A hierarchical transcriptional network activates specific CDK inhibitors that regulate G2 to control cell size and number in Arabidopsis

Yuji Nomoto, Hirotomo Takatsuka, Kesuke Yamada, Toshiya Suzuki, Takamasa Suzuki, Ying Huang, David Latrasse, Jing An, Magdolna Gombos, Christian Breuer, Takashi Ishida, Kenichiro Maeo, Miyu Imamura, Takafumi Yamashino, Keiko Sugimoto, Zoltán Magyar, László Bögre, Cécile Raynaud, Moussa Benhamed & Masaki Ito

How cell size and number are determined during organ development remains a fundamental question in cell biology. Here, we identified a GRAS family transcription factor, called SCARECROW-LIKE28 (SCL28), with a critical role in determining cell size in Arabidopsis. SCL28 is part of a transcriptional regulatory network downstream of the central MYB3Rs that regulate G2 to M phase cell cycle transition. We show that SCL28 forms a dimer with the AP2-type transcription factor, AtSMOS1, which defines the specificity for promoter binding and directly activates transcription of a specific set of SIAMESE-RELATED (SMR) family genes, encoding plant-specific inhibitors of cyclin-dependent kinases and thus inhibiting cell cycle progression at G2 and promoting the onset of endoreplication. Through this dose-dependent regulation of SMR transcription, SCL28 quantitatively sets the balance between cell size and number without dramatically changing final organ size. We propose that this hierarchical transcriptional network constitutes a cell cycle regulatory mechanism that allows to adjust cell size and number to attain robust organ growth.
Results

Identification of a mitotic GRAS-type transcription factor. By analyzing transcripts specific in mitotic cells, we have identified an Arabidopsis GRAS family transcription factor that we designated EIM\(^{25}\). A recent report looking for mitotic genes in the root meristem uncovered the same gene called SCL28\(^{28}\), the name hereafter also adopted in this study. In a synchronized culture of Arabidopsis MM2d cells, we showed that this gene exhibited G2/M-specific transcript accumulation, which closely resembles that of the mitotic cyclin CYCB1;2 (Fig. 1a). As for most G2/M-specific genes, the so-called mitosis-specific activator (MSA) element that serves as a binding motif for MYB3Rs\(^{10,26}\) were repeatedly present within the proximal promoter regions of SCL28 (Fig. 1b). Binding of MYB3R to the SCL28 promoter is supported by our published data from chromatin immunoprecipitation (ChIP) with MYB3R3 followed by high-throughput sequencing (ChIP-Seq; Fig. 1b)\(^{11}\) and DNA affinity purification sequencing (DAP-Seq) data reported for MYB3R1, MYB3R4, and MYB3R5 (Supplementary Fig. 1a)\(^{27}\). In addition, loss of MYB3R activators (myb3r1/4 double mutant) or MYB3R repressors (myb3r1/3/5 triple mutant) resulted in significant down- or up-regulation of SCL28, respectively (Fig. 1c). GUS reporter activity driven by SCL28 promoter decreased significantly in the myb3r1/4 double mutant and became essentially undetectable when the MSA elements in the SCL28 promoter had been deleted (Fig. 1d). Collectively, these observations support the idea that SCL28 is a direct target of both activator and repressor type MYB3Rs in Arabidopsis. Similar to the G2/M-specific CYCB1;1-GFP accumulation\(^{28}\), we observed patchy pattern of SCL28-GFP signal from a construct driven by native SCL28 promoter in root meristem, suggesting cell cycle-regulated protein accumulation (Fig. 1e). Taken together, as has been recently shown\(^{23}\), SCL28 is a mitotic gene directly regulated by MYB3Rs.

To evaluate whether MYB3Rs act as part of DREAM complex on SCL28 transcription, we searched lists of target genes bound by potential DREAM components, RBR and TESMIN/TSO1-LIKE CXC 5 (TCX5), as defined by ChIP-Seq experiments reported previously\(^{15,29}\). This examination revealed that TCX5, but not RBR, showed a significant association to SCL28 locus in vivo. However, SCL28 showed no significant change in expression in neither rbr, tcx5 tcx6 double, nor e2fa e2fb e2fc triple mutants, indicating that SCL28 transcription depends exclusively on MYB3Rs (Supplementary Fig. 1b).

SCL28 strongly affects cell size. To analyze the biological function of SCL28, we generated transgenic plants overexpressing SCL28 under the strong RPSSA promoter (proRPSSA::SCL28). These plants, herein designated SCL28\(^{OE}\), showed general growth retardation both in seedling and adult stages (Fig. 2a). Cell size in these plants was significantly enlarged in all examined organs and tissues, such as root tip, leaf mesophyll, embryo, and inflorescence stem (Fig. 2b and Supplementary Fig. 2). An increased cell size was apparent in both post-mitotic differentiated cells and proliferating cell populations in root and shoot apical meristems (Fig. 2c and Supplementary Fig. 2). As described earlier, the increased cell size in SCL28\(^{OE}\) leaves was associated with elevated levels of cellular ploidy induced by enhanced endoreduplication. The loss-of-function scl28 mutant (Supplementary Fig. 3) showed an opposite cellular phenotype to SCL28\(^{OE}\), having cells with significantly reduced size (Fig. 2b, c and Supplementary Fig. 2). However, unlike SCL28\(^{OE}\), the overall stature of scl28 mutant plants was largely normal and indistinguishable from that of wild type (WT) plants (Fig. 2a).

To explore the developmental origin of cell size differences, we performed kinematic analysis on growing first leaf pairs by monitoring size and number of palisade cells (Fig. 2d–g). This analysis showed that cells in scl28 leaves were smaller than WT already during initial stages of organ development when most cells are actively proliferating (Fig. 2d). During this early

During organ growth and development, cell proliferation is intricately controlled in space and time. Governed by developmental programs\(^{1–3}\) and influenced by active plant responses to environmental conditions\(^{4,5}\), the number of cells produced during organ growth is set by the cell cycle speed during proliferation and the point when cells exit to cellular differentiation. Controlled cell proliferation requires coordinately regulated gene expression within and upon exit from the cell cycle. Generally, there are two main groups of genes showing waves of transcription during the cell cycle; G1/S-specific and G2/M-specific genes\(^{6}\). The G1/S-specific genes facilitate initiation and progression of DNA replication and are typically regulated by the activity of E2F transcription factors\(^{7,8}\). Generally, E2F dimerizes with Dimerization Partner (DP) proteins to activate or repress their target genes, depending on their association with the Retinoblastoma-Related (RBR) repressor protein\(^{9}\). On the other hand, most G2/M-specific genes are positively or negatively regulated by MYB3R family of transcription factors in plants\(^{10,11}\). Some members of MYB3Rs are specifically expressed during G2/M and act as transcriptional activators, while others act as transcriptional repressors of G2/M-specific genes\(^{12–14}\). Recent studies identified the two main groups of transcription factors, E2Fs and MYB3Rs, which had been studied separately, as part of the same multi-protein complex in Arabidopsis\(^{11}\). This E2F-MYB3R complex is evolutionarily related to the DREAM (DP, Retinoblastoma-like, E2F, and MuvB) complex reported in Drosophila and human cells\(^{11,15,16}\). The metazoan DREAM complex plays a predominant role in repressing both G1/S- and G2/M-specific genes, thus promoting cell cycle exit and maintaining cellular quiescence\(^{17,18}\). The DREAM complex in Arabidopsis shows significant differences from metazoan complexes, which include involvement of plant-specific subunits and existence of diversified complexes with different subunit compositions\(^{9,11,15}\).

Transcriptional regulation during cell cycle generally constitutes multi-layered hierarchical networks, in which master regulators regulate other transcription factors, which further regulate each other or downstream genes\(^{19–21}\). Notably, studies in yeasts showed that cell cycle transcriptional activators that function during one stage of the cell cycle regulate transcriptional activators that function during the next stage, forming a connected regulatory network that is itself a cycle\(^{22}\). However, in plants, such a hierarchical network composed of cell cycle transcription factors, E2F and MYB3R, has not yet been uncovered. Exploring the transcription network during cell cycle may uncover missing important factors and hidden mechanisms governing plant-specific cell cycle regulation.

