Functional Conservation in the SIAMESE-RELATED Family of Cyclin-Dependent Kinase Inhibitors in Land Plants

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The best-characterized members of the plant-specific SIAMESE-RELATED (SMR) family of cyclin-dependent kinase inhibitors regulate the transition from the mitotic cell cycle to endoreplication, also known as endoreduplication, an altered version of the cell cycle in which DNA is replicated without cell division. Some other family members are implicated in cell cycle responses to biotic and abiotic stresses. However, the functions of most SMRs remain unknown, and the specific cyclin-dependent kinase complexes inhibited by SMRs are unclear. Here, we demonstrate that a diverse group of SMRs, including an SMR from the bryophyte Physcomitrella patens, can complement an Arabidopsis thaliana siamese (sim) mutant and that both Arabidopsis Sim and P. patens SMR can inhibit CDK activity in vitro. Furthermore, we show that Arabidopsis SIM can bind to and inhibit both CDKA;1 and CDKB1;1. Finally, we show that SMR2 acts to restrict cell proliferation during leaf growth in Arabidopsis and that SIM, SMR1/LGO, and SMR2 play overlapping roles in controlling the transition from cell division to endoreduplication during leaf development. These results indicate that differences in SMR function in plant growth and development are primarily due to differences in transcriptional and posttranscriptional regulation, rather than to differences in fundamental biochemical function.

INTRODUCTION

Proper regulation of the cell cycle is crucial throughout the life cycle for the survival of multicellular organisms. Progression through different cell cycle checkpoints is controlled by serine/threonine kinases known as cyclin-dependent kinases (CDKs).

CDK activity is regulated at the posttranslational level by the binding or proteolytic degradation of regulatory subunits called cyclins (CYCs), by phosphorylation by CDK-activating kinases, and by binding CDK inhibitors (CKIs) (Pines, 1995; Pavletich, 1999). Cyclins and CDKs form heterodimeric CYC/CDK complexes with at least some of the substrate specificity conferred by the cyclin partner (Loog and Morgan, 2005). In yeast, a single CDK binds to different cyclins to regulate cell cycle progression, whereas in plants and mammals multiple CDKs, as well as multiple cyclins, function at different stages of the cell cycle (Mendenhall and Hodge, 1998; Menges et al., 2005; Bloom and Cross, 2007).

Despite having the same core cell cycle mechanism as other eukaryotes, the plant cell cycle has some unique features. For example, plants have a unique class of plant-specific CDKs, the CDKBs (Boudolf et al., 2001; Vandepoele et al., 2002; Dewitte and Murray, 2003). Unlike animal CDKs and plant CDKA;1, these CDKBs do not complement the cell cycle phenotype of cdc2/cdc28 yeast mutants. The CDKBs consist of two subfamilies in Arabidopsis thaliana, with expression of CDKB1 starting during S-phase and peaking in G2 and CDKB2 expression starting later and peaking at the G2-to-M transition (Menges et al., 2005). In plants, unlike mammals, CDK phosphorylation and dephosphorylation does not require WEE1 kinase and CDC25 phosphatase for
inhibition and activation of CDK kinase activity, respectively (De Schutter et al., 2007; Dissmeyer et al., 2010). Plants lack a CDC25 ortholog, and WEE1 is involved in the DNA damage response rather than playing a significant role in the normal mitotic cell cycle in Arabidopsis. Therefore, the G2/M transition in plants appears to be regulated primarily by cell cycle phase-specific transcription of CDKB1s, CDKB2s, and their cyclin partners, rather than by the WEE1 kinase/CDC25 phosphatase pair that regulates entry into M-phase in animals and some fungi (Boudolf et al., 2006).

In plants, two families of CKIs have been identified. One is the INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KIP-RELATED PROTEIN (ICK/KRP) family, named because of their sequence similarity with animal Kip-type CDK inhibitors, which has seven family members in Arabidopsis (Wang et al., 1997; De Veylder et al., 2001). ICK/KRPs are inhibitors of CDKA;1 kinase activity and when expressed at high levels can result in cell death (Schnittiger et al., 2003). A second plant CDK inhibitor family is encoded by the SIAMESE-RELATED (SMR) genes (Churchman et al., 2006). SIAMESE (SIM), the founding member of the family, and other Arabidopsis SMRs have been implicated in binding to several different CYC/CDK complexes, and the rice (Oryza sativa) SMR protein EL2 has been shown to inhibit CDKA;1 kinase activity (Churchman et al., 2006; Peres et al., 2007; Van Leene et al., 2010).

Both SIM and the closely related SMR1/LGO gene were discovered via their roles in promoting a modified version of the cell cycle known as endoreplication in Arabidopsis (Churchman et al., 2006; Roeder et al., 2010). During endoreplication, DNA replicates without subsequent mitosis and cytokinesis, consequently doubling the DNA amount in each round of the cell cycle, resulting in cells with increased ploidy (Edgar and Orr-Weaver, 2001; De Veylder et al., 2011; Fox and Duronio, 2013). In plants, endoreplication typically occurs in tissues that develop mass very quickly or have a higher metabolic rate and is often correlated with cell differentiation and increased cell size. For instance, the trichomes in wild-type plants are large, unicellular, and endoreplicated, while in sim mutants, the trichomes divide during development and become multicellular (Walker et al., 2000; Churchman et al., 2006). Similarly, smr1/lgo mutants lack the giant endoreplicated epidermal cells that are found on the abaxial surfaces of sepals (Roeder et al., 2010).

