Two *Arabidopsis* cyclin A3s possess G1 cyclin-like features

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Abstract

A-type cyclins (CYCAs) are a type of mitotic cyclin and are closely related to cyclin B. Plant CYCAs are classified into 3 subtypes (*CYCA1–CYCA3*), among which *CYCA3* has been suggested to show a biased expression during the G1-to-S phase. We characterised *Arabidopsis CYCA3*s (*CYCA3;1–CYCA3;4*) in terms of expression pattern and protein function. *CYCA3;1* and *CYCA3;2* transcripts were highly accumulated at the G1/S phase, whereas *CYCA3;4* was constantly expressed during the cell cycle. Expressions of *CYCA3;1* and *CYCA3;2* were observed in actively dividing tissues such as root and shoot apical meristems and lateral root primordia. Overexpression of *CYCA3;1* or *CYCA3;2* distorted apical dominance in *Arabidopsis*, indicating that they have critical functions in shoot meristems. In insect cells, *CYCA3;1* formed an active kinase complex with *CDKA;1*, an orthologue of the yeast Cdc2/Cdc28p, and phosphorylated retinoblastoma-related protein, a key regulator in the transition from the G1 to the S phase. Our results suggest that *Arabidopsis CYCA3;1* and *CYCA3;2* are distinct members of the G1 cyclin family that play an important role in meristematic tissues.

Keywords

Cell cycle; Cyclin-dependent kinase; Cyclin A; RBR protein; *Arabidopsis thaliana*; Apical dominance

Abbreviations

CaMV: cauliflower mosaic virus; *CDK*: cyclin-dependent kinase; *CYC*: cyclin; *GFP*: green fluorescent protein; *GST*: glutathione *S*-transferase; *His*: histidine; *MS*: Murashige and Skoog;

ORF: open reading frame; *Rb*: retinoblastoma; *RBR*: retinoblastoma-related; *RT-PCR*: reverse transcription-polymerase chain reaction

Introduction

Plant cells are surrounded by rigid cell walls and thus usually stay where they were generated, unable to move within tissues. This indicates the importance of cell proliferation depending on spatiotemporal regulation of cell division. The strict control of cell proliferation has been suggested previously; for example, *Arabidopsis* transgenic plants that overexpressed a mitotic cyclin produced roots with accelerated growth without inducing neoplasia (Doerner et al. 1996). Cyclins play a dominant role in activating cyclin-dependent kinases (CDKs) during cell cycle progression. In plants, A-, B-, and D-type cyclins, designated as CYCA, CYCB, and CYCD, respectively, were classified according to similarity to animal cyclins (Renaudin et al. 1996; 1998). A- and B-type cyclins are expressed from the S to the M phase and control DNA replication, the G2/M transition, and mitosis; the D-type cyclins, however, are assumed to be sensors of external signals and to be involved in deciding whether cells continue to divide or start to differentiate. In animals, cyclin D forms active kinase complexes with CDK4 and CDK6, which phosphorylate retinoblastoma (Rb) protein and inactivate its suppressor function on the transcription factors E2F and DP; this leads to progression from the G1 to the S phase (Harbour and Dean 2000). Previous reports have suggested that a similar Rb/E2F/DP pathway is also conserved in plants (Nakagami et al. 1999; Shen 2002). Mitogen-induced signals stimulate cyclin D-CDK complexes at multiple levels, including those of gene transcription, translation, protein stability, and assembly and import of these complexes into the nucleus (Sherr and Roberts 2004). Subsequently, active cyclin D-CDK complexes promote progression from the G1 to the S phase and thus enhance cell proliferation. The proliferative functions of cyclin D have been also suggested by using transgenic plants that overexpressed *CYCD2*, *CYCD3*, or *CYCD4* and *Arabidopsis cycd3* or *cycd4* mutants

(Cockcroft et al. 2000; Dewitte et al. 2003; Masubelele et al. 2005; Kono et al. 2007; Qi and John 2007).

CYCAs are a kind of mitotic cyclin and are closely related to CYCBs. In plants, they are further classified into 3 subtypes, namely, *CYCA1*, *CYCA2,* and *CYCA3* (Renaudin et al. 1996; 1998; Chaubet-Gigot, 2000). Reichheld et al. (1996) showed that tobacco *CYCA1* and *CYCA2* are expressed from the mid-S phase to the mid-M phase, whereas *CYCA3* expression is induced at the G1/S transition and continues throughout the S and G2 phases. Similar expression patterns were also observed in *Arabidopsis* suspension cultured cells; *CYCA3* is expressed from G1/S and during the S phase, whereas *CYCA1* and *CYCA2* exhibit a peak of expression at G2/M phase (Menges et al. 2005). Among the 3 types, *CYCA2* has been relatively well studied: *Medicago sativa CYCA2* displayed a first peak of kinase activity in the S phase and a major peak at the G2/M transition (Roudier et al. 2000). Alfalfa *CYCA2;2* is expressed in meristems and in proliferating cells in the lateral root and nodule primordia (Roudier et al. 2000); in contrast, *Arabidopsis CYCA2;1* is expressed not only in dividing cells but also in non-dividing cells, such as pericycle cells and vascular tissues (Burssens et al. 2000). Interestingly, expressions of these *CYCA2*s are upregulated by auxins, suggesting that *CYCA2* may transmit the auxin signal to cell cycle machineries. In *Arabidopsis*, knockout of *CYCA2;3* promoted endocycles and increased the ploidy levels achieved in mature organs, whereas its overexpression restrained endocycles in a dose-dependent manner (Imai et al. 2006). This indicates a suppressive function of *CYCA2* in endocycles that is reminiscent of animal cyclin A, which downregulates the components of pre-replication complex and inhibits re-replication in the cell cycle (Coverley et al. 2000; 2002; Nishitani et al. 2004; Sugimoto et al. 2004).

As described above, a distinct function of *CYCA3* had been assumed based on its early activated expression at the G1/S transition. The promotive function of *CYCA3* in cell proliferation was first observed by local and transient overexpression of *CYCA3;2* in tobacco, which induced cell division in both the shoot apical meristem and the leaf primordia (Wyrzykowska et al. 2002). Yu et al. (2003) reported that antisense expression of *CYCA3;2* in tobacco plants induced defects in embryo formation and impaired callus formation from leaf explants, although it remains unknown whether other tobacco *CYCA3* homologues were also downregulated in these transgenic plants. These studies suggest that plant *CYCA3* may have a specific function at the G1/S phase, whereas a single type of cyclin A is required for cell cycle regulation from the S to M phase in animal somatic cells (Yang et al. 1999). In this paper, we characterised *Arabidopsis CYCA3s* in terms of expression pattern and protein function and observed the overexpression phenotypes of each. We propose that *CYCA3;1* and *CYCA3;2* are possible G1 cyclins that control cell proliferation in meristems.

