A conserved but plant-specific CDK-mediated regulation of DNA replication protein A2 in the precise control of stomatal terminal division

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The R2R3-MYB transcription factor FOUR LIPS (FLP) controls the stomatal terminal division through transcriptional repression of the cell cycle genes *CYCLIN-DEPENDENT KINASE* (*CDK*) *B1s* (*CDKB1s*), *CDKA;1*, and *CYCLIN A2s* (*CYCA2s*). We mutagenized the weak mutant allele *flp-1* seeds with ethylmethane sulfonate and screened out a *flp-1* suppressor 1 (*fsp1*) that suppressed the *flp-1* stomatal cluster phenotype. *FSP1* encodes RPA2a subunit of Replication Protein A (RPA) complexes that play important roles in DNA replication, recombination, and repair. Here, we show that *FSP1/RPA2a* functions together with *CDKB1s* and *CYCA2s* in restricting stomatal precursor proliferation, ensuring the stomatal terminal division and maintaining a normal guard-cell size and DNA content. Furthermore, we provide direct evidence for the existence of an evolutionarily conserved, but plant-specific, CDK-mediated RPA regulatory pathway. Serine-11 and Serine-21 at the N terminus of RPA2a are CDK phosphorylation target residues. The expression of the phosphorylation-mimic variant *RPA2a*$^{S11,21/D}$ partially complemented the defective cell division and DNA damage hypersensitivity in *cdkb1;1 1;2* mutants. Thus, our study provides a mechanistic understanding of the CDK-mediated phosphorylation of RPA in the precise control of cell cycle and DNA repair in plants.

stomatal development | cell division | replication protein A | CDK | DNA damage

Stomata are plant-specific epidermal structures that consist of a pair of Guard Cells (GCs) surrounding a pore. The formation of stomata requires successive asymmetric cell division of the precursor cells, including the Meristemoid Mother Cell (MMC) and the Meristemoid (M), and one symmetric division of the Guard Mother Cell (GMC) to produce two GCs (1). *FOUR LIPS* (*FLP*) and *MYB88* encode R2R3-MYB transcription factors and function in the regulation of symmetric division of the GMCs. In the weak allele *flp-1*, two stomata form abnormally in direct contact. The loss of *MYB88* function dramatically enhances the phenotype of *flp* mutants, leading to tumor-like stomatal clusters (2, 3). The cell cycle genes *CYCLIN-DEPENDENT KINASE* (*CDK*) *B1s* (*CDKB1s*), *CYCLIN A2s* (*CYCA2s*), and *CDKA;1*, as transcriptional targets, are directly suppressed by FLP and MYB88 (4–6).

Replication Protein A (RPA) is a heterotrimeric single-stranded DNA (ssDNA)-binding protein complex that is required for multiple aspects of DNA metabolism, including DNA replication, recombination, repair, and telomere maintenance (7). The homologs of each of the three RPA subunits (RPA1-3) are well conserved in eukaryotes. In humans, phosphorylation of RPA2 at the N-terminal domain is required for the RPA-ssDNA interaction. In mitotic cells, Serine-23 and Serine-29 at the RPA2 N terminus are phosphorylated and activated by Cdc2/CDK to promote DNA replication (8–12). Under a DNA-damaging condition, RPA2 is hyperphosphorylated by the PIKK-family kinases (ATM, ATR, and DNA-PK) that facilitates mitotic exit and the initiation of DNA repair (13–15). All known RPA2 homologs have a conserved N-terminal phosphorylation domain, although the specific residues may be not conserved in different species (11). In contrast to yeast and most mammals, plants carry multiple paralogs for each of the RPA subunit (16). For example, rice has 3 RPA1s, 3 RPA2s, and 1 RPA3 (16, 17). The model plant *Arabidopsis* has 5 RPA1s (RPA1a–e), 2 RPA2s (RPA2a, b), and 2 RPA3s (RPA3a, b). Phylogenetic analysis of the RPA1 sequences suggests that *Arabidopsis* RPA1s diverged into 2 subgroups, the ACE-group (RPA1a, b, c) and the BD-group (RPA1b, d) (18). Previously, genetic analysis confirmed that *RPA2a* plays a critical role in the maintenance of epigenetic gene silencing in plants and abiotic stresses (19–21).

In this study, with an aim of obtaining genetic suppressors of *flp-1*, we identified a genetic mutation in *Arabidopsis RPA2a* that led to inhibited stomatal clustering in *flp-1* and arrested GMC divisions. Our study discovered the existence of an evolutionarily conserved, but plant-specific, CDK-mediating RPA regulatory pathway. Also, by assaying the stomatal development and DNA

Significance

The *Arabidopsis* R2R3-MYB transcription factor FOUR LIPS (FLP) is the first identified key transcription factor regulating stomatal development. By screening and analyzing a genetic suppressor of *flp* stomatal defects, we found that *FSP1/RPA2a*, which encodes a core subunit of Replication Protein A (RPA) complexes, acts downstream of B1-type Cyclin-Dependent Kinases (CDKB1s). This ensures that terminal division during stomatal development will produce a pair of kidney-shaped guard cells to compose a functional stomatal complex. We demonstrate that the CDK-mediated phosphorylation at the N terminus of RPA2a is essential for the RPA functions in cell cycle control and response to DNA damage. We provide direct evidence for the existence of an evolutionarily conserved, but plant-specific, RPA regulatory pathway in plants.

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damage responses, we established the physical and genetic interactions between the RPA and CDKs in the precise control of the cell cycle as well DNA repair in plants.