Recently, additional roles for SMRs in plant growth and development have been identified. Several SMRs have been implicated in regulating root meristem size in response to gibberellin development have been identified (Achard et al., 2009). Additionally, smr triple mutants lacking functional copies of all three CYCD3 genes results in increased endoreplication in Arabidopsis leaves, which indicates that CDKB1;1 restrains endoreplication and promotes maintenance of mitosis (Porceddu et al., 2001; Boudolf et al., 2004).

Although substantial evidence indicates that SIM and SMRs are CKIs, it is not clear yet which specific CYC/CDK complexes are inhibited by SIM to promote endoreplication in Arabidopsis, a mechanism remains unclear. In the latter study, SIM and SMR11 were found to interact with CYCB2;4, an interacting partner of CDKB1;1, while other SMRs were found to interact with CYCDs and CDKA;1. On the basis of these latter results, it has been suggested that the SIM/SMR family members are divided into two functionally distinct groups, with SIM, SMR1, SMR2, and SMR11 being inhibitors of CDKB1;1 complexes and the remaining SMRs being inhibitors of CDKA;1 complexes (Van Leene et al., 2010; Yi et al., 2014). Although multiple SMR genes are present in the genomes of most plants examined so far, little is known about the functions of most of them, and the degree of functional divergence among SMRs remains unclear.

In this study, we show that the SIM/SMR family members are functionally conserved throughout land plant evolution and that even divergent members of the family can replace SIM function in vivo. We also present evidence from both in vitro and in vivo studies that inhibition of CYC3/CDKA;1 complexes is important to SIM function and demonstrate that SIM, SMR1/LGO, and SMR2 play overlapping roles in regulating the balance between cell proliferation and endoreplication during leaf development. Our work suggests that differences in SMR function in plant growth and development are primarily due to differences in transcriptional and posttranscriptional regulation, rather than to differences in fundamental biochemical function.

RESULTS

Divergent Members of the SMR Family Can Functionally Replace SIM

The initial article describing SIM also described three related SMR genes from the Arabidopsis genome, as well as several SMRs from a variety of dicots and monocots (Churchman et al., 2006), and two subsequent articles described three more SMRs (Peres et al., 2007; Yi et al., 2014). The sequence similarity among these SMRs
is limited to a series of short conserved protein sequence motifs that occurred in a consistent order, but with variable spacing. Several other Arabidopsis SMRs have been referred to in the literature (Van Leene et al., 2010; Yi et al., 2014), but the sequence similarities among these genes have not been described, and the functions of most of these genes as cell cycle regulators have not been tested. This is important in light of the limited sequence conservation among putative SMRs and the suggestion by Van Leene et al. (2010) that different SMRs may inhibit different CYC/CDK complexes.

An exhaustive iterative search of the Arabidopsis genome revealed a total of 17 putative SMR genes (SIM and SMR1-16), and a consensus sequence for three conserved protein sequence motifs was derived from the deduced peptide sequences of these genes (Figure 1A). Although most of these SMRs were readily recognizable as being related to SMRs with known functions, other putative members of the family were more divergent. The most strikingly divergent putative Arabidopsis SMR genes were SMR11 and SMR16; a principle component analysis in which components were generated by pairwise alignments of peptide sequences to represent spatial similarities based on a substitution matrix showed that SMR11 clustered with SMR16 and was clearly distinct from the rest of the SMRs (Figure 1B). SMR11 and SMR16 also group together in a neighbor-joining tree of SMR genes (Figure 2; described in more detail in the following section).

We had previously shown that the rice SMR known as EL2 could complement the multicellular trichome phenotype of Arabidopsis sim mutant plants, in spite of having only modest sequence similarity (Peres et al., 2007), but the degree of sequence divergence among putative family members made identifying true SMRs difficult. For this reason, we tested whether a wide variety of Arabidopsis SMRs could complement the multicellular trichome phenotype of sim when expressed under the control of the GL2 promoter (GL2pro), which in leaves is relatively trichome specific. We found that SMR2, SMR4, and SMR11 (Figures 3A to 3E, Table 1; Supplemental Figure 1), as well as SMR1/LGO, SMR3, SMR7, and SMR13 (Table 1; Supplemental Figure 1), could all complement the sim phenotype, as judged both by the number of cells per trichome initiation site in

![Figure 1. Conservation of SMR Sequences and Phylogenetic Analysis.](image-url)

(A) Motifs A, B, and C, derived from alignment of all Arabidopsis SMRs. The alignment is shown in Supplemental Data Set 1.
(B) Principle component analysis of pairwise sequence distances among Arabidopsis SMRs showing that SMR11 and SMR16 are divergent in sequence relative to the other SMRs.
individual complemented lines (Table 1) and by the fraction of lines showing complementation (Supplemental Table 1). These findings indicate that all of these genes are able to functionally replace SIM in suppressing mitosis and promoting endoreplication in developing trichomes.

For SMR5, attempts to complement sim using the annotated open reading frame, with an annotated intron removed, were unsuccessful (Supplemental Figure 2A). We noticed that the predicted protein translated from an unspliced transcript was a better match to the SMR motif consensus than was the annotated predicted product. When this complete coding region was expressed in plants, it fully complemented the sim trichome phenotype (Supplemental Figure 2B), indicating that SMR5 is also functionally equivalent to SIM. The absence of an intron in SMR5 was confirmed by the presence of RNA-seq reads uniformly across the entire gene (Supplemental Figure 2C).