Materials and Methods

Plant material

Arabidopsis plants (ecotype Col-0) were grown in Murashige and Skoog (MS) medium [0.5× MS salts, 1× MS vitamins, and 2% (w/v) sucrose (pH 6.3)] under continuous light conditions at 23°C; they were then transferred onto soil and grown under continuous light conditions at 23°C. Transformation of *Arabidopsis* plants was conducted via *Agrobacterium*-mediated transformation (Clough and Bent 1998). The T-DNA insertion mutants of *CYCA3;1* and *CYCA3;2* were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA); the seed stock numbers of *cyca3;1-1* and *cyca3;2-1* were CS853906 and SALK_57522,

respectively. The insertions were examined by genomic polymerase chain reaction (PCR) with Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) by using a set of primers that hybridise to the T-DNA and the *CYCA3* genes: 5′-AACGTCCGCAATGTGTTATTAAGTTGTC-3′ and 5′-CAGGCATTGTCGCTACACACTTGAA-3′ were used for *cyca3;1-1*, and 5′-GGATTTTCGCCTGCTGGGGCAAACCAGCG-3′ and 5′-CGAACCTATGCTAGCTGCTTTCCAC-3′ were used for *cyca3;2-1*. A suspension culture of the *Arabidopsis* cell line MM2d was maintained and synchronised using aphidicolin, as described by Menges and Murray (2002). A flow cytometry analysis was conducted with a Ploidy Analyzer (PARTEC, Münster, Germany).

Reverse transcription-polymerase chain reaction

Gene expressions in suspension cultured cells were analysed by reverse transcription-PCR (RT-PCR) using the TITANIUM One-Step RT-PCR kit (TaKaRa) according to the manufacturer's protocol. In a total reaction volume of 50 µl, 0.8 µg of RNA was used. The primers used for PCR reactions are listed in Supplemental Table 1. The PCR conditions were as follows: 1 cycle at 50°C for 60 min and at 94°C for 5 min; 20–26 cycles at 94°C for 30 s, at 65°C for 30 s, and at 68°C for 1 min; and 1 cycle at 68°C for 2 min. RT-PCR with *Arabidopsis* seedlings was conducted as follows: cDNA was synthesised with total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA USA). In a total reaction volume of 20 µl, 2 µg of RNA was used. For PCR reactions, Ex Taq DNA polymerase and the primers listed in Supplemental Table 1 were used. PCR conditions were as follows: 1 cycle at 94°C for 2 min; 40 cycles at 98°C for 10 s, at 60°C for 30 s, and at 68°C for 90 s; and 1 cycle

at 68°C for 7 min.

In situ RNA hybridisation

Arabidopsis tissues of 10-day-old seedlings were fixed in FAA (50% (v/v) ethanol, 5% (v/v) acetic acid, and 3.7% (v/v) formaldehyde) and 8-μm paraffin blocks were cut. The sections were hybridised with digoxygenin-labeled probes as described previously (Braissant and Wahli 1998). The *CYCA3;1* probe corresponds to the region 1 to 219 of the *CYCA3;1* open reading frame (ORF). The histone H4 probe was a gift from Dr. Taku Takahashi (Okayama University).

β-Glucuronidase staining

The promoter fragments of *CYCA3;1* and *CYCA3;2* were PCR-amplified from 2000 bp to 1 bp upstream of the start codon, and cloned into the Gateway entry vector pDONR221 (Invitrogen) by an BP reaction. An LR reaction was then performed with the destination vector pGWB3 (Nakagawa et al. 2007) to generate a binary vector carrying the fusion construct with β-glucuronidase (*GUS*). Transgenic plants carrying the *GUS*-fusion constructs were incubated in 90% (v/v) acetone at –20°C overnight and washed in 100 mM sodium phosphate buffer (pH 7.0). For GUS staining, samples were incubated in a solution [100 mM sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 0.5 mg/mL 5-bromo-4-chrolo-3-indolyl-β-D-glucuronide (pH 7.0)] at 37°C for 2–16 h and then mounted in a mixture of chloral hydrate, glycerol, and water (8 g:1 mL:2 mL).

Overexpression in *Arabidopsis*

The ORFs of *CYCA3;1* and *CYCA3:2* without the stop codon were PCR-amplified and cloned into the entry vector pENTR-D/TOPO (Invitrogen). An LR reaction was then performed with the destination vector pGWB5 or pGBW11 (Nakagawa et al. 2007) to produce a binary vector carrying the C-terminal fusion construct with green fluorescent protein (GFP) or FLAG, respectively, under the cauliflower mosaic virus (CaMV) 35S promoter. Overexpression of GFP- or FALG-tagged proteins were examined by RT-PCR with a set of primers: the forward and reverse primers for *CYCA3;1*, and 5′-ATGACAGAGCAAGAGATCTG-3′ and the reverse primer for *CYCA3;2* (Supplemental Table 1).

Protein expression in insect cells

The ORF of *CYCA3;1* was PCR-amplified with a *Bam*HI site at the N-terminal end and a *Not*I site at the C-terminal end. The fragment was cloned into the *Bam*HI/*Not*I site of pFASTBAC1-FLAG1 (Yamaguchi et al. 2000) and was in frame with the FLAG sequence. The plasmids used for histidine (His)-tagged CDK expression were as described previously (Kono et al. 2003; 2006). Transfection of insect Sf9 cells, immunoblotting and kinase assays were performed, as described by Kono et al. (2003). Anti-CDK antibodies were used for immunoprecipitation (Umeda et al. 2000; Kono et al. 2003; Takatsuka et al. 2009). Anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) was used for the detection of *CYCA3;1*-FLAG. For expression of the C-terminal portion of the retinoblastoma-related (RBR) protein in *E. coli*, we used the 465-bp fragment at the 3′-end of the ORF that was cloned into the expression vector pGEX4T-1 (a gift from Dr. Masami Sekine, Ishikawa Prefectural University). The RBR protein fused to glutathione *S*-transferase (GST) was purified with Glutathione Sepharose according to the manufacturer's protocol (GE Healthcare,

Buckinghamshire, UK).