Results

Isolation of *fsp1*, a Suppressor of *flp-1* in Stomatal Development. The *flp-1* mutant is featured by extra terminal divisions during stomatal development, suggesting the role of FLP in restricting cell division (3). To identify new genetic players in regulating the one-time terminal division, we created an ethylmethane sulfonate-mutagenized M2 population of *flp-1* mutants and screened for mutants with altered stomatal phenotypes. *flp-1 fsp1* (*flp-1 s*uppressor1) was isolated for significantly reduced stomatal clusters, compared to *flp-1* (Fig. 1 *A*–*C* and *SI Appendix*, Table S1). In addition, aberrant cells were occasionally found in the epidermis of the *flp-1 fsp1* double mutant (Fig. 1*C*). Using the GC fate marker E1728, we confirmed that the aberrantly shaped single cells in *fsp1* have the GC identity (Fig. 1 *D*, *Inset*), resembling the Single Guard Cell (SGC) phenotype in *cdkb1;1 1;2* and *cyca2s* mutants (4, 5). The Stomatal Index (SI) of the *fsp1* mutant was reduced as well, indicating that *FSP1* promotes stomatal production (*SI Appendix*, Table S2).

***FSP1* Encodes the *Arabidopsis* RPA2a Subunit of the RPA Complex.** Using map-based cloning, we determined that mutation of the *FSP1* gene was located within the bacterial artificial chromosome clone T28124 on chromosome 2. Based on growth defects reminiscent of *rpa2a*/*ror1-1* mutants, such as dwarf seedlings, narrow leaves, and early flowering (*SI Appendix*, Fig. S1*A*) (19, 20), we amplified and sequenced the open reading frames of *RPA2a* from *fsp1* and found that *fsp1* possessed the same point mutation as in *ror1-2*, ag (1343) changed to aa, which induces altered splicing events (Fig. 1*E*). The expression of *RPA2a* (cDNA) fused with GFP under the control of the native promoter fully rescued the stomatal defects of *fsp1* mutants (Fig. 1*F* and *SI Appendix*, Table S2). The functionality of *RPA2a* was further confirmed by the reappearance of *flp-1*–featured stomatal clusters in *flp-1 fsp1* mutants carrying *RPA2a:RPA2a-GFP* (Fig. 1*G* and *SI Appendix*, Table S1). In addition, the introgression of the *fsp1* mutation in *flp-1 myb88* double mutants largely repressed the size and number of stomatal clusters (Fig. 1 *H* and *I* and *SI Appendix*, Fig. S1*B*). We also revisited the T-DNA allele *rpa2-5*/*ror1-3* (Fig. 1*E* and *SI Appendix*, Fig. S1*A*) (19, 20), which displayed a similar stomatal phenotype as *fsp1*. The *flp-1* stomatal phenotype was suppressed in *flp-1 rpa2-5* double mutants (*SI Appendix*, Fig. S1 *C* and *D* and Table S2). Benefiting from easy genotyping, *rpa2-5* was then used in most of the later experiments (renamed as *rpa2a-5*).

***RPA2a* Is Expressed in Specific Stomatal Lineage Stages.** A complemented *ror1-1* transgenic line harboring *ROR1:gROR1-GUS-GFP* (19) (here referred to as *RPA2a-GFP*) was used to investigate the RPA2a expression pattern and localization. RPA2a-GFP fluorescent signals were observed in a subset of stomatal lineage cells and compared with those of three translational reporters of *SPEECHLESS* (*SPCH*), *MUTE*, and *FLP*, which have distinct and sequential expression patterns during stomatal development (Fig. 1*J*). The expression of RPA2a-GFP overlapped with *SPCH-GFP* (22) in MMCs prior to asymmetric entry divisions but not in both newly formed meristemoids (M) and stomatal lineage ground cells after division. In late meristemoids and early GMCs (EGMCs), where MUTE-GFP was turned on (23), diffuse RPA2a-GFP signals reappeared. RPA2a-GFP persisted in late GMCs (LGMCs), but disappeared in young GCs (YGCs) after the terminal symmetric cell division. The expression of *RPA2a* overlapped with *FLP-GFP* (3, 24) only at the LGMC stage, prior to the GMC division, but not in YGCs. Taken together, *RPA2a* shows a cell type- and time-specific expression profile, with the preferences in the precursor cells (actively dividing) prior to either asymmetric or symmetric divisions during stomatal development.

***RPA2a* Functions Together with FLP Downstream Target Genes *CDKB1s* and *CYCA2s*.** The *CDKB1;1* gene is one of the direct transcriptional targets of FLP in controlling stomatal terminal symmetric divisions. In agreement with this, expression of *CDKB1;1-GFP* (24) overlaps with *FLP-GFP* in LGMCs and YGCs and partially with *RPA2a-GFP* in LGMCs (Fig. 1*J*). In comparison to SGC frequencies of 42.0 ± 2.6% in *cdkb1;1 1;2* and 8.0 ± 2.1% in *rpa2a-5*, the *rpa2a-5 cdkb1;1 1;2* triple mutant produced dramatically more SGCs (64.0 ± 1.6%, Fig. 2 *A*–*D*). GMC divisions also require *CYCA2* activity (5). While SGCs in *cyca2;3* mutants were found at a low frequency of 4.0 ± 0.8% (Fig. 2*E*), the occurrence of SGCs in *rpa2a-5 cyca2;3* double mutants was elevated to 47.0 ± 2.0% (Fig. 2*F*). Consistently, the frequency of SGCs in *cyca2;3 2;4* (27.6 ± 3.4%) was greatly elevated to 78.0 ± 8.5% in *rpa2a-5 cyca2;3 2;4* triple mutants

