Plant cell cycle transitions

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Summary

Three decades have passed since the first recognition of restriction checkpoints in the plant cell cycle. Although many core cell cycle genes have been cloned, the mechanisms that control the G1-to-S and G2-to-M transitions have only recently started to be understood in plants. A central role in the regulation of the cell cycle is played by the cyclin-dependent kinases (CDKs), whose activity is steered by regulatory subunits, cyclins. The activity of the CDK/cyclin complexes are further controlled by an intricate panoply of monitoring mechanisms that result in oscillations of CDK activity during the division cycle. These fluctuations trigger the transitions between the different stages of the cell cycle.

Abbreviations

CDK	cyclin-dependent kinase
CYC	cyclin
DEL	DP-E2F-like protein
DP	dimerization partner
E2F	adenovirus E2 promoter-binding protein
KRP	Kip-related protein
MIF	mitosis-inducing factor
RB	retinoblastoma
RBR	retinoblastoma-related protein

Introduction

Several variations in the cell cycle occur in nature, but in its most common form it consists of four different phases. Although the phases of DNA replication (S phase) and mitosis (M phase) are given most attention, cells decide whether to proceed or not into the next S or M phase during the G1 and G2 gap phases, respectively. During the G1 phase, cells monitor their environment and size, whereas one of the tasks performed during G2 phase is to control whether DNA duplication has been completed. The occurrence in plants of the G1-to-S and G2-to-M checkpoints has been discovered already in 1966 by Van't Hof [1], who observed that cells in excised pea root tips stopped dividing and became arrested in the G1 and G2 phase upon carbohydrate starvation. Four years later, the potential of starved cells to resume cell division by re-addition of sucrose was found to depend on RNA transcription and protein synthesis [2], suggesting that the capacity to progress through the cell cycle relies on a molecular identity. Ten years later, Sir Paul Nurse identified the *cdc2* gene of fission yeast, whose gene product was genetically proven to be required to progress through both the G1-S and G2-M transition points [3], a finding rewarded with last year's Nobel Prize. Over the years, *cdc2* has been shown to be part of a evolutionary conserved group of protein kinases whose activation depends on the association with regulatory cyclin subunits, hence their name, "cyclin-dependent kinases" (CDKs).

The first clue suggesting that CDK/cyclin complexes also govern plant cell division came from the discovery of a plant protein related to animal CDKs [4] and the cloning of the first plant CDK-encoding cDNA [5]. Since then, plant homologues of different core cell cycle genes have been

cloned progressively. Nevertheless, although the availability of the complete genome sequence of *Arabidopsis thaliana* allowed us to identify all core cell cycle genes [6•], data on how the G1-to-S and G2-to-M transitions are regulated in plants are only fragmentary and knowledge is lacking on how internal and external signals impinge on these transitions. Here, we briefly overview the most recent findings concerning the mechanistic regulation of the G1-to-S and G2-to-M transitions in plants. Reviews on mitotic exit and endoreduplication in plants, two other important aspects of the cell cycle, have been published by Criqui and Genschik [7••] and Larkins *et al*. [8], whereas the impact of hormones on cell cycle progression has been reviewed by Stals and Inzé [9].

The G1-to-S transition

Regulation of S phase entry through phosphorylation of retinoblastoma-related proteins

Impressive progress has been achieved during the last years on the study of S-phase entry in plants (Figure 1). The mechanism that regulates the G1-to-S transition appears to be conserved between mammals and plants. In mammals, G1-S entry is initiated by the synthesis of D-type cyclins upon mitogenic stimulation by serum-type growth factors. Complexed with specific CDKs, D-type cyclins set off the phosphorylation of the retinoblastoma tumor suppressor protein (RB), a key regulator for the start of DNA replication [10]. In non-phosphorylated state RB binds tightly to the heterodimeric E2F-DP transcription factor, which is required for the transcriptional activation of cell cycle and DNA

synthesis genes. By RB binding, the transcriptional activation domain of the E2F-DP factor is masked, rendering it inactive. Moreover, recruitment by RB of DNA-modifying enzymes, such as histone deacetylases and polycomb proteins, can repress promoter activity of E2F-DP target genes, with chromatin condensation as a result. RB phosphorylation counteracts the inhibitory function of RB and releases transcriptionally active E2F-DP and consequentially onsets DNA replication.

