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Cross-talk between cell-cycle control and the environment

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This thesis is dedicated to my beloved parents.

For their endless love support, and prayers

Abstract:

Even though the understanding of cell-cycle regulators in plants has tremendously increased over the last years, still little is known about cell-cycle regulation in response to environmental signals like DNA damage. A ubiquitous stress for any organism is DNA stress that can either be caused by exogenous sources or internal processes like chromatid separation or DNA strands separation during replication. The posttranslational regulation of Cdk1-type kinases through inhibitory phosphorylation through Wee1-type kinases in the so-called P-loop at the residue Tyr15 or the analogous positions as been found to be of pivotal importance for the arrest of the cell cycle after DNA damage in yeast and animals. But this mechanism is apparently not conserved in plants, as suggested by the hypersensitivity analysis of CDKA;1 dephospho-mutants. The first half of this study focus on possible regulation of CDKA;1 through T-loop phosphorylation upon replication stress in *Arabidopsis.* The positively acting phosphorylation on T161 and analogous residues in the so-called T-loop of the kinase that is required for full CDK activity and serves in substrate recognition. Remarkably, a T-loop phospho-mimicry mutant of CDKA;1, was almost 100% resistant to hydroxyurea (HU) and can partially rescue the hypersensitivity of *wee1* to HU. T-loop phosphorylation is catalysed by CDK activating kinases (CAKs) that are themselves CDKs with typical P- and T-loop regions. Evidence is obtained that WEE1 might inhibit CDKDs (Cdk-activating kinases) that would subsequently result in reduced CDKA;1 activity, and thus, cell-cycle arrest upon DNA damage. It is revealed that dephospho-mimicry mutants of CDKD;2 and 3, which can not be inhibited through WEE1 showed hypersensitivity to HU and not to bleomycin, suggesting their involvement in cell-cycle arrest specifically upon replication stress. Hypersensitivity of *cdkd2cdkd3* to replication stress suggested possible activation of CDKA;1 through CDKD;1 independent of WEE1. An essential role of CDKDs in stabilizing CDKA;1 kinase activity during gamete development has been suggested. Defects observed in *cdka;1*^{VF}*cdkd* mutants during meiosis but not in *cdka;1*^{VF} mutants emphasize on importance of CDKA;1 T-loop phosphorylation for appropriate meiotic division.

In second part of this study interaction between cell-cycle and circadian has been studied. A feedback loop in which the cell cycle could potentially regulate the circadian clock was suggested as a number of circadian genes were found to be deregulated in a microarray experiment with holomorphic CDKA;1 mutants. Thus the circadian gating of cell division of wildtype and *cdka;1* mutants was studied under diurnal growth conditions. The altered time of

division observed in cell-cycle mutants supported the idea of cell-cycle regulation in a time dependent manner. Expression profile of clock genes were analysed in *cdka;1* mutants through luciferase assay system. An altered period and intensity of expression observed in these mutants compared to wild type plants suggested a direct or indirect effect of CDKA;1 activity on clock gene expression.

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1 INTRODUCTION

1.1 Cell Cycle

Cell growth and division are the basis of biological life. Each dividing cell completes an ordered series of events collectively known as the "cell cycle". Typical mitotic cell cycles include the duplication of the genome during DNA synthesis phase (S phase), the segregation of complete sets of chromosomes to each of the daughter cells mitotic phase (M phase) and “gap" phases, known as G1, which connects the completion of M phase to initiation of S phase in the next cycle, and G2, which separates the S and M phases.

External, environmental and intrinsic, developmental and physiological signals together determine whether cells enter a division cycle. This decision is called “START” in yeast and “Restriction point” in mammalian cells. However, the cell-cycle can again come to an halt at so called cell-cycle checkpoints if the cellular conditions are unfavourable for a division. Checkpoints exist for the transition from G1 into S, from G2 into M, during S-phase and also in mitosis (Figure I1)

An important parameter that is assayed at these checkpoints is the integrity of the DNA. Various endogenous and environmental agents that can produce chromosomal aberrations, point mutations or block replication continuously threaten genome integrity. As a result, a sensing mechanism has evolved to delay or even arrest cell-cycle progression to provide time to allow the cell to repair damaged DNA before its entry into new round of DNA replication or mitosis.

The molecular machinery that controls progression through these checkpoints is highly conserved in all the eukaryotes. However, novel mechanisms of cell-cycle regulation in plants, involve plant-specific cyclin-dependent kinases, an unusually vast group of cyclins and plant-specific cell-cycle inhibitors such as the SIAMESE/SIAMESE-RELATED (SIM/SMR) (Vandepoele *et al.,* 2002; Churchman *et al.,* 2006; Peres *et al.,* 2007).

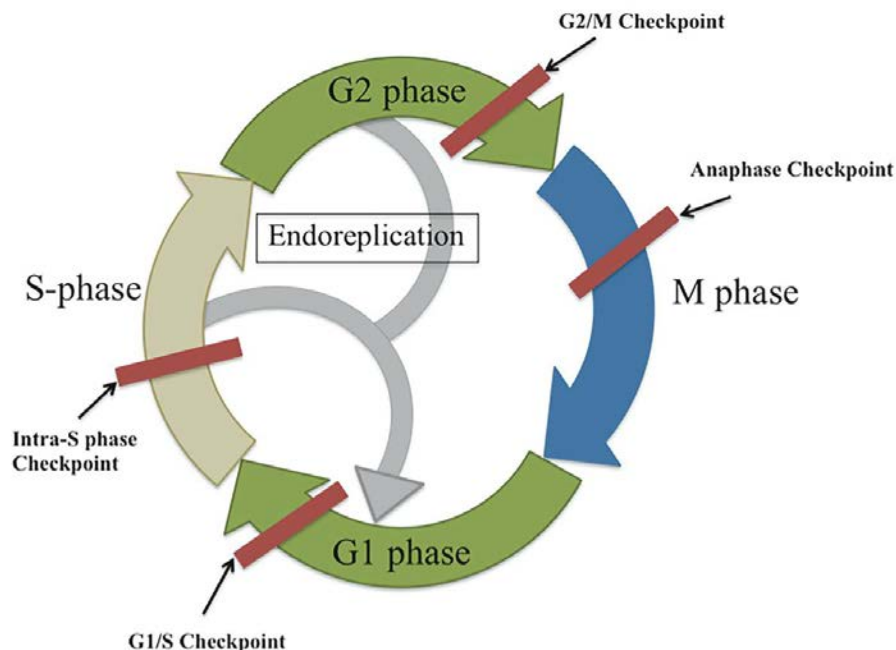

Figure I1: A prototypical cell cycle

Synthesis phase (S) and mitosis phase (M) are separated by two gap phases: G1 and G2. Endoreplication is a cell cycle variant in which nuclear DNA is replicated but division is skipped leading to polyploidy. Checkpoints exist at G1/S and G2/M transition as well as in S-phase and before anaphase.

1.2 Cyclin dependent Kinases

The central regulators of the eukaryotic cell cycle are cyclin-dependent kinases (CDKs). They trigger not only mitosis and DNA replication but also have roles in many other cellular processes such as gene transcription, DNA repair and apoptosis. CDKs typically constitute a family of heterodimeric serine (Ser)/threonine (Thr) kinases that are regulated by binding of cyclins, post-translational modifications and CDK inhibitors (CKIs).

All eukaryotic organisms studied to date possess at least one CDK with the conserved N-terminal PSTAIRE cyclin binding domain (Figure I2). The only PSTAIRE containing CDK in yeast is Cdc2 (*S. pombe)* or Cdc28 (*S. cerevisiae*) homologue of main mammalian CDK: Cdk1 (Morgan, 1997; Solomon et al., 1988; Huang et al., 2007). Cdk1/cyclinA activity is required for the initiation of prophase whereas Cdk1/cyclin B complexes actively participate in and complete mitosis (Riabowol *et al.,* 1989; Furuno *et al.,* 1999). Cdk1 can also drive S phase in the in absence of Cdk2, another important kinase (containing PSTAIRE domain in human, mouse, *Xenopus* and *Drosophila*) (Aleem *et al.,* 2005).

Homozygous *cdk1* mouse embryos die during early development indicating that Cdk1 is essential for early embryonic development (Santamaría *et al.,* 2007; Satyanarayana *et al.,* 2008). Whereas *cdk2* knockout mutants in mice show reduced body size and male and female sterility, indicating a specific role for Cdk2 in meiosis (Berthet *et al.,* 2003; Ortega *et al.,* 2003). Other important CDKs found in mammals are Cdk4 and Cdk6, specifically involved in S phase regulation (Hochegger et al., 2007; Hochegger et al., 2008; Krasinska et al., 2008).

1.2.1 *Arabidopsis* CDKA;1

The plant cell cycle is also governed by CDKs as that of other eukaryotes. For instance, eight classes of CDKs (CDKA to CDKG, and CDK-like kinases—CKLs) have been identified in *Arabidopsis*. A-type CDKs are the best-characterized group in plants. Members of this group bear the highest sequence identity with Cdc2/Cdc28/Cdk1. CDKA;1 is the only PSTAIRE containing CDK identified so far in *Arabidopsis*. It has been found to partially complement the cdc2 mutant of *S. pombe* (Ferreira et al., 1991; Hirayama *et al.,* 1991; Imajuku *et al.,* 1992).

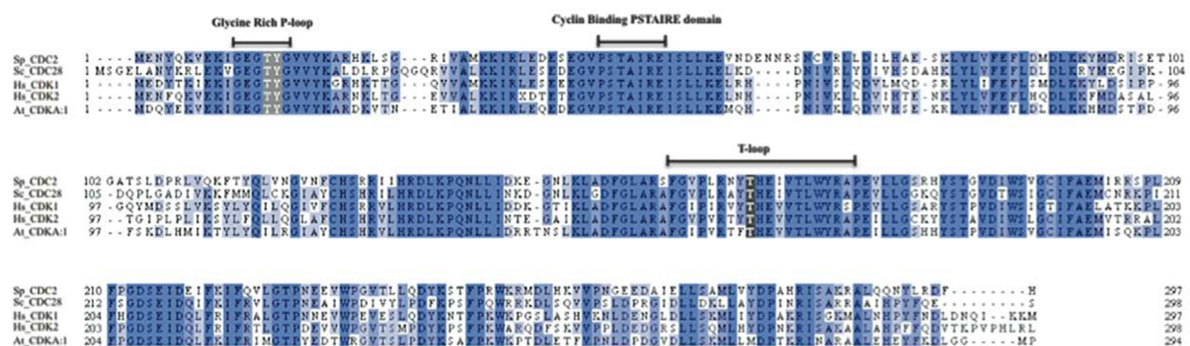

Figure I2: Alignment of the Cdk1 homologue to Arabidopsis CDKA;1
Protein alignment of yeast Cdc2/Cdc28 human Cdk1, Cdk2 and *Arabidopsis* CDKA;1

Heterozygous *cdka;1* mutants fail to undergo the second mitotic division during pollen development resulting in a single instead of a double fertilization that is typical for flowering plants (Iwakawa *et al.,* 2006; Nowack *et al.*, 2006). Subsequently, single-fertilized ovules abort. Occasionally, mutant pollen will execute a second mitotic division allowing double fertilization. When a mutant pollen fertilizes an egg cell that is also mutant for cdka;1, homozygous *cdka;1* embryos are formed that are surprisingly viable and can germinate on agar plates. However, the resulting plants are severely compromised and show for instance no proper root, increased cell size, reduced vegetative growth, reduced trichome branching and

complete sterility (Nowack *et al.,* 2012). These mutant accumulate cells in G1 and have a strong reduction in endoreplication, supporting an important role for CDKA;1 in S phase of the cell cycle (Nowack *et al.,* 2012). Based on biochemical data that showed that CDKA;1 activity also peaks immediately before entry into mitosis similar to human Cdk1, suggesting a likely role of CDKA;1 in mitosis as well (Weingartner et al., 2001). Thus CDKA;1 act as key regulator in plant cell cycle

1.3 Posttranslational Regulation of CDK

Multi-site phosphorylation is an important regulatory feature of CDK-cyclin complexes. This process involves positive and negative regulatory inputs and can generate new protein-binding domains. Phosphorylation of CDK occurs on conserved residues in two regulatory loops called P-loop, also called the ATP-binding loop, and T-loop, also called activation loop. Although the phosphorylation sites and their regulators appear to be conserved between eukaryotes, differences between plants and other eukaryotic species have been observed.

