

Cell cycle controls: genome-wide analysis in *Arabidopsis*

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The first decade of molecular analysis of plant cell cycle control genes revealed how well the important regulators are conserved among eukaryotes. The recent completion of the *Arabidopsis* genome sequence, and the use of increasingly sophisticated biochemical assays and genetic approaches, heralds a period of more detailed functional analysis of cell cycle regulators aimed at resolving their role in plant growth and development.

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Abbreviations

CAK	Cdk-activating kinase
Cdk	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
Cks1	Cdc28 kinase subunit
CycD1	cyclin D1
ICK1	Inhibitor of Cdk 1
SFF	SWI5 factor

Introduction

The basic mechanisms and logic of cell cycle control are highly conserved in eukaryotes, and so are the key genes that mediate cell cycle progression [1,2]. Cyclin-dependent kinases (Cdks) play a central role in mediating cell cycle progression (Figure 1). Cdk activity is regulated by association with cyclin subunits, reversible phosphorylation and association with other regulatory factors. High levels of Cdk activity alternate with high levels of proteolytic activity, which is responsible for the turnover of cyclins and cyclin-dependent kinase inhibitors. In the course of the past decade, the key molecules associated with the control of cell proliferation have been identified experimentally in plants. The majority of these are homologues of or contain domains that are homologous to yeast and animal genes that are known to have a role in the regulation of cell division. However, there is a lack of evidence for an orthologue in plants of the fission yeast Cdc25 phosphatase, an important regulator of the G₂→M transition [3]. The advent of plant genomics now provides excellent opportunities to gain understanding of the mechanisms by which individual cell cycle regulators mediate plant growth and development, and of which processes they regulate.

Recent reviews have discussed much of the latest original literature relating cell division to plant growth and development [4,5]. Here, we will focus on discussing these

advances in the context of the recent completion of the *Arabidopsis* genome and the goal of understanding the function of every (*Arabidopsis*) cell cycle gene before the end of the decade [6].

Exploiting *Arabidopsis* genomics

Plant cell cycle research has suffered from a paucity of gene-specific reagents, particularly null mutants for analysis of gene function and gene-specific antibodies for accurate biochemical analysis. Progress has now been reported in both of these areas [7•,8•]. The *Arabidopsis* genomics toolbox is filling rapidly. Several large collections of insertion mutants are now available [9–11]. Moreover, alternative and very specific methods are now available for the suppression of gene expression [12]. Much effort is being put into developing and exploiting gene arrays to monitor RNA levels on a genome-wide basis. The application of such arrays will lead to a much more comprehensive understanding of the transcriptional regulation of cell division control genes, especially when used to analyse single cells or very small cell groups within meristems [13]. Global analysis of proteins and protein modification is possible with increasingly smaller sample sizes, thereby facilitating the characterisation of the extensive post-translational controls of cell cycle genes.