The presence of an RB-mediated pathway in plants has been supported in the early days by two lines of evidence: the existence of D-type cyclins [11, 12] that contain an LxCxE motif, known to mediate RB binding in human cells and the identification of proteins in geminiviruses that also contain the LxCxE motif required for efficient viral replication in cultured cells [13]. In accordance, RB-related (*RBR*) genes were first discovered in maize and later in other plant species [6•, 14-16]. Plant RBR has been shown to be phosphorylated by A-type CDKs bound to D-type cyclins [17, 18••]. This phosphorylation occurs in a cell cycle-dependent manner, reaches its maximum at the G1-S transition, and remains high until mid/late S phase. In analogy with mammalian systems, this phosophorylation results very probably in an inactivation of RBR, releasing E2F-DP transcription factors in their active form, albeit still to be experimentally proven. CYCD3 cyclins seem to be a rate-limiting factor is this process, because overexpression of *Arabidopsis CYCD3;1* and *Nicotiana tabacum CYCD3;3* stimulates cells to exit the G1 phase [18••, 19••]. On the flipside, Kip-related proteins (KRPs), which are inhibitors of CDK activity [20, 21] might prevent phosphorylation of RBR by inactivating CYCD3-containing complexes. This hypothesis is substantiated by the observation that overproduction of a tobacco KRP protein (NtKIS1)

in *Arabidopsis* completely complements the severe phenotype induced by overproduction of CYCD3;1 [22•]. Also, in trichomes overexpression of a *KRP* gene is able to restore the phenotype induced by *CYCD3;1* overexpression [23••].

Besides D-type cyclins and KRPs, more players are involved in the regulation of RBR activity. Phosphorylation of CYCD3;1 is required for both full kinase activity and localisation in the nucleus, suggesting a still unknown kinase working upstream of CYCD3;1 [18••]. Moreover, overexpression of the CDK-activating kinase CDKD (R2) from rice accelerates S-phase entry [24•], This quickened S-phase entry might occur through the activation of CDKA/CYCD complexes, more rapidly inactivating RBR. Alternatively, CDKD might operate in an RBR-independent manner through the phosphorylation of the C-terminal domain of RNA polymerase II, a known substrate of CDKD [25], which in turn might activate the transcription of specific genes required for S-phase progression.

The E2F-DP gene family

In mammals, six E2Fs exist that can be classified into three groups: the activating E2Fs (E2F1-3), the repressive E2Fs (E2F4-5), and E2F6 [26]. The activating E2Fs function as potent transcriptional activators of E2F-responsive genes and overproduction of one of them is sufficient to drive serum-starved cells into the cell cycle. The repressive E2Fs are mainly found in quiescent cells and are believed to be important in the control of cell cycle exit and induction of terminal differentiation through the recruitment of RBRs to E2F targets genes. E2F6 is characterized by its lack of a transcriptional activation and RB-binding

domain and is believed to function as a competitive inhibitor for E2F-binding sites in an RB-independent manner. All E2Fs have in common that they need to dimerise with DP proteins to bind with high specificity to the E2F-binding sites [27-29].

Plant *E2F* and *DP* genes have been identified in plants [30]. In the *Arabidopsis* genome, three *E2F* genes (*E2Fa*, *E2Fb*, and *E2Fc*) and two *DP* genes (*DPa* and *DPb*) are present [6•] and all display a domain organization similar to those of their mammalian counterparts. In particular, the highly conserved DNA-binding domain suggested that the plant E2F-DP factors bind the same DNA sequence motifs as the animal E2Fs. Indeed, electrophoretic mobility shift experiments showed that plant E2Fs bind to the same E2F-binding site as mammalian E2Fs [31]. Moreover, E2F-binding sites have been mapped in the promoters of several genes [32-35]. An *in silico* search in the *Arabidopsis* genome for genes harbouring the TTTCCCGCC *cis*-acting element in their promoter identified 183 potential E2F target genes, among which genes involved in DNA replication, cell cycle regulation, transcription, stress, and defence or signalling [36]. Many of these and other genes were indeed found to be up-regulated in *Arabidopsis* plants overproducing the E2Fa-DPa transcription factor (K. Vlieghe, D. Inzé, and L. De Veylder, unpublished data).

