Gene expression map of the *Arabidopsis* shoot apical meristem stem cell niche

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Communicated by Elliot M. Meyerowitz, California Institute of Technology, Pasadena, CA, January 27, 2009 (received for review December 9, 2008)

Despite the central importance of stem cells in plant growth and development, the molecular signatures associated with them have not been revealed. Shoot apical meristems (SAMs) harbor a small set of stem cells located at the tip of each plant and they are surrounded by several million differentiating cells. This imposes a major limitation in isolating pure populations of stem cells for genomic analyses. We have developed a system to isolate pure populations of distinct cell types of the SAMs, including stem cells. We have used this system to profile gene expression from 4 different cell samples of SAMs. The cell sample-specific gene expression profiling has resulted in a high-resolution gene expression map to reveal gene expression networks specific to individual spatial domains of SAMs. We demonstrate that the cell sample-specific expression profiling is sensitive in identifying rare transcripts expressed in a few specific subsets of cells of SAMs.

Our extensive RNA in situ analysis reveals that the expression map can be used as a predictive tool in analyzing the spatial expression patterns of genes and it has led to the identification of unique gene expression patterns within the SAMs. Furthermore, our work reveals an enrichment of DNA repair and chromatin modification pathways in stem cells suggesting that maintenance of genome stability and flexible chromatin may be crucial for stem cell function. The gene expression map should guide future reverse genetics experiments, high-resolution analyses of cell–cell communication networks and epigenetic modifications.

Results and Discussion

Gene Expression and Cell–Cell Communication Machinery in *ap1-1;cal1-1* System. We tested whether *ap1-1;cal1-1* SAMs are suitable for cell type specific expression profiling by analyzing the organization of the CZ and the RM. The expression patterns of *CLV3*, and *WUSCHEL* (*WUS*), markers for the cells of the CZ and the RM respectively, were visualized by whole mount RNA in situ analysis. This allowed us to demonstrate that the expression patterns of *CLV3* and *WUS* are similar to the WT expression patterns observed in previous studies (Fig. S1A and B) (9, 10). Furthermore, we tested whether the *ap1-1;cal1-1* SAMs are comparable to WT SAMs with respect to the CLV-WUS-mediated intercellular communication process. Mutations in *chv3* gene result in expansion of the CZ. *CLAVATA3* (*CLV3*), a small extracellular protein synthesized in the CZ activates *CLAVATA1* (*CLV1*)-*CLAVATA2* (*CLV2*) receptor kinase complex in RM cells (5). The active *CLV1-CLV2* receptor kinase complex functions by down regulating the expression of *WUSCHEL* (*WUS*), a homeodomain protein expressed in RM cells. An earlier live-imaging study has revealed that the transient silencing of the *CLV3* gene results in an increased *CLV3* promoter activity in the native domain within 24 h of *CLV3* silencing and followed by the radial expansion of the CZ in the next 48 h. We tested whether a similar CZ reorganization pattern could be observed in the SAMs of *ap1-1;cal1-1* plants. The live-imaging of SAMs of *ap1-1;cal1-1* mutants, upon *CLV3* silencing, revealed a similar temporal sequence of CZ expansion that was observed in WT SAMs (4). This suggested that the intercellular communication-mediated by CLV-WUS network is operational in *ap1-1;cal1-1* SAMs (Fig. S1 C and D). Therefore, we adapted a protoplasting method, followed by FACS analysis to isolate fluorescently-labeled cell populations of distinct cell types from *ap1-1;cal1-1* SAMs (11) (SI Text).

Author contributions: R.K.Y., S.P., M.X., and G.V.R. performed research; R.K.Y., T.G., and G.V.R. analyzed data; R.K.Y., T.G., and G.V.R. wrote the paper; G.V.R. designed research. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0900843106/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.0900843106
Description of Cell Type-Specific Fluorescent Markers. We introduced fluorescent reporters into the ap1-1;cal1-1 background to differentially label 3 distinct cell types of the SAM stem cell niche. The pCLV3::mGFP5-ER was used to label cell types of the CZ (4). The CLV3 expression is restricted to the CZ and the expression extends into the 4th layer starting from the tip (Fig. 1 A and D). pWUS::mGFP5-ER (WUSCHEL promoter driving the expression of endoplasmic reticulum localized-GFP) was used to label cells of the RM and it is expressed only in a few centrally-located cells of the L3 layers (Fig. 1 B and E and Fig. S3D). The pFILAMEN-TOUSFLOWER (FIL)::DsRED-N7 (FIL promoter driving the expression of nuclear-localized dsRED) was used to label specific subsets of cells of the flower organ primordia located within the PZ (Fig. 1 C and F) (12). For simplicity, the 3 cell samples will be referred to as CLV3p, FILp and WUSp. The 3 fluorescent reporters do not express in all cell types of SAMs. Therefore, while collecting GFP positive protoplasts from CLV3p cells, we also collected GFP negative (CLV3n) protoplasts (Fig. S4). The CLV3n cell populations are expected to represent a mixture of SAM cell types that lacks the CLV3p cells.