Dissecting the cyclin gene family

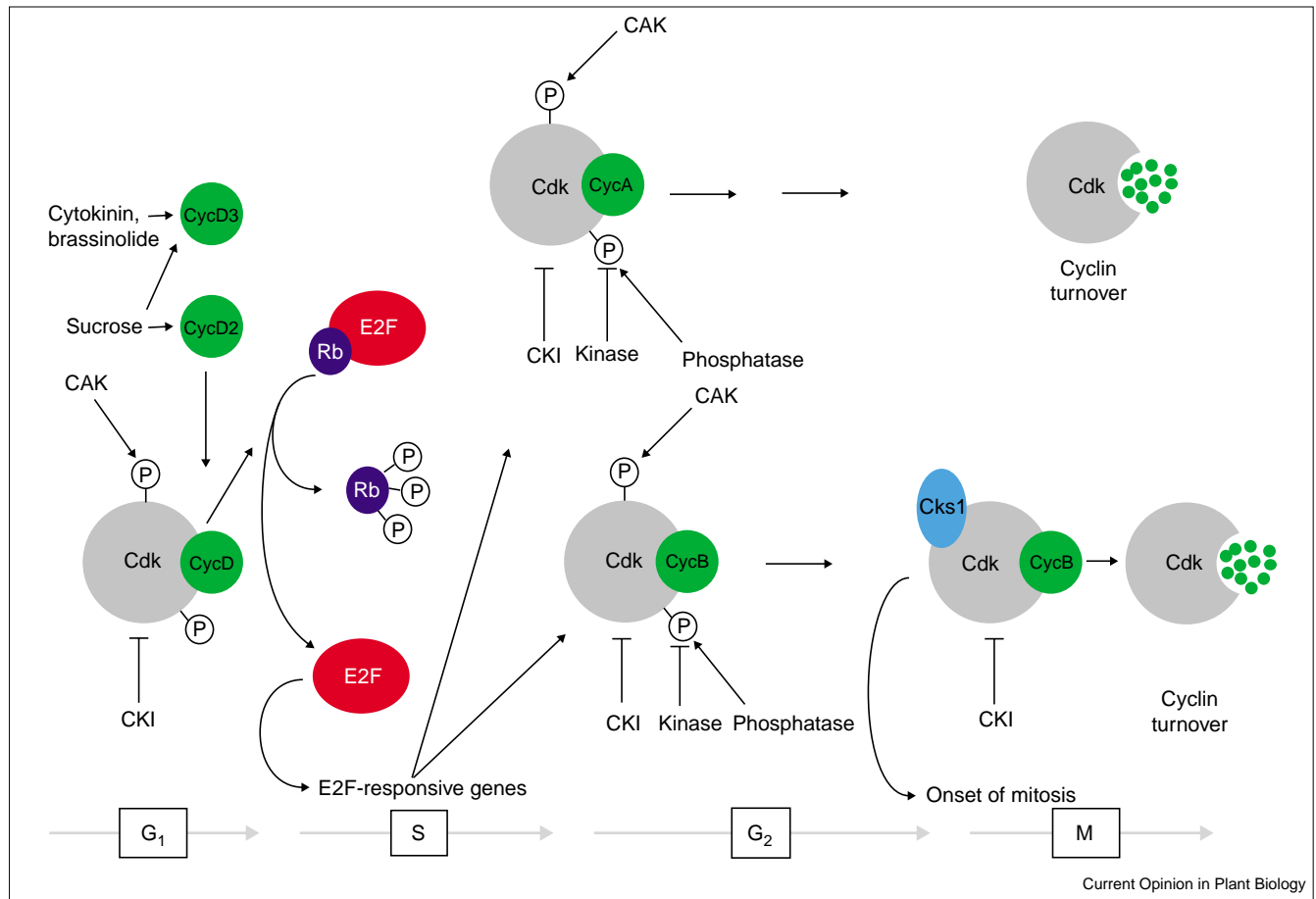
Cyclins are well conserved, and therefore have been comparatively well characterised in plants. Cyclins are essential for Cdk activation and have therefore been the prime suspects for regulators that couple control of proliferation to the multitude of environmental and developmental cues that affect growth.

Control of cyclin activity is complex. Transcriptional control plays an important role, not only for those cyclins that accumulate only during a short period of the cell cycle. Regulated proteolysis and control of sub-cellular localisation have also been shown to be important for the biological function of cyclins in yeast and other systems. A striking feature of the cyclin gene family is the degree to which it has been amplified in plants [14]: the *Arabidopsis* genome contains more than 30 cyclin genes [3]. It is unclear whether this gene amplification primarily reflects a requirement for the differential expression of individual genes with very similar biochemical function or for many genes with distinct biochemical properties. The completion of the *Arabidopsis* genome sequence [3] has provided better conditions than ever before in which to answer this question.