SMRs Are Conserved in All Major Land Plant Lineages

We next conducted a systematic search for all SMR-like genes in the sequenced genomes of the dicots Carica papaya, Citrus × sinensis, and Eucalyptus grandis, the monocot O. sativa, the lycophyte Selaginella moellendorfii, and the bryophyte Physcomitrella patens. Each of these genomes contained multiple SMR-like sequences (Supplemental Figure 3), while no putative SMRs were detected in the genomes of the algal species Chlamydomonas reinhardtii or Ostreococcus tauri. The 74 putative land plant SMR genes that we identified were aligned and used to build a neighbor-joining tree (Figure 2). Although the short length of the conserved region of SMRs, combined with relatively low sequence conservation, left the relationships among many of these genes unresolved, a number of clades with strong bootstrap support were evident. Of particular note, the Arabidopsis SMRs that we found to complement sim included genes in all major clades that contained genes from other angiosperms, as well as a broad sample of genes in unresolved polytomies (Figure 2; At-SMRs are indicated by black dots, and genes demonstrated to complement sim are labeled in red). The bryophyte P. patens was the most phylogenetically divergent lineage for which we obtained putative SMR sequences. We chose one putative P. patens SMR (labeled PpSMR12 in Figure 2) that was in a well-supported P. patens-specific clade and tested it for its ability...
to complement the Arabidopsis sim mutation. As shown in Figure 3F and Table 1, Pp-SMR12 readily complemented sim, indicating that this phylogenetically distant SMR is functionally equivalent to SIM. Combined with the observation that the rice gene EL2, previously shown to complement sim (Peres et al., 2007), was located in a rice-specific clade quite far from SIM in the gene phylogeny, our results suggest that most or all of the putative SMRs we identified encode cell cycle regulators that are functionally equivalent to SIM.

SIM Interacts with Both CDKA;1 and CDKB1;1 in Arabidopsis Protoplasts

As noted above, previous studies have reported conflicting results with regard to the targets of SIM action, i.e., whether SIM inhibits CDKA;1 complexes and/or CDKB1;1 complexes (Churchman et al., 2006; Peres et al., 2007; Van Leene et al., 2010). To address these inconsistencies, we used a different protein-protein interaction assay, the split-luciferase complementation assay, to test interactions between SIM and CDKs in Arabidopsis protoplasts (Fujikawa and Kato, 2007). Because split-protein complementation assays are dependent on the geometry of the interacting proteins, both SIM and CDKA;1 were fused to the N-terminal (Nluc) and C-terminal (Cluc) halves of Renilla reniformis luciferase in all eight possible fusion combinations and tested for functional luciferase activity in protoplasts. Although several combinations gave activity above that of negative controls, the orientation giving the strongest signal, with Cluc at the N terminus of SIM and Nluc at the N terminus of CDKA;1, was used for all other tests of SIM-CDK interactions (Supplemental Figure 4). Interaction of histones H2A and H2B was used as a positive control, and interaction with the transcription factor PERIANTHIA (PAN) was used as a negative control. In this assay, we observed that SIM reproducibly interacted with both CDKA;1 and CDKB1;1 (Figure 4A). The phylogenetically distant P. patens SMR12, used above for complementation, also was tested for interaction with CDKA;1 and was found to interact (Figure 4B).

Table 1. A Diverse Set of SMRs Can Complement the Multicellular Trichome Phenotype of sim

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Nuclei per TIS</th>
<th>Number of TIS</th>
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<tbody>
<tr>
<td>Col-0</td>
<td>1.0 ± 0.00a</td>
<td>60</td>
</tr>
<tr>
<td>sim</td>
<td>2.30 ± 1.18</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR1 sim</td>
<td>1.03 ± 0.18a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR2 sim</td>
<td>1.05 ± 0.22a</td>
<td>60</td>
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<tr>
<td>GL2pro:SMR3 sim</td>
<td>1.07 ± 0.25a</td>
<td>60</td>
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<tr>
<td>GL2pro:SMR4 sim</td>
<td>1.08 ± 0.28a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR5 sim</td>
<td>1.15 ± 0.36a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR7 sim</td>
<td>1.02 ± 0.13a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR11 sim</td>
<td>1.07 ± 0.25a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:SMR13 sim</td>
<td>1.03 ± 0.18a</td>
<td>60</td>
</tr>
<tr>
<td>GL2pro:PpSMR sim</td>
<td>1.13 ± 0.34a</td>
<td>60</td>
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The multicellular trichome phenotype of a complementation line homozygous for a single T-DNA insert for each of the indicated SMRs was assessed by counting the number of DAPI-stained nuclei at each trichome initiation site (TIS) for each genotype. All genotypes with an “a” have significantly fewer nuclei per TIS than the sim mutant (P < 0.0001 in a one-tailed t test, after applying a Bonferroni correction for multiple tests). For each transgenic genotype, at least two additional independent lines were obtained having a phenotype qualitatively equivalent to the line shown here.
Cell Division in sim Mutant Trichomes Depends upon the Function of Both CYCD3s and CDKB1

Our results in Figures 4 and 5 suggest that SIM may inhibit both CDKA;1 and CYCB1;1-containing complexes in vivo. This hypothesis predicts that the cell division observed in sim mutant trichomes (Figures 6A and 6B) should depend on the function of one or both of these CDKs. D3-type cyclins are generally thought to be activators of CDKA;1 complexes (Menges et al., 2006; Van Leene et al., 2010), and CYCD3;1 is capable of activating only CDKA;1 in vitro, not CDKB1;1 (Nowack et al., 2012). Triple mutants lacking all three D3-type cyclins (cycd3;1-3) produce unicellular trichomes resembling those of the wild type (Dewitte et al., 2007; Figure 6C). We constructed sim cycd3;1-3 quadruple mutants and found that they exhibited essentially no cell division in trichomes, indicating that the cycd;1-3 phenotype is epistatic to the sim division phenotype (Figure 6D, Table 2).