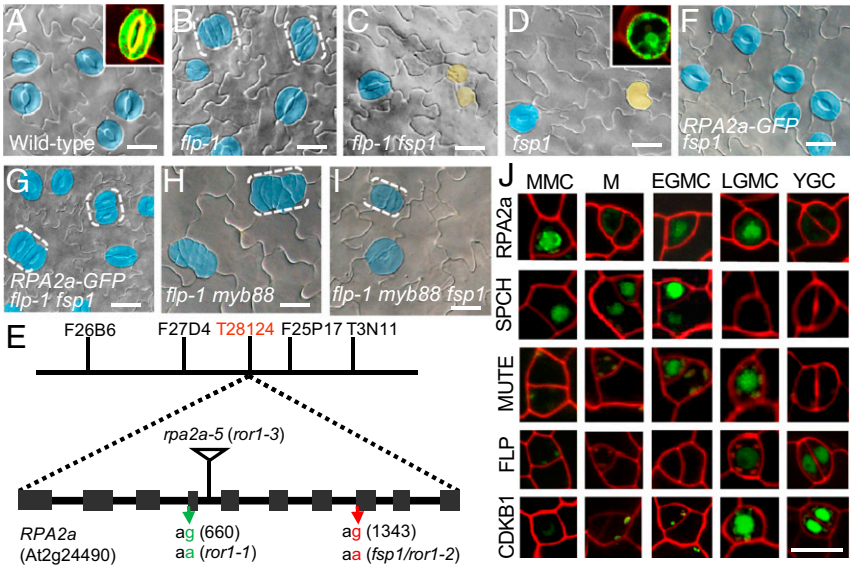

Fig. 1. *FSP1* encodes the RPA2a subunit required for stomatal precursor cell divisions. (*A*–*D*) Differential interference contrast images of epidermis of wild-type (*A*), *flp-1* (*B*), *flp-1 fsp1*(*C*), and *fsp1* (*D*). Brackets indicate the stomatal clusters. SGCs are highlighted with yellow color and normal GCs with blue color. (*Inset* in *A* and *D*) Expression of the mature GC marker E1728 in a SGC. (*E*) Map-based isolation and gene structure of *FSP1*. The mutation site in *fsp1* is the same as *ror1-2*. *rpa2a-5* (*ror1-3*) is a T-DNA insertion line. (*F* and *G*) *RPA2a:RPA2a-GFP* rescued the *fsp1* SGC phenotype (*F*) and induced the reappearance of *flp-1* clusters in *flp-1 fsp1* (*G*). (*H* and *I*) *flp-1 myb88* cluster numbers and size are repressed by *fsp1* mutation. (*J*) *RPA2a-GFP* expression profile in the stomatal linage cells. (Scale bars, 20 μm.)

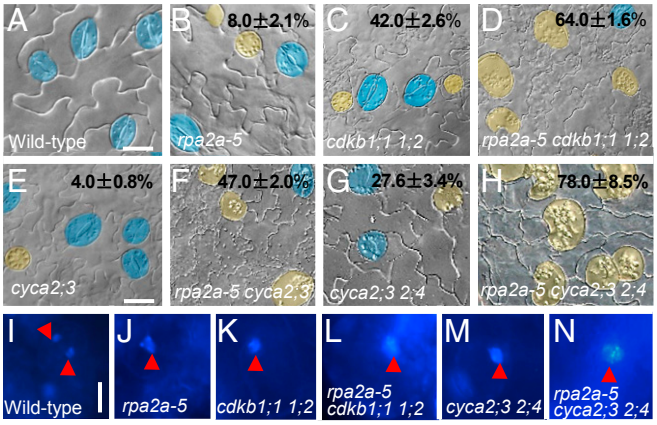

Fig. 2. RPA2a functions synergistically with CYCA2s and CDKB1s in GMC division. (*A–G*) Micrographs of epidermis of wild-type (*A*), *rpa2a-5* (*B*), *cdkb1;1 1;2* (*C*), *rpa2a-5 cdkb1;1 1;2* (*D*), *cyca2;3* (*E*), *rpa2a-5 cyca2;3* (*F*), *cyca2;3 2;4* (*G*), and *rpa2a-5 cyca2;3 2;4* (*H*). SGCs are highlighted with yellow color and normal GCs with blue color. The numbers at the right-upper corner indicate the percentage of SGCs in total stomata. (*I–N*) DAPI fluorescence micrographs. Red arrowheads indicate the nuclei. (Scale bar, 20 μm.)

(Fig. 2 *G* and *H*), indicating that interaction between RPA2a and CDKB1s/CYCA2s functions in GMC divisions.

An ectopic and prolonged expression of RPA2a-GFP was occasionally found in two daughter cells after a GMC division in *flp-1*, suggesting that RPA2a is required for subsequent GMC divisions that end up with two stomata next to each other (*SI Appendix*, Fig. S2 *A–H*). *RPA2a* transcription level slightly but not significantly increased in *flp-1 myb88* (*SI Appendix*, Fig. S2*I*). Although 12 putative FLP/MYB88 binding sequences are present within the *RPA2a* promoter (4–6), neither FLP nor MYB88 showed clear binding activity to *RPA2a* promoter according to the results of yeast one-hybrid assays (*SI Appendix*, Fig. S2 *J* and *K*), indicating that *RPA2a* might not be a direct transcriptional target of FLP/MYB88.

