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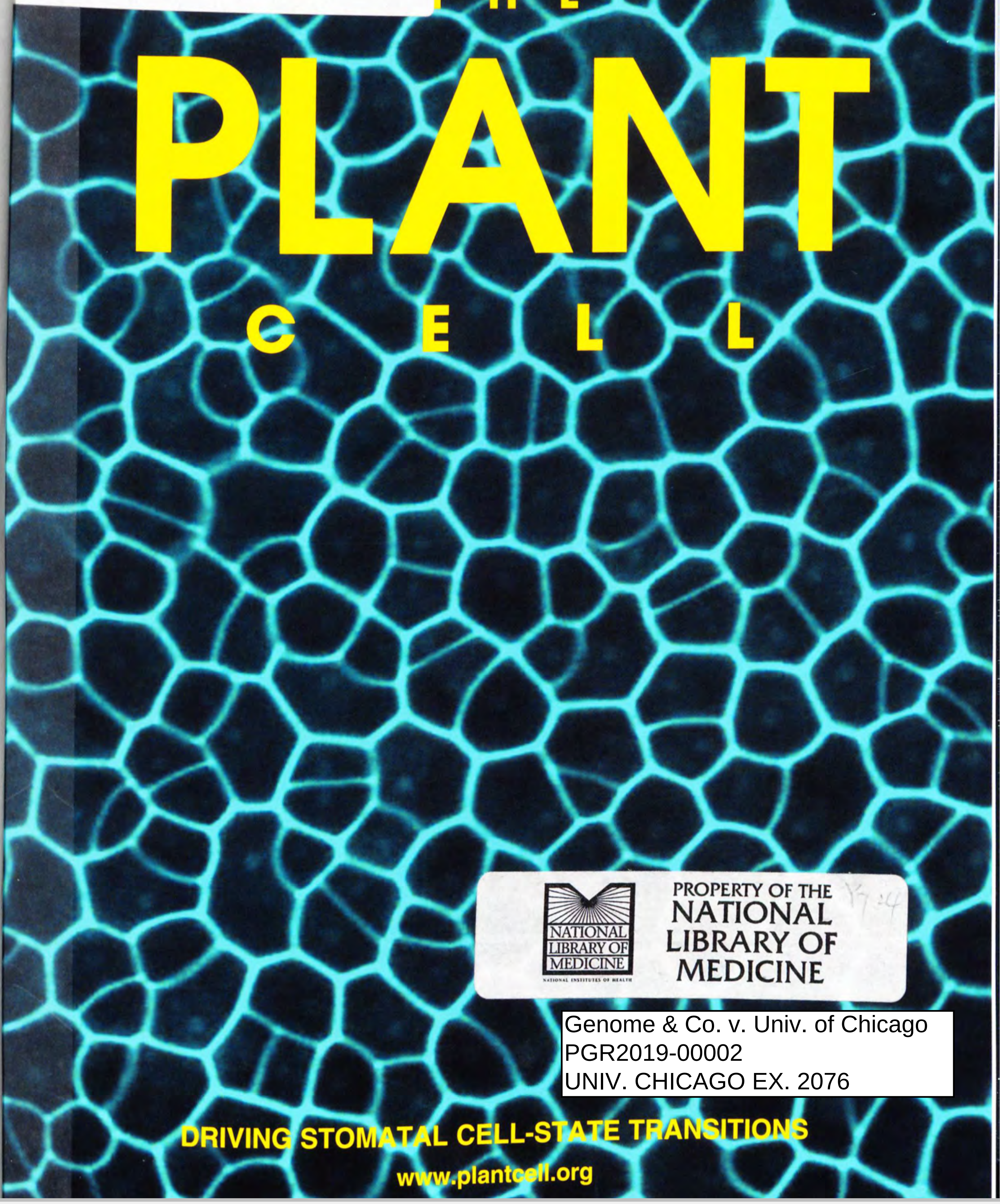

DRIVING STOMATAL CELL-STATE TRANSITIONS

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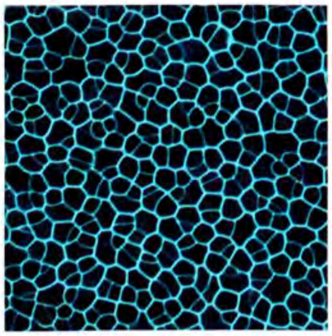
ON THE COVER



Stomata are formed through a series of differentiation events mediated by a trio of basic-helix-loop-helix (bHLH) proteins: SPEECHLESS (SPCH), MUTE, and FAMA. Through characterization of a dominant mutant, *scream-D* (*scrm-D*), which produces an epidermis consisting entirely of stomata, Kanaoka et al. (pages 1775–1785) identified two paralogous *Arabidopsis* bHLH proteins, SCRM and SCRM2, that partner with SPCH, MUTE, and FAMA to drive initiation, proliferation, and terminal differentiation of stomata. The cover shows the rosette leaf epidermis of a *mute scrm-D* double mutant, which is composed of triangular stomatal precursor cells called meristemoids and their sister cells. Surprisingly, SCRM is ICE1, a key upstream regulator of cold-induced gene expression, therefore suggesting a link between the transcriptional regulation of environmental adaptation and development.

