CDKB1;1 Forms a Functional Complex with CYCA2;3 to Suppress Endocycle Onset[1][W][OA]

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The mitosis-to-endocycle transition requires the controlled inactivation of M phase-associated cyclin-dependent kinase (CDK) activity. Previously, the B-type CDKB1;1 was identified as an important negative regulator of endocycle onset. Here, we demonstrate that CDKB1;1 copurifies and associates with the A2-type cyclin CYCA2;3. Coexpression of *CYCA2;3* with *CDKB1;1* triggered ectopic cell divisions and inhibited endoreduplication. Moreover, the enhanced endoreduplication phenotype observed after overexpression of a dominant-negative allele of *CDKB1;1* could be partially complemented by *CYCA2;3* co-overexpression, illustrating that both subunits unite in vivo to form a functional complex. CYCA2;3 protein stability was found to be controlled by CCS52A1, an activator of the anaphase-promoting complex. We conclude that CCS52A1 participates in endocycle onset by down-regulating CDKB1;1 activity through the destruction of CYCA2;3.

In all eukaryotes, oscillations in the phosphorylation status of key proteins control the cell cycle progression through the action of kinases and phosphatases. In particular, Ser-Thr kinase activity of the various cyclin-dependent kinase (CDK) complexes is crucial in determining the cycling state of a cell (Fisher and Nurse, 1996; Inzé and De Veylder, 2006). In plants as well as in mammals, various CDK proteins have been identified and grouped into different classes according to their sequences (Vandepoele et al., 2002). Plants possess a unique group of CDKs, of which the B1-type and B2-type CDKs display a maximum of kinase activity at the G2-to-M transition and during mitosis, respectively (Inzé and De Veylder, 2006). Recently, in Arabidopsis (*Arabidopsis thaliana*), the activity level of CDKB1;1 was found to control whether cells will divide mitotically or will undergo repeated rounds of rereplication in the absence of cytokinesis, a process known as endoreduplication. In cells competent for division, the presence of CDKB1;1 triggers cells to enter into mitosis. By contrast, in the absence of CDKB1;1 activity, cells endoreduplicate (Boudolf et al., 2004b). Hence, the control of CDKB1;1 activity plays a crucial role in determining the timing of the mitosis-to-endocycle transition. However, the mechanisms that regulate CDKB1;1 activity remain unclear.

One essential step in CDK activation involves the association of the kinase with a regulatory cyclin subunit. Cyclins of higher eukaryotes are generally divided into three groups: G1/S (D-type), S/M (A-type), and mitotic (B-type) cyclins. Plants hold multiple members of each cyclin class, but the CDK-binding partner and biological significance have been identified for only a few of them (Inzé and De Veylder, 2006). The abundance of A- and B-type cyclins is controlled by the combined transcriptional activation and proteolytic turnover through ubiquitination (Glotzer et al., 1991).

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Table I. *List of CYCA2;3 copurified proteins identified by mass spectrometry*

Combined peptide mass fingerprint and peptide sequence spectra were searched against The Arabidopsis Information Resource 8.0 database. Threshold values were set for $P = 0.05$.

Locus	Gene Description	Sequence Coverage	Protein Score/ Threshold	Total Ion Score/ Threshold	Best Ion Score/ Threshold
		%			
At2G27960	CKS1	62	168/58	121/24	48/24
At4G16143	Importin α, putative	26	63/58		
At1G01880	DNA repair protein, putative	11	79/58	58/25	20/25
At5G63960	DNA-directed DNA polymerase δ catalytic subunit, putative (POLD1)	23	68/58		
At2G46280	Eukaryotic translation initiation factor 3 subunit 2	21	63/58	30/26	30/26
At1G07890	L-Ascorbate peroxidase 1, cytosolic (APX1)	24	59/58	33/26	33/26
At1G57720	Elongation factor 1B-γ, putative	10		45/24	45/24
At1G61870	Pentatricopeptide repeat (PPR)-containing protein	8		33/26	33/26
At3G54180	CDKB1;1	9		29/24	29/24
At5G16970	NADP-dependent oxidoreductase, putative (P1)	20		28/24	28/24

Protein ubiquitination is a multistep process, requiring at least three different enzymes: the ubiquitin-activating enzyme (E1) that forms a high-energy bond with ubiquitin, which is subsequently transesterified to a ubiquitin-conjugating enzyme (E2), and, finally, to the E3 enzyme that transfers the ubiquitin either directly to the substrate or indirectly by bringing the E2 enzyme near the substrate. E3 ubiquitin ligases determine the specificity of the target protein and timing of polyubiquitination. The anaphase-promoting complex/cyclosome (APC/C) is a highly conserved ubiquitin ligase (E3) formed by 11 subunits in vertebrates and plants

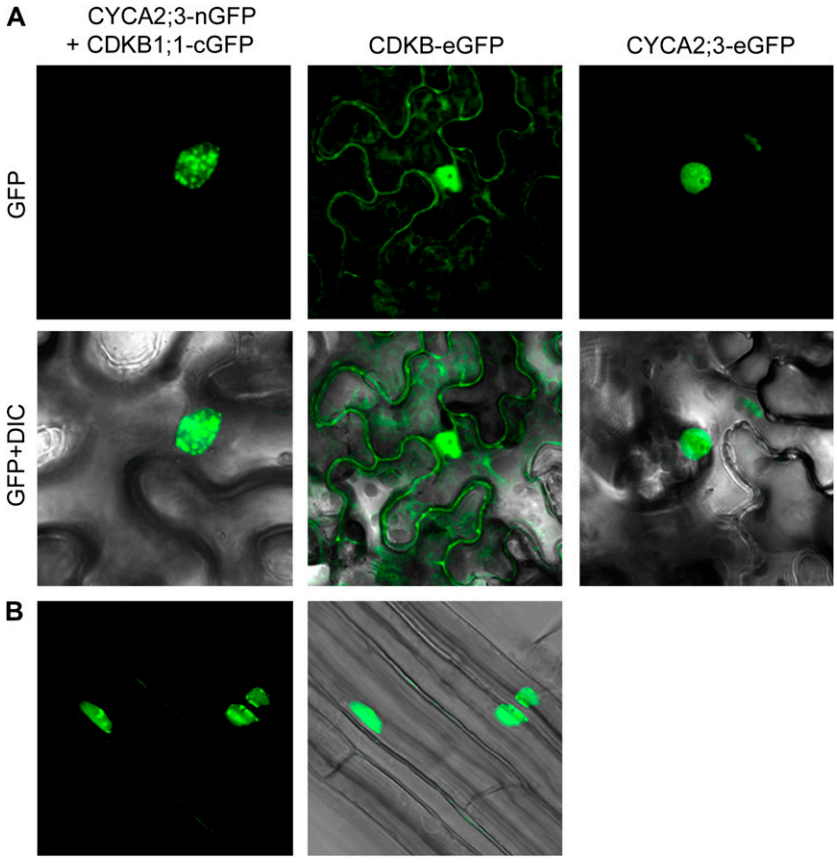

Figure 1. In vivo interaction between CDKB1;1 and CYCA2;3. A, Subcellular localization of CDKB1;1 (CDKB-eGFP), CYCA2;3 (CYCA2;3-eGFP), and the CYCA2;3-CDKB1;1 (CYCA2;3-nGFP + CDKB1;1-cGFP) complex. Tobacco epidermal cells were transfected with constructs encoding the indicated fusion proteins. DIC, Differential interference contrast. B, Confocal images of a root of an Arabidopsis plant coexpressing *CDKB1;1-cGFP* and *CYCA2;3-nGFP*.