Similarly, cdkb1;1 cdkb1;2 double mutant plants had wild-type trichomes showing no division (Figure 6E). Homozygous cdkb1;1 cdkb1;2 sim plants were also primarily unicellular (Figure 6F, Table 2), indicating that cell division in sim mutant trichomes is also dependent on CDKB1 activity.

SMR2 Restricts Cell Proliferation and Cooperates with SIM and SMR1 to Promote Endoreplication during Leaf Development

The preceding work indicated that most, if not all, SMRs are functionally equivalent at the biochemical level, yet all plant genomes tested contain multiple SMR genes. To gain insight into the roles played by SMRs in plant development, we identified T-DNA insertion alleles of two additional Arabidopsis SMRs, SMR1/LGO and SMR2. We noticed that both smr2 alleles identified had larger leaves than the wild type, particularly on the first five leaves of 26-d-old seedlings (Figures 7A and 7B); this phenotype was noticeably stronger in the smr2-1 allele, which has an insertion in the sole exon of the gene. The smr2-1 allele produces no detectable SMR2 transcript (Churchman, 2007). No size increase was detected in smr1/lgo leaves (Figure 7A). A kinematic analysis was conducted to analyze cell division and expansion during growth of the first leaves of wild-type and smr2-1 mutant plants (Figure 8). An increase in leaf cell number was apparent starting at 10 d after stratification (DAS; Figure 8C). This corresponded to a constant cell division rate from 6 to 12 DAS, whereas cell division rates declined during the same time in wild-type leaves. As a result, at 12 DAS, the cell division rate of the mutant was approximately double that of the wild type (Figure 8D).

In contrast, average cell area of mutant leaves by the end of the time period investigated (26 DAS) was slightly less than that of the wild type, indicating that the cause of increased mutant leaf size was primarily increased cell proliferation. Endoreplication levels were also monitored by flow cytometry throughout leaf development; while the time of appearance of 4C, 8C, and 16C cells in mutant leaves was delayed, the mutant leaves ultimately reached ploidy levels similar to those of wild-type leaves (Figure 9A). We also examined the ploidy of mature leaf cells of sim and smr1/lgo single mutants, as well as a sim smr1/lgo smr2 triple mutant. Although the individual single mutants showed only modest effects on the endoreplication index in leaves (the average number of endocycles per cell), the triple mutant exhibited a strongly reduced DNA content and underwent a reduced number of endocycles (Figure 9B; Supplemental Figure 5). Furthermore, mature leaves of both a sim smr1/lgo double mutant and the sim smr1/lgo smr2 triple mutant had significantly greater leaf area, significantly more epidermal cells, and significantly smaller cells than the wild type (Figures 10A to 10C). Additionally, in both the double and the triple mutant, straight cell walls were frequently seen between adjacent epidermal pavement.
cells on mature leaves, in contrast to the “wavy” cell walls typical of wild-type epidermal pavement cells (Supplemental Figures 6C and 6D), which may be indicative of cell divisions late in leaf development.

DISCUSSION

The SMR Gene Family of Land Plants Encodes Functionally Equivalent Cell Cycle Regulators

Here, we identified three previously undescribed putative Arabidopsis SMR genes, SMR14, SMR15, and SMR16, and identified a total of 74 putative SMRs in the sequenced genomes of seven plant species representing a wide range of land plant lineages. Most of these genes contain all three of the key protein sequence motifs defining the SMR family, originally identified as Motifs 1, 2, and 4 by Churchman et al. (2006) and here termed Motifs A, B, and C (Figure 1A). The sequence motifs previously described as Motifs 3 and 5, consisting of short stretches of basic amino acids resembling nuclear localization sequences, are not widely conserved in the wider SMR family. Motif A contains a threonine followed by a proline, which is the minimal consensus site for phosphorylation by CDKs. This pair of amino acid residues has the most highly conserved residues throughout SMR evolution.

Figure 5. Dose-Dependent Inhibition of CYC/CDK Complex Histone H1 Kinase Activity by Arabidopsis SIM and P. patens SMR12.

(A) Inhibition of CYCD3;1/CDKA;1 by GST:SIM.
(B) Inhibition of CYCD2;1/CDKA;1 by GST:SIM.
(C) Inhibition of CYCB1;1/CDKB1;1 by GST:SIM.
(D) Inhibition of CYCD3;1/CDKA;1 by GST:Pp-SMR12.
Cont denotes control kinase assay with no SIM or SMR added. GFP indicates GST:GFP added as a negative control. Note that the HisGST-SIM band appears to be phosphorylated by CYCB1;1/CDKB1;1 (C, two asterisks) and not by the other complexes, although CYCB1;1/CDKB1;1 also appears to phosphorylate HisGST-GFP (C, one asterisk) and thus may be merely phosphorylating the GST domain shared by both proteins.
suggestions that phosphorylation of this threonine may be crucial to SMR function. Motif B is proline-rich and typically contains sequences of the form PXXP, followed by one or more basic residues. This resembles protein interaction domains that interact with partner proteins by forming a PP II helix (Kay et al., 2000). Motif C has previously been reported to be a cyclin-interaction domain in the rice EL2 protein (Peres et al., 2007).

