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The Role of the Anaphase-Promoting Complex/Cyclosome in Plant Growth

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The anaphase-promoting complex/cyclosome (APC/C) is a multi-subunit E3 ubiquitin ligase that plays a major role in the progression of the eukaryotic cell cycle. This unusual protein complex targets key cell cycle regulators, such as mitotic cyclins and securins, for degradation via the 26S proteasome by ubiquitination, triggering the metaphase-to-anaphase transition and exit from mitosis. Because of its essential role in cell cycle regulation, the APC/C has been extensively studied in mammals and yeasts, but relatively less in plants. Evidence shows that, besides its well-known role in cell cycle regulation, the APC/C also has functions beyond the cell cycle. In metazoans, the APC/C has been implicated in cell differentiation, disease control, basic metabolism and neuronal survival. Recent studies also have shed

light on specific functions of the APC/C during plant development. Plant APC/C subunits and activators have been reported to play a role in cellular differentiation, vascular development, shoot branching, female and male gametophyte development and embryogenesis. Here, we discuss our current understanding of the APC/C controlling plant growth.

Keywords Arabidopsis, cell cycle, plant development, ubiquitin-ligase

I. INTRODUCTION TO THE PLANT CELL CYCLE

The cell cycle, the sequence of events comprising DNA replication, cell division and growth, is composed of distinct phases. Replication of nuclear DNA happens in the so-called Synthesis or S-phase, followed by the physical process of cell division referred to as Mitosis or M-phase. S and M are separated by two gap phases, G1 and G2, in which cells prepare for replication and division, respectively. Not surprisingly, the cell cycle machinery is strongly regulated at different points to ensure the fidelity of chromosome duplication and cell division. Highly conserved control mechanisms, known as checkpoints, verify whether or not the cell cycle process has been accurately completed at each phase prior to progression into the next phase (De Veylder *et al.*, 2007). Besides the normal cell division, cells can also undergo a different type of cell cycle, known as endoreduplication or endocycle, which consists of one or several rounds of DNA synthesis without subsequent entry into mitosis (Edgar and Orr-Weaver, 2001; Breuer *et al.*, 2010; De Veylder *et al.*, 2011).

Both the G1-to-S and G2-to-M transitions require the activity of CYCLIN-DEPENDENT KINASEs (CDKs). CDK activity is regulated at multiple levels, such as by association with specific regulatory subunits, called cyclins; by phosphorylation and dephosphorylation; by interaction with inhibitory proteins; and by targeted proteolysis (Inzé and De Veylder, 2006; Sullivan and Morgan, 2007). Eukaryotes use the ubiquitin-dependent proteolysis system to control the abundance of the cyclins and CDK inhibitors (CKIs) (Genschik *et al.*, 1998; Criqui *et al.*, 2000; Jun *et al.*, 2013).

In plants, two major classes of CDKs, known as CDKA and CDKB, directly drive the cell cycle transitions (Boudolf *et al.*, 2004; Iwakawa *et al.*, 2006). A-type CDKs are most closely related to the mammalian CDK1 and CDK2, which contain the evolutionary conserved PSTAIRE motif in their cyclin-binding domain, and regulate both the G1-to-S and G2-to-M transitions. One of the best characterized CDKA substrates is RETINOBLASTOMA RELATED (RBR), a protein that represses the activity of the heterodimeric transcription factor complex E2F/DP (Kuwabara and Gruissem, 2014). The E2Fs represent an important class of transcription factors that retain a conserved DNA-binding domain. Usually, E2F proteins associate with its dimerization partner (DP) protein to form a heterodimeric complex that binds to the promoter of a number of target genes required for multiple processes during the cell cycle (Lammens *et al.*, 2009). In contrast to A-type CDKs, B-type CDKs have a divergent cyclin-binding domain and control the G2-to-M transition (Hemerly *et al.*, 1995; Porceddu *et al.*, 2001; Endo *et al.*, 2012; Nowack *et al.*, 2012).

In order to become active, CDKs must associate with their regulatory partners, the cyclins. In mammals, there are four main types of cyclins that control the cell cycle transition, which are designated A, B, D and E-type cyclins. The entry into the S-phase is under control of D- and E-type cyclins, progression through the S-phase depends on A- and E-type cyclins, whereas the M-phase is coordinated by A- and B-type cyclins (Bloom and Cross, 2007). In Arabidopsis, ten A-, nine B-, and ten D-type cyclins have been described (Vandepoele *et al.*, 2002; Komaki and Sugimoto, 2012). The A-type cyclins are mainly produced from the onset of the S-phase until the middle of G2, the B-type cyclins specifically from G2 until the end of mitosis, while the D-type cyclins mainly operate at the G1-to-S transition (Menges *et al.*, 2005; Inzé and De Veylder, 2006; Menges *et al.*, 2006; Van Leene *et al.*, 2010; Harashima *et al.*, 2013).

The activity of CDK/cyclin complexes is also negatively regulated by CKIs. Based on structural and biochemical features, the mammalian CKIs belong to two very distinct classes. The Inhibitors of CDK4 (INK4) family is characterized by the presence of multiple ankyrin repeats and selectively inhibit G1-specific CDKs, and the Kinase Inhibitor Protein (KIP)/CDK Inhibitor Protein (CIP) family binds and inhibits a broader range of CDKs involved in the control of the G1-to-S transition (Sherr and Roberts, 1999). In plants, no homologs of the mammalian INK4 class of inhibitors have been identified. However, plants encode multiple KIP/CIP-related proteins, known as INHIBITOR OF CDK (ICK) or KIP/CIP RELATED PROTEIN (KRP). Furthermore, plants contain a unique class of CKIs, denominated SIAMASE (SIM) and SIAMESE RELATED (SMR), not found in animals (Churchman *et al.*, 2006; Peres *et al.*, 2007; Yi *et al.*, 2014).

The Arabidopsis genome encodes seven ICK/KRP proteins that all share a short amino acid motif with mammalian KIP/CIP proteins (De Veylder *et al.*, 2001; Torres Acosta *et al.*, 2011). Plants have A-type CDKs associated with A-type or D-type cyclins that are bound and inhibited by ICK/KRP proteins (Lui *et al.*, 2000; Jasinski *et al.*, 2002; Zhou *et al.*, 2002; Nakai *et al.*, 2006; Cheng *et al.*, 2013; Jégu *et al.*, 2013; Wen *et al.*, 2013). Moreover, in agreement with their function as inhibitors of the cell cycle, constitutive overexpression of *ICK1/KRP1, ICK2/KRP2, ICK4/KRP6* or *ICK7/KRP4* results in dwarfed plants with a reduced cell number and organ size (Wang *et al.*, 2000; De Veylder *et al.*, 2001; Zhou *et al.*, 2003; Bemis and Torii, 2007). Knockout mutants of single *ICK/KRP* genes do not show any phenotype, with the exception of the *ick2/krp2* mutant that produces more lateral roots (Sanz *et al.*, 2011). Compared with wild-type plants, the quadruple (*ick1/krp1, ick2/krp2, ick6/krp3, ick7/krp4*) and quintuple (*ick1/krp1, ick2/krp2, ick5/krp7, ick6/krp3, ick7/krp4*) mutants have larger cotyledons; narrower, curled downwards

and larger leaves; and larger petals and seeds. Furthermore, these quadruple and quintuple mutants have more, but smaller, cells in all organs examined. The quintuple mutant also has increased fresh and dry weights (Cheng *et al.*, 2013). CDK activity gradually increases when more *ICK/KRP* genes are down-regulated and this effect is exerted post-transcriptionally (Cheng *et al.*, 2013).

ICK/KRPs also play an important role in the control of endoreduplication. Transgenic plants overexpressing *ICK6/KRP3* have a higher DNA ploidy level in the shoot apical meristem (SAM) and leaves (Jun *et al.*, 2013). Overexpression of *ICK2/KRP2* was reported to have a dosage-dependent effect on the onset of endoreduplication, triggering the mitosis-to-endocycle transition during leaf development (Verkest *et al.*, 2005).