D-type cyclins

D-type cyclins are thought to play important roles in cell cycle responses to external cues. On the basis of phylogenetic analysis, three groups of D-type cyclins exist in

Figure 1



Model of the regulation of cell cycle control genes during the plant cell cycle. Cdk(s) associate with D-type cyclins to promote the $G_1 \rightarrow S$ transition. A crucial substrate is the Rb protein, which upon hyper-phosphorylation is unable to bind to and sequester E2F transcription factors from their target genes. The catalytic activity of the cyclin-Cdk complexes is promoted by phosphorylation by CAK. Although not clearly demonstrated in plants, it is known that inhibitory modification of cyclin-Cdk complexes occurs in animals

and yeast via Wee1 or Myt1 phosphorylation (not shown), which is counteracted by the Cdc25 phosphatase. Cyclin-Cdk complexes can also be inhibited by CKI proteins, this activity has been demonstrated in plants *in vitro* and *in vivo*. D-type cyclins are regulated by sucrose, brassinosteroid and cytokinin. During S phase, A-type cyclins begin to accumulate, joined in late G_2 by B-type cyclins. Cyclin degradation then inactivates the complex and allows the Cdk molecule to be recycled for another cell cycle.

plants: Cyclin D1 (CycD1), CycD2 and CycD3 [14]. The recently completed *Arabidopsis* genome sequence reveals the presence of seven D-type cyclins as well as several more distantly related cyclin-like genes [3].

Multiple pathways control D-type cyclin activity transcriptionally and post-transcriptionally. *CycD3;1* transcription was found to be regulated by cytokinin levels [15]. Interestingly, brassinosteroids also target *CycD3* expression [16]. These findings suggest that the control of *CycD3* expression is a major conduit for plant hormone action. Pathways controlling growth rate in plants appear to act through another D-type cyclin, *CycD2;1* [17••]. The recently described fourth type of D-cyclin [18] also belongs to the D2 group. Perhaps growth rate control is a general function of the D2 class of cyclins, which would be consistent with a proposed role for *CycD2;2* in lateral root initiation [18].

Sucrose has been shown to differentially regulate *Arabidopsis* D-type cyclin expression [19••]. The complex regulation of D-type cyclins further strengthens the notion that these molecules play a role throughout the plant cell cycle and are not restricted to controlling the $G_1 \rightarrow S$ transition. However, direct mechanistic links among environmental cues, cognate plant signalling pathways and the cell cycle machinery have not yet been established, and little progress has been reported in identifying the proteins involved in regulating cyclin abundance.

D-type cyclins also show different spatio-temporal patterns of regulation *in planta*. In snapdragon meristems, the expression of *CycD1*, *CycD3a* and *CycD3b* cyclins was shown to define distinct developmental zones and to be locally regulated by the *CYCLOIDEA* gene [20•]. Although it is not known whether the cyclin-D genes are under the direct control of

CYCLOIDEA, this is a distinct possibility as a related *TCP* (*TEOSINTE-BRANCHED1/CYCLOIDEA/PCF1*) gene is involved in controlling proliferating cell nuclear antigen (PCNA) expression [21]. PCNA is an auxiliary factor for DNA polymerase that is required in S-phase. In *Arabidopsis*, *CycD2;2* (previously known as *CycD4;1*) has been shown to be expressed during the development of vascular tissue, embryogenesis and the formation of lateral root primordia [18].

Recently, the first mutant in a core cell cycle regulator was described [7•]. Although no mutant phenotype could be detected, this approach is likely to be very productive in the future.

Substrates of D-type-cyclin–Cdk complexes

The identification of Cdk substrates has been hampered by the lack of reagents that are specific enough to distinguish between the activity of individual cyclin–Cdk complexes. The number of potential cyclin–Cdk combinations is very large, and such reagents are necessary to understand the function of individual complexes. The empirical nature of antibody generation has slowed progress in this area. Nevertheless, gene-specific antibodies have recently been used to distinguish between *CycD2*- and *CycD3*-dependent Cdk activity [8•,17•]. These two cyclins interact with the PSTAIRE-type Cdk Cdc2a [8•]. In addition, recent reports have validated novel approaches [22•,23] that, when combined with the proteomic methods now possible in *Arabidopsis*, should allow the direct identification of the substrates of protein kinases.

In mammals, the retinoblastoma (Rb) proteins are substrates of cyclin–D–Cdk complexes. Non-phosphorylated retinoblastoma proteins can associate with E2F transcription factors and thereby prevent the expression of genes under the control of E2F. Rb- and E2F-encoding genes have been found in plants, suggesting the conservation of this pathway in multi-cellular eukaryotes, although they are absent from yeast. Retinoblastoma proteins interact with plant D-type cyclins *in vitro* [24,25]. The hypothesis that the $G_1 \rightarrow S$ phase transition is controlled by cyclin–D–Cdk complexes that phosphorylate plant Rb proteins was further strengthened by the work of Chabouté and co-workers [26•]. The observation that cyclinA2 interacts with Rb [27•] suggests that continued phosphorylation of Rb in early S phase might be required to maintain E2F-dependent gene transcription.