Results and Discussion

Expression patterns of *CYCA3* during the cell cycle

Arabidopsis has 4 homologues of *CYCA3* genes, namely *CYCA3;1–CYCA3;4*. Among them, expression of *CYCA3;3* was not detected on Affymetrix 8K or ATH1 arrays (Menges et al. 2005). We also could not detect the *CYCA3;3* transcripts by RT-PCR; thus, here we focused on the other 3 *CYCA3*s to confirm their temporal expression patterns during the cell cycle. An *Arabidopsis* MM2d cell culture was synchronised with aphidicolin. Flow cytometry analysis revealed a prominent peak of cells in G2, 4 h after aphidicolin removal (Fig. 1a). This indicated that the majority of cells proceeded synchronously through the S phase. RT-PCR analysis revealed that *CDKB2;1* transcripts, which accumulate from the G2 to the M phase (Kono et al. 2003; Mészáros et al. 2000), started to increase after 8 h (Fig. 1b), indicating that cells at this stage were in the G2 phase. After 16 h, the G1 phase cells started to accumulate and *CDKB2;1* expression began to decrease. Expressions of *CYCA3;1* and *CYCA3;2* were observed just after the release from the aphidicolin block; the transcripts decreased afterwards but never disappeared (Fig. 1b). They increased again after 22 h; at this point, the G1 phase cells had abundantly accumulated. In contrast, no significant change in the *CYCA3;4* transcript level was observed during the course of this experiment (Fig. 1b). These results indicate that expression of *CYCA3;1* and *CYCA3;2* is upregulated at the G1/S phase while *CYCA3;4* is constantly expressed during the cell cycle. Similar expression patterns have been also observed in microarray analyses (Menges et al. 2005). G1/S-biased expression of *CYCA3* was also suggested in tobacco (Reichheld et al. 1996), indicating that it may be a

characteristic feature of some members of plant *CYCA3*.

Expression patterns of *CYCA3;1* and *CYCA3;2* in *Arabidopsis* tissues

To understand the functional role of *CYCA3;1* and *CYCA3;2* that showed peak expression at the G1-to-S phase, we first examined the expression pattern in plant tissues by generating the *GUS* gene fused to the 2-kb promoter region of *CYCA3;1 or CYCA3;2*. The data obtained for the reporter lines of the 2 *CYCA3*s were almost identical; thus, we report the representative results obtained for the *pCYCA3;1::GUS* plants. As shown in Fig. 2, GUS expression was observed in actively dividing tissues, such as the root apical meristem, lateral root primordia, emerging lateral roots, and vascular bundles. The lamina of young leaves but not of mature leaves was GUS-stained, suggesting a positive correlation with cell proliferation (Fig. 2c). GUS expression was also observed in young flowers, especially in pistils, and some weak expression was detected in anthers (Fig. 2d). However, no staining was observed in sepals, petals, or stamens.

To examine the spatial expression pattern of *CYCA3;1* in shoot apices, we performed *in situ* hybridisations using probes specific for transcripts of *CYCA3;1*. RNA probes were prepared from cDNA and labeled with digoxigenin. As shown in Fig. 3a, hybridisation signals were observed in the vegetative shoot apical meristem, young leaf primordia, and vascular tissues in growing leaves. The control histone H4 probe produced a patchy pattern of signals reflecting S-phase-specific expression (Fig. 3b). No signal was detected with the control sense probe of *CYCA3;1* in shoot apices (data not shown). These results indicate that *CYCA3;1* and *CYCA3;2* control cell proliferation primarily in actively dividing tissues.

Subcellular localisation of *CYCA3;1* and *CYCA3;2*

To examine the localisation of *CYCA3;1* and *CYCA3;2*, both cDNAs were fused in frame to the *GFP* gene and cloned under the CaMV 35S promoter. Roots of transgenic plants carrying the reporter genes were subjected to microscopic observation to detect GFP fluorescence. Both *CYCA3;1*-GFP and *CYCA3;2*-GFP fusion proteins were located exclusively in the nuclei, and dots with strong fluorescence were observed in each nucleus (Fig. 4). Similar results were reported for tobacco *CYCA3;2* that was fused to GFP and expressed in *Arabidopsis* (Yu et al. 2003). These results indicate that plant *CYCA3* may have a specific function in the nuclei by occupying particular nuclear territories. Further analyses with different marker proteins that label distinct nuclear domains will reveal the precise localisation of *CYCA3*s and give a hint of their novel function in the nucleus. As suggested by Yu et al. (2003), GFP-fusion proteins were mainly detected in the proximal portion of the roots while the root tips did not show significant GFP fluorescence (data not shown). This indicates that *CYCA3;1* and *CYCA3;2* are highly expressed in actively dividing cells as described above but that their products may have a short half-life in meristems.

CDKA;1* forms an active kinase complex with *CYCA3;1

Almost no information was available regarding to *CYCA3*-containing kinase complexes and their enzyme activities. Therefore, FLAG-tagged *CYCA3;1* and 6x His-tagged CDKs were expressed in insect cells via a baculovirus-mediated system and examined for complex formation. We also tried to express FLAG-tagged *CYCA3;2* in insect cells, but failed to get a detectable level of protein. Thus we focused on *CYCA3;1*. Protein extract was immunoprecipitated with anti-CDK antibodies that specifically recognise *CDKA*, *CDKB1*,

and *CDKB2*, respectively (Kono et al. 2003; Shimotohno et al. 2003; Umeda et al. 1998; Takatsuka et al. 2009), and assayed by western blotting. His-*CDKA;1*, His-*CDKB1;1* and His-*CDKB2;1* were properly immunoprecipitated by each antibody, and *CYCA3;1*-FLAG was co-precipitated when it was co-expressed with *CDKA;1* but not with *CDKB1;1* or *CDKB2;1* (Fig. 5). This result indicates that His-*CDKA;1* forms a complex with *CYCA3;1*-FLAG. Immunoprecipitates with the anti-*CDKA;1* antibody were then subjected to kinase assay using histone H1 and the *Arabidopsis* RBR protein fused to GST as substrates. We used the C-terminal portion of RBR that contains a cluster of consensus CDK phosphorylation sites (Sherr 1996; Boniotti and Gutierrez 2001). Intense bands of phosphorylation were detected for both substrates with immunoprecipitates containing His-*CDKA;1* and *CYCA3;1*-FLAG, while no phosphorylation was observed with those containing only His-*CDKA;1* (Fig. 5). These results indicate that His-*CDKA;1* was activated by forming a complex with *CYCA3;1*-FLAG in insect cells. It is noteworthy that the *CYCA3;1*-*CDKA;1* complex phosphorylated the RBR protein that functions at the transition from G1 to S, during which *CYCA3;1* and *CYCA3;2* showed expression peaks as described above.