Although in most SMRs all three of these motifs occur in the same order, albeit with variable spacing, some SMRs are exceptions to this pattern. At-SMR11 and At-SMR16, for example, lack a detectable Motif B, although in spite of this sequence divergence, At-SMR11 was able to complement a sim mutation (Figure 3E, Table 1). At-SMR3, which also complemented sim (Table 1; Supplemental Figure 1H), appears to contain a second copy of Motif A located between Motifs B and C (residues 82 to 91, TPVPNVRIP). Finally, a phylogenetically distant SMR from the bryophyte P. patens also complemented the sim mutant phenotype (Figure 3F, Table 1). Taken together, these results indicate that most, if not all, of the SMR sequences that we identified code for functional SMR proteins. These proteins appear to be functionally equivalent, based on their ability to complement the sim trichome phenotype. Thus, the roles played in plant development by different SMRs are most likely maintained primarily by differences in transcriptional and post-transcriptional regulation of individual genes, rather than by differences in their underlying biochemical activity. Indeed, there is already substantial evidence for distinct expression patterns among the SMR genes so far examined (Churchman et al., 2006; Peres et al., 2007; Yi et al., 2014).

**SIM, SMR1, and SMR2 Have Overlapping Roles in Controlling the Transition from Cell Division to Endoreplication during Leaf Development**

Consistent with the overlapping functional roles among SMRs, we found that SIM, SMR1/LGO, and SMR2 cooperate to promote endoreplication during leaf development. The three single mutants affected endoreplication levels only marginally, but the degree of endoreplication was greatly reduced in the triple mutant (Figure 9B; Supplemental Figure 5). Only the smr2 mutant showed an increase in leaf size (Figure 7) and leaf cell number (Figure 8C), due to a prolongation of cell division in the mutant at day 10-12 (Figure 8D), approximately the time when endoreplication is initiated in wild-type leaves (Beemster et al., 2005). Although endoreplication in smr2 mutants lagged behind the wild type initially, it ultimately reached the same level (Figures 9A and 9B).

Both SMR1/LGO and SMR2 could complement the sim trichome phenotype (Figure 3C, Table 1; Supplemental Figures 1A and 1G), indicating that these three SMRs encode similar functions. Although neither the sim nor the smr1/lgo single mutants affected leaf size, the sim smr1/lgo double as well as the sim smr1/lgo smr2 triple mutant all exhibited both increased leaf size and cell number (Figures 10A and 10B), indicating that sim and smr1/lgo play overlapping roles in limiting cell proliferation and promoting endoreplication in the leaf. The differences in phenotype observed among the

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**Table 2. Both CYCD3 and CDKB1 Are Necessary for Cell Division in sim Mutant Trichomes**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Nuclei per TIS</th>
<th>Number of TIS</th>
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<tbody>
<tr>
<td>Col-0</td>
<td>1.0 ± 0.00</td>
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<tr>
<td>sim</td>
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The multicellular trichome phenotype was assessed by counting the number of DAPI-stained trichome nuclei at each trichome initiation site (TIS) for each genotype. Results are presented for two different experiments, one testing the effect of cycd3 mutants on the sim phenotype and the other testing the effect of the cdkb1 mutants on the sim phenotype. The designation cycd3:1-3 indicates plants mutant for all three cycd3 genes.

a In a two-tailed t test, sim cycd3:1-3 differed from sim with P < 0.00001.

b In a two-tailed t test, sim cdkb1;1 cdkb1;2 differed from sim with P < 0.00001.
mutants may be due to differences in the timing of their expression during leaf development. Previously published work shows that while smr2 and sim are most highly expressed early in leaf development, when cell division predominates, smr1/lgo expression is low early in leaf development and increases steadily over the course of leaf development (Beemster et al., 2005). Thus, the transition from mitotic divisions to endoreplication during leaf development may be regulated by the overall level of SMR activity contributed by these three genes over the course of leaf development, with SMR2 expression predominating during the initial transition from proliferation to endoreplication and SMR1 playing a larger role in suppressing proliferation and maintaining endoreplication later in leaf development.

SIM Interacts with and Inhibits Both CDKA;1 and CDKB1;1

In spite of earlier results suggesting that SIM specifically interacts only with either CDKA;1 (Churchman et al., 2006) or CBKB1;1 (Van Leene et al., 2010), the results presented here suggest that SIM interacts with (Figure 4A) and inhibits the activity of (Figures 5A to 5C) both CDKA;1 complexes and CDKB1;1 complexes. Furthermore, SIM was capable of inhibiting the kinase activity of both CYCD3;1-containing and CYCD2;1-containing CDKA;1 complexes (Figures 5A and 5B). The epistasis of cycd3 and cdkb1 mutants to sim shows that cell division in sim mutant trichomes depends upon both CYCD3 and CDKB1 function. It is important to note that while CYCD3;1 can activate kinase activity of CDKA;1, it appears to be incapable of activating CDKB1;1 (Nowack et al., 2012). Thus, this genetic result is consistent with the implications of our interaction and inhibition studies, suggesting that both CYCD3;1/CDKA;1 and CDKB1-containing complexes may be direct targets of SIM inhibition in vivo.

That SIM is capable of inhibiting such a broad range of CDK complexes and depending on their level of expression can inhibit mitosis only, triggering endoreplication (Verkest et al., 2005; Weinl et al., 2005), can reduce both mitosis and endoreplication (De Veylder et al., 2001), or can completely block entry into S-phase when expressed in trichomes from the GL2 promoter, ultimately resulting in cell death (Schnittger et al., 2003). None of our SIM or SMR overexpression lines showed any sign of reduced endoreplication in trichomes or trichome cell death, even though we used the same promoter that was used for overexpression of KRP s in trichomes that triggered cell death (Schnittger et al., 2003). Posttranscriptional regulation by cell cycle stage-specific phosphorylation or protein degradation, limiting the CDK inhibitory activity of SIM to G2/M, provides a possible explanation for the inability to cause cell death, in spite of its ability to inhibit both G1/S and G2/M CDKs.

