ABAP1 is a novel plant Armadillo BTB protein involved in DNA replication and transcription

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In multicellular organisms, organogenesis requires a tight control of the balance between cell division and cell differentiation. Distinct signalling pathways that connect both cellular processes with developmental cues might have evolved to suit different developmental plans. Here, we identified and characterized a novel protein that interacts with pre-replication complex (pre-RC) subunits, designated Armadillo BTB *Arabidopsis* protein 1 (ABAP1). Overexpression of ABAP1 in plants limited mitotic DNA replication and decreased cell proliferation in leaves, whereas ABAP1 downregulation increased cell division rates. Activity of ABAP1 in transcription was supported by its association with the transcription factor AtTCP24. The ABAP1–AtTCP24 complex bound specifically to the promoters of *AtCDT1a* and *AtCDT1b* *in vitro* and *in vivo*. Moreover, expression levels of *AtCDT1a* and *AtCDT1b* were reduced in ABAP1-overexpressing plants and they were increased in plants with reduced levels of ABAP1. We propose that ABAP1 participates in a negative feedback loop regulating mitotic DNA replication during leaf development, either by repressing transcription of pre-RC genes and possibly by regulating pre-RC utilization through direct association with pre-RC components.

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Introduction

In multicellular organisms, organogenesis requires a tight control of the balance between cell proliferation and differentiation. An important regulatory mechanism controlling cell division is licensing DNA for replication at late G1, which allows cells to progress into S phase. This process is regulated by the sequential assembly of ORC, CDC6, CDT1 and MCM onto replication origins, forming the pre-replication complex (pre-RC) (Blow and Dutta, 2005). Differentiation of specialized cells is driven by transcriptional regulatory events involving chromatin remodelling proteins and transcription factors. Therefore, during the transition from a dividing to a differentiating state, cells must tightly integrate transcription regulation with cell cycle controls, such as DNA replication licensing. It is expected that both regulatory pathways might share similar components to facilitate coordination during development. Pre-RC components have been associated with the control of diverse developmental processes in eukaryotes. In mammals and *Drosophila*, ORC seems to have a function in neuronal development (Pinto *et al*, 1999; Huang *et al*, 2005). The involvement of pre-RC components in heterochromatin formation and establishment of transcriptionally repressed DNA regions was well characterized in yeasts, *Drosophila* and mammals (Sasaki and Gilbert, 2007). In animals, a key regulator of pre-RC activity, called geminin, has been implicated in the regulation of cell proliferation and cell differentiation by interacting with components of the pre-RC and transcription factors (Seo and Kroll, 2006).

In plants, a concerted action of DNA replication and gene transcription controls during development is still unidentified. A modular and indeterminate construction of the plant body by self-perpetuating meristems, shaped by external and endogenous developmental signals, imposes an accurate balance of cell division and cell differentiation during plant life to generate organs of the correct form (Castellano and Sablowski, 2005). The extent of the involvement of DNA replication controls in plant development is still not clear as meristematic plant cells commonly exit the mitotic cycle and start differentiation without pausing DNA synthesis. These DNA endoreduplication cycles seem to be very important for some plant cells to undergo differentiation programmes and correct organ development (Gutierrez, 2005). Genetic analyses in *Arabidopsis* have shown that pre-RC components, such as AtCDT1 homologues, AtCDC6, AtORC2 and two AtMCMs can interfere in developmental processes either by directly affecting replication or by indirect effects on endoreduplication and heterochromatin formation (Springer *et al*,

2000; Holding and Springer, 2002; Castellano *et al*, 2004; Collinge *et al*, 2004; Dresselhaus *et al*, 2006).

Here, we addressed whether DNA replication and transcription controls would share regulatory pathways during plant development. We report the identification and characterization of a novel pre-RC-interacting protein with the acronym for Armadillo BTB Arabidopsis protein 1 (ABAP1). ABAP1 is a nuclear G1/early S-specific protein that interacts with members of the pre-RC and the transcription factor AtTCP24 *in vitro* and *in vivo*. We show that the ABAP1–AtTCP24 complex can bind site-specifically DNA regulatory elements and control expression of DNA replication genes *in vivo*. Protein interaction experiments indicated that ABAP1–AtTCP24 might associate with pre-RC subcomplexes or eventually with the full complex in plant cells. The ABAP1–AtTCP24 complex regulates cell proliferation rates during leaf development limiting mitotic DNA replication. ABAP1-overexpressing plants exhibit an overall reduced cell number in leaves and an opposite effect is observed in plants with downregulated levels of ABAP1. Our data demonstrate that ABAP1 function might exemplify a new negative feedback loop regulating mitotic DNA replication during leaf development either by repressing transcription of pre-RC genes and possibly by regulating pre-RC utilization through direct association with pre-RC components.

Results

ABAP1 is a novel plant protein that interacts with the pre-RC

In an attempt to identify protein complexes formed with *Arabidopsis* pre-RC members, a search for pre-RC-interacting proteins was performed. Because AtORC1a harbours two domains involved in transcription regulation—BAH and PHD domains (Masuda *et al*, 2004), its complete open-reading frame was assayed in a two-hybrid screen against an *Arabidopsis* cDNA two-hybrid library. Among the interacting proteins, a novel 737-amino-acid protein (At5g13060) of approximately 81 kDa was identified. It harbours eight predicted repeats of the β -catenin-type Armadillo domain (ARM repeats) in its N-terminal region (amino acids 111–162, 164–212, 213–254, 256–296, 298–338, 340–380, 381–421 and 496–536) as well as a BTB/POZ domain (acronym for Broad complex/Tram-Track/Bric-a-brac/Poxyvirus and zinc-finger), located in the C terminus (amino acids 568–665)

