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The plant specific CDKB1-CYCB1 complex mediates homologous recombination repair in Arabidopsis

Article

The plant-specific CDKB1-CYCB1 complex mediates homologous recombination repair in *Arabidopsis*

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Abstract

Upon DNA damage, cyclin-dependent kinases (CDKs) are typically inhibited to block cell division. In many organisms, however, it has been found that CDK activity is required for DNA repair, especially for homology-dependent repair (HR), resulting in the conundrum how mitotic arrest and repair can be reconciled. Here, we show that *Arabidopsis thaliana* solves this dilemma by a division of labor strategy. We identify the plant-specific B1-type CDKs (CDKB1s) and the class of B1-type cyclins (CYCB1s) as major regulators of HR in plants. We find that RADIATION SENSITIVE 51 (RAD51), a core mediator of HR, is a substrate of CDKB1-CYCB1 complexes. Conversely, mutants in *CDKB1* and *CYCB1* fail to recruit RAD51 to damaged DNA. *CYCB1;1* is specifically activated after DNA damage and we show that this activation is directly controlled by SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), a transcription factor that acts similarly to p53 in animals. Thus, while the major mitotic cell-cycle activity is blocked after DNA damage, CDKB1-CYCB1 complexes are specifically activated to mediate HR.

See also: **B Desvoyes & C Gutierrez** (October 2016)

Introduction

DNA damage is a crucial problem for every organism and many repair pathways exist to recover from the different types of DNA damage. Of key importance after DNA damage is an arrest of cell division to allow sufficient time for repair and to prevent that mutated daughter cells are generated that will propagate incorrect genetic information. One severe type of DNA damage often caused by irradiation or chemical mutagens is double-strand breaks (DSBs), and the signaling cascades from DSBs to cell division arrest are well understood in yeast and animals. In essence, DSBs induce the activity of the kinase ataxia-telangiectasia mutated (ATM) that phosphorylates and activates checkpoint kinase 2 (Chk2). Chk2 in turn inhibits the Cdc25 phosphatase, a central activator of the main cell-cycle regulators Cdk1 and Cdk2 in animals. In addition, the ATM pathway activates Wee1, a negative regulator of Cdk1 and Cdk2 providing a parallel block of the cell cycle (Kastan & Bartek, 2004; Harper & Elledge, 2007; Yata & Esashi, 2009).

Remarkably, plants can cope with very high concentrations of harmful agents in comparison with animals. For instance, a comparative study of tobacco BY-2 and Chinese hamster ovary cells showed that plant cells yielded one-third less double-strand breaks after the same dose of ionizing radiation (IR). Furthermore, the plant cells also tolerated a much higher number of DSBs before they died (Yokota *et al*, 2005). Despite the apparent power and their relevance for agriculture under changing environmental conditions, the plant DNA repair pathways are not very well understood. Moreover, the canonical response pathways of yeast and animals appear to be only partially conserved. While homologs of ATM and its sister kinase ATR (ATM- and Rad3-related) predominantly involved in replication

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stress response by sensing single-stranded DNA have also been identified in *Arabidopsis* (Garcia *et al*, 2000; Culligan *et al*, 2004; Culligan & Britt, 2008), no homologs of Chk2 or its sister kinase Chk1 could be found in plants to date. Furthermore, even though a homolog of the yeast Wee1 kinase exists in *Arabidopsis* and other plants, its function appears to be different as *Arabidopsis* WEE1 was found to act during S phase after hydroxyurea (HU)-induced replication stress and not in repressing CDK activity during mitosis or blocking cell division after DSB formation (De Schutter *et al*, 2007; Cools *et al*, 2011). Moreover, transgenic plants expressing a mutant version of CDKA;1, the *Arabidopsis* homolog of mammalian Cdk1 and Cdk2, in which the putative WEE1 target sites were replaced with non-phosphorylatable amino acids, were not hypersensitive to HU indicated that cell-cycle arrest after DNA damage is differently regulated in plants (Dissmeyer *et al*, 2009, 2010).

Besides CDKA;1, plants contain B-type CDKs that have been implicated in cell-cycle control. While there appears to be only a single B-type CDK in the unicellular algae *Chlamydomonas rheinhardii* that is essential for mitosis (Bisova *et al*, 2005; Tulin & Cross, 2014), B-type CDKs are divided into a B1 and B2 class in *Arabidopsis* and other multicellular plants. B2-type CDKs appear to be major regulators of mitosis in *Arabidopsis* and their loss as well as their overexpression interferes with cell proliferation hinting at a strong dose-dependent action (Andersen *et al*, 2008). However, due to the lack of mutants, a detailed analysis of B2-type kinases is still pending. In contrast, B1-type CDKs have been functionally analyzed, but these studies revealed so far that they apparently act as axillary kinases to A1-type kinases contributing to the refinement of developmental decisions (Xie *et al*, 2010; Cruz-Ramirez *et al*, 2012; Nowack *et al*, 2012; Weimer *et al*, 2012).

Another obvious difference between plants and other well-studied eukaryotes is the presence of a large groups of cyclins, for example, more than 30 cyclins in *Arabidopsis*, most of which are still uncharacterized (Harashima *et al*, 2013). Very little is known about the regulation of these cyclins but remarkably, previous studies have revealed that *CYCB1;1* is upregulated during various treatments of DNA damage-inducing agents or in mutants affected in chromatin organization, DNA metabolism, and/or repair such as *fasciata 1* (*fas1*), *jing he sheng 1* (*jhs1*), and *dna replication factor c1* (*rfc1*) (Chen *et al*, 2003; Culligan *et al*, 2004; Endo *et al*, 2006; Liu *et al*, 2010; Adachi *et al*, 2011; Jia *et al*, 2016). This upregulation is remarkable due to the predicted role of these cyclins in promoting cell division. Up to now, it was not clear what the role of B1-type cyclins in DNA damage response is, especially in which DNA damage pathway they could act.

In plants as well as in other organisms, two major DNA repair pathways are responsible for genomic integrity after DNA double-strand breaks: non-homologous end-joining (NHEJ) and homology-dependent repair, also called homologous recombination repair (HR). With NHEJ, the damaged DNA is repaired by direct ligation of the broken ends. The double-strand break is recognized by a KU70/KU80 heterodimer and then processed by the MRN complex that is composed of MRE11 (MEIOTIC RECOMBINATION 11), RAD50 (RADIATION SENSITIVE 50), and NBS1 (NIJMEGEN-BREAKAGE SYNDROME 1) (Amiard *et al*, 2013). DNA ends are ligated by LIG4 (DNA LIGASE 4) and XRCC4 (X-RAY REPAIR CROSS-COMPLEMENTATION PROTEIN 4) (Bray & West, 2005). Consistently, *ku70* and *ku80* mutants are hypersensitive to the

DSB-inducing agents bleomycin (BLM) and methyl methane sulfonate (MMS) (Riha *et al*, 2002). However, NHEJ can be imprecise, leading to the loss of nucleotides when overlaps are not compatible (Takata *et al*, 1998).

In contrast to NHEJ, HR is highly accurate since it exactly replaces the defective DNA (Shrivastav *et al*, 2008). HR requires a homologous template to repair the damaged DNA and can therefore only occur after DNA replication in S phase and the subsequent G2 phase of the cell cycle when sister chromatids are available. This pathway is initiated by the resection of DNA, also executed by the MRN complex, and formation of long 3′ tails, which are coated by RPA (REPLICATION PROTEIN A) in order to prevent winding of the DNA. Homology search and strand invasion are performed by RAD51 family members (Serra *et al*, 2013), the eukaryotic homolog of the *E. coli* recA protein (Mengiste & Paszkowski, 1999). RAD51 has five paralogs in *Arabidopsis* (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3), all of which function in HR in somatic or meiotic cells and show fewer homologous recombination events after DNA damage (Abe *et al*, 2005; Da Ines *et al*, 2013a,b; Serra *et al*, 2013), which is in line with studies in other eukaryotes (Hosoya & Miyagawa, 2014).

A key question is how cells can decide whether to follow NHEJ or enter an HR pathway. While this decision appears to be complex and likely also involves developmental factors, many studies have revealed that CDKs play an important role in the choice of the repair pathway based on the observation that mitotic CDK activity is rising after S phase and hence allowing a cell to discriminate between a G1 and a G2 phase (Wohlbold & Fisher, 2009; Yata & Esashi, 2009; Trovesi *et al*, 2013). Moreover, CDKs were found to be directly involved in promoting HR. However, the requirement of active CDKs for HR causes an apparent dilemma for a cell since mitotic CDK activity needs to be shut down to arrest the cell division program as a first measure to DNA damage.

Here, we show that plants solve this problem by specifically activating B1-type CDKs at a transcriptional and posttranslational level after DNA damage. With this, we reveal a previously not recognized key function of B1-type CDKs as central regulators of DNA damage response in plants. We show that CYCB1s are the specific partner of CDKB1 during DNA damage and both form active complexes that can phosphorylate RAD51. Moreover, we show that HR and NHEJ pathways act at least partially redundantly on DSB, possibly contributing to the powerful DNA damage repair system of plants.

Results

Mutants for B1-type cyclins are specifically hypersensitive to DNA cross-links

Based on the observation that *CYCB1;1* is upregulated during treatments with DNA damage-inducing agents (Chen *et al*, 2003; Culligan *et al*, 2004, 2006; Ricaud *et al*, 2007; Adachi *et al*, 2011), we isolated mutants in all four B1-type cyclins to address a possible role of these cyclins in DNA stress (Fig EV1A–C). To this end, we monitored root growth of *Arabidopsis* plants on agar plates containing different DNA-damaging drugs (see below). On control plates without DNA damage agents, none of the *cycb1* mutants showed

altered root growth in comparison with the wild type (Figs 1A and B, and EV2A–D and I).

First, we tested root growth on media containing HU, which causes intra-S-phase stress due to the inhibition of the enzyme ribonucleotide reductase and thus a decrease in production of deoxyribonucleotides (Yarbro, 1992). For this analysis, *wee1* was used as a positive control and, consistent with previous data, was found to be highly sensitive to HU, whereas it shows no growth abnormalities on control medium (De Schutter *et al*, 2007; Cools *et al*, 2011). In contrast, root growth on HU of all tested *cycb1* mutants was comparable to the growth of wild-type plants (Fig 1C and D). To address a possible redundant function among the CYCB1 group, we generated the double mutants *cycb1;1 cycb1;2, cycb1;1 cycb1;3, cycb1;1 cycb1;4, cycb1;2 cycb1;4,* and *cycb1;3 cycb1;4.* With the exception of *cycb1;1 cycb1;2,* all double mutants grew indistinguishably from the wild type on media with and without HU (Figs 1A–D and EV3A). The double mutant *cycb1;1 cycb1;2* had shorter roots than the wild type on both media with and without DNA stress-inducing drugs (Fig 1A–D). Comparing the root growth ratios of plants grown on media without and with HU, it became obvious that this double mutant was not more sensitive than the wild type to HU (Fig EV3A).

Next, root growth of single and double mutant combinations of *cycb1s* was tested on media containing the DSB-inducing drug BLM. As a positive control, we used mutants in *ku70* that were shown to grow as the wild type on medium without drugs (Cools *et al*, 2011). Whereas *ku70* mutants were sensitive to BLM and grew only very little consistent with previous reports (Tamura *et al*, 2002; West *et al*, 2002; Cools *et al*, 2011), no significant difference was found between *cycb1* single and double mutants versus the wild type again with the exception of *cycb1;1 cycb1;2* (Fig 1E and F). Comparing root growth ratios on plates with and without BLM indicated that *cycb1;1 cycb1;2* is also not hypersensitive to this drug (Fig EV3A).