RNA samples from 4 different protoplast populations (CLV3p, FILp, WUSp and CLV3n) were hybridized to the Affymetrix GeneChip ATH1 in biological replicates. Correlation coefficients close to 1.0 among the replicates indicated a high reproducibility of the experiments (Table S1). To estimate the extent of gene expression changes in response to the protoplasting method, we compared the gene expression patterns of protoplasted-ap1-1;cal1-1 SAMs with nonprotoplasted-SAMs. Three hundred genes were found to respond to the protoplasting method. In the subsequent analysis these genes were not considered as cell-specific gene candidates (Table S2).

Cell Sample-Specific Expression Profiling Reveals Higher Sensitivity. The cell type-specific transcriptome analysis of Arabidopsis root cell types has revealed higher sensitivity than experiments with whole root samples (7). To assess a similar sensitivity increase for our datasets, we compared the detectable gene sets obtained from cell sample-specific expression profiling dataset (cell-sorted) with that of whole (cell-unsorted) ap1-1;cal1-1 SAMs (Table S3). The comparison of cell-sorted data with that of the whole ap1-1;cal1-1 SAMs revealed 952 genes that could only be identified in the cell type-specific transcriptome (Fig. 2D). This cell type-specific set includes CLV3 (Fig. S5 and Table S4), MEI2 C-Terminal RRM-like1 (MCT1) (Fig. 3E and Fig. S2), B3-TF (Fig. 3H and Fig. S2) and JAGGED LATERAL ORGANS (Fig. S5 and Table S4), which are expressed in small subsets of cells within the SAMs. We also compared the detectable gene sets from our cell sample-specific data with that of the pooled-microarray data of entire AtGenExpress developmental series of WT plants (Fig. 2D and SI Text) (7). This comparison yielded 1031 genes that were unique to cell sample-specific data and it includes PUMILIO10 (Fig. 3A), CLV3, MCT1 (Fig. 3E), MCT2 (Fig. 3B) and BLADE ON PETIOLE2 (Fig. S5 and Table S4), all of them are expressed in very few cells of the SAMs. The comparison of detectable genes in our cell sample-specific dataset with that
of the Arabidopsis roots revealed 3,915 genes specific to the root cell types, whereas 1,739 genes were specific to SAM cell types (Fig. 2D and SI Text) (3, 6). Taken together, the cell type-specific transcriptome, as expected, revealed higher sensitivity in identifying rare transcripts and genes that are unique to either the shoot or root cell-types.

