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Autonomy of cell proliferation and developmental programs during *Arabidopsis* aboveground organ morphogenesis

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Abstract

Elaboration of size and shape in multicellular organisms involves coordinated cell division and cell growth. In higher plants, continuity of cell layer structures exists from the shoot apical meristem (SAM), where organ primordia arise, to mature aboveground organs. To unravel the extent of inter-cell layer coordination during SAM and aboveground organ development, cell division in the epidermis was selectively restricted by expressing two cyclin-dependent kinase inhibitor genes, *KRP1/ICK1* and *KRP4*, driven by the L1 layer-specific *AtML1* promoter. The transgenes conferred reduced plant size with striking, distorted lateral organ shape. While epidermal cell division was severely inhibited with compensatory cell size enlargement, the underlying mesophyll/cortex layer kept normal cell numbers and resulted in small, packed cells with disrupted cell files. Our results demonstrate the autonomy of cell number checkpoint in the underlying tissues when epidermal cell division is restricted. Finally, the L1 layer-specific expression of both *KRP1/ICK1* and *KRP4* showed no effects on the structure and function of the SAM, suggesting that the effects of these cyclin-dependent kinase inhibitors are context dependent.

Keywords: *Arabidopsis*; Cyclin-dependent kinase inhibitor; Cell division; Organ size and shape; L1 layer; Epidermis; Shoot apical meristem

Introduction

Development of multicellular organisms relies on proliferation, growth and differentiation of their building blocks, the cells. Studies have suggested that developing organs may monitor and balance the extent of cell proliferation and cellular growth to accomplish proper final size and form (Conlon and Raff, 1999; Day and Lawrence, 2000; Mizukami, 2001; Nijhout, 2003; Potter and Xu, 2001). This compensatory action of cell proliferation and cellular growth may be observed when cell cycle regulators are manipulated experimentally (Conlon and Raff, 1999; Day and Lawrence, 2000; Tsukaya, 2003). For example, acceleration or reduction of cell proliferation in developing *Drosophila* wing sectors by overexpression of a positive regulator or inhibitor of the cell cycle conferred significant alteration in cell number (Hemerly et al., 1995; Neufeld et al., 1998). However, in either case the altered cell number was offset by a change in cell size, and as a consequence the final wing size was not significantly affected. Similarly, overexpression of *Arabidopsis* cyclin-dependent kinase inhibitor genes *KRP1/ICK1* (*KIP-RELATED PROTEIN1/INHIBITOR1 OF CDC2 KINASE*) and *KRP2*, which act as negative regulators of the cell cycle, led to a compensatory increase in cell volume in response to severely reduced cell numbers that conferred severe growth defects (De Veylder et al., 2001; Wang et al., 2000). The molecular nature of such compensatory mechanisms, along with the extent of coordination between different tissue types, remains elusive.

In higher plants, lateral organs such as leaves are generated reiteratively by the shoot apical meristem (SAM). A unique aspect of plant organogenesis is the continuity of cell layer structures from the undifferentiated SAM to mature aboveground organs. The SAM is composed of three clonally related cell layers, from the outermost L1, L2, to L3 (Howell, 1998; Steeves and Sussex, 1989). Typically, cell division in the L1 and L2 layers occurs anticlinally (i.e., perpendicular to the under-

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neath layers) and maintains the clonal origins. In the shoot organs, the L1 layer differentiates into the epidermis, while the L2 and L3 differentiate into mesophyll/cortex and pith/vasculature, respectively (Howell, 1998; Steeves and Sussex, 1989).

It is not clear whether cells in distinct layers in leaf primordia coordinate division and expansion to form the mature leaf blade. Classic studies from periclinal chimeras, in which the L1 and underlying layers have different genotypic and/or species origins, have shown that leaf shape is determined by the inner layers and that the epidermis has little effect on overall leaf expansion (Marcotrigiano, 2001; McHale and Marcotrigiano, 1998; Szymkowiak and Sussex, 1992, 1996). The results of these genetic mosaic studies, however, are in conflict with the classical biophysical view of cell layer structures within the SAM and leaf primordia, in which the L1 layer provides physical constraints to restrict the growth of inner layers (Green, 1980, 1996).

To understand the extent of cell–cell coordination between the L1 and inner layers during the development of the SAM and aboveground organs, we selectively restricted cell proliferation within the L1 layer of *Arabidopsis* by expressing cyclin-dependent kinase inhibitor genes that are driven by the L1-specific promoter *Arabidopsis thaliana MERISTEM LAYER1* (*AtML1*) (Lu et al., 1996; Sessions et al., 1999). The growth morphology and cellular phenotypes of the transgenic plants uncovered, to our surprise, very limited coordination between the L1 and inner layers during lateral organ development. Our results revealed the autonomy of mesophyll cell proliferation under restricted L1 growth. Furthermore, our study implied the presence of distinct mechanisms for adjusting perturbation of cell proliferation in the SAM versus lateral organs.

Materials and methods

Plant growth condition

The *A. thaliana* accession Landsberg *erecta* (L*er*) was used as a wild type. For phenotypic analysis, plants were grown under a long-day condition (18 h/6 h day–night cycle) at 21 °C.