In Arabidopsis, the SIM/SMR family of CKIs comprises 14 members (Yi *et al.*, 2014). These proteins share a small peptide domain of six amino acids with the ICK/KRP inhibitors, which corresponds to the cyclin-binding motif (Peres *et al.*, 2007). The SIM protein has a central role in the establishment of endoreduplication during trichome development (Churchman *et al.*, 2006). Moreover, the SIM protein interacts with D-type cyclins, as well as with CDKA, and inhibits the kinase activity of CDKA/CYCD complexes (Churchman *et al.*, 2006; Peres *et al.*, 2007; Yang *et al.*, 2011; Yi *et al.*, 2014). In addition to SIM, SMR1 and SMR2 specially copurify with CDKB1;1, whereas SMR4 and SMR5 exclusively copurify with the A-type CDK and D-type cyclins (Van Leene *et al.*, 2010). Recently, for SMR4, SMR5 and SMR7, the cell cycle inhibitory activity was confirmed through the analysis of overexpressing plants, which show reduction in rosette size, serrated leaves, fewer cells with increased cell size, which are all phenotypes characteristic for inhibition of cell division. Furthermore, *SMR5* and *SMR7* knockout plants display an impaired DNA damage checkpoint in leaf cells upon treatment with the replication inhibitory drug hydroxyurea (HU). Moreover, both SMR5 and SMR7 regulate the DNA damage checkpoint in response to reactive oxygen species (Yi *et al.*, 2014).

Protein degradation mediated by ubiquitin is a primary mechanism by which changes in the cell cycle state are achieved. The ubiquitination reaction is a multi-step enzymatic cascade that tags substrates with ubiquitin chains. First, in an ATP-dependent reaction, the E1 activating enzyme binds to and activates an ubiquitin molecule. Then, the ubiquitin molecule is transferred to one of the E2 ubiquitin-conjugating enzymes that, together with the E3 ubiquitin ligase enzyme, transfers the ubiquitin molecule onto a lysine residue of the target protein. This reaction is repeated several times, forming a chain of ubiquitin molecules on the substrate, which will be recognized by the 26S proteasome for subsequent degradation (Hershko, 1997; Peters, 2006). E3 ubiquitin ligases are key components of ubiquitination pathways, because they determine the substrate specificity of the ubiquitination reactions by recruiting the appropriate E2 ubiquitin-conjugating enzyme and possibly also by contributing to E2 activity (Peters, 2006; Van Voorhis and Morgan, 2014). The two central E3 ubiquitin ligases involved in cell cycle regulation are the SKP/CUL/RBX/F-box (SCF) protein complex and the anaphase-promoting complex/cyclosome (APC/C) (Vodermaier, 2004; Genschik *et al.*, 2014).

II. THE SCF E3 LIGASE

The SCF complex is a multi-subunit E3 ubiquitin ligase composed of four components: the S-phase Kinase-Associated Protein SKP1, the cullin-related subunit CUL1, the RING-BOX protein RBX1, and an F-box protein (Figure 1a). Because the F-box protein determines substrate specificity, many different SCF complexes can be distinguished on the basis of the associated F-box protein. The F-box protein forms, together with SKP1, the substrate recognition module (Cardozo and Pagano, 2004). In many cases, the interaction of

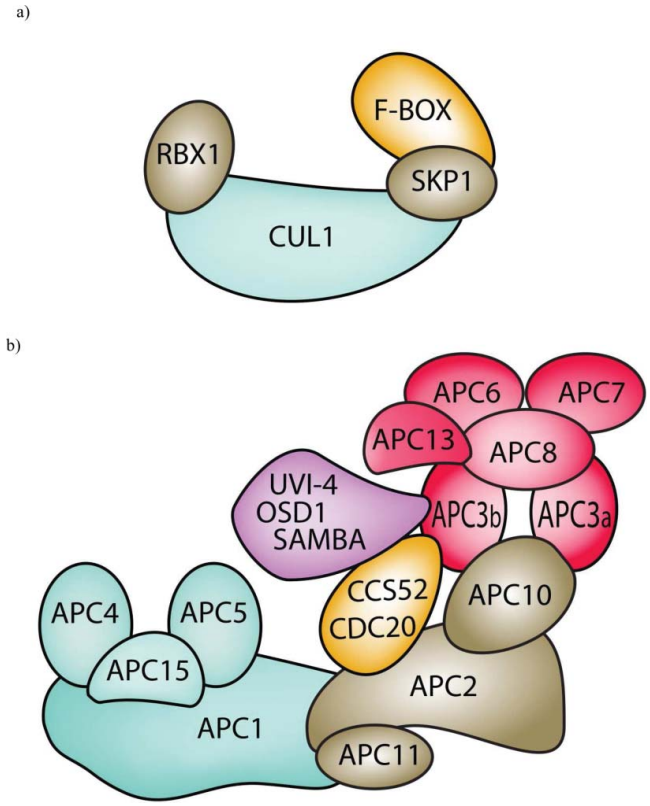

FIG. 1. The two main E3 ubiquitin ligases controlling the cell cycle. a) Schematic representation of the structure of the SCF complex based on the 3D crystal structure (Cardozo and Pagano, 2004). b) Schematic representation of the APC/C complex. The scheme is based on a high-resolution structure using single-particle electron microscopy (EM) of the complex (da Fonseca *et al.*, 2011) and experimentally confirmed interactions of the APC/C subunits from Arabidopsis using two-hybrid interactions (Eloy *et al.*, 2006; Heyman *et al.*, 2011). Green color: platform module; red: structural module; brown: catalytic and substrate recognition module; orange: activators subunit; purple: plant-specific APC/C interactors.

the substrate with the F-box protein depends on the phosphorylation state of the SCF substrate, indicating that substrate modification and F-box protein availability are the most critical steps in SCF-dependent proteolysis (Orlicky *et al.*, 2003; Vodermaier, 2004; Skaar *et al.*, 2013). The Arabidopsis genome encodes about 700 F-box proteins, which implicates that plants have the capacity to create a multitude of F-box containing complexes, possibly controlling the stability of hundreds of substrates involved in numerous biological processes (Gagne *et al.*, 2002; Lechner *et al.*, 2006). During the cell cycle, SCF mainly regulates the G1-to-S transition by degrading CKIs, such as SIC1 in *Saccharomyces cerevisiae* (Dirick *et al.*, 1995), KIP1 in humans (Pagano *et al.*, 1995) and ICK/KRP in plants (Verkest *et al.*, 2005; Ren *et al.*, 2008; Jun *et al.*, 2013). In Arabidopsis, it has been shown that degradation of ICK1/KRP1, ICK2/KRP2, ICK4/KRP6, ICK5/KRP7 and ICK6/KRP3 by the 26S proteasome is mediated by the SCF complex (Verkest *et al.*, 2005; Jakoby *et al.*, 2006; Kim *et al.*, 2008; Jun *et al.*, 2013).

In Arabidopsis, several SCF-associated F-box proteins have been identified. The best studied are SKP2A and SKP2B, which are similar to the metazoan SKP1 and SKP2 (Zheng *et al.*, 2002). In mammals, these F-box proteins regulate proteolysis by acting as a substrate recognition factor forming the SCF^{SKP1} and SCF^{SKP2} complexes (Carrano *et al.*, 1999). In Arabidopsis, SCF^{SKP2A} binds to the transcription factor E2Fc to mediate its proteolysis by the 26S proteasome (del Pozo *et al.*, 2006). E2Fc works as a negative regulator of cell division and is likely necessary for DNA endoreduplication. Plants with reduced levels of E2Fc show lower ploidy levels, while overexpression of a truncated form that lacks the N-terminal region, involved in regulating its stability, affects cell division and cell size (del Pozo *et al.*, 2002). SKP2A also contains an auxin-binding site, explaining a direct involvement of auxin in controlling the stability of E2Fc and cell cycle activity (Jurado *et al.*, 2010; del Pozo and Manzano, 2014). Similarly, SCF^{SKP2B} targets ICK1/KRP1 for degradation (Ren *et al.*, 2008).

Another F-box protein that can form part of an SCF complex, is F-BOX LIKE (FBL17), which was first identified in Arabidopsis. The SCF^{FBL17} complex targets the CKIs ICK4/KRP6 and ICK5/KRP7 for proteasome-dependent degradation (Kim *et al.*, 2008; Gusti *et al.*, 2009) and most likely all seven Arabidopsis ICK/KRP proteins interact with FBL17 (Zhao *et al.*, 2012). FBL17 loss of function leads to the stabilization of ICK4/KRP6 and inhibits cell cycle progression during pollen development. Cellular analysis indicates that SCF^{FBL17} is an essential complex that promotes twin sperm cell production and double fertilization in plants (Kim *et al.*, 2008).

