, the limited effect of the *Arabidopsis dim1A* mutation on plant growth and the lack of a significant dim1A overexpression effect in wild-type plants together make it less likely that catalytically inactive dim1A remains bound to 18S rRNA.

Whereas structural analyses of KsgA in bacteria indicate that the two adenosine dimethylations are essential for optimal conformation of the small ribosomal subunit for translation (Xu et al., 2008; Demirci et al., 2010), we cannot necessarily conclude that this structural change also occurs in the *Arabidopsis* ribosome. In addition, we are unable to ascertain whether the absence of these methylations alone causes the developmental defects observed in the *dim1A* mutant background or if lack of the methylations disrupts binding of a ribosomal protein, affecting ribosome function. Because the adenosine dimethylations occur at helix 45 of the 18S rRNA within the interior region of the ribosome, which is devoid of ribosomal proteins (Decatur and Fournier, 2002), it seems more likely that the *dim1A* mutant phenotypes result from structural changes to the small subunit rather than depletion of a ribosomal protein.

Whereas further analysis is necessary to uncover the mechanistic explanation for *dim1A*’s effect on epidermal cell fate specification and overall plant development, our results document the importance of ribosome biogenesis for many aspects of plant development. Furthermore, they strengthen the notion that precise control of regulatory networks at multiple levels is essential for appropriate establishment of cell-specific gene expression programs in plant development.

**Figure 10.** (continued).

(C) and (D) Leaf vein patterning in cleared cotyledons (C) and first true leaves (D) of the wild type and *dim1A*.

(E) Adaxial surface of wild-type and *dim1A* leaves.

(F) The distribution of trichomes based on number of branches. The mean and SD are indicated for each line.

Note: With the exception of the images in the left panels of (A), all other *dim1A* images were magnified compared with the wild type for ease of comparison. [See online article for color version of this figure.]
METHODS

Plant Material and Growth Conditions

Seeds of Arabidopsis thaliana were surface sterilized and subsequently germinated and grown on mineral nutrient media solidified with 0.6% agarose as previously reported (Schiefelbein and Somerville, 1990). Unless otherwise noted, seedlings were analyzed after 4 d growth at 22°C under continuous illumination. For crosses, propagation, and trichome analysis, seedlings were transferred to soil and grown in growth chambers at 22°C under long-day conditions. The following lines were previously described: wer-1 (Lee and Schiefelbein, 1999), g9-3 (Koornneef, 1981), eg9-1 (Zhang et al., 2003), g2-1 (Koornneef, 1981), tga1-1 (Galway et al., 1994), scrm-2 (Kwak et al., 2005), WERpro:GFP (Lee and Schiefelbein, 1999), GL2pro:GUS (Masucci et al., 1996), EGL3pro:GUS (Zhang et al., 2003), and CPCpro:GUS (Wada et al., 2002). Reporter genes and mutations were introduced by crossing and subsequently verified as homozygous by phenotypic analysis (reporters) or molecular genotyping (mutant lines).

Patterning Mutant Identification and Positional Cloning

Seed mutagenesis of the GL2pro:GUS reporter line (Wassilewskija [Ws] ecotype) with ethyl methanesulfonate was performed as described (Lee and Schiefelbein, 1999). The 45-137 mutant was identified by screening M3 seedlings stained for GUS for defects in root epidermal GL2 promoter activity. 45-137 GL2pro:GUS plants were crossed to Landsberg erecta wild type to generate F2 and F3 offspring for genetic mapping (Lukowitz et al., 2003), WERpro:GFP (Lee and Schiefelbein, 1999), GL2pro:GUS (Masucci et al., 1996), EGL3pro:GUS (Zhang et al., 2003), and CPCpro:GUS (Wada et al., 2002). Reporters genes and mutations were introduced by crossing and subsequently verified as homozygous by phenotypic analysis (reporters) or molecular genotyping (mutant lines).

Cloning and Construction of Transgenic Plant Lines

A 3.2-kb genomic fragment spanning the full length of the Arabidopsis DIM1A gene (At2g47420), including the 5′ and 3′ intergenic spaces as well as the 3′ untranslated region and one exon and one intron of both the 5′ and 3′ neighboring genes, was amplified from DNA isolated from BAC clone T30B22 (ABRC) using PFU Turbo (Stratagene) and the primers 45137COMP-LP and 45137COMP-RP (see Supplemental Table 1 online). The amplified fragment was ligated into the Gateway pENTR/SD/TOPO vector (Invitrogen), and confirmed positives were recombined into Gateway vector pMDC99 using LR clonase (Invitrogen) after linearization of pENTR/DIM1A plasmid DNA. Reporter clones were sequenced, and plasmid DNA was isolated and transformed into Agrobacterium tumefaciens strain GV3101. To obtain the C-terminal GFP-tagged DIM1A protein, a 2.4-kb fragment containing the same upstream and genomic region of the complementation construct but stopping prior to the stop codon was amplified using primers 45137GFP-LP and 45137GFP-RP (see Supplemental Table 1 online) and cloned into Gateway vector pMDC99. To obtain the DIM1A promoter-GUS fusion, a 3.3-kb region upstream of the DIM1A start codon was amplified using the primers 45137PRO-LP and 45137GFP-RP (see Supplemental Table 1 online) and cloned into Gateway vector pMDC43. To obtain the overexpression lines in which DIM1A and 45-137 were expressed from the 35S promoter, a 1.2-kb genomic region of DIM1A from the start codon through the stop codon, which was replaced with Ala (TAA to GCC), was amplified using the primers 45137PRO-LP and 45137GFP-RP (see Supplemental Table 1 online) and subsequently cloned into GATEWAY vector pMDC43. Plants were transformed using a modified version of the Agrobacterium-mediated floral dip transformation protocol (Clough and Bent, 1998), and transformants were selected with hygromycin (50 μg/mL).

