



RESEARCH PAPER

# A2-type cyclin is required for the asymmetric entry division in rice stomatal development

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## Abstract

**In rice, and other major cereal grass crops, stomata are arranged in linear files parallel to the long growth axis of leaves. Each stomatal unit comprises two dumbbell-shaped guard cells flanked by two subsidiary cells. These morphological and developmental characteristics enable grass stomata to respond to environmental changes more efficiently. Cyclin-dependent kinases (CDKs) and their cyclin partners co-ordinate cell proliferation and differentiation during the development of multicellular organisms. In contrast to animals, plants have many more types and members of cyclins. In Arabidopsis, four A2-type cyclins (CYCA2s) function redundantly in regulating CDKB1 activity to promote the asymmetric division for stomatal initiation and the symmetric division of guard mother cells (GMCs). In this study, we examine the function of the single A2-type cyclin in rice, *OsCYCA2;1*, as well the single B1-type CDK, *OsCDKB1;1*. Cross-species complementation tests demonstrated that *OsCYCA2;1* and *OsCDKB1;1* could complement the defective stomatal phenotypes of Arabidopsis *cyca2* and *cdkb1* mutants, but also could suppress DNA endoduplication and cell enlargement. The early asymmetric divisions that establish the stomatal lineages are often missing within the stomatal cell files of *OsCYCA2;1*-RNAi rice transgenic lines, leading to a significantly reduced stomatal production. However, GMC divisions are not disrupted either in *OsCYCA2;1*-RNAi or in *OsCDKB1;1*-RNAi rice transgenic lines as expected. Our results demonstrate a conserved but diverged function and behavior of rice A2-type cyclins, which might be associated with the distinct stomatal development pathways between rice and Arabidopsis.**

**Keywords:** Cyclin-dependent kinases, cell differentiation, cell division, guard mother cells, cyclin, rice, stomata.

## Introduction

Stomata are microscopic valves on aerial surfaces of all land plants regulating the shoot-atmosphere gas exchange. Paleobotanical analyses revealed that stomata originated ~400 million years ago, a key evolutionary innovation formed in the early palaeozoic era (Raven, 2002). Despite the fact that the

distribution pattern and morphology are highly diversified in different plants, stomata arise in the epidermis after a series of cell divisions, cell fate changes, and cell shape controls. In the past decades, results of molecular genetic studies demonstrated that stomatal development is an accessible system to reveal the

Abbreviations: CYCA2, CYCLIN A2; CDKB1, CYCLIN-DEPENDENT KINASE B1; GC, guard cell; GMC, guard mother cell; PC, pavement cell; SGC, single guard cell.

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evolution of genes and signals involved in plant development (Vatén and Bergmann, 2012; Ran *et al.*, 2013; Chater *et al.*, 2017; Qu *et al.*, 2017).

In the dicot model plant *Arabidopsis*, the earliest stomatal precursor, the meristemoid mother cell (MMC), divides asymmetrically (stomatal entry divisions) producing a smaller cell, the meristemoid, as well as a larger sister cell, the stomatal lineage ground cell (SLGC). Meristemoids normally undergo an additional 1–2 rounds of asymmetric divisions (amplifying divisions) to generate new meristemoids before converting into guard mother cells (GMCs). SLGCs can also undergo asymmetric divisions (spacing divisions) to generate satellite meristemoids. Meristemoids differentiate into GMCs after cell fate change. Then, GMCs divide symmetrically to produce paired young guard cells (GCs). During the final stage of stomatal development, GCs undergo cell differentiation, morphogenesis, and pore formation to form functional stomatal units (Bergmann and Sack, 2007).

In contrast to the scattered pattern in dicot *Arabidopsis* leaves, monocot grass stomata are arranged within linear cell files that parallel the growth axis of the leaf. Stomatal lineage cells initiate at the base of the leaf, and divide asymmetrically to produce two daughter cells, a GMC, and a larger sister cell. At the final stage, GMCs divide symmetrically, producing paired dumbbell-shaped GCs. The stomatal subsidiary cells are produced from cell files flanking the stomatal lineage after asymmetric divisions (Franks and Farquhar, 2007; Liu *et al.*, 2009; Serna, 2011; Raissig *et al.*, 2016).

Cyclins form complexes with specific cyclin-dependent kinases (CDKs) to co-ordinate the cell proliferation and differentiation during the development of multicellular organisms (Swenson *et al.*, 1986; Obaya and Sedivy, 2002). Cyclins, acting as the regulator of CDK activity, contribute to the subcellular localization, substrate specificity, and protein stability of the CDK–cyclin complexes (Dewitte and Murray, 2003; Imai *et al.*, 2006; Boudolf *et al.*, 2009; Boruc *et al.*, 2010). A-type cyclins, known as mitotic cyclins, are essential for the mitotic cell cycle. In contrast to animals, plants encode a large family of A-type cyclins that have been classed into A1, A2, and A3 groups (Vandepoele *et al.*, 2002; Dewitte and Murray, 2003; Wang *et al.*, 2004).

The *Arabidopsis* genome has four genes encoding A2-type cyclins. *AtCYCA2* genes display tissue- and cell type-specific and overlapping expression patterns, such as in vascular systems and stomatal lineage cells, which are associated with their redundant functions during plant development (Burssens *et al.*, 2000; Imai *et al.*, 2006; Vanneste *et al.*, 2011; Donner and Scarpella, 2013). Mutants of *AtCYCA2* genes frequently form unpaired single guard cells (SGCs), a similar defect of the terminal GMC division to that also observed in mutants of *AtCDKB1* or *AtCDKA1* genes (Boudolf *et al.*, 2004b; Vanneste *et al.*, 2011; Yang *et al.*, 2014). Overexpression of *AtCYCA2:3* as well as *AtCDKA1* at the late stage of stomatal development induced excessive GC subdivisions (Yang *et al.*, 2014). *Arabidopsis cdkb1;1 1;2* double mutants and *35S:CDKB1;1.N161* dominant negative plants displayed decreased stomatal production and formation of SGCs, indicating that the activity of CDKB1 is required for both meristemoid asymmetric division and

GMC symmetric division in stomatal development. In addition to the function in promoting mitosis, *AtCYCA2*s form functional complexes with CDKs to modulate the cell cycle transition from the mitotic cycle to the endocycle. Genetic suppression of *AtCYCA2* or *AtCDKB1* results in enhanced ploidy levels and enlarged pavement cells (PCs; Vanneste *et al.*, 2011). Co-expression of *CYCA2;3* and *CDKB1;1* induces ectopic cell divisions, limits endoreduplication, and inhibits cell growth (Boudolf *et al.*, 2009).

There are at least 49 putative genes predicted to encode rice cyclins, which were classified into nine types based on evolutionary relationships. Eight of these nine types are common between rice and *Arabidopsis* (Umeda *et al.*, 1999a; Cooper *et al.*, 2003; La *et al.*, 2006). The existence of numerous cyclins implies their diverse regulatory roles in modulating CDK activities during rice development and adaption in response to environmental changes (Cooper *et al.*, 2003; Huang *et al.*, 2008). For example, rice B2-type cyclins, *OsCycB2;1* and *OsCycB2;2*, promote root cell divisions through an association with *OsCDKB2;1* (Lee *et al.*, 2003). *OsCycH;1* specifically binds to R2 and positively controls CDK and CTD kinase activities to adjust the rate of cell proliferation (Fabian-Marwedel *et al.*, 2002).

In this study, we examine the function of the rice single A2-type cyclin *OsCYCA2;1* and *OsCDKB1;1* in rice development. Our results demonstrate a requirement for *OsCYCA2;1* for stomatal and root development. In contrast to its homolog in *Arabidopsis*, *OsCYCA2;1* is exclusively required for the asymmetric entry divisions to produce GMCs at the early stage of stomatal development. In addition, combined with phylogenetic analyses, we are providing new clues for further revealing the evolutionary correlation between cell cycle genes and developmental pathways.

## Materials and methods

### *Plant materials and growth conditions*

The Col-0 ecotype of *Arabidopsis thaliana* L. was used as the wild-type control in the *Arabidopsis* study. The *cdkb1;1 1;2* double mutants were confirmed by PCR-based approaches (Xie *et al.*, 2010). The *cyc2;3/4* double mutants were provided by Steffen Vanneste and Tom Beekman (Vanneste *et al.*, 2011). Seeds were surface sterilized (40 s) in an aqueous solution of 30% (w/v) hydrogen peroxide and 85% (v/v) ethanol in a volume ratio of 1:4 (v/v), and then sown on the surface of half-strength Murashige and Skoog (MS) medium supplemented with 0.8% agar and 1% sucrose. Plants were grown in a controlled temperature and photoperiod chamber at 22 ± 2 °C and 16 h/8 h light/dark illumination cycles. *Oryza sativa* L. spp. *japonica* cultivar Zhonghua 11 was used as the wild-type control and the transformation recipient in the rice study. Rice seeds were soaked in water at 28 °C for 2 d, and then grown in a controlled growth chamber with 30 °C/22 °C day/night temperature cycles, 12 h/12 h light/dark illumination cycles, and 60–70% relative humidity.

