

# Cell cycle regulates cell type in the *Arabidopsis* sepal

Adrienne H. K. Roeder<sup>1,2,\*</sup>, Alexandre Cunha<sup>2,3</sup>, Carolyn K. Ohno<sup>1,\*</sup> and Elliot M. Meyerowitz<sup>1,4</sup>

## SUMMARY

The formation of cellular patterns during development requires the coordination of cell division with cell identity specification. This coordination is essential in patterning the highly elongated giant cells, which are interspersed between small cells, in the outer epidermis of the *Arabidopsis thaliana* sepal. Giant cells undergo endocycles, replicating their DNA without dividing, whereas small cells divide mitotically. We show that distinct enhancers are expressed in giant cells and small cells, indicating that these cell types have different identities as well as different sizes. We find that members of the epidermal specification pathway, *DEFECTIVE KERNEL1* (*DEK1*), *MERISTEM LAYER1* (*ATML1*), *Arabidopsis CRINKLY4* (*ACR4*) and *HOMEODOMAIN GLABROUS11* (*HDG11*), control the identity of giant cells. Giant cell identity is established upstream of cell cycle regulation. Conversely, endoreduplication represses small cell identity. These results show not only that cell type affects cell cycle regulation, but also that changes in the cell cycle can regulate cell type.

**KEY WORDS:** Epidermal specification, Giant cell, Endoreduplication

## INTRODUCTION

One fundamental question is how are complex patterns of specialized cell types self-organized during development? These patterning processes take place while cells are growing and proliferating, so coordination of cell type with cell cycle control is essential, but we are only beginning to understand its mechanism. The current paradigm is that master regulatory transcription factors determine the identity of a cell by activating many downstream genes, including cell cycle regulators such as cyclins and cyclin-dependent kinases (CDKs). The power of this paradigm is demonstrated by recent findings that key transcription factors bind directly to the enhancers of cell cycle genes. For example, the MYB transcription factor FOUR-LIPS binds to the promoter directly and represses *CDKB1;1* and other cell cycle regulators to prevent further division of guard cells (Xie et al., 2010). Likewise, *SHORTROOT* and *SCARECROW* bind directly to the *CYCLIND6;1* regulatory region to promote the asymmetric division of the initial cell daughters to generate cortex and endodermal layers in the root (Sozzani et al., 2010).

Regulation of the cell cycle is essential for creating the characteristic pattern of the outer sepal epidermal cells in *Arabidopsis thaliana* (Roeder et al., 2010). The sepal is the outermost green floral organ, which encloses and protects the developing reproductive organs before the flower blooms (Fig. 1A). The cells in the outer epidermis of *Arabidopsis* sepals exhibit a characteristic pattern, with diverse sizes ranging from giant cells (Fig. 1B) stretching to an average of 360 µm in length (about one fifth the length of the sepal) to the smallest cells reaching only

about 10 µm (Roeder et al., 2010). The giant cells have long been used as a marker for sepal organ identity in the flower (Bowman et al., 1989; Bowman et al., 1991; Ditta et al., 2004; Pelaz et al., 2000), but little is known about the development of this cell type. Giant cells are involved in regulating the curvature of the sepals (Roeder et al., 2010).

Previously, we have shown that variability in the timing of cell division is sufficient to produce the wide variety of cell sizes found within the sepal epidermis (Roeder et al., 2010). Giant cells form very early in the development of the sepal by stopping mitotic division and entering endoreduplication, a cell cycle in which the cell grows and replicates its DNA, but fails to divide (Breuer et al., 2010; Lee et al., 2009; Traas et al., 1998). Concurrently, the smaller cells continue to divide, which reduces their size. The pattern is regulated by CDK inhibitors, which influence the probability with which cells enter endoreduplication early and become enlarged. Overexpression of the CDK inhibitor *KRP1* throughout the epidermis causes many cells to endoreduplicate, resulting in a sepal covered with large cells (Bemis and Torii, 2007; Roeder et al., 2010). Conversely, mutations in the *SIAMESE* family CDK inhibitor *loss of giant cells from organs* (*Igo*) cause an absence of giant cells (Roeder et al., 2010). A computational model in which the decision to divide or endoreduplicate is made randomly can reproduce the cell size distribution within the sepal, suggesting that variability is important in generating the pattern (Roeder et al., 2011; Roeder et al., 2010).

Here, we ask whether developmental regulators interact with the cell cycle to create the characteristic pattern of giant cells and small cells in the sepal epidermis. We find the expression patterns of two enhancers distinguish giant cells from small cells, suggesting that these can be considered to be distinct cell types, as well as cells of different size and ploidy. Through a forward genetic screen, we have identified several members of the epidermal specification pathway, each of which regulates giant cell formation and identity. We find that giant cell identity is established upstream of endoreduplication, but that small cell identity appears to be negatively regulated by endoreduplication directly or indirectly, indicating that cell cycle regulation can control cell identity, just as cell identity can control cell cycle.

<sup>1</sup>Division of Biology, California Institute of Technology, Pasadena, CA 91125 USA.

<sup>2</sup>Center for Integrative Study of Cell Regulation, California Institute of Technology, Pasadena, CA 91125 USA. <sup>3</sup>Center for Advanced Computing Research, California Institute of Technology, Pasadena, CA 91125 USA. <sup>4</sup>Sainsbury Laboratory at Cambridge University, Cambridge CB2 1LR, UK.

\*Present address: Weill Institute for Cell and Molecular Biology and Department of Plant Biology, Cornell University, Ithaca, NY 14853 USA

<sup>†</sup>Present address: Developmental Biology Unit, EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany

<sup>‡</sup>Author for correspondence (ahr75@cornell.edu)

## MATERIALS AND METHODS

### Enhancer trap markers

One marker from the Poethig collection of enhancer trap lines expressed in the flowers (ABRC stock number CS70134) showed the small cell expression pattern. The giant cell marker is enhancer trap line YJ158 from the Bowman collection (Eshed et al., 2004).

### Generation of the fluorescent giant cell marker

The enhancer trap T-DNA driving the giant cell expression pattern is inserted about 4.7 kb upstream of At5g17700, which encodes a MATE efflux family protein, and about 1.4 kb downstream of At5g17710, which encodes a co-chaperone grpE family protein (supplementary material Fig. S1A). To identify the enhancer element that drives giant cell expression, we tested a 1 kb fragment immediately upstream of the trap insertion. The 1 kb fragment was PCR amplified with oAR215 (5'-GCTCGAGCCTGTCCGCTATATCATGCAAATC-3') and oAR214 (5'-CACCTCGAGATACCTTTTGC GTTCGTTGAACCA-3'), and cloned into pCRBlunt II TOPO (Invitrogen) to create pAR108. The 1 kb fragment was cut out of pAR108 with *XhoI* and cloned into a BJ36 plasmid in both orientations in front of the 35S minimal -60 promoter and 3X VenusN7 to create pAR109 (forward) and pAR110 (reverse). The whole reporter fragments were excised with *NotI* and cloned into the binary vector pMLBart to create pAR111 (forward) and pAR112 (reverse) (supplementary material Fig. S1A). Both constructs were transformed into Landsberg *erecta* (*Ler*) by agrobacterium-mediated floral dipping and transgenic plants were selected for Basta resistance. In both forward and reverse orientations, the 1 kb fragment drives strong expression of a nuclear localized fluorescent protein (3× Venus-N7) in sepal giant cells (supplementary material Fig. S1B,C). For ease of imaging, we continued our analysis using the forward 1 kb nuclear localized fluorescent giant cell marker.

We tested whether the entire promoter region of At5g17700 also drives expression in giant cells. The 4.2 kb promoter region from the start of the 5' UTR up to the YJ158 enhancer trap insertion was PCR amplified with oAR217 (5'-CCTCGAGGACTTAACTACAACGCTTGGCT-3') and oAR214, and cloned into pENTR D TOPO (Invitrogen) to create pAR118. The promoter region was recombined into the pBGWFS7 (Karimi et al., 2002) binary vector upstream of eGFP-GUS to create pAR121. pAR121 was transformed into wild-type *Ler* plants via agrobacterium-mediated floral dipping and transgenic plants were selected for Basta resistance. This At5g17700 promoter drives expression in young giant cells in the sepals; however, giant cell expression decreases earlier than in either the 1 kb enhancer or the original giant cell marker (supplementary material Fig. S1D). This promoter drives additional patterns of expression, including petal blades, style, gynoecium and large cells in the stem and petioles. These results suggest that a larger regulatory region modifies the giant cell enhancer.

Combinations of mutants and markers were made by crossing. Mutants were genotyped. Plants homozygous for the markers selected by Basta and/or Kanamycin resistance were imaged.

### Mutations and genotyping

M2 Ethyl methanesulfonate (EMS) mutagenized *Ler* seeds were purchased from Lehle Seeds and examined under a dissecting microscope for the absence of giant cells in the sepal.

The *dek1-4* mutation isolated contains a C to T change at base 6316 of the CDS, which causes a single amino acid substitution of a cysteine for conserved arginine 2106 in domain III of the calpain protease (supplementary material Fig. S2A) (Sorimachi and Suzuki, 2001). The *dek1-4* allele fails to complement the reference *dek1-3* (SAIL\_384\_G07) allele (data not shown), establishing that the absence of giant cells is due to the mutation in the *DEK1* gene.

The *dek1-4* mutation can be PCR genotyped by amplifying with oAR448 (5'-TGTTGGTGAACAGACTATGTGAATTCA-3') and oAR449 (5'-TGAAGACTGAAAGGACAAAAGGTGC-3') with a 60°C annealing temperature followed by digesting the product with *BsaAI* to produce a 108 bp wild-type product or a 137 bp mutant product.

The *atml1-2* allele isolated in this mutant screen contains a C to T change at base 1873 of the CDS, which creates a premature stop codon in place of glutamine 625 truncating C-terminal end of the protein (supplementary material Fig. S2B). Additional *atml1* alleles, *atml1-3* (SALK\_033408) and *atml1-4* (SALK\_128172) (Alonso et al., 2003), also exhibit the absence of giant cells, demonstrating that mutations in *atml1* cause this phenotype. The *atml1-2* allele, which causes a truncation in the C-terminal end of the protein after the START domain, acts semi-dominantly in that heterozygous plants have a variable appearance, ranging from wild-type numbers of giant cells to a complete loss of giant cells. By contrast, *atml1-3*, which is inserted in the homeodomain acts recessively and has the least severe loss of giant cells phenotype, whereas *atml1-4*, which is inserted in the START domain, acts dominantly (heterozygous plants lack giant cells).