III. THE APC/C COMPLEX

The APC/C is one of the most complex molecular machines known to catalyze ubiquitination reactions. Depending on the organism, the APC/C can contain more than a dozen core subunits. The APC/C complex essentially is required at the G2-to-M transition and exit from mitosis by targeting different cell cycle regulators, including mitotic cyclins and PDS1/SECURIN (Irniger *et al.*, 1995; Genschik *et al.*, 1998; Pellman and Christman, 2001; Zheng *et al.*, 2011; Gui and Homer, 2013) for proteolysis. PDS1/SECURIN is an inhibitor of the protease SEPARASE. APC/C-mediated destruction of PDS1/SECURIN leads to the activation of SEPARASE, which in turn cleaves the COHESIN complex that physically attaches to sister chromatids, enabling sister chromatid segregation ((Funabiki *et al.*, 1996; Michaelis *et al.*, 1997; Ciosk *et al.*, 1998). The molecular mechanism of SEPARASE-mediated chromatid segregation is well conserved from yeast to human, but so far remains unclear in plant biology. Direct searches for SECURIN orthologs in plant genomes did not provide any significant hit; however, this might be due to the fact that those proteins are very poorly conserved at the amino acid sequence level between species (Moschou and Bozhkov, 2012).

A. The Core Components of the APC/C

Several high-resolution techniques, such as single-particle electron microscopy (EM), crystallography or nuclear magnetic resonance (NMR), have been used to understand the structure of the APC/C. In budding yeast, single-particle EM revealed the structure of APC/C^{CDH1} and also its substrates. This analysis showed a triangular shape of the APC/C and allowed for the identification of the combined catalytic and substrate recognition module, which is located within the central cavity. Using NMR spectroscopy, the specific interaction between the D-box of the target protein (see further) and APC10 has been demonstrated (da Fonseca *et al.*, 2011; Frye *et al.*, 2013; Zhang *et al.*, 2013; Chang *et al.*, 2014; Yamaguchi *et al.*, 2015).

Although no information is available about the structure of the plant APC/C, its hypothetical structure based on homology with yeast is schematically represented in Figure 1b. The catalytic core is formed by a CULLIN subunit, called APC2, and a RING-H2 protein, denominated APC11. Together, these proteins are sufficient to catalyze an ubiquitination reaction *in vitro*, although without substrate specificity (Gmachl *et al.*, 2000; Leverson *et al.*, 2000; Tang *et al.*, 2001). The APC10/DOC1 subunit is part of the combined catalytic and substrate recognition module located within the central cavity of the APC/C that consists of APC10, APC2 and APC11. APC10 together with CELL DIVISION CYCLE 20 (CDC20) and CCS52/CDH1 (see further) are responsible for substrate recognition (da Fonseca *et al.*, 2011).

The subunits APC3, APC6, APC7 and APC8 all contain tetratricopeptide repeat (TPR) domains that are important for protein-protein interactions and assembly of the structural module of the APC/C. In Arabidopsis, all APC/C subunits are encoded by unique genes with the notable exception of APC3,

for which two genes are present in the Arabidopsis genome, *APC3a*/*CDC27a* and *APC3b*/*HOBBIT* (Blilou *et al.*, 2002). The largest subunit of APC/C, APC1, serves as a scaffold module of the complex, and shares a structural motif, termed proteasome/cyclosome (PC) repeat and consisting of eleven repeats of 35 to 40 amino acid residues, with the two largest subunits of the 26S proteasome, RPN1 and RPN2 (van Leuken *et al.*, 2008; McLean *et al.*, 2011). The subunits APC4 and APC5 are proposed to be so-called connector subunits that, together with APC1, form the platform of the complex to which the structural module formed by TPR-containing subunits is attached together with the catalytic module (Thornton *et al.*, 2006; da Fonseca *et al.*, 2011).

In addition to the subunits described above, the APC/Cs of *Schizoscaccharomyces pombe, S. cerevisiae* and mammals contain two other functional subunits (Barford, 2011), denominated APC13 and APC15. Although plant genomes contain genes that encode proteins resembling APC13 and APC15 (Schwickart *et al.*, 2004; Saze and Kakutani, 2007; Uzunova *et al.*, 2012), both subunits have not been identified by tandem affinity purification (TAP) of the Arabidopsis APC/C, probably due to their small size (Van Leene *et al.*, 2010) (Table 1). In budding yeast, loss of APC13 leads to slow growth and accumulation of G2/M cells, showing the important role of this small subunit for APC/C function (Hall *et al.*, 2003; Schwickart *et al.*, 2004). The homologs of APC13 in human and fission yeast can complement the phenotype of the APC13 deletion mutant of budding yeast (Schwickart *et al.*, 2004). Moreover, APC13 promotes the high affinity association of the TPR-containing subunits APC6 and APC3 within the APC/C complex, and is required for efficient cyclin degradation during the anaphase (Schwickart *et al.*, 2004). Plants with lowered expression levels of *APC13*, also known as *BONSAI*, have an abnormal shoot and inflorescence development (Saze and Kakutani, 2007) (see further).

In human cells, APC15 appears to be part of the APC/C platform domain, where it is located near APC4, APC5 and APC1. APC15 is necessary for rapid auto-ubiquitination and degradation of CDC20 (see further), the APC/C co-activator subunit (Uzunova *et al.*, 2012). Additionally, APC15 depletion delays mitotic progression and cyclin B1 degradation in human cells (Mansfeld *et al.*, 2011). Hitherto, there is no functional data about the plant APC15 homologs.

It is highly likely that the APC/C in eudicots and monocots is similar. Indeed, in the rice (*Oryza sativa*) genome, genes encoding all APC subunits can be identified. For APC1, only a partial sequence is present, possibly due to mis-annotation (Lima *et al.*, 2010) (http://rice.plantbiology.msu.edu/). Homology-based sequence analysis showed that also in maize (*Zea mays*), all APC/C subunits are present (Table 1), and they even contain the conserved domains found in the corresponding subunits of other organisms, strengthening the evolutionary conservation of the APC/C complex. In Table 2, an amino acid identity and similarity comparison are shown

TABLE 1
The APC/C subunits in *Arabidopsis thaliana, Oryza sativa* and *Zea mays*

	A. thaliana	*O. sativa*	*Z. mays*	
APC subunits		Gene Identifier		Motifs
APC1	At5g05560	*	GRMZM2G053980	Rpn1/2
APC2	At2g04660	Os04g40830	GRMZM2G168886	Cullin domain
APC3a	At3g16320			TPR
APC3b	At2g20000	Os06g41750	GRMZM2G392710	
APC4	At4g21530	Os02g54490	GRMZM2G053766	WD40
APC5	At1g06590	Os12g43120	GRMZM2G431251	TPR
APC6a	At1g78770	Os03g13370	GRMZM2G147603	TPR
APC6b			GRMZM2G166684	
APC7	At2g39090	Os05g05720	GRMZM2G089296	TPR
APC8a	At3g48150	Os02g43920	GRMZM2G170591	TPR
APC8b		Os06g46540		
APC10a	At2g18290	Os05g50360	GRMZM2G054247	DOC1
APC10b			GRMZM2G174971	
APC11a	At3g05870	Os03g19059	GRMZM2G162356	RING-H2
APC11b		Os07g22840		
APC13	At1g73177	Os07g44004	GRMZM6G522911	
APC15a	AT5g63135	Os02g38029	GRMZM2G092743	
APC15b			GRMZM2G020201	

Note. *partial sequence.

TABLE 2
Amino acid identity and similarity among the APC/C subunits in *Arabidopsis thaliana* (At), *Oryza sativa* (Os) and *Zea mays* (Zm)

APC subunits	% Identity/Similarity			
	At/Os	At/Zm	Os/Zm	a/b
APC2	64/76	64/77	85/92	
APC3	47/60 (a) 55/71 (b)	48/62 (a) 56/71 (b)	85/91	48/63
APC4	49/67	48/69	71/82	
APC5	53/69	51/67	83/88	
APC6	74/85	73/84 (a) 72/83 (b)	86/92 (a) 87/93 (b)	94/96
APC7	62/77	62/77	90/95	
APC8	65/80 (a) 46/61 (b)	66/80	88/94 (a) 52/61 (b)	58/94
APC10	81/92	82/91 (a) 77/89 (b)	90/94 (a) 84/89 (b)	90/93
APC11	88/91 (a) 87/90 (b)	88/90	96/98 (a) 95/97 (b)	98/98
APC13	58/77	59/77	93/98	
APC15	59/72	57/71 (a) 58/71 (b)	94/96 (a) 94/97 (b)	96/98

Note. a/b: identity and similarity between the subunits a and b of the different species.

between the Arabidopsis, rice and maize core APC/C subunits. A schematic representation of the structure of the maize APC/C subunits is shown in Figure 2.

B. APC/C Co-Activators

The APC/C is regulated by two structurally related co-activator proteins, known as CDC20 and CDC20 HOMOLOG 1 (CDH1) in mammalian, and CDC20 and CELL CYCLE SWITCH 52 (CCS52) in plants. These proteins can bind and activate the APC/C and provide substrate specificity. The co-activators belong to a class of WD-40 repeat proteins that form a β-propeller structure, and represent the major site for protein interactions. The WD-40 domain facilitates the recruitment of target proteins in an F-box protein-dependent manner and is as such essential for substrate recognition in APC/C-dependent proteolysis (van Leuken *et al.*, 2008).