### *Plasmid construction and generation of transgenic plants*

To obtain the construct of gene overexpression, cDNA of *OsCDKB1;1* or *OsCYCA2;1* was cloned into the pH7WG2D.1 vector by using gateway technology and LR Clonase™ II Enzyme Mix (Invitrogen). The recombinant plasmids were confirmed by DNA sequencing before the transformation into *Arabidopsis* wild type and mutants. To generate RNAi transgenic plants against *OsCDKB1;1* and *OsCYCA2;1*, the conserved sequences from base pair 530 to 695 of *OsCDKB1;1* cDNA

and 747 to 979 of *OsCYCA2;1* cDNA were amplified and cloned into pTCK303 vector, respectively. These constructs were electroporated into *Agrobacterium tumefaciens* EHA105 and transformed into rice Zhonghua 11 (Chen *et al.*, 2011). T<sub>1</sub> seeds were collected to screen positive transgenic plants by using 50 µg l<sup>-1</sup> hygromycin B (Roche). Real-time quantitative PCR (RT-qPCR) was conducted to confirm the expression level of target genes in transgenic plants. The primer sequences used in this study are listed in Supplementary Table S1 at JXB online.

#### Real-time quantitative PCR analysis

Rice seedlings were harvested and immediately ground in liquid nitrogen, and the total RNA was isolated using TRNzol reagent (<http://www.tiangen.com>). The first-strand cDNA was synthesized using a Promega Reverse Transcription kit (<http://www.promega.com>). RT-qPCRs were performed by using SYBR Premix Ex Taq™ (TaKaRa) with a Corbett RG3000. The *OsACTIN2* gene was used as an internal control. The primer sequences are listed in Supplementary Table S1.

#### Yeast two-hybrid assay

The full-length cDNA sequences of *OsCYCA2;1* and *OsCDKB1;1* were amplified using the primers listed in Supplementary Table S1 and cloned into pGBKT7 and pGADT7 vectors (Clontech), respectively. These constructs were transformed into *Saccharomyces cerevisiae* yeast strain AH109 and selected on SD/-Leu-Trp or SD/-Leu-Trp-His-Ade plates. X-Gal activity was then detected.

#### Bimolecular fluorescence complementation assay

For bimolecular fluorescence complementation (BiFC) assays, the full-length cDNAs of *OsCYCA2;1* and *OsCDKB1;1* were cloned into pSPYCE-35S and pSPYNE-35S vectors, respectively (Walter *et al.*, 2004). These constructs were transformed into *A. tumefaciens* EH105 and co-injected into tobacco (*Nicotiana benthamiana*). Images were taken after 3 d using a laser scanning confocal microscope (FV1000-MPE, Olympus).

#### Pull-down assay

The *OsCYCA2;1* and *OsCDKB1;1* sequences were cloned into pET-28a and pGEX4T-1 vectors, respectively. The *OsCYCA2;1-pET-28a* and *OsCDKB1;1-pGEX4T-1* constructs were transformed into the BL21 strain of *Escherichia coli*. The transformed strains were grown to OD<sub>600</sub>=0.5 under 37 °C and then placed at 18 °C for 30 min. Fusion proteins were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 20 h. The harvested strains (5000 rpm, 10 min, 4 °C) were re-suspended with ice-cold phosphate-buffered saline (PBS) and lysed by sonication. The lysate was centrifuged at 10000 rpm for 60 min and the supernatant was collected. The glutathione S-transferase (GST)-*OsCDKB1;1* supernatant was loaded on glutathione-Sepharose (GE Healthcare) and washed with PBS. The GST-*OsCDKB1;1* fusion protein on glutathione-Sepharose was incubated with the His-*OsCYCA2;1* supernatant at 4 °C for 2 h. Then the glutathione-Sepharose was washed with PBS and eluted with 10 mM reduced glutathione elution buffer. The samples were loaded on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by using a semi-dry blotting system (Bio-Rad), and then incubated with anti-His6 monoclonal antibodies followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibodies. The color reaction was performed using the Pro-Light HRP Kit (Tiangen). Signals were exposed to X-ray films and developed.

#### DAPI staining and DNA content measurement

Ten-day-old rice roots were fixed in a mixture of 3:1 (v/v) ethanol and acetic acid for 30 min, then rinsed with distilled water. After staining for 30 min with 2 µg ml<sup>-1</sup> DAPI (Roche) in a staining solution (0.1 M sodium phosphate, 1 mM EDTA, 0.1% Triton X-100, pH 7.0), roots were photographed by a fluorescence microscope. The relative fluorescence intensities were measured using ImageJ software (<http://imagej.nih.gov/ij/>).

#### Flow cytometric analysis

About 20–50 mg of fresh tissue were cut into 2–4 mm fragments and then chopped immediately using a razor blade in 1 ml of Galbraith's buffer (45 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 30 mM sodium citrate, 20 mM MOPS, 0.1% Triton X-100, pH 7.0). The cell culture was collected by gentle pipetting and filtered through a cell strainer. The samples were stained with 2 µg ml<sup>-1</sup> DAPI in an ice bath for 30 min before the analysis using a MoFlo-XDP flow cytometer (Beckman) (Dolezel *et al.*, 2007). A total of ~10000 nuclei were measured for each sample.

#### Root semi-thin sections

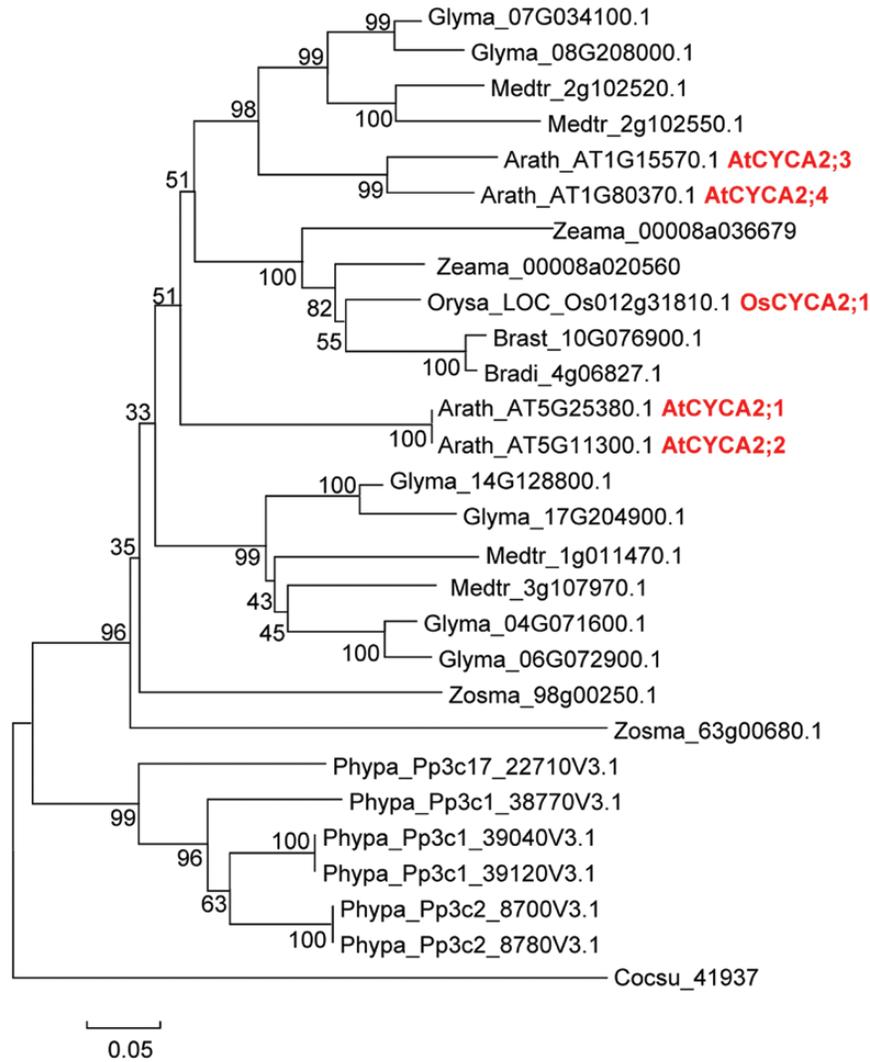
About 1 cm long primary root tips from 6-day-old rice seedlings were harvested and immersed in cold formaldehyde solution. The samples were subjected to a vacuum for 10 min and placed at 4 °C overnight. The materials were washed with 0.1 M PBS (pH 7.2) four times and were fixed in 1% osmic acid for 1 h, followed by a series of dehydration steps, which were performed by using 30, 50, 70, 80, 90, 100, and 100% ethanol (each step lasted 20 min). Ethanol was substituted with 1:1 acetone and ethanol (v/v) as well as pure acetone twice (each step lasted 20 min). Permeation was performed by using a series of 2:1, 1:1, and 1:2 (v/v) of acetone and epoxy (SPURR) mixture solution. Each step lasted for 3 h. After adding pure epoxy for 8 h, samples were embedded and polymerized at 60 °C for 24 h. Semi-thin sections (thickness 1 µm) were obtained by using a Leica UC7 microtome. Before imaging, sections were stained with 0.1% toluidine blue O.