Overexpression of *CYCA3;1* or *CYCA3;2* disturbs apical dominance

To reveal the biological function of *CYCA3;1* and *CYCA3;2*, we identified *Arabidopsis* mutants from T-DNA insertion collections. T-DNAs were inserted into the 2nd exons of *CYCA3;1* and *CYCA3;2* (Supplemental Fig. 1a). RT-PCR with the *CYCA3;1* mutant (hereafter called *cyca3;1-1*) showed that the cDNA upstream to the T-DNA insertion site was amplified from the mRNA of the shoots; however, the downstream region was not amplified at all (Supplemental Fig. 1b). RT-PCR with the *CYCA3;2* mutant (hereafter called *cyca3;2-1*)

showed that the cDNA neither upstream nor downstream to the T-DNA insertion site was amplified (Supplemental Fig. 1b). Both mutants showed no developmental defects throughout the life cycle under normal growth conditions (Supplemental Fig. 1c). We made the double mutants of *cyca3;1-1* and *cyca3;2-1* but did not see a distinct phenotype (Supplemental Fig. 1c).

We then overexpressed FLAG-tagged *CYCA3;1* or *CYCA3;2* in *Arabidopsis* using the constitutive CaMV 35S promoter. RT-PCR showed a high accumulation of transcripts of either cDNA in transgenic plants (Fig. 6a). Although we could not find any obvious change in plant growth or organ shape, distortion of apical dominance was observed in both *CYCA3;1*- and *CYCA3;2*-overexpressing plants: apical buds were terminated earlier than those of wild-type plants, and lateral shoots grew well compared to main shoots (Fig. 6b). A high proliferation of lateral shoots was also observed when tobacco *CYCA3;2* was overexpressed in *Arabidopsis* (Yu et al. 2003). In these transgenic plants, inflorescence ceased elongation by a higher accumulation of *CYCA3;2* protein. This and our results indicate that *Arabidopsis CYCA3;1* and *CYCA3;2* and tobacco *CYCA3;2* may have differential functions in meristems between the main shoot and lateral buds. This may originate from differences in interacting partners that control *CYCA3-CDKA* activity and/or substrates in distinct tissues. Further investigations will be required to reveal how *CYCA3* exerts its functions in controlling shoot architecture during plant development.

So far, *CYCD* has been assumed as the main G1 cyclin that controls the G1-to-S phase progression in plants. The reason would be that only *CYCD*s were reported to interact with *CDKA* and phosphorylate the RBR protein (Huntley et al. 1998; Nakagami et al. 1999; 2002; Boniotti and Gutierrez 2001; Gutiérrez et al. 2005). Most of the plant *CYCD*s retains the

conserved RBR-binding motif (LXCXE), but a few of them have altered sequences; for example, *Arabidopsis CYCD4;2* has the sequences of LVSNF or LESEE (Kono et al. 2006). However, we found that *CYCD4;2–CDKA;1* produced in insect cells can phosphorylate RBR *in vitro* (Kono and Umeda, unpublished results). In this paper, we showed that *Arabidopsis CYCA3;1* and *CYCA3;2*, which do not possess the LXCXE motif either, bind to *CDKA;1* and phosphorylate RBR. These results indicate that the RBR-binding motif may not be necessarily required for plant cyclins to interact with RBR. In animals, it is known that the RBR function depends on its differential phosphorylation state, thus it is likely that *CYCD*- and *CYCA3*-associated kinases are responsible for RBR phosphorylation of distinct sites and provide it with a large variety of functions in the cell cycle and chromatin remodeling.

We also showed that the transcripts of *CYCA3;1* and *CYCA3;2* are highly accumulated at the G1/S phase. The preferential expression in meristematic tissues and distortion of apical dominance by overexpression of *CYCA3;1* or *CYCA3;2* further indicate their critical functions in mitotic division. Therefore, these 2 *CYCA3*s are assumed to be another type of G1 cyclin that plays an important role in controlling cell division and differentiation. On the other hand, *CYCA3;4* is constantly expressed during the cell cycle, and its overexpression did not cause a macroscopic phenotype (Kojima and Umeda, data not shown). This suggests that *CYCA3;4* may have a distinct function from *CYCA3;1* or *CYCA3;2*, thus *Arabidopsis CYCA3*s diverge in terms of molecular function. The double mutants of *cyca3;1-1* and *cyca3;2-1* did not display any distinct phenotype, indicating that some *CYCD*s may have redundant functions. Generation of various combinations of *cyca3* and *cycd* mutants will give us a hint how their differential functions are involved in spatiotemporal regulation of cell proliferation in response to internal and external signals.

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Figure legends

Fig. 1 Expression pattern of *CYCA3* genes during the cell cycle. **a.** Flow cytometry analysis of *Arabidopsis* MM2d suspension culture. Time after release from aphidicolin block is indicated. **b.** RT-PCR analysis of *CYCA3*, *CDKB2;1* and tubulin β (*TUB4*) genes. RNA samples were prepared at 2-h intervals from MM2d cells after aphidicolin removal. Optimized numbers of PCR cycles were 25 cycles for *CYCA3;1* and *CYCA3;4*, 26 cycles for *CYCA3;2*, 24 cycles for *CDKB2;1* and 20 cycles for *TUB4*.

Fig. 2 Spatial expression pattern of *CYCA3;1* showing GUS staining of transgenic plants harboring *pCYCA3;1::GUS*. **a., b.** Three-day-old seedlings and cotyledones, respectively. Bar = 1 mm (a), 0.5 mm (b). **c.** Nine-day-old seedlings. Bar = 5 mm. **d.** Flowers. Bar = 1 mm. **e.** Primary root. Bar = 50 μm. **f.** Lateral root primordium. Bar = 50 μm. **g.** Emerging lateral root. Bar = 100 μm.