In this work, we identified a mitosis specific GRAS family transcription factor, called SCARECROW-LIKE28 (SCL28). In accordance with our work, a recent report identified the same genes being directly regulated by MYB3Rs\(^{23}\). Here, we demonstrate that SCL28 acts in association with the AP2-type transcription factor, AtSMOS1, to directly activate transcription of a specific set of SMR family genes, encoding plant-specific cyclin-dependent kinase (CDK) inhibitors\(^{24}\). This regulatory network inhibits the G2 to M phase transition during the cell cycle and promotes the onset of endoreduplication, an atypical cell cycle consisting of repeated DNA replication without mitosis\(^{4}\). Our study identified a G2/M regulatory pathway that controls cell cycle length, and is likely to be important in optimizing cellular functions by setting cell size and number during organ growth.
Fig. 1 SCL28 expression is cell cycle-regulated under the control of MYB3R transcription factors. a G2/M-specific accumulation of SCL28 transcript. Arabidopsis MM2d cells were synchronized by aphidicolin treatment and used for qRT-PCR analysis to quantify SCL28 mRNA levels (lower). Synchronous progression of cell cycle was monitored by measuring mitotic index (upper). As a representative of G2/M-specific genes, CYCB1;2 was also analyzed in the same way for comparison. Expression data are shown as averages from three technical replicates (±SD). b MYB3R3 binds to the upstream region of SCL28. The ChIP-Seq profile of MYB3R3 indicates its direct binding to the proximal promoter region of SCL28. The nucleotide sequence around the ChIP-Seq peak contains MSA elements repeated four times, of which core motifs (AACGG) are shown by orange (forward orientation) and green (reverse orientation) letters. c Levels of SCL28 transcripts are significantly affected by mutations in myb3r genes. Transcript levels of SCL28 and KNOLLE were analyzed by qRT-PCR in plants 12 days after sowing (DAS) that carry mutations of myb3r genes in the indicated combinations. Expression data were normalized to ACT2 and shown as averages from three biological replicates (±SD). Statistical significance was determined using two-sided Student’s t test. *P < 0.05, **P < 0.01. d Promoter activity of SCL28 requires the presence of MYB3R activators and MSA elements. GUS reporter expression was analyzed in wild type (WT) and myb3r1/4 plants carrying proSCL28::GUS and WT plants carrying proSCL28ΔMSA::GUS, in which all MSA elements in the SCL28 promoter were mutated. GUS staining around shoot apex and root tip regions is shown at higher magnification in the lower panels. Multiple independent lines for each construct showed similar difference in GUS staining. Scale bar indicates 100 µm. e Patchy pattern expression of SCL28-GFP protein. Root meristems of proSCL28::SCL28-GFP and proCYCB1;1::CYCB1;1-GFP plants were analyzed by confocal laser scanning microscopy (CLSM) after counterstaining of the cell wall with propidium iodide (PI). Green and red signals indicate fluorescence of GFP and PI, respectively. Similar expression patterns of SCL28-GFP were confirmed in multiple independent lines. Scale bar indicates 100 µm.

Effects of SCL28 on mitotic cell cycle and endocycle. To directly explore the role of SCL28 in cell cycle progression, we performed live-cell imaging of root meristem cells after introgression of the PCNA-GFP cell cycle marker into SCL28OE and scl28 mutant. The PCNA-GFP line allows quantification of cell cycle stages

Fig. 2 SCL28 regulates cell proliferation and cell size during leaf development. a The number of epidermal pavement cells during leaf development in scl28 mutant leaves is reduced compared with WT (Fig. 2e, f). Conversely, duration of cell proliferation was longer in scl28 mutant leaves than WT, consistent with a higher cell division rate in scl28OE plants (Fig. 2e) due to severely inhibited cell proliferation (Fig. 2f). Although dramatic cell enlargement partially counteracted the reduced cell number, leaf area was still reduced in SCL28OE compared with WT (Fig. 2d, g). We also analyzed cell size and number of epidermal pavement cells during leaf development in scl28 and SCL28OE lines and found the response of this cell type to altered SCL28 activity was comparable to what we have shown for palisade cells (Supplementary Fig. 4). In the root tip, cell size difference was similarly apparent among SCL28OE, scl28, and WT (Fig. 2c, h, i). This cell size difference was maintained along the apical-basal axis of the root from cells adjacent to the quiescent center to those at the transition zone, as well as in differentiated cells at distal positions (Fig. 2h). Therefore, the behavior of cells in the root meristems of SCL28OE and scl28 was generally consistent with those observed in developing leaves.
SCL28 acts together with AtSMOS1 as a heterodimer. Phylogenetic analysis of GRAS family proteins indicated that SCL28 constitutes a unique clade together with SMOS2 in rice (Supplementary Fig. 6a). Rice smos2 was initially identified as a mutant showing reduced organ size with component cells smaller than those in WT. Another rice mutant, smos1, with a phenotype similar to that in smos2, has loss-of-function mutation in a unique gene encoding an AP2-type transcription factor. Because a physical interaction between SMOS1 and SOMS2 has been reported, we postulated that SCL28 may act through interaction with an Arabidopsis protein orthologous to SMOS1. When AP2-type transcription factors were phylogenetically analyzed, we found At2g41710 to be the likely Arabidopsis ortholog.
of SMOS1 (Supplementary Fig. 6b). To test the physical interaction between SCL28 and At2g41710, we performed yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays to obtain clear results showing that they indeed interact in yeast and in plant cells, respectively (Fig. 4a, b). We concluded that the observed protein-protein interaction is evolutionarily related to the SMOS1-SMOS2 interaction in rice and named At2g41710 as AtSMOS1.

To examine AtSMOS1 function, we analyzed a T-DNA insertion mutant for this gene (Supplementary Fig. 7) and found that the observed protein-protein interaction is evolutionarily related to the SMOS1-SMOS2 interaction in rice and named At2g41710 as AtSMOS1.

To test whether the enlarged cells in SCL28OE relies on the presence of AtSMOS1, and indeed in the atsmos1 mutant background, the strong effects of SCL28OE on cell size of leaf palisades (Fig. 4g, h) and root meristem cells (Fig. 4i, j), as well as whole plant growth, completely diminished (Supplementary Fig. 8b). Collectively, these observations strongly suggest that SCL28 and AtSMOS1 function cooperatively to regulate cell size in different plant organs.

To analyze AtSMOS1 expression, we generated plants expressing AtSMOS1-GFP driven by its own promoter. These transgenic plants showed nuclear localization of AtSMOS1-GFP in various tissues and organs such as root meristem, developing leaves, and cotyledons (Fig. 4k). However, unlike SCL28, AtSMOS1-GFP was uniformly expressed in meristematic cells, suggesting a cell cycle-independent AtSMOS1 expression. Nonetheless, cells expressing AtSMOS1 and SCL28 were overlapping, supporting the idea that they interact to form a heterodimer in vivo. We also noted an E2F binding element in the AtSMOS1 promoter, which binds RBR in published ChIP-Seq data. We found that E2F binds to AtSMOS1 promoter in our ChIP-qPCR experiment and that AtSMOS1 is significantly upregulated in the e2fa e2fb e2fc triple mutant (Supplementary Fig. 9), suggesting that AtSMOS1 is indeed regulated by E2Fs. One explanation for
insignificant change of AtSMOS1 mRNA in rbr and tcv5 tcv6 might be that RBR- and DREAM-dependent regulation on E2Fs is developmental specific, and was not apparent at the seedling stage.

**Downstream targets of SCL28.** Studies of smos1 and smos2 rice mutants suggest a role in post-mitotic cell expansion through the regulation of the *Oryza sativa* PHOSPHATE INDUCED1 (OsPHI-1) gene. Because we observed SCL28 expression specific to meristematic cells, we postulated that SCL28 should largely influence actively-proliferating cells before the onset of cell expansion. To identify the downstream targets of SCL28, we analyzed genome-wide gene expression changes in *scl28*, *SCL28OE*, and *atsmos1* by conducting RNA-sequencing (RNA-Seq) and microarray experiments (Supplementary Data 1). We identified the epistatic phenotypes among *scl28*, *SCL28OE*, and *atsmos1* mutants, the critical downstream genes of SCL28 should be affected in *scl28* and *atsmos1* in a similar manner, as well as in *SCL28OE* in an opposing manner. To reveal the genes satisfying these expression criteria, we conducted an overlapping analysis of differentially expressed genes in *scl28*, *atsmos1*, and *SCL28OE*, and identified 21 genes that are significantly (adjusted $P$ value $< 0.05$) downregulated in both *scl28* and *atsmos1*, and upregulated in...
Fig. 5 SCL28 and AtSMOS1 activate transcription of SMR genes. a Venn diagram representing overlap between genes downregulated in scl28 and atsmos1, and upregulated in SCL28OE plants. The SMR2 gene was found in the overlap of these three categories. Upregulated and downregulated genes were defined by a criterion of false-discovery rate (FDR)-adjusted P value < 0.05, which was obtained from expression data of three biological replicates. b Venn diagram representing overlap between genes upregulated in scl28 and atsmos1, and downregulated in SCL28OE plants. Upregulated and downregulated genes were statistically defined as in (a). c-e Transcript levels of SMR genes in scl28 (c), atsmos1 (d), and SCL28OE (e) plants at 8 DAS. qRT-PCR analysis of all 17 genes in the SMR family was performed to compare expression levels with WT plants. Data are averages from three biological replicates (±SD). Statistical significance compared with WT was determined using two-sided Student’s t test. *P < 0.05, **P < 0.01.
SCL28OE (Fig. 5a and Supplementary Fig. 10a), as well as 26 genes upregulated in both scl28 and atsmos1, and downregulated in SCL28OE (Fig. 5b and Supplementary Fig. 10b). Among these genes, we focused on SIAMESE-RELATED2 (SMR2), encoding a member of SMR family proteins, as proteins of this family represent plant-specific CDK inhibitors and some members, such as SIM and SMR1, positively affect cell size and negatively influence cell division, reflecting the observed effect of SCL28.24. Among the genes, we found that SIAMESE-RELATED2 (SMR2), encoding a member of SMR family proteins, as proteins of this family represent plant-specific CDK inhibitors and some members, such as SIM and SMR1, positively affect cell size and negatively influence cell division, reflecting the observed effect of SCL28.24.