Both *E2Fa* and *E2Fb* have been shown to be potent transcriptional activators when coexpressed with *DPa*, suggesting that they belong to the class of activating E2Fs [37-39]. Plants overexpressing the *E2Fa* gene display ectopic cell divisions in all tissues examined [40••]. Extra cells arise because of a delay in cell differentiation. The E2Fa

phenotype was strongly enhanced by the co-expression of *E2Fa* with *DPa*, which can be explained by the ability of DPa to target E2Fa to the nucleus [37]. Whereas *DPa*-overexpressing plants have a normal phenotype, *E2Fa-DPa* transgenic plants have curled leaves along their proximal-distal axis, which correlates with the occurrence of many additional cell divisions [40••]; such a phenotype is highly reminiscent to that of *CYCD3;1* overexpression [19••] and suggests that both E2Fa-DPa and CYCD3;1 function in the same pathway. Nevertheless, also differences exist: in *E2Fa-DPa* transgenic plants, cells are promoted to enter the S phase, driving mitotic cells into another cell cycle and more differentiated cells into the endocycle; this phenotype has been hypothesized to depend on the presence or absence of a mitosis-inducing factor (MIF) [40••]. In contrast, in the *CYCD3;1* transgenic plants, endoreduplication is strongly inhibited, which might be explained by the strong capacity of CYCD3;1 to inhibit cell differentiation [19••].

In certain cases, an E2F promoter element contributes negatively to gene expression, as illustrated for the *PCNA*, *MCM3*, and *RNR2* genes [33, 35, 41•,42]. This negative function is most probably performed by E2Fc that lacks a strong transactivation domain [38]. Therefore, E2Fc might have a function related to that of mammalian E2F4-5, which acts as a repressor of the E2F-DP target genes by recruiting the RBR protein to promoters. This hypothesis is substantiated by the observation that the overproduction of a stable form of E2Fc negatively affects cell division and is correlated with a decrease in expression of *CDC6* gene, a known E2F-DP target gene. Interestingly, the E2Fc protein is degraded rapidly in an ubiquitin-dependent manner after being phosphorylated by CDK/cyclin complexes [43••].

Plants seem to have evolved to use an additional mechanism for modulating the expression of E2F-responsive genes. In the *Arabidopsis* genome, three genes have been identified with a high internal sequence similarity to both E2F and DP, and were, therefore, designated DP-E2F-Like (DEL; [6•]. Sequence similarity is restricted to the DNA-binding domain; surprisingly, this domain is present in tandem, allowing the DELs to bind to the canonical E2F-binding site as a monomer [38,44]. Because of the lack of a transcriptional activation domain, the DEL proteins are postulated to act as repressors of E2F-regulated genes by competing with E2F-DP for the same binding sites. Indeed, in a competition assay, *DEL* expression antagonizes the transcriptional activation by E2F-DP proteins [38, 44]. The expression of *DEL1* and *DEL3* before the transcription of S-phase genes suggests that the DEL proteins might occupy the E2F sites during G1, thereby repressing gene transcription [38]. Whether the DEL proteins can repress gene transcription via the recruitment of DNA-modifying enzymes, such as histone deacetylase and polycomb proteins, and how the E2F-DP proteins at the G1-S transition replace the DELs remain to be determined.