Combined Loss of *RPA2a* and *CDKB1s/CYCA2s* Function Induces Abnormal Cell Enlargement and Endoreduplication. Differing from the interlocking, continuously expanding pavement cells, the mature stomatal GCs, once matured, retain their shape and size for the life time in leaves. The area of the paired GCs ranged mostly from 200 to 500 μm^2 in *Arabidopsis* (*SI Appendix*, Fig. S3). However, about 45 and 71% stomata in *rpa2a-5 cdkb1;1 1;2* and *rpa2a-5 cyca2;3 2;4* triple mutants, respectively, are larger than 500 μm^2 (Fig. 2 *A–H* and *SI Appendix*, Fig. S3). It has been widely accepted that cell size and differentiation are closely correlated to the nuclear DNA levels (25). We therefore measured the nuclear DNA levels in SGCs after DAPI (4′,6-diamidino-2-phenylindole) staining. The DNA content in wild-type GCs was defined as 2C-DNA level. Our results showed that SGCs in the *rpa2a-5*, *cdkb1;1 1;2*, and *cyca2;3 2;4* mutants all displayed a similar DAPI fluorescence intensity (4C-DNA level), indicating that *RPA2a*, similar to *CDKB1s* and *CYCA2s*, is required for the mitosis of GMC divisions. But in the *rpa2a-5 cdkb1;1 1;2* or *rpa2a-5 cyca2;3 2;4* triple mutants, the enlarged SGCs contained single giant nuclei with a mean DNA level of 6C-8C (Fig. 2 *I–N*). The analysis of DNA ploidy distribution in 9- and 14-d-old cotyledons using flow cytometry confirmed that RPA2a may coordinately act with CDKB1s/CYCA2s to restrict the onset of endocycle (*SI Appendix*, Fig. S4).

The above results indicated that the mitosis was blocked but the endoreplication was inappropriately unrestricted in the *rpa2a-5 cdkb1;1 1;2* triple mutants. In the *rpa2a-5* single mutant, the expression levels of the G1-to-S genes *CDT1*, *MCM2*, *ORC3*, and *PCNA1* were significantly increased compared to those in wild type. Most of these elevations, as well as *CDC6a*, *CCS52A*, *ORC1a*, *CDKA;1*, were additively enhanced in *rpa2a-5 cdkb1;1 1;2* triple mutants (*SI Appendix*, Fig. S5*A*). In addition, the expression levels of genes during endoreplication initiation, such as *E2Fa*, *E2Fc*, and *E2Ff*, were largely increased in the triple mutants (*SI Appendix*, Fig. S5*B*). These results demonstrated that RPA2a and CDKB1s act together to suppress the expression of endocycling genes that restrict endoreduplication.

RPA2a Function Is Conservatively Regulated by CDKs through Protein Phosphorylation. Supported by our genetic data above, we tested the hypothesis that RPA2a may directly interact with CDKs in regulating cell division and cell cycle. Results of Bimolecular Fluorescent Complementation (BiFC) assays using the tobacco transient expression system and of the Co-ImmunoPrecipitation (Co-IP) assay using the proteins that were extracted from the infiltrated tobacco leaves suggested that RPA2a directly interacts with CDKB1;1 and CDKA;1 (Fig. 3 *A–D* and *SI Appendix*, Fig. S6).

It was reported that coexpression of the RPA2 subunit with other RPA subunits could enhance the solubility and function of RPA protein complexes (26). When RPA2a-GST was coexpressed with RPA1b-HIS in *Escherichia coli*, RPA1b proteins failed to be pulled down by RPA2a. Only when the RPA3a was present, in which RPA2a-GST-RPA3a-S coexpressed with RPA1b-HIS, the whole RPA complex could be pulled down by GST beads, indicating that the preformation of the RPA2-RPA3 subcomplex is essential for the assembly of the RPA complex (*SI Appendix*, Fig. S7*A*). However, regardless of the presence of RPA3 and RPA1, RPA2a proteins could be phosphorylated

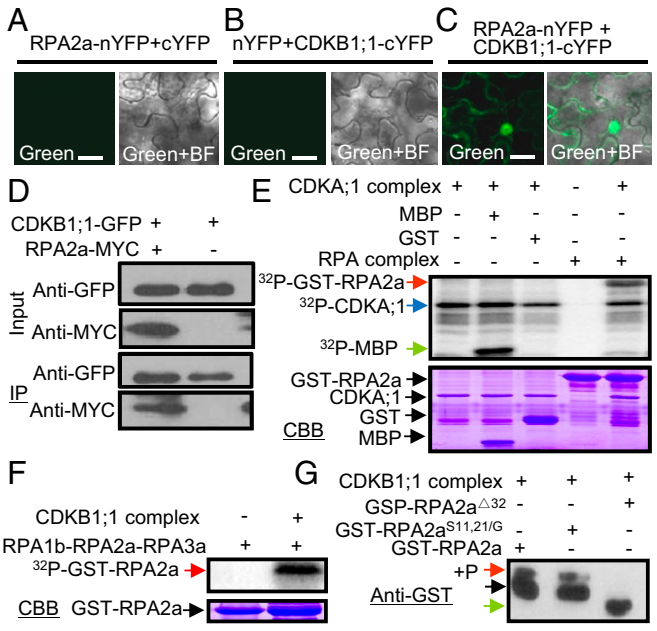

Fig. 3. Phosphorylation of RPA2a by CDK complexes. (*A–D*) The RPA2a protein binds CDKB1;1 protein in BiFC assays (*A–C*) and Co-IP assays (*D*). YFP, yellow fluorescent protein; BF, bright field. (Scale bars, 20 μm.) (*E*) In vitro phosphorylation assays. RPA2a is phosphorylated by the Cak1-CDKA;1-CYCD3;1 complex in the form of the RPA1b-RPA2a-RPA3a complex. (*F*) RPA2a is also phosphorylated by Cak1-CDKB1;1-CYCB1;2 complexes. The bottom box shows the loading controls after Coomassie Brilliant Blue (CBB) gel staining. MBP and GST proteins were used as positive and negative controls, respectively. Red arrows indicate the RPA2a phosphorylation band in phosphorylation autoradiographs. (*G*) Phosphorylation of RPA2a by Cak1-CDKB1;1-CYCB1;2 complexes is confirmed by finding a delayed band in 12.5% SDS/PAGE. The extent of phosphorylation of the RPA2a proteins was reduced when Ser-11 and Ser-21 were substituted or was barely detected when the N terminus was deleted.