As a third drug, the hypersensitivity of *cycb1* mutants to cisplatin was tested. Cisplatin causes in addition to DNA breaks also intra- and interstrand DNA links that require repair by HR in contrast to damage caused by BLM and HU that can also be repaired by NHEJ (Kartalou & Essigmann, 2001; Belenkov *et al*, 2002; De Silva *et al*, 2002; Fuertes *et al*, 2002; Crul *et al*, 2003; Siddik, 2003; Pinato *et al*, 2014). Since cisplatin is unstable in solution, seedlings were germinated on media without the drug and then transferred to plates containing two concentrations of cisplatin (15 and 30 μM) 3 days after germination. On plates with 15 μM cisplatin, the net root growth of the *cycb1* mutants at 3 days after the transfer appeared to be reduced but was not statistically significantly different from the growth of wild-type plants (Fig 1G–I, L and M). However, at 30 μM cisplatin, the roots of all B1-type cyclin mutants were significantly shorter than the roots of wild-type plants (Student's *t*-test *P* < 0.01) (Fig 1J and K). The observation that root growth of the *cycb1* double mutants was not further reduced in comparison with the growth of the single mutants suggested that all four cyclins contribute in a non-additive manner to growth on media with cisplatin (Fig 1G, J, K and N). One exception was the double mutant *cycb1;1 cycb1;4* that, while being shorter than the wild type, grew better than the other double mutants. However, since all single mutants including *cycb1;1* and *cycb1;4* as well as all other double mutant combinations are significantly shorter, we conclude that an indirect effect, for

example, a compensatory action, in the *cycb1;1 cycb1;4* double mutant triggers this response and that the general theme of mutants in B1-type cyclins is a hypersensitivity against cisplatin.

Next, we asked whether the hypersensitivity of the *cycb1* mutants and their reduced growth on cisplatin was due to increased cell death. To this end, we stained the wild type and *cycb1* mutants with propidium iodide to visualize dying cells. Under control conditions, no cell death occurred in all tested genotypes. After cisplatin treatment, dead cells were observed in close proximity to the quiescent center. However, we did not see obvious difference between the wild type and *cycb1* mutants indicating a higher level of cisplatin-induced DNA damage in the *cycb1* mutants (Fig EV4).

Previously, it was reported that *Arabidopsis* root cells entered an endoreplication cycle in which the nuclear DNA is amplified without subsequent cell division as a response to zeocin-induced DNA damage (Adachi *et al*, 2011; De Veylder *et al*, 2011; Edgar *et al*, 2014). Although we cannot exclude long-term effects of cisplatin to promote endoreplication, we did not see a major increase in endoreplication levels in comparison with control plants when we analyzed cells of the root tips of 5-day-old wild-type plants grown for 24 h on media with 50 μM cisplatin. Likewise, a strong increase in endoreplication was not observed in *cycb1;1 cycb1;3* double mutants when treated with cisplatin for 24 h (Fig EV5).

Next, we tested the response to genotoxic stresses of other mutants in cyclins with a mitotic function. To this end, we investigated the group of A2-type cyclins that build a small gene family in *Arabidopsis* with four members (Vanneste *et al*, 2011). The loss of all four members leads to very slow and impaired postembryonic growth, but the triple mutant *cyca2;2 cyca2;3 cyca2;4* (named in the following *cyca2;234*) is viable and was analyzed on media containing HU, BLM, and cisplatin. In contrast to mutants in B1-type cyclins, *cyca2;234* triple mutants were neither sensitive to HU nor BLM and, notably, also not to cisplatin (Fig EV6A–C). Thus, the hypersensitivity to cisplatin is a specific feature of *cycb1* mutants.

To assess whether *cycb1* mutants indeed accumulate more DNA damage after cisplatin treatment, we performed comet assays, which allow the visualization of DNA DSBs through the formation of a DNA "tail" after electrophoresis of isolated nuclei. For this analysis, the double mutant *cycb1;1 cycb1;3* was selected as a representative genotype and assayed after cisplatin treatment and after a recovery phase of 30 min. Quantification of the DNA in the tail showed that *cycb1;1 cycb1;3* double mutants contained significantly more DNA breaks than the wild type during and after withdrawal from cisplatin media (Student's *t*-test *P* < 0.01) (Fig 2A and B).

As a response to DSBs, the histone variant H2AX becomes phosphorylated at serine 139 (designated gamma-H2AX) at the break site (Kuo & Yang, 2008). In mutants impaired in DNA damage repair, such as *atm*, *atr*, *ku70,* and *rad51*, many more gamma-H2AX can be observed. Consistent with a higher rate of DNA lesions, we found that *cycb1;1 cycb1;3* double mutants also showed more gamma-H2AX foci in immunohistological stainings than the wild type after growth on cisplatin-containing media (Student's *t*-test *P* < 0.0001) (Fig 2C and D).

To investigate whether *cycb1* mutants are indeed compromised in HR, we deployed a previously described assay to detect homologous recombination events (Swoboda *et al*, 1994). This assay system makes use of a disrupted gene encoding *beta-glucuronidase*
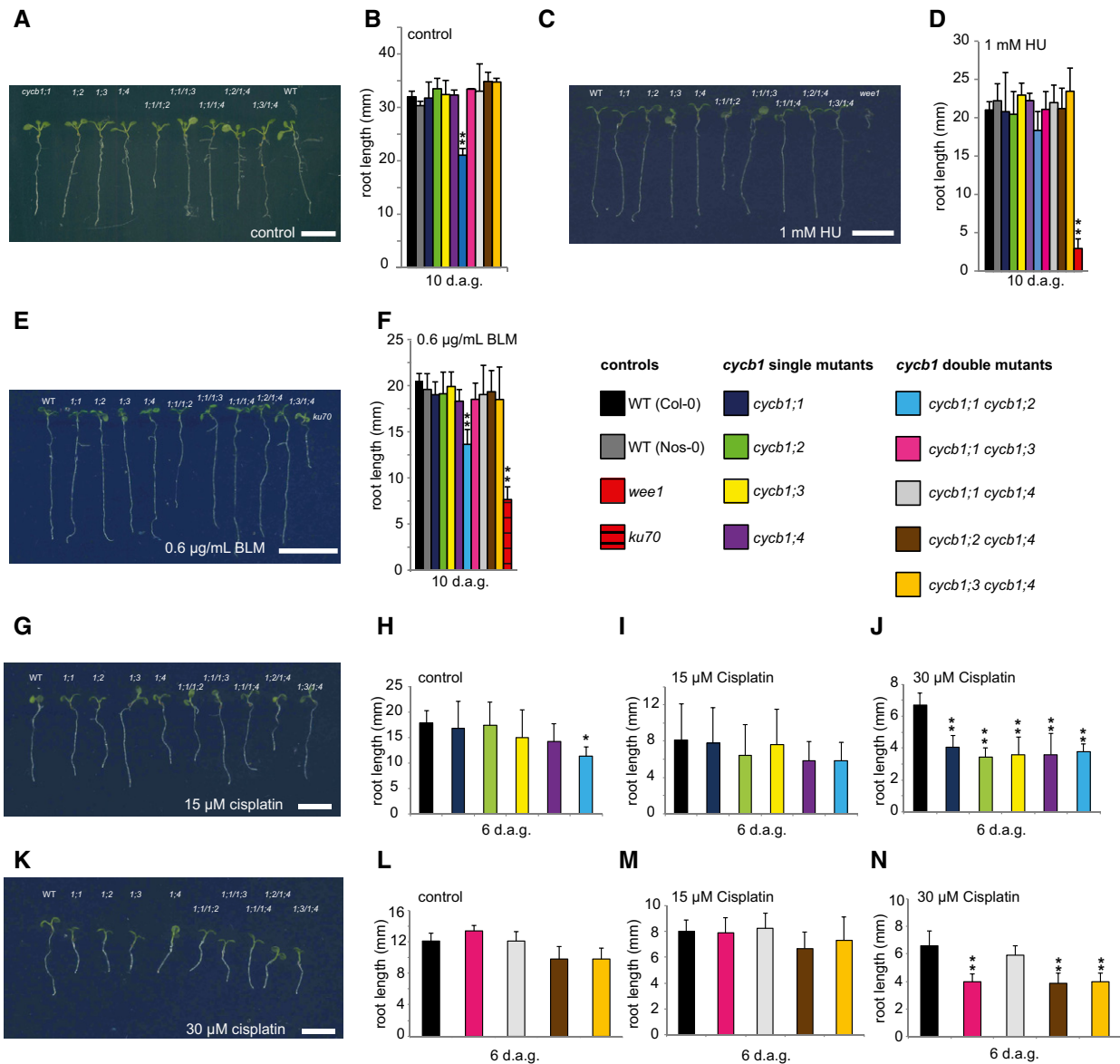
Figure 1. Mutants of B1-type cyclins are hypersensitive to cisplatin.

A   *cycb1;1, cycb1;2, cycb1;3, cycb1;4, cycb1;1/1;2, cycb1;1/1;3, cycb1;1/1;4, cycb1;2/1;4, cycb1;3/1;4,* and the wild type (from left to right) on control plates without genotoxic agent 10 days after germination.

B   The wild type, single, and double mutants of *cycb1* were grown on control plates without genotoxic agent. Root lengths were measured 10 days after germination.

C   The wild type, *cycb1;1, cycb1;2, cycb1;3, cycb1;4, cycb1;1/1;2, cycb1;1/1;3, cycb1;1/1;4, cycb1;2/1;4, cycb1;3/1;4* (from left to right) on plates containing 1 mM hydroxyurea (HU) 10 days after germination. The rightmost plant is the *wee1* mutant that shows high sensitivity to HU.

D   The wild type, single, and double mutants of *cycb1* were grown on plates supplemented with 1 mM HU. Root lengths were measured 10 days after germination.

E   The wild type, *cycb1;1, cycb1;2, cycb1;3, cycb1;4, cycb1;1/1;2, cycb1;1/1;3, cycb1;1/1;4, cycb1;2/1;4, cycb1;3/1;4* (from left to right) on plates containing 0.6 µg/ml bleomycin (BLM) 10 days after germination. The rightmost plant is the *ku70* mutant that shows high sensitivity to BLM.

F   The wild type, single, and double mutants of *cycb1* were grown on plates supplemented with 0.6 µg/ml BLM. Root lengths were measured 10 days after germination.

G   The wild type, *cycb1;1, cycb1;2, cycb1;3, cycb1;4, cycb1;1/1;2, cycb1;1/1;3, cycb1;1/1;4, cycb1;2/1;4, cycb1;3/1;4* (from left to right) on plates containing 15 µM cisplatin 6 days after germination, that is, 3 days after transfer from control plates.

H–J   *cycb1* mutants were germinated on control plates and were transferred to new control plates (H) or plates supplemented with 15 µM (I) or 30 µM (J) cisplatin 3 days after germination. Root lengths were measured 3 days after transfer and the net root growth of 3 days is shown in the graphs.

K   The wild type, *cycb1;1, cycb1;2, cycb1;3, cycb1;4, cycb1;1/1;2, cycb1;1/1;3, cycb1;1/1;4, cycb1;2/1;4, cycb1;3/1;4* (from left to right) on plates containing 30 µM cisplatin 6 days after germination, that is, 3 days after transfer from control plates.

L–N   *cycb1* double mutants germinated on control plates and were transferred to new control plates (L) or plates supplemented with 15 µM (M) or 30 µM (N) cisplatin 3 days after germination. Root lengths were measured 3 days after transfer and the net root growth of 3 days is shown in the graphs.