1.3.1 T-loop Phosphorylation

Phosphorylation of the T-loop is an important step involved in activation of CDK kinase activity since non-phosphorylated T-loop blocks the binding of protein substrate. Consequently, mutation of Thr167 as the phosphorylated residue in the T-loop of Cdc2 hinders the kinase from being fully active (Ducommun *et al.,* 1991; Krek *et al.,* 1992; Solomon *et al.,* 1992) (Figure I3). Similar mutations abolish the kinase activity of human Cdk1 and Cdk2 (Connell-Crowley *et al.,* 1993; De Bondt *et al.,* 1993; Jeffrey et al., 1995; Russo *et al.,* 1996).

In *Arabidopsis,* CDKA;1 also carries a Thr at position 161 in T-loop, which is phosphorylated *in vivo* (Dissmeyer et al., 2007; Harashima et al., 2007). Mutants of CDKA;1 having a phosphomimicry substitution of Thr161 with an aspartate, restored the generation of three-celled pollen and partially rescued homozygous *cdka;1* mutants. Nevertheless, rescued mutants showed reduced kinase activity, larger cells, reduced trichome branching and complete sterility (Dissmeyer *et al.,* 2007; Nowack *et al.,* 2012; Bramsiepe *et al.,* 2010).

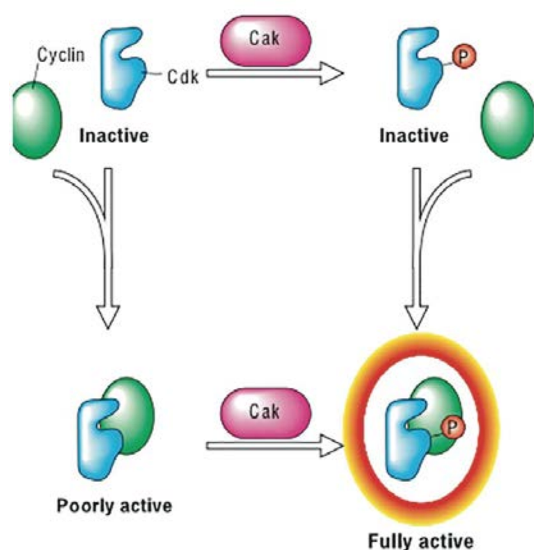

Figure I3: Activation of CDK in association with cyclin subunit and phosphorylation by (CAK)

Evidence from different model system indicates that phosphorylation can proceed independently of CDK-cyclin complex, and conversely that complex assembly can occur either before or after phosphorylation. In either case the cyclin-bound Thr-phosphorylated CDK represents the fully active kinase.

1.3.1.1 CAKs

T-loop phosphorylation of CDKs is catalyzed by CDK-activating kinases (CAKs), which are also members of the CDK family. Consequently, the loss of CAK activity causes cell-cycle arrest (Espinoza *et al.,* 1996; Kaldis *et al.,* 1996; Thuret *et al*., 1996; Larochelle *et al.,* 1998; Lee *et al.*, 1999). In addition to CDKs, CAKs also phosphorylate the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (Serizawa *et al.,* 1995; Shiekhattar *et al.,* 1995; Schwartz *et al.,* 2003).

Cak1/Civ1 is the sole essential CAK identified in budding yeast that possesses Cdc28 activating kinase activity but no direct CTD kinase activity (Table I1). Cak1 is a considerably divergent from other CDKs as it is fully active without a cyclin partner or activating phosphorylation. Furthermore, it lacks the consensus GXGXXG motif that is implicated in nucleotide binding for all classes of protein kinase (Hanks *et al.,* 1995; Solomon *et al.,* 1996; Thuret et al. 1996; Espinoza et al. 1998; Kaldis 1999).

The mammalian CAK comprise Cdk7 that is activated by binding to cyclin H and (Devault et al., 1995; Tassan et al., 1995). The *cdk7* null mutants of Drosophila die before or soon after the initiation of pupation (Larochelle et al., 1998). Similarly, depletion of Cdk7 in Caenorhabditis elegans resulted in an embryonic lethal phenotype (Wallenfang *et al.,* 2002; Rossi *et al.,* 2001) indicating that Cdk7 activity is indispensable during early developmental stages in animals.

Table I1: Protein kinase activation of CDK and basal transcription (Table taken from Umeda *et al.*, 2005)

Organism	Catalytic subunit	Regulatory subunit	Assembly subunit	CAK activity	CTD kinase activity
Human	CDK7	Cyclin H	MAT1	+	+
	p42	–	–	+	–
Sch. pombe	Mcs6/Crk1/Mop1	Mcs2	Pmh1	+	+
S. cerevisiae	Kin28p	Ccl1p	Rig2p/Tfb3p	–	+
	Cak1p/Civ1p	–	–	+	–
Rice	Os;CDKD;1	Os;CycH;1	?	+	+
Arabidopsis	At;CDKD;1	At;CycH;1	?	–	–
	At;CDKD;2	At;CycH;1	?	Low	High
	At;CDKD;3	At;CycH;1	?	High	Low
	At;CDKF;1	?	?	Very high	–

1.3.1.2 *Arabidopsis* CAKs

In plants, two gene classes have been identified as member of CAK family. CDKD and CDKF exhibit CAK activity in rice and Arabidopsis (Chao et al., 2007; Umeda et al., 1998; Yamaguchi et al., 1998) (Table I1). CDKF;1 is a plant-specific CAK that has been identified as a suppressor of the cak1 mutation in budding yeast. It shows similarity to Cdk7 and can phosphorylate human Cdk2 in vitro but unlike vertebrate-type CAKs, it has no CTD kinase activity *in vitro* (Umeda et al., 1998). In *Arabidopsis,* CDKF;1 can phosphorylate CDKA;1 in root protoplasts, and this activity was dependent on T-loop phosphorylation of CDKF;1 (Shimotohno et al., 2006). CDKF;1 also functions as a CAK activating kinase as it phosphorylates CDKD;2 and CDKD;3 and, thereby regulates basal transcription and CDK activation (Shimotohno et al., 2004; Umeda et al., 2005). Significant reduction in kinase activity of CDKD;2 and developmental defects after embryogenesis in *cdkf;1* mutants suggests that CDKF;1 plays an important role in postembryonic development by regulating the protein stability of CDKD;2 in *Arabidopsis*.

```
Hs_CDK7   1 MALD---------VKSRAKRYEKLDFLGEGQFATVYKARDKNTNQIVAIKKIKLGHRSEAKDGINRTALREIKLLQEL----S 70
Os_CDKD;1 1 MASGDGGDD--AGVKRVADRYLKREVLGEGTYGVVFKAVDTKTGNTVAIKKIRLGKY---KEGVNFTALREIKL--------L 70
At_CDKF;1 1 MDKQPATSWSIHTRPEIIAKYEIFERVGSGAYADVYRARRLSDGLIVALKEIFD----------YQSAFREIDALTILN---G 70
At_CDKD;1 1 ME-Q---------PKKVADRYLKREVLGQGTYGVVFKATDTKNGETVAIKKIRLGKE---KEGVNVTALREIKLLKELKHPHI 70
At_CDKD;2 1 MSKS--------GDNQPVDRYLRRQILGEGTYGVVYKATDTKTGKTVAVKKIRLGNQ---KEGVNFTALREIKLLKELNH--P 70
At_CDKD;3 1 MPEQ---------PKKVADRYLKQEVLGQGTYGVVFKATDTKTEQTVAIKKIRLGKQ---REGVNITALREIKMLKELKHP-H 70
```

Figure I4: Alignment of N-terminal segment of human CAK to plant CAKs

The conserved Thr (T) and Tyr (Y) residues in the ATP-binding loop are found only in CAKs in plants. Numbers indicate amino acid positions. Dashes represent gaps introduced to give maximal identity.

CDKD is an orthologue of vertebrate CAK. It is activated by its regulatory partner cyclin H and has both CAK and CTD kinase activities. *Arabidopsis* has three CDKD genes, namely AtCDKD;1, AtCDKD;2, AtCDKD;3. These three CDKDs share a PSTAIRE-like domain: CDKD;1 (NVTALRE), CDKD;2 (NFTALRE) and CDKD;3 (NITALRE) (Figure I4) and CDKD;2 and CDKD;3 have conserved T-loop site that can be phosphorylated by CDKF;1. Plant CDKDs also have phosphorylatable Thr and tyrosine (Tyr) residues in P-loop, which appears to be a unique characteristic of the species as vertebrate CAK, lack these residues (Figure I4). P-loop of CDKD;2 and CDKD;3 can be phosphorylated by WEE1 *in vitro*. CDKD;2 and CDKD;3 phosphorylate human Cdk2 and *Arabidopsis* CTD *in vitro*. The kinase activity of CDKD;3 is higher than that of CDKD;2 *in vitro* whereas CDKD;2 had higher CTD kinase activity than CDKD;3. CDKD;1 was found to be neither phosphorylated by CDKF;1 nor did it show kinase activity towards CDKA;1 *in vitro*, therefore it was suggested as an inactive CAK (Serizawa et al., 1995; Shiekhattar et al., 1995; Shimotohno et al., 2003, 2004, 2006; Takatsuka et al., 2009). However, it has been recently shown that CDKD;1 similarly to CDKD;2 and CDKD;3, phosphorylates all Ser residues of Arabidopsis RNAPII CTD (Hajheidari *et al*., 2012).

Whereas single mutants of *cdkd;1 cdkd;2* and *cdkd;3* show no change in development compared with the wildtype, the double mutants *cdkd;1 cdkd;3*, *cdkd;1 cdkd;2* and *cdkd;2 cdkd;3* are reduced in size and develop curly and serrated leaves. The triple mutants of all three CDKD genes are lethal. However, a hypomorphic triple mutant has been identified that is severely affected in growth and development. These results emphasize the role of CDKD;1 as an active member of CAK family, and its role in cell-cycle regulation (Hajheidari *et al*., 2012).

1.3.2 P-loop Phosphorylation

P-loop phosphorylation of CDKA;1 leads to conformational changes, which reduce the affinity for ATP. This interferes with proper substrate binding, and inhibiting CDKA;1 kinase activity (Endicott et al., 1999).

Early studies in *S. pombe* demonstrated that Cdc2 Tyr15 phosphorylation directly regulates entry into mitosis and is an important element in the control of the unperturbed cell cycle. In animals, phosphorylation at either or both of P-loop Thr/Tyr residues abrogates

Cdk1 kinase activity, and plays a major role in controlling passage through the G2/M transition point. The P-loop of human Cdk1 and Cdk2 comprises of residues 10-17 containing the glycin rich loop (11-GxGxxG-16). Inactivation of CDK is reversed by the dephosphorylation by phosphatases. This mechanism is common in all model organisms so far, except for plants.

Arabidopsis CDKA;1 also contains a glycine rich loop carrying conserved phosphorylatable Thr14 and Tyr15 residues. Substitution of Thr14 and Tyr15 with Asp and Glu respectively mimic P-loop phosphorylation thus results in constitutively restricted CDKA;1 activity. These variants can partially rescue homozygous *cdka;1* mutants and result in smaller plants with larger cells, reduced kinase activity and sterility (Dissmeyer et al., 2009). De-phosphomimicry version of CDKA;1 (T14V; Y15F) can also rescue *cdka;1* homozygous mutants and these plants are indistinguishable from WT plants, suggesting under standard greenhouse conditions phosphorylation of the P-loop is dispensable to arrest the cell cycle.

1.3.2.1 Wee1

Wee1 is an evolutionarily conserved Tyr kinase, which executes the inhibitory phosphorylation in the P-loop of CDKs in animals and yeast. It is a key regulator of cell-cycle progression that is markedly active during the G2/M phase of the cell cycle. In *S. pombe* Wee1 and its homologue Mik1 dual-specific (Thr/Tyr) kinase repress Cdc2. Yeast *wee1* cells enter prematurely entry into mitosis resulting progressively smaller cells (Nurse, 1990; Russell, 1991; Muller *et al.,* 1995). In mammals, Wee1 and the related kinase Myt1 catalyze the phosphorylation of Thr14 and Tyr15 of Cdk1 (Figure I5, A). Wee1 deletion causes premature entry into mitosis in cells that are still too small for division (Krek and Nigg, 1991; Lundgren et al., 1991; Lee et al., 1994; Muller *et al.,* 1995; Nurse, 2004; Morgan, 2007). Cell cycle arrest during G2 upon DNA damage is Wee1 dependent in mammals and *S. pombe.* This allows the cell to repair the damage before a new cell cycle round (Michael and Newport, 1998; Rhind and Russell, 2001).