(Figure 1A). Therefore, the gene was designated ABAP1. Yeast two-hybrid assays revealed that ABAP1 interaction with AtORC1a was mediated by the ARM repeats and by AtORC1a N terminus (amino acids 1–341) that contained the BAH and PHD domains (Supplementary Figure 1A). β -Catenin ARM repeats are approximately 40-amino-acid-long tandemly repeated sequence motifs and occur in various

Figure 1 Characterization of ABAP1 protein interactions and localization. (A) Schematic representation of ABAP1 protein domains with the eight β -catenin-type Armadillo (white boxes) and BTB/POZ (oval grey) domains. Nuclear localization signal (NLS) is represented as an asterisk. (B) Subcellular localization of GFP::ABAP1 in roots of 6-day-old *Arabidopsis* seedlings by confocal microscopy. Merged images of rhodamine/DAPI (B1 and B3) and rhodamine/GFP (B2 and B4). White arrowheads in (B1) indicate mitotic cells. (C) GST-pulldown assay between ABAP1-GST and radiolabelled AtORC1a (left panel) and AtORC1b (right panel). (D) GST-pulldown assay with ABAP1-GST and radiolabelled AtCDT1 homologues. (E) GST-pulldown assay with ABAP1-GST and radiolabelled AtORC1a-N'Term and AtORC4. (F) Triple GST pull-down with AtORC1a-GST and radiolabelled ABAP1 and AtCDT1b. (G) Immunoprecipitation of *Arabidopsis* protoplasts expressing ABAP1, AtORC1a-GST, AtORC3-HA and AtCDT1a-FLAG. Anti-ABAP1 immunoprecipitated proteins were assayed with antibodies against ABAP1 and tags.

families of eukaryotic proteins involved in different cellular processes, such as cell–cell adhesion, nuclear import, cell signalling and transcriptional regulation (Coates, 2003; Hatzfeld, 2005). The BTB/POZ domain is usually found in leucine zipper proteins involved in transcriptional repression with a possible role as E3 ligase (Perez-Torrado *et al*, 2006).

In agreement with the presence of a putative nuclear localization signal in ABAP1 N terminus (Figure 1A), confocal and fluorescent microscopy of *Arabidopsis* plants producing GFP::ABAP1 indicated that ABAP1 was exclusively located in the nucleus, homogeneously distributed or enriched in nuclear domains, and GFP::ABAP1 was absent in mitotic cells (Figure 1B). Analyses of endogenous levels of ABAP1 in synchronized *Arabidopsis* cell cultures indicated that it accumulated during G1 and/or early S phase (Supplementary Figure 2B–E). This result was corroborated by the studies on BY2 tobacco cells producing GFP::ABAP1 after treatment with inhibitors of cell cycle progression (Supplementary Figure 2A and Supplementary Video 1).

A genomic search revealed the existence of at least 108 ARM domain and 80 BTB domain proteins in the *Arabidopsis* genome (Mudgil *et al*, 2004; Gingerich *et al*, 2005). ABAP1 has the unique feature to harbour both Armadillo and BTB domains in its primary sequence. *Arabidopsis* has an ABAP1 homologue (At5g19330), characterized earlier as ARIA (ARM repeat protein interacting with ABF2) that shares 59% identity of the deduced amino-acid sequence with ABAP1 and is involved in abscisic acid response through interaction and regulation of the transcription factor ABF2 (Kim *et al*, 2004). Remarkably, no ABAP1 homologue was found in eukaryotes other than plants.

In a yeast two-hybrid assay against all other pre-RC proteins, except MCM2–7, ABAP1 associated directly with AtORC1b, AtCDT1a and AtCDT1b (Supplementary Figure 1B). The interactions between ABAP1 and the pre-RC components were confirmed in GST-pulldown experiments with ABAP1–GST and radiolabelled AtORC1a^{35S}, AtORC1b^{35S}, AtCDT1a^{35S} and AtCDT1b^{35S}. The pre-RC components bound to ABAP1 but not to GST alone (Figure 1C and D). The specificity of the interactions was demonstrated in an assay where ABAP1–GST did not bind to radiolabelled AtORC4^{35S} and bound to AtORC1a N terminus^{35S} (Figure 1E). The formation of a triple complex between AtORC1a, ABAP1 and AtCDT1b was shown in a GST-pulldown experiment with AtORC1a–GST, ABAP1^{35S} and AtCDT1b^{35S} (Figure 1F). As AtCDT1b and AtORC1a did not bind directly, the presence of two radioactive bands indicated that ABAP1 can associate with pre-RC subcomplexes or eventually with the full complex.

The association between ABAP1 and pre-RC was further confirmed *in vivo* by immunoprecipitations with the polyclonal anti-ABAP1 antibody (Supplementary Figure 3) and protein extracts of *Arabidopsis* cell suspension LMM-1 protoplasts that produced ABAP1, AtORC1a–GST, AtORC3–HA and AtCDT1a–FLAG (Figure 1G). In two-hybrid assays with pre-RC proteins, AtORC3 did not interact either with ABAP1 (Supplementary Figure 1B) or with the AtCDT1 homologues, but with all AtORCs, except AtORC1a and itself, possibly having a central function in maintaining ORC associations (Masuda *et al*, 2004). Similar interaction profile of AtORC3 with the other ORC proteins was reported by GST-pulldown experiments (Diaz-Trivino *et al*, 2005). In immunoprecipitation

experiments with anti-ABAP1, ABAP1 interacted *in vivo* with AtORC1a–GST, AtCDT1a–FLAG and also with AtORC3–HA (Figure 1G), suggesting that ABAP1 might associate with pre-RC subcomplexes or eventually with the full complex in plant cells. Possibly, ABAP1 can form different complexes with different affinities to anti-ABAP1, as the immunoprecipitations depleted the interacting AtORC3 and AtCDT1a, but not AtORC1a and ABAP1 itself.