According to RNA-Seq and microarray data, in addition to SMR2, we found other SMR family members as candidates of downstream genes, with decreased expression levels in both scl28 and atsmos1 and increased levels in SCL28OE. To further analyze SMR family genes, we conducted quantitative reverse-transcription PCR (qRT-PCR) analysis to examine expression of all members of this family in scl28, atsmos1, and SCL28OE, and identified at least seven members (SMR1, SMR2, SMR6, SMR8, SMR9, SMR13, and SMR14) out of all 17 as potential target genes of SCL28 and AtSMOS1 (Fig. 5c–e). Consistent with this conclusion, we also observed reduced expression of proSMR2::SMR2-GFP and proSMR13::SMR13-GFP in root meristems in scl28 or atsmos1 mutant backgrounds compared with WT (Supplementary Fig. 11).

KIP-RELATED PROTEINS (KRP) constitute another family of CDK inhibitors in addition to SMRs in plants. The general view is that KRP proteins primarily connect with CDKA to negatively regulate G1/S34, while SMR proteins inhibit G2/M by associating with both CDKA and CDKB124.35. There are seven KRP members encoded in the Arabidopsis genome, and some we did find deregulated in the scl28, atsmos1, and SCL28OE plants. However, in contrast to the SMRs, the change in expression of KRP genes were generally moderate and inconsistent with the activating function of SCL28-AtSMOS1 heterodimer, and thus likely to be an indirect pleiotropic effect (Supplementary Fig. 12). Therefore, the SCL28 and AtSMOS1 may directly affect expression of SMR members, but not those of the KRP family, consistent with our results that SCL28 preferentially affects G2 duration in meristematic cells.

Genome-wide mapping of SCL28 and AtSMOS1 binding sites. In order to define direct targets for SCL28 and AtSMOS1, combined with transcriptome analysis, we performed ChIP-Seq assays using proRPS5A::SCL28-GFP and proAtSMOS1::AtSMOS1-GFP lines to identify their binding genomic loci. Consistent with our emphasis on downregulation of SMR genes, which suggests that SCL28 and AtSMOS1 act as transcriptional activators, the majority of binding sites are located in promoter regions proximal to transcription start sites (TSS) (Supplementary Fig. 13) and located within nucleosome-free and highly accessible chromatin regions (Supplementary Fig. 14a–d). Their enrichment levels at TSS correlated well with mRNA levels of corresponding genes, suggesting a positive correlation between their binding and transcriptional activity (Supplementary Fig. 14e, f).

The ChIP-Seq data analysis uncovered 463 and 4,287 genes as targets of SCL28 and AtSMOS1, respectively (Supplementary Data 2). Comparing these target genes revealed the presence of a significant overlap, a set of 308 common targets that accounts for 66% of SCL28 targets (Fig. 6a). On one hand, this result confirms our genetic analysis, suggesting that SCL28 function largely relies on the interaction with AtSMOS1. On the other hand, AtSMOS1 may show a wider range of functions both dependent on and independent of SCL28. This interpretation is also supported by our RNA-Seq data showing larger number of downregulated genes in atsmos1 compared to scl28 and these downregulated genes have a relatively small overlap (see Fig. 5a). We also found only a limited overlap between common targets identified by ChIP-Seq and those that show regulated expression in transcriptome analysis of scl28, atsmos1 and SCL28OE lines (Fig. 6b).

Gene ontology (GO) enrichment analysis of the 308 common targets revealed overrepresented GO terms related to cell cycle, such as “regulation of mitotic nuclear division” and “regulation of DNA endoreduplication” (Supplementary Fig. 15). This over-representation of cell cycle-related GO terms largely relies on the presence of five common genes that belong to such GO categories. All these genes were found to be members of SMR family genes, SMR2, SMR4, SMR6, SMR8, and SMR9, all of which, except SMR4, showed significant downregulation in both scl28 and atsmos1 and upregulation in SCL28OE in our qRT-PCR experiments described above (see Fig. 5). Though three SMR genes—SMR1, SMR13, and SMR14—with significant expression changes in all lines failed to fulfill the criteria of ChIP-Seq data analysis, visual inspection revealed recognizable peaks for both SCL28 and AtSMOS1 in ChIP-Seq profiles, suggesting these SMRs are also direct common targets (Fig. 6c and Supplementary Fig. 16). The ChIP-Seq peaks were observed at either 5' (SMR1, SMR2, and SMR4), 3' (SMR6, SMR9, and SMR14) or both 5' and 3' (SMR8 and SMR13) regions of the target SMR loci. In most cases, we found that the positions of ChIP-Seq peaks adjacent to SMR loci coincided for SCL28 and AtSMOS1, suggesting that they associate with the same sites. Our ChIP-Seq data also showed that none of the KRP genes were found as common targets of SCL28 and AtSMOS1, further confirming that SCL28-AtSMOS1 directly regulates some SMRs but not KRPCs.

To find common binding sites and sequence motifs for SCL28 and AtSMOS1, we compared the precise positions of ChIP-Seq peaks along the gene structure. Dot-plot analysis of the common targets showed frequent clustering of SCL28 and AtSMOS1 peaks at the same positions relative to TSS and transcription end site (TES; Fig. 6d). Similar analysis at a genome-wide scale confirmed that SCL28 frequently targets the same sites as AtSMOS1 around the TSSs (Supplementary Fig. 17a). Within the common peaks for SCL28 and AtSMOS1, a DNA motif of C(a/t)T(a/t)GGATNC(t)/a(t) could be identified as an overrepresented cis element (Fig. 6e). This motif is indeed present among more than 40% of the common targets, but is much less frequent among targets of either SCL28 or AtSMOS1, which contain quite different cis elements based on sequence overrepresentation analysis (Supplementary Fig. 17b, c). This suggests that the formation of SCL28-AtSMOS1 heterodimer generates a specific sequence preference for DNA binding to regulate transcription of a specific set of targets. This or closely related motifs are present in all seven SMR target loci at positions frequently coinciding with the peak summits of SCL28 and AtSMOS1 (Fig. 6c and Supplementary Fig. 16), suggesting that SCL28 and AtSMOS1 recognize this motif and bind to their targets as a heterodimer.

We then tested whether chromatin association of SCL28 depended on AtSMOS1 and vice-versa by conducting ChIP-qPCR experiments in scl28 and atsmos1 backgrounds (Fig. 6f). As expected, both SCL28 binding on SMR2 promoter in the atsmos1 and AtSMOS1 binding in the scl28 mutant backgrounds were abolished. Collectively, our data supports SCL28 and AtSMOS1 functional heterodimer binding to specific sets of SMR target genes to activate transcription and negatively regulate cell cycle progression at the G2 to M cell cycle transition.