The involvement of D3-type cyclins in the regulation of mitosis in this context is particularly intriguing. CYCD3s have generally been considered to be G1/S cyclins. In vitro, CYCD3;1 can activate the kinase activity of CDKA;1, considered to be the main G1/S CDK, and not the kinase activity of the mitotic CDK CDKB1;1, and CYCD3;1/CDKA;1 complexes can phosphorylate RBR, the gatekeeper of the G1/S checkpoint (Nowack et al., 2012). In cell culture, CYCD3;1 overexpression promotes the G1/S transition, and cells accumulate in G2, consistent with a primary role in regulating entry into S-phase (Menges et al., 2006). In support of a role for CYCD3/CDKA;1 complexes in initiating the G2/M program, within 6 h of induction of CYCD3;1 in Arabidopsis seedlings, expression of a suite of genes directly related to mitosis is significantly upregulated (de Jager et al., 2009). These mitotic genes are not upregulated by induced expression of either E2Fa or E2Fc, key transcription factors downstream from RBR in the G1/S checkpoint. Elimination of CYCD3 function in a triple mutant defective for all three D3-type cyclin genes (the same triple mutant used in this study) results in increased endoreplication, indicating that wild-type CYCD3s suppress endoreplication and promote division in the leaf (Dewitte et al., 2007). Finally, and most telling for our work, ectopic
Figure 8. Kinematic Analysis of Wild-Type (Col-0) and smr2-1 Leaves Indicates That SMR2 Restricts Cell Proliferation.

(A) Leaf area. Inset shows same data on a linear scale.
(B) Leaf expansion rate.
(C) Cell number.
(D) Cell division rate.
(E) Cell area.
(F) Stomatal index.

For (A) and (B), n = 4 to 28, with larger sample sizes for the earlier developmental time points. For (C) to (F), n = 3 to 6. All error bars indicate se.
expression of CYCD3;1 in developing trichomes causes the trichomes to divide, phenocopying the sim mutant phenotype (Schnittger et al., 2002). Recent work indicates that CYCD3 is part of a complex web of interactions involving RBR and multiple E2F transcription factors that control the balance between cell division and endoreplication (Magyar et al., 2012, 2013). Other D-type cyclin-containing CDK complexes may also be targets of inhibition by SIM during the establishment of endoreplication in developing trichomes, as indicated by the ability of SIM to inhibit CYCD2;1/CDKA;1 complexes in vitro (Figure 5B). Although ectopic expression of CYCD2;1 in trichomes was reported to have no effect on trichome development (Schnittger et al., 2002), the CYCD2;1 cDNA used in that study is known to undergo an aberrant splicing event resulting in a nonfunctional protein, whereas expression of genomic CYCD2;1 reduces the duration of S-phase, causes division at smaller cell size, and limits the degree of endoreplication (Qi and John, 2007). The in vivo significance of inhibition of CYCD2;1/CDKA;1 complexes

Figure 9. Endoreplication Is Delayed in an smr2 Mutant and Greatly Reduced in a sim smr1/lgo smr2 Triple Mutant.

(A) Nuclear DNA ploidy of cells of first leaf pairs was assessed by flow cytometry at the indicated times after sowing for Col-0 and smr2-1. n = 3, and error bars represent se.

(B) Ploidy of mature first leaf pairs was assessed by flow cytometry for the indicated genotypes. Data are expressed as endoreplication indices, the average number of replication cycles above 2C. Asterisk indicates that the ploidy of sim smr1 smr2 differs from Col-0 (P < 0.0001) as determined by a pairwise two-tailed t test. n = 3, and error bars indicate se.
The overall P values listed in parentheses were determined by ANOVA. The Col-0 ecotype of Arabidopsis thaliana was used as the wild-type control for all experiments. The sim-1 allele has been previously described (Churchman et al., 2006). The cdkb1;1-1 (SALK_073457), cdkb1;2-1 (SALK_133560), ccd3;1-1 (SET_4061), ccd3;2-1 (SALK_3580), and ccd3;3-1 (N74667) mutants have all been described previously (Devitte et al., 2007; Nowack et al., 2012). The cyclin D3 triple mutant ccd3;1-3 was a kind gift from Walter Devitte and James Murray (University of Cambridge, UK) (Devitte et al., 2007). The T-DNA insertions in smr1 (SALK_033905) and smr2 (SALK_124828C and SALK_006096C) were obtained from the NASC. All T-DNA and Ds insertion genotypes were confirmed by PCR using the primers specific for the wild-type allele and for the T-DNA allele, using primers described in Supplemental Table 2. The sim-1 allele was also genotyped via PCR. The sim-1 allele changes the start codon to ATG (Churchman et al., 2006), creating a new BglII restriction site. Unfortunately, another BglII restriction site is located only 22 bp away from this new site. Therefore, primers were designed such that a 180-bp PCR fragment was produced from both wild-type and mutant DNA, and the preexisting BglII restriction site was destroyed by a mismatch in one of the primers (Supplemental Table 2). Digestion of the PCR product from the mutant allele with BglII results in two fragments of 144 and 36 bp, while the PCR product of the wild-type allele is not cleaved by the enzyme.

For complementation experiments, crosses, and most other experiments, plants were grown on soil as previously described (Larkin et al., 1999). For kinematic analysis of leaf growth, plants were grown for 26 d as follows: Seeds were sterilized in 70% ethanol for 1 min and in 50% bleach for 10 min then rinsed four times with deionized water. The seeds were sown on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% plant tissue culture agar. At 2 DAS at 4°C, the plates were placed horizontally on cooled benches in a growth chamber kept at 22°C under long-day conditions (16 h of light/8 h of darkness, 80 to 90 µmol m⁻² s⁻¹ PAR, supplied by fluorescent tubes; Osram Lumilux cool white).