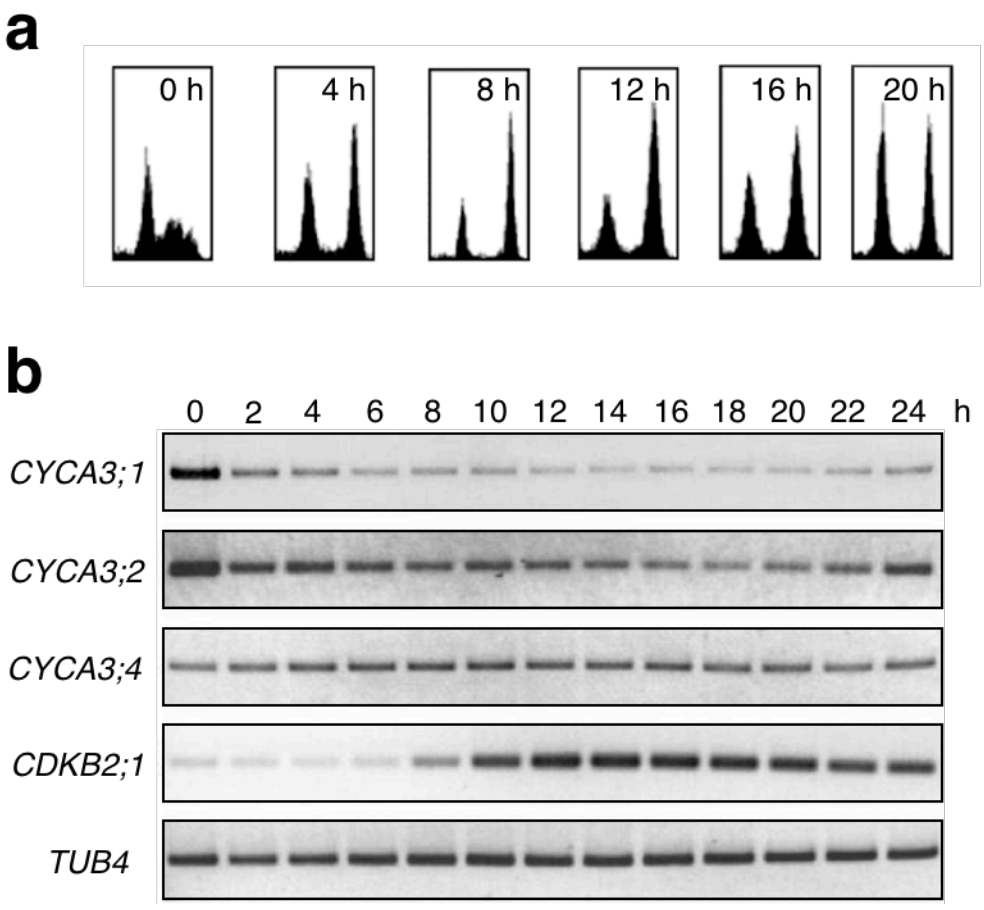
Fig. 3 *In situ* hybridization of *CYCA3;1* (a) and histone H4 (b). Antisense riboprobes were labeled with digoxygenin and hybridized with longitudinal sections through shoot apices of 10-day-old seedlings. Bars = 100 μm.

Fig. 4 Subcellular localization of GFP-fused *CYCA3;1* and *CYCA3;2*. GFP fluorescence in transgenic plants harboring *p35S::CYCA3;1-GFP* (a) or *p35S::CYCA3;2-GFP* (b) was observed in the root elongation zone (right). Bright-field images are shown in the left. Arrowheads indicate corresponding cells. Bars = 50 μm.

Fig. 5 Activation of *CDKA;1* by interaction with *CYCA3;1* in insect cells. His-tagged CDKs and FLAG-tagged *CYCA3;1* were expressed in insect Sf9 cells and protein extract was immunoprecipitated with anti-CDK antibody (IP). Immunoprecipitates were then immunoblotted with anti-CDK or anti-FLAG antibody (IB) and subjected to the kinase assay using histone H1- or GST-fused RBR as a substrate.

Fig. 6 Overexpression of *CYCA3;1* or *CYCA3;2* in *Arabidopsis*. FLAG-tagged *CYCA3;1* or *CYCA3;2* was overexpressed under the CaMV 35S promoter. **a.** RT-PCR analysis of transgenic lines using primers specific to *CYCA3;1* or *CYCA3;2*. Total RNA prepared from wild-type plants (WT) was used as a control. **b.** Plant architecture of *CYCA3;1*- or *CYCA3;2*-overexpressing lines (35DAS). Arrowheads indicate positions of apical buds. Bars = 1 cm.