The *atml1-2* mutation can be PCR genotyped by amplifying with oAR316 (5'-AAACAGAGTGGGAAGTCAAGCG-3') and oAR299 (5'-CACTCAGGACAACGTTTCATAGCT-3') followed by digesting the product with *HhaI* to produce a 103 bp wild-type product or a 124 bp mutant product.

The extracellular domain of the receptor kinase ACR4 contains seven crinkly repeats and three cysteine-rich repeats with homology to the tumor necrosis factor receptor (TNFR). The *acr4-23* allele isolated in this mutant screen contains a G to A mutation at base 300 of the CDS, which creates a premature stop codon at amino acid 100 in the extracellular crinkly repeats (supplementary material Fig. S2C). The *acr4-24* allele, which was also isolated in this mutant screen, contains a G to A change at base 935 of the CDS, which causes the substitution of a tyrosine for a conserved cysteine at amino acid 312. This substitution is predicted to disrupt the formation of a disulfide bond that is involved in the folding of the seventh crinkly repeat (Gifford et al., 2005). Transformation of *acr4-24* with a wild-type copy of *ACR4* rescues both giant cell formation and the fertility and ovule defects, indicating that all of these phenotypes are caused by mutations in *acr4*.

The *acr4-23* mutation can be PCR genotyped by amplifying with oAR304 (5'-GCTATCTCATCAGCCATATTGTTG-3') and oAR305 (5'-GTAATCACCAGCACTAAGTTCTAA-3') followed by digesting the product with *BstXI* to produce a 90 bp wild-type product or a 109 bp mutant product. The *acr4-24* mutation can be PCR genotyped by amplifying with oAR302 (5'-ATAGAAGTCCCTGTGAGAACTGCG-3') and oAR303 (5'-TATGATCATAGTGC GTTCTGTTGG-3') followed by digesting the product with *HhaI* to produce a 105 bp wild-type product or a 128 bp mutant product.

The *hdg11-3* allele isolated in this screen contains a C to T change at base 415 of the CDS, which creates a premature stop codon at amino acid 139 of the protein. The reduction in giant cells in *hdg11-3* mutants is strongest immediately following bolting and becomes less pronounced with age. The reference *hdg11-1* (SAIL\_865\_G09) allele also exhibits a subtle reduction in giant cells (data not shown). Both alleles exhibit increased trichome branching as described previously (Nakamura et al., 2006). Similar to the *atml1* alleles, *hdg11-3*, which truncates the protein in the zipper loop zipper domain, acts semi-dominantly with heterozygous plants having a range of phenotypes from wild type to mutant.

The *hdg11-3* mutation can be genotyped by PCR amplifying with oAR300 (5'-GTGAAGATCCTTACTTTGATGAT-3') and oAR301 (5'-TCAAGCTATGCAAAAAGATCAAA-3') and cutting with *BclI* to produce a 129 bp wild-type fragment or a 153 bp mutant fragment.

The *hdg11-1* allele can be genotyped by PCR amplification with oAR282 (5'-ATTCTATCACCGGAAGGGAAG-3'), oAR283 (5'-TGAAGAGAAAGAGACACCCAG-3') and SLB1 (5'-GCCTTTTCAGAAATGGATAATAGCCT-3'). The wild-type product will be 546 bp and the mutant product 753 bp.

### Microscopy

To analyze fluorescent reporters, stage 12 medial adaxial sepals were removed with a needle, stained with 0.1 mg/ml propidium iodide for 10 minutes and mounted in 0.01% Triton X-100 on a slide under a cover slip. Sepals were imaged with 10× and 20× objectives on Zeiss 510 Meta or Zeiss 710 laser scanning confocal microscope. The small cell marker was

excited with a 488 nm laser line and collected by reflection from either a 635 nm or 545 nm primary dichroic, reflection from a 545 nm secondary dichroic and passing a 505–530 nm band pass filter such that wavelengths 505–530 nm were collected. The giant cell enhancer was excited with a 514 nm laser line and collected by reflection from a 635 nm dichroic mirror, reflection from a 545 nm dichroic mirror and passage through a 530–600 nm band pass filter such that wavelengths 530–545 nm were collected. Propidium iodide was excited with either a 488 nm or 514 nm laser line and collected by reflection from a 635 nm dichroic, passage through a 545 nm dichroic and passage through a 585–615 nm band pass filter such that wavelengths 585–615 nm were collected. Some bleed through was observed from the giant cell enhancer into both the small cell marker and the propidium iodide channels; however, this bleed-through signal could be distinguished by its nuclear localization. Multiple images from the same sepal were merged in Adobe Photoshop CS and a small white line was drawn to indicate the location of the border between images.

Live imaging of the sepals expressing the markers was conducted with the settings above as described (Cunha et al., 2012; Roeder et al., 2010) with 12- or 24-hour intervals. Images were volume rendered and registered in Amira 5.4.2 (www.amira.com). Cells were tracked using colored dots in Adobe Photoshop CS5.1 as described (Roeder et al., 2010).

GUS staining was performed with 5 mM ferricyanide and 5 mM ferrocyanide as described (Sessions et al., 1999). Flowers and GUS-stained tissue was imaged with a Zeiss Stemi SV 11 dissecting microscope. Images were taken with a Canon Powershot A640 digital camera. Whole-plant images were taken with an Olympus C-2040Zoom digital camera.

Scanning electron microscopy was performed as described using a Zeiss 1550VP or a Leica 440 (Roeder et al., 2010). Giant cells identified by their slight protrusion from the sepal were false colored red by hand with Adobe Photoshop CS.

#### Nuclear segmentation using Costanza ImageJ plug-in

Confocal projection images of the fluorescent giant cell marker in 10 sepals for each mutant and double mutant combination were used to count the number of cells expressing the giant cell enhancer. We used the ImageJ plug-in Costanza (<http://home.thep.lu.se/~henrik/Costanza/>) with the following pre-processors: background extraction, intensity threshold 40; mean filter, radius 2 and number of times 2. Post-processors were: peak remover, size threshold 10, intensity threshold 10; peak merger, radius 10. Occasional large nuclei that were split in two were hand corrected. A threshold area of 231  $\mu\text{m}^2$  was set for separating small nuclei from large nuclei.

#### Cell area measurements

For measuring the cell size distribution in the mutants, each mutant genotype was transformed with the epidermally specific plasma membrane marker (pAR169 *ATML1p::mCitrine-RC12A*) by agrobacterium-mediated floral dipping (Roeder et al., 2010). Stage 12 sepals were dissected and mounted in 0.01% triton X-100. mCitrine was excited with a 514 nm laser line and collected by reflection from a 635 nm dichroic mirror, reflection from a 545 nm dichroic mirror, and passage through a 530–600 nm band pass filter such that wavelengths 530–545 nm were collected. Six confocal images tiling each of five sepals were taken with the 20 $\times$  objective and the projections were merged with the Adobe Photoshop function Photomerge. Plasma membranes were segmented, hand corrected and processed as described (Cunha et al., 2010; Roeder et al., 2010). The histogram of areas was normalized to the total sepal area segmented.

For measuring cell size and marker expression, five 2 $\times$ 3 tiled images were taken with a 20 $\times$  objective on a Zeiss 710 confocal microscope as described above. Cells were outlined by hand using a Wacom Bamboo tablet in Adobe Photoshop CS5.1. The hand segmentation was narrowed to a single pixel width as described previously (Cunha et al., 2010). The giant cell marker was segmented using thresholding after non-local means noise reduction and image sharpening. The giant cell marker was subtracted from the small cell marker owing to bleed-through into that channel. Stomata were marked by hand. Matlab R2011B (<http://www.mathworks.com/products/matlab/>) was used to measure the area of each cell, to quantify the small cell marker expression and to detect

whether the cell expressed the giant cell marker or was a stoma. The data were sorted and analyzed in Microsoft Excel for Mac 2011.

#### Flow cytometry

The ploidy of stage 12 sepal epidermal cells for each of the mutants was measured as described previously (Roeder et al., 2010). The ploidy of cells expressing the giant cell enhancer were measured similarly, except that the giant cell enhancer was used to drive expression of Histone 2B-GFP (supplementary material Fig. S1E,F). First, a giant cell promoter gateway destination vector was created (pAR201). The 1 kb giant cell enhancer and –60 minimal promoter were amplified from pAR109 with oAR244 (5'-gggtcgacgtacgCCTGTCCGCTATATCATGCAAATC-3') and oAR245 (5'-agtcgacGGTTCGATACCCGATCCCCCGT-3') and cloned into pCR Blunt II TOPO (Invitrogen) to make pAR136. The 3' OCS terminator from BJ36 was PCR amplified with oAR446 (5' ggatctTCCTGCTTTAA-TGAGATATGCGA-3') and oAR445 (5'-CAGCGGCCGcgagctc-AGATTAGGTGACACTATA-3'), cut with *EcoRV* and *NotI*, and cloned into pAR136 to create pAR199. The gateway conversion cassette (Invitrogen) was cloned into the *EcoRV* site of pAR199 to create pAR200. The Giant Cellp-GW-3'OCS fragment was cut out with *SacI* and cloned into the binary vector pMOA34 (Barrell and Conner, 2006). The H2B-mGFP entry clone pAR179 (Roeder et al., 2010) was LR recombined into pAR201 to generate pAR202 Giant Cellp::H2B-GFP. pAR202 was transformed into Ler plants by agrobacterium mediated floral dipping and transgenic plants were selected for Hygromycin resistance. The expression pattern of pAR202 was examined with confocal microscopy and was similar to pAR111.

#### Cell cycle regulator overexpression

The *CYCD1;1* gateway cDNA (G60123) (Yamada et al., 2003) was LR recombined into the ATML1p gateway vector pAR176 (Roeder et al., 2010) to generate pWS109 *ATML1p::CYCD1;1*. pWS109 and pAR178 *ATML1p::LGO* (Roeder et al., 2010) were individually transformed into plants homozygous for the small cell marker by agrobacterium-mediated floral dipping.

#### Accession numbers

DEK1, AT1G55350; ATML1, AT4G21750; ACR4, AT3G59420; HDG11, AT1G77360; giant cell enhancer trap marker, YJ158; small cell enhancer trap marker, CS70134; LGO, AT3G10525; KRP1, AT2G23430; CYCD1;1, AT1G70210; *dek1-3*, SAIL\_384\_G07; *atml1-3*, SALK\_033408; *atml1-4*, SALK\_128172; and *hdg11-1*, SAIL\_865\_G09.