ABAP1 controls cell proliferation in leaves

Studies on localization of ABAP1 expression in plant tissues showed that ABAP1 was weakly expressed in the emerging lateral roots (Figure 2A; Supplementary Figure 2B), being mainly expressed in the shoot apex, young leaves (Figure 2B; Supplementary Figure 2C) and flower buds (Supplementary Figure 4A and D), suggesting a possible function in these organs. Strong GUS expression was detected in developing leaves of 7- and 9-day-old seedlings, as observed for other pre-RC components, such as *PROLIFERA* (AtMCM7), *AtCDC6*, *AtCDT1* homologues and *AtORC1b* (Springer *et al*, 2000; Castellano *et al*, 2001; Castellano *et al*, 2004; Diaz-Trivino *et al*, 2005). As the leaf grew, GUS expression decreased in a tip-to-base gradient (Figure 2C and D). In agreement, ABAP1 mRNA levels were higher in developing leaves during proliferation stage (9-day-old plants) and rapidly declined as observed in leaves of 13 till 21-day-old plants (Figure 2E). GUS expression in the meristemoid and young stomatal cells illustrates ABAP1 expression during stomatal cell differentiation (Supplementary Figure 4E).

To assess the function of ABAP1 during development, plants with increased or reduced expression levels of ABAP1 were characterized. Seventeen lines of *Arabidopsis* plants expressing higher levels of ABAP1 mRNA and protein under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter were generated (here denoted as ABAP1^{OE}). The increase in mRNA and protein levels varied among the different overexpressing lines and the developmental stage, ranging from 5- to 17-fold and 2- to 5-fold, respectively (Supplementary Figure 5A and B). A heterozygous enhancer trap line (ET13614, here denoted as ABAP1^{ET}) with interference T-DNA inserted into the first exon of ABAP1 gene (10 base pairs after the initial ATG) was analysed (Supplementary Figure 6). The plants showed an average of two- and five-fold reduction in ABAP1 mRNA and protein levels, respectively (Supplementary Figure 6A and B). Homozygous plants could not be rescued in ABAP1^{ET}, suggesting that the absence of ABAP1 causes a lethal phenotype (Supplementary Figure 6C).

Comparative phenotype analyses between ABAP1 overexpressor lines (ABAP1^{OE}) and control lines showed that plants with higher levels of ABAP1 developed normally, except that rosette area and leaf growth were moderately diminished during development (Figure 2F–H). Quite the opposite, reduction of ABAP1 levels in ABAP1^{ET} plants caused an increase in the rosette and leaf growth (Figure 2I–K). Kinematics studies of developing leaves showed that cell division rates were higher in ABAP1^{ET} plants and lower in ABAP1^{OE} plants during early leaf development when compared with wild-type controls (Figure 3A and C). Though leaf cell organization (data not shown) and cell sizes were similar to those of control plants, cell numbers were significantly reduced in ABAP1^{OE} and increased in ABAP1^{ET} plants

Figure 2 *ABAP1* expression and function in plants. (A–D) Detection of GUS activity of pro*ABAP1::GUS* in *Arabidopsis*. (A) Emerging lateral root of a 22-day-old plant. (B) Shoot apex of 7-day-old seedling. (C) Leaf of 9-day-old seedling. (D) Leaf of 11-day-old plants. (E) Real-time RT–PCR analyses of *ABAP1* expression in leaf number 2 at different developmental stages. The transcript level is represented as a ratio of the absolute value of the studied gene to the absolute value of *AtUBI14* gene. The data are the result of at least two experiments and representative results are shown. (F–K) Phenotypic analysis of *ABAP1*^{OE} and *ABAP1*^{ET} lines. (F) Time course of average rosette area of wild-type ecotype Col (white circles) and *ABAP1*^{OE} lines (black circles). All trait variables were transformed to Ln. All points of the *ABAP1*^{OE} plants are significantly different from wild type (ANOVA, $P < 0.05$). (G) Three-week-old soil-grown wild-type Col (upper panel) and *ABAP1*^{OE} plants (lower panel). (H) Leaf number 4 of 4-week-old wild-type Col (upper leaf) and *ABAP1*^{OE} (lower leaf) plants. (I) Time course of average rosette area of wild-type ecotype Ler (white circles) and *ABAP1*^{ET} lines (black circles). All trait variables were transformed to Ln. All points of the *ABAP1*^{ET} plants are significantly different from wild type (ANOVA, $P < 0.05$). (J) Three-week-old soil-grown wild-type Ler (upper panel) and *ABAP1*^{ET} plants (lower panel). (K) Leaf number 4 of 4-week-old wild-type Ler (upper leaf) and *ABAP1*^{ET} (lower leaf) plants.

(Figure 3E). Our data suggested that *ABAP1* negatively regulated overall cell divisions in leaves, differing from the pre-RC subunits *AtCDT1* and *AtCDC6* that could specifically affect stomata formation (Castellano *et al*, 2004). An effect of *ABAP1* levels in early leaf development is consistent with its high expression levels in shoot meristem and young leaves.