In maize, a partial WEE1 homologue was cloned that inhibits CDK activity *in vitro* (Sun *et al.,* 1999) whilst a full-length WEE1 has been identified in the *Arabidopsis* genome, which is highly expressed in meristems. However, no homolog of Mik1 or Myt1 has been identified in *Arabidopsis* (Sorrell *et al.,* 2002). *AtWEE1* can regulate cell size when inducibly

expressed in fission yeast hence is capable of regulating G2/M transition in fission yeast (Sorrel *et al.,* 2002, 2005). Furthermore, WEE1 might be involved in endoreplication given that its transcript levels is high in the endosperm of *Z. mays* and in tomato fruit, two endoreplicating tissues (Sun *et al.,* 1999; Gonzalez *et al.,* 2004, 2007).

In *Arabidopsis*, Tyr15 in the P-loop of CDKA;1 can be phosphorylated by WEE1 *in vitro,* suggesting a similar specificity as in yeast and vertebrates (Shimotono *et al.,* 2006; Schutter et al., 2007) (Figure I5, B) . A distinct feature of *Arabidopsis* WEE1 is that it can inhibit CDKD;2 kinase activity in root protoplast and also phosphorylates the P-loop of CDKD;3 *in vitro* (Shimotono *et al.,* 2006).

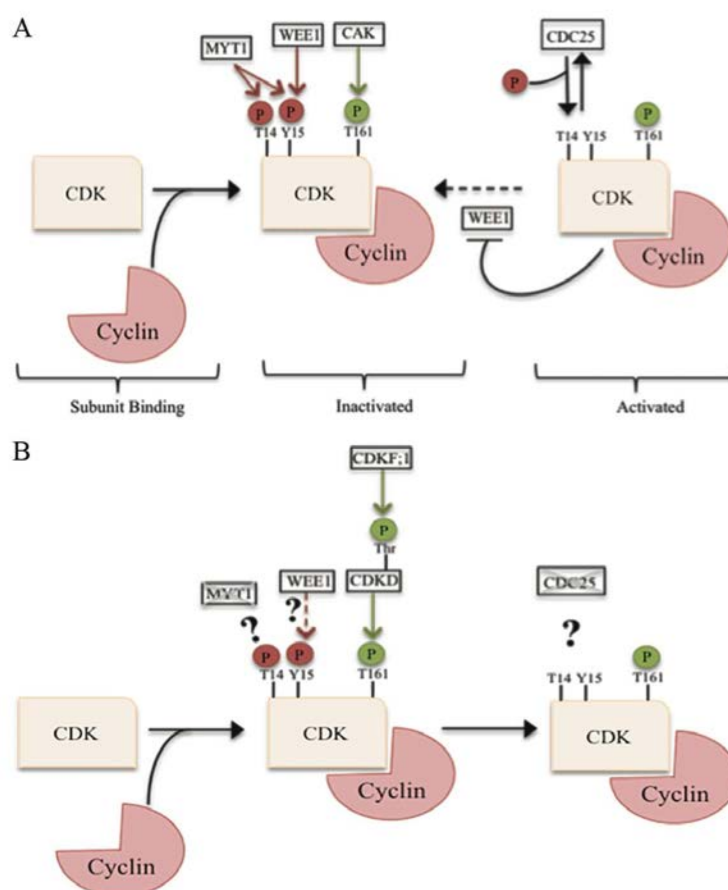

Figure I5: Scheme of posttranslational modifications of CDKs

(A) In mammals, following CDK-cyclin subunit binding, Myt1/ Wee1 phosphorylates the P-loop in order to inhibit the CDK kinase activity. CAK phosphorylate the T-loop for activation. Cdc25 phosphatases remove phosphate groups in the P-loop and active CDKs. (B) Plant specific circuits of posttranslational CDK modifications. It appears that P-loop phosphorylation by WEE1 is not of great importance in plants. CDKDs activate CDKA;1 via T-loop phosphorylation and are activated through CDKF;1..CDC25 is not involved in mitotic cell-cycle regulation in *Arabidopsis.*

Arabidopsis wee1 mutants display no growth defects under green house conditions. (De Schutter et al., 2007; Spadafora *et al.,* 2011). However, *wee1* mutants are hypersensitive to replication blocking agents suggesting a role of WEE1 in S-phase. By contrast, overexpression of WEE1 results in phenotypes consistent with permanent activation of cell cycle checkpoints, including cell-cycle arrest, differentiation of stem cells and shrinkage of the meristem (De Schutter et al., 2007; Ricaud et al., 2007).

1.3.2.2 Cdc25

Cdc25 is highly conserved protein with dual phosphatase specificity that activates CDKs by dephosphorylating P-loop residues. Only one Cdc25 phosphatase has been identified in yeast. In mammals, three isoforms of Cdc25 have been identified: Cdc25A, Cdc25B and Cdc25C2–4, whereas *X. laevis* and *G. gallus* each have two orthologues of Cdc25. The catalytic domains of Cdc25 proteins are conserved among the different species (Bell *et al.,* 1993; Orchard *et al.,* 2005).

In *Arabidopsis,* only a presumptive homologue of Cdc25 has been identified. However, a large N- terminal region usually found in cell-cycle related Cdc25-like phosphatases is missing (Landrieu *et al.,* 2004a; Landrieu *et al.,* 2004b; Boutros *et al.,* 2006). Surprisingly, *Arabidopsis* knockout mutants of *AtCDC25* showed no phenotype under greenhouse conditions and none after DNA damage induction (Dissmeyer et al., 2009, 2010). CDC25 overexpressing plants do not show susceptibility to genotoxic stress even though it can be expected due to a faster cell cycle and reduced checkpoint control resulting in premature entry into mitosis (Dissmeyer et al., 2010).

These results suggest that plants might lack a phosphatase for activating the P-loop.

1.4 Cell cycle regulation by internal and external Signals

1.4.1 Cell cycle regulation upon DNA damage

To survive during various changes in the environment, organisms need to adapt their growth behavior through altering the rate of cell proliferation and differentiation. Therefore, expression of many cell-cycle regulators is affected by internal or external cues.

Living organisms are continuously exposed to genotoxic threats, induced by environmental conditions, for example UV radiation) or by endogenous factors, for example, replication errors and reactive oxygen intermediates (Ciccia and Elledge, 2010). Cells coordinate DNA-repair machinery with cell-cycle regulators through DNA damage checkpoints to delay or even arrest the cell cycle until the damage is repaired.

Upon damage detection, a signaling cascade of checkpoint protein gets activated which transmit the damage signal to the downstream targets such as the DNA-repair apparatus and the cell-cycle machinery. The activation of these targets is achieved by different phosphorylation events that regulate the cell-cycle transitions by activating or inhibiting the proteins involved in checkpoint maintenance and cell-cycle progression. The importance of these checkpoints is highlighted by the fact that they are present in almost all the eukaryotes, playing their role to delay cell cycle progression in response to DNA stress. Due to the sessile nature of plants cannot skip the environmental threats to DNA and they might have developed specific adjustments of these checkpoints.

1.4.1.1 Activation of signalling cascade

In eukaryotes, DNA replication is initiated at numerous origins along chromosomes, and many obstacles to the progressing fork appear during each S-phase. Any hindrance to fork progression can be caused by DNA lesions, by non-histone proteins tightly bound to DNA, by nucleotide pool imbalance or by conflicts with the transcription machinery. This can lead to accumulation of stalled forks structures pausing of DNA replication. At this point intra S-phase checkpoint stops the cell cycle.

The ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases are triggered in response to different types of DNA stress in mammals and plants. Double-strand breaks (DSBs) activate ATM, whereas activation of ATR occurs upon the generation of lesions containing single-stranded DNA (ssDNA), which evolve at stalled replication forks.

In mammals, DNA damage activated ATM and ATR phosphorylate and activate Chk2 and Chk1 kinases, respectively (Figure I6, A) . Chk1 is an essential Ser/Thr kinase involved in S- and G2/M-phase checkpoints, replication initiation and fork stability, homologous recombination repair, and entry into mitosis in normal cycling cells. Importantly, Chk1 is necessary for unperturbed DNA replication and cell-cycle coordination even in the absence of any exogenous insult. ATR phosphorylates Chk1 on Ser residues and stimulates its auto-

phosphorylation. Phosphorylated Chk1 can dissociate from chromatin and ATR regulation of Chk1 may thereby control transition of DNA damage signals from chromatin to its targets. The molecular events in DNA damage response are governed by p53 transcription factor (Rozan and El-Deiry, 2007).

Although starting and end point of signaling cascade are conserved among yeast mammals and plants however the inner frame- work of the pathway seems to be distinct appears to be vastly different. In plants, no homologs of Chk1, CHK2 and p53 have been identified. However, plants have a specific central regulator of the DNA damage checkpoint, "SOG1", a NAC-domain-containing transcription factor that appears to function analogously to p53 (Yoshiyama et al., 2009) (Figure I6, B).

Another striking difference between animals and plants is that checkpoint genes are not essential during normal growth in plants. Except for partial sterility of *atm* mutants, checkpoint mutants are phenotypically normal under non-stressed conditions in *Arabidopsis.* However, *atm* mutants show hypersensitivity to gamma irradiation and other DNA-damaging agents such as DNA-alkylating chemicals. Furthermore, they show defects in meiosis (Garcia et al., 2003). Whereas *atr* mutants are hypersensitive to hydroxyurea (HU), aphidicolin, and UV-B, which block DNA replication but it shows only mild sensitivity to gamma irradiation (Culligan et al., 2004). A *sog1* mutant fails to arrest leaf development after gamma irradiation, and *atr* but not *atm* mutants have a similar phenotype.

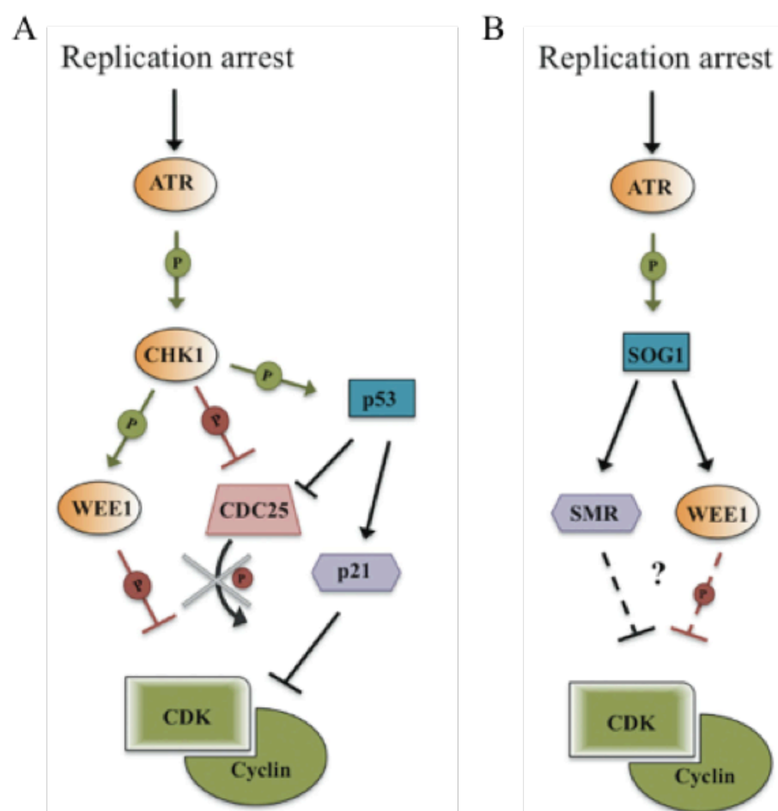

Figure I6: DNA damage checkpoint pathways in mammals and plants upon replication stress

(A) Activation of DNA damage checkpoint through a signaling cascade involves the kinases ATR Chk1 Wee1 which leads to cell cycle arrest in mammals (B) In *Arabidopsis*, ATR presumably activates SOG1 and WEE1 however many intermediate regulator are absent like Chk1 and the detailed mechanism leading to cell cycle arrest remains unclear.

1.4.1.2 Cell cycle arrest upon DNA damage

Once the DNA damage signaling cascade is activated in animals, it leads to cell-cycle inhibition to provide the time for repair. Downstream of CHK kinases are Wee1 and Cdc25 phosphatase, which are also ultimately controlled by Cdk1-mediated multi-site phosphorylation. Following its activation ATM-CHK2 and ATR-Chk1 kinase phosphorylate Cdc25 phosphatase, leading to its inhibition or ubiquitin dependent degradation. The degradation of Cdc25A interferes with the dephosphorylation of the CDK Tyr15 residue. This results in inhibition of CDK activity thus leading to cell cycle arrest. Chk1 can also phosphorylate Cdc25B and Cdc25C, which may also contribute to restrain CDK activity.