**Differential Expression Analysis and Validation of Microarray Data.**

To identify differences in gene expression programs among the individual cell types, we identified differentially-expressed genes (DEGs) (SI Text). Systematic comparisons among all 3 cell samples identified a union set of 2515 DEGs \( P < 0.01 \) (Table S5 and SI Text). Next we generated 3 lines of evidence to validate the cell sample specific transcriptome data. First, we measured the relative abundance of transcripts of 3 marker genes (CLV3, WUS, and FIL), in isolated protoplasts by quantitative RT-PCR (qRT-PCR). The results showed an increased expression of the transcripts for CLV3, WUS, and FIL in the respective cell samples, which correlates well with the microarray expression data (Fig. 1J and K). The detection of WUS transcripts in CLV3p cells suggested that the expression of 2 genes overlap with each other. We generated a double transgenic plant carrying pCLV3:mGFP5-ER and pWUS::dksRED-N7 (WUS-CHEL promoter attached to nuclear-localized dsRED) to test whether the expression domains of CLV3 and WUS overlap with each other. A high resolution microscopic analysis revealed that the 2 genes are expressed in overlapping sets of cells located in L3 layers of SAMs (Fig. S3A-I). Although we detected high levels of WUS transcripts in CLV3p cells, however, a similar enrichment of WUS transcripts in CLV3p cells was not observed (Fig. 1J and K). This could be due to the dilution of CLV3-expressing cells because of a...
higher ratio of WUSp:CLV3p cells in regions where the 2 domains overlap. Enrichment of WUS transcripts in CLV3p cells, although the overlap is restricted to very few cells, could be because of higher levels of WUS expression in the most centrally-located cells of the WUS expression domain that overlap with the CLV3 expression domain. Our whole-mount WUS RNA in situ expression patterns reveal that WUS is expressed in a graded fashion with higher levels of expression detected in the centrally-located cells (Fig. S3 I and J). Second, we tested the transcript abundance of 7 genes by qRT-PCR that appeared to be differentially-expressed among CLV3p and CLV3n cell samples and found that the relative transcript abundance in the respective cell types was in agreement with the microarray data (Fig. S4). Third, we analyzed the relative expression differences of genes for which RNA in situ expression patterns and promoter reporter expression data are available from earlier studies (Fig. 2B, Fig. S5, and Table S4). The relative enrichment of characterized differentially-expressed genes across cell samples was in agreement with our DEG analysis results (Table S5 and Fig. S5).

Hierarchical cluster analysis grouped these genes largely according to their expression patterns observed on tissue sections, such as the PZ, the L1 layer, the CZ (includes narrow and broad) and the overlapping region between the CZ and the RM (Fig. 2A and B).

Comparison of Differential Gene Expression Among 3 Cell Samples Reveals Unique and Overlapping Sectors of Gene Activity. We organized the set of 2515 DEGs into 3 subcategories by determining genes that exhibited a significant up-regulation in 1 of the 3 cell samples (CLV3p, WUSp, and FILp) relative to at least 1 of the 2 other cell samples. The 3-way Venn diagram representation of these 3 DEG lists revealed 7 possible overlap/uniqueness sectors (Fig. 2C, Table S5, and SI Text). To mine this dataset, we evaluated the distribution of characterized marker genes in the individual Venn diagram sectors. The sector S5 (CLV3 expression domain, which excludes WUS and FIL expression domains) is expected to represent the central part of superficial cell layers (the L1 or the L2 or both the L1 and the L2) of the SAM and it includes CLV3, AINTEGUMENTA-LIKE (AIL5, AIL6, and AIL7) and TERMINAL EAR LIKE1 (Table S5 and Table S4). The sector S4 (shared between CLV3 and WUS expression domains, which excludes FIL expression domain) is expected to represent central part of the SAM including the superficial cell layers of the Rib-meristem and it includes SPATULA, REPRODUCTIVE MERISTEM1, HANBA TARANUMONOPOLE, CLAVATA1, WUS, SHOOTMERISTEMLESS, TERMINAL FLOWER1, CYTOKININ OXIDASE5, PIN FORMED1 (Table S5 and Table S4). The S6 (WUS expression domain, which excludes CLV3 and FIL domains) is expected to represent deeper cell layers of differentiating region of the Rib-meristem. The S2 sector (shared between CLV3 and FIL expression domains, which excludes WUS domains) is expected to represent superficial cell layers (the L1 or the L2 or both the L1 and the L2) of the entire SAM surface including differentiating cells of the organ primordia and it includes ARABIDOPSIS CRINKLY4, ARABIDOPSIS THALIANA MERISTEM LAYER1, PROTODermal FACTOR 1 and 2, and BODYGUARD (Table S5 and Table S4). The S7 sector (exclusive FIL expression domain) is expected to represent cells of the PZ including differentiating organ primordia and it includes FIL, ASYMMETRIC LEAVES1 and 2, CUP SHAPED COTYLEDON3 and many such genes (Table S5 and Table S4). The S3 (shared between FIL and WUS expression domains, which excludes CLV3 domain) is expected to represent a relatively broad SAM region encompassing the differentiating cells of the organ primordia and the deeper cell layers of the Rib-meristem, and BREVIPEDICELLUS is associated with this sector (Table S5 and Table S4). Because a large part of the CLV3n cell sample is expected to be a part of FILp and WUSp cell samples, it was not included in the DEG analysis. However, a side-by-side comparison of expression values of genes in CLV3n cell sample with other cell samples has enabled us in assessing the radial dimensions of expression domains of genes that are part of the central region of SAMs (Fig. S2 and Fig. S5). Taken together, our analysis demonstrates that the dataset is suitable for analyzing the gene expression programs enriched in distinct spatial domains of SAMs.