Figure 1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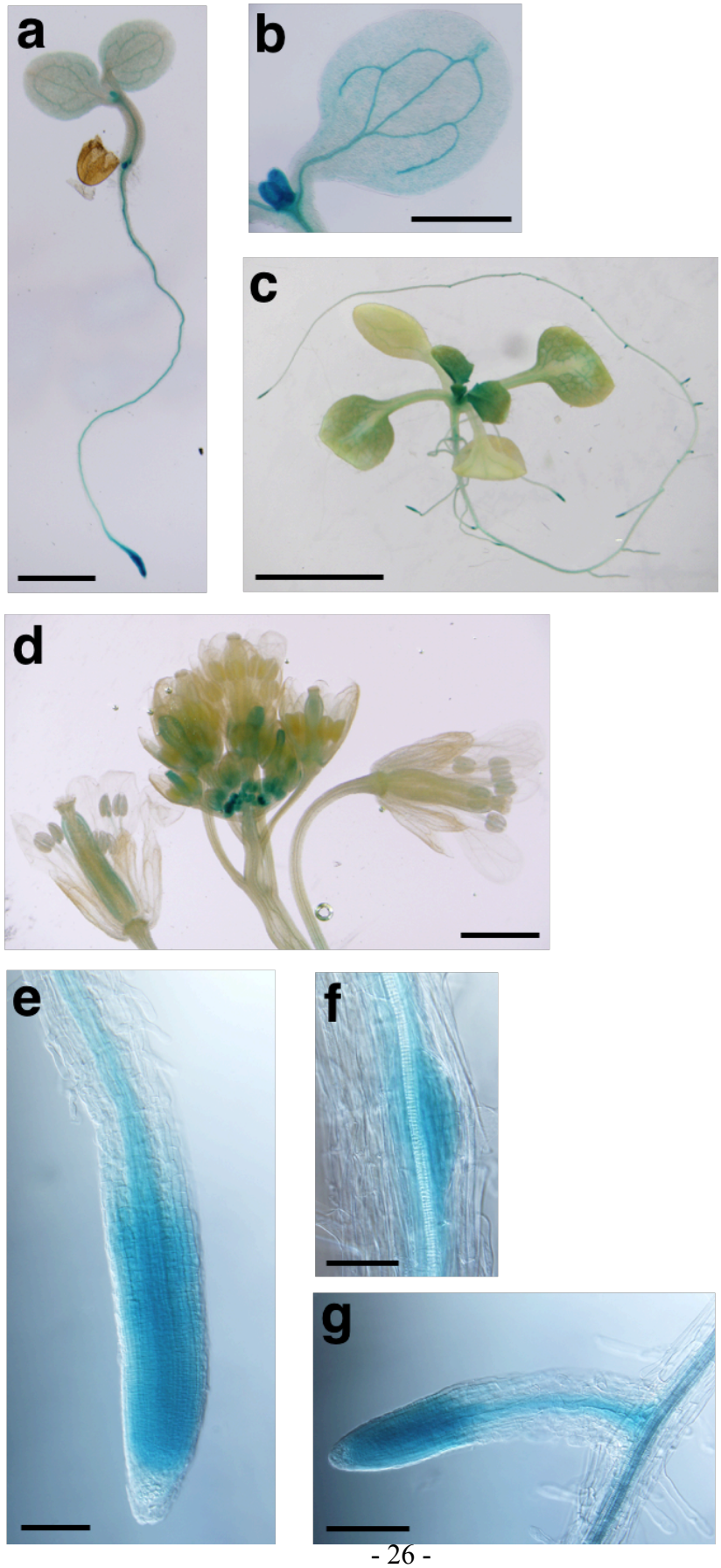
CYCA3;1
CYCA3;2
CYCA3;4
CDKB2;1
TUB4

Figure 2



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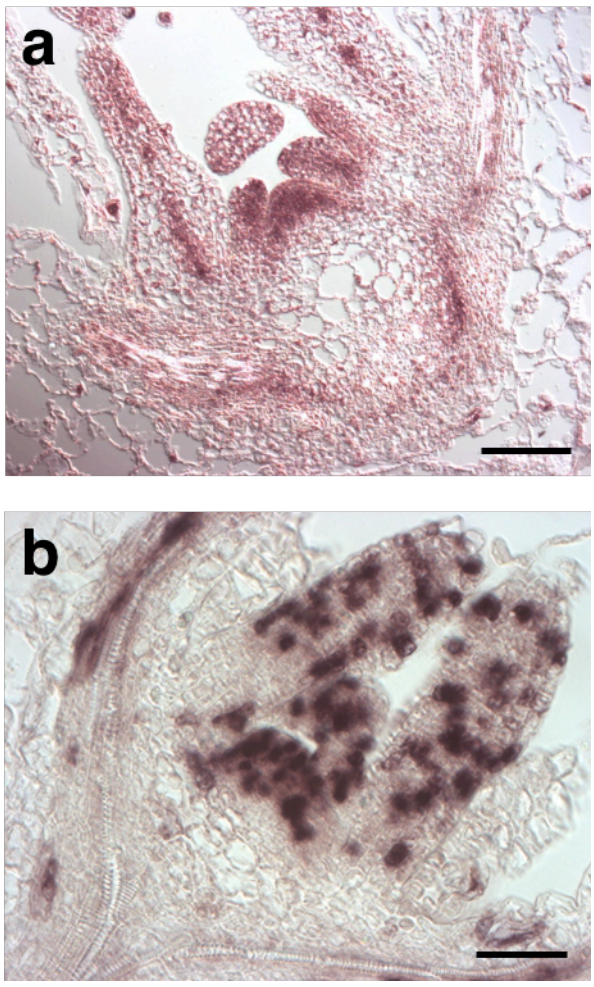

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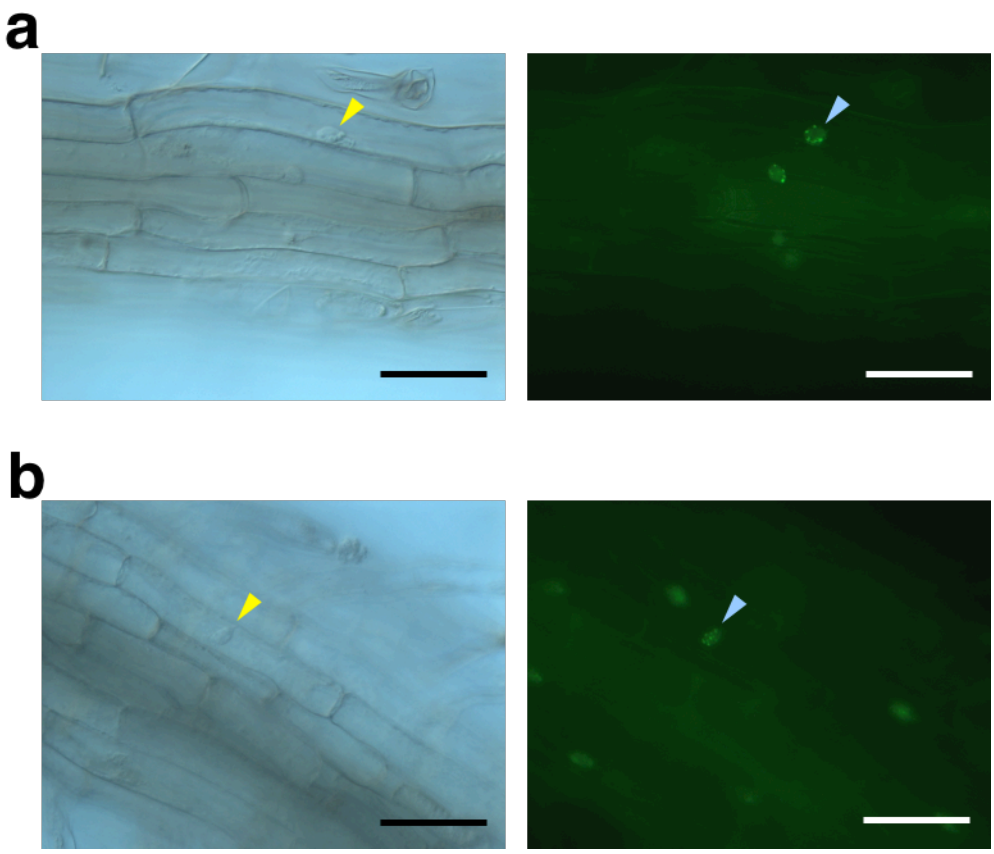