Data information: One or two asterisks indicate significant differences within a 5 and 1% confidence interval, respectively (Student's *t*-test). Scale bars: 1 cm. Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. Graphs represent mean ± SD.
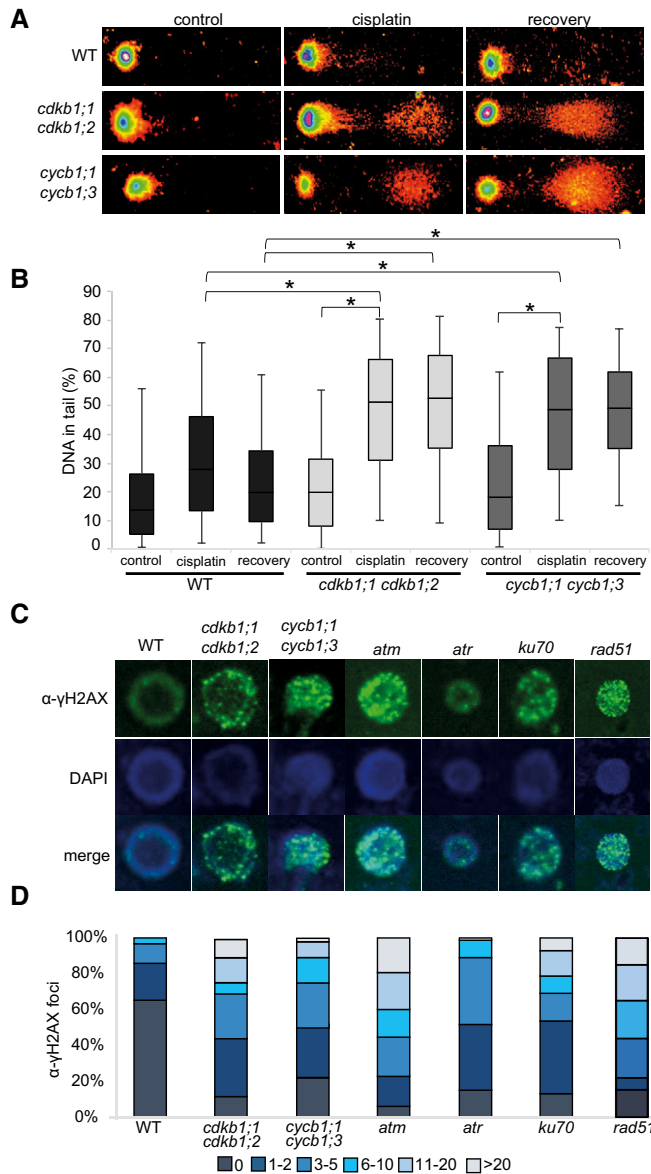
Figure 2. Mutants of *cdkb1* and *cycb1* show increased number of DSBs and delayed DNA repair upon cisplatin treatment.

A Representative examples of comets of 21-day-old wild-type plants, *cdkb1;1 cdkb1;2* and *cycb1;1 cycb1;3* double mutant seedlings in full spectrum view of the TriTek Comet Score software. Shown are comets of plants incubated with 50 μM cisplatin for 1 h and then transferred to medium without cisplatin for 30 min (recovery) and plants incubated without cisplatin for 1 h (control), respectively.

B Box plot of percentage of tail DNA of wild-type cells, *cdkb1;1 cdkb1;2* and *cycb1;1 cycb1;3* double mutants under cisplatin treatment. Plots are based on analyses of 200 cells per sample from random microscopic fields of three independent biological replicates. The percentage of DNA fragments in the comet tail was calculated by the TriTek Comet Score software. The box represents the interquartile range, the line across the box indicates the median values, and whiskers represent 5–95 percentile values. Brackets connect plots of sample groups that are significantly different with a confidence level higher than 99.99% calculated with Student's *t*-test.

C Immunostaining of γ-H2AX foci in wild-type plants and mutant cells after 2 h of treatment with 50 μM cisplatin.

D Counted numbers of γ-H2AX foci per cell detected after 2 h of treatment with 50 μM cisplatin in wild-type and mutant plants. For each sample, the γ-H2AX foci of 100 cells were counted and grouped into six categories: cells with no, 1–2, 3–5, 6–10, 11–20, and more than 20 foci per cell.

Mutants for B1-type CDKs are specifically hypersensitive to DNA cross-links

Cyclins usually act together with a CDK partner. Among the five CDKs in *Arabidopsis* that have been implicated in direct regulation of cell-cycle progression (i.e. CDKA;1, CDKB1;1, CDKB1;2; CDKB2;1, and CDKB2;2), CDKA;1 has been found to be of central importance controlling both the G1-S and G2-M transition (Nowack *et al*, 2012). Although homozygous *cdka;1* mutants exist, they are so severely compromised that an analysis, especially under stress conditions, faces the danger of giving ambiguous results due to pleiotropic and indirect effects (Nowack *et al*, 2012). We therefore made use of a previously described weak loss-of-function allele, designated *CDKA;1^{T14D;Y15E}* or short *DE* (Dissmeyer *et al*, 2009), to address whether CDKA;1 plays a role in the response to cisplatin. *DE* displayed shorter roots than the wild type on plates without DNA damage-inducing agents (Fig 4A, G, J and M) as well as on media supplemented with BLM (Fig 4C and L). Previously, we found that *DE* is not sensitive to HU (Dissmeyer *et al*, 2009) (Fig 4B and K). Taking the root growth ratio into account, we determined that *DE* was neither more sensitive to HU nor to BLM than the wild type (Fig EV3B). Mutants in *ku70* and *wee1* were used as a control for BLM and HU treatments, respectively, and, consistent with previous reports, showed hypersensitivity on the respective drugs (Fig 4B, C, E and F). In addition, analyzing the growth ratio of *DE* roots on 15 and 30 μM cisplatin-containing medium compared to roots grown on control plates without DNA damage-inducing drugs, it became obvious that *DE* was also not hypersensitive to cisplatin (Fig 4G–I, M and N). This result suggested that other CDKs might be involved in the response to cisplatin damages and operate together with B1-type cyclins.

Putative candidates are the plant-specific CDKB1s since the expression pattern of B1-type cyclins in G2 phase overlaps with the expression pattern of *CDKB1s* during the cell cycle (Menges *et al*, 2005). In addition, it was shown that CYCB1;2 has kinase activity in complex with CDKB1s *in vitro* (Harashima & Schnittger, 2012). Since CDKB1;1 and CDKB1;2 were found to have overlapping

(*uidA/GUS*) gene, which serves as a substrate for homologous recombination (Fig 3A). In cells, where homologous recombination events occur, the *uidA* gene is restored and subsequently, GUS activity can be detected as blue spots after histochemical staining (Fig 3B and C). The line with the disrupted *uidA* was crossed to all four *cycb1* single mutants and plants homozygous for the reporter and the respective mutant were identified. Already under control conditions, *cycb1* mutants appeared to have significantly fewer blue sectors, average of 0.5 per seedling plant, in comparison with the wild type with on average 1 spot (Student's *t*-test *P* < 0.001) (Fig 3D). After incubation on 15 and 30 μM cisplatin for 3 days, recombination rates increased in the wild type reaching 6 and 13 spots per plant, respectively (Fig 3B, E and F). However, all *cycb1* mutants showed fewer blue spots indicative for reduced number of homologous recombination events (Student's *t*-test *P* < 0.001) (Fig 3C, E and F). We therefore concluded that B1-type cyclins are required for homologous recombination repair in *Arabidopsis*.
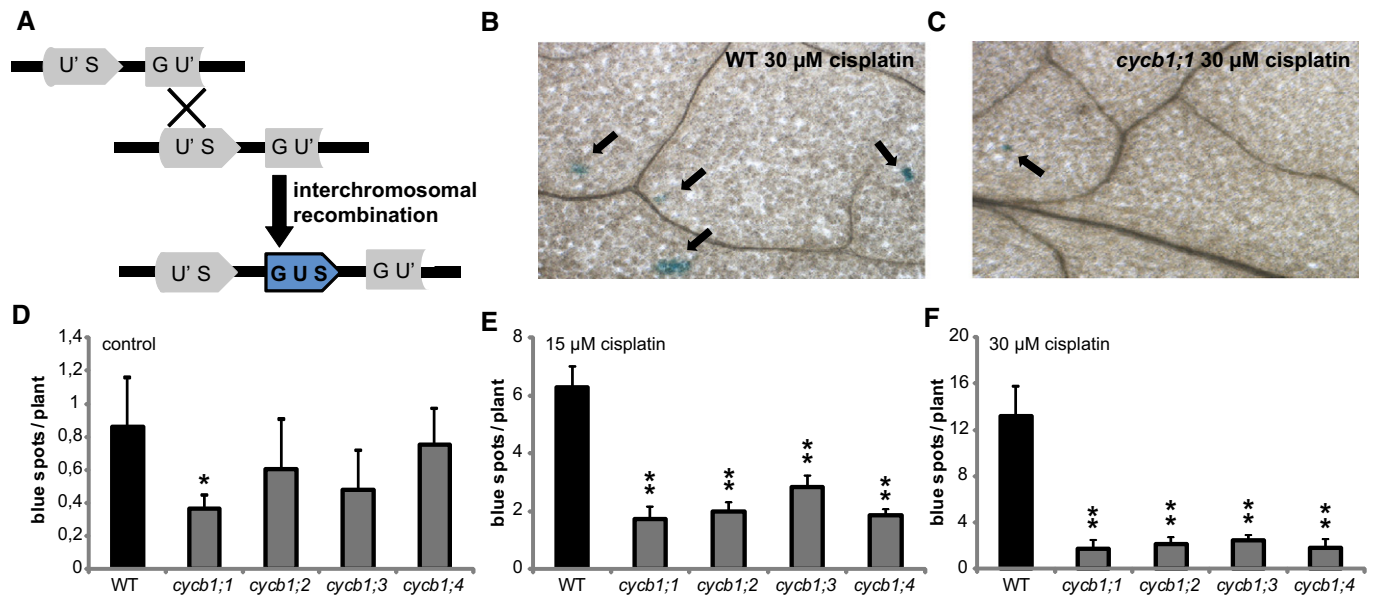
Figure 3. Homologous recombination frequencies are strongly reduced in *cycb1* mutants.

A   Schematic drawing of homologous recombination assay. Restoring the functional GUS gene from two disrupted parts (GU' and US') is restricted to an intermolecular homologous recombination event. Homologous events occur only when a sister chromatid or homolog is available as a template, that is, in G2 phase of the cell cycle.

B   Wild-type plants show blue spots on the leaves after 3 days of incubation on 30 μM cisplatin. Arrows indicate representative blue sectors.

C   *cycb1;1* plants show blue spots on the leaves after 3 days of incubation on 30 μM cisplatin. Arrows indicate representative blue sectors.

D–F   Graphs show numbers of blue sectors per plant grown without drug treatment (D) or after incubation on 15 μM (E) or 30 μM (F) cisplatin for 3 days. One or two asterisks indicate significant differences within a 5 and 1% confidence interval, respectively (Student's *t*-test). Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. Graphs represent mean ± SD.

functions (Xie *et al*, 2010; Cruz-Ramirez *et al*, 2012; Nowack *et al*, 2012; Weimer *et al*, 2012), we used the previously generated double mutant *cdkb1;1 cdkb1;2* in the following studies, referred to as *cdkb1*. We first tested *cdkb1* mutants on HU but the single as well as the double mutants grew indistinguishably from the wild type (Fig 4D, E and K). As a control, we monitored again *wee1* mutants that were found to be highly susceptible to HU in the media. Next, we analyzed root growth on BLM-containing media with *ku70* mutants as a control (Fig 4F and L). While both *cdkb1* single mutants were not hypersensitive, the double mutant *cdkb1;1 cdkb1;2* showed a reduction by approximately 25% in root growth when compared to wild-type plants grown on BLM (Student's *t*-test $P < 0.05$). This indicated a minor but distinguishable role of CDKB1s in DSB repair induced by BLM (see below).

Next, we assayed root growth of *cdkb1* mutants on two different concentrations of cisplatin. Under these conditions, the roots of *cdkb1;1 cdkb1;2* were dramatically compromised and grew only up to a third of the size of the wild type (Student's *t*-test $P < 0.001$) (Fig 4G–I, M and N). Consistent with this growth reduction, we could detect very high levels of DNA damage in *cdkb1;1 cdkb1;2* during cisplatin treatment and 30 min after recovery (Fig 2A and B). Quantification of gamma-H2AX foci even showed higher levels of DNA damage in *cdkb1;1 cdkb1;2* double mutants than in *cycb1;1 cycb1;3* double mutants reaching a level comparable to the one seen in *atm* mutants (Fig 2C and D). Similar to *cycb1* mutants, we also did not observe strongly increased endoreplication levels in *cdkb1*

mutants grown on media with cisplatin in comparison with mutants grown on media without cisplatin (Fig EV5).