A-type cyclins

In mammals, a single type of cyclin A is sufficient to promote Cdk activity in S and G_2 phase. The *CycA*–Cdk2 complex plays a role in DNA replication and in transcriptional regulation during S phase. In contrast, multiple A-type cyclins exist in plants. They can be grouped into three subgroups, A1–A3, according to sequence [28]. The functional significance of their complex expression patterns remains unclear [27•,29,30].

B-type cyclins

B-type cyclins are expressed specifically in late G_2 and early M phase of the cell cycle [31], and transcriptional control is crucial for regulation. In yeast, the MADS-box transcription factor *Mcm1* forms a ternary complex with *SWI5* factor (SFF) to regulate G_2/M specific expression of a cluster of genes, including the main B-type cyclins. SFF contains the transcription factors Forkhead 1 and 2, which target SFF to the appropriate promoters [32••–34••]. Forkhead expression in S phase sets up a transcription factor cascade that in succession regulates $G_2 \rightarrow M$ and $M \rightarrow G_1$ gene expression. It is yet unclear whether this ‘domino’ mode of gene regulation applies universally to the regulation of B-type cyclin expression.

The *Arabidopsis* genome contains no obvious homologues of either *Mcm1* or *Forkhead* genes, indicating that plants have developed mechanisms that differ from those in yeast to control B-type cyclin expression. Ito *et al.* [35] described an element from a periwinkle B-type cyclin promoter that is sufficient to trigger specific expression of a luciferase reporter gene in late G_2 and M. This *cis*-element has strong homology to the cognate binding site of animal *MYB* transcription factors, for which homologues were recently discovered in *Arabidopsis* [36,37] (see also Update). A similar promoter sequence from a tobacco cyclin promoter has been described as an upstream activating sequence in that promoter [38]. These reports suggest that regulation of B-type cyclin expression in plants is different from that in yeast. Sharp periodic peaks of gene activity can also be achieved by the feedback-regulated interaction of positive and negative regulatory proteins, analogous to the model proposed for the circadian regulator [39].

In plants, cyclin B1 proteins are turned over, as in other eukaryotes, via the Destruction box pathway [40,41]. Expression of a non-destructible *CycB1* in tobacco suspension cultures did not prevent exit from mitosis [41], whereas similar experiments in yeast result in an inability to exit mitosis.

Atypical cyclins

The recently described *Arabidopsis* cyclin *CycJ18* was isolated by complementation of a G_1 -cyclin-deficient yeast strain, as were *Arabidopsis* cyclins *CycD1*, *CycD2* and *CycD3*. However, *CycJ18* cannot be grouped with any previously characterised cyclin class [42]. It remains to be tested whether *CycJ18* acts as a genuine G_1 cyclin or whether it has a novel function. The *Arabidopsis* genome contains many more genes that have weak homology to cyclins.

Modulators of Cdk activity

In yeast and animals, Cdk activity is not only determined by its association with individual cyclin subunits but also depends on both stimulatory and inhibitory phosphorylation of specific amino-acid residues within the enzyme. Cdk activity is positively regulated by the Cdk-activating kinase (CAK), which is required to change the conformation

around the active site by phosphorylating a residue in the T-loop. Two protein kinases, Wee1 and Myt1, are responsible for inhibitory phosphorylation in an amino-terminal domain of the Cdk. A specific phosphatase, Cdc25, counteracts the latter phosphorylation events, resulting in the activation of Cdk. Suc1/Cks1 (Cdc28 kinase subunit) proteins bind to Cdks, thereby stimulating the ability of the Cdk complex to be reversibly phosphorylated by regulatory proteins such as Wee1 and CAK. A further class of proteins, the cyclin-dependent kinase inhibitors (CKIs), can interact with the fully assembled and active complex to suppress its kinase activity (Figure 1).