As *ABAP1* interacted with members of the pre-RC and accumulated at G1 and early S phase of the cell cycle, the cell proliferation phenotypes observed in *ABAP1*^{OE} and *ABAP1*^{ET} leaves could be at least partly triggered by a mis-regulation of the DNA replication machinery. Cell proliferation assays with [³H]thymidine in *ABAP1*^{OE} and *ABAP1*^{ET} seedlings showed that the DNA replication levels were significantly lower in *ABAP1*^{OE} plants than those in the wild-type plants (Figure 3B). In contrast, *ABAP1*^{ET} seedlings showed higher levels of DNA replication than wild type (Figure 3D). A direct role of *ABAP1* in pre-RC assembly and association with chromatin was investigated by measuring MCM loading onto chromatin, the hallmark step in the formation of the pre-RC. Cells were separated into cytoplasmic, nucleus-soluble and chromatin-enriched fractions and levels of MCM7 (PROLIFERA) and *ABAP1* were assayed by immunoblot analysis (Figure 3F). PROLIFERA association with chromatin was lower in 6-day-old *ABAP1*^{OE} plants than in wild-type plants, and the excess of *ABAP1* was mostly found as a soluble nuclear protein. When *ABAP1* levels were reduced in *ABAP1*^{ET}, higher levels of PROLIFERA were found loaded onto chromatin. Ploidy levels in developing leaves of *ABAP1*^{OE} plants were similar as control, suggesting that *ABAP1* might have a function in mitotic replication but not in endocycles (Supplementary Figure 7). Altogether, the data on the characterization of plants with modified levels of *ABAP1* indicated that *ABAP1* exert a negative role in cell proliferation in leaves, possibly by inhibiting mitotic DNA replication.

***ABAP1* interacts with plant transcription factors, the complex binds DNA and regulates gene expression**

To unravel protein complexes in which *ABAP1* takes part, a yeast two-hybrid screen under high stringency selection conditions was performed with an *Arabidopsis* cDNA library as a bait. Several transcription factors, belonging to the NAC, AP2 and TCP families (unpublished results) were identified. One of the *ABAP1*-associated transcription factors was *AtTCP24* (At1g30210) that belongs to the class-II TCP transcription factor family (Cubas *et al*, 1999), the members of which negatively regulate plant cell proliferation and leaf morphogenesis (Nath *et al*, 2003; Palatnik *et al*, 2003).

To address a possible cooperation of *ABAP1* and *AtTCP24* in the control of gene transcription, the association between both proteins was characterized. The *AtTCP24*–*ABAP1* interaction was confirmed in a GST-pulldown assay with *AtTCP24*–GST and radiolabelled *ABAP1*^{35S}. *ABAP1* bound to *AtTCP24*–GST but not to GST alone (Figure 4A). *In vivo* interaction was observed in immunoprecipitation experiments of protein extracts of *Arabidopsis* cell suspension LMM-1 protoplasts that produced *ABAP1* and *AtTCP24*–FLAG, with the anti-*ABAP1* antibody (Figure 4B). The ability of the *ABAP1*–*AtTCP24* heterodimer to recognize the consensus sequence for class-II TCP (TGGGCC/T) (Trémousaygue *et al*, 2003) was confirmed in electrophoretic mobility shift assays (EMSAs) (Supplementary Figure 8).

The data demonstrated that the association of ABAP1 with AtTCP24 did not affect the DNA-binding properties of the latter and that ABAP1 could participate in mechanisms of transcriptional regulation. Other class-II TCP members (AtTCP3, AtTCP5, AtTCP13 and AtTCP17) did not interact

with ABAP1 in yeast two-hybrid assays (Supplementary Figure 9A), indicating some specificity in the ABAP1 association with class-II TCP family.

To investigate a possible role of ABAP1 in the control of gene expression, putative target genes regulated by ABAP1–AtTCP24 complex were examined by searching for class-II TCP-interacting motifs in the promoters of *Arabidopsis* genes. Surprisingly, four boxes for class-II TCP were found in the 500 bp upstream of the initial methionine of the *AtCDT1a* promoter region, making it a good candidate for AtTCP24-mediated expression. One class-II TCP box was also found in the *AtCDT1b* promoter. In EMSA performed with the TCP consensus motif of *AtCDT1a* (ATGGGCCT, –215 to –223) and its homologue *AtCDT1b* (ATGGGCC, position –129 to –137), both AtTCP24 and AtTCP24–ABAP1 were associated with the wild type (Figure 4C, lanes 1, 5, 10 and 14) but not with the mutated probes (Figure 4C, lanes 4, 9, 13 and 18). ABAP1 alone was associated with neither of the probes (Figure 4C, lanes 6 and 15). The presence of AtTCP24–GST and ABAP1 in the protein–DNA complex was confirmed by a supershift assay with anti-ABAP1 and anti-GST antibodies (Figure 4C, lanes 3, 7, 8, 12, 16 and 17). The data indicate that both *AtCDT1a* and *AtCDT1b* expression might be regulated by ABAP1–AtTCP24 complex. To test this hypothesis, we first confirmed that ABAP1 associates with *AtCDT1* promoters *in vivo* in chromatin immunoprecipitation (ChIP) experiments with anti-ABAP1 antibody (Figure 4F). The results revealed that ABAP1 was associated with regions of the *AtCDT1a* and *AtCDT1b* promoters harbouring the TCP consensus motif. This interaction was site-specific as ABAP1 was not detected in a 350-bp region inside the *AtCDT1a*-coding region. In addition, *AtCDT1a* and *AtCDT1b* mRNA levels were analysed in 6-day-old *ABAP1^{OE}* and *ABAP1^{ET}* versus wild-type seedlings by real-time PCR (Figure 4D and E). Both genes were downregulated in plants with higher levels of ABAP1 mRNA and protein (Figure 4D) and they were upregulated in plants where ABAP1 mRNA and protein levels were diminished (Figure 4E). These data suggest a direct effect of ABAP1 on *AtCDT1a* and *AtCDT1b* expression.