To test whether CDKB1s operate in the same or in a parallel genetic pathway as CYCB1s, we generated the homozygous triple mutant *cycb1;1 cdkb1;1 cdkb1,2* and analyzed its response to the DNA-damaging drugs HU, BLM, and cisplatin. Our hypothesis was that, if CDKB1s and CYCB1s act in different pathways, *cycb1;1 cdkb1;1 cdkb1;2* triple mutants should be more compromised on media containing DNA-damaging drugs than *cdkb1* mutants. However, the triple mutant was not significantly different from *cdkb1;1 cdkb1;2* double mutants grown on control plates and plates supplemented with 1 mM HU, 0.6 μg/ml BLM, and 30 μM cisplatin (Fig EV7).

Taken together, we conclude that B1-type CDKs are involved in the response after DNA damage, especially damage induced by cisplatin. Our data show that CDKB1s do not carry out this function in combination with A2-type cyclins (Fig EV6) that were previously identified as specific partners of B1-type CDKs (Boudolf *et al*, 2009) but largely in conjunction with B1-type cyclins.

RAD51 localization depends on CDKB1s and CYCB1s

RAD51 is a homolog of the bacterial *RecA* recombinase and a key factor in HR in eukaryotes. *RAD51* and its homologs build a small gene family in *Arabidopsis* similar to other eukaryotes (Lin *et al*, 2006). Previous studies revealed hypersensitivity of mutants in *Arabidopsis RAD51* family members to DNA-cross-linking agents
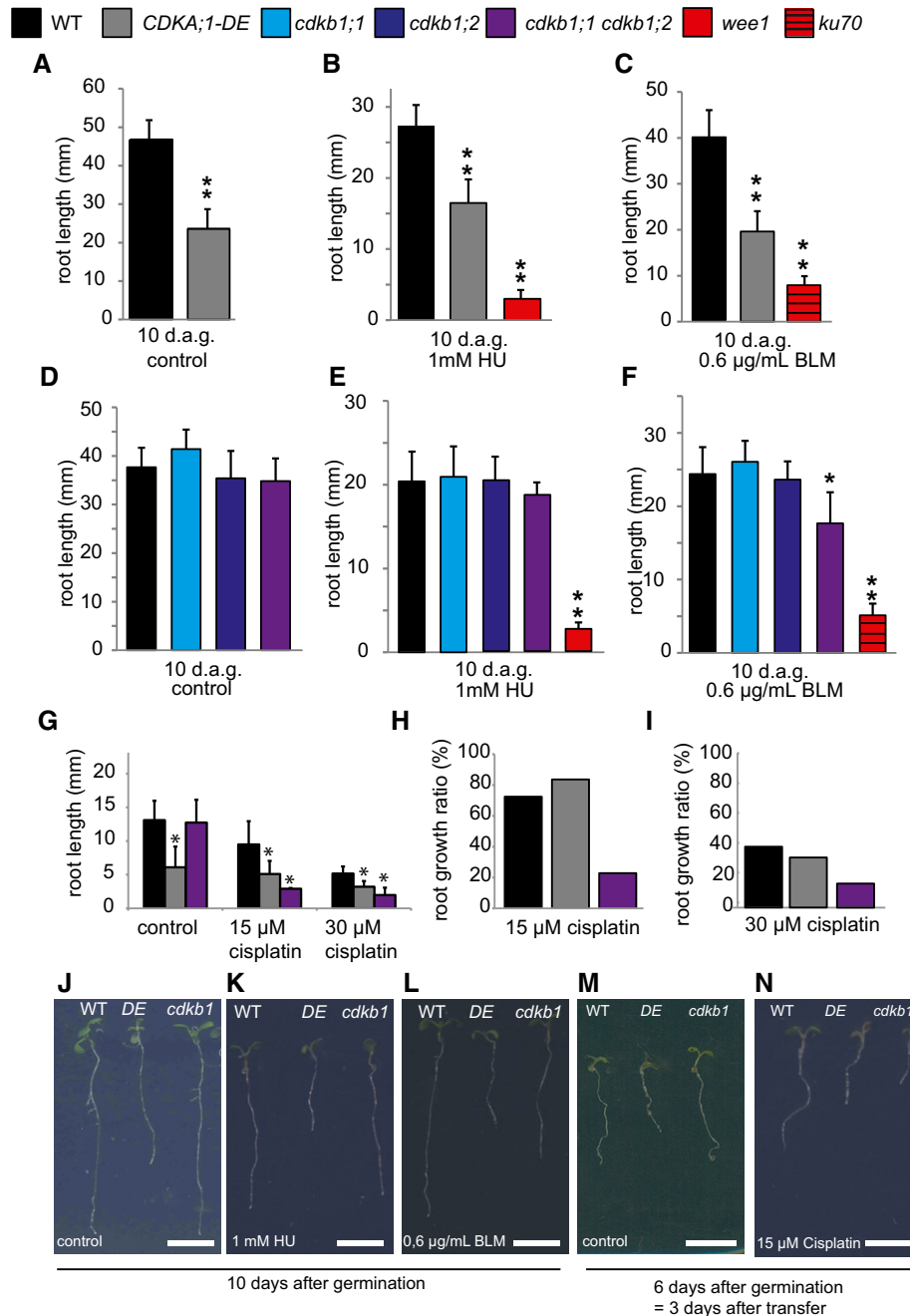
Figure 4. Mutants of *cdkb1* but not *cdka;1* are hypersensitive to cisplatin.

A–C   The wild type and *CDKA;1-DE* mutants were grown on control plates (A) or containing 1 mM hydroxyurea (B) or 0.6 μg/ml bleomycin (C) for 10 days. The mutants *wee1* and *ku70* were used as positive controls for hydroxyurea or bleomycin sensitivity, respectively. Root lengths were measured 10 days after germination.

D–F   The wild type, *cdkb1;1*, *cdkb1;2*, and the double mutant *cdkb1;1 cdkb1;2* were grown on control plates (D) or plates containing 1 mM hydroxyurea (E) or 0.6 μg/ml bleomycin (F) for 10 days. The mutants *wee1* and *ku70* were used as positive controls for hydroxyurea and bleomycin sensitivity, respectively. Root lengths were measured 10 days after germination.

G   The wild type, *CDKA;1-DE*, and the double mutant *cdkb1;1 cdkb1;2* were grown on control plates and were transferred to plates containing 15 or 30 μM cisplatin 3 days after germination. Root lengths were measured 3 days after transfer and the net root growth of 3 days is shown in the graphs.

H, I   Graphs represent the ratio of the mean growth rate on 15 μM (H) or 30 μM (I) cisplatin compared to control experiments on plates lacking cisplatin for the wild type, *CDKA;1-DE*, and *cdkb1;1 cdkb1;2*.

J–N   Images show a wild-type plant, *CDKA;1-DE*, and the double mutant *cdkb1;1 cdkb1;2* (from left to right) on the indicated day after germination and the indicated drug treatment. Scale bars: 1 cm.

Data information: One or two asterisks indicate significant differences within a 5 and 1% confidence interval, respectively (Student's *t*-test). Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. Graphs represent mean ± SD.

such as cisplatin and mitomycin C, as seen, for example, by a reduced number of true leaves formed in *rad51b* and *rad51c* mutants in comparison with the wild type (Osakabe *et al*, 2002, 2005; Bleuyard & White, 2004; Abe *et al*, 2005; Bleuyard *et al*, 2005; Li *et al*, 2005; Charbonnel *et al*, 2010). Consistent with these reports, we found that the root growth of *rad51* mutants is impaired in the presence of cisplatin (Fig 5A–C). Correspondingly, we found that *rad51* mutants accumulated a large number of gamma-H2AX foci after treatment with cisplatin (Fig 2C and D).

Matching the previously reported localization patterns after DNA damage (Da Ines *et al*, 2013b), we found that RAD51 builds foci in the nuclei of root cells after treatment with cisplatin as seen with an antibody raised against RAD51 (Fig 5D and E). These foci depend on the presence of the checkpoint kinase ATM and to a lesser degree on ATR activity while they are absent, as expected, in the *rad51* mutant (Fig 5D). In accordance with their key role in HR, we found that RAD51 foci are strongly reduced in *cycb1;1 cycb1;3* double mutants. A reduction of RAD51 foci was even more pronounced in the *cdkb1;1 cdkb1;2* double mutant consistent with its severe mutant phenotype on cisplatin-containing media (Fig 5D and E).

Next, we asked whether RAD51 could be a direct target of CDKB1-CYCB1 complexes. To this end, we expressed and purified from bacterial extracts next to CDKB1;1-CYCB1;1 several other CDK-cyclin complexes as controls, that is, CDKA;1-CYCA2;3, CDKA;1-CYCB1;1, CDKB1;1-CYCA2;3, CDKB1;1-CYCD2;1 (Fig 5F). Protein blots confirmed that all complexes contained similar amount
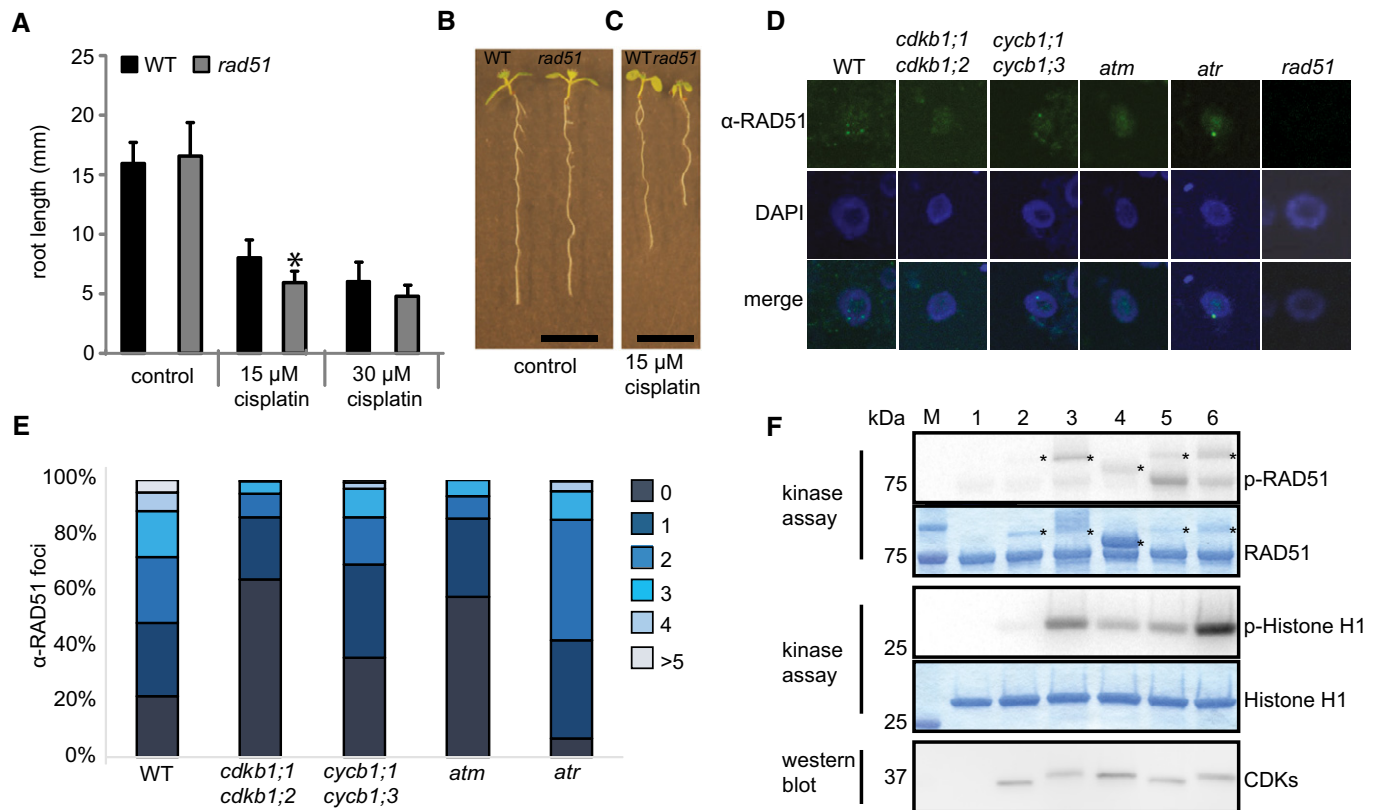

Figure 5. The CDKB1;1-CYCB1;1 complex phosphorylates RAD51.