## Results

### Evolutionary analysis of A2 cyclins in plants

Phylogenetic analysis indicates that homologs of A2-type cyclin are found in lineages that diverged early in the evolution of land plants, before the appearance of stomata. For example, the unicellular green alga *Coccomyxa subellipsoidea* has a *CYCA2* gene in its genome. In the non-vascular land plant moss *Physcomitrella patens*, stomata are exclusively found on the diploid sporophyte (Chater *et al.*, 2016); there are six putative orthologs of *CYCA2*. In the vascular dicot plants *Arabidopsis*, soybean (*Glycine max*), and alfalfa (*Medicago truncatula*), the number of *CYCA2* genes was four, six, and four, respectively (Fig. 1). In contrast, the rice genome contains only one copy of the *CYCA2* gene, *Os012g31810*, which is predicted to encode *OsCYCA2;1* protein consisting of 490 amino acid residues. Multiple sequence alignment reveals that *OsCYCA2;1* shows 40.5% amino acid sequence identity with *Arabidopsis CYCA2s*, and contains a CDK-binding cyclin box, which is highly conserved among mitotic cyclins (Supplementary Fig. S1) (Umeda *et al.*, 1999a). Interestingly, similar to rice, *Brachypodium stacei*, *Brachypodium distachyon*, *Zea mays* (Fig. 1), and many other monocot grasses, *Hordeum vulgare*, *Oropetium thomaeum*, *Panicum hallii*, *Sorghum bicolor*, *Setaria italica*, and *Setaria viridis*, only have 1–2 *CYCA2* genes (Supplementary Fig. S2).

The seagrass *Zostera marina* belongs to basal monocots that returned to the sea. The absence of stomata in *Z. marina* is consistent with the evolutionary loss of entire genes that are required for stomatal development (Olsen *et al.*, 2016). However, like the above grass plants, *Z. marina* possesses two *CYCA2* genes, suggesting that the A2-type cyclin is fundamentally important for plant growth and development, and is not solely linked to stomatal development. The low number of *CYCA2* genes in grasses indicates that *CYCA2* gene



**Fig. 1.** Phylogenetic tree shows that A2-type cyclin-like proteins are conservatively present in green land plants. The phylogenetic tree was constructed using amino acid sequences of Arabidopsis CYCA2 family members based on Phytozome V12.1, using the Neighbor-Joining method in MEGA4. Bootstrap values for 1000 replicates are given in nodes as percentages. Amino acid sequences were used from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Brachypodium stacei*, *Coccomyxa subellipsoidea*, *Glycine max*, *Medicago truncatula*, *Oryza sativa*, *Physcomitrella patens*, *Zea mays*, and *Zostera marina*.

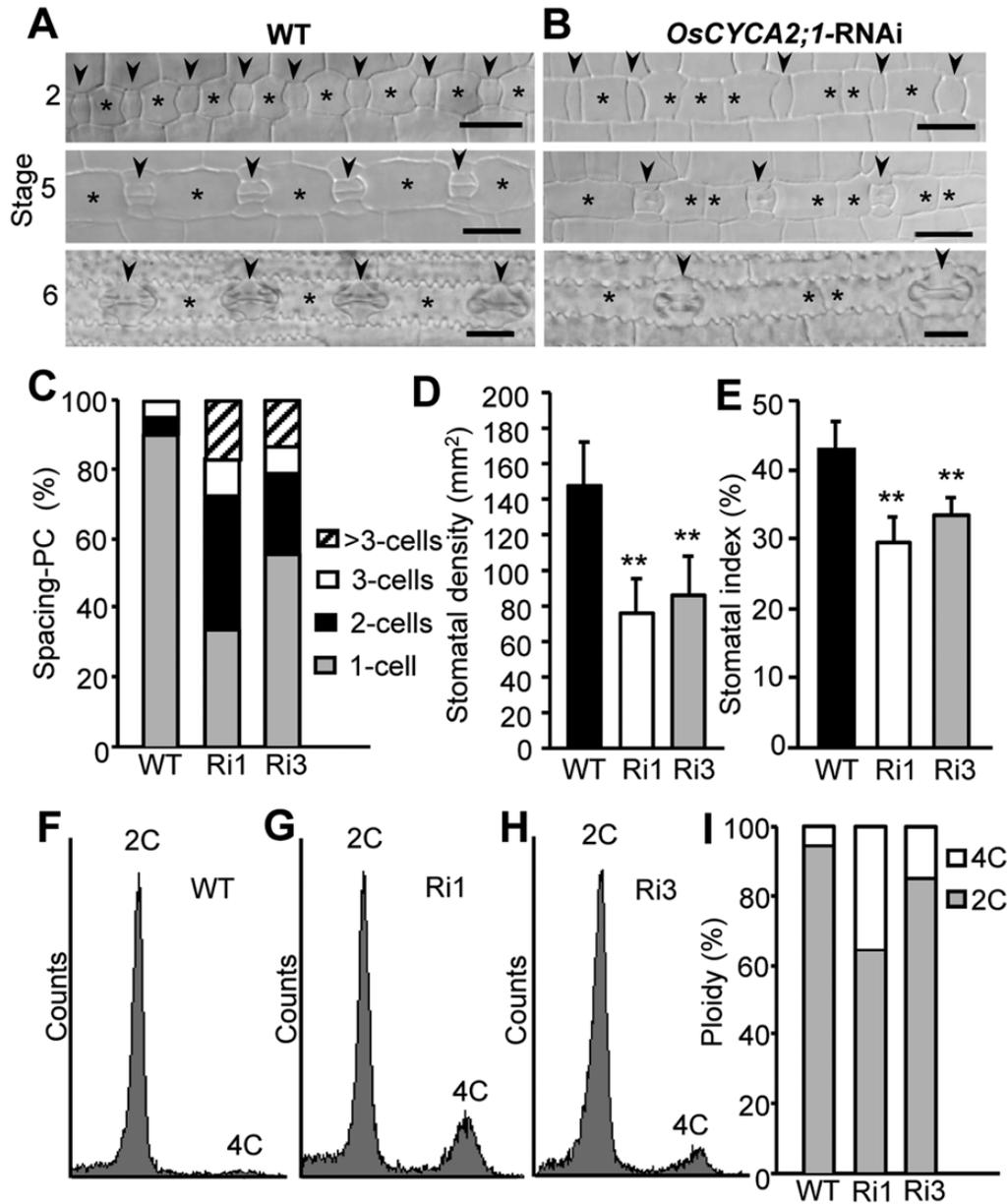
duplication might not be necessary, which is associated with their unique developmental pathways and morphogenesis.

#### Requirement of *OsCYCA2;1* for stomatal initiation

To elucidate the function of A2-type cyclin in rice development, RNAi transgenic rice lines targeting *OsCYCA2;1* were generated. Transcript levels of *OsCYCA2;1* in two lines, Ri1 and Ri3, were suppressed to 28% and 61%, respectively, in relation to the level in wild-type rice seedlings (Supplementary Fig. S3A). In rice leaf epidermis, stomata form within the stomatal lineage files following a gradual base to tip maturation pattern; the developing stomata can only be found at the proximal end (base) of the leaf. Unlike in Arabidopsis, GMCs in rice are produced directly by asymmetric entry divisions without the precursor stage of the meristemoid. Each undifferentiated cell close to the base of the leaf divides asymmetrically and generates one smaller GMC and one larger sister cell (Stage 2, upper panel of Fig. 2A). Subsidiary mother cells (SMCs) flanking the GMCs

are produced by the cells in the neighboring cell files. The terminal division of GMC produces a pair of immature GCs (Stage 5, middle panel of Fig. 2A). Within the wild-type stomatal lineage cell files, stomatal complexes are spaced by one lobbed PC (Stage 6, lower panel of Fig. 2A). However, in *OsCYCA2;1*-RNAi transgenic plants, more than two spacing cells were often observed between two neighboring GMCs/stomata within the same cell file (Fig. 2B, C), leading to a decreased stomatal density and stomatal index (Fig. 2D, E).