(Peters, 2002; Capron et al., 2003) and 13 subunits in yeast (Yoon et al., 2002), although its minimal ubiquitin ligase module comprises only APC2 and APC11 (Gmachl et al., 2000; Tang et al., 2001). The two latter subunits interact with each other and with the E2 ubiquitin-conjugating enzymes and have been shown to be sufficient for ubiquitin transfer in vitro, without any substrate specificity (Gmachl et al., 2000; Tang et al., 2001). In vivo, substrates are selected through binding of the APC/C activator proteins: CDC20/FZY from late G2 to late anaphase and CDH1/FZR/CCS52 from late mitosis to the G1-to-S transition (Harper et al., 2002). Proteins carrying characteristic degradation motifs, such as D box, KEN, GXEN, A, C, O, and the recently identified TEK boxes, are recognized by these APC activators and brought to the core complex for ubiquitination (Burton and Solomon, 2001; Burton et al., 2005; Kraft et al., 2005).

Recently, the APC/C activator genes *CDH1*, *FZR*, and *CCS52A* have been found to promote the endocycle onset and progression in human, fruit fly (*Drosophila melanogaster*), and the plants *Medicago truncatula* and Arabidopsis, respectively (Sigrist and Lehner, 1997; Cebolla et al., 1999; Schaeffer et al., 2004; Lasorella et al., 2006; Binné et al., 2007; Lammens et al., 2008; Narbonne-Reveau et al., 2008; Larson-Rabin et al., 2009). In fruit fly and mammals, the mitotic cyclins degraded by $APC/C^{FZR/CDH1}$ at the endocycle onset have been identified. However, in plants, this identification turned out to be difficult because of the enormously expanded number of cyclins. In vitro binding assays yielded a subset of potential cyclin-CCS52 interactions (Fülöp et al., 2005), but, unfortunately without placing them in a developmental context.

Here, we report on the interaction of CDKB1;1 with A2-type cyclins. Biochemical and genetic studies revealed that CDKB1;1 and CYCA2;3 form a functional complex whose activity drives the mitotic cell cycle and prevents cells from entering the endocycle program. Moreover, we identified CYCA2;3 as an in vivo substrate of $APC/C^{CCS52A1}$ but not of $APC/C^{CCS52A2}$. We conclude that the controlled inactivation of CDKB1;1-CYCA2;3 by $APC/C^{CCS52A1}$ directs the endoreduplication process in Arabidopsis.

RESULTS

CYCA2;3 Interacts with CDKB1;1

Previously, we have demonstrated that CDKB1;1 activity, together with the E2Fa-DPa transcription factor, controls the balance between proliferation and endoreduplication (Boudolf et al., 2004b). However, the regulatory cyclin subunit that interacts with CDKB1;1 in this defined developmental context remained to be characterized. To find interaction partners of the mitotic CDKB1;1 kinase, a yeast two-hybrid screen was used with an Arabidopsis cell suspension cDNA library fused to the GAL4 sequence-encoding activation domain. The screening was carried out with a dominant negative allele of the *CDKB1;1* gene (*CDKB1;1.N161*), because initially no cyclin interactors had been identified with the wild-type gene as bait. This strategy turned out to be successful, probably thanks to stabilization of the kinase-cyclin complex, and resulted in the identification of CYCA2;2 and CYCA2;3 as potential CDKB1;1 interactors (data not shown). To confirm these interactions, we applied the tandem affinity purification (TAP) technique that has been proven to be a powerful tool to characterize protein complexes in plants (Van Leene et al., 2007). *CYCA2;2* and *CYCA2;3* were fused with the TAP tag and expressed in Arabidopsis cell cultures. The resulting immunological complexes were purified (Van Leene et al., 2007). Mass spectrometry-driven peptide sequencing allowed the identification of the CDKB1;1 protein as part of the CYCA2;3, but not of the CYCA2;2, complexes (Table I; data not shown). As only the interaction of CYCA2;3 with CDKB1;1 was observed in both the yeast two-hybrid and TAP analyses, we decided to focus on this interaction.

CDKB1;1 and CYCA2;3 Colocalize in the Nucleus

To ascertain the interaction of CYCA2;3 and CDKB1;1 in planta, bimolecular fluorescence complementation (BiFC) was utilized. The sequences coding for CYCA2;3 and CDKB1;1 were fused in frame with

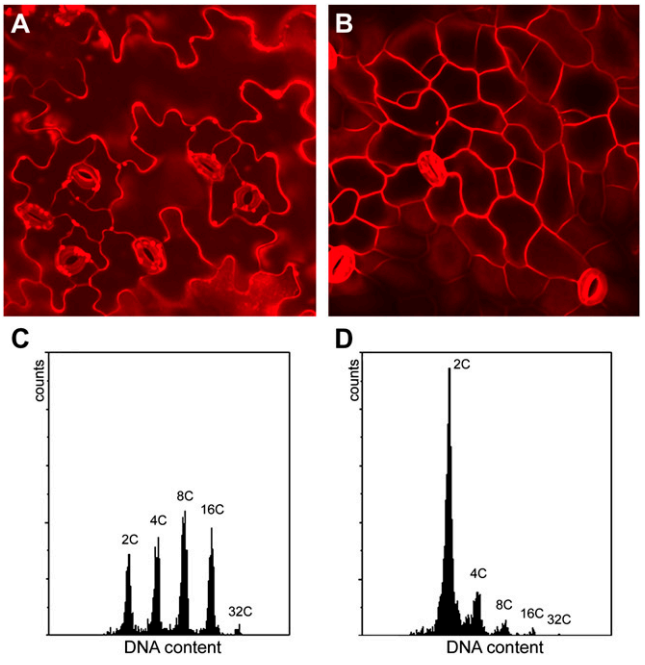

Figure 2. Effect of coexpression of *CYCA2;3-nGFP* and *CDKB1;1-cGFP*. Col-0 seedlings (A and C) and plants coexpressing *CYCA2;3-nGFP* and *CDKB1;1-cGFP* (B and D) were grown for 12 DAG on agar medium, and cotyledons were harvested. The cotyledons were stained with PI and observed with a confocal laser-scanning microscope (A and B) or subjected to flow cytometry (C and D).

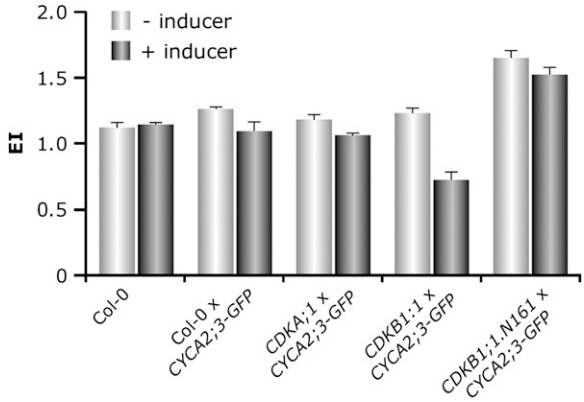

Figure 3. Effect of *CYCA2;3-GFP* induction on the EI in transgenic plants ectopically expressing *CDKA;1*, *CDKB1;1*, or *CDKB1;1.N161*. Cotyledons were harvested at 7 DAG. Seedlings were grown on agar medium without (− inducer) or with (+ inducer) 10 μM β-estradiol and subjected to flow cytometry. Col-0 was used as a control. Values are means ± SD (n = 3). Each biological repeat was carried out with seedlings obtained from independent F1 crosses.

the N-terminal and C-terminal fragments of the enhanced GFP (eGFP), namely *CYCA2;3-nGFP/CDKB1;1-cGFP* and *CDKB1;1-nGFP/CYCA2;3-cGFP*, respectively. The interaction between the different fusion proteins was tested by transient expression in leaf epidermal cells of tobacco (*Nicotiana benthamiana*). No fluorescence was detected when any combination with empty vectors was introduced into tobacco cells (*CYCA2;3-nGFP* and *cGFP*, *nGFP* and *CDKB1;1-cGFP*, *CDKB1;1-nGFP* and *cGFP*, and *nGFP* and *CYCA2;3-cGFP*; data not shown). By contrast, eGFP fluorescence was observed in the nuclei of cells transfected with *CYCA2;3-nGFP* and *CDKB1;1-cGFP* (Fig. 1A) or with *CDKB1;1-nGFP* and *CYCA2;3-cGFP* (data not shown), demonstrating that the CYCA2;3 protein interacted with CDKB1;1 in the plant nucleus. Fluorescence was most intense at localized foci, probably corresponding to the chromocenters. When the subcellular localization of CYCA2;3 and CDKB1;1 was examined in tobacco leaf epidermal cells, the fusion protein CDKB1;1-eGFP resided in both the nucleus and the cytoplasm, whereas the fusion protein CYCA2;3-eGFP was found exclusively in the nucleus (Fig. 1A).