A   WT and *rad51* mutants were grown on control plates or transferred to plates supplemented with 15 or 30 μM cisplatin, respectively, 3 days after germination. Root lengths were measured 3 days after transfer and the net root growth of 3 days is shown in the graph. Asterisk indicates significant differences within a 5% confidence interval (Student's *t*-test). Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. Graphs represent mean ± SD.

B   Image shows a wild-type plant (left) and *rad51* mutant (right) grown on control plates. Images were taken 6 days after germination. Scale bar: 1 cm.

C   Image shows a wild-type plant (left) and *rad51* mutant (right) germinated on control plates and transferred to plates supplemented with 15 μM cisplatin 3 days after germination. Images were taken 6 days after germination, that is, 3 days after transfer to cisplatin. Scale bar: 1 cm.

D   Immunostaining of RAD51 foci in the wild-type and indicated mutant cells after 2 h of treatment with 50 μM cisplatin.

E   Counted numbers of RAD51 foci per cell detected after 2 h of treatment with 50 μM cisplatin in wild-type and mutant plants. For each sample, the RAD51 foci of 100 cells were counted and grouped into six categories: cells with 0, 1, 2, 3, 4, or > 5 foci per cell.

F   *In vitro* kinase assay of purified CDK complexes phosphorylating RAD51. RAD51 and histone H1 kinase assays were performed with [γ-^{32}P]ATP as a phosphate donor. Proteins were subjected to SDS–PAGE after the kinase reaction and stained with Coomassie Brilliant Blue demonstrating the equal loading of the substrates (lower panels). Phosphorylated proteins were detected by autoradiography (upper panels). The reactions were normalized by using equal amounts of CDKs assuring equal levels of active CDK-cyclin complexes; the protein blot indicates the relative amounts of the CDKs in the reaction (bottom panel). Abbreviation: p-RAD51 and p-histone H1 for [^{32}P]-phosphorylated MBP-RAD51-His6 and recombinant human histone H1, respectively, resulting from kinase assays with radiolabeled ATP. Asterisks indicate varying amounts of cyclins that can be in the reaction due to purification procedure. 1: without kinase, 2: CDKA;1-CYCA2;3, 3: CDKB1;1-CYCA2;3, 4: CDKB1;1-CYCD2;1, 5: CDKA;1-CYCB1;1, 6: CDKB1;1-CYCB1;1.

of CDKs (Fig 5F). All kinase reactions were incubated with comparable amounts of RAD51 and histone H1 as an alternative substrate.

In general, all CDK complexes tested here were active although some complexes had a much higher activity against histone H1 than others with CDKB1;1-CYCA2;3 and CDKB1;1-CYCB1;1 performing the best and CDKA;1-CYCA2;3 the poorest (Fig 5F). Importantly, we found that CDKB1;1-CYCB1;1 could phosphorylate RAD51 *in vitro*. Moreover, the substrate preference toward RAD51 was different with CDKA;1-CYCB1;1 and CDKB1;1-CYCB1;1 now having the highest activity (Fig 5F). Thus, it appears that especially CYCB1;1-containing complexes are active against RAD51.

Currently, it remains difficult to further test the functional relevance of RAD51 phosphorylation by CDKB1-CYCB1 complexes since a genomic RAD51 reporter was not functional during DNA damage response (Da Ines *et al*, 2013b). Nevertheless, the *in vitro* phosphorylation identified here suggests a direct regulation of RAD51 *in vivo* by mitotic kinases and corroborates the conclusion that CDKB1 and CYCB1 act together in the control of HR in *Arabidopsis*.

CYCB1;1 upregulation upon DNA damage is directly controlled by SOG1

Seeing that CDKB1 and CYCB1 are involved in the control of HR, we explored next how this complex is regulated under DNA damage conditions. To obtain a cellular resolution, we created reporter lines for all four *CYCB1* genes. For this purpose, the N-terminal parts of the respective cyclin (CYCB1;1 to CYCB1;4) were cloned, including the destruction box that was fused to GFP. These reporter genes were placed under the control of ~1.2 kb 5′ region of the respective cyclin. We found more cells expressing GFP in the root tips of the *CYCB1;1* reporter line compared to plants carrying the reporter grown on medium without cisplatin (Fig 6A and D). Noteworthy, only cells in the root tip, that is, cells with mitotic potential, showed the accumulation of the *CYCB1;1* reporter. This effect was specific to *CYCB1;1* as the other B1-type cyclins, *CYCB1;2*, *CYCB1;3,* and *CYCB1;4* were not upregulated based on our reporter lines (Figs 6B and E, and EV8).
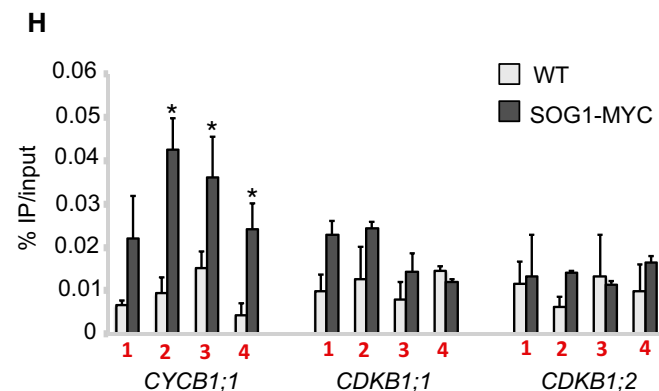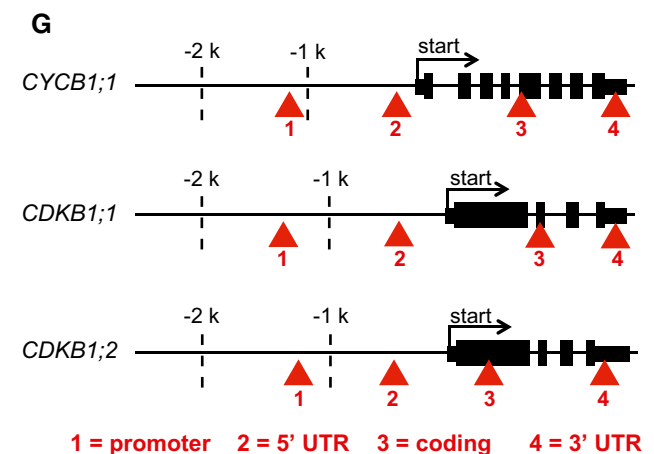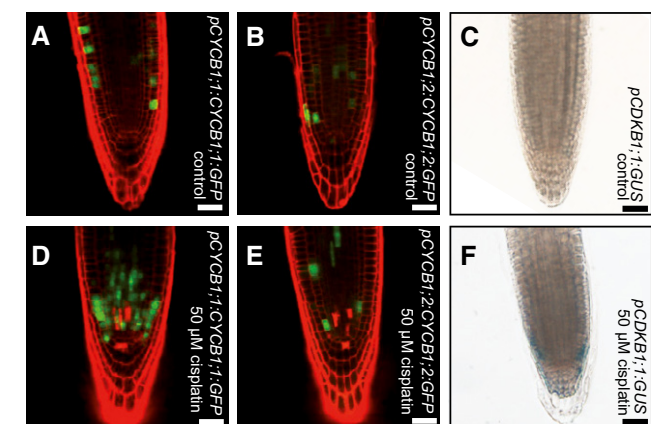

Figure 6. CYCB1;1 is upregulated after cisplatin treatment.

A Transgenic *Arabidopsis* plants harboring the *CYCB1;1* promoter and a GFP fused to the N-terminal part of *CYCB1;1* including the destruction box grown on control plates and imaged 5 days after germination.

B Transgenic *Arabidopsis* plants harboring the *CYCB1;2* promoter and a GFP fused to the N-terminal part of *CYCB1;2* including the destruction box grown on control plates and imaged 5 days after germination.

C Transgenic *Arabidopsis* plants harboring *CDKB1;1* promoter fused to GUS grown on control plates and were stained and imaged 5 days after germination.

D Transgenic *Arabidopsis* plants harboring the *CYCB1;1* promoter and a GFP fused to the N-terminal part of *CYCB1;1* including the destruction box germinated on control plates and were transferred 4 days after germination to plates supplemented with 50 μM cisplatin and were imaged 24 h after drug application.

E Transgenic *Arabidopsis* plants harboring the *CYCB1;2* promoter and a GFP fused to the N-terminal part of *CYCB1;2* including the destruction box germinated on control plates and were transferred 4 days after germination to plates supplemented with 50 μM cisplatin and were imaged 24 h after drug application.

F Transgenic *Arabidopsis* plants harboring *CDKB1;1* promoter fused to GUS germinated on control plates and were transferred 4 days after germination on plates supplemented with 50 μM cisplatin and were stained and imaged 24 h after drug application.

G Structure of genes tested by ChIP with an anti-MYC antibody in PRO_{SOG1}:*SOG1-Myc* and wild-type plants. A total of four regions were tested as indicated by arrowheads. Asterisks indicate significant differences within a 5% confidence interval (Student's *t*-test).

H Chromatin immunoprecipitation (ChIP) of wild-type plants and PRO_{SOG1}:*SOG1-Myc* lines and anti-MYC antibody. The promoter region of *CYCB1;1* is enriched in *SOG1-Myc* after cisplatin treatment. Red arrowheads indicate the primer binding sites for PCR.

Data information: Scale bars: 20 μm. Three biological and three technical replicates were performed. Graphs represent mean ± SD.

The fact that the *CYCB1;2, CYCB1;3,* and *CYCB1;4*, although showing the typical patchy pattern of mitotically expressed genes, were not altered in their expression pattern already indicated that the upregulation of *CYCB1;1* cannot be largely due to a cell-cycle phase effect, that is, a potential enrichment of G2 cells due to an active G2-M checkpoint. This conclusion was consistent with our flow cytometry analyses of wild-type plants on media with and without cisplatin and in comparison with *cycb1;1 cycb1;3* double mutants (Fig EV5). Next, we analyzed the expression of *CDKB1;1* using a previously generated promoter reporter line (Boudolf *et al*, 2004). Similar to *CYCB1;1*, we found that the promoter of *CDKB1;1* is activated upon cisplatin treatment (Fig 6C and F).

SOG1 is a central regulator of DNA damage response in plants that mediates checkpoint signaling (Yoshiyama *et al*, 2009, 2013). Previously, it was found that *CYCB1;1* upregulation depends on ATM and SOG1 (Culligan *et al*, 2006; Yoshiyama *et al*, 2009). To test therefore whether *CYCB1;1* and *CDKB1s* are directly regulated by SOG1, we performed ChIP experiments with plants expressing a tagged SOG1 version grown on cisplatin. While we could not detect SOG1 on the *CDKB1;1* nor *CDKB1;2* genomic region, SOG1 was found to bind to the *CYCB1;1* genomic region, especially the 5′ and 3′ UTR (Fig 6G and H). Thus, we conclude that SOG1 directly controls *CYCB1;1* expression where *CDKB1* appears to be controlled by another, yet unknown pathway.