These results indicate that ABAP1 might exert a function together with transcription factors to regulate cell division in plants. Various class-II TCP genes have been shown to negatively regulate cell proliferation in leaves (Nath *et al*, 2003; Palatnik *et al*, 2003); nevertheless, their mechanism of

Figure 3 ABAP1 function in DNA replication control. (A, C) Kinematic analysis of cell division rates of (A) wild-type Col (black circles) and *ABAP1^{OE}* (white circles) rosette leaves and (C) wild-type Ler (black circles) and *ABAP1^{ET}* (white circles). (B, D) [³H]Thymidine incorporation assay with (B) wild-type Col and *ABAP1^{OE}* plants and (D) wild-type Ler and *ABAP1^{ET}* plants. *ABAP1^{OE}* and *ABAP1^{ET}* [³H]thymidine incorporation are significantly different from wild type (Student's *t*-test, *P* < 0.05). (E) Number and size of cells per leaf in leaf number 2 of wild-type (Col), *ABAP1^{OE}*, wild-type (Ler) and *ABAP1^{ET}* 21-day-old plants estimated by kinematic analyses. (F) Chromatin fractionation assay. Pipeline of assay (upper panel, left). S1, first low-speed supernatant containing non-chromatin-binding proteins; P1, crude chromatin pellet; S2, supernatant from nuclear fraction; P2, high-speed pellet containing polynucleosomes. Western blot of the fractions obtained from wild-type Col, *ABAP1^{OE}*, wild-type Ler and *ABAP1^{ET}* were assayed with anti-PROLIFERA and anti-ABAP1 antibodies (lower panel) and PROLIFERA P2 band intensity was quantified (upper panel, right). As loading control, a Ponceau staining of the nitrocellulose membrane is shown.

Figure 4 Characterization of ABAP1 and AtTCP24 transcription factor interaction and ABAP1 function in transcription. **(A)** Confirmation of ABAP1–AtTCP24 interaction through the *in vitro* GST-pulldown interaction assay. **(B)** Immunoprecipitation of protein extracts of *Arabidopsis* protoplasts producing ABAP1 and AtTCP24–FLAG. Total protein extracts were immunoprecipitated with anti-ABAP1 and analysed in a protein gel blot with antibodies against FLAG and ABAP1. **(C)** EMSA of ABAP1, AtTCP24 and the heterodimer AtTCP24–ABAP1 with wild-type (WT) and mutated (mut) radiolabelled probes of *AtCDT1b* and *AtCDT1a* promoter regions harbouring TCP recognition box. Anti-GST and anti-ABAP1 were used for ABAP1 and AtTCP24–GST supershift assays (lanes 3, 7–9 and 12, 16–18). **(D, E)** Real-time RT–PCR analyses of *AtCDT1a* and *AtCDT1b* expression in **(D)** wild-type Col (white bars) and ABAP1^{OE} 6-day-old seedlings (black bars), and **(E)** in wild-type Ler (white bars) and ABAP1^{ET} 6-day-old seedlings (black bars). The transcript levels are represented as a ratio of the absolute value of the studied gene to the absolute value of *AtUBI14* gene. Gene expression was normalized by values obtained for wild-type controls. Asterisks indicate values statistically different from the wild type (Student's *t*-test, *P* < 0.05). Inset in **(D, E)**, western blot analysis using anti-ABAP1 in total protein extracts of ABAP1^{OE} and wild-type Col 6-day-old seedlings; and ABAP1^{ET} and wild-type Ler 6-day-old seedlings, respectively. **(F)** Chromatin immunoprecipitation of 14-day-old wild-type Col leaves with anti-ABAP1. In upper panel, schematic representation of the amplified promoter regions of *AtCDT1a* and *AtCDT1b*; *AtCDT1a* CDS amplifies the coding sequence of *AtCDT1a*; arrows indicate each primer set used in PCR; TCP consensus motif is represented as an asterisk. In lower panel left side, PCR results using primer sets for *AtCDT1a* and *AtCDT1b* promoter regions and *AtCDT1a* coding sequence; and right side, graph of PCR band intensities normalized using lane 2.

action is still not understood. Because cell division rates were lower in ABAP1^{OE} plants and higher in ABAP1^{ET} plants, one hypothesis is that, during leaf development, ABAP1 might collaborate with AtTCP24 in mediating repression of genes that promote cell proliferation, such as *AtCDT1*. If so, down-regulation of *AtCDT1* could be one of the pathways used by class-II TCP genes to negatively regulate cell division. To test

this hypothesis, plants overexpressing *AtTCP24* (*AtTCP24*^{OE}) were generated (LM Cabral, unpublished data). Remarkably, these plants exhibited a phenotype similar to that of ABAP1^{OE} plants showing reduced rosette areas and smaller leaves with normal cell sizes but fewer cells (Supplementary Figure 9B–D), pointing to an inhibition of cell proliferation in *AtTCP24*^{OE} leaves. Gene expression analyses of 9-day-old *AtTCP24*^{OE}

seedlings with real-time RT-PCR showed that *AtCDT1a* and *AtCDT1b* levels were downregulated in *AtTCP24^{OE}* plants (Supplementary Figure 9E). These data are consistent with ABAP1 and AtTCP24 functioning together during leaf development, to negatively regulate *AtCDT1a* and *AtCDT1b* expression levels.