Subsequently, we also tested whether the CDKB1;1-CYCA2;3 complex was formed in Arabidopsis plants. For this purpose, we supertransformed Arabidopsis plants carrying the *CDKB1;1-cGFP* gene construct with a T-DNA harboring the *CYCA2;3-nGFP* gene. Fifteen out of 20 double transformed lines showed detectable fluorescence, and four of the most intense ones were selected for further analysis. The fluorescent signal of the complex was detected in the nucleus in roots and hypocotyls (Fig. 1B; data not shown).

Co-overexpression of *CDKB1;1* and *CYCA2;3* Induces Ectopic Cell Divisions

Interestingly, both the overexpression of *CDKB1;1.N161* and the knockout of *CYCA2;3* result in an increased DNA ploidy level (Boudolf et al., 2004b; Imai et al., 2006), suggesting that they might operate together to suppress the endocycle onset. To test this hypothesis, we analyzed phenotypically the plants coexpressing the *CDKB1;1-cGFP* and *CYCA2;3-nGFP* constructs. Microscopic analysis of the cotyledons revealed much smaller cells with newly formed cell walls in the plants coexpressing the *CDKB1;1-cGFP* and *CYCA2;3-nGFP* constructs when compared with the wild-type (Columbia-0 [Col-0]) plants, indicating induction of ectopic cell divisions (Fig. 2, A and B). Furthermore, as seen by flow cytometry of the cotyledons, the pool of endoreduplicating cells had decreased, resulting in an 85% reduced endoreduplication index (EI), which is the mean number of endoreduplication cycles per nucleus (Fig. 2, C and D). Similar results were obtained for leaves and roots (Supplemental Fig. S1).

To investigate whether the ectopic cell division and reduced endoreduplication phenotype were due to a specific action of the CDKB1;1-CYCA2;3 complex, plants overexpressing *CDKB1;1* or *CDKA;1* were crossed with lines harboring a β-estradiol-inducible

Table II. *Effect of CYCA2;3-GFP induction on DNA ploidy level distribution in transgenic plants expressing CDKA;1, CDKB1;1, or CDKB1;1.N161*

Cotyledons were harvested at 7 DAG. After flow cytometry, the EI was calculated. Col-0 was used as a control. Values are means ± SD (n = 3).

Ploidy	Col-0	Col-0 × *CYCA2;3-GFP*	*CDKA1;1* × *CYCA2;3-GFP*	*CDKB1;1* × *CYCA2;3-GFP*	*CDKB1;1.N161* × *CYCA2;3-GFP*
Without β-estradiol					
2C	28.1 ± 0.6	26.4 ± 0.3	26.6 ± 0.8	27.4 ± 0.9	11.8 ± 1.0
4C	36.8 ± 0.4	30.6 ± 0.8	35.1 ± 0.7	30.2 ± 1.4	28.7 ± 1.2
8C	29.8 ± 0.4	33.8 ± 0.7	33.3 ± 1.0	35.5 ± 0.4	42.2 ± 4.0
16C	4.9 ± 0.7	9.1 ± 1.2	5.1 ± 0.3	6.9 ± 1.0	17.0 ± 4.4
+10 μM β-estradiol					
2C	26.9 ± 0.3	28.9 ± 1.9	28.0 ± 1.2	47.1 ± 1.4	12.1 ± 0.8
4C	37.1 ± 0.2	37.1 ± 0.0	40.7 ± 1.2	35.2 ± 1.0	32.0 ± 2.0
8C	30.2 ± 0.4	31.1 ± 1.0	27.8 ± 1.0	16.5 ± 2.0	48.4 ± 1.3
16C	5.2 ± 0.1	2.8 ± 0.9	2.9 ± 1.0	1.0 ± 0.4	7.4 ± 1.8

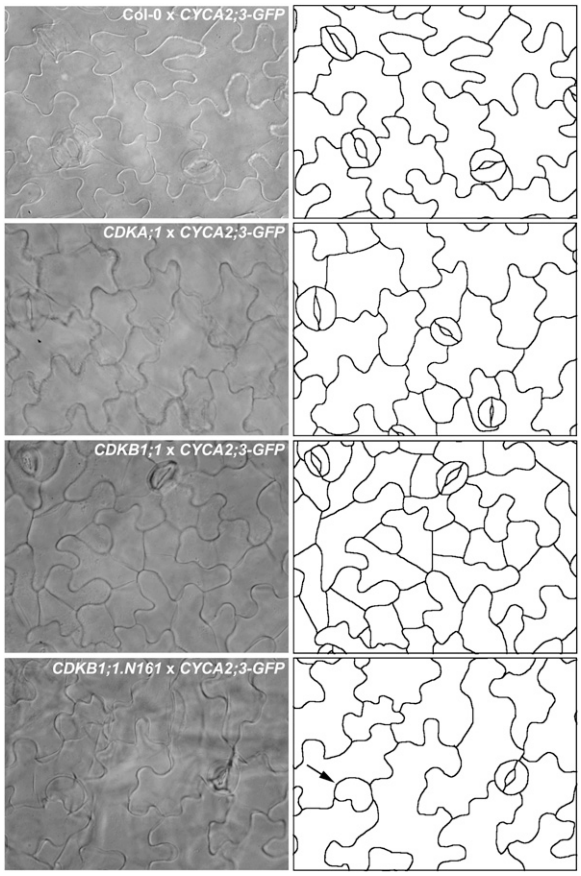

Figure 4. Phenotypes of cotyledon epidermal cells in Col-0 × *CYCA2;3-GFP* plants (control) and plants coexpressing *CYCA2;3-GFP* and *CDKA;1* (*CDKA;1* × *CYCA2;3-GFP*), *CDKB1;1* (*CDKB1;1* × *CYCA2;3-GFP*), or *CDKB1;1.N161* (*CDKB1;1.N161* × *CYCA2;3-GFP*). Seedlings were grown for 14 DAG on agar medium containing 10 μM β-estradiol. At 7 DAG, seedlings were transferred to fresh 10 μM β-estradiol-containing medium in order to sustain the induction of *CYCA2;3-GFP*. At left are images obtained with differential interference contrast microscopy; at right are drawings of the outlines of the epidermal cells obtained by tracing the microscopy images with Adobe Photoshop 6. The arrow marks aberrant stomatal morphology.

construct overexpressing *CYCA2;3-GFP* (Imai et al., 2006) and the F1 generation was characterized phenotypically. Cotyledons of seedlings grown for 7 d on agar medium with and without 10 μM β-estradiol were subjected to flow cytometry to analyze the DNA ploidy content (Fig. 3; Table II). Induction of *CYCA2;3-GFP* expression in a wild-type or *CDKA;1*-overexpressing background resulted in a similar, slightly reduced EI. By contrast, when *CYCA2;3-GFP* expression was induced in a *CDKB1;1*-overexpressing background, an important increase in the proportion of 2C cells ($P < 0.05$ for the proportion of 2C cells in *CYCA2;3-GFP* × *CDKB1;1* versus in *CYCA2;3-GFP*) and a reduction of the EI were observed.

Overexpression of solely the wild-type *CDKA;1* or *CDKB1;1* gene does not affect the cell cycle (Hemerly et al., 1995; Boudolf et al., 2004a). Analogously induced *CYCA2;3-GFP* expression in a wild-type or *CDKA;1*-overexpressing background did not affect profoundly cell division (Fig. 4). By contrast, microscopic analysis of mature cotyledons clearly showed the induction of ectopic cell divisions in plants coexpressing *CDKB1;1* and *CYCA2;3-GFP*, as illustrated by the occurrence of smaller cells and newly formed cell walls with a straight appearance (Fig. 4). Moreover, CYCA2;3-associated kinase activity was higher in plants coexpressing *CDKB1;1* and *CYCA2;3-GFP* than in plants expressing *CYCA2;3-GFP* or coexpressing *CYCA2;3-GFP* and *CDKA;1* (Fig. 5). Taken together, these results imply that CDKB1;1 and CYCA2;3 might form a functional complex in planta.