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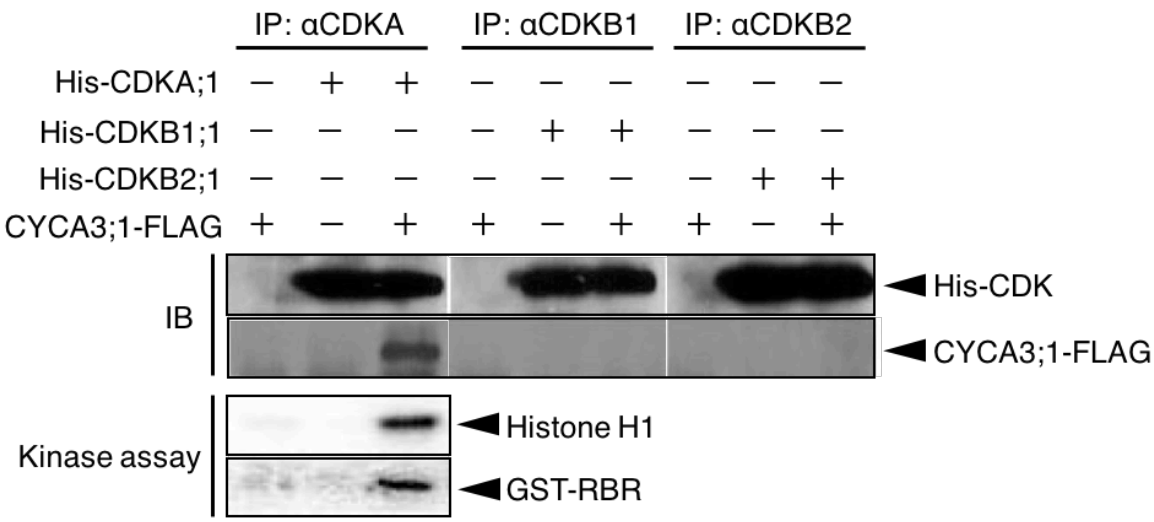
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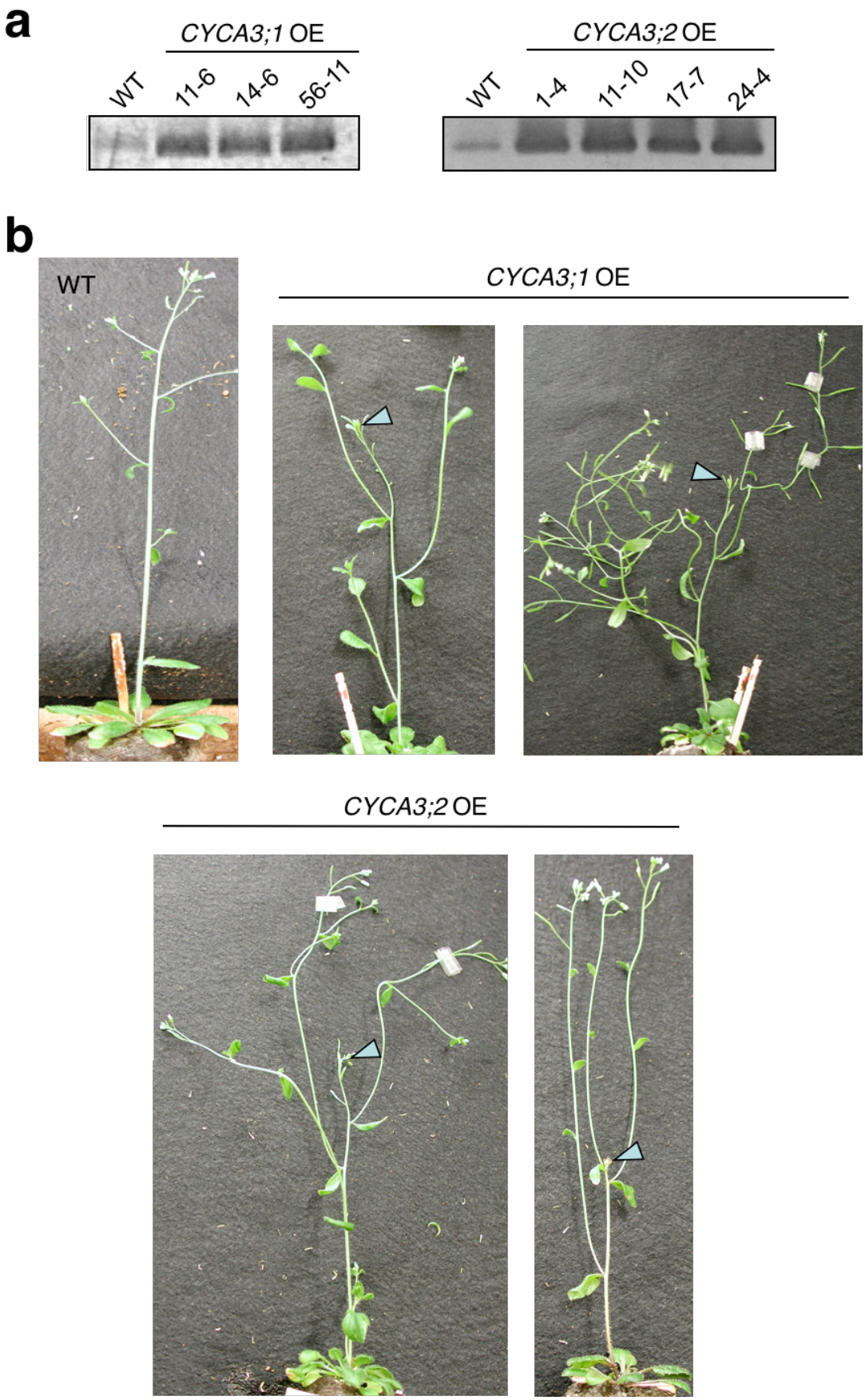
Kinase assay
Histone H1
GST-RBR

Figure 6


a
CYCA3;1 OE
WT
11-6
14-6
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CYCA3;2 OE
WT
1-4
11-10
17-7
24-4
b
WT
CYCA3;1 OE
CYCA3;2 OE