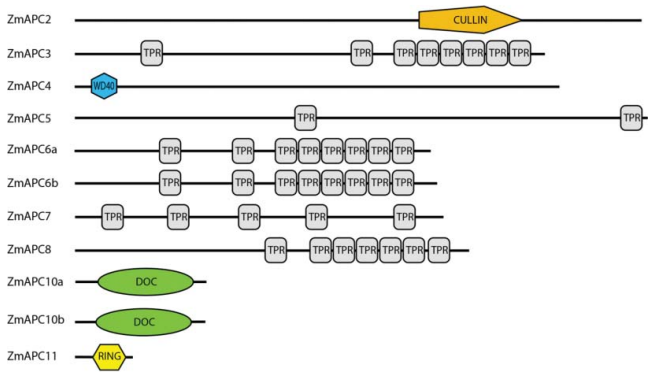

FIG. 2. Schematic representation of the conserved domains of APC/C subunits from *Zea mays*. All domains and full-length protein sequences are represented by colored boxes and gray lines, respectively.

In mammals, the APC/C is activated during early mitosis by CDC20, and the APC/C^{CDC20} complex is essential for SECURIN degradation at the metaphase/anaphase transition (Cohen-Fix *et al.*, 1996; Gorr *et al.*, 2005). Later in the telophase and the G1-phase, the APC/C associates with CDH1, and APC/C^{CDH1} marks B-type cyclins (CYCB) for degradation, facilitating exit from mitosis (Schwab *et al.*, 1997; Visintin *et al.*, 1997; Zachariae *et al.*, 1998; Kramer *et al.*, 2000).

The Arabidopsis genome harbors five genes encoding putative CDC20 proteins (Capron *et al.*, 2003a; Kevei *et al.*, 2011). Two of them, *CDC20.1* and *CDC20.2*, execute conserved and redundant functions in the mitotic cell cycle, while the three others, *CDC20.3, CDC20.4* and *CDC20.5*, might be pseudogenes (Kevei *et al.*, 2011). *CCS52*, the plant ortholog of *CDH1*, was first identified in alfalfa (*Medicago sativa*). In Medicago, there are two CCS52 co-activators, CCS52A and CCS52B, and partial suppression of *CCS52A* gene expression in *M. truncatula* reduces endoreduplication and cell size (Cebolla *et al.*, 1999). Arabidopsis has three genes encoding the CCS52 co-activators, *CCS51A1, CCS52A2* and *CCS52B*. *CCS52A1* and *CCS52A2* share high homology with each other and both are closely related to *CCS52A* of Medicago, while *CCS52B* is more similar to *CCS52B* (Fülöp *et al.*, 2005). Mutation of either *CCS52A1* or *CCS52A2* in *Arabidopsis* results in rosette leaves with a reduced endoreduplication index, which represents the mean number of endoreduplication cycles that a typical nucleus undergoes (Lammens *et al.*, 2008). In addition, down-regulation of *CCS52A* in tomato also reduces endoreduplication and results in a decreased cell size in developing tomato fruits. Curiously, ectopic overexpression of *CCS52A* in tomato plants triggers an initial delay in

endoreduplication, which is later resumed and even enhanced (Mathieu-Rivet *et al.*, 2010b). In Arabidopsis, ectopic expression of *CCS52A2* or *CCS52B* results in slightly swelled roots with cells that have higher ploidy levels, up to 32C and 64C. Similarly, leaf trichomes of plants overexpressing *CCS52A2* or *CCS52B* have four or five branches in contrast to three branches of wild-type trichomes. This phenotype is also the result of an enhanced level of endoreduplication (de Almeida Engler *et al.*, 2012). Simultaneously down-regulating *CCS52A2* and *CCS52B*, or down-regulating only *CCS52B* results in decreased ploidy levels in cotyledon cells (de Almeida Engler *et al.*, 2012). Moreover, when *CCS52A2*- or *CCS52B*-overexpressing plants are infected by root-knot or cyst nematodes, the feeding cells normally induced in the roots become gigantic with fewer, but enlarged nuclei, due to precocious endoreduplication and hampered mitosis, causing decreased nematode growth and reproduction. In contrast, down-regulation of *CCS52A* and *CCS52B* results in smaller feeding cells containing little cytoplasm, showing a delay in nematode development (de Almeida Engler *et al.*, 2012). In Arabidopsis, in the root apex at the transition zone, which is located between the meristem and the cell elongation zone, cytokinin signaling activates the transcription factor ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2), which directly induces the expression of *CCS52A1*. The presence of CCS52A1 is postulated to activate APC/C and to promote the degradation of mitotic regulators, causing cell division to arrest, thus controlling the onset of endoreduplication and meristem size (Takahashi *et al.*, 2013).

Similarly to vertebrates, the plant APC/C co-activators also display temporal cell cycle regulation. In animals, CDC20 starts to accumulate in the S-phase, peaks at mitosis and drops as cells exit mitosis, while the CDH1 activity is elevated in the late anaphase and persists throughout the G1-phase (Hu *et al.*, 2011). In synchronized *Arabidopsis* cell cultures, the two functional *CDC20*s, *CDC20.1* and *CDC20.2*, are expressed from the S-phase until the M-phase exit. Similarly, *CCS52B* is expressed from G2/M- to M-phase, whereas *CCS52A1* and *CCS52A2* transcripts are present from late M- until early G2-phase (Menges *et al.*, 2003; Fülöp *et al.*, 2005; Kevei *et al.*, 2011).

C. APC/C Inhibitors

Vertebrates utilize inhibitory proteins to restrict APC/C activity until the appropriate time in mitosis and meiosis. One of these inhibitors is EARLY MITOTIC INHIBITOR1 (EMI1), which plays an essential function during cell proliferation by preventing re-replication of DNA, and the destruction of EMI1 triggers the activation of APC/C at mitosis (Grosskortenhaus and Sprenger, 2002; Di Fiore and Pines, 2007; Machida and Dutta, 2007). *In vitro* experiments have shown that EMI1 inhibits CYCB ubiquitination and an excess of EMI1 added to Xenopus egg extracts prevents CYCA, CYCB, SECURIN and GEMININ degradation (Reimann *et al.*, 2001). In Xenopus and mouse, EMI2, a homolog of EMI1, plays an important role in maintaining a balanced CDK activity at meiosis. In Xenopus oocytes, meiosis is driven by the CDK/CYCB complex. At the end of meiosis I, CYCB is only partially degraded and the decrease in CDK/CYCB activity is essential for entry into meiosis II (Furuno *et al.*, 1994). The partial CYCB degradation required for the progression of meiosis I to meiosis II is obtained through temporally controlled inhibition of the APC/C by the EMI2 protein (Madgwick *et al.*, 2006; Ohe *et al.*, 2007; Tang *et al.*, 2008). The mechanism of APC/C inhibition by EMI1 and EMI2 proteins is through competitive pseudosubstrate binding. Both proteins compete with proper APC/C substrates, thereby preventing their ubiquitination and degradation (Miller *et al.*, 2006; Ohe *et al.*, 2007). In budding yeast, ACM1 (APC/C^{CDCH1} MODULATOR 1) plays a similar role as EMI1 in inhibiting APC/C^{CDH1} during mitosis. ACM1 binds to CDH1 via a D- and KEN-box (see further), acting as an APC/C pseudosubstrate, thereby preventing substrate binding. ACM1 is then degraded in late mitosis by APC/C (Martinez *et al.*, 2006; Burton *et al.*, 2011). Although ACM1 is ubiquitinated by APC^{CDC20} during mitosis (Enquist-Newman *et al.*, 2008), it is not an APC^{CDH1} substrate. ACM1 normally binds to CDC20 via D-box 1, leading to its ubiquitination by APC^{CDC20}, whereas it binds to CDH1 via D-box 3 and the KEN-box, leading to inhibition of APC^{CDH1}. Thus, ACM1 appears to bind to CDC20 and CDH1 in two very different orientations, with different consequences: ubiquitination or APC/C inhibition, respectively (Burton *et al.*, 2011).