Overexpression of the dominant negative *CDKB1;1.N161* allele competes with its endogenous wild-type counterpart for regulatory proteins, resulting in a decrease in endogenous CDKB1;1 activity, increased DNA ploidy levels, and formation of aberrant stomata (Boudolf et al., 2004a, 2004b). Induction of *CYCA2;3-GFP* in a *CDKB1;1.N161*-overexpressing background partially complemented the endoreduplication phenotype ($P < 0.05$, EI for *CYCA2;3-GFP*$_{\text{induced}}$ × *CDKB1;1.N161* versus EI for *CYCA2;3-GFP*$_{\text{not-induced}}$ × *CDKB1;1.N161*; Fig. 3; Table II), mainly as a consequence of the suppression of the 16C population, but it could not restore the aberrant stomatal phenotype (Fig. 4).

APC/C^{CCS52A1}, But Not APC/C^{CCS52A2}, Determines CYCA2;3 Stability in Vivo

One of the most defined characteristics of cyclins is their periodicity and concomitant instability. The CYCA2;3 protein contains a D box, which was demon-

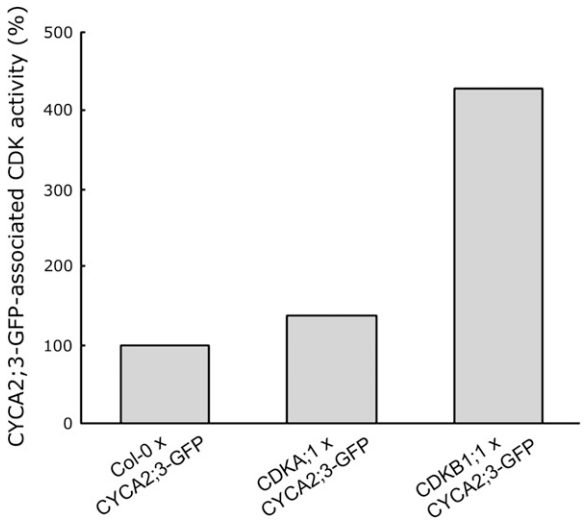

Figure 5. High kinase activity in *CDKB1;1* × *CYCA2;3-GFP* plants. Kinase activity assays of purified CYCA2;3-GFP complexes in Col-0 × *CYCA2;3-GFP*, *CDKA;1* × *CYCA2;3-GFP*, and *CDKB1;1* × *CYCA2;3-GFP* plants (F1 generation of approximately five independent crosses). Relative CYCA2;3-GFP-associated CDK activity was measured with histone H1 as substrate. For quantification, the control was arbitrarily set at 100%.

strated to determine the protein's half-life (Capron et al., 2003; Imai et al., 2006). D box motifs have been shown to mediate interaction with the APC/C activators CDC20/FZY and CDH1/FZR/CCS52, which bring the substrate to the APC/C (Burton and Solomon, 2001; Burton et al., 2005; Kraft et al., 2005). The recent identification of CCS52A1 and CCS52A2 as essential regulators of the endoreduplication program in Arabidopsis (Lammens et al., 2008; Larson-Rabin et al., 2009) and the observed decrease in ploidy in *CYCA2;3-GFP* plants led us to investigate whether CYCA2;3 and each of these activators might interact. Plants with a homozygous mutation for *CCS52A1* (*ccs52a1-1*) or *CCS52A2* (*ccs52a2-1*; Lammens et al., 2008) were crossed with plants harboring a β-estradiol-inducible *CYCA3;2-GFP* construct or a similar construct with a nonfunctional D box sequence, designated *mDB-CYCA2;3-GFP* (Imai et al., 2006). The intensity of the nucleus-localized GFP signal was stronger than that of the control (*CYCA2;3-GFP*) when *CYCA2;3-GFP* was induced in a *ccs52a1-1* background (*ccs52a1-1* × *CYCA2;3-GFP*; Fig. 6), indicative of an increase in CYCA2;3 stability upon loss of *CCS52A1*. The fluorescence intensity resembled that of plants expressing *mDB-CYCA2;3-GFP* in both Col-0 and *ccs52a1-1* backgrounds (Fig. 6). Remarkably, no change in CYCA2;3 stability could be observed in roots when *CYCA2;3-GFP* expression was induced in a *ccs52a2-1* background (Fig. 6), suggesting that CYCA2;3 degradation in roots is specifically achieved by CCS52A1-activated, and not by CCS52A2-activated, APC/C. CYCA2;3 stabilization in CCS52A1 was most clear in the columella cells and around the transition zone, marking the transition of mitotic division to endoreduplication (Fig. 7). No difference in GFP fluorescence could be observed at the meristematic zone (Fig. 7).

Next, it was tested whether the observed stabilization of CYCA2;3 in the *ccs52a1-1* genotype had any effect on the endoreduplication levels. Although the induction of *CYCA2;3-GFP* reduces the endoreduplication levels in both roots and cotyledons (Imai et al., 2006), under our experimental conditions, this effect was more pronounced in cotyledons (Fig. 8). When induced in a *ccs52a1-1* background, the stabilization of CYCA2;3-GFP further decreased the endoreduplication levels (Fig. 8A; Table III; $P < 0.005$, EI *CYCA2;3-GFP*$_{\text{induced}}$ × *ccs52a1-1* versus EI *CYCA2;3-GFP*$_{\text{induced}}$). The important increase in the proportion of 2C cells in 7-d-old cotyledons of the induced *CYCA2;3-GFP* plants suggested a CYCA2;3-dependent induction of ectopic cell divisions. Indeed, microscopic analysis of cotyledons grown for 14 d after germination (DAG) on β-estradiol indicated ectopic cell divisions, as illustrated by the presence of smaller, less differentiated cells (Fig. 9). In agreement with the decrease in endoreduplication, this ectopic cell division phenotype was enhanced upon induction of *CYCA2;3* in the *ccs52a1-1* background (Fig. 9).