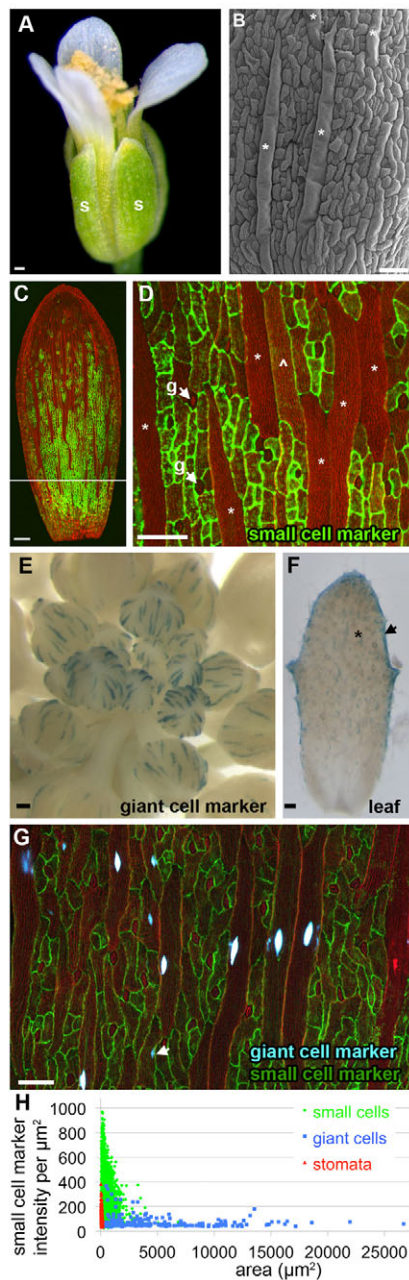
## RESULTS

### Giant cells and small cells are different cell types

We first asked whether giant cells and small cells in the outer (abaxial) sepal epidermis are different cell types (using the criterion of different patterns of gene expression) or are merely extremes in a continuum of cell size distinguishable only by size and ploidy. To address this question, we identified two enhancer trap markers: one primarily expressed in small cells (Fig. 1C,D) and one in giant cells (Fig. 1E). The giant cell marker is also expressed in leaf giant cells as well as leaf margin cells and elongated root cells (Fig. 1F; supplementary material Fig. S1G-I) (Eshed et al., 2004).

To test whether the expression patterns of giant cell and small cell markers are unique or overlapping, we recreated the giant cell marker as a fluorescent marker (supplementary material Fig. S1A-D). The fluorescent giant cell marker shows strong expression in sepal giant cells, as well as expression in some of the smaller cells, particularly towards the top of the sepal (Fig. 1G; supplementary material Fig. S1B,C,E,F). The difference in expression between the original marker and the fluorescent marker may be either due to increased sensitivity in detecting low levels of expression with the fluorescent reporter, or to differences in the enhancer region driving the markers.





We characterized the correlation between marker expression and cell size (Fig. 1H). Ninety five percent of large cells ( $>5000 \mu\text{m}^2$ ) express the giant cell marker, whereas expression of the small cell marker in these cells is minimal ( $<200$  intensity units /  $\mu\text{m}^2$ ). The small cell marker is strongly expressed in 48% of cells less than  $5000 \mu\text{m}^2$ . The small cell marker is generally not expressed in stomatal cells and in cells towards the tip of the sepal. Most cells expressing a marker exclusively express either the giant cell marker or the small cell marker; however, 0.2% of cells (8/4644) express both markers (Fig. 1G,H). These cells fall within the smaller size range ( $<5000 \mu\text{m}^2$ ). The expression patterns of these two markers suggest that giant cells and small cells have different identities, but that these identities are not necessarily mutually exclusive.

Next we tested whether these markers are general markers of cell division. First, these markers do not report cell ploidy, because they

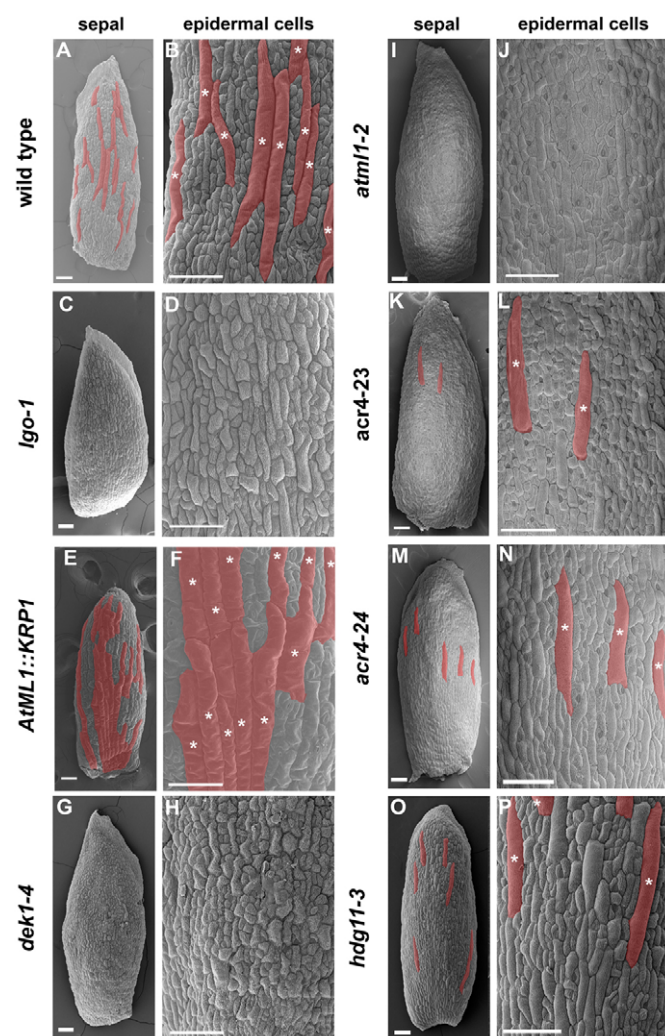
**Fig. 1. Giant cells and small cells have distinct enhancer expression.** (A) Wild-type flower with sepals (s). (B) Scanning electron micrograph of the sepal epidermis showing the cell size pattern of giant cells (\*) interspersed between smaller cells in a large range of sizes. (C) The small cell marker (ER localized GFP, green) expressed in the small pavement cells on the outer (abaxial) sepal epidermis. Generally, the small cell marker is more strongly expressed towards the base of the sepal. The cell walls (red) have been stained with propidium iodide (PI). (D) Expression of the small cell marker is reduced (^) or absent (\*) in the giant cells, and also absent in guard cells (g). (E) The original giant cell enhancer trap marker YJ158 is expressed (GUS staining blue precipitate) in the giant cells on the abaxial side of young sepals in the inflorescence. (F) The original giant cell enhancer trap marker YJ158 is also expressed (blue precipitate) in the giant cells on the abaxial side of leaves (\*) as well as in the leaf margin cells (arrow). (G) Sepal cells expressing the re-engineered fluorescent giant cell marker (light-blue nuclei;  $3\times\text{Venus-N7}$ ) together with the small cell marker (green ER). The large cells express the giant cell marker, the small cells express the small cell marker and a few cells express both (arrow). The cell walls (red) have been stained with PI. (H) Scatter plot showing the relationship between cell area and small marker expression per  $\mu\text{m}^2$  for 4644 cells from five sepals expressing both the small cell marker and fluorescent giant cell marker. Cells expressing the giant cell marker (blue squares) and stomata (red triangle; two guard cells counted as one unit) express low levels of the small cell marker. The cells that are not stomata and do not express the giant cell marker are labeled small cells (green diamond). Scale bars:  $100 \mu\text{m}$  in A,C,E,F;  $50 \mu\text{m}$  in B,D,G.

are specific to epidermal pavement cells; the giant cell marker is not expressed in the highly endoreduplicated trichomes (Fig. 1F), and the small cell marker is not expressed in diploid guard cells (Fig. 1D,H). Furthermore, the small cell marker is not expressed in sepal mesophyll cells, in cells in the inner (adaxial) epidermis of the sepal or in leaf epidermal cells, which all contain many diploid cells (supplementary material Fig. S3B-D). To test whether the small cell marker is expressed in cells that will divide, we imaged live developing sepals expressing the markers (supplementary material Movies 1-4). None of the 193 cells expressing the small cell marker that were tracked divided in at least 60 hours. Previously, we have shown that almost all dividing cells in the sepal have cell cycles less than 60 hours (Roeder et al., 2010). Furthermore, the small cell marker was not expressed until after division occurred (supplementary material Fig. S3A). Likewise, none of the cells expressing the giant cell marker divided. These results suggest that these are late markers of differentiating giant and small cells in the sepal.

### The epidermal specification pathway controls giant cell formation

To identify genes involved in giant cell development, we screened for mutant plants lacking visible giant cells in M2 progeny of an EMS chemical mutagenesis. We isolated mutations in five genes that caused either a strong reduction or a complete loss of large cells (Fig. 2). We have previously shown that one of these mutants, *lgo-1*, disrupts the regulation of the cell cycle, causing cells to divide and become small instead of entering endoreduplication early and becoming giant (Fig. 2C,D) (Roeder et al., 2010). The LGO CDK inhibitor promotes early endoreduplication of giant cells (Churchman et al., 2006; Peres et al., 2007; Roeder et al., 2010).

Surprisingly, positional cloning revealed that the remaining four mutants isolated in the screen could all be associated with the epidermal specification pathway in plants. However, each of these mutants has an intact epidermis expressing a reporter for the



**Fig. 2. The epidermal specification pathway controls giant cell formation.** Scanning electron micrographs of the abaxial side of stage 14 sepals. Giant cells identified by morphology (length, width generally twice that of small cells and bulging from the sepal) have been false colored red and marked with asterisks. (A,B) Wild-type (*Ler*) sepals have giant cells interspersed among smaller cells. (C,D) *lgo-1* mutant sepals lack large cells. (E,F) Overexpression of *KRP1* throughout the epidermis (*ATML1P::KRP1*) produces sepals that are nearly covered by large cells, although patches of small cells are present. (G,H) *dek1-4* sepals lack giant cells. (I,J) *atml1-2* sepals lack giant cells. (K,N) *acr4-23* and *acr4-24* sepals have a few giant cells. (O,P) *hdg11-3* sepals show a subtle reduction in giant cells compared with wild type. Scale bars: 100  $\mu$ m.

epidermal promoter *MERISTEM LAYER1* (*ATML1::H2B-mFYP*) and containing specialized epidermal cell types, including trichomes and guard cells (Fig. 2; data not shown). As loss of the epidermis is lethal, screening for defects in giant cells provides a genetically sensitized background in which subtle defects are easily observed. These mutants indicate that the epidermal specification pathway not only initiates epidermal development, but also continues to shape its subsequent patterning of cell sizes.