Mutations of Arabidopsis *CYCA2* genes caused a failure of GMC division, leading to the formation of aberrant stomatal units (SGCs) (Vanneste et al., 2011). However, the structure and morphology of mature stomata in *OsCYCA2;1*-RNAi rice transgenic plants are indistinguishable from those of wild-type stomata, indicating that the subsequent GMC symmetric divisions as well as the subsidiary cell asymmetric divisions are not interrupted by the suppression of *OsCYCA2;1* (lower panels in Fig. 2A and B). Taken together, the above observations indicate that *OsCYCA2;1* is essentially required for the asymmetric



**Fig. 2.** Suppression of *OsCYCA2;1* causes defective cell division in rice. (A, B) Differential interference contrast micrographs of epidermal cells from 6-day-old rice seedlings grown in darkness. Stomata were initiated at the proximal end (base) of young leaves. Asymmetric cell divisions produce a smaller GMC and one larger sister cell (Stage 2, upper panels). The terminal symmetric division of the GMC produces a pair of immature GCs (Stage 5, middle panels). Mature stomatal complexes (a pair of dumbbell-shaped GCs and two flanking SCs) are spaced by one pavement cell (PC) (Stage 6, lower panels). Arrowheads indicate the GMCs or stomatal complexes. Asterisks indicate the PCs that separate stomata in the same cell file. Scale bar=20  $\mu$ m. (C) The numbers of PCs between two adjacent stomata within the same cell file are often increased in RNAi lines. (D, E) Leaf stomatal density and index of two *OsCYCA2;1*-RNAi lines and the wild type (WT;  $n=12$ ). Data represent the mean  $\pm$ SD. Asterisks indicate a significant difference from WT controls (Student's *t*-test, \*\* $P<0.01$ ). (F–H) Flow cytometric analysis of nuclei in shoot cells. (I) Quantitative analysis of DAPI fluorescence. *OsCYCA2;1*-RNAi transgenic lines have a higher average 4C DNA content than the WT. For each line, ~10000 cell nuclei were measured.

entry division during stomatal initiation at the early stage of stomatal development, but not for the terminal GMC symmetric divisions and subsidiary cell asymmetric divisions.

It has been demonstrated that *Arabidopsis* CYCA2s not only promote cell proliferation but also negatively regulate endo-cycle onset (Imai *et al.*, 2006; Yoshizumi *et al.*, 2006; Vanneste *et al.*, 2011). Flow cytometric analysis showed that in wild-type rice, only 6% of cells showed a 4C DNA content, whereas most cells were 2C (diploid). However, in *OsCYCA2;1*-RNAi transgenic rice lines Ri1 and Ri3, the fraction of 4C

cells markedly increased to 36% and 18%, respectively. Higher ploidy levels, like in *Arabidopsis cyca2* mutants (8C, 16C, 32C), were barely detectable in rice *OsCYCA2;1*-RNAi plants (Fig. 2F–I).

#### *OsCYCA2;1* complements epidermal defects of *Arabidopsis cyca2* mutants

The *Arabidopsis* epidermis is an ideal system to identify gene functions in plant development programs and morphogenetic

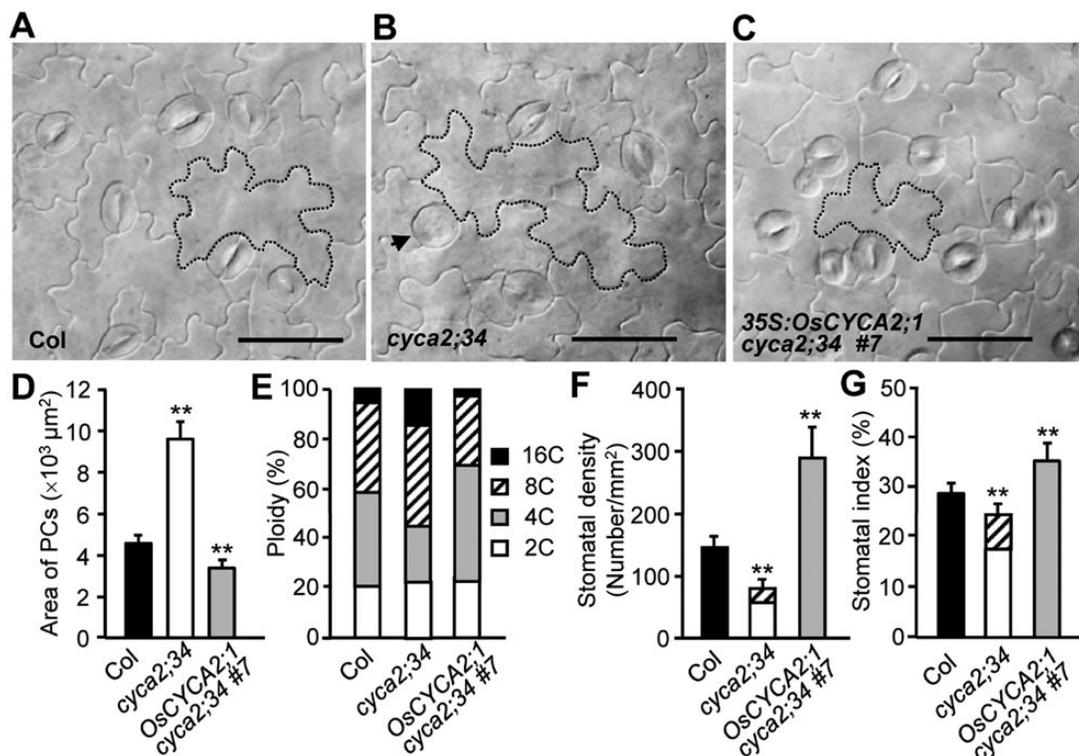
patterns. To identify further the function of *OsCYCA2;1* in epidermal development, *OsCYCA2;1* coding sequences driven by 35S promoters were transformed into Arabidopsis *cyca2;34* mutants. Compared with the wild type, *cyca2;34* mutants display enlarged pavement cells and enhanced ploidy levels. Cross-species expression of *OsCYCA2;1* (line #7) inhibits the abnormal PC enlargement in *cyca2;34* epidermis, to a cell size even smaller than in the wild type (Fig. 3A–D). Moreover, revealed by flow cytometric analysis, expression of *OsCYCA2;1* is able to inhibit the high DNA ploidy levels in *cyca2;34* mutants (Fig. 3E; Supplementary Fig. S4). This result is consistent with the previous findings that overexpression of *CYCA2* genes could restrain endoreduplication in Arabidopsis (Imai *et al.*, 2006; Boudolf *et al.*, 2009).

In *cyca2;34* mutants, ~10% of GMCs failed to divide symmetrically and formed into SGCs (Fig. 3B, arrow). Strikingly, *OsCYCA2;1* expression can fully rescue the defective GMC division in *cyca2;34* epidermis, suggesting that rice *OsCYCA2;1* remains a conserved function in promoting the GMC symmetric divisions. In addition, expression of *OsCYCA2;1* in the *cyca2;34* mutant background induced formation of excessive stomata, reflected by an increased stomatal density and stomatal index (Fig. 3F, G). In another 35S:*OsCYCA2;1 cyca2;34* transgenic line (line #8), the relative transcript level of *OsCYCA2;1* is much lower than in line #7; the defective GMC division and reduced stomatal production are partially rescued, indicating that *OsCYCA2;1* quantitatively promotes stomatal development depending on its expression level (Supplementary