To date, orthologs of EMI1 and ACM1 proteins have not been described in plants. However, several APC/C inhibitors that appear to act similarly to EMI proteins have been identified. Tandem affinity purification using APC/C subunits as bait and subsequent protein identification by mass spectrometry allowed the identification of two plant-specific APC/C inhibitors, known as ULTRAVIOLET-B-INSENSITIVE 4 (UVI4) and OMISSION OF SECOND DIVISION 1 (OSD1)/GIGAS CELL1 (GIG1) (Van Leene *et al.*, 2010). The former protein has previously been reported to affect endoreduplication in leaves (Hase *et al.*, 2006). UVI4 regulates APC/C activity by binding to the CCS52A1 co-activator, thereby inhibiting the destruction of CYCA2;3. The *uvi4* mutant fails to accumulate CYCA2;3 during the S-phase and prematurely aborts the cell cycle, triggering the onset of the endoreduplication (Imai *et al.*, 2006; Heyman *et al.*, 2011). Similarly, OSD1/GIG1, an UVI4 homolog that shares partial structural and functional similarities with the vertebrate EMI2, was also found to be an APC/C inhibitor (Cromer *et al.*, 2012). As detailed above, a balanced APC/C activity is essential to promote meiotic progression. This also is the case in plants, and OSD1/GIG1 appears to have an important role in this process. Arabidopsis mutants in *OSD1*/*GIG1* fail to enter the second meiotic division during both male and female gametogenesis,

thus producing diploid gametes (d′Erfurth *et al.*, 2009; Bulankova *et al.*, 2010; d′Erfurth *et al.*, 2010). In addition, OSD1/GIG1 also has a function during the mitotic cell cycle. Whereas *UVI4* expression levels increase at the G1-to-S transition, suggesting that UVI4 exerts its role as an APC/C inhibitor during the S-phase, *OSD1/GIG1* expression levels peak at the G2-to-M transition, acting later during mitosis. Mutant *osd1/gig1* plants show abnormal cotyledon development, displaying different types of giant guard cells, some of which are reminiscent of single-celled stomata. The latter are typically generated when guard mother cells fail to undergo cytokinesis or are arrested at the G2-phase (Iwata *et al.*, 2011). Moreover, in plants overexpressing *OSD1/GIG1*, an accumulation of CYCB1;2 is observed, supporting the idea that OSD1/GIG1 affects cyclin stability. Overexpression of the APC/C activator *CDC20* in *osd1/gig1* mutant plants results in a severe endomitosis phenotype. Endomitosis is a cell cycle form in which cells undergo various aspects of mitosis, but fail to execute cytokinesis. Thus, OSD1/GIG1 may prevent the ectopic occurrence of endomitosis by repressing the activity of APC/C^{CDC20} during mitosis (Heyman *et al.*, 2011; Iwata *et al.*, 2011).

D. Novel Proteins Interacting with APC/C

In Arabidopsis, TAP using APC/C subunits as bait was used to isolate the entire APC/C from cell cultures (Van Leene *et al.*, 2010). The cell cultures used for this TAP showed an equivalent distribution of cells in the G1- and G2-phase, leaving the possibility that interacting proteins might be part of different alternative cell phase-specific complexes (Van Leene *et al.*, 2007). The twelve APC/C subunits that constitute the core complex (Van Leene *et al.*, 2010) and novel APC/C interactors not described so far, were identified by mass spectroscopy. Many of these proteins were used in turn as bait for novel TAP experiments (Van Leene *et al.*, 2007; Van Leene *et al.*, 2010).

One of the identified novel APC/C interactors, denominated SAMBA, was characterized as a plant-specific negative regulator of growth. The *SAMBA* gene is highly expressed in developing seeds and during early plant development, indicating a specific regulatory role at initial developmental stages. In agreement with this prediction, *samba* mutants have an enlarged meristem size and show growth-related phenotypes, including the formation of large seeds, leaves, and roots (Eloy *et al.*, 2012). This growth-enhancing phenotype is further, often synergistically, enhanced when *SAMBA* is combined with other growth-regulatory genes (Vanhaeren *et al.*, 2014).

Using the information available from the interactome identified by TAP, yeast two-hybrid and bimolecular complementation (Eloy *et al.*, 2006; Boruc *et al.*, 2010; Van Leene *et al.*, 2010; Heyman *et al.*, 2011), an interaction network around the twelve known APC/C subunits was built (Figure 3). The combined network has 111 interactions, including all APC/C subunits, well-known cell cycle proteins such as CDKA, CYCA2;3, CAK (CDK ACTIVATING KINASE), and APC/C-associated proteins such APC/C co-activators (CDC20, CCS52A and CCS52B) and APC/C regulators (UVI-4, UVI4-like/OSD1/GIG1 and SAMBA). Moreover, the network contains many proteins belonging to several functional categories, including unknown proteins, hormone metabolism-related and RNA regulation proteins. As mentioned above, the auxin hormone can directly bind to SKP2, thus controlling SCF activity. Some proteins of the APC/C interaction network are related to auxin and ethylene signaling, hinting at a possible role of APC/C activity in hormone metabolism. Moreover, some novel interactors could be potential APC/C substrates or regulators.

IV. TARGET PROTEINS OF THE APC/C

The capacity of the APC/C to recognize and target specific proteins for destruction depends on the presence of short conserved amino acid motifs known as degrons. The classical APC/C degron is the destruction box or D-box, which occurs as a nine-residue motif (RxxLxxxxN). The D-box was first characterized in B-type cyclins and contains at least an arginine and a leucine separated by two residues (Glotzer *et al.*, 1991). The KEN-box (KENxxxN/D) is the second well-characterized APC/C degron, often occurring together with the D-box in APC/C target proteins (Pfleger and Kirschner, 2000).

Besides the well-known D- and KEN-boxes, two additional degron motifs are recognized by the APC/C, the A-box and GxEN-box. The A-box has been found in the N-terminal region of Aurora A (Aur-A), a kinase that is required for the formation of a bipolar mitotic spindle and accurate chromosome segregation. The A-box consists of a short sequence, Q^{47}RILGPSNVPQRV, which is highly conserved in all vertebrate Aur-As, and is absolutely required for its CDH1-dependent destruction (Littlepage and Ruderman, 2002). In Arabidopsis, there are three Aurora kinases, denominated AURORA-1, -2 and -3, having a conserved degron A-box that resembles the one found in human Auroras. In mammals, this motif has been shown to be essential for Aurora degradation, indicating that Arabidopsis Auroras may be subject of proteasome-dependent proteolysis as well (Demidov *et al.*, 2005; Petrovská *et al.*, 2012). The GxEN-box has been identified in the C-terminus of the Xenopus chromokinesin kid (Xkid) protein, which is essential for chromosome alignment on the metaphase spindle. The motif is required for Xkid degradation by the APC/C (Castro *et al.*, 2003). The GxEN-box degron is also found in the Ama1 protein, which is an APC/C activator specific for meiotic progression. In yeast, mutation of the GxEN-box degron leads to the stabilization of Ama1 (Tan *et al.*, 2013). To date, no GxEN-box motif was found in plants. In all cases, the mutation or deletion of one of above-described degrons leads, in the absence of other degrons, to a failure in the APC/C-mediated destruction of the substrates by the 26S proteasome. In plants, there is experimental evidence that D- and