Cdc25 and Wee1 act as respective ‘‘on’’ and ‘‘off’’ switches on CDK activity. Thus, CHK kinases prevent the degradation of Wee1 kinase at the same time until DNA repair is completed so that CDKs are inactivated by inhibitory phosphorylation (Harper and Elledge,

2007). Wee1 directly phosphorylates and inhibits Cdk1 and Cdk2 effecting entry into mitosis as well as coordination of DNA replication events. Wee1 is therefore critical for properly timing cell division in unperturbed cells, and loss of Wee1 results in chromosomal aneuploidy and accumulated DNA damage. Wee1 is inhibited both by phosphorylation and degradation allowing rapid increase in CDK activity at entry into mitosis or upon repair completion.

The loss of each of ATR Chk1 and Wee1 leads to increased CDK activity resulting in loss of control of the replication coordination. Inhibition of Chk1 leads to an increased loading of replication factor Cdc45L onto chromatin, which is followed by a dramatic increase in replication initiation. Deletion of ATR, Chk1 or Wee1 in mice causes embryonic lethality demonstrating that these checkpoint kinases are essential for embryonic development.

1.4.1.3 What is different in plants?

Even though plants have no functional CDC25 homolog, cell cycle inhibition in response to DNA damage apparently involves phospho control. Down-regulation of CDK in response to DNA stress is controlled by the Cdc25-counteracting WEE1 kinase. WEE1 functions downstream of ATR and is transcriptionally upregulated in response to DNA replication stress in *Arabidopsis* (De Schutter et al., 2007; Takahashi et al., 2008). Cell cycle arrest is affected in *wee1* upon HU treatment (Cools *et al.,* 2011).

In plants, WEE1 is an essential intra-S-phase checkpoint gene as demonstrated by the accumulation of the gene product in replicating nuclei of mutant roots upon HU treatment, leading to their accumulation in S-phase in *Arabidopsis* (Cools *et al.,* 2011). Dissmeyer *et al.,* 2009 analyzed CDKA;1 P-loop variants in which conserved residues were substituted to either mimic phosphorylation or prevent phosphorylation under replication stress. It was shown that *DE* mutants grew slightly better on HU than *wee1,* suggesting that slower cell cycle might provide time to repair the stalled replication fork. *wee1 DE* mutants resembled *DE* under green house condition. However, *wee1 DE* showed shorter root lengths than *DE* but root growth was slightly better than *wee1* on HU suggesting that *wee1* is epistatic over *DE* under replicative stress condition (Dissmeyer *et al.,* 2009).

Surprisingly, plants having Thr14 and Tyr15 of CDKA;1 substituted with Val and Phe, respectively, to mimic dephosphorylation of CDKA;1 (*VF*), did not show the *wee1* phenotype on HU. These results demonstrated that WEE1 probably arrest the cell-cycle upon DNA

replication stress independently of the inhibitory phosphorylation of CDKA;1 in *Arabidopsis.* It is possible that WEE1 phosphorylates proteins other than CDKA;1 to preserve genome integrity after DNA replication stress. Analysis of *Arabidopsis* CDKA;1 T-loop variants having Thr161 substituted by Asp (*D*) for hypersensitivity to replication stress. Theses mutants were 100% resistant to HU. Unexpectedly when introgressed in a *wee1* background *D* mutants were able to rescue hypersensitivity of *wee1* mutants to HU (Annika Weimer, personal communications).

1.4.2 Cell cycle and circadian clock interaction

1.4.2.1 The circadian clock

To anticipate daily environmental changes, especially light-dark cycle and temperature oscillations, most organisms including plants, have developed an internal timing system, called circadian clock. External signals like light play a major role in the synchronization of the circadian clock with the diurnal (day-night) cycle. These signals entrain or set the pace of the clock, which in turn has the capacity to drive self-sustained oscillations with a near 24-hour periodicity (Pittendrigh, 1960).

The general principle of a circadian clock is its composition of three modules: a central clock, an internal oscillator that generates body time, an input unit that keeps the clock in phase with environmental cues, foremost the light-dark cycle, and an output module that couples the clock to biological processes. This basic scheme appears to apply to all known circadian clocks, regardless of the species involved (Pittendrigh, 1960; Stephan and Zucker, 1972; Moore and Eichler, 1972).

In *Arabidopsis*, the central feedback loop is based on the regulation between of two morning- expressed transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPO- COTYL (LHY). CCA1 and LHY directly activate the transcription of PSEUDO-RESPONSE REGULATOR9 (PRR9) and PRR7 in the morning. At the same time, CCA1 and LHY directly repress TIMING OF CAB EXPRESSION1 (TOC1) by binding a motif within its promoter region, called the EVENING ELEMENT (EE). CCA1 and LHY simultaneously suppress the expression of the other clock genes with afternoon to evening peaks, such as CCA1 HIKING EXPEDITION (CHE), GIGANTEA (GI), LUX

ARRHYTHMO (LUX), and EARLY FLOWERING4 (ELF4) (Farré EM 2005; Harmer SL 2009; Imaizumi T 2010) (Figure I7).

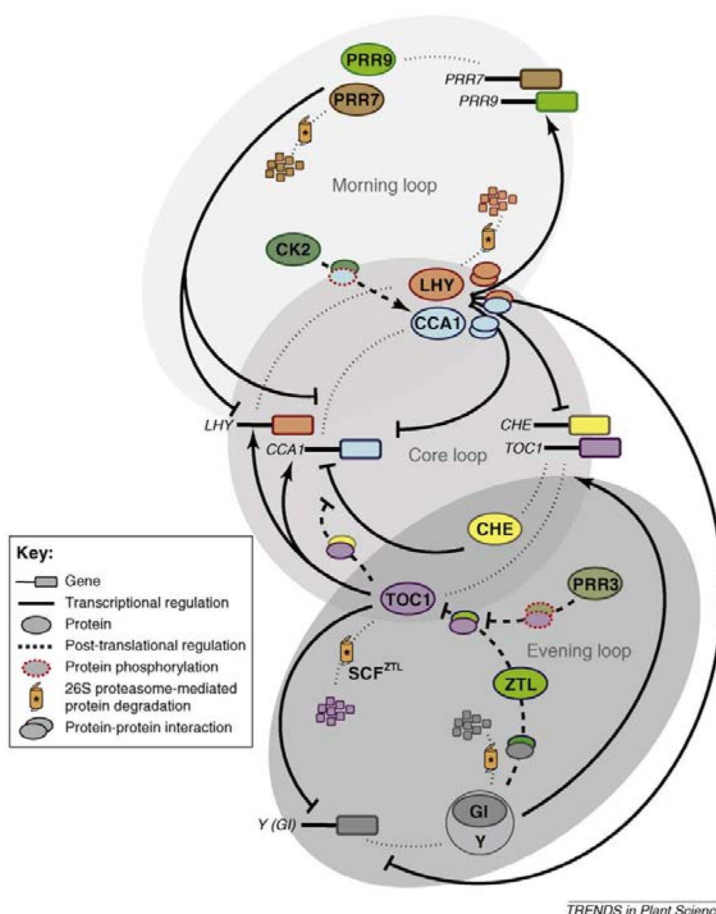

Figure I7: Model of the circadian clock in Arabidopsis.

The core feedback loop consists of two transcription factors, CCA1 and LHY, which negatively regulate the expression of TOC1. TOC1 has been proposed to activate the expression of CCA1 and LHY. An additional module within this loop includes the reciprocal repression between CCA1 and CCA1 HIKING EXPEDITION (CHE). TOC1 probably antagonizes CHE through direct interaction. Two additional phase-specific feedback loops have been proposed. In the morning loop, CCA1 and LHY activate the expression of PSEUDORESPONSE REGULATORS 7 and 9 (PRR7 and PRR9), which in turn repress CCA1 and LHY. In the evening loop, TOC1 represses an unknown component generically named Y [GIGANTEA (GI) appear to be part of Y], which in turn activates the expression of TOC1. TOC1 levels are controlled by proteasomal degradation mediated by the F-box protein ZEITLUPE (ZTL). GI and the competitive interaction between ZTL and PRR3 with TOC1 modulate this mechanism. The interaction between TOC1 and PRR3 is probably favored by the phosphorylation of these proteins (Figure taken from Jose *et al.,* 2010).

1.4.2.2 Cell cycle interaction with circadian clock

There is substantial evidence in metazoans and unicellular algae that the circadian clock controls cell-cycle progression (Goto and Johnson, 1995; Smaaland, 1996; Mori and Johnson, 2000; Dekens et al., 2003). The first evidence of circadian influence onto cell

division came from unicellular dinoflagellate *G. polyedra,* which divides at the end of darkness in a 12hrs light/12hrs dark cycle. Strikingly, the periodicity of cell division remained in continuous light for several days indicating that this rhythm is under circadian control (Sweeney *et al.*, 1958).

Obviously, the situation in multicellular organisms is much more complicated, as the timetable of physiological events and cell division is not necessarily synchronous among various tissues. None-the-less, circadian influence of cell cycle division was reported in cats already more than a century ago, and followed by reports in a wide range of mammalian species (including humans) and single-cell organisms (Fortuyn-van Leyden, 1917; Pilgrim *et al.,* 1963; Edmunds, 1964; Scheving *et al.,* 1978; Brown, 1991; Smaaland *et al.,* 1991; Goto and Johnson, 1995; Mori *et al.,* 1996; Bjarnason and Jordan, 2000; Dekens *et al.,* 2003; Tamai *et al.,* 2008; D'Autilia *et al.,* 2010).

Many of the core clock genes have also been linked to cell-cycle related phenotypes, both *in vitro* and *in vivo*. When exposed to ionizing radiation (IR), clock gene *Period2* mutants (*per2)* of mouse displayed higher tumor formation, impaired DDR in thymocytes and altered cell-cycle gene expression (Fu *et al.,* 2002; Gauger *et al.*, 2005; Miller *et al.,* 2007). In rodents, regularly scheduled meal hours synchronize the timing of S-phase in the cornea, bone marrow, lymphoid system and intestine (Canaple *et al.,* 2003; Li *et al.,* 2009).

The first direct link between the circadian clock and the cell cycle was found in mammals when it was shown that molecular components of the circadian clock directly regulate the expression of the cell-cycle gene *WEE1* (Matsuo *et al.,* 2003). The expression of Wee1 is controlled through E-box elements in its promoter, leading to circadian oscillations of Wee1 kinase activity and resulting in circadian phosphorylation of its constitutively expressed target proteins (Matsuo *et al.*, 2003). In zebrafish, there is data indicating that the cell cycle is gated through a systemic mechanism involving circadian glucocorticoid secretion (Dickmeis *et al.,* 2007).

Many aspects of plant physiology exhibit circadian behavior including leaf movement, flower opening, stomata opening, metabolic pathways and gene expression (Engelmann *et al.,* 1992; Jouve *et al.,* 1998; Dowson-Day *et al.*, 1999; Webb *et al.,* 2003). In *Arabidopsis*, a computer-based identification of potential *cis* elements in promoter region of CDK genes

showed that almost all promoters have several copies of light responsive and circadian rhythms elements (Inzé., 2008).

Table I2: Putative *cis*-element in the 2-kb-long promoter region of Rice and *Arabidopsis* CDK genes

Genes	Light Response	Circadian Rhythms
OsCDKA;1	1367	335, 366, 1783
OsCDKD1;1	524, 556, 629, 1612, 1858, 1991	1156
OsCDKF1;1	1020, 1315, 1380, 1539	
AtCDKA;1	49, 308, 890, 1180	73,255,109
AtCDKB1;1	24, 1134, 1072, 1079, 1769	532, 541, 638, 1238, 1359, 1484
AtCDKB1;2	21,162,243,772,819,1528	1709
AtCDKB2;1	65, 890, 1072, 1570, 1575, 1611	1413
AtCDKB2;2	64,263, 1831	1725
AtCDKC;1	948, 1376, 1800, 1848	
AtCDKC;2		876, 1940, 1986
AtCDKD;1		1139,1235, 1868
AtCDKD;2		70,87,1189
AtCDKD;3	376	101, 1679
AtCDKE;1		1136, 1435
AtCDKF;1	299, 508, 649	286, 536, 941, 1269, 1701

Number and position of potential transcription factor-binding sites are provided. The position is indicated as base pairs from the ATG start in 5´direction. Light response: INRNTPSADB (YTCANTYY); Circadian rhythm: CIACADIANLEHC (CAANNNNATC), EVENINGAT (AAAATATCT).