#### defective kernel1 (*dek1*)

The new allele of *defective kernel1* (*dek1-4*), a missense mutation, caused a near absence of giant cells in sepals (Fig. 2G,H;

supplementary material Fig. S2A). *DEK1* encodes a protein with two regions of multiple transmembrane domains separated by a loop in the N terminus and an intracellular calpain protease domain in the C terminus (Lid et al., 2002; Wang et al., 2003). Thus, *DEK1* has been hypothesized to receive an extracellular signal and transmit that signal by cleaving intracellular targets (Javelle et al., 2011).

*DEK1* orthologs are involved in the specification of epidermal identity in both *Arabidopsis* and maize. Loss-of-function T-DNA insertion alleles in *Arabidopsis* cause embryonic lethality and a loss of epidermal specification in the aborted embryos (Johnson et al., 2005; Lid et al., 2005). Reduction of *DEK1* activity via RNAi produces *Arabidopsis* seedlings that lack an epidermal layer (Johnson et al., 2005). Likewise, in maize, strong *dek1* alleles produce arrested embryos and the aleurone layer of the mutant seed fails to differentiate (Becraft and Asuncion-Crabb, 2000; Lid et al., 2002). In weak *dek1-D* maize allele mutants, the specification of epidermal cell types is disrupted (Becraft et al., 2002). Wild-type maize leaves contain files of bulliform cells (specialized cells that allow the leaves to fold or roll in response to drought stress) in the epidermis, but in the *dek1-D* mutant all of the epidermal cells adopt a partial bulliform cell identity (Becraft et al., 2002). Thus, in both *Arabidopsis* and maize, *DEK1* plays a role in epidermal specification as well as more specific roles in the development of epidermal cell types.

#### meristem layer1 (*atml1*)

Giant cells are also absent in mutants homozygous for the new allele of *meristem layer1* (*atml1-2*) (Fig. 2I,J; supplementary material Fig. S2B). *ATML1* encodes a transcription factor in the class IV homeodomain leucine zipper (HD-ZIP) family (Abe et al., 2003; Nakamura et al., 2006). Two additional T-DNA insertion alleles also lack giant cells (*atml1-3* and *atml1-4*) (supplementary material Fig. S2). *ATML1* is redundantly required with *PDF2* to specify epidermal identity; the epidermis is absent and the surface cells appear similar to the underlying mesophyll cells in *atml1 pdf2* double mutants (Abe et al., 2003).

#### *Arabidopsis crinkly4* (*acr4*)

Positional cloning showed that fewer giant cells are present in sepals of plants homozygous for each of the two new alleles of *Arabidopsis crinkly4* (*acr4-23* and *acr4-24*) (Fig. 2K-N; supplementary material Fig. S2C). *ACR4* encodes a transmembrane receptor kinase. *ACR4* is expressed in the epidermis, and is thought to promote epidermal identity through intercellular signaling (Gifford et al., 2003; Ingram, 2007). Although the defect in epidermal development of *acr4* mutants is subtle (Gifford et al., 2003; Watanabe et al., 2004), a role in epidermal development is clearly apparent from the highly abnormal morphology of epidermal cells in mutants of the maize homologue *crinkly4* (*cr4*) (Becraft et al., 2001; Becraft et al., 1996). In addition, both of these new *acr4* mutants exhibit reduced fertility and abnormal ovule development, as described for previously characterized *acr4* alleles (Gifford et al., 2003; Watanabe et al., 2004). The previously characterized *acr4-2* allele also exhibits a reduction in giant cell numbers.

#### homeodomain glabrous11 (*hdg11*)

A subtle reduction in the number of giant cells is found in *homeodomain glabrous11-3* mutant sepals (*hdg11-3*) (Fig. 2O,P; supplementary material Fig. S2D). *HDG11* encodes a class IV HD-ZIP in the same family with *ATML1* (Nakamura et al., 2006).



Previously, *hdg11* mutants have been shown to have trichomes with increased branching and our new mutant also exhibits this phenotype.

*HDG11* is expressed in the epidermis (Nakamura et al., 2006). Although the *hdg11* single mutant does not exhibit general epidermal defects, it is possible that any role in specification of epidermal identity is covered by redundancy with closely related family members such as *ATML1* and *PDF2*. However, *atml1-2* *hdg11-3* sepals have a similar phenotype to *atml1-2* sepals (data not shown).

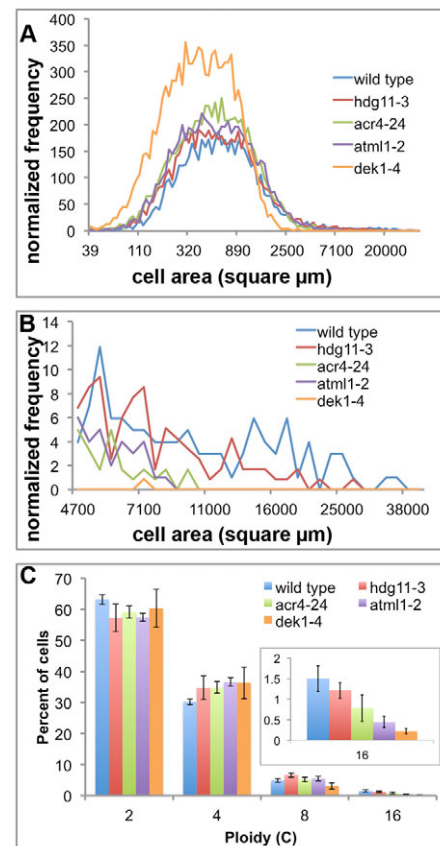
### Epidermal specification factors promote endoreduplication

Quantification of the effect of each mutation on the cell size pattern through using semi-automated image processing to measure the cell areas (see Materials and methods) revealed that there is a progression in the severity of the reduction in large cells: (strongest) *dek1-4>atml1-2>acr4-24>hdg11-3* (weakest) (Fig. 3A,B). We have shown previously that the area of sepal epidermal cells correlates roughly with their DNA content and that giant cells have generally undergone three endocycles to reach 16C (Roeder et al., 2010). Measuring the ploidy distribution in the sepal epidermis of each mutant showed a corresponding progressive reduction in 16C nuclei (Fig. 3C), indicating that these members of the epidermal specification pathway control the cell size distribution in the sepal epidermis by promoting endoreduplication.

### The epidermal specification pathway promotes giant cell identity

We next asked whether these members of the epidermal specification pathway control the identity of giant cells, as well as cell size and DNA content. To test this, we quantified the effect of the mutants on the expression of the fluorescent giant cell enhancer marker. As we engineered the marker to localize to the nucleus, we used image processing to count the number of fluorescent nuclei and measure their size (Fig. 4A-C) (see Materials and methods for details). As mentioned previously, the fluorescent giant cell enhancer is expressed both in the giant cells and a few of the smaller cells primarily near the top of the sepal (Fig. 1G; Fig. 5A,B). Therefore, we classified nuclei by their size, which correlates with the ploidy and consequently with cell size (Jovtchev et al., 2006; Kowles et al., 1992; Melaragno et al., 1993). Wild-type sepals have  $14.9 \pm 1.4$  cells with large nuclei expressing the giant cell enhancer (Fig. 4A), which corresponds well with our observation of about 14 cells per sepal that we would classify as giant based on their morphology. In addition to these cells with large nuclei,  $47.2 \pm 5.3$  small cells with small nuclei express the giant cell enhancer in wild-type sepals (~3% of sepal epidermal cells) (Fig. 4A). Whereas 98±2% of the highest ploidy cells in the sepal express the giant cell enhancer, fewer than  $0.6 \pm 0.3\%$  of the diploid cells in the sepal express the giant cell marker (supplementary material Fig. S1F).

In the *dek1-4* mutant, expression of the giant cell enhancer is strongly reduced (Fig. 4A; Fig. 5C,D). Large giant cell nuclei expressing the enhancer are nearly absent ( $0.2 \pm 0.3$ ) and only  $10.6 \pm 3.7$  small cell nuclei expressing the giant cell enhancer remain. Thus, *DEK1* promotes giant cell identity. The large giant cell nuclei expressing the giant cell enhancer are also absent ( $0.1 \pm 0.2$ ) in *atml1-2* mutants, corresponding with the observed loss of giant cells; however, the number of small nuclei expressing the marker remains unchanged compared with wild type ( $41.5 \pm 5.1$ ) (Fig. 4A; Fig. 5E,F). The number of large giant cell nuclei