The G2-to-M transition

Although recent data have strongly expanded our understanding of the molecular events controlling S-phase entry in plants, regulation of entry into mitosis is still poorly understood (Figure 2). In mammals and insects, the G2-to-M transition is specifically regulated by CDKs that associate with A- and B-type cyclins. A-type cyclins are produced and degraded earlier in the cell cycle than B-type cyclins and provide

distinct and non-redundant functions in cell cycle progression. In plants, kinase activity assays suggest that two types of CDKs (A and B) control the G2-M transition [45, 46], where the B-type CDKs most probably regulate plant-specific features of the cell cycle, because no counterparts of these particular CDKs can be recognized in other species. When grown in the dark, transgenic *Arabidopsis* plants containing an antisense *CDKB1;1* construct display a short hypocotyl and open cotyledons. This phenotype is related to reduced cell size rather than cell number. In addition, cotyledons of antisense lines fail to green when transferred from dark to light, a failure attributed to the conversion of etioplasts to amyloplasts. These data indicate a role for CDKB1;1 in hypocotyl cell elongation and cotyledon cell development [47]. We found that *CDKB1;1* expression is highly specific to stomata and stomatal precursors of cotyledons, suggesting a prominent role for CDKB1;1 in stomata development as well. In accordance, transgenic *Arabidopsis* plants with reduced B-type CDK activity have a decreased stomatal index and aberrant guard cells, which are blocked in their G2 phase (V. Boudolf, D. Inzé, and L. De Veylder, unpublished data).

Based on their peak of transcription at G2 and M phases, both A- and B-type cyclins are probably responsible for the mitotic events. Ectopic expression of alfalfa *CYCB2;2* and *Arabidopsis CYCB1;2* cyclins were indeed shown to stimulate G2-to-M transition [48, 49]. Plant B-type cyclin promoters contain a common *cis*-acting element, called the M-specific activator (MSA) element, which is necessary and sufficient for the periodically specific promoter activation. This motif was also identified in the promoter of a G2-M-specific gene that encodes a kinesin-like protein, *NACK1*, suggesting that a defined set of G2-M-specific genes are

co-regulated by a common MSA-mediated mechanism in plants [50-52••]. In a search for MSA-binding factors, three different Myb-like proteins were identified, NtmybA1, NtmybA2, and NtmybB. The latter one is expressed constitutively during the cell cycle, whereas the two others are produced specifically at the G2-to-M transition, when B-type cyclin genes are transcribed. Furthermore, NtmybB is a repressor of MSA-containing promoters, whereas NtmybA1 and NtmybA2 are activators. Hence, plants, like animals, contain Myb proteins with a role in cell proliferation; however, plant Myb proteins seem to be involved in G2/M transition whereas mammalian ones in G1/S transition [52••].

Accumulation of mitotic cyclins is a gradual process, whereas the onset of mitosis is an abrupt and dramatic change. Hence, the CDK/cyclin complex activity is mainly achieved by changes in the phosphorylation state of the kinase. In yeast and mammals, the CDK/cyclin activity is promptly regulated by the dephosphorylation of a single tyrosine 15 (yeast) or both tyrosine 15 and threonine 14 (higher eukaryotes) at the G2-to-M boundary. The CDK/cyclin complexes formed during S and G2 phase are maintained in an inactive form through inhibitory phosphorylation of these conserved residues by a family of kinases, designated WEE1 (WEE1, MIK1, and MYT1). The phosphorylation of Tyr15 and Thr14 of the CDK subunit inhibits ATP fixation and blocks substrate binding, preventing protein kinase activation for the remainder of the interphase. A homologue to WEE1 has been characterized in maize and *Arabidopsis* [53, 54]. Overexpression of plant *WEE1* genes in *Saccharomyces pombe* inhibit cell division. Interestingly, purified WEE1 can inhibit $p13^{Suc1}$-absorbed mitotic CDK activity. Furthermore, *WEE1* transcripts are more abundant in actively dividing tissues and in maize endosperm during the

endoreduplication period, suggesting that inhibition of mitotic CDK activity by WEE1 could be one of the mechanisms inhibiting G2-to-M transition and activation of the endocycle [53].