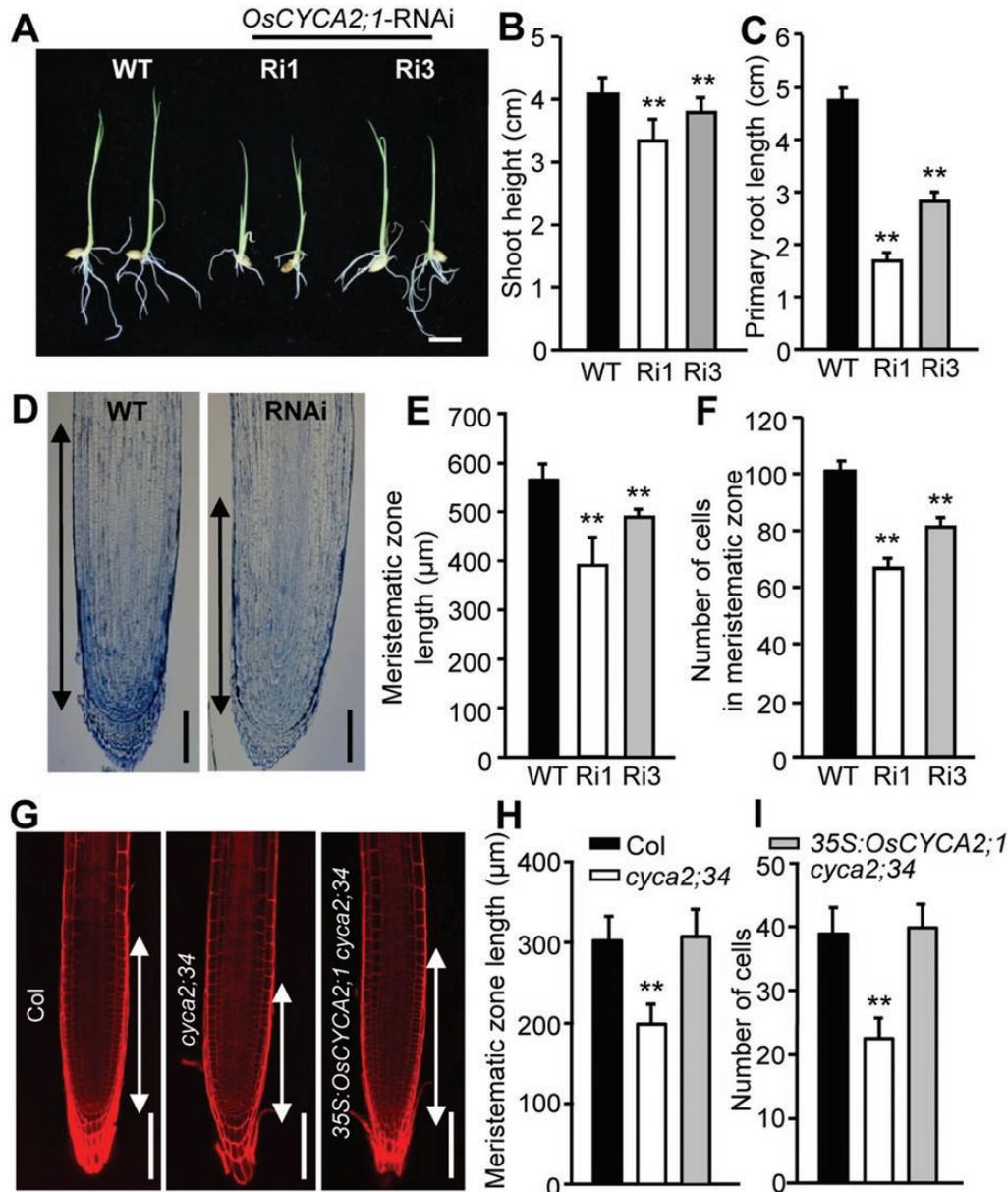
Fig. S5). Taken together, our results of cross-species complement tests demonstrate the conserved abilities of *OsCYCA2;1* in limiting cell endoreduplication and PC size, as well as in rescuing *cyca2;34* defective asymmetric entry divisions (for stomatal initiation) and symmetric GMC divisions (for guard cell formation), despite *OsCYCA2;1* being functionally required only for stomatal entry divisions in rice.

#### *OsCYCA2;1* is also required for cell division and differentiation in roots

*OsCYCA2;1* is preferentially expressed in proliferating tissues. Besides in the base dividing zone (proximal end) of leaves, a higher transcript level of *OsCYCA2;1* is found in rice root tips, implying that a similar *OsCYCA2;1* regulatory mechanism exists in rice roots (Supplementary Fig. 3B). Therefore, we probed the impact of down-regulated expression of *OsCYCA2;1* on root growth. As shown in Fig. 4, the overall growth of shoots and roots in 10-day-old *OsCYCA2;1*-RNAi transgenic seedlings is much less than in the wild type (Fig. 4A–C). To determine whether the root growth defects arose from a defective cell proliferation, we compared the root longitudinal sections of the wild type and the RNAi line. The shorter meristematic zone in *OsCYCA2;1*-RNAi is correlated with a considerably fewer number of cells within its meristematic zone (Fig. 4D–F). Similarly, Arabidopsis *cyca2;34* mutants exhibit a short meristematic zone and fewer cells than Col wild type. Ectopic expression of *OsCYCA2;1* restored the



**Fig. 3.** Cross-species expression of *OsCYCA2;1* complements the epidermal defects of Arabidopsis *cyca2;34* mutants. (A–C) Differential interference contrast micrographs of cotyledon epidermal cells of 14-day-old Arabidopsis seedlings of the Col, *cyca2;34*, and *cyca2;34* harboring 35S:*OsCYCA2;1*, Line #7. An arrow points to a single guard cell (SGC). Representative pavement cells (PCs) are traced with dashed lines. Scale bar=50  $\mu\text{m}$ . (D) Comparison of PC area ( $n=30$ ). (E) Proportions of cells with different ploidies. (F and G) Stomatal density and index. The diagonal line-filled box indicates the SGCs. Data in (D, F, G) represent the mean  $\pm$ SD. Asterisks indicate a significant difference from Col wild-type controls (Student's *t*-test, \*\* $P<0.01$ ).



**Fig. 4.** Suppression of *CYCA2* expression causes defective cell proliferation within the meristematic zone of roots. (A) Ten-day-old *OsCYCA2;1*-RNAi and wild-type (WT) rice seedlings. Scale bar=1 cm. (B, C) The length of shoots and primary roots ( $n=24$ ). (D) Longitudinal sections of the primary root tips. Scale bar=100  $\mu\text{m}$ . (E, F) Length and cell number of the meristematic zone in roots ( $n=20$ ). (G) Propidium iodide-stained images of Arabidopsis root tips. Scale bar=100  $\mu\text{m}$ . (H, I) Length and cell number of the meristematic zone in Arabidopsis roots ( $n=20$ ). Double-headed arrows in (D, G) indicate the extent of the meristematic zone. Data in (B, C, E, F, H, I) represent the mean  $\pm$ SD. Asterisks indicate a significant difference from WT controls (Student's *t*-test, \*\* $P<0.01$ , \* $P<0.05$ ). (This figure is available in colour at JXB online.)

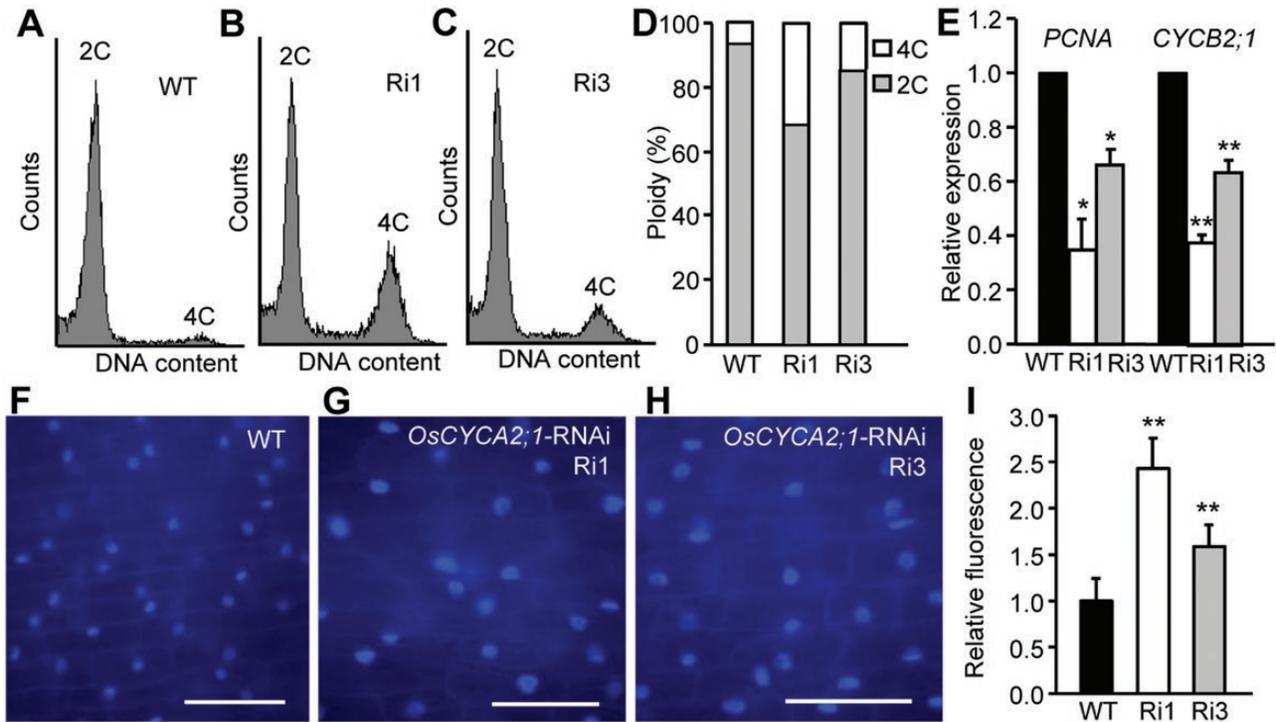
length of and cell number within the meristematic zone to the wild-type level (Fig. 4G–I), supporting that *OsCYCA2;1* is an evolutionarily conserved regulator that is required for cell proliferation in roots.