In microarray data analyses of *Arabidopsis*, circadian fluctuations in the expression levels of *TOC1* with two peaks at 36 and 60h were observed. The *Arabidopsis CDKG;1* gene displayed a very similar expression pattern, with moderate amplitude. *CDKA;1* expression was elevated, however, expression pattern were different from *TOC1* (Inzé, 2008) (Figure I8).

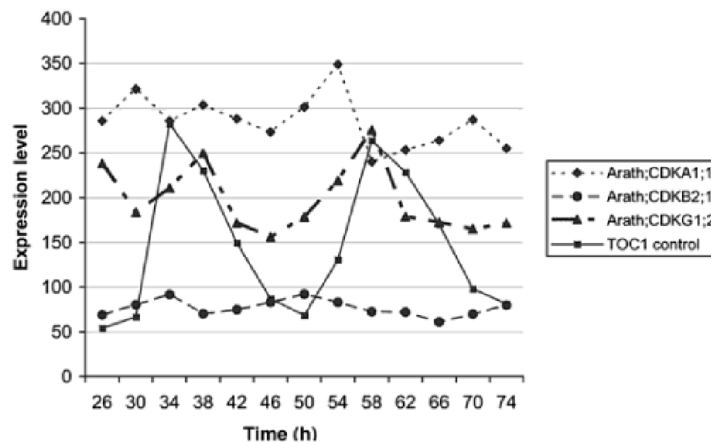

Figure I8: Expression of *Arabidopsis* CDK gene during circadian rhythms

Circadian microarray analysis showed that expression of TOC1 and CDKG;1 peaks at two different time points in *Arabidopsis* seedlings. CDKA;1 showed higher level of expression. However, its expression pattern differs from TOC1 whereas CDKB2;1 gene expression was reduced. (Circadian Microarray data analysis NASCArray experiment, De Kireon Edwards, University of Warwick, UK).

The core circadian oscillator is dependent on light perception and a feed-back system by the clock genes (*CCA1, LHY* and *TOC1)*. Expression of cell-cycle regulated genes such as histone *H4* and of central cell-cycle genes such as *CYCLIN B1;1* occurs at the end of the day and is under circadian control in the *Arabidopsis* shoot apical meristem (Bouget and Boudolf, 2004; Inzé, 2008).

A proposal for the coupling of the circadian and cell-division cycle is supported by data pointing to the diurnal variation of plant hormones or metabolites known to influence the cell cycle in plants directly. Nováková *et al.,* 2005 reported diurnal changes in the level of cytokinins indole-acetic acid and abscisic acid. In *Arabidopsis* rosettes, the sucrose glucose fructose and starch levels follow diurnal cycle in coordination with the diurnal expression patterns of sugar responsive genes (Blasing *et al.,* 2005). However, until now no data is available about a possible circadian regulation of the cell cycle in plants.

2 OBJECTIVES

Even though plants contain homologs of CDKs that have important roles in cell cycle regulation, little is known about the regulatory pathways through which CDKs interact with other cellular processes. In this work regulation of CDKs in response to environmental signal like DNA damage or circadian gene regulation has been studied.

In the first part of this study, the interconnection between cell cycle and DNA damage response is explored. DNA damage response has been intensively studied in animals however many regulators of this pathway still remains to be found in plants. A number of studies have shown that plants control their cell cycle differently after DNA damage compared to yeast and animals. Thus the objective of this study is to investigate these differences in detail. The major objectives of this work were:

1) To explore the alternative pathway of CDKA;1 regulation through WEE1 upon DNA damage induction and the involvement of CAKs, that control T-loop phosphorylation of CDKA;1, in this regulation.

2) To find out the interaction of P- and T-loop phosphorylation of CDKA;1 during meiosis and effect of CDKDs in this division.

In second half of this study the interaction of cell cycle regulators with circadian clock regulators is studied. Circadian clock regulation is very well understood in mammals and recent studies have shown an interaction of circadian clock and cell cycle processes. The aim of this work was

3) To reveal the existence of feedback loop between cell-cycle regulators and circadian clock regulators in *Arabidopsis.*

3 DISCUSSION

3.1 Regulation of CDKA;1 upon DNA damage

Cell-cycle regulation in response to DNA damage has been extensively studied in eukaryotes. The major cellular response upon DNA damage is not only the activation of the DNA repair machinery but also the inhibition of the cell cycle to provide cells sufficient time to repair the damage. Due to its role in diseases, especially cancer, the understanding of DNA damage control pathways is much further advanced in animal model systems and yeast than in plants. In mammals and yeast the activation of Wee1-type kinases by a DNA damage-signalling cascade represents a direct link of DNA damage to cell-cycle regulation (Perry and Kornbluth, 2007; Yata and Esashi, 2009). Wee1-type kinases phosphorylate Cdk1-type kinases in the P-loop at Thr and Tyr residues leading to the inhibition of Cdk kinase activity.

However, observations from yeast and plants open the possibility for the existence of alternative functions of WEE1. *S. cerevisiae* Swe1, a Wee1 homologue, acts in a morphogenesis checkpoint monitoring actin cytoskeleton integrity during bud formation. Mutations impairing actin organization or affecting septin organization lead to Swe1-mediated cell-cycle delays (Barral et al. 1999; Longtine et al. 2000; Lew, 2003). In some strains HU treatment also promote Swe1-dependent bud elongation (Jiang and Kang 2003; Liu and Wang 2006), possibly indicating that replication stress activates Swe1. However, in other strains, it appears that DNA checkpoint proteins prevent Swe1 from causing bud elongation in response to replication stress (Enserink et al. 2006). *S. cerevisiae* Cdc28 variants deprived of Tyr19 phosphorylation (equivalent to Tyr15) do not show any problem in cell-cycle arrest in response to DNA damage (Amon *et al.,* 1992 ; Sorger *et al.,* 1992).

Similarly, the current data on CDKs regulation through WEE1 in plants does not lead to a coherent model. Arabidopsis WEE1 appears to be involved in an intra-S phase checkpoint seen by the hypersensitivity of *wee1* mutants to HU and the accumulation of mutant cells in S phase with incompletely replicated or damaged DNA (De Schutter et al., 2007; Cools *et al.,* 2011). However, the mechanism of the WEE1 dependent cell-cycle arrest in response to this replication stress is not well understood. On the one hand, WEE1 can efficiently phosphorylate Tyr15 in the ATP-binding loop of monomeric CDKA;1 *in vitro* (Shimotono *et al.,* 2006; De Shutter *et al.,* 2007).

Phosphoproteomics data from *Arabidopsis* suggest that P-loop phosphorylation of CDKA;1 is not generally employed during cell proliferation since no phosphorylated Tyr 15 on CDKA;1 has been identified while T-loop phosphorylation is readily observed (de la Fuente van Bentem *et al.,* 2008; Sugiyama *et al.,* 2008). Importantly, P-loop dephospho-variant of CDKA;1, designated *VF* (*T14V;Y15F*), can completely rescue *cdka;1* mutants and do not mimic wee1 mutants on HU, i.e., they are not more sensitive to replication stress than the wildtype (Dissmeyer *et al.,* 2009). Better growth of CDKA;1 phospho-mimicry mutants *DE* on HU compared to *wee1,* but hypersensitivity of *wee1DE* stressed on role of WEE1 in an alternative pathway of cell-cycle inhibition upon replication stress, than in CDKA;1 P-loop inhibition (Dissemeyer *et al.,* 2009).

One possibility is that WEE1 could regulate cell-cycle progression in plants by targeting other CDKs than CDKA;1. One target could be B1-type CDKs, a plants specific class of CDKs. CDKB family members also contain conserved P- and T-loop segments and show high sequence similarities to CDKA;1. CDKB1s are expressed in a cell-cycle dependent manner: CDKB1 transcript accumulates in late S, G2 and M phase whereas CDKB2 seems to be specific for G2/M transition. Since CDKB1s are expressed during S phase they can be possible candidate for WEE1 phosphorylation. The rescue of *cdka;1* by $PRO_{CDKA;1}$*:CDKB1;1* (Nowack et al., 2012) and the hypersensitivity of *cdkb1;1 cdkb1;2* double mutants to the Cdk inhibitor roscovotine showed that CDKB1;1 and CDKA;1 have at least some overlapping functions.

Through analyzing the growth of plants expressing CDKB1 phosphomimicry mutants in a wee1 mutant background on HU this hypothesis can be tested. Rescue of *wee1* phenotype can be expected in *wee1* $CDKB;1^{T14D:Y15E}$ mutants if CDKB1 is a downstream target of WEE1 upon DNA damage. Also $CDKA;1^{VF}$ $CDKB;1^{VF}$ can be combined and tested on HU. Given that WEE1 cannot inhibit both CDKA;1 and CDKB;1 in these mutants, a severe hypersensitivity can be expected.

3.2 Role of T-loop in regulating CDKA;1 upon DNA damage

The resistance of the CDKA;1 T-loop phosphomimicry mutant *D* (*T161D*) to genotoxic stress and the partial rescue of the *wee1* phenotype on HU suggested a possible role of T-loop regulation in DNA damage response. The root-growth rate of homozygous *D* plants remained unaffected on HU versus MS without HU. Since these mutants have reduced CDKA;1 activity, one possible explanation of the observed rescue of *wee1* by D was that cells in this mutant combination may have more time to repair and reinitiate stalled replication forks. However, the P-loop phosphomimicry mutant DE has similarly reduced kinase activity levels and slow cell proliferation rates, but fails to rescue *wee1* mutants (Dissmeyer *et al.,* 2009). Growth analyses of *wee1* in combination with another weak allele of *CDKA;1* (*F80G*) conducted in this study showed that the rescue of the *wee1* phenotype on HU is specific to the T-loop phospho-mimicry variant of CDKA;1.

Based on these observations, CDKDs might be another target of WEE1 action after DNA damage. *Arabidopsis* CDKDs have Tyr residues in their P-loops, which is a specific feature of CAKs in plants but not in animals or yeast. Indeed, Arabidopsis WEE1 can efficiently phosphorylate these residues *in vitro* (Shimotono et al., 2006). Consequently, the inhibition of CDKD kinase activity through WEE1 mediated phosphorylation of their P-loops should result in the arrest of T-loop phosphorylation of CDKA;1.

To test this, dephospho-variants of CDKDs were constructed with non-phosphorylatable P-loop residues. These should mimic *wee1* mutants and indeed the here-analyzed *CDKD;2*^{Y24F} and *CDKD;3*^{Y23F} variants showed hypersensitivity to HU. At least in *in vitro* assays, the kinase activity of CDKD;3 on CDKA;1 is higher than that of CDKD;2 (Shimotono *et al.,* 2003) which may explain the stronger hypersensitivity of *CDKD;3*^{Y23F} compared to *CDKD;2*^{Y24F} on HU. In contrast, the dephospho-variant of *CDKD;1*, *CDKD;1*^{Y22F}, did not show hypersensitivity to HU. However, CDKD;1 was not shown to be phosphorylated by WEE1, thus suggesting that CDKD;2 and in particular CDKD;3 have majors role in cell-cycle inhibition upon replication stress.

The hypersensitivity of CDKD dephospho-variants was shown to be not simply due to promotion of cell-cycle activity since the expression of the full length CDKD version did not result in hypersensitivity to HU. The absence of a *wee1*–like severe hypersensitive phenotype in single *CDKD* $^{P\text{-}loop}$ variants may be due to a dosage effect. If so an increased sensitivity can

be expected in plants carrying increased number of copies of CDKD dephospho variants. Indeed combined effect of *CDKD;2*^{Y24F} and *CDKD;3*^{Y23F} when studied under replication stress, was stronger hypersensitivity compared to single *CDKD* $^{P\text{-}loop}$ mutants. Thus the regulation of the cell cycle through CDKDs appears to be a dose-dependent effect as increased number of copies of mutated gene resulted in stronger reduction of CDKA;1 activation, leading to much lower kinase activity.