Genetic interaction between NHEJ and HR pathways

The transcriptional upregulation of *CYCB1;1* not only on cisplatin but also on medium containing other DNA-damaging drugs raised the hypothesis that CYCB1;1 also promotes HR in response to DSB formation. A second hint for a role of HR in DSB repair comes from the observation that the *cdkb1;1 cdkb1;2* double mutant was also sensitive to BLM (see above, Fig 4F and L). In addition, mutants in the plant *RAD51* family are well known to be sensitive to DSB-inducing drugs such as BLM and MMC (Bleuyard *et al*, 2005; Da Ines *et al*, 2013b; Wang *et al*, 2014). Previous studies have shown that NHEJ is the most prominent repair mechanism in plants and hence a role of CDKB1-CYCB1 complexes in DSB repair might be largely masked by a very efficient execution of NHEJ (Knoll *et al*, 2014). We therefore asked which role CYCB1-mediated HR plays in a genetic background in which the NHEJ pathway is severely compromised, that is, in mutants of the DNA binding protein KU70 that is required during NHEJ. Therefore, *ku70* was introgressed into *cycb1* mutants (Fig EV2) resulting in lines compromised in both NHEJ and presumably also in HR. Root growth of *cycb1;1 cycb1;2 ku70* on medium without BLM was similar to *cycb1;1 cycb1;2* (Fig 7A and B). The *cycb1;1 ku70* and *cycb1;2 ku70* double mutants were as sensitive to BLM as *ku70*. However, the generated triple mutant *cycb1;1 cycb1;2 ku70* was much more sensitive to BLM than *ku70* by itself with roots growing approximately only one-tenth as long as the wild-type control plants (Fig 7B, C and E). These results suggest that some BLM-induced damage in *ku70* is repaired by homologous recombination in a CYCB1-dependent manner. Thus, the presence of KU70 might drive repair toward NHEJ but a HR pathway is activated at the same time and functions in parallel to back up NHEJ. Likely, this setup also contributes to the power of DNA repair in plants.

Discussion

In this study, we have shown that B1-type cyclins together with B1-type CDKs play a major role in HR-dependent DSB repair in plants. Our work assigns a new and important function to B1-type CDKs, which have been previously thought to just act as auxiliary kinases with a minor and largely redundant role with the major cell division kinase CDKA;1. Seeing the impact of CDKB1s during DNA damage, as visualized by the growth retardation, the accumulation of gamma-H2AX foci, and the pronounced formation of a tail of fragmented DNA in comet assays of the mutant, puts these kinases in the group of major DNA damage response regulators in *Arabidopsis* such as ATM, ATR, and SOG1 (Culligan *et al*, 2004, 2006; Yoshiyama *et al*, 2009).

Inhibition versus activation of CDKs under DNA damage

The requirement of CDK activity for DNA damage pathways stands in apparent contradiction to the general theme in DNA damage repair to arrest cell division. Indeed, CycB1 expression was reported to be suppressed in HeLa cells after DNA damage resulting in a G2 delay (Muschel *et al*, 1991; Maity *et al*, 1995). Conversely, DNA damage response has been found to be repressed by high levels of Cdk1 activity in dividing mammalian cells (Zhang *et al*, 2011). However, a key role for active CDK complexes in DNA damage response matches recent reports in yeast and metazoans (Wohlbold & Fisher, 2009; Yata & Esashi, 2009; Trovesi *et al*, 2013).

In *S. cerevisiae*, cell-cycle arrest is executed with high CDC28p activity (Sorger & Murray, 1992; Enserink *et al*, 2009). Furthermore, high CDK activity is needed in G2 to concurrently promote HR and to repress NHEJ (Zhang *et al*, 2009). In particular, it was shown that CDC28p is required for processing of DNA ends to produce single-stranded DNA essential for HR (Aylon *et al*, 2004; Ira *et al*, 2004). Conversely, *S. cerevisiae* cells with reduced CDK activity are highly sensitive to DNA-damaging agents (Enserink *et al*, 2009). Thus, it appears that DNA damage repair and the subsequent choice of repair pathways in *S. cerevisiae* is dependent on the activity level of CDC28p as HR can operate exclusively after DNA replication in S and G2 phase of the cell cycle (Zhang *et al*, 2009). However, *S. cerevisiae* has only one cell-cycle CDK and it is still an open question how inhibition of mitosis can be reconciled with an activation of CDKs to promote DNA damage repair.

In metazoans, CDK activity has also been found to be important for DNA repair and the application of chemical CDK inhibitors such as roscovitine was reported to increase sensitivity of cells to DNA-damaging compounds such as ionizing radiation and cisplatin (Ongkeko *et al*, 1995; Maggiorella *et al*, 2003). The closest homologs of CDC28p in metazoans are Cdk1 and Cdk2. The general dogma is that Cdk2 complexes promote entry and progression through S phase while Cdk1 controls mitosis although Cdk1 can almost completely compensate for the loss of Cdk2 (Santamaría *et al*, 2007; Malumbres *et al*, 2009). In particular, Cdk2 was found to play an active role in DNA repair (Deans *et al*, 2006; Wohlbold *et al*, 2012). Likewise, CycA1 and CycA2, two cyclin partners of Cdk2, have been reported to promote DNA DSB repair by HR in mice (Müller-Tidow *et al*, 2004). However, Cdk2 is inhibited by the CDK inhibitor p21 after occurrence of DNA damage (Bartek & Lukas, 2001). Thus, it is not clear how Cdk2 complexes could execute DNA
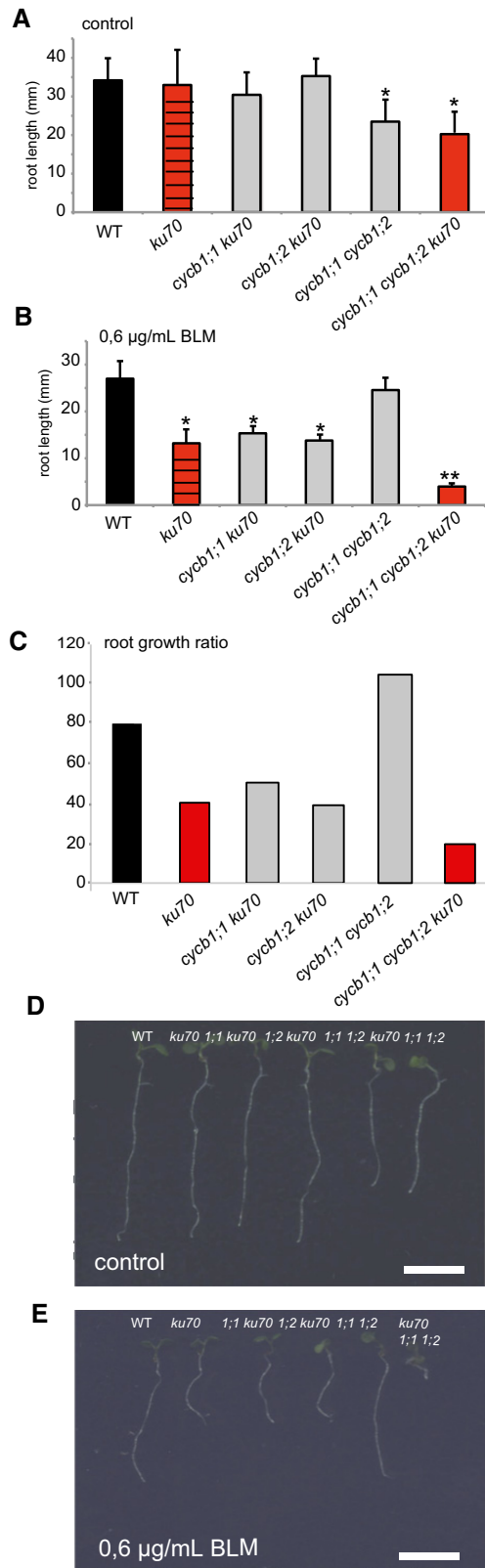
A control

B 0,6 μg/mL BLM

C root growth ratio

D

E

Figure 7. **Mutants of *cycb1 ku70* are hypersensitive to BLM: genetic interaction between NHEJ und HR.**

A, B   The wild type, *ku70*, the double mutants *cycb1;1 ku70*, *cycb1;2 ku70*, and *cycb1;1 cycb1;2*, and the triple mutant *cycb1;1 cycb1;2 ku70* were grown on control plates (A) or plates containing 0.6 μg/ml BLM (B) and root lengths were measured 10 days after germination.

C   Graph represents the ratio of the mean growth rate on 0.6 μg/ml BLM compared to control experiments on plates lacking BLM for the wild type, *ku70*, *cycb1;1 ku70*, *cycb1;2 ku70*, and *cycb1;1 cycb1;2*, and the triple mutant *cycb1;1 cycb1;2 ku70*.

D, E   Image shows the wild type, *ku70*, the double mutants *cycb1;1 ku70*, *cycb1;2 ku70*, and *cycb1;1 cycb1;2*, and the triple mutant *cycb1;1 cycb1;2 ku70* (from left to right) grown on control plates (D) or plates containing 0.6 μg/ml BLM (E) 10 days after germination.

Data information: One asterisk indicates significant differences within a 5% confidence interval between the wild type and the mutants (Student's *t*-test). Two asterisks represent significant differences within a 5% confidence interval between the wild type and the triple mutant *cycb1;1 cycb1;2 ku70* as well as a significant reduction of root growth between *ku70* and the triple mutant *cycb1;1 cycb1;2 ku70*. Scale bars: 1 cm. Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. Graphs represent mean ± SD.

Arabidopsis has only one Cdk1/Cdk2 homolog, namely CDKA;1, which also contains functional aspects of both CDKs in metazoans, that is, CDKA;1 function in S and M phase control (Nowack *et al*, 2012). Here, we have shown that plants with a reduced activity level of CDKA;1 are neither hypersensitive to BLM nor cisplatin. In addition, we have previously found that a weak loss-of-function allele of *CDKA;1* also does not sensitize plants to HU (Dissmeyer *et al*, 2009). Although we cannot fully exclude a role of CDKA;1 in DNA repair, our combined data suggest that this kinase is largely not required for a proper DNA damage response in *Arabidopsis*. Instead, we found that especially CDKB1 complexes control HR.

Remarkably, A2-type cyclins, which are well-known partners of CDKB1 activity, for instance, during stomata development (Vanneste *et al*, 2011), were not found to play a major role during DNA repair on cisplatin media in *Arabidopsis*. Instead, we found that B1-type cyclins, which can also form an active complex with CDKB1s, are hypersensitive to cisplatin. However, *Arabidopsis* has eight D-type cyclins next to six further A- and six B-type cyclins that all belong to the core cell division machinery. The slightly weaker mutant phenotype of *cycb1* mutants in comparison with the double mutant *cdkb1;1 cdkb1;2* suggests that one or a combination of different cyclins acts partially redundant with the class of B1-type cyclins. Further studies are required to work out the minor role of other cyclins in HR.

B1-type cyclins also form active complexes with CDKB2 (Harashima & Schnittger, 2012). Based on their expression pattern and the phenotype of their downregulation, it is conceivable that CDKB2s are the major mitotic regulators in *Arabidopsis* (Menges *et al*, 2003; Andersen *et al*, 2008). It has been observed that CDKB2s are degraded and transcriptionally downregulated upon DNA damage in *Arabidopsis* (Adachi *et al*, 2011). However, whether the function and regulation of CDKB2 is conserved in plants is not clear yet since the only member of the CDKB2 group in rice, CDKB2;1, accumulates upon DNA damage (Endo *et al*, 2012). Moreover, *CDKB2;1* knockdown lines in rice were more sensitive to radiation possibly hinting at a conserved role for mitotic CDKs in plants for HR. However, it remains to be seen whether CDKB2;1 from rice is functionally more related to CDKB2s than CDKB1s from

repair when they are in an inhibited state as a response to DNA damage. It is also not clear how HR can be promoted in late G2 and early M phase when presumably only Cdk1 is active.

Arabidopsis, especially since expression of *CDKB2;1* in rice is not restricted to mitosis resembling more the *CDKB1* expression pattern in *Arabidopsis* (Menges *et al*, 2003; Endo *et al*, 2012).

In *Arabidopsis*, the transcriptional repression of *CDKB2s* depends on the putative transcription factor SOG1 that plays a key role in DNA damage response in *Arabidopsis* (Yoshiyama *et al*, 2009). At the same time, it was previously found that upregulation of *CYCB1;1* after DNA damage requires SOG1 (Yoshiyama *et al*, 2009). Here, we have shown that SOG1 binds to the promoter of *CYCB1;1* and hence appears to directly activate its expression presumably promoting CDKB1 activity and the execution of HR.

Taken together, we propose a division of labor among the CDKs in *Arabidopsis* (Fig 8). The major mitotic force represented by CDKB2 and possible other kinases might be shut down in a SOG1-dependent mechanism, while SOG1 promotes the expression of *CYCB1* and hence stimulates the activity of CDKB1 complexes required for HR.