The ABAP1–AtTCP24 heterodimer associates with pre-RC members and the complex binds DNA

To test whether ABAP1 could have a dual function in mechanisms controlling DNA replication and transcription, or

whether it executes separate functions in these complexes, we addressed if the ABAP1–AtTCP24 complex could associate with pre-RC members in plants. The formation of a triple complex between AtORC1a, ABAP1 and AtTCP24 was shown *in vitro* in a GST-pulldown experiment with AtORC1a–GST, ABAP1^{35S} and AtTCP24^{35S} (Figure 5A). A triple complex between ABAP1, AtTCP24 and AtCDT1b was also observed in a GST-pulldown experiment with AtTCP24–GST, ABAP1^{35S} and AtCDT1b^{35S} (Figure 5B). As AtTCP24 did not bind directly to AtORC1a or AtCDT1b (Figure 5A, lane 5 and Figure 5B, lane 1), the presence of two radioactive bands

Figure 5 ABAP1–AtTCP24 heterodimer associates with pre-RC members and the complex binds DNA. (A) *In vitro* GST-pulldown assay between AtORC1a–GST and radiolabelled ABAP1 and AtTCP24. (B) *In vitro* GST-pulldown assay between AtTCP24–GST and radiolabelled ABAP1 and AtCDT1b. (C) GST-pulldown competition assay using ABAP1–GST, radiolabelled AtTCP24^{35S} and increasing amounts of AtORC1a–GST. Excess of free GST was added as control for nonspecific binding. (D) *In vivo* GST pulldown between AtORC1a–GST, ABAP1, AtTCP24–CBP, AtORC3–HA and AtCDT1b–FLAG. Protoplasts transformed with AtORC1a–GST, AtTCP24–CBP, AtORC3–HA and AtCDT1b–FLAG were purified in a GST column and tested for the presence of recombinant proteins with tag-specific antibodies. (E) EMSA of the AtTCP24–ABAP1–AtORC1a complex with wild-type (WT) and mutated (mut) radiolabelled probes of *AtCDT1b* and *AtCDT1a* promoter regions harbouring TCP recognition box. Anti-ABAP1 was used for supershift assays (lanes 5 and 11). Arrows indicate the different delays in mobility.

indicated that ABAP1 might work as a bridge connecting pre-RC proteins and AtTCP24 in a complex. The ability of ABAP1 to bind both AtTCP24 and AtORC1a concomitantly was further tested in a GST-pulldown competition assay, where increasing amounts of AtORC1a-GST were mixed with ABAP1-GST and AtTCP24^{35S} (Figure 5C). There were no differences in binding intensity between ABAP1 and AtTCP24 in the absence or presence of either 2.5, 10 and 50 µg of AtORC1a-GST, suggesting that there was no competition between AtORC1a and AtTCP24 for ABAP1-binding sites. The association of ABAP1 with AtTCP24 and pre-RC proteins in the same complex *in vivo* was revealed in a GST-pulldown experiment with protein extracts from protoplasts transformed with AtORC1a-GST, AtTCP24-CBP, AtCDT1a-FLAG and AtORC3-HA (Figure 5D, lane 4). The specificity of the interactions was demonstrated in an assay where ABAP1, AtORC1a-GST, AtTCP24-CDB, AtCDT1a-FLAG and AtORC3-HA were not rescued when an excess of free GST was added prior to column purification (Figure 5D, lane 2). In addition, an EMSA supershift assay revealed that the triple complex formed by AtTCP24, ABAP1 and AtORC1a was still able to recognize and bind to the TCP consensus motif of *AtCDT1a* and *AtCDT1b* promoters (Figure 5E). Neither AtORC1a alone nor AtORC1a-ABAP1 was associated with *AtCDT1a* and *AtCDT1b* probes (data not shown). The triple complex formed by AtORC1a, AtTCP24 and ABAP1 interacted with the wild type but not with the mutated probes of *AtCDT1a* and *AtCDT1b* promoters, as the migration of the probes in the gel was delayed, indicating the presence of the triple complex interacting with the DNA probes (Figure 5E, lanes 1 and 7). The presence of ABAP1 in the complex was confirmed by an extra shift in the gel when anti-ABAP1 antibody was added (Figure 5E, lanes 5 and 11).

Discussion

ABAP1 is a novel pre-RC-interacting protein involved in DNA replication and gene transcription controls

Pre-RC assembly, an important event that regulates G1-to-S transition in eukaryotes (Blow and Dutta, 2005), has led many groups to study which elements define a replication origin and how they are activated during development (Costa and Blow, 2007). Nevertheless, few studies have been carried out to understand the molecular mechanisms involving other proteins that control pre-RC assembly and functioning, and how it is coupled with developmental signalling pathways. In addition to the regulatory mechanisms involving protein degradation and CDK activity (Arias and Walter, 2007), few proteins were shown to interact with pre-RC and directly regulate DNA replication (Nishitani and Lygerou, 2004; Iizuka *et al*, 2006). Here, we identified and characterized a novel pre-RC interactor—ABAP1—and we provided data on a dual role of ABAP1 in DNA replication and transcription controls. Our data demonstrate that ABAP1 associates with pre-RC members and the transcription factor AtTCP24 exert a role in a negative feedback loop regulating mitotic DNA replication during leaf development either by reducing *AtCDT1* expression mediated by AtTCP24 or possibly by direct association with pre-RC.