Recently a CAK from *Arabidopsis* was studied using transgenic plants overexpressing either sense or antisense transcripts of the CAK under inducible control [43]. Interestingly, both sense and antisense expression led to a decrease in Cdk activity that was followed by the differentiation of the root initial cells; subsequently, cell division activity in the root meristem was shutdown. Although these findings are not fully understood at present, they seem to indicate that CAK activity in plants affects growth control in more complex ways than had been anticipated.

A Suc1/Cks1 homologue of *Arabidopsis* was studied using overexpressing transgenic lines [44•], which showed growth inhibition. It is not yet clear whether overexpression of Cks1 leads to altered steady-state phosphorylation at the Cdk amino-terminus or whether it interferes with CAK-mediated activation of Cdks. Both mechanisms could underpin the observed effects on growth.

The CKI genes constitute a small gene family in *Arabidopsis* [3] (see also Update). The plant Cdk inhibitor *Inhibitor of Cdk 1 (ICK1)* had previously been shown to restrict Cdk activity *in-vitro* [45]; and the *in vivo* effects of overexpressing *ICK1* in *Arabidopsis* were reported recently [46•]. The observed inhibition of growth in *ICK1*-overexpressing plants confirms that this gene is involved in controlling proliferation *in planta*, and that exquisite control of proliferation is important in controlling organ morphogenesis. Perhaps such genes are involved in controlling heteroblasty of leaf organs. A second CKI, *ICK2*, has been isolated and shown to interact with the CDK Cdc2a but not with Cdc2b [47]. *ICK2* is a potent inhibitor of CDK activity *in vitro*. The *in planta* expression pattern of *ICK2* was distinct from that of *ICK1* expression, suggesting that the members of this small gene family have non-overlapping functions.

Conclusions

Recent work has begun to shed light on the complex patterns of regulation of plant cyclins. Three recent developments augur excellent opportunities for the future. First, the completion of the *Arabidopsis* genome sequence will give rise to much more rigorous experimental designs, and in the short term, will likely lead to a further concentration of efforts upon this model plant, despite its limitations. Second, the increasing number of

reports utilising gene-specific antibodies bodes well for the development of much more detailed biochemical analyses. Finally, the availability of large mutant collections will accelerate the genetic analysis of plant cell-division regulators.

Therefore, we can soon expect answers to the question of why many plant cell cycle regulator genes have been amplified to such an extent. One possible explanation is to allow for the differential expression of gene products that have very similar biochemical activity. A recently revealed example of this mechanism is the ability of two different MYB transcription factors, *WEREWOLF* and *GLABROUS1*, to functionally substitute for each other when their promoters are swapped [48]. As an alternative explanation, gene amplification also provides the starting material for the evolution of gene products with non-overlapping biochemical function. Currently, there is evidence that both hypotheses could be true for cell cycle regulators: whereas ectopic expression *CycB1;1* cannot convert endoreplication cycles into mitotic cycles, ectopic expression of *CycB1;2* can (Schnittger A, Schöbinger U, Stierhof Y-D, Steigele H, Hülskamp M, Abstract 237, 12th International *Arabidopsis* Meeting, 23–27 June 2001, Madison, WI). In contrast, the reported lack of an obvious phenotype in an enhancer trap line of *CycD3;2* [7••], suggests that *CycD3;2* functions in parallel with other D-type cyclins. Once hypomorphic alleles for cell cycle regulators are available, we will be in a better position to understand the evolutionary mechanisms driving the amplification of cell cycle regulator genes in plants.

Update

Recent work has described the isolation of three *MYB* transcription-factor-related genes from tobacco that interact with the MSA elements found in some genes that are highly expressed at the G₂→M transition [49••]. Two of these genes appear to be activators of transcription whereas the third suppresses the expression of target genes, suggesting that these genes might interact to stringently control the timing of target gene expression.

A further report now delineates the size of the cyclin-dependent kinase inhibitor gene family in *Arabidopsis* [50••], and confirms the potential of these genes to inhibit growth and affect morphogenesis *in planta*. Elevated levels of one *CKI* gene affected leaf morphology and endoreplication.

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