The dephosphorylation of Thr14 and Tyr15, accompanied with the activation of the CDK/cyclin activity at the G2/M transition is achieved by a dual specificity phosphatase CDC25. No homologue of CDC25 could be identified so far in the *Arabidopsis* genome. Nevertheless, the presence of the antagonistic WEE1 kinase and accumulating biochemical data as well suggest the existence of a CDC25-like regulation of CDKs in plants. First, tobacco cell suspension that is arrested in G2 by cytokinin starvation can resume mitosis by inducible expression of the yeast *cdc25* [55]. Secondly, overexpression of the *S. pombe cdc25* gene in tobacco plants leads to an increase of lateral root primordium formation and a new threshold size for cell division in the primordia [56, 57]. Furthermore, microinduction techniques recently have been used to locally and transiently manipulate the expression of the yeast *cdc25* in tobacco plants [58], clearly demonstrating that the local expression of *cdc25* on the flanks of young leaf primordia leads to changes in cell division patterns. Probably, the *CDC25* gene undergoes such dramatic changes over evolution that it no longer can be recognized as a *CDC25* phosphatase in the *Arabidopsis* genome.

Conclusions

Although our understanding increases on how cell cycle transitions are regulated we still have no clue how the different transitions are communicating to each other. For budding yeast, the combination of

chromatin immunoprecipitation assays with DNA microarray analysis revealed that proteins that operate as activators during one stage of the cell cycle can contribute to the transcriptional activation of proteins that function during the next stage, forming a fully connected regulatory circuit [59]. For example, activators of the G1-to-S transition control the expression of G1-S cyclin genes, but also regulate the expression of the G2-M-specific cyclin *clb2*, which subsequently inhibits further expression of the G1-S cyclin genes and promotes entry into mitosis. The observation that overexpression of the *CYCD3;1* not only triggers S-phase entry, but also activates expression of the mitose-specific genes *CYCB1;1* and *CYCB1;2*, suggests that similar regulatory networks might exist in plants as well [60••]. In concert, plants that display ectopic cell division as a result of *CYCD3;1* or *E2FA-DPa* overexpression have increased levels of negative regulators of the E2F-RBR pathway [19••; K. Vlieghe, D. Inzé, and L. De Veylder, unpublished data]. First steps have been undertaken to unravel this genetic network by the identification of cell cycle-modulated genes in *Arabidopsis* and tobacco BY2 cells [61•, 62•]. Both data sets resulted in the identification of a large group of genes with a still unknown function. The major future challenge will be to design experiments that can help us understand how the already known cell cycle genes interact with each other, to assign functions to proteins of the newly discovered genes, and to identify the proteins that operate together to carry out particular processes.

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extracts. Depending on the developmental stage of the plant, the E2F sites contribute either to gene expression or repression.

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The authors demonstrate that the *cis*-acting M-specific activator (MSA) element in plant promoters is necessary and sufficient for directing G2-M-specific gene expression. Via a one-hybrid approach, three genes were isolated, encoding MYB proteins (MybA1, MybA2, and MybB), which bind to the MSA element. MybA1 and MybA2 activate MSA-containing promoters, whereas MybB represses them.

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Ectopic expression of *CYCD3;1* is demonstrated to promote S phase entry. Surprisingly, overexpression also triggers muticellularisation of trichomes that is accompanied with transcriptional induction of M-phase-specific genes, such as *CYCB1;1* and *CYCB1;2*. These data provide evidence that overexpression of *CYCD3;1* is able to trigger the G2-to-M transition.

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The authors describe the identification of cell cycle-regulated transcripts using a synchronizable *Arabidopsis* cell line and microarrays. The high number of cell cycle-regulated genes demonstrate the integration

of the plant cell cycle with many cellular pathways.

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Tobacco BY-2 cells are highly synchronizable and, as such, well suited to identify cell cycle-regulated transcripts. The authors of this paper used cDNA-AFLP-based transcript profiling to build a comprehensive inventory of plant cell cycle-modulated genes. Approximately 10% of all visualized transcripts were found to be expressed periodically.