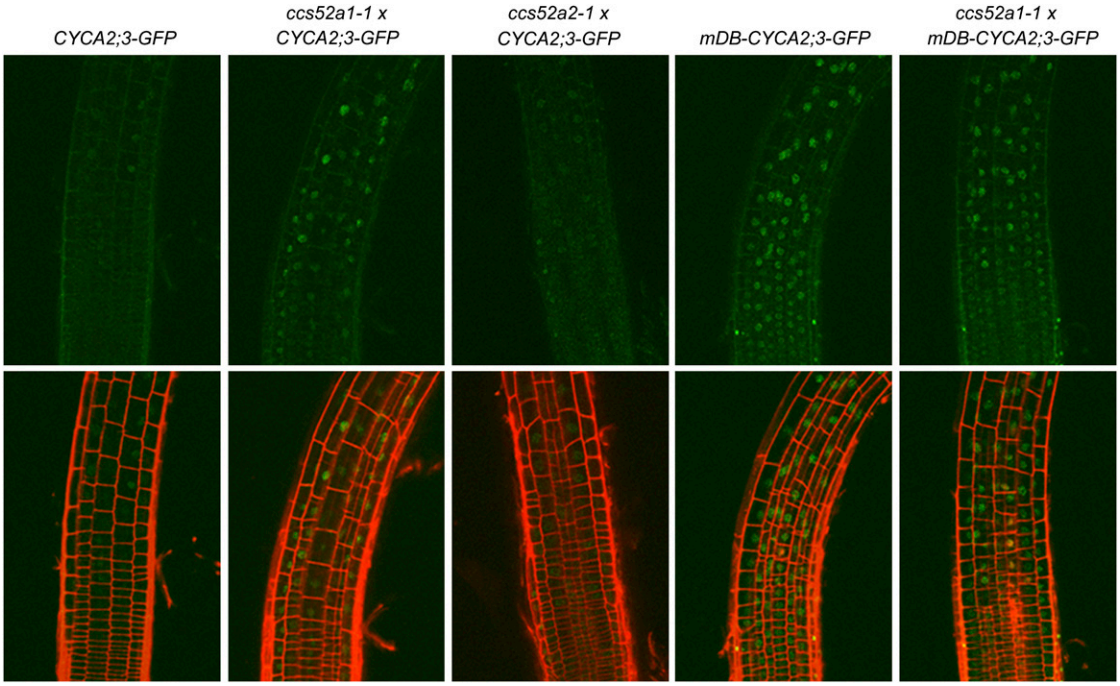

Figure 6. Stabilization of CYCA2;3 in the absence of CCS52A1. Fluorescence in inducer-treated roots expressing *CYCA2;3-GFP* or *mDB-CYCA2;3-GFP* in Col-0 or *ccs52a1-1* (*CYCA2;3-GFP* × *ccs52a1-1* and *mDB-CYCA2;3-GFP* × *ccs52a1-1*) background and *CYCA2;3-GFP* in *ccs52a2-1* (*CYCA2;3-GFP* × *ccs52a2-1*) background. Seedlings were grown for 5 d on agar without β-estradiol and for 2 d on agar medium with 10 μM β-estradiol. Cell walls were stained with PI. The fluorescence from the GFP fusion proteins was observed using confocal laser-scanning microscopy.

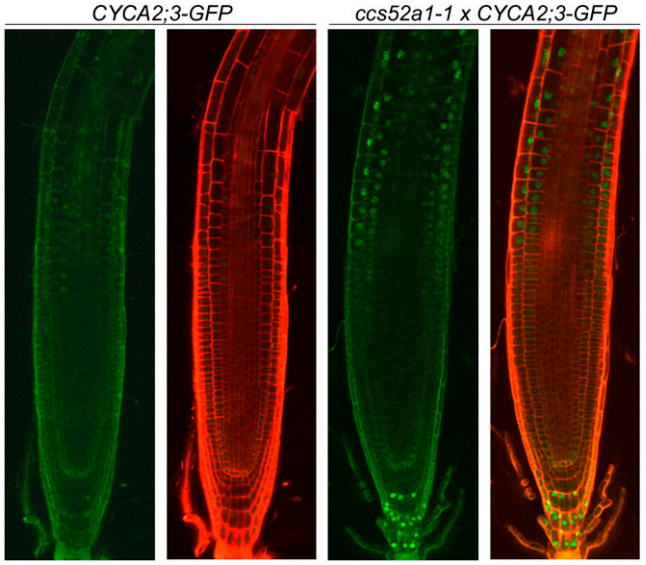

Figure 7. CYCA2;3 stabilization in *ccs52a1-1* roots. Fluorescence in inducer-treated roots expressing *CYCA2;3-GFP* or in Col-0 or *ccs52a1-1* (*CYCA2;3-GFP* × *ccs52a1-1*) background. Seedlings were grown for 5 d on agar medium without β-estradiol and for 2 d on agar medium containing 10 μM β-estradiol. Cell walls were stained with PI. The fluorescence from the GFP fusion proteins was observed with confocal laser-scanning microscopy.

DISCUSSION

CDKB1;1 and CYCA2;3 Control the Mitotic-to-Endocycle Transition

The regulated synthesis and destruction of cyclins provide waves of CDK activity, governing two crucial transitions within the cell cycle program: the entry into S phase from G1 and into M phase from G2. Because no M phase is seen within the endoreduplication program, entrance into and maintenance of the endoreduplication cycle are thought to require the elimination of M phase-associated kinase activity, which has been identified as the plant-specific mitotic CDKB1;1 (Boudolf et al., 2004b). Plants overproducing a dominant negative form of this CDK have increased endoreduplication levels and aberrant stomatal morphology (Boudolf et al., 2004a, 2004b). Although cyclins affecting endoreduplication levels in plants have been identified (Schnittger et al., 2002; Imai et al., 2006), no consensus exists on which cyclins interact with CDKB1;1, and constitute an integral part of the M phase CDK activity, which inhibits endoreduplication. By combining yeast two-hybrid, TAP purification, and BiFC assays, we showed that CYCA2;3 interacts with CDKB1;1. The enhanced endoreduplication phenotype induced by overexpression of *CDKB1;1.N161* could be partially complemented by overexpression of *CYCA2;3*, demonstrating that CYCA2;3 and CDKB1;1 form a functional complex whose activity inhibits the endoreduplication program. Interestingly, the stomatal phenotypic trait of the *CDKB1;1.N161*-overexpressing plants could not be complemented by induced expression of *CYCA2;3*, indicating that during stomatal development cyclins other than CYCA2;3 might form active complexes with CDKB1;1. Alternatively, the levels of *CYCA2;3* induction obtained in these experiments might be sufficient to trigger pavement cell divisions but insufficient to compete with the dominant negative *CDKB1;1* to drive stomatal divisions. This hypothesis would imply that the required dose of CDKB1;1 activity would be higher for a stomatal lineage than that for a normal proliferative division. Similarly, in fruit fly, levels of CDK activity necessary for asymmetric divisions of neuroblast cells are higher than those for symmetric divisions (Chia et al., 2008).

Notably, the coexpression of *CYCA2;3* and *CDKB1;1* triggered more ectopic cell divisions than were observed after overexpression of *CYCA2;3* alone. As no effect on cell division is seen in *CDKB1;1*-overexpressing plants, these observations indicate that *CYCA2;3* is the rate-limiting factor in determining the level of CDKB1;1 activity. By contrast, no ectopic divisions were seen upon the coexpression of *CYCA2;3* and *CDKA;1*, although both proteins have been reported to

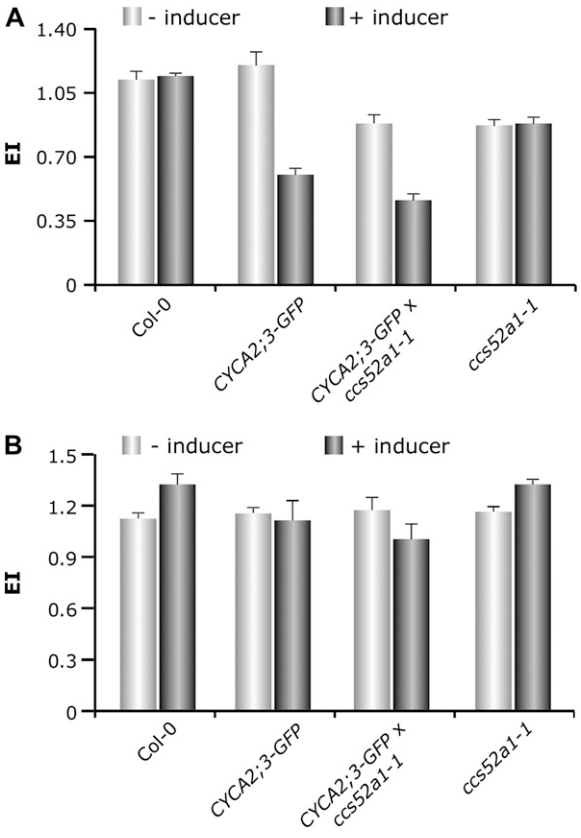

Figure 8. Effect on endoreduplication of CYCA2;3-GFP stabilization in *ccs52a1-1*. A and B, Cotyledons (A) and roots (B) from seedlings harvested at 7 DAG. Seedlings were grown on agar medium without (− inducer) or with (+ inducer) 10 μM β-estradiol; the respective tissues were subjected to flow cytometry. Values are means ± SD ($n = 3$).

Table III. *Effect of CYCA2;3-GFP stabilization in ccs52a1-1 on DNA ploidy level distribution*

Cotyledons and roots were harvested at 7 DAG and subjected to flow cytometry. Values are means ± SD ($n = 3$).