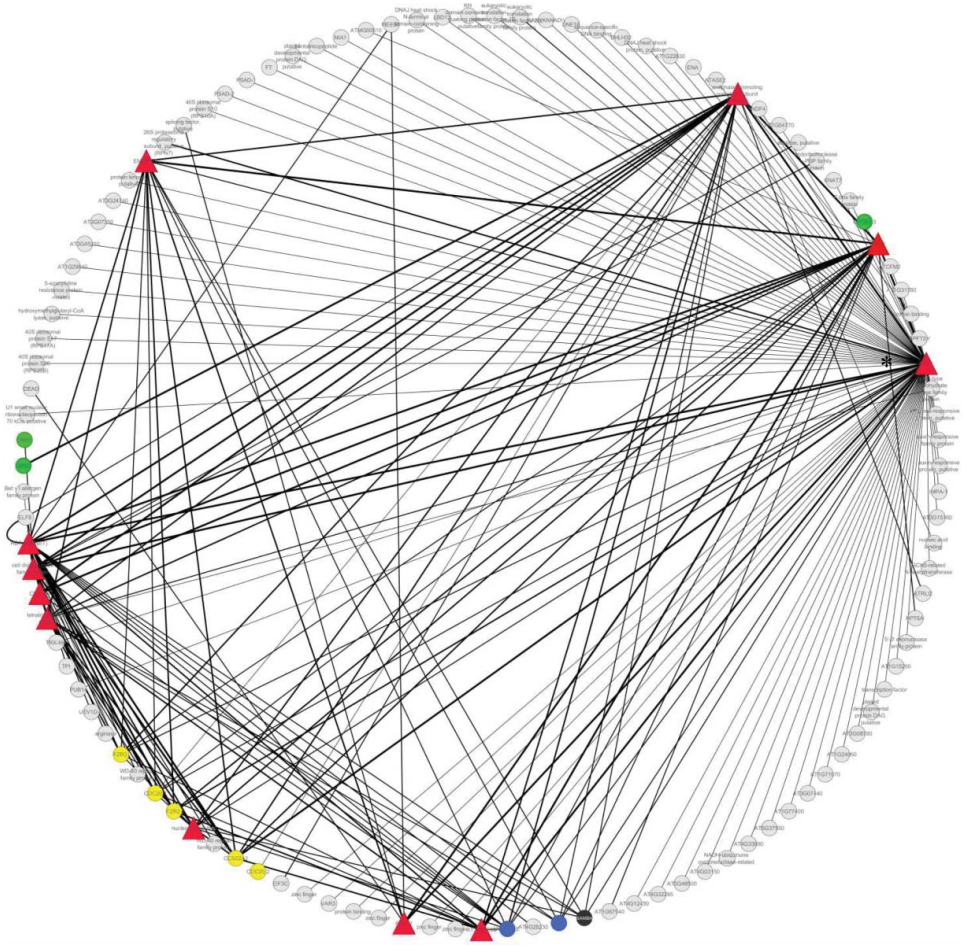

FIG. 3. Protein-protein interaction network of all APC/C subunits from Arabidopsis. The interaction map was created using the experimentally confirmed interactions identified by affinity purification coupled with mass spectrometry, yeast two-hybrid and/or bimolecular fluorescence complementation using the APC/C subunits (Boruc *et al.*, 2010; Van Leene *et al.*, 2010; Heyman *et al.*, 2011; Eloy *et al.*, 2012). The APC/C subunits used as bait are shown as red triangles. The circles show the interacting proteins with the APC/C proteins. The yellow color indicates the APC/C activators (CDC20, CCS52A and CCS52B), the blue color represents the APC/C-regulatory proteins (UVI4 and UVI4-like/OSD1GIG1), the green color indicates cell cycle-regulatory proteins, including CYCA2;3, CDKA and CAK (CDK ACTIVATING KINASE), and the black color designates the SAMBA protein. The most connected protein is the APC8 subunit (*), which is part of the structural core and contains TPR motifs. The network was accomplished using the program CORNET 2.0 (De Bodt *et al.*, 2012).

KEN-boxes are functional, demonstrating the conserved role of both degrons for APC/C-mediated degradation by the 26S proteasome.

In yeast and vertebrates, many APC/C substrates are known and the best studied are the cell cycle regulators such as cyclins, CKIs and DNA replication proteins. Other known APC/C targets are very diverse, including transcription factors, kinesin motor proteins and other microtubule-associated proteins, actin-binding proteins, regulators of sister chromatid cohesion and chromatin remodeling, and even metabolic enzymes such as 6-phosphofructo-2-kinase with a role in the glycolytic pathway (Goto and Eddy, 2004; Rankin *et al.*, 2005; Stewart and Fang, 2005; Zhao and Fang, 2005; Colombo *et al.*, 2011; Lim *et al.*, 2013; Singh *et al.*, 2014). In plants, the number of verified APC/C targets is limited and, as detailed later, more research is required to identify them.

As a first effort to study cyclin proteolysis during the plant cell cycle, CYCA and CYCB stability was investigated in transgenic tobacco BY-2 cells and later on in Arabidopsis plants. When cyclins are mutated within the D-box, cell cycle-specific proteolysis is eliminated, showing that mitotic cyclins in plants, as in others organisms, are subjected to destruction via the 26S proteasome in a D-box-dependent manner (Genschik *et al.*, 1998; Criqui *et al.*, 2000; Boudolf *et al.*, 2009). In agreement, loss of function of the APC/C activator *CCS52A1* stabilizes CYCA2;3 in Arabidopsis (Boudolf *et al.*, 2009). Similarly, in tomato plants, down-regulation of *CCS52A* also stabilizes CYCA3;1, an S-phase-specific A-type cyclin likely involved in the control of endoreduplication (Joubès *et al.*, 2000; Mathieu-Rivet *et al.*, 2010b).

The recently identified APC/C interactor, SAMBA, has been show to bind CYCA2;3 in a D-box-dependent manner.

Moreover, loss of function of SAMBA stabilizes CYCA2;3 in Arabidopsis plants during early developmental stages, suggesting that SAMBA can also act as a potential activator of the APC/C during the initial stages of development (Eloy *et al.*, 2012). The higher CYCA2;3 levels in *samba* mutants is likely to shorten cell cycle duration.

Several APC/C target proteins with a role other than the control of cell division have been described. One of these proteins is MONOCULM 1 (MOC1), which has been found to be a key regulator of rice tillering and shoot branching (Lin *et al.*, 2012; Xu *et al.*, 2012). MOC1 stability is controlled by TILLERING AND DWARF 1 (TAD1), the rice ortholog of CCS52. MOC1 harbors a canonical D-box at the N-terminus, and the replacement of the two conserved arginine and leucine residues by alanine, abolishes the interaction with TAD1 and reduces its degradation, showing that MOC1 is destroyed in a D-box-dependent manner. Moreover, in the absence of TAD1, the APC/C fails to recruit MOC1 for degradation, which results in the accumulation of MOC1 (Lin *et al.*, 2012; Xu *et al.*, 2012).

Another APC/C target protein is the rice RAA1 (ROOT ARCHITECTURE-ASSOCIATED 1). RAA1 is a small GTP-binding protein, encoded by a gene homologous to the FLOWERING PROMOTING FACTOR 1 (FPH1) protein in Arabidopsis, and was identified as a putative cell cycle inhibitor and a negative regulator of primary root growth (Xu *et al.*, 2010). APC/C-mediated degradation of RAA1 is D-box-dependent and is essential for the transition from metaphase to anaphase during cell division (Han *et al.*, 2008; Xu *et al.*, 2010). Yet another APC/C substrate is RICE SALT SENSITIVE 1 (RSS1), a key protein for the maintenance of meristem activity and viability under salt stress environments. The stability of RSS1 is D- and KEN-box-dependent, likely through APC/C^{CDC20}. The loss of function of *rss1* exhibits an extreme dwarf and short root phenotype under high-salt conditions (Ogawa *et al.*, 2011).

In Arabidopsis, the DOUBLE-STRANDED-RNA-BINDING 4 (DRB4) protein is involved in the biogenesis of different classes of small RNAs. In a two-hybrid assay, the protein interacts with APC10, and the reduction of APC/C activity by down-regulation of either *APC6* or *APC10* leads to a strong accumulation of DRB4, showing that DRB4 is proteasome-dependently degraded (Marrocco *et al.*, 2012).

Recently, the ETHYLENE RESPONSE FACTOR 115 (ERF115) has been identified as a rate-limiting transcription factor for cell division and stem cell renewal at the root quiescent center. Proteolysis of ERF115 was shown to be affected specifically in *ccs52a2* knockout plants, but not in the *ccs52a1* mutant. ERF115 has two putative D-box motifs, and inactivation of both degrons stabilizes the protein, showing that ERF115 is a novel proteolytic target of APC/$C^{CCS52A2}$ in a D-box-dependent manner (Heyman *et al.*, 2013).

V. APC/C FUNCTION DURING MEIOSIS AND GAMETOGENESIS

Meiosis reduces the ploidy level of the original cell by halving the nuclear DNA content in two subsequent chromosome segregation steps, without an interfering S-phase, leading to the formation of haploid gametes (Wijnker and Schnittger, 2013). Furthermore, the process allows for exchanges of genetic material by recombination, generating more diversity. To enter the meiotic program, cells exit the cell cycle early in the G1-phase prior to the accumulation of G1 cyclins. In yeast, the transition between mitotic and meiotic cell division is promoted by APC/C^{CDC20} through degradation of the transcriptional repressor Ume6 (Mallory *et al.*, 2007). Unlike mitosis, meiosis has two distinctive phases, known as meiosis I and meiosis II. In meiosis I, which is divided in prophase I, metaphase I, anaphase I, and telophase I, the homologous chromosomes are separated, producing two haploid cells. During meiosis II, the cell will generate four haploid cells, in a similar process to that of mitosis. The four main processes of meiosis II are: prophase II, metaphase II, anaphase II and telophase II. During meiosis I, for the proper progression from metaphase I to anaphase I, APC/C needs to destroy the separase inhibitor SECURIN, similar to its function during the normal mitotic cell cycle (Cooper and Strich, 2011).