By means of flow cytometry approaches, we found that, in contrast to the 6% of 4C cells in the wild type, the fractions of cells with 4C DNA content in *OsCYCA2;1*-RNAi lines Ri1 and Ri3 are dramatically increased to 32% and 15%, respectively (Fig. 5A–D). Moreover, the relative expression levels of an S-phase-specific gene, *PCNA*, and a M-phase cyclin gene, *CYCB2;1*, were suppressed in *OsCYCA2;1*-RNAi plants (Fig. 5E). Consistently, epidermal cells in the maturation zone of *OsCYCA2;1*-RNAi roots showed stronger

DAPI fluorescent signals than in the wild type (Fig. 5F–H). Quantitative analysis of the DAPI fluorescence intensities further confirmed that a higher DNA level (~2-fold) is present in *OsCYCA2;1*-RNAi roots (Fig. 5I). The higher DNA content in *OsCYCA2;1*-RNAi root cells might be due to delayed or arrested  $G_2$  to M transition, a result supporting the idea that *OsCYCA2;1* is required for cell mitosis.

#### *OsCYCA2;1* conservatively interacts with *OsCDKB1;1*

*CYCA2*s play their regulatory roles through interacting with multiple CDKs, such as by forming *CYCA2;3*-*CDKB1;1* or *CYCA2;3*-*CDKA;1* protein complexes. Arabidopsis



**Fig. 5.** *OsCYCA2;1* is required for rice root cell mitosis. (A–C) Profiles of distribution of cells with different DNA content after flow cytometric analysis. Roots of *OsCYCA2;1*-RNAi lines Ri1 (B) and Ri3 (C) have more 4C cells than wild-type (WT) roots (A). (D) Quantitative analysis of the cell DNA ploidy levels. (E) Relative expression levels of *PCNA* and *CYCB2;1* in *OsCYCA2;1*-RNAi lines and WT roots. (F–H) DAPI staining of the epidermal cells in the maturation zone of the WT (F), *OsCYCA2;1*-RNAi line Ri1 (G), and Ri3 (H). Scale bar=50  $\mu$ m. (I) Quantitative analysis of DAPI fluorescence revealed that *OsCYCA2;1*-RNAi transgenic lines have a higher average DNA content than the WT. Data represent the mean  $\pm$ SD. Asterisks indicate a significant difference from WT controls (Student's *t*-test, \*\**P*<0.01). (This figure is available in colour at *JXB* online.)

*AtCYCA2;3* interacts with *AtCDKB1;1* to form a functional complex which promotes the formation of a two-celled stoma and prevents entry into the endocycle program (Boudolf *et al.*, 2009; Vanneste *et al.*, 2011). According to the sequence blasting results in the rice genome, *Os01g67160* encodes the putative *OsCDKB1;1*. The deduced amino acid sequence of *OsCDKB1;1* shares 88.5% sequence identity with the Arabidopsis *CDKB1s*. A B1-type-specific cyclin interaction motif 'PPTALRE' is highly conserved in rice *OsCDKB1;1* (Supplementary Fig. S6). Yeast two-hybrid assays showed that *OsCYCA2;1* can interact with *OsCDKB1;1* (Fig. 6A). Consistently, pull-down assays verified the direct protein interaction between *OsCYCA2;1* and *OsCDKB1;1* (Fig. 6B). To determine the subcellular localization, *OsCYCA2;1* or *OsCDKB1;1* fused with GFP were transiently expressed in tobacco (*N. benthamiana*) leaves. The fluorescent signals from *OsCYCA2;1*-GFP were exclusively detected in nuclei, while *OsCDKB1;1*-GFP was found in both the cytoplasm and nuclei (Fig. 6C). BiFC analysis confirmed that *OsCYCA2;1* directly interacts with *OsCDKB1;1* in nuclei (Fig. 6D). These results suggest that *OsCYCA2;1* may act as a conserved activator regulating the activity of *OsCDKB1;1* kinase in rice.

#### Suppression of *OsCDKB1;1* has no obvious effects on rice development

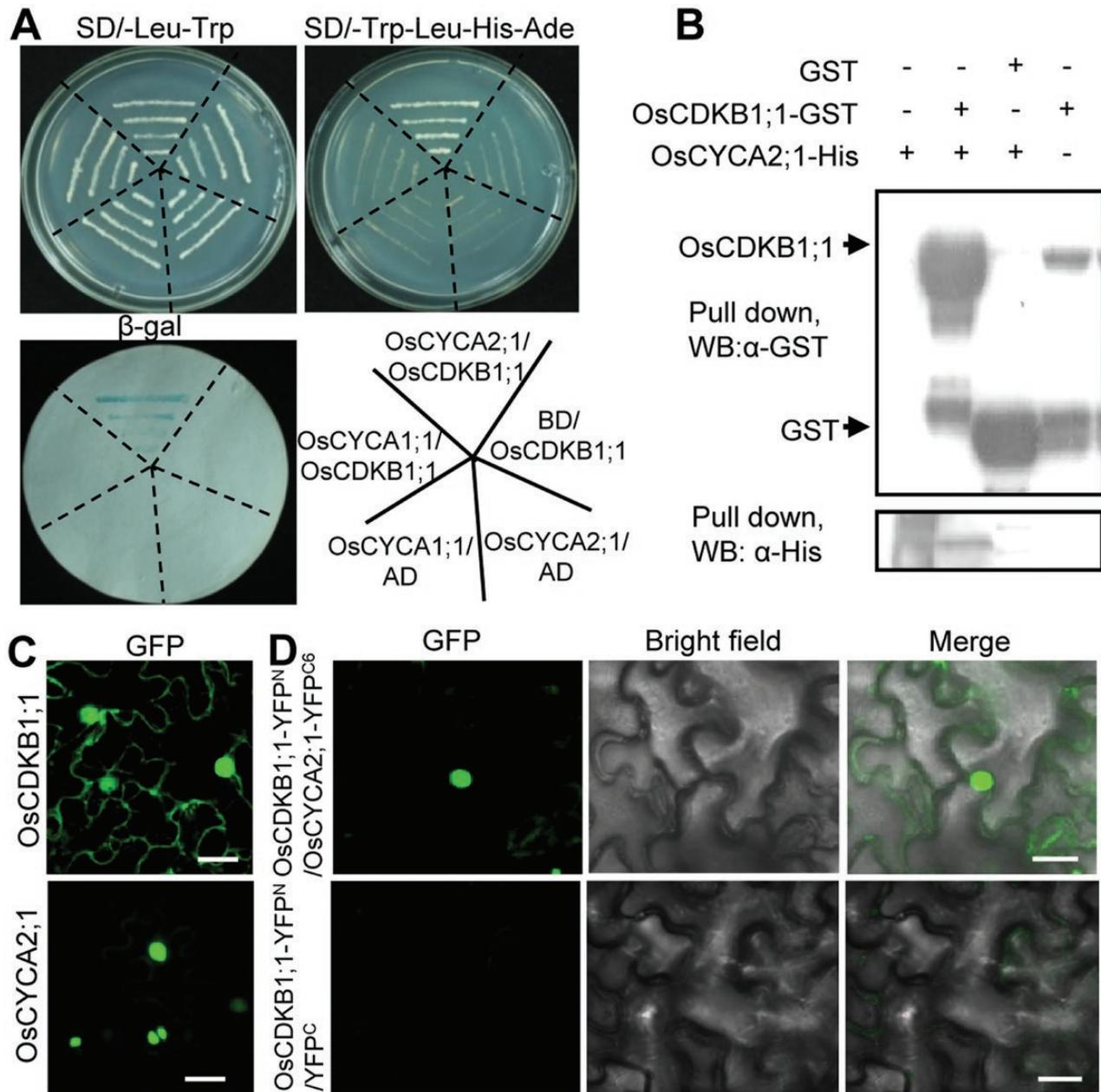
To determine whether *OsCDKB1;1*, like its partner *OsCYCA2;1*, is required for rice development, we generated

and selected two *OsCDKB1;1*-RNAi transgenic lines, Ri2 and Ri3, in which *OsCDKB1;1* transcript levels were significantly suppressed (Supplementary Fig. S7A). However, the overall growth of these two transgenic lines is comparable with that of the untransformed controls (Supplementary Fig. S7B–D). Longitudinal sections of roots demonstrate that the suppression of *OsCDKB1;1* has no significant impact on cell numbers of the root meristematic zone (Supplementary Fig. S7E–G). In addition, we found that neither the stomatal production (stomatal density) nor the stomatal complex morphology has been affected in *OsCDKB1;1*-RNAi transgenic lines (Supplementary Fig. S7H–J).