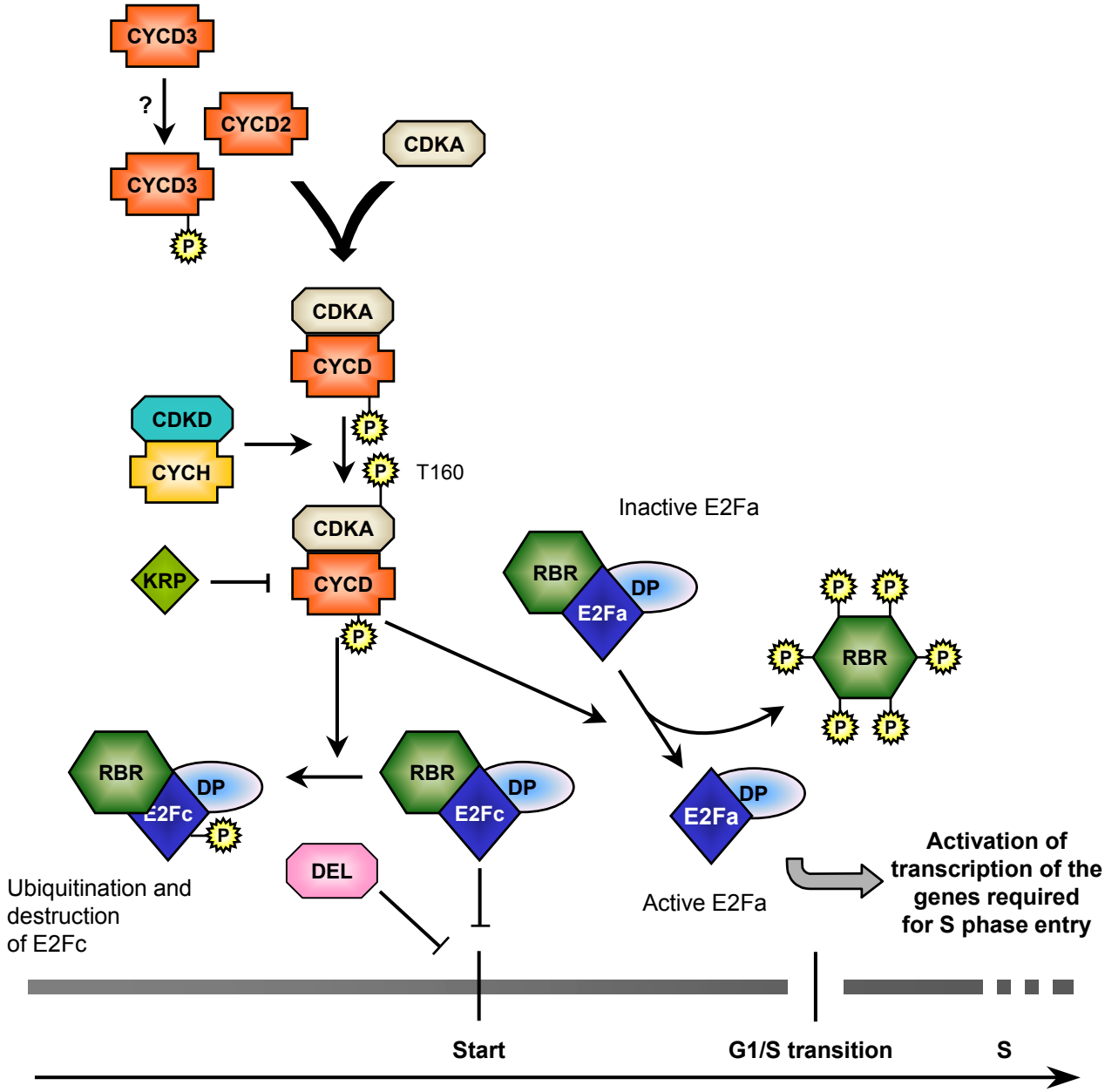
LEGEND TO FIGURES

Figure 1. Schematic overview of how the G1-to-S transition is mechanistically regulated in plants. Pathways that are still not fully experimentally demonstrated are marked by question marks.

Figure 2. Schematic overview of how the G2-to-M transition is mechanistically regulated in plants. Question marks indicate pathways that are still not fully experimentally demonstrated.

Teaser: Many cell cycle genes have been cloned years ago, but only now we start to understand how they all cooperate to drive the cell cycle.