Targets of CDK-CYC complexes in HR versus NHEJ

How do active CDK complexes, such as CDKB1-CYCB1, control HR? A first step to answer this question is the identification of substrates of CDK complexes during DNA damage conditions. An early and decisive step in HR is the resection of the 3′ ends at the site of the DSB to allow subsequent strand invasion of the damaged into the undamaged chromatid. In animals and yeast, it has been found that CDKs phosphorylate proteins in the MRN complex that process 3′ ends, for example, Nbs1, and proteins that work in concert with this complex, such as the nuclease Sae2/CtIP/Com1 (Huertas *et al*, 2008; Huertas & Jackson, 2009; Wohlbold *et al*, 2012; Simoneau *et al*, 2014). Conversely, mutants of Nbs1 in which the phosphorylation site of Cdk2 was eliminated were hypersensitive to radiation in a similar manner as cells in which Cdk2 was chemically inhibited (Wohlbold *et al*, 2012).

Here, we identified RAD51 as a possible target of DNA damage-induced CDK activity. To our knowledge, RAD51 has not been identified outside of plants as a possible target of CDKs under DNA damage conditions. However, animal RAD51 also contains a consensus CDK phosphorylation site S/T-X-R/K and it has been previously observed in animals and yeast that the formation of Rad51 foci after DNA damage indirectly depends on CDK activity via BRCA1 and BRCA2 (Scully *et al*, 1997; Ira *et al*, 2004; Johnson *et al*, 2009; Quennet *et al*, 2011). At least in plants,
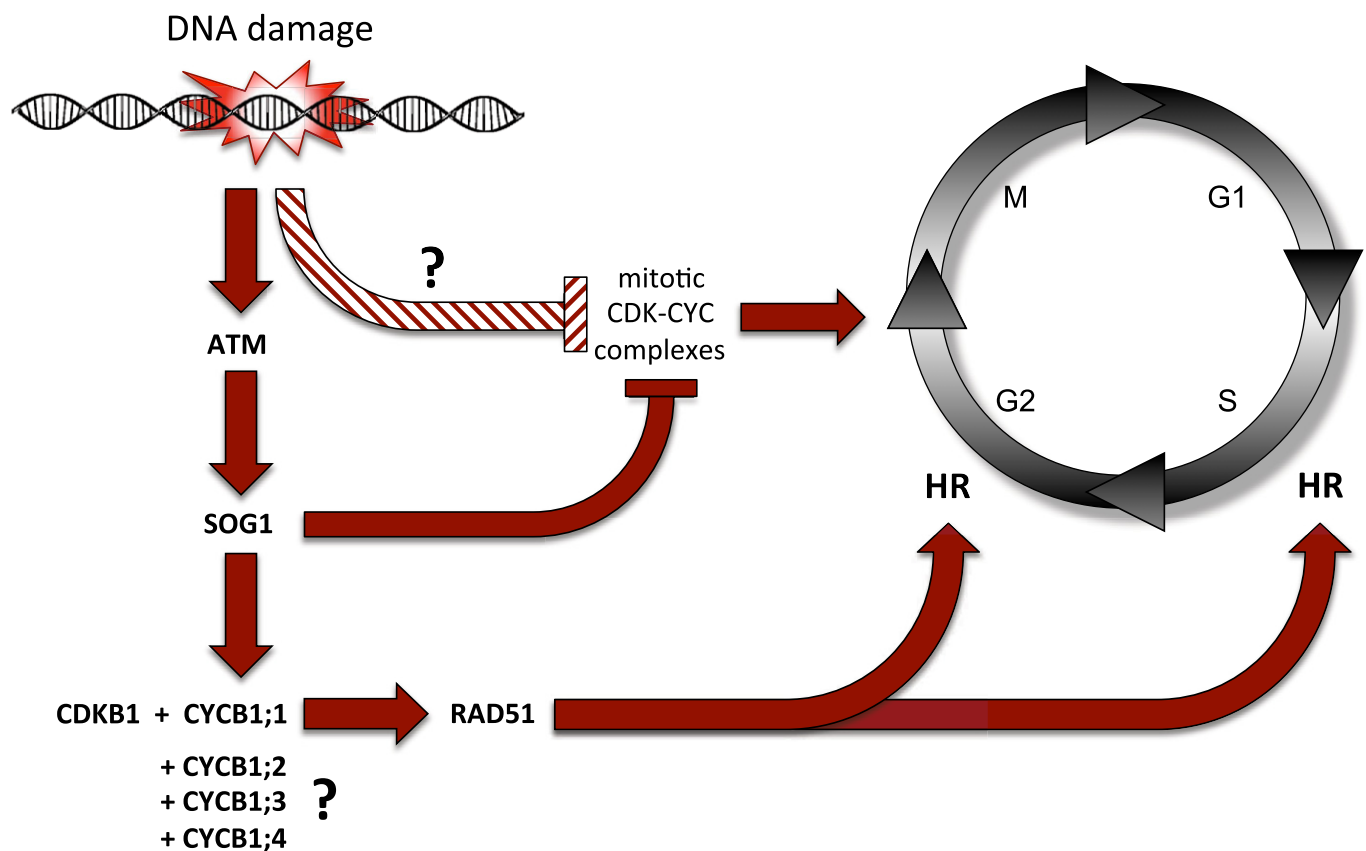

Figure 8.   Division of labor model of the regulation of cell proliferation and DNA damage response in *Arabidopsis*.
DNA damage, for example induced by chemical mutagens, is followed by the activation of the checkpoint kinase ATM (ataxia-telangiectasia mutated) that activates the transcription factor SOG1 (SUPPRESSOR OF GAMMA RESPONSE 1). On the one hand, SOG1 represses, directly or indirectly, the expression CDKB2 and possible other CDKs as the major driving force of mitosis to allow a cell time for DNA repair. On the other hand, SOG1 directly binds to the promoter of *CYCB1;1* and activates its expression. CYCB1;1 builds an active complex together with CDKB1. This complex phosphorylates the DNA binding protein RAD51 (RADIATION SENSITIVE 1) that gets recruited to the DNA damage site. This cascade is required for HR in S and G2 phases of the cell cycle in meristematic cells. To trigger this response, the action of all four B1-type cyclins is necessary, possibly by providing a threshold of mitotic CDK activity that then gets amplified through SOG1-dependent stimulation of *CYCB1;1* expression.

recruitment of RAD51 to damaged DNA might not only be an indication for HR but could directly be dependent on CDK activity.

Arabidopsis also contains COM1 and NBS1 homologs and it remains to be seen whether these proteins are also phosphorylated by CDKB1 complexes upon DNA damage, at least both proteins contain several CDK consensus phosphorylation sites. Hence, one alternative possibility to explain the failure to recruit RAD51 in *cdkb1* mutants is an insufficient resection of DSBs due to compromised MRN and/or COM1 activity. In addition, there could also be other plant-specific phospho-targets of CDKB1 during HR. The few targets of CDK action identified in *Arabidopsis* so far suggest a rather large number of species-specific CDK targets (Pusch *et al*, 2012). Hence, an unbiased forward approach to identify additional CDK targets under DNA damage conditions might be powerful to further understand how HR is controlled by CDKs.

Several components of the NHEJ pathway have also been identified as targets of CDKs. However, CDK phosphorylation inhibits the recruitment of NHEJ factors, for instance, YKu70 (the yeast KU70 homolog) and Lif1 to DNA breaks in *S. cerevisiae* (Zhang *et al*, 2009) and XRCC4/XLF1 in *S. pombe* (Hentges *et al*, 2014). Thus, there appears to a reciprocal relationship between these two repair pathways where NHEJ is high in G1 and low in G2 phase, whereas HR is not possible in G1 and high in G2 phase (Ferreira & Cooper, 2004). Whether such an antagonistic relationship between these two different classes of DNA repair mechanisms exists in plants as well needs to be shown.

Previous experiments suggested that NHEJ and not HR is the predominant type of DNA repair in plants, a situation reminiscent to mammals but different to yeast in which HR is preferred over NHEJ (Puchta *et al*, 1996; Kempin *et al*, 1997; Gisler *et al*, 2002). However, our work clearly indicates that HR is executed under DNA damage conditions in plants and depends on the presence of CDKB1s and CYCB1s. This is consistent with previous studies that have reported an upregulation of *CYCB1;1* after DNA damage (Chen *et al*, 2003; Culligan *et al*, 2006; Ricaud *et al*, 2007). Our finding that *CYCB1;1* is upregulated outside of mitosis but still limited to the meristematic region of the root where cells are in a proliferative mode suggests a developmental stage dependent choice of DNA repair pathways. We postulate that cells with mitotic potential prefer the error-free HR over NHEJ after progression through S phase.

The execution of HR might be very sensitive to the dosage of mitotic activity as seen by the reduced growth and decreased blue spots in our recombination assays already in all single *cycb1* mutants. We speculate that a threshold level of mitotic activity is needed for HR after DNA damage and all CYCB1 proteins contribute to this threshold in a non-redundant manner. A second, yet not mutually exclusive explanation for the sensitivity of all cyclins could by that different CYCB1 complexes have to some degree non-overlapping substrates that are still required for HR, for example, RAD51 by CDKB1s-CYCB1;1 and a different substrate, possibly COM1, by another CDKB1s-CYCB1 complex. Alternatively, HR substrates might require the concomitant phosphorylation of different CDKB1-CYCB1 complexes.

Nonetheless, loss of mitotic activity in *cycb1* or *cdkb1* mutants does not immediately result in reduced repair activity and reduced

growth upon the formation of DSBs presumably due to remaining NHEJ activity as seen by the unperturbed growth of *cycb1* mutants and only a slight reduction of *cdkb1* mutants on BLM-containing media. Such a parallel role of NHEJ and HR matches the observation that the concomitant inactivation of both NHEJ and HR as presented in *ku70 cycb1;1 cycb1;2* triple mutant results in severe growth reduction on BLM.

An apparent dominance of NHEJ over HR could further be explained if terminal differentiated cells or endoreplicating cells that comprise the vast majority of all cells of a plant might preferentially execute NHEJ. One reason for this could simply be the fact that mitotic genes are generally inactive in differentiated cells and hence mitotic CDK activity required for HR is missing. The importance of the developmental state in DNA damage response has already been observed by the preferential cell death of stem cells that is not found in other cells of the root after mild induction of DSBs (Fulcher & Sablowski, 2009).

However, DNA repair in plants appears to be even more complex. The fact that *cycb1;1 cycb1;2 ku70* showed severe reduction in root growth on BLM but not a complete growth stagnancy, suggests that a third pathway might back up the DNA repair to a certain extent. This could be the KU70-independent NHEJ repair pathway (Charbonnel *et al*, 2010), which depends on the action of XRCC1 (XRAY REPAIR CROSS-COMPLEMENTATION PROTEIN 1). KU80 has a similar function as KU70 and *xrcc1 ku80* double mutants display more defects after irradiation than the single mutants, which leads to the conclusion that both proteins act in independent repair pathways (Charbonnel *et al*, 2010). Most likely, this pathway remains functional in *cycb1;1 cycb1;2 ku70* after BLM treatment but has only a minor contribution due to the severe root growth defects of the triple mutant. Further work is now required to understand how an XRCC1-dependent pathway is integrated with HR and NHEJ. Likely, the interplay of all these pathways is the reason for the enormous power of plant DNA repair systems.

Materials and Methods

Plant material and growth conditions

The *Arabidopsis thaliana* plants used in this study were either grown on soil (16 h light) or *in vitro* on half-strength (½) Murashige and Skoog (MS) medium (Sigma-Aldrich) containing 0.5% sucrose (16 h light) in a growth chamber. The accessions Columbia (Col-0) and Nossen (No-0) were used as the wild-type control. *cycb1;1* and *cycb1;2* T-DNA insertion lines were isolated from the Koncz collection (Rios *et al*, 2002). T-DNA insertion line *cycb1;3* (pst15850) was obtained from the RIKEN collection (Yokohama, Japan) and *cycb1;4* from the GABI-KAT collection (Bielefeld, Germany) (Kleinboelting *et al*, 2012). The *ku70* (Riha *et al*, 2002), *wee1* (De Schutter *et al*, 2007), and *rad51* (Li *et al*, 2004) mutants were described previously. The reporter line uidA/IC9C was used as described in Molinier *et al* (2004) and the *pCDKB1;1:GUS* line as in Boudolf *et al* (2004). The mutant $CDKA;1^{T14D;Y15E}$ is published in Dissmeyer *et al* (2009), the *cdkb1;1 cdkb1;2* double mutant in Nowack *et al* (2012). All genotypes were determined by PCR and primers are indicated in Table EV1.