Ploidy	Col-0	*CYCA2;3-GFP*	*ccs52a1-1*	*CYCA2;3-GFP* × *ccs52a1-1*
Cotyledons				
Without β-estradiol				
2C	28.1 ± 0.6	27.8 ± 1.1	30.2 ± 1.9	30.3 ± 1.8
4C	36.8 ± 0.4	30.7 ± 2.7	52.4 ± 2.8	51.2 ± 0.4
8C	29.8 ± 0.4	35.9 ± 2.7	16.8 ± 1.2	17.9 ± 1.4
16C	4.9 ± 0.7	5.4 ± 1.1	0	0
+10 μM β-estradiol				
2C	26.9 ± 0.3	51.2 ± 2.1	28.4 ± 0.5	57.5 ± 1.5
4C	37.1 ± 0.2	38.3 ± 1.7	55.6 ± 1.5	39.5 ± 0.8
8C	30.2 ± 0.4	9.8 ± 0.6	15.6 ± 1.8	2.6 ± 0.7
16C	5.2 ± 0.1	0.4 ± 0.0	0	0
Roots				
Without β-estradiol				
2C	35.2 ± 0.8	33.6 ± 0.8	30.7 ± 1.8	30.2 ± 2.3
4C	30.1 ± 1.4	31.4 ± 0.7	31.6 ± 0.7	31.9 ± 1.1
8C	22.7 ± 1.6	21.4 ± 1.0	29.6 ± 1.7	28.1 ± 2.1
16C	11.8 ± 0.8	13.2 ± 1.2	7.9 ± 0.4	9.4 ± 1.1
+10 μM β-estradiol				
2C	27.2 ± 2.5	35.7 ± 5.1	24.5 ± 1.1	40.2 ± 5.0
4C	26.9 ± 1.7	25.0 ± 2.6	29.4 ± 1.9	24.0 ± 1.4
8C	27.7 ± 1.6	31.9 ± 1.6	36.5 ± 1.4	31.9 ± 3.0
16C	16.2 ± 0.5	7.2 ± 2.6	9.5 ± 1.9	3.9 ± 0.7

form a complex (Imai et al., 2006). These data suggest that the CDKA;1-CYCA2;3 complex is inactive or, alternatively, that CDKA;1 and CDKB1;1 are controlled differently at the posttranscriptional level. A-type CDK activity is regulated negatively by inhibitory phosphorylation (Da Costa et al., 2006; Harashima et al., 2007) and association with inhibitory proteins, such as Kip-related proteins (KRPs; Schnittger et al., 2003; Verkest et al., 2005) and SIAMESE (Churchman et al., 2006). By contrast, no such regulatory mechanisms have been demonstrated convincingly for CDKB1;1, indicating that cyclin binding might be the only requirement to activate it, whereas additional steps might be needed for CDKA;1 activation.

Generally, CDKs fulfill three major roles during mitotic cell cycle progression: entry into S phase, promotion of mitosis, and prevention of DNA rereplication (Porter, 2008). In fission yeast (*Schizosaccharomyces pombe*), the mitotic CDK complex hinders relicensing of replicated DNA by its association with the replication complex during chromosome duplication and prevents reinitiation of DNA synthesis (Wuarin et al., 2002). Similarly, CDKB1;1 might negatively regulate prereplication complex assembly and, thereby, repress the endocycle. Because CDKB1;1 activity peaks at the G2-to-M transition, it is presumed to play a role only during this transition point. However, *CDKB1;1* transcription is regulated through E2Fa and E2Fb transcription factors (Boudolf et al., 2004b; Magyar et al., 2005; Sozzani et al., 2006). Accordingly, *CDKB1;1* transcript levels increase from the G1-to-S transition onward, followed by an increase in CDKB1;1 activity during S phase (Porceddu et al., 2001; Sorrell et al., 2001; Breyne et al., 2002; Menges et al., 2002). These data indicate that CDKB1;1 might already be functional before its observed expression peak at the G2-to-M transition. In yeast that has only one single CDK to control the cell cycle, a quantitative model describes three levels of CDK activity: low, which allows licensing of DNA replication; intermediate, which initiates DNA replication but prevents relicensing; and high, which enables mitosis (Stern and Nurse, 1996; Porter, 2008). Although cell cycle regulation is more complex in higher eukaryotes, a quantitative model for CDK action is still partially applicable (Krasinska et al., 2008; Porter, 2008). From this point of view, an intermediate CDKB1;1 activity during the S phase could be a working mechanism that represses relicensing.

In synchronized cell cultures, the expression of *CYCA2;3* is restricted to the G2-to-M transition (Menges et al., 2005), which could argue against a possible function during S phase. However, the promoter of *CYCA2;3* is not only active in proliferating tissue but also in differentiated cells, such as stomatal guard cells, and in endoreduplicating cells, such as trichomes (Imai et al., 2006), suggesting a broader expression profile. In endoreduplicating cells, the CDKA;1-CYCA2;3 complex might negatively regulate the endocycle succession through down-regulation of prereplication complex function (Imai et al., 2006). In vitro interaction of CYCA2;2 and CDT1 (for chromatin licensing and DNA replication factor 1) supports this working mechanism (Castellano et al., 2004). Moreover, E2F transcription factors have been found to interact with A2-type cyclins (J. Boruc, L. De Veylder,

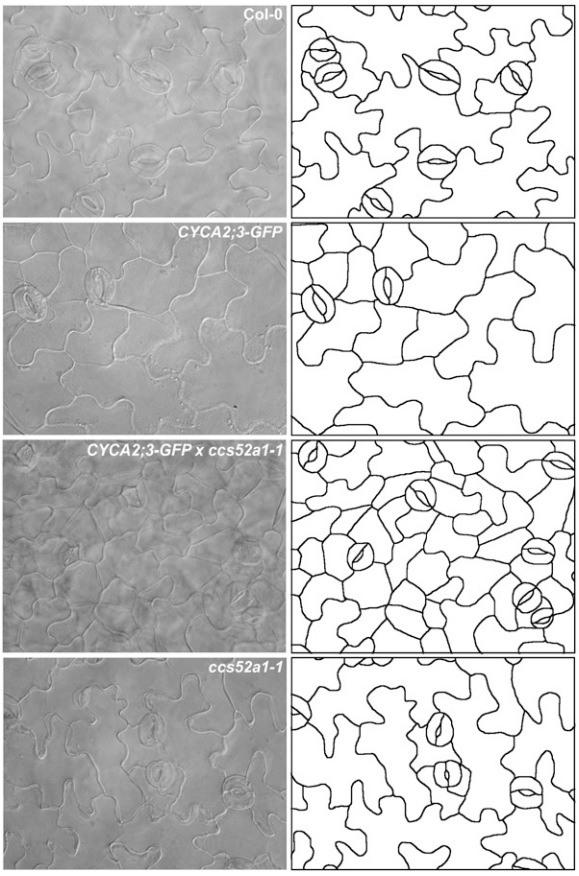

Figure 9. Phenotype of cotyledon adaxial epidermal cells in Col-0 (control), *CYCA2;3-GFP*, *ccs52a1-1*, and *CYCA2;3-GFP* × *ccs52a1-1* plants. Seedlings were grown for 14 d on agar medium containing 10 μM β-estradiol. At 7 DAG, seedlings were transferred to fresh 10 μM β-estradiol-containing medium to sustain the induction of *CYCA2;3-GFP*. At left are images obtained with differential interference contrast microscopy; at right are drawings of the outlines of the epidermal cells obtained by tracing the microscopy images using Adobe Photoshop 6.

and E. Russinova, unpublished data), another indication that A2-type cyclins might play a role during the S phase itself. In mammals, E2F activity is regulated by CDK2-cyclin A phosphorylation (Krek et al., 1994; Xu et al., 1994; Kitagawa et al., 1995; Hayashi and Yamaguchi, 1999; Morris et al., 2000). Inactivation of E2Fs by CDK2 phosphorylation promotes the mitotic cell cycle by driving cells into the G2 phase. We have shown that E2Fa-DPa regulates both the mitotic and endoreduplicating cycles in Arabidopsis (De Veylder et al., 2002) and that the decision to divide mitotically or to undergo endoreduplication depends on the presence of CDKB1;1 (Boudolf et al., 2004b). Thus, CDKB1;1-CYCA2;3 might regulate the E2F-DP transcription factor in mitotically dividing cells, which might explain why *CDKB1;1-CYCA2;3* overexpression triggers ectopic cell divisions.