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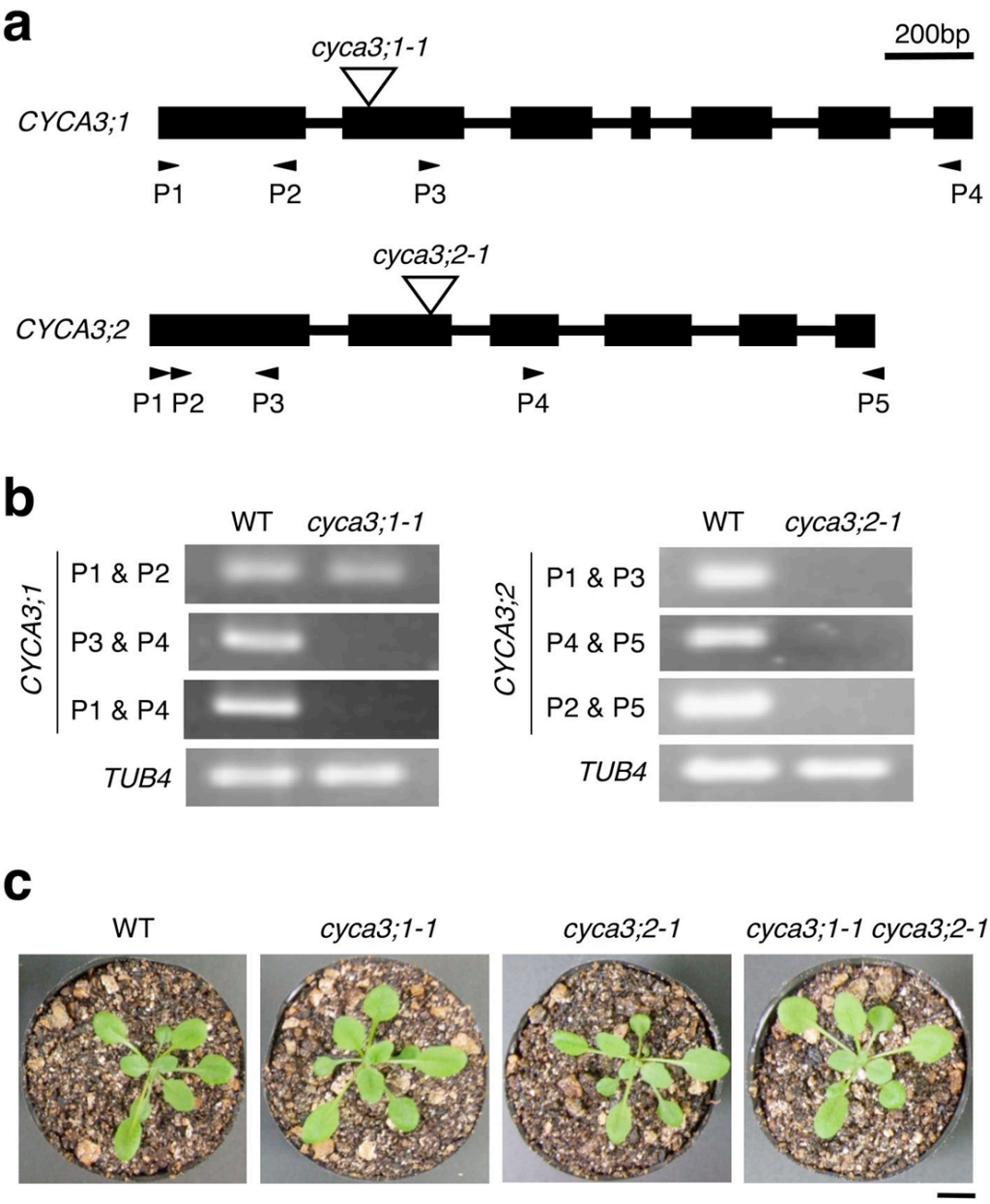
Supplemental Table 1 Primers used for RT-PCR

Name	Sequence (5' > 3')
For MM2d cells and transgenic plants	
CYCA3;1 forward	GTCGTACTCGGAGAGCTTCCAAACT
CYCA3;1 reverse	CAGGCATTGTCGCTACACACTTGAA
CYCA3;2 forward	CGATCTCTCAATCCTCTCAAATCTCAAACT
CYCA3;2 reverse	GGTCAATAATAGCCACCGGAGCT
CYCA3;4 forward	CGGAGAATCAGAACTGTGCGAGGAT
CYCA3;4 reverse	GCTTCAAGCTTCTTTTACGCCATTCCTC
CDKB2;1 forward	ATGGACGAGGGAGTTATAGCAGTTTC
CDKB2;1 reverse	TCAAAAACCAGGTACAGTACAGTTTTGCC
TUB4 forward	CTCTGTGCATCAGCTTGTCGAAAACG
TUB4 reverse	CCGAGGGAGCCATTGACAACATCTT
For the *cyca3* mutants	
CYCA3;1 P1	ATGGCCGACGAAAAAGAGAA
CYCA3;1 P2	GTCTGAATTTAAGGTTTCGA
CYCA3;1 P3	AGAGGAGTTTTGGTTGATT
CYCA3;1 P4	CAGGCATTGTCGCTACACACTTGAA
CYCA3;2 P1	ATGACAGAGCAAGAGATCTG
CYCA3;2 P2	AGTCAGCTCCGGTGGCTATTATTGACCTA
CYCA3;2 P3	TCTGAACTTTCTCTATGTAATCTG
CYCA3;2 P4	GAAGCTGATATACTTCTTGC
CYCA3;2 P5	AGCATAACCTCAGATCAAATGGTAACATCC



Supplemental Fig. 1 T-DNA insertion mutants of *CYCA3;1* and *CYCA3;2*.

a. Schematic diagrams of the *CYCA3;1* and *CYCA3;2* genes. Exons and introns are indicated by boxes and solid bars, respectively, on which the T-DNA insertion sites are shown. Arrowheads indicate primers that were used for RT-PCR. **b.** Expression analysis of the mutants. RT-PCR was conducted with total RNA from whole wild-type (WT) or mutant seedlings by using the indicated sets of primers. The amplified cDNAs were stained with ethidium bromide. Tubulin β (*TUB4*) was used as a control. **c.** Eighteen-day-old seedlings of WT and T-DNA insertion mutants. Bar = 1 cm.