SMRs are critical downstream effectors for SCL28 and AtSMOS1. If SMR genes directly bound by SCL28 and AtSMOS1 are crucial for mediating their effects on cellular phenotype, transcript levels of those SMR genes should be altered accordingly with cell size phenotype observed in our epistasis analysis (see Fig. 4c–j). Confirming this idea, downregulation of target SMR
genes—SMR2, SMR9 and SMR13—were quantitatively equivalent in scl28, atsmos1, and double scl28 atsmos1 mutants (Fig. 7a). In addition, strong activating effects of SCL28OE on target transcription was totally abolished under atsmos1 mutant background, providing the molecular basis for AtSMOS1-dependent action of SCL28 on cell size (Fig. 7b). To further elucidate that AtSMOS1 acts together with SCL28 on SMR genes, we also analyzed the proSMR2::LUC reporter co-transfected in protoplasts with pro35S::SCL28 and/or pro35S::AtSMOS1 constructs. The most prominent and significant activation of luciferase was only observed when both plasmids expressing SCL28 and AtSMOS1 were simultaneously transfected, supporting the conclusion that SCL28 and AtSMOS1 fulfill their function as a transcriptional activator by forming a heterodimer (Fig. 7c).

To directly address whether these SMR genes are critical for SCL28 function, we performed genetic analysis focusing on SMR1, SMR2 and SMR13, which are significantly downregulated in scl28 and upregulated in SCL28OE. For each smr mutant, we could not detect any abnormalities in cell size (Supplementary Fig. 18). However, when these mutations were combined in a
Fig. 7 Cell size control by SCL28-AtSMOS1 is mediated by SMRs. a Double scl28 atsmos1 mutation downregulates SMR genes, equivalently to each single mutation. qRT-PCR analysis of SMR2, SMR9 and SMR13 transcripts was performed for comparing expression levels in WT, scl28, atsmos1, and scl28 atsmos1 plants. Data are shown as averages from three biological replicates (±SD). Statistical analysis was performed for each SMR gene based on one-way ANOVA and Tukey’s test, and significant differences (P < 0.05) were shown in different letters above the bars. b Upregulation of SMR genes in SCL28OE requires presence of AtSMOS1. qRT-PCR analysis was performed for comparing expression levels between atsmos1, SCL28OE plants, and those carrying atsmos1 and SCL28OE in combination. Two independent lines were used for analyzing SCL28OE/ atsmos1. Data are shown as averages from three biological replicates (±SD). Statistical analysis was performed as in (a). c Co-expression of SCL28 and AtSMOS1 activates the SMR2 promoter in the protoplast transient assay. proSMR2::LUC reporter plasmid was transfected into protoplasts prepared from T87 cells together with expression plasmids of SCL28 and AtSMOS1 as indicated. Schematic presentation of reporter and expression plasmids as shown on the left. Data are shown as averages from four biological replicates (± SD). Statistical analysis was performed as in (a). d DIC observations of leaf palisade cells in the first leaf pairs from plants with indicated genotypes. Boxplot was generated using data collected from leaves of ten different plants, in each of which more than 60 cells were analyzed (midline = median, box = IQR, whiskers = 1.5 × IQR). Different letters above the boxplots indicate significant differences (P < 0.05) based on one-way ANOVA and Tukey’s test. e CLSM observation of cortical cell files in root meristems from WT, scl28, smr1/2/3, and smr1/2/3 scl28 plants at 7 DAS. Scale bar indicates 20 µm. f Quantification of cortical cell length in root meristems from plants with indicated genotypes. Boxplot was generated using data collected from roots of multiple individual plants (midline = median, box = IQR, whiskers = 1.5 × IQR). Numbers of plants analyzed were 10, 12, 12, and 11 for WT, scl28, smr1/2/3, and smr1/2/3 scl28, respectively. In each plant, more than 40 cells were analyzed for calculating average, which were further used for statistical analysis. Different letters above boxplots indicate significant differences as in (e). 

smr1/2/3 triple mutant, there was a significant reduction in cell size in multiple tissues, including leaf palisades (Fig. 7d, e) and root meristem (Fig. 7f, g), suggesting functional redundancy among SMR genes. To address the link between SCL28 and SMRs, we studied the cell size phenotype when these mutations were combined. In leaf palisade cells, the triple smr1/2/3 mutant showed stronger reduction in cell size than the scl28 mutant, which did not further reduce when all mutations combined in the smr1/2/3 scl28 line, supporting the idea that cell size regulation by SCL28 is mediated by SMR1/2/13 (Fig. 7d, e). Conversely, scl28 mutation still significantly decreased cell size in the root meristem when combined with smr1/2/3 (Fig. 7f, g). Therefore, SMR1/2/3 contribution downstream of SCL28 appears to be larger in palisade tissue than root meristem, where additional SCL28-regulated SMRs may play a role in cell size control. These tissue-specific differences indicate the contribution of developmental regulation that positions different sets of SMRs downstream of SCL28.

Dose-dependent control of cell size and cell number by SCL28 to maintain organ size homeostasis. In the scl28 mutant, leaf growth is essentially normal, but the constituent cells became small and more numerous (see Fig. 2d, e, g), suggesting that SCL28 is pivotal to maintain organ size homeostasis by regulating the balance between cell size and cell number. For a regulator designed to tune the balance between cellular parameters, one assumes that it acts dose dependently. To explore whether or not SCL28 levels quantitatively affect cell size, we analyzed plants heterozygous for scl28 (scl28/+ plants). Heterozygous plants showed reduced SCL28 transcript levels that is approximately half of that in WT (Fig. 8a). This SCL28 downregulation resulted in a clear reduction of cell size in root meristem and leaf palisade tissue, with an intermediate cell size in heterozygous plants between those in WT and scl28 homozygous plants (Fig. 8b, c and Supplementary Fig. 19a, b).

Overexpression of SCL28 causes a phenotype opposite to scl28, dramatically reducing the number of cells that became
indicate significantly different as in (d). b Correlation between SCL28 mRNA levels and palisade cell size analyzed by scatterplots, where each dot corresponds to each individual plant from different transgenic lines carrying proSCL28::SCL28-GFP under SCL28 background. In addition to proSCL28::SCL28-GFP plants (shown in gray dots), WT (blue dot), scl28 (red dot), and SCL28OE plants (green dot) were also analyzed. e Correlation between SCL28 mRNA levels and number of palisade cells per leaf was analyzed by scatterplot as in (d). Symbols are same as in (d). f Correlation between SCL28 mRNA levels and leaf area was analyzed by scatterplot as in (d). Symbols are same as in (d). Values in (d-f) represent Pearson correlation coefficients ($r_p$) and its two-sided $P$ value without adjustment ($p$).

enlarged. To examine the outcome when SCL28 expression is only moderately increased, we utilized plants from different T2 lines carrying proSCL28::SCL28-GFP in scl28 mutant background and showing varying levels of SCL28-GFP expression. We quantitatively compared levels of SCL28 transcript and cellular parameters of palisade tissues in each individual plant (Fig. 8d, e and Supplementary Fig. 19c). This showed that SCL28 expression positively correlates with cell size and negatively with leaf cell number. Both positive and negative correlations were statistically significant. Due to compensatory changes in cell number and size, overall leaf size was not dramatically affected by moderate alteration of SCL28 expression (Fig. 8f). Therefore, we concluded that a finely-graded expression of SCL28 sets the balance between cell size and number without altering organ growth as a whole. This mechanism may enable plants to achieve an optimal balance between cell size and number, possibly depending on developmental status and environmental conditions.

**Discussion**

Cell size at division depends on coordination between cell growth and cell cycle progression, which is actively maintained in a cell autonomous manner in both uni- and multicellular organisms. Here, we identified a molecular mechanism for cell size regulation in Arabidopsis that relies on a hierarchical transcriptional activation of CDK inhibitors. In this pathway, SCL28 expression is specifically confined to mitosis through the MSA element in its promoter controlled by MYB3Rs. In turn, SCL28 associates with AtSMOS1, a transcription factor with cell cycle-independent expression, and this dimer defines the binding site present in a set of SMR genes encoding CDK inhibitors to activate their expression and thereby act as a brake against the cell cycle engine predominantly driven by CDK activity (Fig. 9). The SCL28-AtSMOS1-SMR axis uniquely affects only cell cycle progression and cell doubling time, but not the exit from cell proliferation. Therefore, it acts to set the balance between cell number and size during organ development without significantly
Formation of the SCL28-AtSMOS1 heterodimer generates specific DNA damage response in Arabidopsis. Similar to SCL28, SOG1, acting as a central regulator of GAMMA RESPONSE 1 (SOG1), is a primary regulator of cell growth and proliferation and is involved in cell size regulation in yeast. In Arabidopsis, TOR, acting through YAK1 kinase, regulates the transcription of SMR genes and thereby G2/M transition. However, the set of YAK1-targeted SMRs overlap with those regulated by SOG1 rather than those regulated by SCL28. Therefore, our study places SCL28 as part of a novel pathway for regulating G2/M progression that determines cell size and cell number during plant organ development.