Root growth analysis

Elongation of the roots was marked daily for 10 days after germination on vertical plates in 16 h/8 h growth chamber and measured with ImageJ. Root length was measured from the root tip to the root–hypocotyl border. Three biological replicates, each containing at least 15 plants, were analyzed. The mean of the root length of each individual experiment was determined and again averaged for the three biological replicates. In order to measure root growth on DNA damage-inducing media, plants germinated on 1 mM hydroxyurea (Sigma-Aldrich) or 0.6 µg/ml bleomycin (Duchefa) and were measured daily for 10 days. For analyzing the sensitivity on cisplatin, plants were sown on ½ Murashige and Skoog medium with 0.5% sugar and transferred to either control plates or plates containing 15 or 30 µM cisplatin (Sigma-Aldrich) 3 days after germination. Roots were measured 3 days after transfer (figures show elongation of the roots within 3 days).

Cloning of CYCB1 marker lines

The fragments were first cloned in pGEM-T easy and sequence validated, then excised and cloned into pTV50-GUS or pTV50-GFP: These two vectors are derived from pBIB (Becker, 1990): First, the NOS terminator in pBIB was replaced by the OCS terminator, and then all sequences between the unique HindIII and SacI sites in pBIB were excised and replaced by the modified uidA gene from pRAJ260 or GFP6, respectively, with the following polylinker preceding the respective start codon: aagcttgaggtcgactctagA, giving rise to pTV50-GUS or pTV50-GFP, respectively. Cyclin promoter-gene fragments were cloned in frame using either SalI or HindIII on the 5′ end and an XbaI-compatible restriction site at the 3′ end. Primer sequences are indicated in Table EV1. The CYCB1;1 construct was described in Ubeda-Tomás et al (2009).

Root cell wall staining

Entire seedlings were stained with propidium iodide (Invitrogen, 1 mg/ml, 100× dilution) in H$_2$O for 3–4 min and rinsed afterward two times in H$_2$O.

Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on an inverted Zeiss LSM 510 confocal microscope. Excitation and detection windows were set as follows: GFP excitation at 488 nm, emission at 500–600 nm; propidium iodide excitation at 488 nm, emission at 500–550 nm.

Homologous recombination assay

For the homologous recombination assay, all *cycb1* single mutants were crossed to the IC9C reporter line (kindly provided by Holger Puchta, KIT, Karlsruhe, Germany) and double homozygous lines were obtained in the F3 generation. Plants were germinated on ½ Murashige and Skoog medium with 0.5% sugar. Five days after germination seedlings were transferred to plates containing either 15 or 30 µM cisplatin (Sigma-Aldrich) and control plates without cisplatin. Three days after transferring, seedlings were incubated in

staining solution for 48 h and afterward destained in 70% ethanol at 60–70°C. Staining solution for 5 ml: 100 µl of 10% Triton X-100, 250 µl 1M NaPO$_4$ (pH 7.2, make 1 M NaH$_2$PO$_4$ and titrate with NaOH), 100 µl 100 mM potassium ferrocyanide, 100 µl potassium ferricyanide, 400 µl 25 mM X-Gluc, 4,050 µl dH$_2$O. Blue sectors were counted using a binocular Leica S4E. Images of leaves with blue spots were taken with an Olympus BX51 light microscope.

Immunostaining

Ten-day-old seedlings were treated with 50 µM cisplatin for 2 h. γ-H2AX immunostaining of root tip spreads was performed as described earlier (Friesner et al, 2005). A rabbit anti-plant γ-H2AX antibody (kindly provided by Charles White, CNRS, Clermont-Ferrand, France) was used in a 1:600 dilution (Charbonnel et al, 2010). As secondary antibody, a goat Alexa Fluor 488 anti-rabbit (Life Technologies, Carlsbad, CA, USA) was used in a 1:300 dilution. Imaging of the nuclei was done with a LSM700 confocal microscope (Carl Zeiss, Jena, Germany). Immunostaining of RAD51 foci was performed with the Rad51 (H-92) sc-8349 antibody (Santa Cruz Biotechnology, Texas, USA) with a dilution of 1:400. A goat Alexa Fluor 488 anti-rabbit (Life Technologies, Carlsbad, CA, USA) was used in a 1:300 dilution as a secondary antibody.

Comet assay

The evaluation of DNA damage in cisplatin treated plants was done by an *N*-methyl-*N*-nitrosourea (N/N) comet assay. Therefore, seedlings were grown for 21 days under sterile conditions on ½ MS medium, 0.5% sugar. The plantlets were transferred to ½ MS liquid medium (control) or ½ MS liquid medium containing 50 µM cisplatin. After 1 h of incubation, a fraction of the cisplatin treated plants were separated. The remaining plants were shortly dried on paper towels and immediately frozen in liquid nitrogen. The separated plants were washed three times with ½ MS and transferred to ½ MS liquid medium without cisplatin for recovery. After 30 min of incubation, these plants were also briefly dried and frozen. The preparation of the comet slides was performed according to Menke et al (2001) and stained with 3× GelRed Nucleic Acid stain (Biotium, Hayward, CA, USA) diluted in 0.1 M NaCl. The comets were observed and images were taken on an AXIO Imager Z1 fluorescence microscope with an AXIOCam MRm (Carl Zeiss, Jena, Germany). The analysis of the images was done utilizing the TriTek Comet Score software and 200 comets per sample were measured.

Ploidy analysis

Root tips of 5-day-old plants treated and untreated with 50 µM cisplatin for 24 h were chopped with a razor blade in 200 ml of Cystain UV Precise P nuclei extraction buffer, supplemented with 800 ml staining buffer (Partec). The mix was filtered through a 50-mm column and read by the Cyflow MB flow cytometer (Partec). Nuclei were analyzed using the Cyflogic software.

Kinase assay

To clone RAD51, total RNA was extracted from inflorescences by using the NucleoSpin RNA plant kit (Macherey-Nagel).

First-stranded cDNA was synthesized by SuperScript III reverse transcriptase (Life Technologies) with oligo dT-AP_M13 according to the manufacturer's instruction. RAD51 cDNA was amplified first with primers RAD51_s1 and M13 forward, followed by primers RAD51_s2 and RAD51_as with Phusion DNA polymerase (Thermo Scientific). The PCR product was cloned into pJET1.2 (Thermo Scientific), followed by sequence confirmation. After RAD51 was subcloned into pDONR223 (Invitrogen), a recombination reaction was performed between the resulting entry clone and a destination vector pMGWA (Busso *et al*, 2005). CDKB1;1-CYCB1;1 complexes were expressed and purified as described previously (Harashima & Schnittger, 2012). To express recombinant proteins, *E. coli* BL21-AI cells (Invitrogen) were transformed with the resulting vector. *Escherichia coli* cells were grown in LB medium containing 100 mg/l ampicillin at 37°C until OD600 = 0.6 and the production of the fusion protein was induced by adding 0.3 mM IPTG (Nacalai Tesque) and 0.2% (w/v) L-arabinose (Wako) overnight at 18°C. Cells were harvested by centrifugation and re-suspended in Ni-NTA binding buffer (50 mM NaH2PO4, 100 mM NaCl, 10% (v/v) glycerol, 25 mM imidazole, pH 8.0) and lysed by sonication (Digital Sonifier 450D, BRANSON). After addition of Triton X-100 to 0.2% (w/v), the cell slurry was incubated at 4°C and clarified by centrifugation. The supernatant was passed through a column with Ni-NTA resin (Qiagen), which was washed sequentially with Ni-NTA binding buffer, and eluted with Ni-NTA elution buffer (Ni-NTA binding buffer containing 200 mM imidazole). The eluate was sequentially purified with a column packed with amylose resin (NEB), which had been equilibrated with Ni-NTA binding buffer. The column was washed with Ni-NTA binding buffer followed by kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM EGTA). MBP fusion proteins were eluted with kinase buffer containing 10 mM maltose (Wako).

Kinase reactions were normalized so that equal amount of the respective CDKs (and hence functional CDK-cyclin complexes) were present in the reaction mixture. The assays were carried out with equal amounts of MBP-RAD51-His6 as a substrate and 92.5 kBq of [γ-^{32}P]ATP (PerkinElmer) as previously described (Harashima & Sekine, 2011; Harashima & Schnittger, 2012). After the kinase reaction, proteins were subjected to SDS–PAGE on a 10% TGX gel (Bio-Rad) and stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad). The gel was dried with HydroTech Gel Drying System (Bio-Rad). The radioisotopic signals were detected with Typhoon FLA 7000 (GE Healthcare).

ChIP

ChIP experiments were carried out as previously described with minor modifications (Saleh *et al*, 2008). Seeds of *PRO$_{SOG1}$:SOG1-Myc*, described in Yoshiyama *et al* (2013), were germinated and cultured in ½ MS medium containing 0.5% sucrose (pH 5.7) under continuous light at 22°C with gentle shaking (50 rpm). The 2-week-old seedlings were treated with 50 μM cisplatin for 2 h. Sonicated chromatin solution (corresponding to 0.7 g tissue) was used for immunoprecipitation with anti-Myc antibodies (Santa Cruz Biotechnology). The ChIP products were used for real-time quantitative PCR analysis. qPCR was performed using primers (Table EV1) on a LightCycler system (Roche) with Thunderbird SYBR qPCR Mix (Toyobo) according to the following reaction conditions: 95°C for

1 min; 70 cycles at 95°C for 10 s, at 60°C for 10 s, and at 72°C for 20 s.

qPCR

The entire inflorescences of Col-0, Nos, *cycb1;1*, *cycb1;2*, *cycb1;3*, and *cycb1;4* were immediately frozen in liquid nitrogen after collection and stored temporarily at −80°C. RNA was extracted using NucleoSpin RNA Kit (MACHEREY-NAGEL). RNA concentration and purity was tested using nanodrop-photometric quantification (Thermo Scientific). RNA integrity was verified by running 1 μl of total RNA on 1.5% agarose TBE gels to detect the 28S and 16S rRNA bands. 1 μg of total RNA was processed to obtain cDNA using polyT primer and SuperScript III RNase H reverse transcriptase. As negative control, all steps were followed in the same manner, except for adding the reverse transcriptase. The resulting cDNA was used for quantitative real-time PCR (qRT–PCR) using the Roche LightCycler 480 system. Three to four biological with three technical replicates each were processed. Cq calling was done using the Second Derivative Maximum method. Reaction-specific efficiencies were deduced using LinRegPCR 7.4 (http://LinRegPCR.nl). Data were quality-controlled, normalized against 3 reference genes, and statistically evaluated (unpaired *t*-test) using qbasePLUS 2.3 (http://biogazelle-qbaseplus.software.informer.com/2.3/).

Accession numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: CYCB1;1 (AT4G37490); CYCB1;2 (AT5G06150); CYCB1;3 (AT3G11520); CYCB1;4 (AT2G26760); RAD51 (AT5G20850); KU70 (AT1G16970); WEE1 (AT1G02970); CYCA2;2 (AT5G11300); CYCA2;3 (AT1G15570); CYCA2;4 (AT1G80370); CDKB1;1 (AT3G54180); CDKB1;2 (AT2G38620); CDKA;1 (AT3G48750); SOG1 (AT1G25580).

Expanded View for this article is available online.

Author contributions

AKW, SB, HH, FR, NT, CK, PD, MU, and AS conceived and designed the experiments. AKW, SB, HH FR, NT, JF, YG, and GP performed the experiments. DVD, KS, CK, PD, MU, and AS contributed material and reagents. AKW, SB, HH, NT, MH, PD, MU, and AS analyzed the data. AKW and AS wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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