Subclassification of DEGs Based on Expression Intensity. The organization of SAMs has been described with the help of a limited set of molecular markers. New molecular markers are required to gain a better understanding of the spatial organization of SAMs. Therefore, we sought to identify markers for cell types of SAMs by mining the datasets obtained from DEG analyses. DEG analyses, however, provide information across a continuous range of expression levels. To predict genes that are only expressed in discrete subdomains of the SAM, we subclassified the identified DEGs by their expression
levels in the individual cell samples (Fig. 2C and 4A). For this, their normalized intensity values from the 3 cell samples, CLV3p, FILp, and WUSp, were subgrouped into the 4 expression states absent (A), low (L), medium (M), and high (H) (Fig. 4A and SI Text). The 4 expression intervals for the 3 samples provide 81 possible intensity interval clusters (IICs), of which only 49 were observed in the dataset of the 2515 DEGs (Table S5 and Table S6). Approximately 42% of the DEGs could be classified into 3 IICs with the most uniform expression patterns. AAA, LLL, and MMM (Fig. 4B and Table S6) represented 58% of all DEGs surveyed. The remaining 42% of DEGs exhibited different intensity intervals, among cell types, and they could be organized into 2 subclasses (Fig. 4B and Table S6). The first one contained 12 IICs accounting for 47% of DEGs and the rest 11% of DEGs belonging to the remaining 34 IICs suggesting that their spatial expression is relatively restricted to specific subsets of cells (Fig. 4B and Table S6). Consistent with this hypothesis, a majority (40/47) of characterized differentially-expressed genes could be found in the latter 2 subclasses, suggesting that this approach can be applied to identify genes that are expressed in specific subsets of cells (Table S5 and Table S6).

Validation of DEGs by RNA in Situ Analysis Reveals Markers for Stem Cell Niche and Unique Expression Domains. Next, we mapped the 49 IICs onto the 6 overlap/unique sectors of the DEG analysis (Fig. 4C and Table S6). This allowed us to predict genes with discrete spatial expression patterns and confirm their expression patterns by RNA in situ analysis (Fig. 3). For example, we hypothesized that the genes belonging to the LAA (Low in CLV3p, Absent in FILp and Absent in WUSp) cluster in the S4 and S5 sectors should exhibit an overlapping expression with the CLV3 domain (Fig. 4C and Table S5). Indeed, the in situ experiments confirmed such an overlapping expression pattern within the CLV3 domain (Fig. 3A–F and Fig. S2). The subtle variations to the CLV3 pattern could be observed, with gene expression restricted specifically to the L1 layer of the CZ (Fig. 3 C and F) or to the L2 layer of the CZ (Fig. 3D). The genes that are shared between CLV3 and WUS domains could be identified in LAL, MAM, MAL, LAH or MLM cluster of S4 sectors (Fig. 3). These genes exhibited the expression that overlaps with both the CLV3 and the WUS domains (Fig. 3 G–Q and Fig. S2). The genes that were part of ALM, LLC and AAL clusters exhibited expression within the Rib-meristem (Fig. 3 R–T). Genes expressed in specific subsets of cells of the CZ could be found in the S7 sector (Fig. 3U and Fig. S2). A few genes that are part of the CLV3n cell sample, as expected, were excluded from 3 cell types and their expression was detected in the vasculature cells (Fig. 3 V and W and Fig. S2). Taken together, differential expression analysis and subsequent classification of DEGs based on expression levels can provide a better idea about their spatial expression patterns. However, the approach may not resolve finer differences in spatial expression patterns of genes that belong to the same IIC (Fig. 3 K and Q). This could be because of the inherent differences in gene expression levels and the rigid cut-off scale used for defining the intensity intervals. Nevertheless, our extensive in situ experiments demonstrate that the 49 IICs are a valuable data source for predicting spatial expression patterns. By using the markers that are expressed in distinct cell layers of the CZ (Fig. 3 C and D), we may be able to improve the spatial resolution and predictive capability of our expression map in the future.