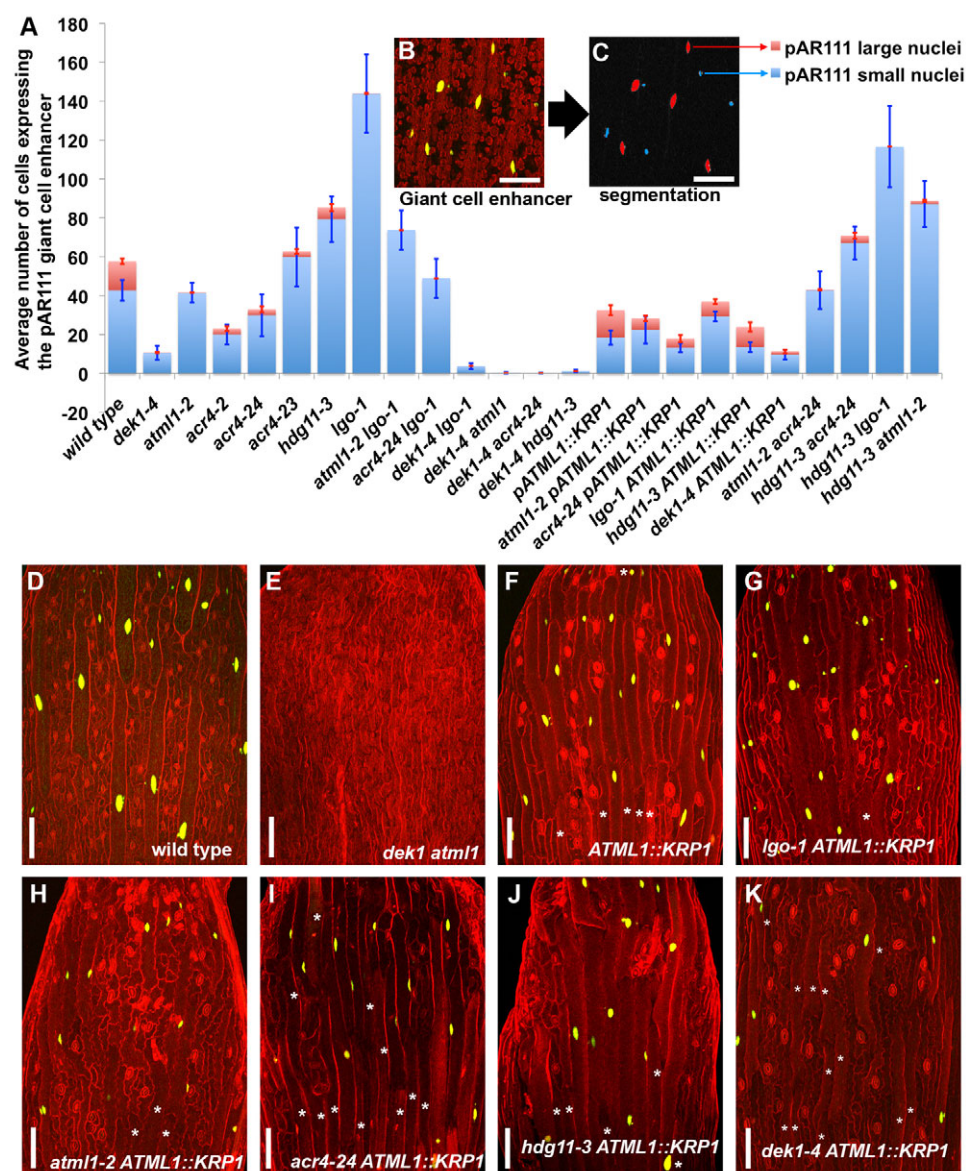


**Fig. 3. Epidermal specification factors promote endoreduplication.**

(A) Histogram of cell area measurements for wild-type (blue), *dek1-4* (orange), *atml1-2* (purple), *acr4-24* (green) and *hdg11-3* (red) sepal epidermal cells. Frequencies are normalized to the total segmented area for each mutant. The cell area is shown on a log scale. Over 5000 cells were measured per genotype from five sepals. (B) Histogram of cell size measurement showing only the cells larger than  $4700 \mu\text{m}^2$  from A. There are almost no cells in this size range in *dek1-4* sepals. *atml1* and *acr4-24* mutants do not have cells larger than  $11,000 \mu\text{m}^2$ . Both wild type and *hdg11-3* have cells as large as  $38,000 \mu\text{m}^2$  but *hdg11-3* consistently has fewer of these large cells. (C) The average percent of sepal epidermal cells with each DNA content measured with flow cytometry. Corresponding with the affect on cell size, *dek1-4* mutations cause the greatest loss of 16C giant cells (0.22% of sepal epidermal cells are 16C versus 1.50% for wild type significantly different at the  $P < 0.01$  level in *t*-test), followed by *atml1-2* (0.44%  $P < 0.01$ ), *acr4-24* (0.78%  $P < 0.05$ ) and *hdg11-3* (1.22%). Only *dek1-4* also significantly decreases the number of 8C cells (3.09% of *dek1-4* sepal epidermal cells are 8C versus 4.88% or wild type,  $P < 0.05$ ). The graph shows mean of four replicates with >5000 cells per sample and the error bars represent 95% confidence interval of the mean.

expressing the giant cell enhancer is decreased in both *acr4* ( $3.0 \pm 1.5$  for *acr4-24*) and *hdg11-3* ( $6.0 \pm 1.8$ ) single mutants, corresponding with the reduction in giant cells observed (Fig. 4A; Fig. 5G-J).

We further tested whether *ATML1*, *ACR4* and *HDG11* contribute to giant cell specification by testing whether they contribute to the remaining expression of the giant cell marker in *dek1-4* mutants. The cells of *atml1-2* *dek1-4* and *acr4-24* *dek1-4* sepals are physically small in size, like those of *dek1-4* sepals (compare Fig. 6L-O with Fig. 2G,H). Giant cell marker expression is nearly



**Fig. 4. Quantification of giant cell identity.** (A) The average number of cells expressing the giant cell enhancer per sepal in each genotype ( $n=10$  sepals). The cells have been split into two categories: typical giant cells (red; large nuclei, which correlates with highly endoreduplicated cells) and small cells expressing the giant cell enhancer (blue; small nuclei, which correlates with lower ploidy). Error bars represent the 95% confidence interval on the mean. (B) Sample portion of an image used for quantification of the number of cells expressing the giant cell enhancer (yellow). Chloroplast autofluorescence for contrast is in red. (C) Automatic detection of giant cell enhancer nuclei. Red, large; blue, small, as graphed in A. (D) Top center of a wild-type sepal showing expression of the giant cell enhancer (yellow). Cell walls stained with PI are visualized in red. The giant cells have large nuclei expressing the marker. Some smaller cells especially near the tip express the marker. (E) Giant cell enhancer expression is absent in *dek1 atml1* double mutants. (F-K) Overexpression of *KRP1* (*ATML1p::KRP1*) (F) is sufficient to cause the formation of large cells in *lgo-1* (G), *atml1-2* (H), *acr4-24* (I), *hdg11-3* (J) and *dek1-4* (K) mutants. However, some of the large cells induced by *ATML1p::KRP1* do not express the giant cell enhancer (asterisks), indicating that endoreduplication is insufficient to induce giant cell identity. This is particularly evident in the *dek1* mutant background where giant cell identity is severely reduced (K). Scale bars: 100  $\mu$ m.

absent in *atml1-2 dek1-4* double mutants, as well as in *acr4-24 dek1-4* and *hdg11-3 dek1-4* double mutants (Fig. 4A; Fig. 4D,E), suggesting that *ATML1*, *HDG11* and *ACR4* also promote giant cell specification.

Unlike the single mutants (supplementary material Fig. S4), double mutants homozygous for both *dek1-4* and a second epidermal specification mutation exhibit defects in overall plant morphology. In both *atml1-2 dek1-4* and *hdg11-3 dek1-4* double mutants, the sepals and cauline leaves (leaves on the stem, which also lose giant cells in these mutants) roll inwards (Fig. 6A,C-E,G-I). Cauline leaves of *acr4-24 dek1-4* mutants roll inwards as well (Fig. 6F). Conversely, *ATML1p::KRP1* sepals, which are nearly covered by large cells, bend outwards (Fig. 6B) (Bemis and Torii, 2007; Roeder et al., 2010). In both cases, the defects in organ curvature mean that the sepals do not fully enclose the developing flower as do wild-type sepals (Fig. 6A-C). These defects further support the conclusion that giant cells control the curvature of both sepals and leaves (Roeder et al., 2010).

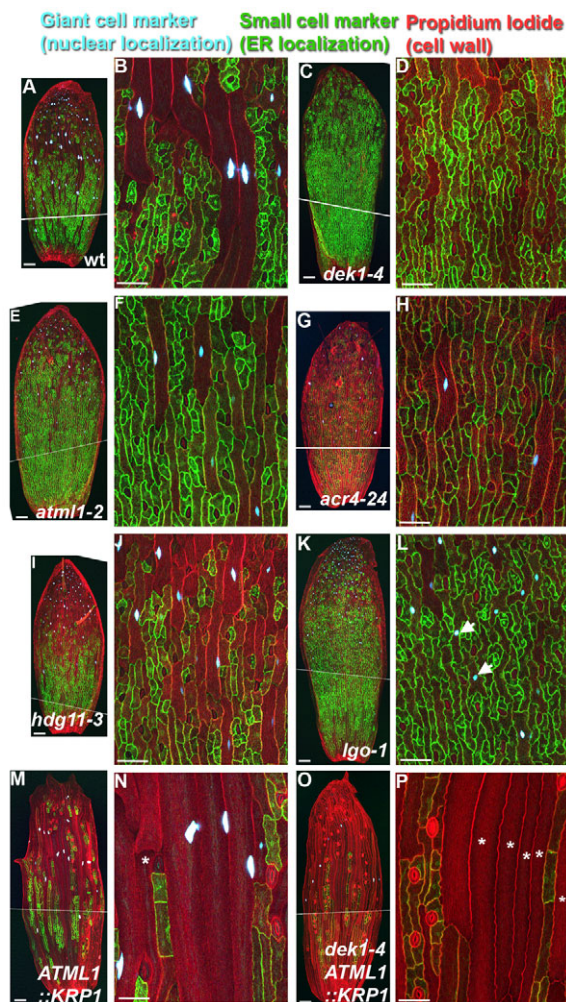
The *acr4-24 dek1-4* double mutant plants are dwarfed and organs within the inflorescence become fused to one another (Fig.

6J,K). The organ fusion appears similar to mutants such as *hothead* and *fiddlehead*, which have defects in the biosynthesis of the cuticle overlying the epidermis (Krolikowski et al., 2003; Pruitt et al., 2000) and suggests that epidermal identity is further reduced in *acr4-24 dek1-4* double mutants, resulting in a defective cuticle. The cuticular ridges characteristic of sepal cells are variably reduced in *acr4-24 dek1-4* sepals, whereas no defect is observed in *atml1-2 dek1-4*, suggesting that *ACR4* and *ATML1* have distinct roles (Fig. 6P-S).

### Giant cell identity is established upstream of endoreduplication

As giant cells are highly endoreduplicated to 16C, we next investigated the connection between endoreduplication and the establishment of giant cell identity by altering the regulation of the cell cycle. We first asked whether endoreduplication is necessary for establishing giant cell fate. Loss of function of the CDK inhibitor *LGO* results in sepals lacking highly endoreduplicated 16C cells, as well as a shift of the overall sepal cell size distribution towards smaller cells (Fig. 2C,D) (Roeder et al., 2010). We





examined the expression of the fluorescent giant cell marker in *lgo* mutants. Surprisingly, despite the absence of endoreduplicated cells, the number of small cells expressing the giant cell enhancer was greatly increased in *lgo* ( $149.3 \pm 20.1$ ) (Fig. 4A; Fig. 5K,L). This suggests that giant cells are specified upstream of endoreduplication. We further asked whether these additional small cells expressing the giant cell enhancer were produced via the epidermal specification pathway. Giant cell marker expression is strongly reduced in *dek1-4 lgo-1* double mutants ( $3.8 \pm 1.5$ ) (Fig. 4A), indicating that these small giant cells are specified as giant through the epidermal specification pathway.