in vitro by active CDK-CYCLIN protein complexes (27) (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S7*B*). Additionally, a faint delayed band next to the RPA2a-GST was observed when the products of the CDK phosphorylation reaction were separated on SDS/PAGE gels (Fig. 3*G*). Once alkaline phosphatases were added to the products, the delayed band disappeared, indicating that the phosphorylation is the cause of the slower mobility of RPA2a-GST proteins in gels (*SI Appendix*, Fig. S7*B*). Elimination of the N-terminal 32 amino acids abolished the delayed band, suggesting that this region contains the primary phosphorylation target sites (Fig. 3*G*).

To predict the CDK-phosphorylation target residues in RPA2a protein, the sequence of the N terminus of RPA2a protein was compared with those in human, yeast, and rice RPA2 proteins. Among the 12 Serine residues within the first 32 amino acids of the RPA2a N terminus, Ser-11 is the consensus CDK phosphorylation site, corresponding to Ser-23 of human RPA2 protein. Ser-21 is another predicted phosphorylation target site of CDKs (www.cbs.dtu.dk/services/) that may be conserved in plants (*SI Appendix*, Fig. S7 *C*–*F*). When Ser-11 and Ser-21 were substituted with nonphosphorylatable Glycines, the extent of the phosphorylated band was reduced (Fig. 3*G*), indicating that these two residues are predominant CDK-phosphorylation targets, but other sites in RPA2a might become phosphorylated in vitro as well.

To assess the function of phosphorylation sites in the RPA2a protein *in planta*, RPA2a variants were generated and introduced into the *rpa2a-5* mutant to evaluate their ability to rescue stomatal defects. *RPA2a-GFP* and individual variants with a single mutation, *RPA2a*$^{S11/G}$*-GFP*, and *RPA2a*$^{S21/G}$*-GFP* could fully rescue the stomatal production and GMC division defects of the *rpa2a-5* mutant (*SI Appendix*, Table S2). By contrast, further mutation of *RPA2a*$^{S11,21/G}$*-GFP* or the N-terminal deletion variant *RPA2a*$^{\triangle 32}$*-GFP* showed a reduced rescuing ability (Fig. 4 *A* and *B* and *SI Appendix*, Table S2). However, the phosphorylation-mimic variant *RPA2a:RPA2a*$^{S11,21/D}$*-GFP* (Ser-11 and Ser-21 were substituted with aspartic acids) rescued *rpa2a-5* GMC division defects (Fig. 4*C* and *SI Appendix*, Table S2).

We further examined how RPA2a phosphorylation variants perform in *flp-1 fsp1* double mutants. While the wild-type RPA2a-GFP fully complemented the loss function of *RPA2a* and restored the *flp-1*–like stomatal clustering, the expressions *RPA2a:RPA2a*$^{\triangle 32}$*-GFP* and *RPA2a:RPA2a*$^{S11,21/G}$*-GFP* led to a mild stomatal clustering phenotype (Fig. 4 *D* and *E* and *SI Appendix*, Table S1). *RPA2a*$^{S11/G}$*-GFP* and *RPA2a*$^{S21/G}$*-GFP* fully complemented the stomatal phenotype of *flp-1 fsp1* (*SI Appendix*, Table S1). Therefore, the phosphorylation status of Ser-11 and Ser-21 within the N-terminal 32 residues is critical for the full function of RPA2a.

We introduced *RPA2a-GFP*, *RPA2a:RPA2a*$^{S11/D}$*-GFP*, *RPA2a*$^{S21/D}$*-GFP*, and *RPA2a*$^{S11,21/D}$*-GFP* into *cdkb1;1 1;2* double mutants to assess the genetic interaction between *RPA2a* and *CDKB1s* in vivo. *RPA2a*$^{S11,21/D}$*-GFP* showed strong effects in reducing the formation of SGCs in *cdkb1;1 1;2*, while the remaining 3 transgenes had certain effects (Fig. 4 *F*–*H* and *SI Appendix*, Table S3). Therefore, these results suggest that the phosphorylation status of RPA2a is epistatic to *CDKB1* mutations.