Sequence Collection

We performed BLAST and PSI-BLAST searches among all land plant and algal genomes in the GenBank Refseq (http://www.ncbi.nlm.nih.gov/), Phytozome (http://www.phytozome.net), and PLAZA (http://bioinformatics.psb.ugent.be/plaza/) databases for putative SMRs using the known Arabidopsis SMR genes. Using custom scripts, we filtered the data set to give a unique nonredundant set. Based on their taxonomic diversity, we selected 74 putative SMRs from seven plant genomes for further analyses. The Illumina reads for the RNA-seq data are from Oh et al. (2014) and are deposited at the NCBI-SRA database under accession number SRX877897 (PMID:24563282).

Multiple Sequence Alignment and Phylogenetic Analysis

We performed multiple sequence alignment initially with Clustal Omega (Sievers et al., 2011) and further improved it using Rascal v1.34 (Thompson et al., 2003). The final alignment is available as Supplemental Data Set 1. A neighbor-joining tree was constructed using MEGA 6 (Tamura et al., 2013) with 74 unique sequences. The distance matrix from the pairwise sequence comparisons was used to create the distribution for the principal component analysis. The evolutionary distances were computed using p-distance and pairwise deletions. All ambiguous positions were removed for each sequence pair. Bootstrap analysis was performed with 1000 replicates.

by SIM remains an open question. Nonetheless, the results presented here point directly toward both CYCD3/CDKA;1 and CDKB1-containing complexes as being important targets of inhibition by SIM and other SMRs to inhibit mitosis and promote endoreplication, although substantial work remains to be done before we understand the control of this crucial developmental transition. Furthermore, the biochemical function of SMRs appears to have been highly conserved in land plant evolution, despite significant sequence divergence.
Generation of Transgenic Lines

Coding regions of SMRs were synthesized by Integrated DNA Technologies, PCR amplified with appropriate primers (Supplemental Table 2), and inserted into the vector (pENTR-D-TOPO) using a pENTR Directional TOPO Cloning Kit (Life Technologies). Error-free entry clones were confirmed by sequence analysis. An LR clonase reaction was performed to insert genes into the Gateway binary T-DNA destination vector pAMPAT-PROGL2, which contains the GL2 promoter (Weinl et al., 2005). The resulting SMR expression constructs were introduced into Agrobacterium tumefaciens strain GV3101 pMP90RK by electroporation and subsequently used to transform sim-1 homozygous mutant Arabidopsis plants via the floral dip method (Clough and Bent, 1998). Seeds were planted on soil and transgenic plants were selected with 1 mM BASTA spray. Complementation of the sim trichome phenotype was initially scored in T1 generation transgenic plants, and 12 to 18 primary transformants per construct were screened for segregation of a single BASTA-resistant insert in the T2 generation. The three most strongly complementing single insert lines were used to produce homozygous T3 lines. In all complementation experiments, the plants were confirmed to be sim-1 homozygotes as described above.

Microscopy

For scanning electron microscopy, first leaves of 2-week-old Arabidopsis plants were mounted on the specimen stubs using double-sided tape and observed under high vacuum mode at 5.0 kV in a JEOL JSM 6610LV scanning electron microscope, working quickly to avoid drying and damage from the beam.

Light microscopy was performed with a Leica DM RXA2 light microscope equipped with differential interference contrast and epifluorescence optics, using either the 100× or the 200× objective. Images were captured using a SensiCam QE 12-bit, cooled CCD camera and analyzed with Slidebook software from 3i. Nuclei per trichome initiation site were counted using first leaves stained with 4',6-diamidino-2-phenylindole (DAPI), as described previously (Walker et al., 2000).

Split-Luciferase Assays

The dual expression series vectors pDuEx-AN6, pDuEx-DN6, pDuEx-AC6, and pDuEx-DC6 (Fujikawa and Kato, 2007), which carry sequences encoding either the N terminus (amino acids 1 to 229, Nluc) or the C terminus (amino acids 230 to 311, Cluc) of Renilla reniformis luciferase, were used for the split-luciferase complementation assays. SIM and CDKs were introduced into their respective vectors by Gateway cloning (Life Technologies). Proper orientation and correct sequence of the inserts in all constructs was confirmed by sequence analysis. The assays were performed in 96-well plates. Plasmids carrying coding regions of proteins to be tested were introduced into protoplasts using polyethylene glycol-mediated transfection and incubated overnight at room temperature (Fujikawa and Kato, 2007). After 14 to 18 h incubation, a coelenterazine derivative, ViviREN Live Cell substrate (Promega), was added to the protoplasts, and luminescence was detected with a Vertocam microplate luminometer as described previously (Fujikawa and Kato, 2007).