Variability in cell size at birth or stochastic changes in key regulators necessitate mechanisms that achieve cell size homeostasis by linking cell cycle progression to cell growth. The way cell size variations are corrected has long been debated. The possible co-existence of a mix of these mechanisms that may act at multiple control points of the cell cycle.

We provide multiple lines of evidence supporting the conclusion that SCL28 acts as a brake on the G2/M transition to set cell size: (1) measurements of cell cycle phases by following PCNA-GFP using live-cell imaging showed that scl28 mutant and SCL28OE showed a clear alteration in cell doubling time without any effect on cell cycle exit; (2) the molecular mechanisms identified here are fully consistent with the cell cycle inhibitory function, SCL28, forming a dimer with AtSMOS1 and directly activating the transcription of SMR genes whose products inhibit CDK activity; (3) in support of this, the scl28 mutant phenocopied and interacted in an epistatic fashion with the triple smr1/2/13 mutant; and 5) SCL28 regulates the number of endoreplication cycles, which is typically enhanced by G2 inhibition. Previous reports suggest that SCL28 plays a positive role in root growth and promotes the G2/M cell cycle progression. These conclusions are inconsistent with our data and the small cell size phenotype observed in both studies, which may originate from using different methods relying on fixed samples.

Another example of a transcription factor inhibiting cell cycle at G2/M is a NAC-type transcription factor, SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), acting as a central regulator of DNA damage response in Arabidopsis. Similar to SCL28, SOG1 transcriptionally activates SMR family genes to inhibit cell cycle progression upon DNA damage, but its targets, SMR5 and SMR7, are not regulated by SCL28, which instead directly regulates seven other SMR genes. Notably, in contrast with SOG1, which is activated specifically upon DNA damage, SCL28 negatively regulates cell cycle, thereby affecting cell size during normal plant development. The target of rapamycin (TOR) signaling pathway is a primary regulator of cell growth and proliferation and is involved in cell size regulation in yeast. In Arabidopsis, TOR, acting through YAK1 kinase, regulates the transcription of SMR genes and thereby G2/M transition. However, the set of YAK1-targeted SMRs overlap with those regulated by SOG1 rather than those regulated by SCL28. Therefore, our study places SCL28 as part of a novel pathway for regulating G2/M progression that determines cell size and cell number during plant organ development.

Variability in cell size at birth or stochastic changes in key regulators necessitate mechanisms that achieve cell size homeostasis by linking cell cycle progression to cell growth. The way cell size variations are corrected has long been debated. The possible co-existence of a mix of these mechanisms that may act at multiple control points of the cell cycle.

Live-cell imaging of cell cycle markers in Arabidopsis shoot apical meristem demonstrated that both G1/S and G2/M transitions are regulated in a cell size-dependent manner. Accumulation of waves of CDK activities underpinned by D-type cyclin and mitotic CDKB1, their interactors or downstream effectors were proposed as molecular components for cell size-dependent G1/S and G2/M regulation, respectively. SCL28 may participate in regulating G2/M transition in this model, in which dilution of an inhibitor rather than accumulation of an activator is the principle behind. How SCL28 is connected to monitoring of cell size remains to be determined. Stochastic variability in the accumulation of key regulators can also set cell size and cell fate differences in a developing organ. An example is ATML1, a transcription factor that is necessary for the patterning of giant cells within the sepals of Arabidopsis by overcoming a threshold in G2 cells and activating the downstream SMR1, also called LOSS OF GIANT CELLS FROM ORGANS (LGO). Similar to ATML1, SCL28 shows a finely-tuned dose-dependent effect on cell size by accurately distinguishing gene copy numbers of SCL28 and reflecting its graded overexpression. It is possible that the molecular ruler in an inhibitor dilution model potentially involving SCL28 is set by the synthesis rate limited by gene copy number, as shown for Whi5 in budding yeast.

The human functional orthologue of Whi5 is pRB, which also controls cell size via its synthesis mostly after G1 and subsequent dilution during growth. In Arabidopsis, both RBR and E2FB were shown to strongly affect cell size in proliferating cells, but as opposed to scl28 that solely acts on cell proliferation rate,
the RBR-E2F pathway also regulates cell cycle exit, establishment of quiescence, and cellular differentiation. The repressortype MYB3Rs—MYB3R1, MYB3R3 and MYB3R5—when combined with RBR and E2FC form the DREAM complex that represses mitotic genes during or after developmental cessation of cell proliferation. Whether the RBR/E2F/DREAM pathway directly regulates the cell size in proliferating cells or its cell size effect is through the regulation of cell cycle exit, remains to be established. It will be also important to know how the RBR/E2F/DREAM and the SCL28/AtSMOS1 pathways are coordinated.

In addition to cell size during proliferation, SCL28 also strongly affects the size of terminally differentiated cells. In the scl28 mutant, we observed a reduced final cell size in various organs, whereas moderately higher SCL28 expression led to its increase. Reduced cell size caused by increased cell division, as observed in scl28, has been repeatedly reported in Arabidopsis organ development. Examples include plants with inhibited RBR expression, those overexpressing CYCD3;1, or co-overexpressing E2FA and DPA. In many of these cases, the cellular phenotype is due to prolonged cell proliferation and delay in cell differentiation, leading to additional cell division that is not balanced by cell size increase due to developmental decrease of cell expansion. On the contrary, we showed with kinematic analysis during leaf development that the increased number of smaller cells observed in scl28 leaves is caused by accelerated cell proliferation, but not by prolonged cell proliferation. Therefore, it is puzzling how loss of SCL28 affects final cell size that is largely determined by post-mitotic cell expansion occurring in the absence of SCL28.

Our observations are reminiscent of the phenomena reported in Drosophila wings, where the alteration of cell number is compensated by changes in cell size such that final organ size is essentially unchanged. Based on this and similar observations, it has been proposed that animal organ size may be controlled by ‘total mass checkpoint’ mechanisms, which operate at the level of whole organs, although the underlying molecular details remain unknown. Instead, the well-established mechanism of ‘compensation’ in plants shows post-mitotic cell expansion that is enhanced by reduced cell proliferation. A small number of larger cells observed in SCL28OE leaves may be a prime example of compensation. One possible explanation for compensatory alteration of cell expansion is that, during cell proliferation, cells establish memory that is maintained during the elongation phase. An example of this can be seen in plants overexpressing KRP2, where final cell size may be influenced by the size of mitotic cells that are already altered during proliferation. We observed larger and smaller mitotic cells both in root meristem and young leaves of SCL28OE and scl28, respectively, which may be maintained until terminal differentiation, thus affecting final cell size. A possible molecular mechanism to create such memory is cell size-dependent alteration in gene expression. It was shown in yeast that cell size changes due to ploidy levels are associated with altered gene expression related to functions of cell wall and extracellular matrix. Similarly, in Arabidopsis root, ploidy levels were correlated with expression of genes related to chromatin and cell expansion, such as ion transport and cell wall modifications. Therefore, it is plausible that SCL28 action in meristematic cells has long-lasting effects through cell expansion on final cell size in differentiated tissues. Another possible mechanism for cell size memory involves a monitoring system for cellular dimensions that shares components in proliferating and expanding cells. Supporting this idea, it has been suggested that cells expand to a target size by a mechanism requiring a cell size measurement device, from an approach combining quantitative analysis and mathematical modeling of variability in final cell size of Arabidopsis root. Further careful studies are required to clarify how final cell size is affected by expression levels of SLC28 that is confined to proliferating cells.

In summary, we identified a novel hierarchical MYB3R-SCL28/AtSMOS1-SMR transcriptional regulatory pathway leading to cell cycle inhibition at G2/M that regulates cell size and number during organ development without dramatically altering organ size. This mechanism may help to adjust cell size to optimize cellular and tissue performance such as metabolic functions or mechanical properties.

**Methods**

**Plant materials.** Arabidopsis thaliana Columbia (Col) was used as the WT plant. The accession and transgenic lines used in this study were obtained from the ABRC online catalog. The mutant alleles scl28-1 (SALK_205284), atsmos1-1 (SALK_111056), smr1-1 (SALK_033905), smr2-1 (SALK_006998C), and smr13-1 (SALK_053256C) were identified from SALK T-DNA collection, and used for phenotype analysis and generating multiple mutant combinations. Other mutants and transgenic lines, namely myb3r1-1, myb3r5-2, myb3r5-1, PCNA-RFP, and CYCB1;1-GFP, were described previously. Sterilized seeds were germinated on one-half strength Murashige and Skoog (1/2 MS) medium containing 2% sucrose and 0.6% agar. Plants were grown on 1/2 MS agar medium or soil under continuous light at 22 °C. For root phenotype analysis, plants were grown on a vertical surface of 1/2 MS medium containing 1.0% agar.