EDITORIAL

IN THIS ISSUE

IN BRIEF

LETTERS TO THE EDITOR

REVIEW

RESEARCH ARTICLES

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LETTER TO THE EDITOR

Eleven Golden Rules of Quantitative RT-PCR

Reverse transcription followed by quantitative polymerase chain reaction analysis, or qRT-PCR, is an extremely sensitive, cost-effective method for quantifying gene transcripts from plant cells. The availability of nonspecific double-stranded DNA (dsDNA) binding fluorophors, such as SYBR Green, and 384-well-plate real-time PCR machines that can measure fluorescence at the end of each PCR cycle make it possible to perform qRT-PCR on hundreds of genes or treatments in parallel. This has facilitated the comparative analysis of all members of large gene families, such as transcription factor genes (Czechowski et al., 2004). Given the relatively low cost of PCR reagents, and the precision, sensitivity, flexibility, and scalability of qRT-PCR, it is little wonder that thousands of research labs around the world have embraced it as the method of choice for measuring transcript levels. However, despite its popularity, we continue to see systematic errors in the application of methods for qRT-PCR analysis, which can compromise the interpretation of results. The letter to the editor by Gutierrez et al. in this issue highlights one of many common sources of error, namely, the inappropriate choice of reference genes for normalizing transcript levels of test genes prior to comparative analysis of different biological samples. The following are 11 golden rules of qRT-PCR that, when observed, should ensure reproducible and accurate measurements of transcript abundance in plant and other cells. These rules are for relative quantification of RNA using two-step RT-PCR (where the product of a single RT reaction is used as template in multiple PCR reactions), SYBR Green to detect gene-specific PCR products, and reference genes for normalizing transcript levels of test genes before comparing samples. Further details can be found elsewhere (Czechowski et al., 2004, 2005). Most of these rules also apply to relative quantification methods that employ sequence-specific fluorescent probes, such as TaqMan probes, and to absolute quantification methods (http://www.gene-quantification.info/).

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(1) Harvest material from at least three biological replicates to facilitate statistical analysis of data, freeze immediately in liquid nitrogen, and store at −80°C to preserve full-length RNA.

(2) Use an RNA isolation procedure that produces high-quality total RNA from all samples to be analyzed. Check RNA quality using an Agilent 2100 Bioanalyzer (RNA integrity number, RIN > 7 and ideally > 9) or by electrophoretic separation on a high-resolution agarose gel (look for sharp ethidium bromide–stained rRNA bands) and spectrophotometry ($A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 2.0$). Quantify RNA using A_{260} values.

(3) Digest purified RNA with DNase I to remove contaminating genomic DNA, which can act as template during PCR and lead to spurious results. Subsequently, perform PCR on the treated RNA, using gene-specific primers, to confirm absence of genomic DNA.

(4) Perform RT reactions with a robust reverse transcriptase with no RNaseH activity (like SuperScriptIII from Invitrogen or ArrayScript from Ambion) to maximize cDNA length and yield. Use ultraclean oligo(dT) primer of high integrity. qRT-PCR gene expression measurements are comparable only when the same priming strategy and reaction conditions are used in all experiments and reactions contain the same total amount of RNA (Ståhlberg et al., 2004).

(5) Test cDNA yield and quality. Perform qPCR on an aliquot of cDNA from each sample, using primers to one or more reference genes that are known to be stably expressed in the organ(s)/tissue(s) under the range of experimental conditions tested. Threshold cycle (Ct) values should be within the range mean ±1 for each reference gene across all samples to ensure similar cDNA yield from each RT reaction. Quality of

primers for a reference gene that are ~1 kb apart. Typically, the Ct value for the primer pair at the 5′-end of a cDNA will be higher than the Ct value of the primer pair at the 3′-end, as reverse transcription begins at the 3′ [poly(A)] end of the template mRNA and does not always extend to the 5′-end of the template. Ideally, the Ct value of the 5′-end primer pair should not exceed that of the 3′-end pair by more than one cycle number.

(6) Design gene-specific PCR primers using a standard set of design criteria (e.g., primer $T_m = 60 \pm 1$°C, length 18 to 25 bases, GC content between 40 and 60%), which generate a unique, short PCR product (between 60 and 150 bp) of the expected length and sequence from a complex cDNA sample in preliminary tests, to facilitate multiparallel qPCR using a standard PCR program. The 3′-untranslated region is a good target for primer design because it is generally more unique than coding sequence and closer to the RT start site.

(7) Reduce technical errors in PCR reaction setup by standardizing (robotize if possible) and minimizing the number of pipetting steps. Mix cDNA with qPCR reagents, then aliquot a standard volume of this "master mix" into each reaction well containing a standard volume of specific primers. Set up reactions in a clean environment free of dust, preferably under a positive airflow hood. Routinely check for DNA contamination of primer and reagent stocks by performing PCR reactions on no template (water) controls.

(8) For relative quantification of transcript levels, design and test gene-specific primers for at least four potential reference genes selected from the literature (e.g., Czechowski et al., 2005) or from your own experience that are likely to be stably expressed throughout all organs and treatments to be compared. Validate reference genes in preliminary experiments on the range of tissues and treatments you wish to compare using a foreign cRNA added to

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