We next tested whether endoreduplication is sufficient to promote giant cell identity. Overexpression of the CDK inhibitor KRP1 throughout the epidermis (*ATML1p::KRP1*) produces sepals nearly covered with large cells (Fig. 2E,F) (Bemis and Torii, 2007; Roeder et al., 2010). However, the number of large cells expressing the giant cell enhancer is not increased ( $14.2 \pm 2.6$  versus  $14.9 \pm 1.4$  in wild type) (Fig. 4A; Fig. 5M,N). Large cells that do not express the giant cell enhancer are present in *ATML1p::KRP1* sepals (Fig. 4F), suggesting that endoreduplication is not sufficient to induce giant cell identity. The total number of sepal epidermal cells is reduced in *ATML1p::KRP1* sepals and thus these large cells represent a higher proportion of the cells than giant cells in a wild-type sepal. We further tested whether forcing cells to endoreduplicate could restore giant cell identity in epidermal specification mutants. We found that overexpression of *KRP1* in

**Fig. 5. Epidermal specification factors promote giant cell identity and endoreduplication represses small cell identity.** Two images are fused (white line at junction) to show the expression of the giant cell fluorescent marker (light-blue nuclei) and small cell marker (green ER localization) in the whole stage 12 mature abaxial (outer) sepal epidermis for each genotype. Cell walls have been stained with PI (red) to show cell size. A magnified image shows cellular resolution.

(A,B) Wild-type sepal showing the expression of the giant cell marker in the large giant cells, as well as a few smaller cells, towards the tip of the sepal. The small cell marker is expressed in the small cells, particularly near the base of the sepal. (C,D) *dek1-4* sepal showing a strong reduction in the number of cells expressing the giant cell marker and an expansion of the small cell marker expression throughout the sepal. (E,F) *atml1-2* sepal lacking large giant cells, but with smaller sized cells expressing the giant cell marker. (G,H) *acr4-24* sepal with fewer giant cells and a corresponding reduction in the giant cell marker expression. (I,J) *hdg11-3* sepal with a subtle reduction in the number of giant cells. The number of smaller cells expressing the giant cell enhancer is slightly increased. (K,L) *lgo-1* sepal with a large number of small cells expressing the giant cell marker, particularly near the top of the sepal. The small cell marker expression expands to most of the cells of the sepal, including many of those small cells expressing the giant cell marker (arrow). (M,N) *ATML1p::KRP1* sepal showing an increase in the number of large cells, no change in the number of cells expressing the giant cell marker and limitation of the small cell marker to the remaining small cells. (O,P) *dek1-4 ATML1p::KRP1* sepal showing a strong reduction in the number of cells expressing the giant cell marker similar to *dek1-4* single mutants. Large cells are formed similar to *ATML1p::KRP1* sepals, but many of these cells do not express the giant cell marker (asterisks). The small cell marker is expressed in the few remaining small cells. Scale bars: 100  $\mu$ m in A,C,E,G,I,K,M,O; 50  $\mu$ m in B,D,F,H,J,L,N,P.

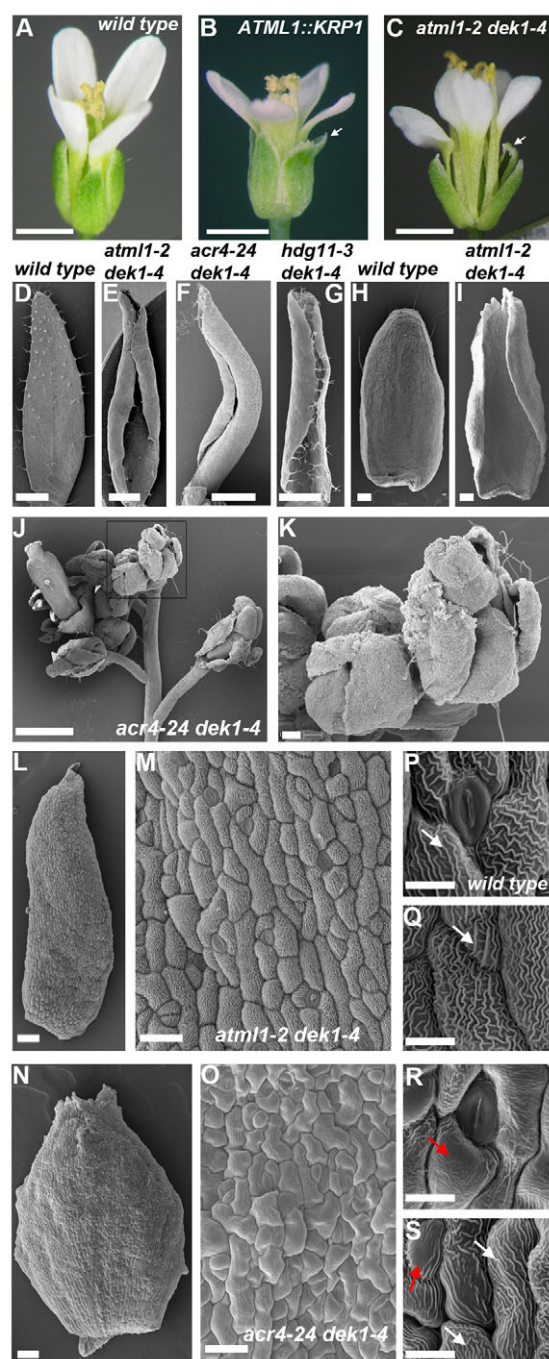
*dek1-4*, *atml1-2*, *acr4-24* or *hdg11-3* was sufficient to produce large cells (Fig. 4F-K; Fig. 5O,P). However, overexpression of *KRP1* in the *dek1-4* mutant, where very few giant cells are specified, clearly showed that these additional large endoreduplicated cells did not express the giant cell enhancer (Fig. 4K; Fig. 5O,P). Therefore, driving endoreduplication is not sufficient to specify giant cell identity, confirming that the epidermal specification pathway establishes giant cell identity upstream of endoreduplication.

### Endoreduplication represses small cell identity

We next tested how endoreduplication affects the establishment of small cell identity. Overexpression of *KRP1* to induce endoreduplication is sufficient to repress small cell identity in most large cells (Fig. 5M,N). This is true even when *KRP1* is overexpressed in the *dek1* mutant background (Fig. 5O,P), where most cells would otherwise default to small cell identity (Fig. 5C,D). Conversely, when endoreduplication is reduced in *lgo* mutants, the small cell enhancer expression expands to most cells (Fig. 5K,L). Many of the small cells expressing the giant cell enhancer also express the small cell marker, indicating that loss of endoreduplication is sufficient to promote small cell identity. Small cell identity is similarly expanded in *dek1-4* mutants, which have a reduction in endoreduplication similar to *lgo* (Fig. 5C,D), supporting repression of small cell identity by endoreduplication.

To confirm that altering cell cycle regulation could change small cell identity, we expressed *CYCLIN D1;1* to promote cell division instead of endoreduplication throughout the epidermis (*ATML1p::CYCD1;1*). T1 transformants phenotypically ranged from a small reduction in the formation of large cells to a complete loss of large cells. The expression of the small cell marker





**Fig. 6. Loss of giant cells affects organ curvature.** (A) A wild-type flower showing that the sepals straighten to protect the base as the flower opens. (B) *ATML1p::KRP1* flower showing that the sepals curve outwards (arrow). (C) *atml1-2 dek1-4* flower showing that the sepals curl inwards (arrow). The sepals do not cover the base of the flower. (D) Scanning electron micrograph of a wild-type cauline leaf, which is fairly flat. (E) *atml1-2 dek1-4* cauline leaves roll inwards. (F) *acr4-24 dek1-4* cauline leaves curl inwards. (G) *hdg11-3 dek1-4* cauline leaves also roll inwards. (H) Scanning electron micrograph of the adaxial (inner) side of a wild-type sepal. (I) *atml1-2 dek1-4* sepal edges curl inwards, similar to the mutant leaves. (J) Flowers of the *acr4-24 dek1-4* inflorescence stick together. (K) Fused flowers from the *acr4-24 dek1-4* inflorescence. Magnified view of the region boxed in J. (L, M) Abaxial (outer) side of an *atml1-2 dek1-4* sepal showing the cell size pattern consists of small epidermal cells similar to *dek1-4*. (N, O) Abaxial (outer) side of an *acr4-24 dek1-4* sepal showing that the cell size pattern also consists of small epidermal cells. (P) Wild-type sepal epidermal cells have characteristic cuticular ridges (e.g. arrow). (Q) The cuticular ridges (arrow) of *atml1-2 dek1-4* sepal epidermal cells appear similar to wild type. (R, S) *acr4-24 dek1-4* sepal cells show a variable reduction in cuticular ridges (white arrows). Patches without ridges (red arrows) are present. Scale bars: 1 mm in A-G, J; 100  $\mu$ m in H, I, K, L, N; 30  $\mu$ m in M, O; 10  $\mu$ m in P-S.

## DISCUSSION

Our results suggest that regulation of the cell cycle can act both upstream and downstream of the establishment of cell identity (Fig. 7G). The epidermal specification pathway promotes giant cell identity, which in turn promotes early entry into the endocycle, causing the enlargement of giant cells. Endoreduplication directly or indirectly leads to the repression of small cell identity.

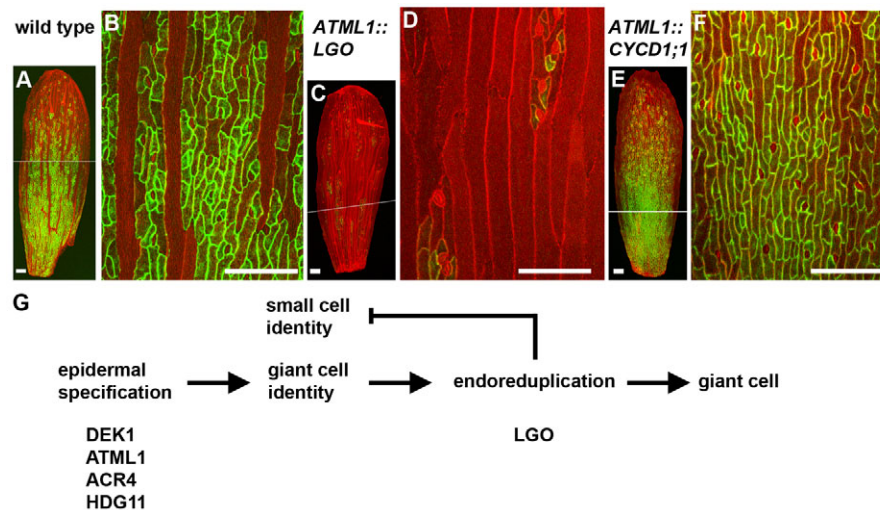
In this context, the smaller cells expressing the giant cell marker can be explained as cells that were specified as giant cells, but then did not endoreduplicate extensively. In some of those cells, endoreduplication does not repress small cell marker expression, leading to the rare cells that express both markers.