Another possible working mechanism of the CDKB1;1-CYCA2;3 complex might be that it is directly responsible for the activation of CDKA;1 activity. A model in which CDKB1;1 controls the levels of CDKA;1 activity by phosphorylation has been proposed (Verkest et al., 2005) and, as a consequence, proteasomal degradation of the CDKA;1 inhibitor ICK2/KRP2. The overproduction of the CDKB1;1-CYCA2;3 complex might inhibit the action of ICK2/KRP2 and allow the cells to enter mitosis.

CDKB1;1-CYCA2;3 Abundance Is Controlled by APC/C^{CCS52A1}

The CYCA2;3 protein contains a clear D box, which has been demonstrated to be essential to the protein's half-life (Capron et al., 2003; Imai et al., 2006). D box motifs occur in many proteins targeted for destruction by the APC/C and are thought to mediate target binding by APC/C activator proteins, such as CDC20/FZY and CDH1/FZR/CCS52 (Burton and Solomon, 2001; Burton et al., 2005; Kraft et al., 2005). The stabilization of CYCA2;3-GFP in *CCS52A1* loss-of-function plants clearly indicates that CYCA2;3 is a CCS52A1 substrate. Moreover, the same stabilization could be observed upon mutation of the D box of CYCA2;3, indicating that CCS52A1-mediated destruction of CYCA2;3 happens in a D box-dependent manner. Both CYCA2;3 and CCS52A1 have been linked before to the control of the endoreduplication program (Imai et al., 2006; Lammens et al., 2008; Larson-Rabin et al., 2009). Thus, our observations suggest that CYCA2;3 stabilization, at least partially, contributes to the decreased endoreduplication levels observed upon loss of *CCS52A1*. Interestingly, CYCA2;3-GFP was not stabilized in the roots of *CCS52A2* knockout plants, although both CSS52A1 and CCS52A2 regulate the DNA ploidy level of leaf cells (Lammens et al., 2008).

The control of the APC/C activity plays an important role in the correct progression through the cell cycle (Acquaviva and Pines, 2006; Peters, 2006). In mammals, the APC/C complex inhibits CDK activity during late M and G1 phases, allowing licensing. During the remainder of the cell cycle, the APC/C must be inactivated for mitotic cyclins to accumulate. Inactivation of APC/C is achieved in part by the CDK-dependent phosphorylation of the APC/C activator CDH1 (the mammalian counterpart of CCS52A). The CDK-dependent inhibition of CDH1 seems to be conserved in plants, because mutations mimicking phosphorylation of the CDK phosphorylation sites in the *Medicago* CCS52A inhibit the interaction with the APC/C (Tarayre et al., 2004). In both animals and plants, the APC/C activator genes *CDH1*, *FZR*, and *CCS52A* have been found to promote endocycle onset (Sigrist and Lehner, 1997; Cebolla et al., 1999; Schaeffer et al., 2004; Lasorella et al., 2006; Binné et al., 2007; Lammens et al., 2008; Narbonne-Reveau et al., 2008; Larson-Rabin et al., 2009). In plants, no data are available on the timing of APC/C activation by CCS52A, but the expression of *CCS52A1* and

CCS52A2 is known to be transcriptionally regulated, with expression levels peaking between late M and early G2 (Fülöp et al., 2005). Previously, we found that *CCS52A2*, but not *CCS52A1*, transcription is regulated by the E2Fe/DEL1 transcription factor (Lammens et al., 2008), indicating that both *CCS52A* genes are under different genetic controls. Moreover, in roots, CCS52A1, rather than CCS52A2, has been found to control endocycle onset (M. Vanstraelen and E. Kondorosi, unpublished data). Furthermore, stabilization of CYCA2;3-GFP within a *ccs52a1-1* context is most evident in the transition zone, a region with high *CCS52A1* expression (M. Vanstraelen and E. Kondorosi, unpublished data), and probably marks the site of endoreduplication onset in Arabidopsis roots. Altogether, these data indicate that CCS52A1 and CCS52A2 might operate in a tissue-specific manner, rather than being redundant. Unfortunately, the low levels of CYCA2;3-GFP did not allow us to test the effects of *CCS52A1* and *CCS52A2* knockout on CYCA2;3-GFP stability in leaves.

In conclusion, CDKB1;1 complexed with CYCA2;3 prevents cells from exiting the mitotic cell cycle, probably by repressing relicensing of DNA replication during the S-to-G2 transition and boosting CDK activity at the G2-to-M transition. Furthermore, we demonstrated that CDKB1;1-CYCA2;3 complex activity is mainly determined by the availability of the CYCA2;3 protein, whose abundance is controlled by $APC/C^{CCS52A1}$.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Col-0) were grown at 22°C and a 16-h photoperiod (65 μE m^{-2} s^{-1}) on half-strength Murashige and Skoog medium containing 0.5 g L^{-1} MES, 10 g L^{-1} Suc, and 0.8% plant tissue culture agar, unless noted otherwise. The *CDKB1;1* and *CDKB1;1.N161* lines (Boudolf et al., 2004a), *ccs52a1-1* and *ccs52a2-1* lines (Lammens et al., 2008), and *CYCA2;3-GFP* and *mDB-CYCA2;3-GFP* lines (Imai et al., 2006) have been described elsewhere.

Yeast Two-Hybrid Analysis

For a yeast two-hybrid screening, a fusion between the GAL4 DNA-binding domain and *CDKB1;1.N161* was used as bait. The cDNA for *CDKB1;1.N161* was obtained by mutation of the codon GAT (Asp-161) for AAT (Asn-161) by in vitro mutagenesis (Porceddu et al., 1999). Vectors and strains used were provided with the Matchmaker Two-Hybrid System (Clontech). The baits were constructed by inserting the *CDKB1;1.N161* PCR fragment into the pGBT9 vector. The *CDKB1;1.N161* PCR fragment was created from the cDNA using primers to incorporate *Eco*RI restriction enzyme sites (5′-CGGATCC-GAATTCATGGAGAACGAG-3′ and 5′-CGGATCCGAATTCTCAGAACT-GAGA-3′). The *CDKB1;1.N161* PCR fragment was cut with *Eco*RI and cloned into the *Eco*RI site of pGBT9, resulting in the plasmid pGBTCDKBDN. The GAL4 activation domain cDNA fusion library was obtained from mRNA of Arabidopsis cell suspensions harvested at various growing stages: early exponential, exponential, early stationary, and stationary phases (De Veylder et al., 1999). For the screening, a 500-mL culture of the *Saccharomyces cerevisiae* strain HF7c (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3 URA3::$GAL4_{17mers(3x)}$-$CyC1_{TATA}$-LacZ*) was cotransformed with 250 μg of pGBTCDKBDN, 500 μg of DNA from the library, and 20 μg of herring testes carrier DNA, according to the lithium acetate method (Gietz et al., 1992). To estimate the number of independent cotransformants, 1‰ of the transformation mix was plated on Leu and Trp medium. The rest of the transformation mix was plated on medium to select for His prototrophy (Trp, Leu, and His). After 4 d of growth at 30°C, the colonies larger than 2 mm were streaked on His-lacking medium. Of the His^+ colonies, the activation domain of the plasmids was isolated as described (Hoffman and Winston, 1987). The pGAD10 inserts were amplified by PCR using the primers 5′-ATACCACTACAATGGATG-3′ and 5′-AGT-TGAAGTGAACTTGCGGG-3′. Plasmid DNA was electroporated into *Escherichia coli* XL1-Blue, and the DNA sequences of the inserts were determined. Extracted DNA was also used to retransform HF7c to test the specificity of the interaction.