Microscopy

Histochemical analysis of plants containing the GUS reporter gene was performed as previously described (Masucci et al., 1996). For quantitative analysis of reporter-expressing cells, epidermal cell location was determined relative to an anticlinal cortical cell wall (ACCW), and cells within one hair file (bordering and underlying ACCW) and one non-hair file (not bordering and underlying ACCW) were counted from the meristem initial up to the elongation zone. The pattern of root hair and non-hair epidermal cell types was determined by examining seedling roots following staining with toluidine blue. Ten cells in each the H position and N position were counted for at least 20 seedling roots per line. An epidermal cell was scored as a root hair cell if any protrusion was visible, regardless of its length. All fluorescence imaging was done using a TCS SP5 DM-6000B broadband confocal microscope (Leica) with a HCX PL APO CS ×20 dry lens or HCX PL APO CS 100.0 ×1.40 oil lens. Image capture and analysis was done with LAS AF software (Leica Microsystems). GFP and propidium iodide (PI) were observed sequentially on separate channels. The GFP signal was excited using the argon 488-nm laser and captured using PMT2 between bandwidth 493 and 557 nm. The PI signal was excited using a diode-pumped solid state 561-nm laser and captured using PMT3 between 591 and 765 nm. Image capture was performed with a pinhole of 60.8 nm, a line and frame average both of two, and a scan speed of 200 Hz. Just prior to imaging, seedlings were counteredstained for 2 min with light shaking in 10 μg/mL PI in water. After washing twice in double distilled water, roots were mounted on slides, with cotyledons removed, in double distilled water. WERpro:GFP intensity was quantified using the return on investment tool to measure total pixels from individual cells demarcated by PI staining. Meristem length was measured from the first visible epidermal cell at the tip of the root to the cell previous to the first that was longer than wider (indicating entry into rapid elongation zone and exit from the meristematic region). Pixel sum analysis was performed on cells within a region one-third the length of the meristem measured toward the root tip down from the last meristematic cell. The average pixel sum was calculated for each cell file. One N file and one H file were analyzed from five different roots for three biological repeats (a total of 15 wild-type and 15 dim1A roots). Analysis of meristem cell size and cell number was measured from medial longitudinal confocal sections. Root length was measured from seedlings grown vertically on minimal media plates. Individual seedlings were tracked and imaged every day for 6 d after germination. Leaf surface area and trichome characteristics were determined for the first pair and second pair of fully expanded leaves from 21-month-old soil-grown plants. Leaf surface area was measured using Magnification (Orbicle). Trichome counts and branching were analyzed for individual leaves using a dissection scope. Leaf venation and stomatal pattern were analyzed on leaves cleared overnight in 70% ethanol. Unless otherwise indicated, all measurements were done with Adobe Photoshop CS3.

RNA Isolation, RT-PCR, and Primer Extension

Total cellular RNA was extracted from whole 5-d-old seedlings using Trizol (Invitrogen) as described (Weigel and Glazebrook, 2002). RNA was treated with TURBO-Free DNase (Ambion) following the manufacturer’s protocol. DNase-treated total RNA was used to make cDNA using random hexamer primers with a First-Strand cDNA Synthesis Kit (GE Healthcare) according to the manufacturer’s instructions. cDNA was diluted to a final concentration of 1/10,000, for the processing assay and 1/200 for DIM1A expression analysis and subjected to PCR using specific primers (see Supplemental Table 1 online). The poisoned primer extension procedure was adapted from Richter et al., (2010). A total of 50 pmol of PAGE-purified DNA oligonucleotide primer 18SPE (see Supplemental Table 1 online) was end-labeled with 150 μCi [32P]dATP using T4-polynucleotide kinase (Fermentas) then separated from unincorporated nucleotides using a G-50 sephadex column (GE Healthcare). End-labeled primer (2 pmol) was annealed to 4 μg RNA in the presence of 1 mM nucleotides (dATP, dCTP, dGTP, and dTTP) by incubating at 95°C for 1 min, 65°C for 5 min, and ice for 15 min. The extension reaction were performed using AMV reverse transcriptase (Promega) in the presence of 10 mM sodium pyrophosphate.
Accession Numbers
Arabidopsis sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: DIM1A (At2g47420), CPC (At2g46410), EGL3 (At1g63650), GL2 (At1g79840), GL3 (At5g14750), MYB23 (At5g40330), TTG1 (At5g24520), and WER (At5g14750). Sequence data for DIM1A-related genes from other species can be found under the following accession numbers: NP_001150423 (Zeama), 5,715,604 (Chlamydomonas reinhardtii), XP_001769214.1 (Physcomirella patens), XP_638864.1 (Dictyostelium discoideum), NP_015057.1 (Saccharomyces cerevisiae), NP_567269.1 (Cryptococcus neoformans), NP_01089685.1 (Xenopus laevis), NP_001003556.1 (Eleusino reio), EAW13861.1 (Homo sapiens), NP_651660.1 (Drosophila melanogaster), and XP_001704233.1 (Giardia lamblia).

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

Supplemental Figure 1. Expression of DIM1A is Not Altered in the dim1A Mutant.
Supplemental Figure 2. The Effect of Overexpression of DIM1A or GL668 dim1A.
Supplemental Figure 3. Reduced Epidermal Cell Division by Antibiotic Treatment Does Not Significantly Alter Cell-Type Expression of GL2pro:GUS.
Supplemental Table 1. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS
Y.W. and J.S. designed the research, analyzed the data, and wrote the article. Y.W. performed the research.

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