In Arabidopsis, two cyclins were shown to have a meiotic function, the A-type cyclin, CYCA1;2, also known as TARDY ASYNCHRONOUS MEIOSIS (TAM), and a cyclin with properties of both A- and B-type cyclins, the so-called SOLO DANCERS (SDS). Arabidopsis knockout plants of *CYCA1;2/TAM* exit the meiotic cycle after the first division, generating diploid gametes instead of haploids (Wang *et al.*, 2004; d'Erfurth *et al.*, 2010). The *sds* mutant exhibits defects in homolog chromosome pairing and crossover formation during prophase I, leading to reduced levels of meiotic recombination (Azumi *et al.*, 2002; De Muyt *et al.*, 2009; Bulankova *et al.*, 2010).

B-type cyclins have prominent roles during mitosis, and currently CYCB3;1 is the only B-type cyclin detected during meiosis by promoter-GUS fusion reporter lines (Bulankova *et al.*, 2013). Two CYCB3;1 mutants alleles, *cycb3;1-1* and *cycb3;1-2*, produce pollen mother cells (PMCs) with unusual structures, resembling incomplete cell walls formed at ectopic locations. These cellular observations indicate that CYCB3;1 activity contributes to the spatial and temporal regulation of cell wall formation in PMCs (Bulankova *et al.*, 2013).

Functional characterization of APC/C subunits in Arabidopsis has revealed that all subunits investigated so far are essential for gametophytic development and/or embryogenesis. Mutations in *APC1, APC2, APC4* or *APC6* arrest female gametogenesis due to the inability to destroy CYCB, as revealed by CYCB accumulation in the mega-gametophyte (Capron *et al.*, 2003b; Kwee and Sundaresan, 2003; Wang *et al.*, 2012; Wang *et al.*, 2013). Hypomorphic *apc6* lines

show CYCB accumulation in developing leaves as well (Marrocco *et al.*, 2009). Similarly, disruption of *APC8* or *APC13* function causes the failure of CYCB1;1 degradation during the male gametophyte development (Zheng *et al.*, 2011). Both *apc8* and *apc13* mutants are affected in pollen development, leading to an increased proportion of uni-nucleated mature pollen and indicating that the APC/C is required at mitosis during male gametophytic development (Zheng *et al.*, 2011).

Single *apc10* and double *apc3a/apc3b* mutants show that APC10 and APC3 are also essential for female gametophyte development (Pérez-Pérez *et al.*, 2008; Eloy *et al.*, 2011), whereas APC1 and APC4 are also critical for embryogenesis (Wang *et al.*, 2012; Wang *et al.*, 2013).

VI. THE ROLE OF THE APC/C DURING PLANT DEVELOPMENT

Whereas the role of the APC/C in mitosis and meiosis is reasonably well characterized, little is known about its function during plant development. Evidence shows that the APC/C complex is also involved in plant growth, since overexpression of core APC/C subunits leads to enhanced growth.

In tobacco plants, ectopic expression of *APC3a*, also known as *CDC27a*, results in an increased growth rate and organ size. The transgenic plants are taller, displaying a larger leaf size and significant increases in stem and root dry matter. Moreover, analysis of the meristem revealed a smaller cell size with increased cell numbers compared to the wild type. The observed phenotype appears to be the effect of enhanced APC/C activity, which was demonstrated by an elevated ubiquitination of the mitotic cyclin, CYCB1;1 (Rojas *et al.*, 2009). In addition, the APC3b subunit has been implicated in the maintenance of cell division at meristems during post-embryonic development (Blilou *et al.*, 2002; Pérez-Pérez *et al.*, 2008).

In Arabidopsis, constitutive overexpression of *APC10* results in increased leaf size due to a faster rate of cell division during the early stages of leaf development. The average time to complete an entire cell cycle in leaves shortens from 21 hours to 19 hours (Eloy *et al.*, 2011). Moreover, the overexpression of *APC10* in Arabidopsis causes an increased protein degradation of CYCB1;1, possibly accelerating the transition through mitosis (Eloy *et al.*, 2011). Also in tobacco, overexpression of *APC10* produces taller plants with larger leaves due to an increased cell number. Additionally, the transgenic plants produce more seed capsules and have an augmented biomass accumulation. In addition, a cross between *APC10*- and *APC3a*-overexpressing tobacco plants results in T1 plants that have an enhanced growth phenotype compared to the overexpression of the single APC/C subunits (de Freitas Lima *et al.*, 2013).

Whereas overexpression of APC/C subunits can stimulate plant growth, reduced expression levels of *APC6* or *APC10* provoke several defects in vascular development in Arabidopsis. Transgenic plants with diminished *APC6* or *APC10* expression show a reduced leaf size and curled leaves, and at later developmental stages, mutant plants develop severe morphological aberrations, such as shorter inflorescences and a remarkable formation of clusters of siliques (Marrocco *et al.*, 2009). Consistently with the phenotype observed in Arabidopsis, rice mutants of *APC6* are dwarfed and have smaller seeds (Kumar *et al.*, 2010). Moreover, knockdown of *APC6* in *Medicago truncatula* results in defective primary root growth, and fewer lateral roots (Kuppusamy *et al.*, 2009). *APC3b* is required for postembryonic progression of cell differentiation in the shoot and root meristem, and for maintenance of cell division. Mutation in *APC3b* interferes with postembryonic cell division and differentiation of the distally located quiescent centrum, columella root cap and lateral root cap cells (Willemsen *et al.*, 1998). Additionally, perturbation in *APC3b* expression leads to severe dwarfism, a phenotype called HOBBIT, which is characterized by the limited capacity of meristematic cells to divide post-embryonically (Blilou *et al.*, 2002).

In Arabidopsis, a weak allele of *APC8, apc8-1*, shows pleiotropic phenotypes, including distorted leaf shapes and abnormal shoot meristem development. Furthermore, *apc8-1* displays defects in flower and silique development, exhibiting bushy inflorescences and shorter siliques (Zheng *et al.*, 2011). Loss of function of *APC13* results in a so-called Bonsai-like phenotype, which is characterized by an inhibition of internode elongation and termination of shoot growth, leading to dwarfed plants (Saze and Kakutani, 2007). Moreover, mutation of either *APC8* or *APC13* results in a reduced transcription of microRNA159 (*miR159*) and lessened accumulation of mature *miR159* (Palatnik *et al.*, 2007; Brownfield *et al.*, 2009; Zheng *et al.*, 2011).

Altered expression of *CCS52A1* or *CCS52A2* negatively affects plant development. Transgenic tomato plants with reduced expression levels of *CCS52A* display a smaller fruit size. Similarly, tomato plants overexpressing *CCS52A* have an underdeveloped root system and small and curly leaves, and they also hardly produce viable flowers and few very small fruits with an irregular shape (Mathieu-Rivet *et al.*, 2010a; Mathieu-Rivet *et al.*, 2010b). In addition, overexpression of *CCS52A1* in Arabidopsis was shown to completely suppress the formation of multicellular trichomes in *siamese* mutants, demonstrating that the CDK inhibitor SIAMESE cooperates with CCS52A1 to control the endoreduplication in trichomes (Kasili *et al.*, 2010). *CCS52A2* mutant plants also have a reduced organ size and biomass. Whereas strong overexpression of *CCS52A1* or *CCS52A2* negatively affects organ size, mild overexpression of *CCS52A1* or *CCS52A2* under the control of the ubiquitin promoter results in a significant increase in the organ sizes, showing that engineering of the expression of *CCS52* genes in a dose-dependent manner is a possible new route for biomass production (Baloban *et al.*, 2013). Moreover, *CCS52A1*, but not *CCS52A2*, is transcriptionally regulated by the trihelix transcription factor, GT2−LIKE 1 (GTL1). GTL1 directly represses the transcription of

CCS52A1 to arrest the progression of endoreduplication and cell growth by down-regulating the APC/$C^{CCS52A1}$ activity (Breuer *et al.*, 2012).

In rice, modification of the activity of the CCS52 ortholog, TAD1, also causes severely impaired plant development. The *tad1* mutant plants show a reduced plant height and an increased number of tillers, whereas *TAD1*-overexpressing plants display reduced tiller numbers (Lin *et al.*, 2012; Xu *et al.*, 2012).

In Arabidopsis, simultaneous down-regulation of both *CDC20.1* and *CDC20.2* leads to a severe delay in plant development. These *CDC20* RNAi lines show a reduced meristem size and root length, and smaller leaves with less cells. Moreover, the transgenic plants display reduced fertility, with shorter siliques and many aborted ovules, as a consequence of male sterility (Kevei *et al.*, 2011).