Tandem Affinity Purification and Analysis

Essentially, all steps were as described (Van Leene et al., 2007), including the Arabidopsis cell suspension cultivation, transformation and selection, protein extraction, and subsequent TAP. In short, the sequences coding for *CYCA2;2* and *CYCA2;3* were cloned by recombination into the pKNTAP vector, generating Pro-35S:TAP-CYCA2;2 and Pro-35S:TAP-CYCA2;3 cassettes (pKNTAPCYCA2;2 and pKNTAPCYCA2;3). Arabidopsis cell suspension cultures were stably transformed by *Agrobacterium tumefaciens*-mediated cocultivation with pKNTAPCYCA2;2 and pKNTAPCYCA2;3. Transformed Arabidopsis cells were selected and transferred to a liquid medium for upscaling. Expression levels of TAP-tagged proteins were checked by protein blotting with an anti-calmodulin-binding protein antibody (data not shown). In the first round of affinity purification, protein extracts of 15 g of plant material were incubated with an IgG resin. Bound complexes were released and eluted from the resin by tag cleavage with tobacco etch virus protease. In the second affinity step on a calmodulin agarose column, coeluting noninteracting proteins and the tobacco etch virus protease were removed with the flow through. Finally, both the CYCA2;2 and CYCA2;3 baits and interacting proteins were eluted from the calmodulin agarose through EGTA-mediated calcium removal. Eluted proteins were separated on 4% to 12% NuPAGE gels, excised, and analyzed by matrix-assisted laser-desorption ionization time-of-flight/time-of-flight mass spectrometry as described (Van Leene et al., 2007). To increase the stringency of the data set, contaminating proteins due to the experimental background as determined by Van Leene et al. (2007) were systematically subtracted from the lists of copurified proteins.

BiFC Construct Cloning

The BiFC constructs were obtained through recombinational Gateway cloning (Invitrogen). The GFP moieties were created by PCR for N- and C-terminal parts of the fluorescent molecule. DNA sequences were generated that encoded an N-terminal part of the eGFP, designated nGFP (1–465 bp, amino acids 1–155) and a C-terminal part, cGFP (466–717 bp, amino acids 156–239). No linker sequence was included. All full-length open reading frames (ORFs) of the cell cycle proteins of interest were recombined into the pDONR221 entry vector (Invitrogen) by a BP reaction. A multisite Gateway reaction resulted in translational fusions between the cell cycle ORFs and the moieties of GFP driven by the 35S promoter. The expression clones were generated in the pH7m34GW and pK7m34GW destination vectors (for the ORF::cGFP and ORF::nGFP fusions, respectively).

Tobacco Infiltration

Wild-type tobacco (*Nicotiana benthamiana*) plants were grown under a normal light regime (14 h of light, 10 h of darkness) at 25°C and 70% relative humidity. All BiFC constructs were transferred into the *A. tumefaciens* LBA4404 strain harboring the VirG plasmid. The obtained *A. tumefaciens* strains were used to infiltrate tobacco leaves. The transient expression assay on tobacco leaves was done according to Batoko et al. (2000) with minor modifications. The transformed agrobacteria harboring the constructs of interest were grown for 2 d in a shaking incubator (200 rpm) at 28°C in 5 mL of yeast extract broth medium supplemented with appropriate antibiotics (100 μg mL^{-1} spectinomycin and 40 μg mL^{-1} gentamycin). After incubation, 2 mL of well-grown bacterial culture was transferred to Eppendorf tubes and centrifuged (10 min, 5,200*g*). The pellets were washed twice with 1 mL of infiltration buffer (50 mM MES, 2 mM Na_3PO_4, and 0.5% Glc, pH 5.6). The final pellet was resuspended in the infiltration buffer supplemented with 100 μM acetosyringone. The bacterial suspension was diluted with the same supplemented buffer to adjust the inoculum concentration to the final optical density at 600 nm value (dilution series from 0.5 to 0.1). For coexpression experiments,

500 mL of each bacterial culture was mixed prior to the leaf infiltration, with the inoculum of each construct adjusted to the required final optical density at 600 nm value. The inoculum was delivered to tobacco leaves by gentle pressure infiltration of the lower epidermis with a 1-mL syringe without a needle. The infiltrated area of the leaf was delimited and labeled with an indelible pen. The plant was incubated under normal growing conditions, and results were analyzed 3 to 5 d after infiltration.

Confocal Microscopy

Transgenic Arabidopsis plants and transfected tobacco leaves were assayed for fluorescence with a confocal microscope (100M) with software package LSM 510 version 3.2 (Zeiss) and a confocal microscope (Olympus FluoView FV1000) equipped with a 63× water-corrected objective (numerical aperture of 1.2) to scan the cells. Emission fluorescence was captured in the frame-scanning mode alternating GFP fluorescence via a 500- to 550-nm band-pass emission filter.

Flow Cytometry

Flow cytometry was done according to Boudolf et al. (2004b). The EI was calculated from the number of nuclei of each represented ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level.

Light Microscopy

Cotyledons were harvested, cleared overnight in ethanol, and stored in lactic acid for further use. Cotyledons were mounted on slides, covered, and observed with a microscope fitted with differential interference contrast optics (DMLB; Leica). Digital images were handled with Adobe Photoshop 6.

Propidium Iodide Staining

The vital dye propidium iodide (PI) was used to stain cell walls. Plant material was incubated for 2 min in a working solution of 10 μM PI in water. Accumulation of PI was observed under epifluorescence light with the appropriate filter set (excitation filter, 535–550 nm; dichroic mirror, 565 nm; barrier filter, 590 nm).

Kinase Assay

Agarose-conjugated anti-GFP monoclonal antibody was washed three times with 500 μL of cold lysis buffer containing 50 mM Tris-HCl (pH 7.2), 250 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA, 10% glycerol, and one tablet 10 mL^{-1} protease inhibitor cocktail (Roche). Protein extracts were prepared from 7-d-old seedlings, induced for 2 d on agar medium containing 10 μM β-estradiol, of Arabidopsis Col-0 and from F1 crosses between lines harboring a β-estradiol-inducible construct overexpressing *CYCA2;3-GFP* and Col-0 (Col-0 × *CYCA2;3*) and plants overexpressing either *CDKA;1* or *CDKB1;1* (*CDKA;1* × *CYCA2;3* and *CDKB1;1* × *CYCA2;3*). In a total volume of 200 μL of homogenization buffer containing 25 mM Tris-HCl (pH 7.6), 60 mM β-glycerophosphate, 15 mM nitrophenyl phosphate, 15 mM EGTA (pH 8), 15 mM $MgCl_2$, 85 mM NaCl, 1 mM dithiothreitol, 0.1 mM vanadate, 1 mM NaF, 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, one tablet 10 mL^{-1} protease inhibitor cocktail (Roche), and 530 μg of protein extract was loaded on 30 μL of 50% gel slurry of agarose-conjugated anti-GFP monoclonal antibody and incubated on a rotating wheel for 2 h at 4°C. After a brief centrifugation at 2,500*g* and removal of the supernatant, the beads were carefully washed three times with cold lysis buffer and once with kinase buffer (50 mM Tris-HCl [pH 7.8], 15 mM $MgCl_2$, 5 mM EGTA, and 2 mM dithiothreitol). The supernatant was removed carefully. The histone H1 kinase reactions were initiated by resuspending the anti-GFP agarose pellets with 35 μL of the reaction mixture containing 5 μCi of [γ-^{33}P]ATP (3,000 Ci $mmol^{-1}$), 0.5 mg mL^{-1} histone H1, 50 mM Tris-HCl (pH 7.8), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 60 μg mL^{-1} cAMP-dependent kinase inhibitor, and 10 μM ATP. After 20 min of incubation at 30°C, the kinase reactions were stopped by the addition of 10× SDS-PAGE loading buffer. Aliquots were boiled, loaded on a 12% (w/v) acrylamide gel, and stained by Coomassie Brilliant Blue. The gel was dried overnight, and incorporation of [γ-^{33}P]ATP into histone H1 was detected by autoradiography. The background signal measured in the Col-0 sample was subtracted from the signals obtained with the other samples.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of coexpression of *CYCA2;3-nGFP* and *CDKB1;1-cGFP*.

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