The establishment of giant cell identity upstream of endoreduplication parallels trichome development, suggesting that this is a commonly used developmental pathway. Although distinct, the pathways incorporate members of the same gene families (the class IV HD-ZIP transcription factors *ATML1* and *HDG11* in giant cells, and *GL2* in trichomes; the CDK inhibitors *LGO* in giant cells and *SIAMESE* in trichomes) (Grebe, 2012), hinting that these genes are involved in a conserved regulatory module for the differentiation of endoreduplicated cell types.

## Cell cycle control of cell identity

That the cell cycle can contribute to the identity of a cell is somewhat unexpected. Future research will show whether this is a general trend in development, but there are a few other examples. In *Arabidopsis*, endoreduplication is important for the maintenance of trichome identity. In the *siamese* mutant in which trichome cells divide instead of endoreduplicate, some trichomes lose their identity and revert to pavement cells (Bramsiepe et al., 2010). As *SIAMESE* is the CDK inhibitor most closely related to *LGO*, it will be interesting to further investigate the relationship between *LGO* and the maintenance of giant cell identity. CDK inhibitors also play a role in specification and differentiation in other systems. In the development of the *Xenopus* retina, it has been shown that the CDK inhibitor p27<sup>Xic</sup> controls both cell cycle exit and specification of Müller glia, and that separate domains of p27 mediate cyclin-CDK complex binding and Müller glial identity (Ohnuma et al.,

corresponded with the observed cell size phenotype: the small cell marker domain expanded with the loss of enlarged cells (Fig. 7A,B,E,F). Conversely, we further tested whether driving cells to endoreduplicate through overexpression of *LGO* in the epidermis (*ATML1p::LGO*) was sufficient to repress small cell identity. T1 transgenic plants also showed a range of phenotypes from a slight increase in the number of large cells to sepals covered in large cells. Again, loss of small cell marker expression correlated with an expansion of large endoreduplicated cells covering the sepal (Fig. 7A-D). It appears that altering the cell cycle is sufficient to change small cell identity, as defined by expression of this cell-type specific reporter.



**Fig. 7. Feedback between cell cycle and cell identity.** (A-F) Ectopic expression of cell cycle regulators throughout the epidermis is sufficient to alter the expression pattern of the small cell marker (A,B, green, ER localization; propidium iodide, red). *ATML1::LGO* (C,D) causes the formation of ectopic large cells and limits the expression of the small cell marker to the remaining small cells, similar to overexpression of the cell cycle inhibitor *KRP1* (see Fig. 5M,N). Conversely, overexpression of *CYCD1;1* (E,F) is sufficient to promote mitotic cell cycles causing the absence of large endoreduplicated cells and the expanded expression of the small cell marker. Scale bars: 100  $\mu$ m. (G) Schematic representation of the giant cell pathway. The epidermal specification genes *DEK1*, *ATML1*, *ACR4* and *HDG11* promote giant cell identity specification, which acts upstream of endoreduplication promoted by *LGO* to form a giant cell. Endoreduplication in turn directly or indirectly inhibits small cell identity.

1999). Likewise, separate domains of p27<sup>Xic</sup> are required to inhibit the cell cycle and promote cardiomyocyte differentiation in *Xenopus* (Movassagh and Philpott, 2008). Furthermore, a number of core cell cycle regulators are being shown to have roles in regulating the differentiation, migration and synaptic plasticity of neurons after cell cycle exit (Frank and Tsai, 2009; Nguyen et al., 2006). However, the cyclin E/Cdk2 complex from *C. elegans* inhibits differentiation of germ cells directly by phosphorylating the translational repressor GLD-1 (Jeong et al., 2011). Finally, the cell cycle regulator cyclin D1 has recently been shown to interact with transcriptional regulatory machinery in mouse and associate with the promoters of abundantly expressed genes during development (Bienvenu et al., 2010). Through this association, cyclin D1 regulates the expression of *Notch1* in the developing mouse retina, indicating that cell cycle regulators can directly control developmental gene expression. In the sepal epidermis, it will be interesting to determine whether the repression of small cell identity by endoreduplication is directly regulated by the cell cycle machinery or indirectly the result of some property of endoreduplicated cells such as their size or growth.

### Epidermal specification factors regulate proliferation

Cell specification factors have been shown to regulate the cell cycle in many systems, including plants; developmentally regulated transcription factors bind upstream regulatory elements of cell cycle genes and regulate their expression to cause the cell to divide at the appropriate time (Duman-Scheel et al., 2002; Lee and Orr-Weaver, 2003; Sozzani et al., 2010; Xie et al., 2010). Here, we show that epidermal specification mutants lack highly endoreduplicated giant cells. Although epidermal specification factors promote the establishment of giant cell identity, future experiments will determine whether they also have a direct role in regulating the cell cycle. Previous work suggests it is plausible that these epidermal specification factors inhibit proliferation.

Transcriptional inhibition of *NbDEK1* through virus-induced gene silencing (VIGS) in *N. benthamiana* (a tobacco relative) induces transcriptional activation of cell cycle genes and hyperproliferation (Ahn et al., 2004). Similarly, strong alleles of maize *cr4* often exhibit overproliferation of leaf cells, creating disorganized outgrowths (Jin et al., 2000). In the root, *ACR4* constrains the number of divisions in the pericycle cell layer during lateral root formation and in the columella lineage at the root tip (De Smet et al., 2008; Stahl et al., 2009). In *acr4* mutant sepals, we have shown there is increased proliferation in the place of endoreduplication, which is also consistent with a role for *ACR4* in limiting proliferation. In the root tip, the small signaling peptide CLE40 acts upstream of *ACR4* to activate restriction of columella divisions (Stahl et al., 2009). It will be interesting to determine whether CLE signaling peptides (Jun et al., 2010) also play a role in giant cell development in the sepal.

The pathway between the epidermal specification genes remains somewhat unclear in both the specification of epidermis and giant cells. For example, *ATML1* was thought to act downstream of *DEK1* because *ATML1* expression is absent from the outer cell layer of *dek1*-null mutant embryos, which lack an epidermis (Johnson et al., 2005). However, we see that the *ATML1* promoter is active in *dek1-4* sepals, suggesting that pathway is more complex. In the future, the giant cell system may help us unravel the complexities of this pathway.

### Acknowledgements

We thank Kaoru Sugimoto, Zack Nimchuk, Yun Zhou, Erich Schwarz and Xian Qu for helpful comments and discussions. This work was initiated in the laboratory of Martin Yanofsky at UCSD and has benefited greatly from his insights. We thank John Bowman and Yuval Eshed for providing the giant cell marker YJ158 and information on the insertion site; Gwyneth Ingram at the University of Edinburgh for providing the *acr4-2* allele; Keiko Torii for providing *ATML1p::KRP1* seeds; and the Salk Institute Genomic Analysis Laboratory and Arabidopsis Biological Resource Center for *dek1-3*, *hdg11-1*, *atml1-3*, *atml1-4* and the small cell marker CS70134. We thank Henrik Jönsson and Pawel Krupinski for the Costanza ImageJ plug-in for segmentation of nuclei. We



thank Rochelle Diamond and the Caltech Flow Cytometry Facility for expertise in flow cytometry, Aida Sun for technical assistance with mapping, Will Suh for technical assistance with cloning, and The Arabidopsis Information Resource (TAIR) for essential genome information.

### Funding

This work made use of the Cornell Center for Materials Research Facilities supported by the National Science Foundation [DMR-0520404]. The authors acknowledge the Department of Energy Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the US Department of Energy [DE-FG02-88ER13873 to E.M.M.] for funding the experimental work described; the Gordon and Betty Moore Foundation Cell Center (<http://www.cellcenter.caltech.edu/>) (A.H.K.R. and A.C.) for funding the computational image analysis and the salary for A.H.K.R. to finish the project; and a Helen Hay Whitney Foundation postdoctoral fellowship to A.H.K.R. for her salary in initiating the project.

### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.082925/-/DC1>

### References

- Abe, M., Katsumata, H., Komeda, Y. and Takahashi, T. (2003). Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in Arabidopsis. *Development* **130**, 635-643.
- Ahn, J. W., Kim, M., Lim, J. H., Kim, G. T. and Pai, H. S. (2004). Phytocalpain controls the proliferation and differentiation fates of cells in plant organ development. *Plant J.* **38**, 969-981.
- Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653-657.
- Barrell, P. J. and Conner, A. J. (2006). Minimal T-DNA vectors suitable for agricultural deployment of transgenic plants. *Biotechniques* **41**, 708-710.
- Becraft, P. W. and Asuncion-Crabb, Y. (2000). Positional cues specify and maintain aleurone cell fate in maize endosperm development. *Development* **127**, 4039-4048.
- Becraft, P. W., Stinard, P. S. and McCarty, D. R. (1996). CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* **273**, 1406-1409.
- Becraft, P. W., Kang, S. H. and Suh, S. G. (2001). The maize CRINKLY4 receptor kinase controls a cell-autonomous differentiation response. *Plant Physiol.* **127**, 486-496.
- Becraft, P. W., Li, K. J., Dey, N. and Asuncion-Crabb, Y. (2002). The maize dek1 gene functions in embryonic pattern formation and cell fate specification. *Development* **129**, 5217-5225.
- Bemis, S. M. and Torii, K. U. (2007). Autonomy of cell proliferation and developmental programs during Arabidopsis aboveground organ morphogenesis. *Dev. Biol.* **304**, 367-381.
- Bienvenu, F., Jirawatnotai, S., Elias, J. E., Meyer, C. A., Mizeracka, K., Marson, A., Frampton, G. M., Cole, M. F., Odom, D. T., Odajima, J. et al. (2010). Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen. *Nature* **463**, 374-378.
- Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1989). Genes directing flower development in Arabidopsis. *Plant Cell* **1**, 37-52.
- Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1991). Genetic interactions among floral homeotic genes of Arabidopsis. *Development* **112**, 1-20.
- Bramsiepe, J., Wester, K., Weinl, C., Roodbarkelari, F., Kasili, R., Larkin, J. C., Hülkamp, M. and Schnittger, A. (2010). Endoreplication controls cell fate maintenance. *PLoS Genet.* **6**, e1000996.
- Breuer, C., Ishida, T. and Sugimoto, K. (2010). Developmental control of endocycles and cell growth in plants. *Curr. Opin. Plant Biol.* **13**, 654-660.
- Churchman, M. L., Brown, M. L., Kato, N., Kirik, V., Hülkamp, M., Inzé, D., De Veylder, L., Walker, J. D., Zheng, Z., Oppenheimer, D. G. et al. (2006). SIAMESE, a plant-specific cell cycle regulator, controls endoreplication onset in Arabidopsis thaliana. *Plant Cell* **18**, 3145-3157.
- Cunha, A., Roeder, A. and Meyerowitz, E. M. (2010). Segmenting the sepal and shoot apical meristem of Arabidopsis thaliana. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* **2010**, 5338-5342.
- Cunha, A., Tarr, P. T., Roeder, A. H., Altinok, A., Mjolsness, E. and Meyerowitz, E. M. (2012). Computational analysis of live cell images of the Arabidopsis thaliana plant. *Methods Cell Biol.* **110**, 285-323.
- De Smet, I., Vassileva, V., De Rybel, B., Levesque, M. P., Grunewald, W., Van Damme, D., Van Noorden, G., Naudts, M., Van Isterdael, G., De Clercq, R. et al. (2008). Receptor-like kinase ACR4 restricts formative cell divisions in the Arabidopsis root. *Science* **322**, 594-597.
- Ditta, G., Pinyopich, A., Robles, P., Pelaz, S. and Yanofsky, M. F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. *Curr. Biol.* **14**, 1935-1940.
- Duman-Scheel, M., Weng, L., Xin, S. and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature* **417**, 299-304.
- Eshed, Y., Izhaki, A., Baum, S. F., Floyd, S. K. and Bowman, J. L. (2004). Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. *Development* **131**, 2997-3006.
- Frank, C. L. and Tsai, L. H. (2009). Alternative functions of core cell cycle regulators in neuronal migration, neuronal maturation, and synaptic plasticity. *Neuron* **62**, 312-326.
- Gifford, M. L., Dean, S. and Ingram, G. C. (2003). The Arabidopsis ACR4 gene plays a role in cell layer organisation during ovule integument and sepal margin development. *Development* **130**, 4249-4258.
- Gifford, M. L., Robertson, F. C., Soares, D. C. and Ingram, G. C. (2005). ARABIDOPSIS CRINKLY4 function, internalization, and turnover are dependent on the extracellular crinkly repeat domain. *Plant Cell* **17**, 1154-1166.
- Grebe, M. (2012). The patterning of epidermal hairs in Arabidopsis – updated. *Curr. Opin. Plant Biol.* **15**, 31-37.
- Ingram, G. C. (2007). Signalling during epidermal development. *Biochem. Soc. Trans.* **35**, 156-160.
- Javelle, M., Vernoud, V., Rogowsky, P. M. and Ingram, G. C. (2011). Epidermis: the formation and functions of a fundamental plant tissue. *New Phytol.* **189**, 17-39.
- Jeong, J., Verheyden, J. M. and Kimble, J. (2011). Cyclin E and Cdk2 control GLD-1, the mitosis/meiosis decision, and germline stem cells in *Caenorhabditis elegans*. *PLoS Genet.* **7**, e1001348.
- Jin, P., Guo, T. and Becraft, P. W. (2000). The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response. *Genes* **27**, 104-116.
- Johnson, K. L., Degnan, K. A., Ross Walker, J. and Ingram, G. C. (2005). AtDEK1 is essential for specification of embryonic epidermal cell fate. *Plant J.* **44**, 114-127.
- Jovtchev, G., Schubert, V., Meister, A., Barow, M. and Schubert, I. (2006). Nuclear DNA content and nuclear and cell volume are positively correlated in angiosperms. *Cytogenet. Genome Res.* **114**, 77-82.
- Jun, J., Fiume, E., Roeder, A. H., Meng, L., Sharma, V. K., Osmont, K. S., Baker, C., Ha, C. M., Meyerowitz, E. M., Feldman, L. J. et al. (2010). Comprehensive analysis of CLE polypeptide signaling gene expression and overexpression activity in Arabidopsis. *Plant Physiol.* **154**, 1721-1736.
- Karimi, M., Inzé, D. and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* **7**, 193-195.
- Kowles, R., Yerk, G., Srien, F. and Phillips, R. (1992). Maize endosperm tissue as an endoreduplication system. *Genet. Eng.* **14**, 65-88.
- Krolkowski, K. A., Victor, J. L., Wagler, T. N., Lolle, S. J. and Pruitt, R. E. (2003). Isolation and characterization of the Arabidopsis organ fusion gene HOTHREAD. *Plant J.* **35**, 501-511.
- Lee, L. A. and Orr-Weaver, T. L. (2003). Regulation of cell cycles in Drosophila development: intrinsic and extrinsic cues. *Annu. Rev. Genet.* **37**, 545-578.
- Lee, H. O., Davidson, J. M. and Duronio, R. J. (2009). Endoreplication: polyploidy with purpose. *Genes Dev.* **23**, 2461-2477.
- Lid, S. E., Gruis, D., Jung, R., Lorentzen, J. A., Ananiev, E., Chamberlin, M., Niu, X. M., Meeley, R., Nichols, S. and Olsen, O. A. (2002). The defective kernel 1 (dek1) gene required for aleurone cell development in the endosperm of maize grains encodes a membrane protein of the calpain gene superfamily. *Proc. Natl. Acad. Sci. USA* **99**, 5460-5465.
- Lid, S. E., Olsen, L., Nestetog, R., Aukerman, M., Brown, R. C., Lemmon, B., Mucha, M., Opsahl-Sorteberg, H. G. and Olsen, O. A. (2005). Mutation in the Arabidopsis thaliana DEK1 calpain gene perturbs endosperm and embryo development while over-expression affects organ development globally. *Planta* **221**, 339-351.
- Melaragno, J. E., Mehrotra, B. and Coleman, A. W. (1993). Relationship between endopolyploidy and cell size in epidermal tissue of Arabidopsis. *Plant Cell* **5**, 1661-1668.
- Movassagh, M. and Philpott, A. (2008). Cardiac differentiation in Xenopus requires the cyclin-dependent kinase inhibitor, p27Xic1. *Cardiovasc. Res.* **79**, 436-447.
- Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K. T. and Takahashi, T. (2006). Characterization of the class IV homeodomain-Leucine Zipper gene family in Arabidopsis. *Plant Physiol.* **141**, 1363-1375.
- Nguyen, L., Besson, A., Heng, J. I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J. M. and Guillemot, F. (2006). p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev.* **20**, 1511-1524.
- Ohnuma, S., Philpott, A., Wang, K., Holt, C. E. and Harris, W. A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. *Cell* **99**, 499-510.

- Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E. and Yanofsky, M. F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* **405**, 200-203.
- Peres, A., Churchman, M. L., Hariharan, S., Himanen, K., Verkest, A., Vandepoele, K., Magyar, Z., Hatzfeld, Y., Van Der Schueren, E., Beemster, G. T. S. et al. (2007). Novel plant-specific cyclin-dependent kinase inhibitors induced by biotic and abiotic stresses. *J. Biol. Chem.* **282**, 25588-25596.
- Pruitt, R. E., Vielle-Calzada, J. P., Ploense, S. E., Grossniklaus, U. and Lolle, S. J. (2000). FIDDLEHEAD, a gene required to suppress epidermal cell interactions in Arabidopsis, encodes a putative lipid biosynthetic enzyme. *Proc. Natl. Acad. Sci. USA* **97**, 1311-1316.
- Roeder, A. H. K., Chickarmane, V., Cunha, A., Obara, B., Manjunath, B. S. and Meyerowitz, E. M. (2010). Variability in the control of cell division underlies sepal epidermal patterning in Arabidopsis thaliana. *PLoS Biol.* **8**, e1000367.
- Roeder, A. H., Tarr, P. T., Tobin, C., Zhang, X., Chickarmane, V., Cunha, A. and Meyerowitz, E. M. (2011). Computational morphodynamics of plants: integrating development over space and time. *Nat. Rev. Mol. Cell Biol.* **12**, 265-273.
- Sessions, A., Weigel, D. and Yanofsky, M. F. (1999). The Arabidopsis thaliana MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *Plant J.* **20**, 259-263.
- Sorimachi, H. and Suzuki, K. (2001). The structure of calpain. *J. Biochem.* **129**, 653-664.
- Sozzani, R., Cui, H., Moreno-Risueno, M. A., Busch, W., Van Norman, J. M., Vernoux, T., Brady, S. M., Dewitte, W., Murray, J. A. and Benfey, P. N. (2010). Spatiotemporal regulation of cell-cycle genes by SHORTROOT links patterning and growth. *Nature* **466**, 128-132.
- Stahl, Y., Wink, R. H., Ingram, G. C. and Simon, R. (2009). A signaling module controlling the stem cell niche in Arabidopsis root meristems. *Curr. Biol.* **19**, 909-914.
- Traas, J., Hülskamp, M., Gendreau, E. and Höfte, H. (1998). Endoreduplication and development: rule without dividing? *Curr. Opin. Plant Biol.* **1**, 498-503.
- Wang, C. X., Barry, J. K., Min, Z., Tordsen, G., Rao, A. G. and Olsen, O. A. (2003). The calpain domain of the maize DEK1 protein contains the conserved catalytic triad and functions as a cysteine proteinase. *J. Biol. Chem.* **278**, 34467-34474.
- Watanabe, M., Tanaka, H., Watanabe, D., Machida, C. and Machida, Y. (2004). The ACR4 receptor-like kinase is required for surface formation of epidermis-related tissues in Arabidopsis thaliana. *Plant J.* **39**, 298-308.
- Xie, Z., Lee, E., Lucas, J. R., Morohashi, K., Li, D., Murray, J. A., Sack, F. D. and Grotewold, E. (2010). Regulation of cell proliferation in the stomatal lineage by the Arabidopsis MYB FOUR LIPS via direct targeting of core cell cycle genes. *Plant Cell* **22**, 2306-2321.
- Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., Southwick, A. M., Wu, H. C., Kim, C., Nguyen, M. et al. (2003). Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* **302**, 842-846.