Assessment of Overall Accuracy of the Technique and the DEG Analysis. To assess the accuracy of the technique, we considered a total of 70 documented differentially-expressed genes, including 23 expression patterns described in this study, whose spatial expression patterns could be judged with a fair degree of confidence. A comparison of DEG-predicted sectors of these genes with their observed spatial expression patterns on tissue sections revealed that 4 genes were incorrectly assigned to different DEG sectors and the other 4 genes were not identified as differentially-expressed, resulting in a combined error of 11% (Table S4). In contrast to our expectations, BARELY ANY MERISTEM1, CUP SHAPED COTYLEDON2, REVOLUTA and ULTRAPETALAI1 were not identified as DEGs, although a relative enrichment of these genes in expected cell samples could be detected (Table S5, Table S4, and Fig. S5). A notable exception is CUP SHAPED COTYLEDON1, which was associated with the S5 sector (CLV3p) instead of the S7 (FILp) (Table S4 and Table S5).

Overrepresentation of DNA Repair and Epigenetic Pathways in Stem Cells. It has been speculated, based on the organization of cell types, that the plant stem cell niches may share common properties with that of animal stem cell niches (13). However, molecular evidence to support this view is largely lacking because of the nonavailability of stem cell specific gene expression profile. To assess the molecular composition and pathways enriched stem cells, we performed enrichment analyses of Gene Ontology (GO) terms in our dataset (Fig. 2E and Table S7). First we tested the DEG sectors for overrepresented- GO terms that are characteristic for certain cell types of SAMs. The sector S2, which contained genes, expressed in the L1 layer was enriched with genes that are involved in wax biosynthesis (P < 1.9 × 10−2) and lipid metabolism (P < 1.2 × 10−2), which is consistent with one of the expected function of the L1 layer cells in secretion of epi-cuticular wax (14). As expected, the differentiating cells of the rib-meristem (S6) and differentiating cells of developing organs (S7) were over-represented with genes involved in various metabolic processes including that of carbohydrate metabolism, lignin deposition and photosynthetic genes (Fig. 2E and Table S7).

The sector S5, which is enriched in meristematic genes is over-represented with genes involved in DNA metabolism (P < 1.17 × 10−1), and DNA replication and repair (P < 8.41 × 10−7, 6.72 × 10−5) (Fig. 2E and Table S7). This includes MUTLI PROTEIN HOMOLOG 1 (ATMSH1), MUTLS HOMOLOG 2 (MSH2), MUTS LIKE PROTEIN 4 (MSH4), MUTS HOMOLOG 6-2 (MSH6-2), RADIATION SENSITIVE (ATRAD17), RADIATION SENSITIVE (ATRAD51), ARABIDOPSIS THALLAIA BREAST CANCER ASSOCIATED RING1 (ATBAR1), ARABIDOPSIS THALLAIA BREAST CANCER SUSCEPTIBILITY1 (ATBCRA1), and ARABIDOPSIS THALLAIA TELOMERASE REVERSE TRANSCRIPTASE (ATTERT), involved in telomere maintenance. The enrichment of both the enzymes of the mismatch repair and nonhomologous end-joining (NHEJ) DNA repair pathways suggest
that the SAM stem cells exhibit typical characteristics of cells experiencing stress (15). It is conceivable that error-free DNA replication is critical to prevent incorrect cellular amplification of mutations, and amplification of stem cell daughters that carry double-strand breaks (DSBs) can be deleterious to plant development.

Genes involved in chromosome organization and biogenesis (P < 4.84 × 10⁻⁴) were also over-represented in stem cells suggesting that the stem cell chromatin is maintained in a flexible state (Fig. 2E and Table S7). They include genes involved in HISTONE and DNA modifications such as centromeric HISTONE H3, HISTONE ACETYL TRANSFERASE (HAT), HISTONE DEACETYLASE 18 (HD18), HISTONE LYSINE AND METHYL TRANSFERASES (SUVRH, SUVRH9, and SUVR2), ARABIDOPSIS TRITHORAX-LIKE 1 (ATXI) and SIRTIUNS (SIR1 and SIR2), NAD-dependent deacetylases. Recent studies on murine embryonic stem cells (ES) have identified bivalent domains of both H3 lysine 27 methylation (a repressive chromatin mark) and H3 lysine 4 methylation (a chromatin mark that positively regulates transcription) within genes that encode developmentally important transcription factors. This suggested that the bivalent domains are responsible for keeping differentiation genes silent in ES cells, while keeping them primed for activation (16, 17). The enrichment of DNA and HISTONE modifications in stem cells of SAMs suggests that they might play a similar role in maintaining flexible chromatin to facilitate the dynamic balance of gene expression. This can be tested, in future experiments, by profiling DNA and HISTONE modifications of individual cell types of SAMs with the method described here.