Flow cytometric assays also indicate that DNA ploidy levels were not changed in either the roots or shoots of *OsCDKB1;1*-RNAi (Supplementary Fig. S8A–H). Consistent with this, the expression of S-phase *PCNA* and M-phase cyclin *CYCB2;1* was not different between wild-type and *OsCDKB1;1*-RNAi transgenic plants (Supplementary Fig. S8I). Taken together, it seems that cell division was not interrupted by down-regulation of the *OsCDKB1;1* transcript level in transgenic rice, though we could not exclude the possibility that the remaining activity of *OsCDKB1;1* protein is sufficient to function.

#### *OsCYCA2;1* and *OsCDKB1;1* enable complementation of Arabidopsis *cdkb1;1 1;2*

Arabidopsis *cdkb1;1 1;2* mutants, like the *cyca2;34* mutants, display a decreased stomatal production, formation of SGCs,



**Fig. 6.** *OsCYCA2;1* directly interacts with *OsCDKB1;1*. (A) Yeast two-hybrid assay. (B) Protein pull-down assay. (C) Transient expression of *OsCDKB1;1*-GFP and *OsCYCA2;1*-GFP in tobacco leaves. Scale bar=20  $\mu$ m. (D) Bimolecular fluorescence complementation assay shows that *OsCDKB1;1* interacts with *OsCYCA2;1* in nuclei. Scale bar=20  $\mu$ m. (This figure is available in colour at *JXB* online.)

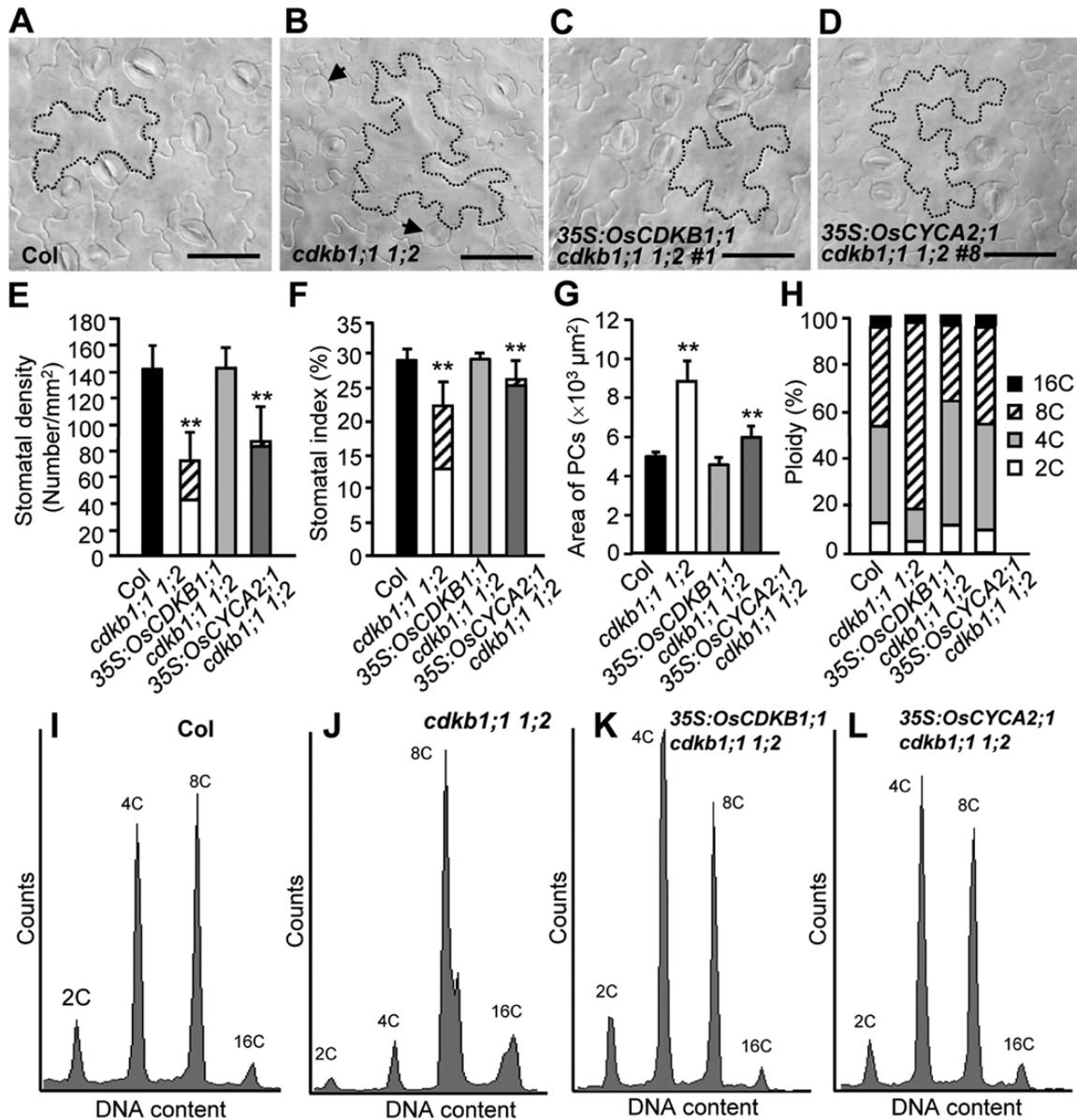
enlarged PCs, and increased cell ploidy levels (Boudolf *et al.*, 2004a; Xie *et al.*, 2010). Introduction of *OsCDKB1;1* fully complements the impaired GMC division in *cdk1;1 1;2*, and restores stomatal production, indicating that *OsCDKB1;1* has the ability to promote both symmetric and asymmetric division. Meanwhile, expression of *OsCKDB1;1* could efficiently prevent the occurrence of enlarged PCs and increased DNA ploidy levels in *cdk1;1 1;2* mutants (Fig. 7; Supplementary Figs S9, S10).

Interestingly, the defective stomatal production, impaired GMC division, and abnormal cell enlargement and DNA levels in *cdk1;1 1;2* could be partially rescued by overexpression of *OsCYCA2;1* (Fig. 7; Supplementary Figs S9, S10). It is therefore possible that *OsCDKB1;1* and *OsCYCA2;1* have evolved

from the common ancestor genes with Arabidopsis *AtCDKB1* genes and *AtCYCA2* genes, even though the developmental pathways of the two species have been diverged.

## Discussion

The control of cell division and differentiation is the core of the development and morphogenesis of multicellular organisms. Cyclins, known as conserved activators for the activity of CDKs, play a crucial regulatory role in cell cycle progression in diverse species. The functional pathway of CYCA2s and CDKB1s has been well investigated in the model plant Arabidopsis (Boudolf *et al.*, 2004b, 2009; Imai *et al.*, 2006;



**Fig. 7.** Ectopic expression of *OsCYCA2;1* and *OsCDKB1;1* complements Arabidopsis *cdkb1;1 1;2* mutant phenotypes. (A–D) DIC images of the epidermis of 14-day-old Arabidopsis cotyledons. Arrows indicate the formation of SGCs. Representative PCs are traced with dashed lines. Scale bar=50 μm. (E–G) Comparison of stomatal density, stomatal index, and area of PCs from the cotyledons. The diagonal line-filled box indicates the fraction of SGCs. Data represent the mean ±SD (n=24). Asterisks indicate a significant difference from Col after Student’s *t*-test, \*\**P*<0.01. (H) Proportions of cells with different ploidies. (I–L) Results from the flow cytometric analysis; ~10 000 cell nuclei were measured for each sample.

Xie *et al.*, 2010; Vanneste *et al.*, 2011; Yang *et al.*, 2014). In this study, we generated RNAi transgenic rice lines and performed cross-species complement tests to explore the function of the single rice A2-type cyclin, *OsCYCA2;1*, as well the single rice B1-type CDK, *OsCDKB1;1*. Cross-species expression of *OsCYCA2;1* or *OsCDKB1;1* enables rescue of the defective asymmetric entry divisions for stomatal initiation and GMC symmetric divisions for GC production in Arabidopsis *cyca2;34* and/or *cdkb1;1 1;2* mutants, suggesting that both *OsCYCA2;1* and *OsCDKB1;1* might have evolved from the common ancestor genes with Arabidopsis.