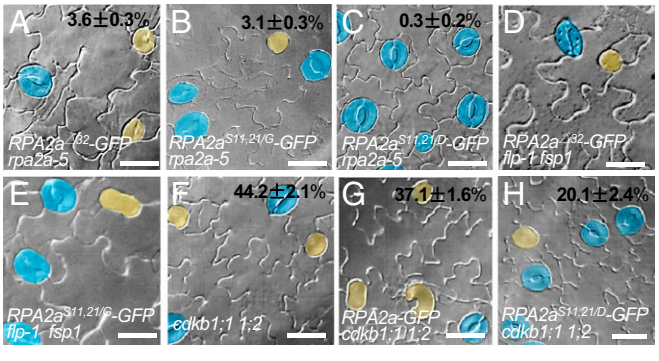

Fig. 4. Rescue stomatal phenotypes by *RPA2a* variants. (*A*–*C*) The formation of SGCs in the *rpa2a-5* mutant was partially rescued by the *RPA2a*$^{\triangle 32}$*-GFP* construct (*A*) or *RPA2a*$^{S11,21/G}$*-GFP* (*B*) and was mostly rescued by *RPA2a*$^{S11,21/D}$*-GFP* (*C*). (*D* and *E*) The formation of SGCs in *flp-1 fsp1* was barely affected by *RRPA2a*$^{\triangle 32}$*-GFP* (*D*) or *RPA2a*$^{S11,21/G}$*-GFP* (*E*). (*F*–*H*) The formation of SGCs in the *cdkb1;1 1;2* mutant is partially rescued by *RPA2a-GFP* (*G*), but is greatly repressed by *RPA2a*$^{S11,21/D}$*-GFP* (*H*). The numbers at the right-upper corner indicate the percentage of SGCs in total stomata. SGCs are highlighted with yellow color and normal GCs with blue color. (Scale bars, 10 μm.)

CDKB1 Activity Promotes RPA2a Nuclear Localization. The strong expression of complementary RPA2a-GFP was found mainly in the nucleus, but also was present in the cytoplasm. RPA2a-GFP signals were more diffuse in *cdkb1;1 1;2* epidermal cells (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S8 *A* and *B*). Western blot analysis confirmed that the abundance of nuclear RPA2a-GFP proteins decreased in *cdkb1;1 1;2* (Fig. 5*C*). RPA2a$^{\Delta 32}$-GFP and the phosphorylation-deficient version RPA2a$^{S11,21/G}$-GFP displayed pronounced fluorescent signals in the cytoplasm of epidermal cells (Fig. 5 *D* and *E* and *SI Appendix*, Fig. S8 *C* and *D*). By contrast, the signals of RPA2a$^{S11,21/D}$-GFP, which fully complemented the stomatal defects of *rpa2a-5*, displayed a high degree of colocalization with DAPI in the nucleus, with a Pearson's *Rr*-value of 0.89 (Fig. 5*F* and *SI Appendix*, Fig. S8*E*). Quantitative analysis of relative GFP fluorescent intensity in the nucleus consistently supported that CDK-mediated phosphorylation of RPA2a promotes the nuclear accumulation of RPA2a (*SI Appendix*, Fig. S8*K*).

We fused the Nuclear Localization Sequence (NLS) or Nuclear Export Signal (NES) to the C terminus of CDKB1;1 and coexpressed with RPA2a-mCherry in tobacco leaves. A Non-Nuclear Localization Sequence (NNLS, a control sequence to the NLS) and a Non-Nuclear Export Signal (NNES, a control sequence to the NES) (28) were also fused with CDKB1;1 as controls. When individually expressed, RPA2a-mCherry remained in the nucleus and cytoplasm (*SI Appendix*, Fig. S9*A*). However, RPA2a-mCherry signals were predominantly retained in the nucleus when coexpressed with CDKB1;1-NLS-GFP (*SI Appendix*, Fig. S9*B*). By contrast, when coexpressed with CDKB1;1-NES-GFP, in addition to the nucleus, abundant RPA2a-mCherry signals were accumulated in the cytoplasm (*SI Appendix*, Fig. S9*C*). CDKB1;1-NNLS-GFP or CDKB1;1-NNES-GFP did not change the subcellular localization pattern of RPA2a-mCherry (*SI Appendix*, Fig. S9 *D* and *E*). Thus, the physical interaction with CDKB1;1 either in the nucleus or in the cytoplasm seems to affect the subcellular localization of RPA2a.

To further clarify whether the subcellular localization is associated with the function of RPA2a, we generated NLS-fused *RPA2a:RPA2a-NLS-GFP* and NES-fused *RPA2a:RPA2a-NES-GFP* and transformed them into a *rpa2a-5* mutant. The fluorescent signals of RPA2a-NLS-GFP were exclusively accumulated in the nuclei (Fig. 5*H* and *SI Appendix*, Fig. S8 *G* and *L*), in which the defective overall growth and GMC division of *rpa2a-5* mutants were fully recovered (Fig. 5*L* and *SI Appendix*, Table S4). However, a transgenic line carrying *RPA2a-NES-GFP* line #12, in which the diffuse GFP signals were detected in both cytoplasm and nucleus (Fig. 5*J* and *SI Appendix*, Fig. S8 *I* and *L*), the growth and stomatal defects in *rpa2a-5* were partially rescued (Fig. 5*L* and *SI Appendix*, Table S4). Whereas *RPA2a-NES-GFP* transgenic line #5, in which GFP signals were restricted mostly to the cytoplasm, barely showed a colocalization with DAPI in the nucleus and a representative low value of Pearson's *R* at −0.23 (Fig. 5*K* and *SI Appendix*, Fig. S8 *J* and *L*). Neither the plant growth defect nor the stomatal defect of the *rpa2a-5* mutant was rescued in line #5 (Fig. 5*L* and *SI Appendix*, Table S4). The high expression level of *RPA2a-NES-GFP* in line #5 (*SI*