**qRT-PCR analysis.** Total RNA was extracted using TRIzol reagent (Thermo Fisher) and used for synthesis of first-strand cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo), according to the manufacturer’s instruction. A real-time qPCR was conducted using Thunderbird SYBR qPCR Mix (Toyobo) on StepOnePlus Real-Time PCR Systems (Applied Biosystems). Three biological replicates were run for each measurement, and results were normalized to the expression of UBQ10 as an endogenous control. Primers used for qRT-PCR are listed in Supplementary Table 1.

**Yeast two-hybrid assay.** For yeast two-hybrid assays, Saccharomyces cerevisiae strain L40 [MATa his3-200 trpl-901 leu2-3,112 ade2-10 lys2::(LeuA)-His3 URA3::(LexA-op)-LexA-2] was transformed with pBTM116- and pVP16-based plasmids carrying coding sequences encoding for SCL28 or AtSMOS1 using S.c. EasyDAK Transformation Kit (Thermo Fisher). Transformants were selected on the synthetic medium lacking Ura, Leu and Trp at 30 °C for 2–3 days. A single colony was diluted with water and spotted on synthetic medium lacking Ura, Leu, Trp and His supplemented with 3-AT (50 or 100 mM). After incubation at 30 °C for 2 days, the yeast transformants grown on the medium were photographed.

**BiFC assay.** For construction of plasmids used for BiFC assays, the entire coding regions of SCL28 and AtSMOS1 were amplified by PCR, and cloned into donor vectors pDONR201 and pDONR207 using BP Clonase II (Thermo Fisher), respectively. The cloned fragments were then transferred into the destination vectors pGWY1 and pGWCY1 using LR clonase II (Thermo Fisher) to generate cell- and fusion of YFP fragments. Transient gene expression using Arabidopsis leaf mesophyll protoplasts was performed as described previously. The transfected protoplasts were incubated overnight in the dark at 22 °C. YFP fluorescent was observed with fluorescent optics on a BX54 microscope (Olympus).

**LUC reporter assay.** Preparation of protoplasts from Arabidopsis T87 suspension cultured cells and polyethylene glycol-mediated gene transfer were performed as described previously. An empty vector containing 35S promoter (pIT-60) and 35SrHrLUC plasmid expressing the humanized Renilla LUC (hRLUC) were used as a negative control and an internal control, respectively. Protoplasts (1.5 × 10^5 for each transfection) were co-transfected with 15 μg each of the LUC reporter and expression plasmids, then incubated at 22 °C for 20 h before measuring LUC activities, which was performed using the Dual-Luciferase Reporter Assay system (Promega) and Luminoskan Ascent luminometer (Thermo Fisher). LUC activity was normalized according to hRLUC activity in each assay, and the relative ratio was determined.

**Plasmid construction.** To construct GUS fusion reporters, the upstream region of SCL28 (2.2 kb) was amplified by PCR and cloned into the pENTR/D-TOPO vector (Thermo Fisher), then transferred to pBGGS5 through Gateway LR reaction to create proSCL28:GUS. The PCR-based site-directed mutagenesis was performed to change all four MSA core motifs, AAGCG, in the SCL28 promoter to ATTGG, resulting in generation of proSCL28:MSA:GUS.

To prepare the construct expressing SCL28 fused to GFP at its C-terminus (SCL28-GFP) under the control of the native promoter, the entire SCL28 genomic region containing 2.2 kb of 5′ upstream and an 1.1-kb genomic DNA from Arabidopsis (Col), and cloned into pENTR/D-TOPO. The resulting plasmid was then used for In-Fusion reaction (Takara Clontech) to insert PCR-
amplified GFP fragments at the C-terminus of the SCL28 coding sequence, to create entry plasmid containing the proSCL28:SCL28-GFP fusion construct, which was then used for transformation of Arabidopsis. Total RNA was extracted using Trizol reagent and subjected to ethanol precipitation at −20°C for 20 min. The precipitated RNA was resuspended in 1 mL of Extraction buffer II (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5 M NaCl) and then loaded on equal volume of 3′ IVT Express Kit (Affymetrix), and used for hybridization to the chip according to the supplier’s protocol. Data analysis was performed using Microarray Suite ver. 5 (Affymetrix) and GeneSpring 7.1 (Agilent Technologies). For transcriptome profiling in WT and SCL28 knockout plants, whole seedlings at 9 DAS were analyzed with three biological replicates. Genes with FDR ≤ 0.05 were defined as differentially expressed genes in SCL28® compared with WT plants. For RNA-Seq analyses, total RNA was extracted as above and used to construct cDNA libraries with the TruSeq RNA Library Preparation Kit v2 (Illumina, United States) according to the manufacturer’s protocol. For transcriptome profiling in SCL28 knockout plants, whole seedlings at 9 DAS were analyzed in three biological replicates. The libraries were sequenced using the NextSeq500 sequencer (Illumina, United States). Raw reads containing adapter sequences were trimmed using bcftools mpileup (Illumina, United States), and nucleotides with low-quality (QV < 25) were masked by N using the original script. Reads shorter than 50 bp were discarded and remaining reads were aligned to the cDNA reads using Bowtie with the following parameters: “–very-sensitive”. The reads were counted by transcript models. Differentially expressed genes were selected based on adjusted P-value calculated using edgeR (version 3.20.9) with default settings.

ChIP-qPCR analysis. Whole seedlings were cross-linked in 20 mL of 1% formaldehyde solution under vacuum for 30 min. Plants were then washed twice with 0.125 M glycine solution, ground to powder in liquid nitrogen and resuspended in 15 mL Extraction buffer I (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl2, and complete protease inhibitor cocktail tablets [Roche]). After filtration with Miracloth (Millipore), samples were centrifuged and resulting pellets were resuspended in 1 mL of Extraction buffer II (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl2, and complete protease inhibitor cocktail tablets [Roche]). After centrifugation, the pellets were resuspended in 300 µL of Extraction buffer III (1.7 M sucrose, 10 mM Tris-HCl, 2 mM MgCl2, 0.15% Triton-X-100, and 0.2% SDS), once with high salt buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 400 mM NaCl, 0.5% Triton-X-100 and 0.2% SDS) and once with LiCl buffer (10 mM Tris-HCl, 1 mM EDTA, 250 mM LiCl, 1% NP-40 and 1% sodium deoxycholate). Chromatin was then eluted from the beads using elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 300 mM NaCl, and 1% SDS). Cross-linking of chromatin was reversed by incubating at 65°C for 12 h followed by digestion with Proteinase-K (9033). The reaction was purified by NucleoSpin (Macherey-Nagel) according to the manufacturer’s instruction and used for quantitative PCR with specific primer pairs (Supplementary Table 1).

Kinematic analysis of leaf growth. After plants were fixed in a 9:1 of ethanol and acetic acid solution and cleared with Hoyer’s solution (a mixture of 100 g chloral hydrate, 10 g glycerol, 15 g gum arabic, and 25 mL water), we performed microsections using 1st blades of leaves using 1×1 mm sections. The sections were then dehydrated in acetone series, and whole leaf images were captured, palisade cells at positions one-fourth and three-fourth from the tip of each leaf were observed with differential interference contrast (DIC) microscope (BX51, Olympus). The captured images were analyzed using ImageJ (version 2.1.0; rsb.info.nih.gov/j) and the average size of palisade cell, the number of cells in the uppermost layer of palisade tissue per leaf, and cell division rate were calculated according to methods described previously.