The genomic instability and changes in gene expression are regarded as hallmarks of the aging process in eukaryotes. A recent study, on mouse embryonic stem cells, has revealed that the mammalian deacetylase, Sir2 (SIRT1), in response to DNA damage, relocalizes to the sites of DNA breaks to mediate repair. The over-expression of SIRT1 has been shown to promote survival of early embryonic stem cells. In stem cells of SAMs it suggests that they might play a similar role in maintaining flexible chromatin to facilitate the dynamic balance of gene expression. This can be tested, in future experiments, by profiling DNA and HISTONE modifications of individual cell types of SAMs with the method described here.

Conclusions

We have described a method to generate a cell-sample-specific gene expression map of SAMs. Our work has identified many genes with cell-sample-specific expression profiles within the SAM stem cell niche. The gene expression patterns identified in this study can be used to refine the spatial organization of cell types of the SAMs. Identification of molecular signatures associated with these cell types may provide clues to their function. The markers for cells of the stem cell niche, identified in this study, can be a valuable resource in following the dynamic regulation of stem cell maintenance by cell–cell communication. The dataset is a valuable resource for guiding future functional analyses of stem cell enriched genes including evolutionarily conserved PUMILIO class of translational repressors and genes homologous to LONELY GUY (LOG) of rice, which has been shown to function in meristem development by regulating cytokinin biosynthesis (Fig. 3A and K) (19, 20). More significantly, it is now possible to analyze cell–cell communication networks at a single cell type resolution, similar to the studies carried out in the Arabidopsis root system (21, 22). Elucidation of molecular networks at higher spatiotemporal resolution may provide a molecular framework to model cell–cell communication processes within the SAMs (23).

Materials and Methods

Transgenic Lines. The cell-type specific, fluorescent reporter constructs used for expression profiling experiments have been described in the following studies, CLV3:mGFP-ER (4) and FLC:dsRED-N7 (12), pWUS:mGFP-ER reporter line was assembled in pZP222 vector by using a 4.5-kb fragment upstream of WUSCHEL ORF and a 1.5-kb fragment downstream of the stop codon. The transgenic lines were generated using floral dip method (24). The double transgenic line expressing CLV3:mGFP-ER and WUS:dsRED-N7 has been described earlier (25). The transient silencing of CLV3 was achieved by using the constructs described in earlier study (4).

Protoplasting of SAM Cells. Approximately 200 SAMs of 24-day-old ap1-1,crl1-1 plants were harvested within 15 min and placed in protoplasting solution for ~1 h and 15 min. The concentration and composition of cell wall digesting enzymes were optimized to obtain maximum protoplast yield (11, 26). Harvested shoot apices were incubated in protoplasting solution [Solution B = Solution A + 1.5% cellohexaose (YAKULT; catalog no. 030260), 1% Pectolyase (YAKULT pharmaceutical Ltd, Tokyo Japan, Catalog no. 20047) and 1% Hemicellulase (Sigma USA, catalog no. H2125)], within a ~0.5 μm cell strainer placed in a small Petri-plate. Subsequent steps involved in harvesting protoplasts for FACS-mediated separation were carried out according to an earlier study (6). The modified protoplasting method yielded ~150,000 to 200,000 fluorescently labeled protoplasts. The purity of sorting events was independently confirmed through microscopic observation of sorted protoplasts.

Acknowledgments. We thank Barbara Walter (Institute of Invertebrate Geobiology, University of California, Riverside) for help with the FACS sorting and microarray hybridizations, Zhenhua Deng for help with qRT-PCR analysis, Mayana Hegde for help in generating marker lines, and Elliot Meyerowitz for providing FLC:dsRED-N7 DNA construct, Nicholas Provart and Zhenbiao Yang for comments on the manuscript. DNA sequencing/quantitative PCR/Microarray data were generated by the Genomics Core at the Institute for Integrative Genome Biology at the University of California, Riverside. This work was funded by National Science Foundation Grant IOS-0718046 (to G.V.R.).