Construction of plasmids and transgenic plants

The *AtML1* expression cassette was constructed as follows. First, the CaMV35S terminator was amplified from pAVA319 (von Arnim et al., 1998) using the primer pair: CaMVterm5 (5′ CCCAAGCTTGAATTCACTAGTGGATCCGTCCGCAAAAATCAC-CAGT 3′) and CaMVterm3rc (5′ GGGGTACCGTCACTGGATTTTGGTTTTA 3′). The amplified fragment was inserted into *Kpn*I/*Hin*dIII-digested pKUT565, which lacks the *Eco*RI site from pPZP222 (Hajdukiewics et al., 1994). This insertion creates unique restriction sites *Eco*RI, *Spe*I and *Bam*HI in front of the terminator. The plasmid was named pKUT566. Next, the 3.6-kb *AtML1* promoter was cleaved from pAS99, which was kindly provided by the Marty Yanofsky lab (Univ. California-San Diego), and inserted into the *Hin*dIII site of pKUT566 to generate pKUT567. Finally, the full-length cDNA clones of *KRP1/ICK1* and *KRP4* were inserted into *Eco*RI digested pKUT567 to generate pLYS003 (*AtML1*::*KRP1/ICK1*) and pLYS004 (*AtML1*::*KRP4*), respectively. The plasmids were transformed into Agrobacterium GV3101 (pMP90) by electroporation. Transformation of *Arabidopsis* by vacuum-infiltration and selection of transgenic plants were performed as described in Shpak et al. (2003).

RNA and RT-PCR analysis

Total RNA was extracted from 5-week-old L*er* and T2 generation transgenic inflorescence tissue using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were subsequently treated with DNaseI (Invitrogen) prior to the RT-PCR reaction. cDNA was synthesized from 4 μg of the total RNA using Thermoscript reverse transcriptase-mediated PCR system (Invitrogen) with a random hexamer as a primer. For detection of transcripts, 1 μl of cDNA was used for PCR with the following primer pairs and PCR cycles. *KRP1/ICK1* endogenous: ICK1-478 (CGCACACGTAACC-TAAATCG) and ICK1-1369rc (CCCATTCGTAACGTCCTTCT); 34 cycles. *KRP1/ICK1* transgene: AtML1_prom1.62 (TGAAGAGTGATATATTCTACC) and ICK1-1369rc; 28 cycles. *KRP4* endogenous: KRP4-439 (TCCGATT-TTTCTCACACCTCCCAATC) and KRP4-2121rc (TTACTAGTTTCAGCAC-CCGAGAAAAACT); 28 cycles. *KRP4* transgene: AtML1_prom1.62 and KRP4-2121rc; 28 cycles. The *Arabidopsis* actin (*ACT2*) gene was amplified as a template control as described by Shpak et al. (2003).

In situ hybridization

Probe template construction

An inflorescence apex-derived cDNA was used for in situ hybridization probe template construction. The sequence of PCR primer pairs and corresponding cDNA positions for each template plasmid are indicated below.

KRP1/ICK1: ICK1-501 (GTGAATTCATATGGTGAGAAAATATAGAA-AAGC) and ICK1-1072rc (TCTAGACATCGTTTTCCTCCCGCTACAACA); 1–384 bp.

KRP4: KRP4-717 (GAATTCTCGTTACCACCGACTTCTGCT) and KRP4-1977rc (TCTAGATGTGGATTCCCTTGTGGTCCT); cDNA 217–606 bp.

The *CLAVATA3* (*CLV3*) probe template (pNB4/35) was a gift from Rüdiger Simon (University of Dusseldorf). Amplified fragments for the two *KRP* genes were initially cloned into pCR2.1-TOPO (Invitrogen) to generate pEJH201 (for *KRP1/ICK1*) and pEJH204 (for *KRP4*), and then into the *Eco*RI and *Xba*I-digested pSP73ΔAatII (Shpak et al., 2003) to generate pEJH211 (for *KRP1/ICK1*) and pEJH214 (for *KRP4*). The sense- and antisense probes for each gene were generated as follows: pEJH211 and pEJH214 were linearized with *Xba*I or *Eco*IR and transcribing in vitro with SP6 and T7 RNA polymerase, respectively; pNB4/35 was linearized with *Eco*RI or *Xba*I and transcribed in vitro with T7 or T3 RNA polymerase, respectively. Probe synthesis was performed as described in McAbee et al. (2005).

Tissue sectioning and in situ hybridization

Tissue preparation and in situ hybridizations were performed as described in McAbee et al. (2005) with the following modifications. Probe concentration of 60 pg/μL were used for *KRP1/ICK1* and *KRP4* probes, and 100 pg/μL for *CLV3* probes, and slides were incubated with hybridization buffer overnight at 50 °C.

Tissue clearing, sectioning, microscopy and image analysis

Pictures of plants were taken under an Olympus SZX12 dissecting microscope attached to an Olympus C-2020Z digital camera (Olympus, Center Valley, PA). Plastic embedding and sectioning were performed as described previously (Shpak et al., 2003) with the exception that tissue was fixed in 25 mM PIPES (1,4 Piperazine bis 2-ethanosulfonic acid; Sigma) buffer (pH 7.2) containing 1.5% glutaraldehyde, 0.3% paraformaldehyde and 0.1% Tween 20. For visualization of nuclear DNA, plastic-embedded tissue was sectioned at 6 μm, and incubated in 0.5 μg/mL DAPI (4′,6-Diamidino-2-phenylindole; Sigma) in 1× PBS for 1 h in the dark. Slides were viewed using a DAPI filter (D360ext/460emt) on a Nikon Microphot-FX epi-fluorescence microscope equipped with a QImaging Retiga 1300 digital camera (QImaging, Burnaby, BC, Canada). Images were acquired using Open*lab*™ image capture and analysis software (Improvision Inc., Lexington, MA). Tissue clearing by chloral hydrate was performed as described previously (Woodward et al., 2005). Specimens were viewed under differential-interference-contrast (DIC) microscopy using an Olympus BX51 equipped with a DP70 digital camera. The scanning electron microscopy was performed as described in Shpak et al. (2003) using Joel JSM-840A. Photographic images were processed by Adobe