Keywords: CDK, cell cycle, cyclin, DNA replication, E2F, mitosis, restriction point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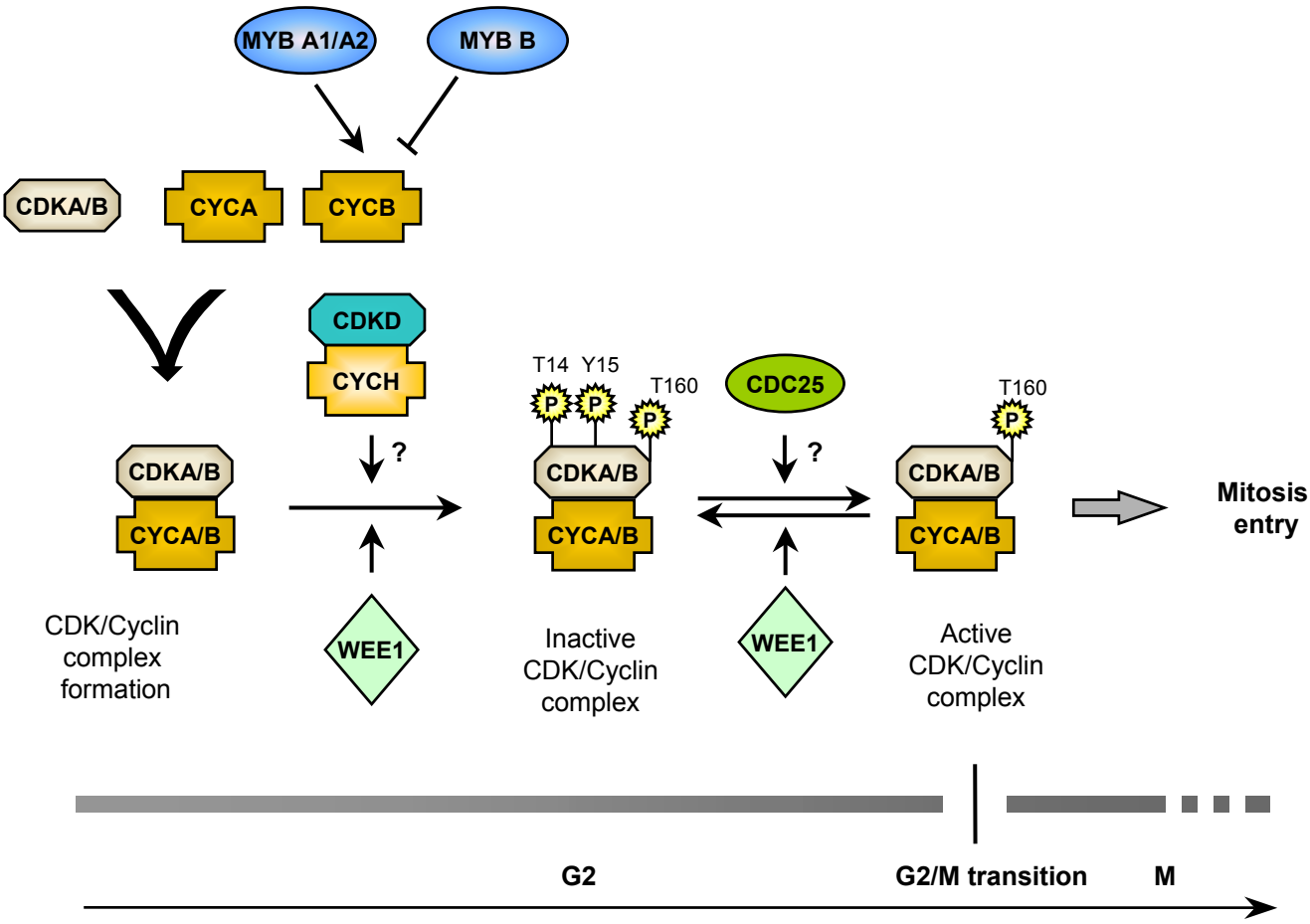
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