In Arabidopsis, asymmetric divisions generated the early stomatal precursor cells, meristemoids. Meristemoids then differentiate into GMCs after a cell fate change. In grasses, GMCs

are created directly by stomata initiating asymmetric divisions (entry division) without the prior precursor stage of meristemoid. Orthologs of Arabidopsis stomatal transcriptional regulators SPCH, MUTE, FAMA, ICE1, and SCRM2 have been identified in grasses (Liu *et al.*, 2009; Vatén and Bergmann, 2012; Ran *et al.*, 2013; Chen *et al.*, 2017). Instead of a single copy in Arabidopsis, the rice genome has duplicated *SPCH* genes, *OsSPCH1* and *OsSPCH2*. Similar to the weak allele of Arabidopsis *spch*, the rice mutant *ossph2* exhibits a reduced number of stomata (Liu *et al.*, 2009). In Arabidopsis, SPCH heterodimerizes with SCRM/ICE1 or AtSCRM2 to promote stomatal lineage initiation (Kanaoka *et al.*, 2008; Horst *et al.*, 2015). In contrast, in the grass *B. distachyon*, *BdICE1* and *BdSCRM2* show a functional diversity in regulating stomatal

pattern and morphology (Raissig *et al.*, 2016). It is already known that Arabidopsis SPCH activity or stability is modulated by multiple kinases, including MPKs, GSK3/BIN2, and CDKs (Lampard *et al.*, 2009; Gudesblat *et al.*, 2012; Kim *et al.*, 2012; Le *et al.*, 2014). Phosphorylation of Ser186 of SPCH, which might be the target residue of CDKs, positively regulates stomata production (Yang *et al.*, 2015). Thus, it will be interesting to establish if there is a conserved regulatory mechanism between CDK–cyclin and SPCH-ICE1/SCRM2 in grasses.

Besides the involvement in stomatal initiation, Arabidopsis AtCYCA2s and CDKB1s are synergistically required for the GMC symmetric division that is a prerequisite for the final stomatal development (Vanneste *et al.*, 2011). Arabidopsis and rice share common GMC–GC processing; GMCs divide symmetrically to produce the paired GCs of stomata, though the GC shapes are distinct. Thus, a role for OsCDKB1–OsCYCA2 in rice GMC divisions has been highly expected. However, suppression of OsCYCA2;1 transcription in rice by RNAi does not affect the rice GMC symmetric division. It has been identified that transcription of *CDKB1;1*, *CYCA2;3*, and *CDKA;1* in Arabidopsis is repressed by FOUR LIPS (FLP)/MYB88 MYB transcription factors during the GMC–GC transition stages (Xie *et al.*, 2010; Vanneste *et al.*, 2011; Yang *et al.*, 2014). FAMA, like FLP/MYB, also binds to the *CDKB1;1* promoter (Hachez *et al.*, 2011) to limit the GMC divisions to one. In contrast to the tumor-like phenotype in Arabidopsis *fama-1* mutants, the loss-of-function rice allele *osfama-1* did not undergo excessive division except the appearance of misshaped GCs and showing a lack of stomatal pores (Liu *et al.*, 2009). These observations suggest that GMC–GC differentiation is uncoupled from GMC division, in which the putative downstream FAMA/FLP/MYB88, CDKB1;1, and CYCA2;1 are not essential.

According to the phylogenetic analysis, CYCA2 and CDKB1 widely exist in diverse plant species, both in plants bearing stomata and in plants lacking stomata, indicating that CYCA2 and CDKB1 might function as fundamental regulators of the mitotic cell cycle, as well as outside stomatal development. High expression of *OsCYCA2;1* is associated with a high activity of cell proliferation, such as in the proximal end of leaves or root tips (Supplementary Fig. S3B).

Endoreduplication often occurs in cell types that undergo specialized differentiation. In Arabidopsis, the highly differentiated epidermal cells, such as mature PCs and trichomes, usually undergo multiple rounds of DNA replication without mitosis, resulting in polyploid cells (Bursdens *et al.*, 2000). In contrast to the differentiated cells in Arabidopsis, polyploid cells in rice can only be found in the endosperm (Sabelli and Larkins, 2009). In *Drosophila*, it has been reported that cyclin A is one of the key components of chromosomal DNA replication that prevents re-initiation of DNA replication. Overexpression of *Drosophila* cyclin A caused a reduction in ploidy levels and inhibition of the endocycle (Hayashi and Yamaguchi, 1999). Here we found that the fraction of 4C cells remarkably increased in *OsCYCA2;1*-RNAi transgenic plants, while most cells keep a 2C DNA content. However, the expression levels of an S-phase gene *PCNA* and a M-phase gene *CYCB2;1* were suppressed in *OsCYCA2;1*-RNAi plants. Therefore, we speculated that

the increase of 4C cells might be caused by the arrested G<sub>2</sub> to M transition, similar to the observation in the rice knockdown lines of *OsCDKB2;1* (Endo *et al.*, 2012).

However, the *OsCDKB1;1*-RNAi transgenic rice plants, in which *OsCDKB1;1* transcript levels were significantly decreased, display phenotypes comparable with wild-type rice seedlings regarding the stomatal density, root cell division, and DNA content. CDKBs are plant-specific cyclin-dependent kinases that can be subdivided into two groups according to the different cyclin-binding motifs, namely ‘PPTALRE’ in CDKB1s and ‘PPTTLRE’ in CDKB2s (Joubès *et al.*, 2001). In Arabidopsis, each CDKB1 and CDKB2 subgroup contains two members (Vandepoele *et al.*, 2002). It has been predicted that the rice genome has a single *CDKB1* gene and a single *CDKB2* gene, encoding *OsCDKB1;1* and *OsCDKB2;1*, respectively (Supplementary Fig. S11). However, the amino acid sequence alignment revealed that rice *OsCDKB1;1* and *OsCDKB2;1* share the same ‘PPTALRE’ cyclin-binding motif (Supplementary Fig. S12). Expression of *OsCDKB2;1* has been detected in the dividing region of the rice root apex (Umeda *et al.*, 1999a). Transcription of rice *OsCDKB2;1* is abundant during the G<sub>2</sub> to M phase. Knockdown of the *OsCDKB2;1* gene in rice induces an increase of the 4C cell population (Umeda *et al.*, 1999b; Endo *et al.*, 2012). In addition, *OsCDKB2;1* promotes cell division in the root meristem probably through the association with *OsCYCB2s* (Lee *et al.*, 2003). Thus, we cannot rule out that *OsCDKB1;1* might function redundantly with *OsCDKB2;1*, such as forming active CDK–cyclin complexes via binding to the same type cyclins (i.e. *OsCYCA2;1*). Previous *in situ* hybridization results showed that both *OsCDKA;1* and *OsCDKA;2* are expressed in dividing root cells of rice (Umeda *et al.*, 1999b). Thus, further characterization of rice CDK–cyclin pairing and activity can help to reveal the regulatory mechanisms of cell division and differentiation during rice development.

## Supplementary data

Supplementary data are available at *JXB* online.

Table S1. List of primers used in this study.

Fig. S1. Amino acid sequence comparison of A2-type cyclins from rice and Arabidopsis.

Fig. S2. In contrast to dicot Arabidopsis, only one or two copies of genes encoding CYCA2 are found in monocot grasses.

Fig. S3. Relative expression of *OsCYCA2;1* in rice RNAi transgenic plants and in different tissues of wild-type plants.

Fig. S4. Overexpression of rice *OsCYCA2;1* suppresses the enhanced endoreduplication levels in Arabidopsis *cyca2;34*.

Fig. S5. Correlation between stomatal phenotypes and *OsCYCA2;1* expression levels in Arabidopsis *cyca2;34* mutants harboring *OsCYCA2;1*.

Fig. S6. Comparison of the amino acid sequence of *OsCDKB1;1* with that of Arabidopsis CDKB1;1 and CDKB1;2.

Fig. S7. Suppression of *OsCDKB1;1* has no obvious impact on rice root and stomatal development.

Fig. S8. Suppression of *OsCDKB1;1* has no obvious impact on the distribution of DNA ploidy.

Fig. S9. Expression analysis of *OsCDKB1;1*-OE and *OsCYCA2;1*-OE transgenic plants in Arabidopsis *cdkb1* mutants.

Fig. S10. Ploidy distribution analysis of Arabidopsis *cdkb1;1;2* mutants carrying rice *OsCDKB1;1* or *OsCYCA2;1* genes.

Fig. S11. Phylogenetic tree of CDKB1 and CDKB2 in monocots.

Fig. S12. CDKB1 and CDKB2 contain the same cyclin-binding domain in most monocots.

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