Several experimental evidences support a role of ABAP1 in DNA replication and cell cycle progression. ABAP1 directly interacts with pre-RC components *in vitro*, and the *in vivo*

protein interaction data suggest that ABAP1 might associate with pre-RC subcomplexes or eventually with the full complex in plant cells. ABAP1 is exclusively located in the nucleus and is highly regulated during the cell cycle, accumulating in G1 and early S. Our results illustrated reduced cell division rates in *ABAP1^{OE}* plants and an opposite phenotype in *ABAP1^{ET}* plants, suggesting that ABAP1 has a negative function in cell proliferation in leaves. A more direct role on DNA replication was demonstrated by decreased levels of thymidine incorporation and reduced pre-RC loading onto chromatin in *ABAP1^{OE}* plants and a reverse observation in *ABAP1^{ET}* plants. Altogether, the data indicate that ABAP1 has a function as an inhibitor of DNA replication.

Strong evidence for a role of ABAP1 in gene expression control comes from the identification of different putative transcription factors that associate with ABAP1, of which some are members of families known to be involved in the regulation of different developmental programmes, such as AtTCP24. Although ABAP1 houses two domains that are involved in transcriptional regulation, ARM repeats and BTB, it does not exhibit any clear DNA-binding signature. Therefore, ABAP1 might exert a function by taking part in transcriptional protein complexes, together with transcription factors and other regulators. The site-specific binding of the ABAP1-AtTCP24 complex with DNA regulatory elements further corroborates this hypothesis.

The relevance of the interaction between ABAP1 and AtTCP24 was investigated by assessing possible gene targets of the complex *in vivo*. The presence of TCP-binding motifs in the promoter regions of *Arabidopsis* *CDT1* homologues, together with the site-specific binding of the ABAP1-AtTCP24 complex with these DNA regulatory elements in EMSA, indicates that *AtCDT1a* and *AtCDT1b* might be targets of ABAP1-AtTCP24 regulation. Confirming this hypothesis, ChIP experiments have shown that ABAP1 binds to promoter regions of the *AtCDT1* homologues and the levels of *AtCDT1a* and *AtCDT1b* mRNA are lower in *ABAP1^{OE}* and higher in *ABAP1^{ET}* plants than those in wild-type plants, indicating that ABAP1 regulates AtTCP24 targets *in vivo*. A similar effect on *AtCDT1* gene expression occurs in plants overexpressing *AtTCP24*.

ABAP1 and AtTCP24 function together regulating cell proliferation rates during leaf development

TCP family of transcription factors has a central function in leaf development, transcriptionally regulating genes involved in the cell cycle and thus defining marginal configuration and the overall leaf geometry (Li *et al*, 2005; Barkoulas *et al*, 2007). Class-II TCP genes from *Antirrhinum majus* and *Arabidopsis* have been shown to be negative regulators of cell proliferation (Nath *et al*, 2003; Palatnik *et al*, 2003). This observation, together with our findings, corroborates the hypothesis that ABAP1 and AtTCP24 might work together to repress cell division in specific developmental contexts. Because class-I TCP factors have been reported earlier to stimulate growth and positively regulate expression of the cell cycle regulator *CYCB1;1* (Kosugi and Ohashi, 2002; Li *et al*, 2005), organ growth might be controlled by the balance of opposite activities of class-I and -II TCPs (Li *et al*, 2005). The mechanism by which class-II TCP factors negatively regulate cell proliferation might involve the repression of pre-RC gene expression such as *CDT1*. Both class-I and -II

TCP factors probably require interaction with other proteins to activate or repress transcription (Kosugi and Ohashi, 2002; Li *et al*, 2005). Interaction with ABAP1 might represent one mechanism to assist repression of gene expression mediated by class-II TCP factors. During leaf development, ABAP1–AtTCP24 complex might be part of a mechanism to maintain homeostasis of AtCDT1a and AtCDT1b levels, and assure proper rates of cell division.

In *Arabidopsis*, as mitotic divisions cease in leaves from tip to base, subsequent growth depends primarily on cell expansion and differentiation accompanied by endocycles of DNA replication (Donnelly *et al*, 1999; Beemster *et al*, 2005). Ectopic expression of *AtCDT1a* was shown to increase ploidy levels in leaves (Castellano *et al*, 2004). Nevertheless, *ABAP1^{OE}* leaves did not show reduced ploidy levels compared with wild-type plants, suggesting that ABAP1 has a role controlling only mitotic DNA replication in leaves. Corroborating this hypothesis, ABAP1 expression in leaves decreases from tip to base and is opposite to the increase in rates of cell expansion and endocycles.

ABAP1 mechanism of action connects gene transcription and DNA replication controls

Our data raise the intriguing observation that ABAP1 function might exemplify a new negative feedback loop between cell proliferation and control of DNA replication by both repressing transcription of pre-RC genes and possibly by regulating pre-RC utilization through direct association with pre-RC components (Figure 6). The conception of this dual role in the control of pre-RC could represent a strategy to ensure an efficient repression of cell cycle progression at G1-to-S transition. This model for the action mechanism of ABAP1 has similarities to another G1-to-S regulator, the retinoblastoma (RB). RB negatively regulates cell cycle progression through interaction with the transcription factor E2F/DP, inhibiting transcriptional activation of E2F/DP target genes, including pre-RC genes (Bracken *et al*, 2004). In *Drosophila*, RB-E2F associates with DmORC at replication origins, limiting DNA replication (Bosco *et al*, 2001). RB was also linked to the Myb transcription factor, forming a protein complex that participates in cell differentiation through transcription regulation (Lewis *et al*, 2004).