T-loop phosphorylation of Cdks appears to be highly dose-dependent in other organisms. In yeast, Cak1 has been shown to repress defect caused by deletion of Smk1, a MAP kinase required for response to environmental stimuli. Increased copies of Cak1 suppress the developmental defect of *smk1* mutants (Wagner *et al.,* 1997). The phosphorylation of both Cdk1 on Thr161 and Cdk2 on Thr 160 decreased after Cdk7 inhibition in dose-dependent fashion (Ramanathan et al., 2001). Also Cdk9 activating-phosphorylation through Cdk7 was shown to be dosage dependent. Immuno-blot experiments showed that T-loop phosphorylation of Cdk9 decreases upon inhibition of Cdk7 activity in a dose-dependent manner (Larochelle *et al.,* 2012). In *Arabidopsis,* single mutants of CDKD do not show any defects. However, growth defects observed in double mutants and heterozygous triple mutants suggested an overlapping function of these genes. This can explain increased hypersensitivity of mutants having increased copies of dephospho-variants of CDKD.

Hypersensitivity of CDKD dephospho-mutants to HU suggests that WEE1 can repress CDKA;1 activity to delay/arrest cell-cycle progression upon replication stress by inhibition CDKD through inhibitory phosphorylation. This can be verified by comparing P-loop phosphorylation levels of CDKD in wild-type plants versus *wee1*. Whereas in wild-type plants an increase of inhibitory P-loop phosphorylation levels of CDKDs is expected following HU treatment, such an increase should not occur in *wee1* mutants. The laboratory of Masaaki Umeda in NAIST has generated CDKD antibodies that could be used to test these predictions in future.

Conversely, CDKA;1 T-loop phosphorylation should diminish following replication stress whereas in *wee1* mutant the phosphorylation should remain at the same level as seen in plants grown on MS media without HU. This can also be tested now by Western blots using an antibody that recognizes phospho-Thr160 of Cdk1 and that cross-reacts with the phosphorylated Thr161 in Arabidopsis (Harashima et al. 2007).

Finally, if the hypersensitivity observed in the CDKD;2 and CDKD;3 P-loop variants is caused by constitutive active CDKA;1, the growth of these dephospho-variants should be rescued by expressing *D* similarly to the rescue observed of *wee1* by *T161D*. For this purpose, I have introgressed *D* into *CDKD;2*^{Y22F} and *CDKD;3*^{Y23F} and these plants are ready now for growth analyses on HU media.

Assuming that *CDKD*$^{P\text{-}loop}$ variants are hypersensitive to HU due to the constitutive phosphorylation of the CDKA;1 T-loop, *cdkd* null mutants can be used as negative control due to presumable reduce T-loop phosphorylation activity. Single mutants of *cdkd;1* and *cdkd;3* showed no hypersensitivity to replication stress. The wild-type like growth of these mutants on HU indicated no significant alteration T-loop phosphorylation. Similarly, the rate of growth reduction of the *cdkd;1 cdkd;2* double mutants was not more than the control plants on HU. Surprisingly, *cdkd;2 cdkd;3* double mutants showed stronger reduction in root length compared to wild-type plants on HU. However, the reduction in root length of *cdkd;2 cdkd;3* mutants was not as severe as observed in *CDKD;2*^{Y24F}*CDKD;3*^{Y23F} (Figure D1).

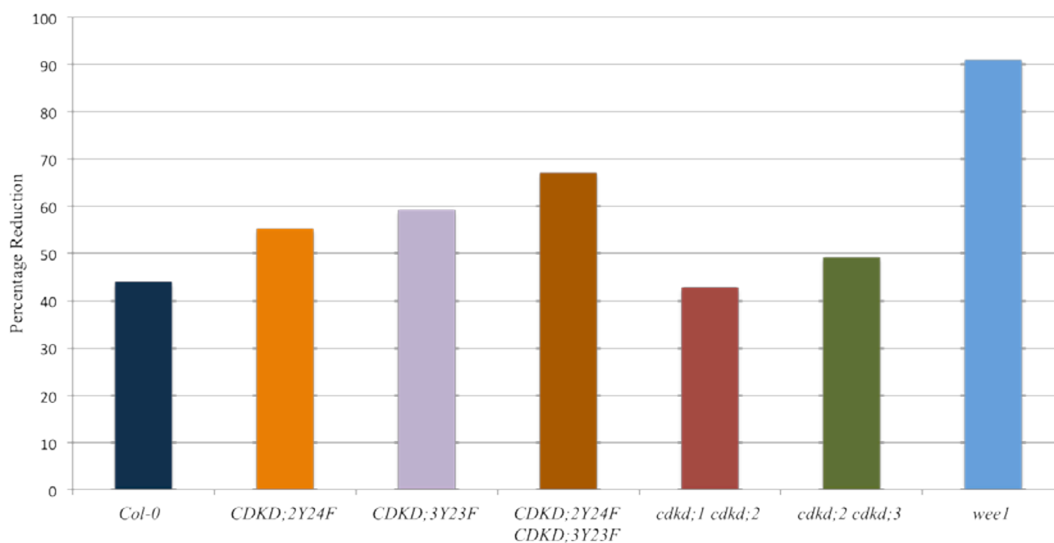

Figure D1: Percentage of the reduction in root growth of WT, *CDKD*$^{P\text{-}loop}$ and *cdkd* mutants
The bars represent the reduction of root growth between MS and 1mM HU in %.

Unaltered growth of *cdkd* single mutants and *cdkd;1 cdkd2* on control plates suggested that T-loop activity of CDKA;1 is not significantly reduced in these mutants to effect the growth. No hypersensitivity of these mutants to HU can be attributed to this unaltered T-loop phosphorylation level. Reduced root growth of *cdkd;2 cdkd;3* mutants on HU could be due to the activity of CDKD;1. CDKD;1 has been found to be an active CAK as it can phosphorylate

all Ser residues of *Arabidopsis* RNAPII CTD (Hajheidari *et al.,* 2012). An essential role of CDKD;1 in cell-cycle regulation can be perceived by the fact that *cdkd;2 cdkd;3* double mutants are viable but triple homozygous mutants of *cdkd* are lethal. CDKD;1 could also not be phosphorylated by WEE1 *in vitro*. Thus, it can be assumed that in single *cdkd* mutants and *cdkd;1 cdkd;2 and cdkd;1 cdkd;3* double mutants at least one CDKD gene, which can still be inhibited through WEE1, is present. Inhibition of this CDKD gene upon replication stress can reduce CDKA;1 activity as the proper response to replication stress. However, in *cdkd;2 cdkd;3,* activation of the CDKA;1 T-loop might still be possible through CDKD;1, which can not or perhaps less efficiently be inhibited through WEE1. This can result in reduced root growth of these double mutants on HU (Figure D2).

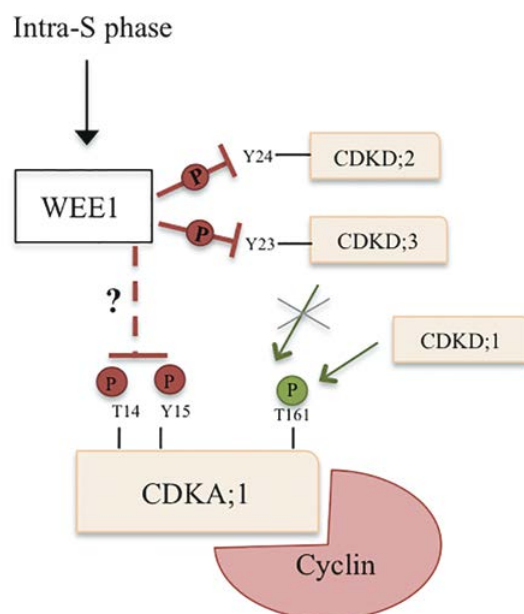

Figure D2: Proposed model for CDKA;1 inhibition via CDKDs through WEE1

This can be further investigated by checking the T-loop phosphorylation level of CDKA;1 in *cdkd;2 cdkd;3*. If CDKD;1 is involved in CDKA;1 activation, CDKA;1 T-loop phosphorylation should still be detected in these mutants. By expressing *T161D* in triple *cdkd* mutants it can be decided whether phosphorylated T-loop residues can rescue lethality of these mutants.

Interestingly, Ostapenko *et al.,* (2003, 2005) showed that deletion of Cak1 in yeast can indirectly disturb DNA damage response. Ctk1, a kinase associated with RNAPII, has been

shown to be involved in DNA damage induced transcription. Ctk1 is phosphorylated by Cak1 and *cak1* mutants are sensitive to DNA damage due to the failure of Ctk1 phosphorylation.

Even though plants and animals have some regulators of DNA damage response pathway in common, many key regulators like Chk1, Chk2, and p53 are absent in plants. Also due to their inability to avoid many constantly damaging influences, plants need to utilize efficient ways to cope with these stresses. Thus, they might have evolved alternative pathways to respond to external and internal stresses.

In mammals and fission yeast, Cdk7 and Mcs6 kinases, respectively, have dual CAK activity. This single CAK functions in regulating CDK kinase activity, and phosphorylating CTD of RNAPII. In budding yeast, on the other hand, two different orthologs of CAK, Cak1 shows kinase activity toward Cdc28 and Kin28, which is involved in phosphorylation of CTD of RNAPII. However, a special feature of *Arabidopsis* CAK is that instead of acting in distinct pathways they are involved in phosphorylation of CDK and CTD together (Shimotono *et al.,* 2004; Takatsuka *et al.,* 2009).

A possible involvement of T-loop phospho-regulation in DNA damage response has also been found in animals. Liang *et al.,* (2003) suggested that Cdk7 could act as an inducible G2 checkpoint by arresting the cell cycle under stress conditions. It was shown that an artificially induced inhibition of Cdk7 through drugs can arrest the cell cycle, thus preventing tumor formation in mammals. This negative regulation was shown to be independent of P-loop inhibition. This inhibition was very potent since even in the absences of P-loop phosphorylation of Cdk1, the cell cycle could be blocked through inhibition of Cdk7.

It has also been suggested in a number of studies that induction of the CDK inhibitor p21 in mammals upon DNA damage, inhibits T161 phosphorylation of Cdk1/Cdc2 and Cdk2 rendering the Cdk/cyclin complex inactive (Ohta *et al.,* 1998; Smits *et al.,* 2000; Bipin *et al.,* 2005). Expression of p21 is activated by p53, a transcription factor acting downstream ATM/ATR in DNA damage signaling cascade (Dulic *et al.,* 1994; el-Deiry *et al.,* 1994). In plants, a NAC-domain-containing transcription factor, called SOG1, has been identified that may function analogously to p53. SOG1 has been found to act downstream ATM/ATR and upstream WEE1 (Yoshiyama *et al.,* 2009). Even though regulatory relationship between SOG1 and WEE1 kinase is not clear yet, one can extrapolate that SOG1 activates WEE1 that than inhibit CDK activity by inhibiting T-loop phosphorylation by inhibiting CAKs.

However, p21 does not affect CAK activity rather it blocks access of CAK by binding to Cdk/cyclin complex. This block in G2 is maintained for up to 24 h, indicating that p21 can sustain a G2 arrest for a significant amount of time (Aperlikova et al., 1995; Hitomi et al., 1998; Smits *et al.,* 2000). Still, it has not been suggested to be the sole pathway regulating this arrest, rather it function appear to be secondary i.e., to ensure or sustain the arrest caused via P-loop phosphorylation (Bunz *et al.,* 1998; Smits *et al*., 2000).

Taken into account that WEE1 can phosphorylate the P-loop of monomeric CDKA;1 at least *in vitro,* and *CDKD$^{P\text{-}loop}$* mutants are not as sensitive as *wee1* mutants to HU, it might still be possible that WEE1 has dual function by regulating both P- and T-loop To explore the interaction between P- and T-loop phosphorylation, if any dephospho variants of *VF* in *cdkd* mutant background can serve as an important tool Triple *cdkd;3-cdka;1^{VF}* and *cdkd;1-cdka;1^{VF}* mutants have un-phosphorylatable P-loop residues and null *cdkd* mutation that can play a role in T-loop activation. Thus, in these mutants CDKA;1 activity was expected to be unaltered. Homozygous *cdka;1* null mutants can not only be fully rescued by *VF* but can also grow like wild-type plants under green house conditions (Dissmeyer *et al.,* 2009). However, the absence of double homozygous *cdka;1^{VF} cdkd;3* and *cdka;1^{VF} cdkd;1* in segregating progeny implies that CDKA;1 activity is significantly altered especially T-loop phosphorylation. This was illustrated by the fact that mutant CDKA;1 with non-phosphorylatable Val at Thr161 (*T161V*) can not rescue the homozygous *cdka;1* mutant (Dissmeyer *et al.,* 2007).