ChIP-qPCR assay. ChIP-seq assays were performed on whole seedlings using anti-GFP antibody (Abcam, ab290). Seedlings at 14 DAS (5 g) from proAtMS1::AtMS1-GFP and proSCL28::SCL28-GFP were crosslinked in 1% (v/v) formaldehyde at room temperature for 15 min. Crosslinking was then quenched with 0.125 M glycine for 5 min. The crosslinked seedlings were ground, and nuclei were isolated and lysed in nuclei lysis buffer (1% SDS, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Crosslinked chromatin was sonicated using a water bath Bioruptor UCD-200 (Diagenode) (15 s on/15 s off pulses; 15 times). The complexes were immunoprecipitated with antibodies (1 µg for each immunoprecipitation), overnight at 4°C with gentle shaking, and incubated for 1 h at 4°C with 40 µL of Protein A/G beads. The beads were washed 2 × 5 min in ChIP Wash Buffer 1 (0.1% SDS, 1% Triton-X-100, 20 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, pH 8.0), 2 × 5 min in ChIP Wash Buffer 2 (0.1% SDS, 1% Triton-X-100, 20 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, pH 8.0, 2 × 5 min in ChIP Wash Buffer 3 (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and twice in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). ChIPed DNA was eluted by two 15 min incubations at 65°C with 250 µL elution buffer (1% SDS, 0.1 M NaHCO3). Chromatin was reverse-crosslinked by adding 20 µL of 10 M NaOH and 50 µL of 1 M Tris·HCl (pH 8.0) at 37°C overnight. The sample was subjected to RNase and proteinase K digestion and extracted with phenol-chloroform. DNA was ethanol precipitated in the presence of 20 µg of glycogen and resuspended in 50 µL of nuclease-free water (Ambion) in a DNA low-bind tube. We used 10 ng of IP or input DNA for ChiP-seq library construction using the NEBNext Ultralow Input DNA Library Prep Kit for Illumina (New England Biolabs) according to manufacturer’s recommendations. For all libraries, twelve cycles of PCR were used. Library quality was assessed with Agilent 2100 Bioanalyzer (Agilent).

Computational analysis of ChIP-seq data. Single-end sequencing of ChIP samples was performed using Illumina NextSeq 500 with a read length of 76 bp. Reads were quality controlled using FASTQC (http://www.bioinformatics.ualberta.ca/projects/fastqc/). Trimmomatic was used for quality trimming. Parameters for read quality filtering were set as follows: minimum length of 50 bp, mean Phred quality score greater than 30; and leading and trailing bases removal with base quality below 5. Reads were mapped onto the TAIR10 assembly using Bowtie with mismatch permission of 1 bp. To identify significantly enriched regions, we used MACS2. Parameters for peak detection were set as follows: number of duplicate reads at a location: 1; mfold of 5:0 q value cutoff: 0.05; extsize: 200; and sharp peak. Visualisation and analysis of genome-wide enrichment profiles were performed with Integrated Genome Browser. Peak annotations such as proximity to genes and overlap of genomic features, including transposons and genes were performed using BEDTOOLS INTERSECT. NCSpot was used to profile enrichment at TSSs and along the gene. Spatial binding of the AtMS1 and SCL28 peaks were performed by position-wise comparison using a binning approach and plotted in hexplot. De novo motif analysis of both SCL28 and AtMS1 binding regions were screened using HOMER.

Histochemical analysis. Excised plant organs were fixed in FAA solution (100% ethanol/30%glacialacetic acidwater: 20:19:1) under vacuum, stained with 1% Safranin O, dehydrated with 50%, 70%, 100% ethanol series, and embedded in Technovit 7100 (Heraeus Kulzer). 2 µm-thickness sections were cut with 1% Safranin O, dehydrated with 30%, 50%, 70%, and 100% ethanol series, and embedded in Technovit 7100 (Heraeus Kulzer). The resulting sections were stained in pENTR/D-TOPO cells as a template.

Kinematic analysis of leaf growth. After plants were fixed in a 9:1 of ethanol and acetic acid solution and cleared with Hoyer’s solution (a mixture of 100 g chloral hydrate, 10 g glycerol, 15 g gum arabic, and 25 mL water), we performed microsections using 1st blades of leaves using 1×1 mm sections. The sections were then dehydrated in acetone series, and whole leaf images were captured, palisade cells at positions one-fourth and three-fourth from the tip of each leaf were observed with differential interference contrast (DIC) microscope (BX51, Olympus). The captured images were analyzed using ImageJ (version 2.1.0; rsb.info.nih.gov/j) and the average size of palisade cell, the number of cells in the uppermost layer of palisade tissue per leaf, and cell division rate were calculated according to methods described previously.
Analysis of meristem cell number and cell size in roots. To visualize cell outlines, roots from plants at five DAS were stained with 0.05 mg mL−1 propidium iodide and observed with confocal laser scanning microscopy (CLSM), using an inverted fluorescence microscope (Eclipse T2, Nikon) equipped with a confocal scanning unit (A1, Nikon). The resulting images were processed using ImageJ software to measure cell length in the root meristem. Root meristem size was measured by counting the number of cortical cells between the quiescent center and the first elongated cell.

Cell cycle analysis by time-lapse imaging. Seedlings at six DAS carrying PCNA-GFP were transferred onto an MS medium in a glass-bottom dish, and root meristem cells were observed by CLSM as described above. Time-lapse images were acquired every 30 min for 15 h. To avoid long-time imaging that possibly damages the samples, the duration of the G1/S and G2/M phases was measured separately. Based on PCNA-GFP fluorescence patterns, duration of each cell cycle phase was analyzed for epidermal cells in hair cell lineage.

GUS staining. Tissue was pre-fixed in 90% (v/v) acetone for 20 min on ice, rinsed with 100 mM sodium phosphate solution (pH 7.0), and transferred to staining solution (100 mM sodium phosphate buffer pH 7.0, 0.1% Triton-X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, 0.5 mg mL−1 5-bromo-4-chloro-3-indolyl-β-D-galacturonic acid) and incubated at 37°C for 12 h. GUS-stained samples were then transferred to 70% ethanol, incubated to remove chlorophyll and then photographed or used for microscopic observation.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. RNA-Seq data for WT, sqcl2, and atsMos1 can be accessed from the DDBJ database under accession number DRA012786. ChIP-seq data of SCL28 and ATSMOS1 can be accessed at Gene Expression Omnibus database under accession number GSE183209. Arabidopsis mutants and transgenic lines, as well as plasmids generated in this study, are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Received: 16 October 2021; Accepted: 7 March 2022; Published online: 29 March 2022.

References

1. Roeder, A. H. et al. Variability in the control of cell division underlies sepal chlorophyll and then photographed or used for microscopic observation. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-29316-2 • www.nature.com/naturecommunications

2. Polyn, S., Willems, A. & De Veylder, L. Cell cycle entry, maintenance, and exit during plant development. EMBO J. 34, 1992–2007 (2015).

3. Ito, M. et al. G2/M-phase-specific transcription during the plant cell cycle is mediated by c-Myc-like transcription factors. Plant Cell 13, 1891–1905 (2001).

4. Haga, N. et al. Mutations in MYB3R1 and MYB3R4 cause pleiotropic developmental defects and preferential down-regulation of multiple G2/M-specific genes in Arabidopsis. Plant Physiol. 157, 706–717 (2011).

5. Kobayashi, K. et al. Transcriptional repression by MYB3R proteins regulates plant organ growth. EMBO J. 34, 1992–2007 (2015).

6. Ito, M. et al. G2/M-phase-specific transcription during the plant cell cycle is mediated by c-Myc-like transcription factors. Plant Cell 13, 1891–1905 (2001).

7. Haga, N. et al. R1R2R3-Myb proteins positively regulate cytokinesis through activation of KNOXE transcription in Arabidopsis thaliana. Development 134, 1011–1110 (2007).

8. Okumura, T. et al. MYB3R-mediated active repression of cell cycle and growth under salt stress in Arabidopsis thaliana. J. Plant Res. 134, 261–277 (2021).

9. Ning, Y. Q. et al. DREAM complex suppresses DNA methylation maintenance genes and precludes DNA hypermethylation. Nat. Plants 6, 942–956 (2020).

10. Lang, L. et al. The DREAM complex represses growth in response to DNA damage in Arabidopsis. Life Sci. Alliance 4, e20211141 (2021).

11. Sadasivam, S. & DeCaprio, J. A. The DREAM complex: master coordinator of cell cycle-dependent gene expression. Nat. Rev. Cancer 13, 585–595 (2013).

12. Fischer, M. & Müller, G. A. Cell cycle transcription control: DREAM/MuvB complexes. Crit. Rev. Biochem. Mol. Biol. 52, 638–662 (2017).

13. Lee, T. L. et al. Transcriptional regulatory networks in Saccharomyces cerevisiae. Science 298, 799–804 (2002).

14. Zhu, W., Giangrande, P. H. & Nevin, J. R. E2F's link the control of G1/S and G2/M transcription. EMBO J. 23, 4615–4626 (2004).

15. Millour, J. et al. ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. Mol. Cancer Ther. 10, 1046–1058 (2011).

16. Simon, I. et al. Serial regulation of transcriptional regulators in the yeast cell cycle. Cell 106, 697–708 (2001).

17. Goldy, C. et al. The Arabidopsis GRAS-type SCL28 transcription factor controls the mitotic cell cycle and division plane orientation. Proc. Natl Acad. Sci. USA 118, e2000256118 (2021).