In multicellular eukaryotes, proteins called geminins have also been implicated in the regulation of cell proliferation and cell differentiation by interacting with components of the pre-RC (CDT1) and with transcription factors (Six and Hox) (Luo *et al*, 2004; del Bene *et al*, 2004). The interaction of geminin with CDT1 inhibits replication licensing, by recruiting this protein and avoiding the complete pre-RC assembly. Binding with the transcription factors can displace geminin from CDT1, allowing proliferation to occur. Curiously, so far no putative geminin homologue could be found in plants. In *Arabidopsis*, a CDT1 interactor GEM was identified, the mechanism of action of which shares similarities with that of geminins (Caro *et al*, 2007). GEM mediates histone H3 modifications and binds to TTG1—member of a transcription regulator complex that modulates the expression of the homeobox GLABRA (GL2) gene involved in root epidermal cell fate. Competition of GEM for CDT1 and TTG1 might determine GL2 expression and cell division control (Caro *et al*, 2007). Our data indicated that transcription factors do not compete with pre-RC components for binding ABAP1, but

Figure 6 Proposed model for ABAP1 role in a negative feedback loop to control mitotic DNA replication. Transcription factors, and maybe also ABAP1, sense intra- and extracellular signals triggering changes in gene expression patterns. In AtTCP24-mediated signaling, ABAP1 associates with AtTCP24 and controls AtCDT1a/b homeostasis by enhancing transcription repression of *AtCDT1a/b*. Lower levels of AtCDT1a and AtCDT1b lead to defects on pre-RC assembly and/or functioning, limiting DNA replication, consequently blocking cell cycle progression at G1 to S. ABAP1 also directly interacts with AtORC1a/b and AtCDT1 homologues, so ABAP1 might possibly hamper DNA replication through direct interaction with pre-RC.

could possibly participate in the same complex, implying different mechanisms for ABAP1 and geminin function.

In other experimental models, transcription factors have been found to be part of protein complexes that bind to replication origins that control DNA replication. Transcription factors could exert an effect redundantly to recruit the general replication machinery for site-specific origin utilization during replication, facilitating pre-RC localization and formation (Danis *et al*, 2004; MacAlpine *et al*, 2004). Conversely, in *Drosophila*, E2F–RB interaction with the pre-RC inhibits ORC to bind origins, limiting DNA replication initiation at origins (Bosco *et al*, 2001). Finally, it is possible that both machineries are working together in processes other than DNA replication licensing, such as gene regulation or establishment of heterochromatin (Suter *et al*, 2004; Sasaki and Gilbert, 2007). In this scenario, our data did not exclude that ABAP1 can be shared between replication licensing and transcription machineries to exert a dual function in these processes. EMSA revealed that AtORC1a is also present in the AtTCP24–ABAP1 complex bound to the class-II TCP element. Furthermore, protein interaction experiments indicated that AtTCP24–ABAP1 might associate with pre-RC subcomplexes or eventually with the full complex in plant cells.

Therefore, ABAP1 appears to be involved in the regulation of two vital cellular processes—replication and transcription. Transcription factors are well-known targets of signal transduction that regulates development; and pre-RC assembly and function are essential requirements for cell cycle progression. Therefore, we propose that ABAP1 participates in a

novel signalling network that controls cell cycle progression in plants, by integrating plant developmental signals with DNA replication and transcription controls (Figure 6). The identification of ABAP1 specifically in plants raises the question whether this signalling pathway has evolved to satisfy peculiar strategies of the plant development. ABAP1 probably has different functions in plant development, depending on the proteins it interacts with. Future biochemical analysis of multiprotein complexes containing ABAP1 will be important to better understand the complexity of the mechanisms that lead to the precise balance of cell division and cell differentiation in various plant morphogenetic events.

Materials and methods

Expression analyses

Expression analyses using real-time PCR, transgenic plants production, GUS activity assay and subcellular localization in *Arabidopsis* are described in Supplementary data.

Analysis of proABAP1::GUS, 35S::GFP::ABAP1, ABAP1^{OE} and ABAP1^{ET} plants

Transgenic lines were identified by selection in 50 mg/l kanamycin in germination medium (for proABAP1::GUS and GFP::ABAP1) or by enhanced green fluorescence (for ABAP1^{OE} and AtTCP24^{OE} plants). At least, 10 transgenic lines were analysed for each construct. ABAP1^{ET} belongs to the Cold Spring Harbor Enhancer trap bank (seed code ET13614). Lines were selected in 50 mg/l kanamycin in germination medium. Thirty plant genotypes were determined by PCR with specific primers for Enhancer Trap DS element and for ABAP1. Primers ABAP1 5 and ABAP1 3, DS5-4 and ABAP1 3 were used for WT and DS insertion alleles, respectively (Supplementary data—constructs). For details on phenotypic analysis of ABAP1^{OE} and ABAP1^{ET} plant, see Supplementary data.

Yeast two-hybrid assay

Yeast two-hybrid screening of an *Arabidopsis* cDNA expression library was carried out according to De Veylder *et al* (1999). For details, see Supplementary data.

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In vitro and in vivo protein interaction assays

ABAP1-GST, AtTCP24-GST and AtORC1a-GST were produced in cells of *Escherichia coli* strain BL21 (Supplementary data). *In vitro* transcription and translation of ³⁵S-methionine (GE Healthcare) was performed using the TNT Quick Coupled Transcription/Translation Systems (Promega) according to the supplier's instructions. GST-pulldown analyses were carried out according to Tarun and Sachs (1996). Protoplast transformation, immunoprecipitation and protein gel blots were carried out by standard techniques, according to protocols described in the Supplementary data.

EMSA

EMSAs were performed as described earlier (Kosugi and Ohashi, 1997) with modifications. For details, see Supplementary data.

Protein chromatin-binding assay

Wild-type, ABAP1^{OE} and ABAP1^{ET} plants were collected at 6 days after sowing and submitted to lysis in liquid nitrogen. Plant cell lysates and chromatin fractionation were performed as described by Mendez and Stillman (2000), with minor adaptations. For details, see Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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