3.3 Role of CDKA;1 activity in meiosis

The combination of null *cdka;1* mutants carrying the *VF* variant in a *cdkd* mutant background did not result in defects during vegetative growth. Dissmeyer *et al.,* 2007 showed that reduced T-loop phosphorylation in *T161D* results in sterility due to meiotic defects. Similar sterile phenotype observed in *cdka;1^{VF} cdkd;3$^{+/-}$* and *cdka;1^{VF} cdkd;1$^{+/-}$*. Impaired male meiosis observed in *cdka;1^{VF} cdkd* mutants emphasize that certain levels of CDKA;1 T-loop activation are required for proper regulation and progression through meiosis but vegetative cell division is not sensitive enough to altered level of CDKA;1 activity.

Bulankova *et al.,* 2010 showed that CDKA;1 is present in meiosis in *Arabidopsis*. It was shown that the activating phosphorylation in CDKA;1 T-loop oscillates during meiosis, with peaks in metaphases I and II. Crossover formation between non-homologous

chromosomes and the occasional segregation of univalents at metaphase-I *cdka;1*^{VF} *cdkd;3*$^{+/-}$ but not in *VF* mutants, is consistent with role of T-loop activation in proper progression through meiosis.

Cell wall formation in interkinesis was observed in *D* mutants due to reduced T-loop activity (Dissmeyer et al., 2007). Presence of the phosphorylated and hence likely active CDKA;1 at the organelle band that separates the two cell poles suggested that active CDKA;1 is required to inhibit cytokinesis after meiosis-I. High kinase activity was shown to be required to prevent premature exit from meiosis (Bulankova et al. 2010; Zhao et al. 2011 ; Cromer et al. 2012; Nowack et al. 2012). Thus, formation of dyads due to cytokinesis during first meiosis division in *cdka;1*^{VF} *cdkd;3*$^{+/-}$meiocytes can be explained due to reduced phosphorylation of CDKA;1 in these mutants. Similar phenotypes observed in *cdka;1*^{VF} *cdkd;1*$^{+/-}$ mutants suggest the involvement of CDKD;1 in T-loop regulation of CDKA;1 in meiosis. However, this activity might not be as strong as CDKD;3 as suggested by reduced frequency of dyads in *cdka;1*^{VF} *cdkd;1*$^{-/-}$ compared to *cdka;1*^{VF} *cdkd;3*$^{+/-}$.

Meiotic defects observed in *cdka;1*^{VF} *cdkd;3*$^{+/-}$ and *cdka;1*^{VF}*cdkd;1*$^{+/-}$ occurred only in plants having no wild-type allele of CDKA;1. Whereas *VF* variants having either one or two wild type CDKA;1 alleles, even in a homozygous *cdkd* background, did not show sterility, suggests that the *VF* mutation is in particular sensitive to T-loop phosphorylation. This is consistent with the observation that CDKA;1 triple phospho-site mutants *VFD* which have a non phosphorylatable P-loop and constitutively active T-loop, showed severe growth defects (Annika Weimer and Nico Dissmeyer, personal communication).

The production of triploid and especially tetraploid plants in the progeny of *cdka;1*^{VF} *cdkd* indicates that female meiosis is also affected. Thus, a detailed analysis of the female meiosis in these mutants might shed new light on the role of CDKA;1 T-loop phosphorylation in reproduction. Analysis of *wee1 cdka;1*^{VF} for these meiotic defect will also be interesting. If the regulation of both the P- and T-loop of CDKA;1 is downstream of WEE1, a *cdka;1*^{VF} *cdkd* like phenotype can be expected in *wee1 cdka;1*^{VF}. Analysis of the meiosis in *cdkd* double and triple mutants will also be conducted in near future to investigate the reasons of sterility observed in these mutants.

3.4 Interaction between CDKA;1 and circadian clock

DNA damage is only one out of many stresses and environmental conditions to which the cell cycle has to respond and that need to be integrated with physiological and developmental responses at the organisms level. Especially for plants as photosynthetically active organisms, day and night cycles are one of the major environmental parameters. To ensures that responses to the environment occur at the precise time of the day, even if primary light cues are absent or delayed, plants as most other organisms have developed a circadian clock that can generate oscillation with an approximately 24 hour rhythmicity. In this work, I have explored whether the circadian clock also controls the cell cycle in Arabidopsis and especially whether there exist a feedback mechanism from the cell cycle to the circadian clock.

A direct regulation of cell cycle by the circadian clock has been demonstrated in animals (Matsuo *et al.*, 2003; Fu et al., 2005). Daily patterns of cell divisions are tightly tied to the endogenous time provided by the circadian clock in *Euglena*, *Synechococcus* and various animal such as mouse and zebrafish (Schevingt *al.,* 1998; Cardone *et al.,* 2003; Matsuo *et al.,* 2003; Dekens *et al.,* 2003). A possible advantage that has been proposed for the observed connection between the time of the day and the cell cycle is the prevention of DNA damage, e.g., cell division would be timed so that cells are in a G2 phase when UV radiation is the highest and hence DNA damage could be repaired though rather error-free homologous DNA recombination. Little is known about the existence of such a co-ordination in plants. Moulager *et al.,* (2007) even showed that cell division in *Chlamydomonas* is regulated in a time-dependent manner.

To explore a diurnal control of the cell cycle in *Arabidopsis,* DNA profiles of young seedlings were obtained at different times of the day. Wild-type plants showed a significant increased in the percentage of cells residing in G1 six hours after dawn, suggesting that divisions are preferably executed in the morning. This pattern is different from the one observed in other organisms. In mouse liver, for example, *Wee1* mRNA displayed a peak at eight hours after dawn with a subsequent decrease. Similar patterns were observed in regenerated liver, where *Wee1* trancript levels increase till ZT8 (ZT light turn on) suggesting a maximum occurrence of mitosis during these hours (Matsuo, *et al.,* 2003; Fu et al., 2005). Similarly, a peak of Cdc2 activity, indicative for the maximum of mitotic activity, was observed at ZT8 (ZT light turn on) in cells of regenerated livers cultured under light and dark cycle (Matsuo *et al.,* 2003). In mice neurogenic region number of S-phase cells were

quantified by BrdU staining and a no alterations were observed during the day and but an increase was observed preferably during night (Tamai *et al.,* 2008). It can be suggested as an internal mechanism to avoid enhanced environmental threats during sunlight and thus preventing damages during replication.

The timing of the cell cycle appeared to be altered in *cca1* compared to wild type. *cca1* mutants showed a shift of mitoses towards evening since the largest fraction of cells residing in G1 was found around dusk. Similarly *cdka;1* mutants showed a shift in time of division, as in *cdka;1* 2C contents were comparable in morning and evening, with maximum 2C content around dawn. Thus suggesting that cell division time is altered either by disturbing cell regulators or clock players. These results can now be verified by quantification of the mRNA levels of cell-cycle-phase specific genes at various time points of day in wild-type plants versus cdka;1 mutants.

Microarray analysis of an allelic series of *cdka;1* mutants showed altered expression level of clock genes in these mutants compared to wild-type plants. Not only the expression of core clock regulators like CCA1, LHY and CCR2 was down regulated but also expression of a number genes related to circadian responses like flowering time were also altered in *cdka;1* mutants. To test whether indeed circadian genes are deregulated in these mutants, the expression pattern of *CCA;1* and *GI* genes was investigated in weak loss-of-function mutants of *CDKA;1*. To this end, luciferase markers for both theses genes were introgressed in D and DE mutant version and the luminescence was analyzed in constant light conditions. The luminescence profile of *CCA1:LUC* in D and DE showed that this gene is still still rhythmically expressed in *cdka;1* mutants but has a reduced amplitude. Remarkably, the period of CCA1 rhythmicity was decreased in these mutants compared to wildtype. Reduced period length observed is in in consistence with down regulation of CCA1, as found in the microarray experiments. The reduced phase amplitude of *CCA1* in *cdka;1* mutants suggest a possible positive regulation of the clock gene through cell-cycle kinase. CCA1 has been shown to be direct target of phosphorylation and can interact with casein kinase 2 (CK2) (Sugano *et al.,* 1999).

On the other hand, *GI:LUC* expression profiles showed an increase in period length in *cdka;1* mutants, suggesting that expression of central and evening-phased-loop regulators are decoupled in cell-cycle mutants. Such phenomena have already been described in *Arabidopsis*. In shoots, the period of *CCA1* and *LHY* was longer in constant dark compared to

constant light whereas in roots, the period length was the same under both conditions. This suggested that genes of the core loop could be decoupled from oscillation in roots. Dalchau *et al.,* (2010) showed that period length of *GI:LUC* rhythms was slightly decreased after sucrose treatment in *Arabidopsis* plants whereas similar treatment resulted in longer period for *TOC1:LUC,* suggesting the existence of uncoupled oscillators. However in this cases observed decoupling was attributed to induced sucrose stress. But how the mutation in cell-cycle regulator function can lead to decoupling can be an interesting area to explore. Also despite the decoupling of morning and evening genes, these mutants still seem to maintain rhythms for both factors. It would be interesting to investigate how this can be possible.

These preliminary data indicate that cell-cycle kinases can have an effect on the regulation and expression of clock genes. Whether this effect is direct or indirect needs to be addressed in the future.

4 MATERIALS AND METHODS

4.1 Materials

Table M1: List of primers used for genotyping

Gene	Primer	Sequence 5' to 3'
Salk_LB	S.7	GCGTGGACCGCTTGCTGCAACTCTCTCAGG
cdka;1 T-DNA	S.27	CCAGATTCTCCGTGGAATTGCG
CDKA;1 ss	S.29	TGTACAAGCGAATAAAGACATTTGA
CDKA;1 as	S.38	TTTGGCTGGCTGCATTCCTTA
Gabi T-DNA	S.61	CCCATTTGGACGTGAATGTAGACAC
WEE1 ss	S.59	TCAATAAGGCTTGGTTTCTTCAGT
WEE1 as	S.60	AGGCATGTAACGTGCATCTC
Ku70 ss	S.62	AACCCTTACTTAGATATGATTTAC
KU70 as	S.63	AGGGTGTTATTCCGAGGCTTACT
CDKD1WT as	S.79	GATGTGGCCGTACATTGGTCTTTAGAA
CDKD1 WT/mut ss	S.77	GTTGTGGCAATTTGTAGAATGG
Cdkd1-5 mut as	S.78	CTGGGAATGGCGAAATCAAGGCATC
Cdkd3-3 WT as	S.80	GCATTTGGAAACAGAGCTCAC
Cdkd3-3 WT/mut ss	S.81	GAGTCGTCTTCAAAG CCA CTG
Cdkd3-3 mut as	S.82	GCGTGGACCGCTTGCTGCAACTCTCTCAGG
CDKA;1 ss1	S.326	CCGAGCACCAGAGATACTCCTAGG
CDKA;1 ss2	S.327	GAATTTGTTGATTGATCGCCGC
CDKA;1 as	S.328	CTAAGGCATGCCTCCAAGATCC
Fetch CDKA;1	S.324	TCAGCTGGCTTGTTTGATTG
Fetch CDKA;1	S.325	AACGGAGGATCACCACTTTG
CDKD1 Y22F ss	S.311	GATAATATATGAGTTCGCTGCTCTGG
CDKD1 Y22F as	S.312	GAGTTTACTTCAGCTTTATTATTCAGG
CDKD2 Y24F ss1	S.205	GAAGCAGTGATTCGTGATCG
CDKD2 Y24F ss2	S.206	GAAGGAACATTCGGTGTCGT
CDKD2 Y24F as	S.207	GGGGGTCAAGTGAATCCTTC
CDKD3 Y23F ss	S.201	CCACGATAAATGGGTTTTGC
CDKD3 Y23F as	S.203	CCCCCTTACTGGAACTCAAGAT

Table M2: T-DNA insertion lines used for study

Gene Locus	T-DNA lines	AGI code	Ecotype
CDKA;1	SALK_106809	At3G48750	Col-0
WEE1	GABI_270E05	At1G02970	Col-0
KU70	*ku-70*	At1g16970	Col-0
CDKD;1	Salk_114643	At1G73690	Col-0
CDKD;2	Salk_065163 Salk_053029	At1G66750	Col-0
CDKD3	Salk_120536 Salk_007756	At1G18040	Col-0

Table M 3: List of Crosses generated and used for this study

Crosses	Crosses
wee1-1 X *CDKA;1* ^{F80G}	*VF* X *CDKD3* ^{Y23F}(*cdkd1-1)*
wee1-1 X *cdkd;1-1*	*VF* X *CDKD2* ^{Y24F}(*cdkd3-1*)
wee1-1 X *cdkd;3-1*	*VF* X *CDKD3* ^{Y23F}(*cdkd1-1)*
cdkd1-1 X *cdkd3-1*	*CDKD2* ^{Y24F} X *CDKD3* ^{Y23F}
cdkd2-1 X *cdkd3-1*	*CDKD2* ^{Y24F}(*cdkd1-1*) X *CDKD3* ^{Y23F}(*cdkd3-1*)
cdkd1-1X cdkd2-1cdkd3-1	*D7* X *CDKD2* ^{Y24F}
VF X *cdkd1-1*	*D7* X *CDKD3* ^{Y23F}
VF X *cdkd3-1*	*D7* X *CCA1:LUC*
VF X *CDKD2* ^{Y24F}	*D7* X *CCR2:LUC*
VF X *CDKD3* ^{Y23F}	*D7* X *GI:LUC*
VF X *CDKD2* ^{Y24F}(*cdkd3-1*)	

4.1.1 Plant material

Arabidopsis thaliana (L.) Heynh plants were used for this study. All experiments were done in the ecotype Columbia (Col-0) background.