CDK Kinase Assays

The coding regions of At-SIM and Pp-SMR12 were inserted into the destination vector pHGGWA, a HIS-GST-tag expression vector (Busso et al., 2005) via a Gateway (Life Technologies) recombination reaction. The entry clone pDONR221-GFP(S65T) was obtained from Akira Iwase, and the GFP insert from this clone was inserted into pHGGWA for expression of the negative control His:GST:GFP fusion protein. Error-free destination clones were confirmed by sequence analysis. To express His:GST-fused proteins, Escherichia coli SoluBL21 cells (AMS Biotechnology) were transformed with the resulting destination clones. E. coli cells were grown in Luria-Bertani medium containing 100 mg/L ampicillin at 37°C until OD600 = 0.6 and the production of the fusion protein was induced by adding 0.3 mM isopropyl β-D-thiogalactopyranoside overnight at 18°C. Cells were harvested by centrifugation and resuspended in Ni-NTA binding buffer (50 mM NaH2PO4, 100 mM NaCl, 10% [v/v] glycerol, and 25 mM imidazole, pH 8.0) containing protease inhibitors (Complete EDTA-free; Roche) and lysed by sonication (Digital Sonifier 450D; Branson). After addition of Triton X-100 to 0.2% (w/v), the cell slurry was incubated at 4°C and clarified by centrifugation. The supernatant was passed through a column packed with Ni-NTA agarose resins (Qiagen), which was washed sequentially with Ni-NTA binding buffer and eluted with Ni-NTA elution buffer (Ni-NTA binding buffer containing 200 mM imidazole), and the buffer was exchanged to kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM EGTA) with a PD-10 column (GE Healthcare). After each complex was concentrated with a Vivaspin 2 (Sartorius), the concentration of proteins was calculated using BSA as a standard.

After CDK complexes were expressed and purified as described previously (Harashima and Schnitter, 2012), ATP was added to 2 mM, and the complexes were incubated for 1 h at 30°C. The reaction was then further purified with a column packed with Strept-Tactin sepharose resins (IBA), which had been equilibrated with kinase buffer. CDK complexes were eluted with kinase buffer containing 2.5 mM desthiobiotin. After measuring the concentration of the complexes with Bradford reagent (Sigma-Alrich) using BSA as a standard, the aliquoted complexes were frozen in the liquid nitrogen and stored at −80°C until use.

The kinase assays were performed 20-μL reactions containing 15 nM kinase, 2 μg histone H1° (NEB) as a substrate, 92.5 kBq [γ-32P]ATP (Perkin-Elmer), and 150 nM, 750 nM, or 1.5 μM of At-SIM or Pp-SMR12 protein where indicated. To assay for the inhibitory activity of SIM, purified His:GST-SIM, His:GST-PpSMR1, or His:GST-GFP fusion proteins were added to the kinase reactions before the addition of the substrate.

After incubation for 30 min at 30°C, kinase reactions were stopped by adding Laemmli sample buffer (Bio-Rad) and boiled. Samples were separated on 12% TGX gels (Bio-Rad), and after the gels were stained with BioSafe Coomassie G-250 Stain (Bio-Rad), they were dried with HydroTech Gel Drying System (Bio-Rad). Radioactive histone H1 proteins were detected using a Typhoon FLA-7000 system (GE Healthcare).

Leaf Growth Analysis

Growth analysis was performed on the first leaf pair. Leaves were fixed and cleared with 70% ethanol for 24 h and subsequently in 100% lactic acid, which was also used as a mounting agent. Leaf images were obtained with a Nikon Eclipse E600. Leaf samples of later days were cleared with 70% ethanol for 24 h and subsequently in 100% lactic acid, then stained with propidium iodide (Wuyts et al., 2010), and cell measurements were used for locally fitting a five-point quadratic function (Ericksen, 1976) of which the first derivative was used to determine the relative expansion rate.

Cellular Measurements

We performed a kinematic analysis (De Veylder et al., 2001) of the abaxial epidermal cells of three to six average leaves from 6 to 26 DAS as described previously (Nelissen et al., 2013). Leaves of early days, i.e., 6 to 10 DAS, were stained with propidium iodide (Wuyts et al., 2010), and cell images were obtained with a Nikon C1 confocal microscope using a Nikon Eclipse E600. Leaf samples of later days were cleared with 70%
Flow Cytometric Analysis

The first leaf pair of the wild type and mutants was harvested from 9 to 26 DAS, frozen in liquid nitrogen, and kept at −80°C until analysis. For flow cytometry analysis, nuclei were extracted by chopping 3 to 30 leaves with a razor blade in 200 μL Cystain UV Precise P Nuclei extraction buffer (Partec), supplemented with 800 μL staining buffer. The mix was filtered through a 50-μm filter and read through the Cyflow MB flow cytometer (Partec). The nuclei were analyzed with the CyFlow flow cytometer and the FloMax software (Partec).

Accession Numbers

Accession numbers for all SMR genes and gene ID numbers for all Arabidopsis SMR genes described or referred to in this study can be found in Supplemental Data Set 2.

Supplemental Data

Supplemental Figure 1. Representative fields of trichomes from complementation experiments.

Supplemental Figure 2. SMR5 (At1g07500) does not contain an intron annotated in TAIR10.

Supplemental Figure 3. The number of SMR genes detected in the genomes of various land plant species.

Supplemental Figure 4. Cluc:SIM and NLuc:CDKA;1 give the strongest interaction signal for testing SIM-CDK interactions in split-luciferase complementation assays.

Supplemental Figure 5. Quantification of nuclear DNA content of mature Col-0 and sim smr1 smr2 triple mutant mature leaves by flow cytometry.

Supplemental Figure 6. Differential interference contrast microscopy images of the abaxial epidermis of Columbia wild type, smr2, sim smr1, and sim smr1 smr2.

Supplemental Table 1. Frequency of completion of sim by various SMRs in independent transgenic lines is similar to the frequency of completion of sim by SIM.

Supplemental Table 2. Synthetic DNA primers used for ORF amplification or genotyping.

Supplemental Data Set 1. Text file of the alignment used for the phylogenetic analysis in Figure 2.

Supplemental Data Set 2. Accession numbers of SMR genes.

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