Also several APC/C substrates have been shown to have a role in development. The rice MOC1 protein, which has been demonstrated to be an APC/C substrate (see above), controls the initiation and outgrowth of axillary meristems at both vegetative and reproductive stages. *Moc1* rice plants are characterized for having a single main culm without any tillers and reduced panicles branches (Li *et al.*, 2003; Lin *et al.*, 2012; Xu *et al.*, 2012).

VII. PERSPECTIVES FOR FUTURE RESEARCH

In vertebrates, APC/C has been reported to function in differentiated cells. Functional analyses of APC/C^{CDH1} and APC/C^{CDC20} have uncovered critical roles for these two distinct APC/C complexes in the post-mitotic regulation of axon and dendrite morphogenesis, respectively (Konishi *et al.*, 2004). The APC/C^{CDH1} complex restricts axon growth and controls their patterning in the mammalian brain, while APC/C^{CDC20} promotes dendrite growth and arborization (Yang *et al.*, 2010). Furthermore, it has been shown that loss of APC^{CDH1} activity enhances glycolysis, suggesting that the APC/C might control components of metabolic pathways as well (Almeida *et al.*, 2010). In plants, genes encoding APC/C subunits also show strong expression in non-dividing tissues like leaves, roots and siliques (Figure 4), suggesting that in plants they could also have functions not only during cell division, but also during differentiation and development. As detailed above, overexpression and loss-of-function data on APC/C and its substrates indicate such regulatory role.

The further elucidation of the role of the APC/C during development will require the identification and functional analysis of APC/C substrates. The first attempt in plants to identify APC/C interactors using TAP revealed novel proteins (Van Leene *et al.*, 2010), of which some were shown to be essential for the control of the APC/C in plants (see above; Blomme *et al.*, 2014). At least one of the interactors, ERF115, was shown to be an APC/C substrate (Heyman *et al.*, 2013). However, TAP might not be the best method to identify potential APC/C targets, as it requires a strong interaction of proteins with the purified complexes (Dedecker *et al.*, 2015).

In human cells, the identification of several new APC/C substrates was performed using what is known as

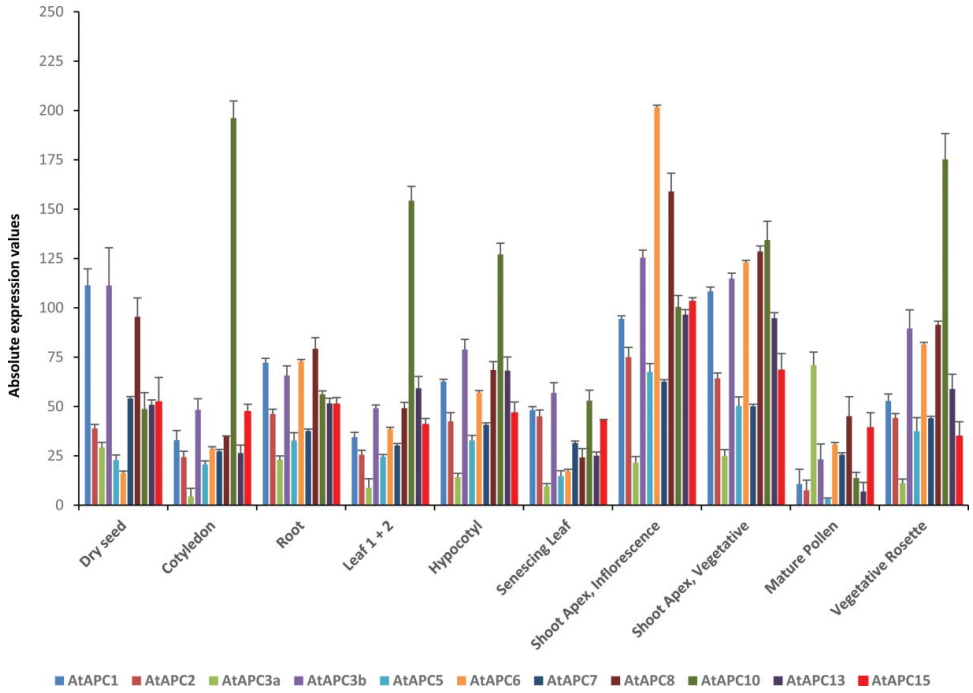

FIG. 4. Genevestigator Arabidopsis organ-specific expression of APC/C subunits. Expression values obtained from compiled Arabidopsis microarray experiments as reported by Genevestigator. Data are reported as absolute expression values.

co-regulation proteomics. The underlying hypothesis is that all proteins ubiquitinated by the APC/C for degradation by the proteasome in a cell cycle-dependent manner would show similar abundance profiles over the course of the cell cycle. Thus, a time-course experiment was conducted to identify substrates degraded by the APC/C during mitosis (Singh *et al.*, 2014). By comparing the profile and abundance with the known APC/C substrates, this approach generated a list of eight putative new APC/C substrates, all showing high similarity of protein accumulation with the known APC/C substrates, like CYCB and SECURIN. The list generated shows enrichment for microtubule-associated proteins of the kinesin motor family. The identified kinesins are KIFC1, KIF2C, KIF4A, KIF13A, KIF14, KIF18A, KIF22 and KIF23. Among the five kinesins tested for *in vitro* degradation, four of them show a time-dependent degradation profile, similar to that of the known APC/C substrates, CYCB and SECURIN. Moreover, the kinesin proteins are stabilized in the presence of two APC/C inhibitors, the EMI1 as a non-competitive inhibitor and SECURIN as a competitive inhibitor, or by mutation in their D-box, validating the used approach for the identification of new APC/C substrates (Singh *et al.*, 2014). A similar approach could be used to identify APC/C substrates in plants. Some cell cultures, such as tobacco BY-2 cells, are highly synchronizable (Nagata and Kumagai, 1999), but limited information is available about the proteome of this plant (Laukens *et al.*, 2004; Duby *et al.*, 2010).

It is expected that several different APC/C complexes operate in specific plant tissues or during specific phases of plant development. By performing TAP on specific tissues/organs and/or plants in specific developmental stages, such APC/C subcomplexes could be identified. A TAP performed with SAMBA as bait in Arabidopsis cell cultures and seedlings showed some differences in the purified proteins (Van Leene *et al.*, 2010; Eloy *et al.*, 2012). TAP on cell cultures yielded 16 proteins, and eight common proteins were found in both experiments. All eight were the core APC/C subunits APC1, APC2, APC3b, APC4, APC5, APC6, APC7 and APC8; the cell culture-specific proteins were the two APC/C inhibitors, UVI-4 and UVI4-like, the replication license factor, MCM6, and five other proteins not related to the cell cycle; and the seedling-specific proteins were the remaining APC/C core subunits, APC3a and APC10, and the activator CCS52A2 (Van Leene *et al.*, 2010; Eloy *et al.*, 2012). Due to the limitation of having enough Arabidopsis material to perform such experiments, a similar approach could be followed using different plants with more biomass that will give enough material from different organs and tissues.

Above-described methods can also be combined with strategies that specifically enrich ubiquinated proteins, as recently demonstrated in Arabidopsis (Kim *et al.*, 2013). This affinity-based technology makes use of concatenated ubiquitin-interacting domains that increase the purification stringency and yield of ubiquitin-containing proteins followed by high-sensitivity mass spectrometry. This proteomics approach has elucidated processes in Arabidopsis affected by ubiquitination (Kim *et al.*, 2013).

Measurements of the APC/C activity *in vivo* can be accomplished using reporter proteins fused to the target proteins. One can take advantage of several reporter proteins, such as GFP (Green Fluorescence Protein) and YFP (Yellow Fluorescence Protein) to be fused to the potential substrates (with and without degrons) and evaluate the level of degradation by treating it with the proteasome inhibitor MG132 (Heyman *et al.*, 2011; Iwata *et al.*, 2011; Zheng *et al.*, 2011). Recently, new specific chemical inhibitors of the APC/C, such as apcin, have been developed (Sackton *et al.*, 2014) and it will be of interest to test whether these molecules also abolish plant APC/C activity.

The APC/C has an essential role during both mitosis and meiosis, as exemplified by the often lethal phenotypes when one of the core subunits is inactivated. These essential functions render the analysis of the role of APC/C at later developmental stages (e.g., in differentiating cells) difficult, because it cannot easily be separated from the effect in dividing cells. A possible solution is the use of conditional expression systems, for example those based on CRE-LOX recombination (Kawade *et al.*, 2010). In such a system, a simple treatment, either chemical (e.g., dexamethasone) or physical (e.g, heat shock), causes the excision of a functional APC/C subunit in a respective mutant background, thus producing tissue sectors in which a given APC/C subunit is inactivated (Kawade *et al.*, 2010). Such systems can also be used to analyze the effect of specific mutations in core APC/C subunits or their essential substrates.

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