4.1.2 Soil mixture

Soil mixture for Arabidopsis cultivation, selection and propagation: 8 bags of MiniTray (70 L/bag, Balster Einheitserdewerk); added 50L water containing 800mL Osmocote Start (Scotts International BV) and 250 g BioMükk (Sautter & Stepper).

4.1.3 Plant Growing Media

0.5% MS: 2,16 g Murashige and Skoog Basal Salt (Sigma Alderich), 5 g Sucrose, pH 5.7 adjusted with 1M KOH, added H_2O up to 1L, 8 g Bactoagar,.

MS3: 4.4g Murashige and Skoog Basal Salt (MS), 30g sucrose, 5g 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7 adjusted with KOH, added H_2O up to 1L,15g Phytoagar.

Media was sterilized by autoclaving at 120^0C for 30 min.

4.1.4 Buffers for DNA work

Extraction buffer for genomic plant DNA ("Magic buffer"): 50 mL 1 M Tris-Cl pH 7.5 (for 50 mM), 60 mL of 5 M NaCl (for 300 mM), 100 g sucrose (for 300 mM), added H_2O up to 1L. Sterilized by autoclaving.

10x direct PCR buffer (with gel-tracking dyes): 24.23 g Tris (for 200 mM), 37.275 g KCl (for 500 mM), 4.07 g $MgCl_2$ hexahydrate (for 20 mM), pH 8.7 adjusted with HCl. 1.5 g/L xylene cyanol FF and 1.5 g/L Orange G added H_2O up to 1L. Sterilized by autoclaving.

10X TBE Buffer (Tris-borate): 108 g Tris and 55 g boric acid in 900 mL H_2O. pH adjusted up to 8.0 with 40 mL 0.5M Na_2EDTA, added H_2O up to 1L.

Alexander stain: 10mL of Ethanol 95%, 1mL Malachite green (1% in 95% Ethanol), 5mL Fuchsin acid (1% in water), 0.5 mL Orange G (1% in water), 5g Phenol, 5g Chloral hydrate, 2mL Glacial acetic acid, 25mL Glycerol, 50 mL dH_2O.

DAPI solution : 2.5 µg/ml DAPI in 50 mM PBS pH 7.2 with 0.01 % Tween20 and 5 % DMSO

4.1.5 Buffers used for Luciferase imaging

0.1M Triphosphate Buffer for Luciferin : 3.56g of Na_2HPO_4, 2.76g of NaH_2PO_4, pH 8.0 adjusted with Na_2HPO_4, added H_2O up to 200mL.

50 mM Luciferin stock solution: 1g of firefly D-luciferin, 71.3mL of 0.1M of the triphosphate Buffer, 100µL of 0.01%Triton X-100, up to 1L of dH_2O.

5 mM Luciferin working solution: 1.5mL of 50 mM luciferin stock, 13.5mL of Triton-X

solution. Filter sterilized.

4.2 Methods

All the bench work was performed according to Standard laboratory techniques (Sambrook and Russell, 2001).

4.2.1 Plant work

Plant growth conditions

Plants were grown on soil under 16hr light and 8hr dark conditions for crossings, pollen analysis and seed propagation. For *in vitro* analysis plants were grown under 14hr light and 10hr dark conditions on 0.5 % MS media. For periodicity assays plants were grown under 12hr light and 12hr dark conditions on MS3 media.

Seed sterilization

The seeds were sterilized in a small vacuum container. Of the different seed stocks 50 to 100 seeds were provided in opened 2 mL round- bottom micro-centrifuge tubes (eppendrof) in a desiccator or similar container, provided with a small beaker containing approx. 30 mL Bleach (FLOREAL Haagen). Under the fume hood, 3mL concentrated HCL was added and the lid immediately closed to keep the produced chlorine gas in the compartment. The chlorine produced by this reaction, was used to surface sterilize the seeds for three to four hours. The dry seeds were either directly plated or aseptically stored for further use.

Plant selection

For selection of transformed plants in T1, plants were sprayed with 0.001 % BASTA as soon as the two cotyledons were visible. Spraying of BASTA was repeated two to three times. For establishing of T2 and T3 lines seeds of from individual plants were sown on MS plates containing PPT (Phosphinothricin-N-acetyltransferase)

Crossing

At a stage when the flowers were closed and the pollen were not mature, the anthers of the receptor flowers were carefully opened and emasculated with fine forceps (Dumont). All remaining older and younger flowers were also removed. After two days the stigma of the

carpels were pollinated with pollen from the donor plant and separately bagged after the siliques had formed. Then, approx. two weeks after crossing, watering was terminated. Plants were transferred to a much warmer greenhouse where maturation takes place much faster, seeds for the F1 generation were collected, dried for a couple of days at 37°C and cleaned.

Root measurements

For root growth measurements 12-15 seeds were sown in line at equal distance, in square plates and were placed at 4°C for three to four days for stratification. The plates were then put in growth chamber vertically. Measurements of root growth rates were started one day after the germination of seeds. Each day a small scratch at the back of the plate perpendicular to the growth direction of the root marking the position of the tip was made during 10 days.

The plates were scanned on a Toshiba E scanner. The scanned images of plates were opened with image-analysis software ImageJ (rsb.info.nih.gov/ij). For calibration of the scale image of ruler taken under same settings was used. The 'Freehand' tool was used to measure the distance between two marks. Root length was measured from the root tip to the root-hypocotyl border. Three biological replicates, each containing at least 12 plants, were analysed. The mean of the root lengths of each individual experiment was determined and again averaged for three biological replicates.

Plant treatments for root growth analysis

All the experiments with various drugs were additionally performed on control plates (0.5 % MS) and control plants at the same time under the same light conditions.

For preparing media containing 1mM HU, 1000 ml of 1M HU (Sigma-Alderich) stock solution was dissolved in 1L hand warm 0.5% MS media. For plates containing media with 0.6 µg/mL Bleomycin (Duchefa), 1000 ml of 0.6 mg Bleo stock solution was added per 1L of 0.5% MS media.

4.2.2 DNA work

Genomic DNA preparation

To determine the presence of the respective T-DNA insertions, a quick method (Berendzen et al., 2005) was carried out. One or two young leaves were harvested and put into a 2 ml deep well of a 96 well storage plate (Abgene). The plant samples were treated directly with 500 µl

of DNA extraction buffer and small stainless steel beads (3.175 mm, Mühlmeier) were added to facilitate homogenisation. After closing the storage plate with collection microtube caps (Qiagen), the plant tissues were homogenised at a high frequency in the Tissue Lyser II (Qiagen) for 2-3 min. The plates were centrifuged for 3 min to spin down the liquid from the microtube caps to avoid cross contamination. For liquid transfer in case of low volumes within the storage plates used pipette tips with extended length (Starlab TipOne). For long-term storage at -20°C, used 96- well silicone sealing mats (Abgene). 1.5 µL of the suspension were directly used as a template in a 20 µL PCR reaction

Polymerase chain reaction

PCR for high-throughput genotyping was directly performed using 1.5 µl genomic DNA to make 20 µl PCR reactions. PCRs were done with homemade Taq polymerase and the following protocol

Master Mix for 10 samples	µl
H_2O	660
10x direct PCR buffer	25
2.5mM dNTPs (Promega)	25
BSA (10mg/ml)	25
PVP-40 (5%)	50
Primer 1 (100mM)	10
Primer 2 (100mM)	10
Homemade Taq	70

A detailed protocol is described in (Dissmeyer and Schnittger, 2011).

Gel-electrophoresis

20 µl pf the PCR reaction was used for gel electrophoresis with 1.5 % agarose in 0.5 X TBE. 250 bp DNA ladder was used from Invitrogen, Life Technologies.

4.2.3 Cytology and Microscopy

Flow cytometry/ Ploidy Analysis

For Flow cytometry analysis, plant material (seedlings or flower buds) were chopped up finely with a razor blade in nuclear extraction buffer (CyStain UV-precise kit by Partec

GmbH, Muenster, Germany). All preparations were filtered through a 30 µm nylon mesh and stained with nuclear staining solution (CyStain UV- precise kit by Partec GmbH, Muenster, Germany) containing 4',6- Diamidino-2-phenylindole (DAPI). Flow cytometry was performed on a Cy Flow® Ploidie Analyser (PARTEC) using the 405 nm solid state laser for excitation and a 440/40 nm band pass filter for recording of DAPI fluorescence. The ploidy level, represented by the mean peak position in a DAPI fluorescence intensity histogram, was calibrated against the 2C nuclear DNA content peak derived from a preparation of young flowers of Col-0 plants.

Pollen Size Measurements:

For pollen size measurements unopened flower buds were used to stain the pollen. Imaging of mature pollen were done using Axiophot microscope (Zeiss). For size measurement, pollen surface area was measured using ImageJ/pollen. Data was analysed using StatPlus software.

Meiotic Cell Spread:

Meiotic cell spreads were made from whole flower buds, using standard protocols (Ross *et al.* 1997). Young flower buds were collected in the morning and fixed in Ethanol +Acetic Acid mix (3:1 v/v) and stored at 4°C for 24 hours. After 24 hours mix was changed to 70% ethanol and material was stored at 4°C. A Zeiss Axiophot microscope equipped with Apotome was used for imaging. Images were analysed with ImageJ and Adobe PhotoShop.

4.2.4 Periodicity assay

Entrainment Conditions

Around 100 seeds were sterilized and plated on the MS3 media containing appropriate antibiotics. The seeds were then stratified for 3 days at 4° and transferred to the growth cabinet under light dark. For LD entrainment, the growth cabinet was set for 12 hour light and 12 hour darkness (12h:12h LD) cycles under constant 22°C. The plants were entrained for ten days under these conditions. On 10th day plants were prepared for imaging.

Bioluminescence Imaging :

Tend ays after germination seedlings were transferred to black 96-well Microplates (OPTIPLATE TM- 96F, PerkinElmer) containing 200 µl of MS3 agar. 15 µl of 5mM

Luciferin was added to each well and the plates were sealed with transparent film (Packard Topseal). Finally, each well was perforated using a needle. Plates were transferred back to their respective cabinet for an additional day of entrainment before plates were transferred to the TOPCOUNT® scintillation counter (PerkinElmer), at subjective dusk. Luminescence values were recorded as the average count of 5 second and monitored every 30-60 min for 5-6 days. When using constant-light conditions, reflector plates were placed in between the seedling plates, and an additional count delay of one minute was applied before the start of the luminescence measurements. The light source was tri-chromatic LED panels (Mark Darby, MD Electronics, UK) attached to the TOPCOUNT® stackers. A minimum of 49 plants per genotype was used for each experiment.

Period estimation:

Luminescence values obtained by TOPCOUNT were visualized using EXCEL macro TOPTEMP II (http://millar.bio.ed.ac.uk/Downloads.html). Rhythmic traces were analysed by the Biological Rhythms Analysis Software System (BRASS) macro (Southern and Millar, 2005) in EXCEL that uses the FAST FOURIER TRANSFORMATION NONLINEAR LEAST SQUARES (FFT-NLLS) method to estimate period (Plautz et al., 1997). A 90 h window starting from the beginning of the free-running condition was selected to calculate period. All period values with a weighted real amplitude error (RAE) below 0.45 were considered. R.A.E. defines the extent to which the mathematic model of FFT-NLLs analysis for a perfect curve fits to the actual data. Hence, R.A.E estimates the precision of rhythmicity ranging from 0 (a perfect oscillation) to 1 (arrhythmic oscillation).

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