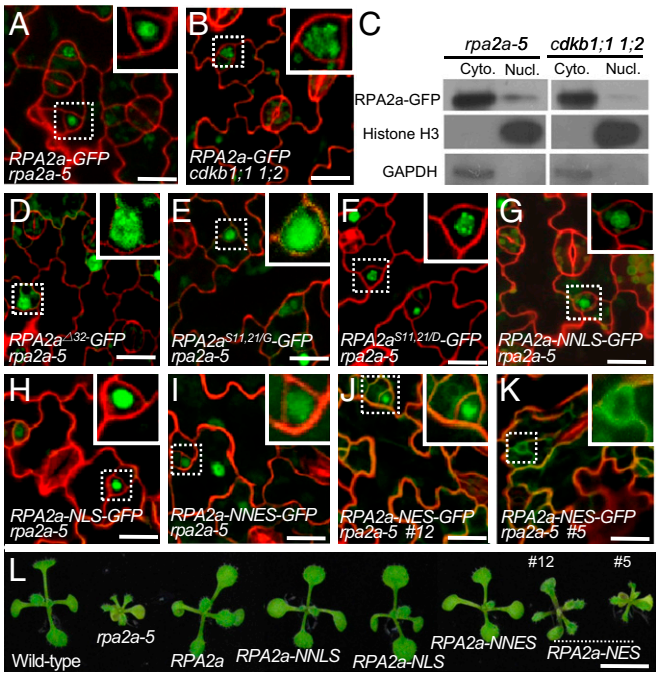

Fig. 5. The function of RPA2a is associated with its phosphorylation status and subcellular localization. (*A*) Nuclear expression of RPA2a-GFP in a complemented *rap2a-5*. (*B*) Diffuse RPA2a-GFP in *cdkb1;1 1;2*. (*C*) RPA2a-GFP proteins were found in both the cytoplasm and the nucleus by Western blot analysis. The amount of RPA2a-GFP in the nucleus is reduced in *cdkb1;1 1;2*. (*D* and *E*) Diffuse GFP signals in RPA2a$^{\triangle 32}$-GFP (*D*) and RPA2a$^{S11,21/G}$-GFP (*E*). (*F*) Predominant GFP signals in the nucleus of RPA2a$^{S11,21/D}$-GFP. (*G*–*K*) The RPA2a subcellular localization in RPA2a-NNLS-GFP (*G*), RPA2a-NLS-GFP (*H*), RPA2a-NNES-GFP (*I*), and two RPA2a-NES-GFP transgenic lines [line #12 (*J*) and line #5 (*K*)]. (*Insets*) The enlarged region highlighted by the white dashed-line boxes. (Scale bars, 20 μm.) (*L*) The retarded overall growth of *rpa2a-5* was not fully rescued by *RPA2a-NES-GFP*. (Scale bar, 1 cm.)

Appendix, Fig. S10) might provide higher nuclear export signals (from the NES), which drive RPA2a proteins into the cytoplasm against the endogenous nuclear "stay force," such as being phosphorylated by CDKs. The control lines *RPA2a-NNLS-GFP* and *RPA2a-NNES-GFP* phenocopied the protein localization and complementation ability of the wild-type RPA2a-GFP (Fig. 5 *G*, *I*, and *L* and *SI Appendix*, Fig. S8 *F* and *H* and Table S4).

The N-Terminal Domain Is also Required for RPA2a's Function in Response to DNA Damage. It has been reported that *Arabidopsis rpa2a* alleles exhibited hypersensitivity to the genotoxic agent Methyl Methane Sulfonate (MMS) treatment (19–21). However, how the RPA2a protein and its subdomains are related to the DNA damage responses in plants remains unknown. The 5-d MMS treatment generally arrested plant growth and induced seedling chlorosis. The introduction of *RPA2a-GFP* or *RPA2a$^{S11,21/D}$-GFP* recovered *rpa2a-5* growth to the wild-type level. However, the N-terminal defective RPA2a$^{\triangle 32}$-GFP and the phosphorylation-deficient RPA2a$^{S11,21/G}$-GFP only partially complemented the chlorosis phenotype of *rpa2a-5* (*SI Appendix*, Fig. S11). Furthermore, *cdkb1;1 1;2* and *rpa2a-5 cdkb1;1 1;2* displayed hypersensitivity to MMS treatment as well. The phosphorylation-mimic variant RPA2a$^{S11,21/D}$ alleviated the sensitivity of *cdkb1;1 1;2* to MMS (Fig. 6 *A* and *B* and *SI Appendix*, Fig. S12), which is consistent with its stronger capability than wild-type RPA2a in rescuing *cdkb1;1 1;2* GMC divisions (*SI Appendix*, Table S3). Therefore, our finding observation is consistent with the previous findings in yeast and humans that the phosphorylation of the N terminus is important for RPA2 function in response to DNA damage.

Discussion

In this study, we found that a genetic mutation in *Arabidopsis RPA2a* not only suppressed *flp-1* stomatal clustering phenotype but also induced the formation of SGCs. *RPA2a* functions together with *CDKB1s* and *CYCA2s* in ensuring the stomatal lineage cell divisions and maintaining a normal DNA content and guard cell size. Furthermore, we found that Ser-11 and Ser-21 of *Arabidopsis* RPA2a are consensus sites for CDK phosphorylation. Being phosphorylated by CDKs at these sites is tightly associated with the RPA2a subcellular localization and function in cell cycle control and the response to DNA damage.