18. Kumar, N. et al. Functional conservation in the SIAMESE-RELATED family of cyclin-dependent kinase inhibitors in land plants. Plant Cell 27, 3065–3080 (2015).

19. Ito, M., Kodama, H. & Komamine, A. Gene expression and its regulation during the cell cycle of higher plants in synchronous cell culture systems. Vitr. Cell. Dev. Biol. 35, 388–395 (1999).

20. Ito, M. et al. A novel cis-acting element in promoters of plant B-type cyclin genes activates M phase-specific transcription. Plant Cell 10, 331–341 (1998).

21. O'Malley, R. C. et al. Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. Cell 165, 1280–1292 (2016).

22. Ubeda-Tornás, S. et al. Gene expression in the endosperm controls Arabidopsis root meristem size. Curr. Biol. 19, 1194–1199 (2009).

23. Bouyer, D. et al. Genome-wide identification of RETINOBLASTOMA RELATED 1 binding sites in Arabidopsis reveals novel DNA damage regulators. PLoS Genet. 14, e1007797 (2018).

24. Yokoyama, R., Hirakawa, T., Hayashi, S., Sakamoto, T. & Matsunaga, S. Dynamics of plant DNA replication based on PCNA visualization. Sci. Rep. 6, 29657 (2016).

25. Jones, A. R. et al. Cell-size dependent progression of the cell cycle creates homeostasis and flexibility of plant cell size. Nat. Commun. 8, 15060 (2017).

26. Hirano, K. et al. Small organ size 1 and small organ size 2 dwarf and low-tiltering form a complex to integrate auxin and brassinosteroid signaling in rice. Mol. Plant 10, 590–604 (2017).

27. Aya, K. et al. A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. Plant Cell Physiol. 55, 897–912 (2014).

28. Verkest, A. et al. The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf development through inhibition of mitotic CDKAs kinase complexes. Plant Cell 17, 1723–1736 (2005).

29. Wang, K. et al. The CDK Inhibitor SIAMESE Targets Both CDKAs and CDKB1 Complexes to Establish Endoreplication in Trichomes. Plant Physiol. 184, 165–175 (2020).

30. Zatulovskiy, E. & Skotheim, J. M. On the Molecular Mechanisms Regulating Animal Cell Size Homeostasis. Trends Genet. 36, 360–372 (2020).

31. Jones, A. R., Band, L. R. & Murray, J. A. Double or Nothing? Cell Division and Cell Size Control. Trends Plant Sci. 24, 1083–1093 (2019).

32. D’Ario, M. & Sablowski, R. C. Cell Size Control in Plants. Annu. Rev. Genet. 53, 45–65 (2019).

33. Cho, J. et al. Characterization of the GRAS transcription factor SCARECROW-LIKE 2’s role in Arabidopsis root growth. J. Plant Biol. 60, 462–471 (2017).

34. Yi, D. et al. The Arabidopsis SIAMESE-RELATED cyclin-dependent kinase inhibitors SMR5 and SMR7 regulate the DNA damage checkpoint in response to reactive oxygen species. Plant Cell 26, 296–309 (2014).

35. Davie, E. & Petersen, J. Environmental control of cell size at division. Curr. Opin. Cell Biol. 24, 838–844 (2012).

36. Barrada, A. et al. A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis. Development 146, dev172398 (2019).

37. Willis, L. et al. Cell size and growth regulation in the Arabidopsis thaliana apical stem cell niche. Proc. Natl Acad. Sci. USA 113, E8238–E8246 (2016).

38. Fantes, P. A., Grant, W. D., Pritchard, R. H., Sudbery, P. E. & Weeks, A. E. The regulation of cell size and the control of mitosis. J. Theor. Biol. 50, 213–244 (1975).

39. Schindler, K. M., Turner, J. J., Kövomagi, M. & Skotheim, J. M. Dilution of the cell cycle inhibitor Wh1 controls budding-yeast cell size. Nature 526, 268–272 (2015).
46. Zatulovskiy, E., Zhang, S., Berenson, D. F., Topacio, B. R. & Skotheim, J. M. Cell growth dilutes the cell cycle inhibitor Rb to trigger cell division. Science 369, 466–471 (2020).
47. D’Ario, M. et al. Cell size controlled in plants using DNA content as an internal scale. Science 372, 1176–1181 (2021).
48. Meyer, H. M. et al. Fluctuations of the transcription factor ATML1 generate the pattern of giant cells in the Arabidopsis sepal. Elife 6, e19131 (2017).
49. Magyar, Z. et al. The role of the Arabidopsis E2F transcription factor in regulating auxin-dependent cell division. Plant Cell 17, 2527–2541 (2005).
50. Gutzeit, R. et al. Retinoblastoma-related protein controls the transition to autotrophic plant development. Development 138, 2977–2986 (2011).
51. Brgyzh, L. et al. Arabidopsis Retinoblastoma-related is required for stem cell maintenance, cell differentiation, and lateral organ production. Plant Cell 22, 1735–1747 (2010).
52. Dewitte, W. et al. Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3. Plant Cell 15, 79–92 (2003).
53. De Veylder, L. et al. Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J. 21, 1360–1368 (2002).
54. Potter, C. J. & Xu, T. Mechanisms of size control. Curr. Opin. Genet. Dev. 11, 279–286 (2001).
55. Hisanaga, T., Kawade, K. & Tsukaya, H. Compensation: a key to clarifying the internal scale. Plant Cell 25, 4662–4672 (2013).
56. Suyama, T., Nishiyama, R., Morikyo, K., Ishikawa, M. & Machida, Y. NOK1/NIK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes Dev. 17, 1055–1067 (2003).
57. Hino, T. et al. Two Sec13p homologs, AtSec13A and AtSec13B, redundantly contribute to the formation of COPII transport vesicles in Arabidopsis thaliana. Biosci. Biotechnol. Biochem. 75, 1848–1852 (2011).
58. Yoshida, H. et al. DELLA protein functions as a transcriptional activator through the DNA binding of the indeterminate domain family proteins. Proc. Natl Acad. Sci. USA 111, 7861–7866 (2014).
59. Maoe, K. et al. An AP2-type transcription factor, WRINKLED1, of Arabidopsis thaliana binds to the AW-box sequence conserved among proximal upstream regions of genes involved in fatty acid synthesis. Plant J. 60, 476–487 (2009).
60. Nakagawa, T. et al. Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Biosci. Biotechnol. Biochem. 74, 2095–2100 (2007).
61. Kozgunova, E., Suzuki, T., Ito, M., Higashiyama, T. & Kurihara, H. Daopin has Multiple Functions in the Plant Cell Division Regulatory Network. Plant Cell Physiol. 57, 848–861 (2016).
62. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
63. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2010).
64. Zhang, Y. et al. Model-based Analysis of ChiP-Seq (MACS). Genome Biol. 9, R137 (2008).
65. Shen, L., Shao, N., Liu, X. & Nestler, E. ngsplot: Quick mining and visualization of next-generation sequencing data by integrating genomic databases. BMC Genom. 15, 284 (2014).
66. Kim, S. et al. GCN5 modulates salicylic acid homeostasis by regulating HSL1-ac levels at the 5’ and 3’ ends of its target genes. Nucleic Acids Res. 48, 5953–5966 (2020).
67. De Veylder, L. et al. Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell 13, 1653–1667 (2001).

Acknowledgements

The authors thank Kyoko Kato, Akiko Nakahara, Tsukasa Ono, Akiko Yamamoto, Chikako Inoue, Nanako Ishibashi, Satoko Nasu, Yuiko Tachikawa, Asako Segawa, Ayami Furuta, Tomomi Shinagara, Akiko Nakamura, and Ayumi Yamada for technical assistance. This study was funded by The Japan Society for the Promotion of Science KAKENHI (20H05408 and 17H03696 to M.I., 20H05911 and 20H05905 to K.S.), Japan Science and Technology Agency (IST grant number JPMJPF2102 to M.I.) and the Institut Universitaire de France (to M.B.). Y.H. was supported by China Scholar Council fellowships (201806690005).

Author contributions

M.I. conceived the study; C.R., M.B., Z.M., K.S., T.Y., and M.I. designed the experiments; J.A., Takamasa. S. analyzed the data; M.I., L.B., Z.M., and M.B. wrote the paper. H.T. and Y.N. contributed equally.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29316-2.

Correspondence and requests for materials should be addressed to Masaki Itô.

Peer review information Nature Communications thanks John Larkin and the other anonymous, reviewer for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party materials in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022