RPA2a and Core Cell Cycle Genes Coordinately Control Cell Cycle Procession. FLP and MYB88 directly repress the transcription of several core cell cycle genes, such as *CDKB1;1*, *CYCA2;3*, and *CDKA;1*, to ensure that only 2 GCs are produced during the terminal GMC divisions (2–6). FLP, as an atypical R2R3-MYB transcription factor, recognizes downstream targets through a *cis*-regulatory element that overlaps with E2Fs (4, 29, 30). There are 12 putative FLP/MYB88/E2F-binding sequences within the *RPA2a* promoter. However, neither FLP nor MYB88 displayed a clear binding activity to the *RPA2a* promoter (*SI Appendix*, Fig. S2). It was proposed that rice *OsRPA1* and *OsRPA2* might be the target genes of the S-phase transcription factor E2Fs (31). It will be interesting to test whether FLP and MYB88 compete with E2Fs in regulating *RPA2a* transcription.

RPA stabilizes ssDNA and interacts with replication proteins at the initiation site of DNA replication to facilitate the formation of replication forks (12, 32). CDT1 and CDC6 are DNA replication licensing components that help the loading of the MCM DNA helicases to the DNA replication origins. Similar to human Cdt1 proteins, the *Arabidopsis* CDT1 is degraded through a CDK-mediated phosphorylation. *CDT1* and *CDC6* are also regulated by E2F transcription factors (33). In *rpa2a cdkb1;1 1;2* or *rpa2a cyca2;3 2;4*, transcription levels of a set of G1-to-S phase genes, including *CDT1*, *CDC6*, *E2F*-family genes, are significantly

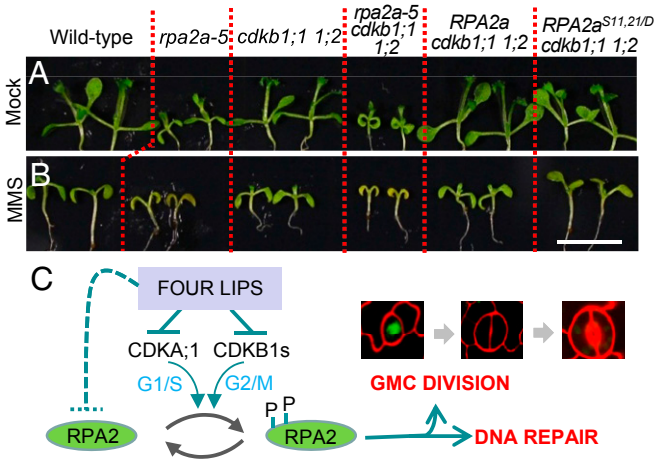

Fig. 6. Phosphorylation-mimic *RPA2a$^{S11,21/D}$* alleviated the MMS effects in *cdkb1;1 1;2*. (*A* and *B*) Seven-day-old seedlings were transferred to liquid Murashige–Skoog (MS) medium (*A*) or liquid MS medium containing 0.01% MMS (*B*) for 5 d before imaging. Seedlings of *rpa2a-5*, *cdkb1;1 1;2*, and *rpa2a-5 cdkb1;1 1;2* displayed hypersensitivity to MMS. Phosphorylation-mimic *RPA2a$^{S11,21/D}$* alleviated the MMS effects on *cdkb1;1 1;2*. (Scale bar, 1 cm.) (*C*) Model for RPA2 function in stomatal terminal division regulation and DNA repair progression. T-bars and dashed lines indicate negative and positive regulation, respectively.

up-regulated, indicating that *RPA2a* and core cell cycle genes coordinately restrict the G1-to-S transition and prevent the entry of endocycle. Recently, the roles of *CYCD5;1* and *CYCD7;1* in regulating GMC division and differentiation have been elegantly described (34, 35). However, the expression of *CYCD5;1* and *CYCD7;1* was not altered either in *rpa2a-5, cdkb1;1 1;2*, or in *rpa2a-5 cdkb1;1 1;2* mutants (*SI Appendix*, Fig. S5*C*).

The Functions of RPA2a Are Regulated by CDKs in a Plant-Specific Manner. RPA2 proteins are phosphorylated during the G1-to-S transition and M-phase and dephosphorylated at the end of the M-phase (8, 36–38). In yeast, a single CDK (cdc2) regulates DNA replication licensing, DNA replication initiation, and cell mitosis by changing its activity levels from low to intermediate to high, respectively (34, 39). CDK-mediated RPA2 phosphorylation at Ser-23 occurs during S-phase while phosphorylation at Ser-29 happens only during mitosis (12). In *Arabidopsis*, generally, CDKA;1 activity is more important for the G1-to-S transition, and CDKB1s is required for the G2-to-M progression (40). Here, we found that RPA2a proteins could be phosphorylated by CDKA;1 in vitro as well, suggesting that RPA2a might be regulated by both A1- and B1-type CDKs in *Arabidopsis*. Therefore, in contrast to the single CDK in yeast, the divergent functions of RPA2a from DNA replication to mitosis might be regulated by both A1-type CDKA;1 and the plant-specific B1-type CDKB1s (Fig. 6*C*).

RPA2a and CDK-CYCLIN Are Involved in DNA Repair. DNA double-strand breaks also can occur during DNA replication. The *Arabidopsis atm* mutant displays chromosomal fragmentation and reduced fertility (41). SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) is a functional analog of mammalian tumor suppressor p53 in plants, which is directly phosphorylated and activated by ATM upon DNA damage (42, 43). The activated SOG1 induces an increase of *CYCB1;1* transcript (44). In *rpa2a-5*, the transcription level of *CYCB1;1*, but not of *CYCA2;3*, was dramatically up-regulated (*SI Appendix*, Fig. S12*B*), suggesting the existing of a regulatory circuit during DNA repair.

Materials and Methods

Details on plant growth conditions, positional cloning, plasmid construction, flow cytometric analysis, protein–protein interaction, and BiFC and Co-IP assays are provided in *SI Appendix, Materials and Methods*.

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