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Genome based identification and analysis of the pre-replicative complex of *Arabidopsis thaliana*

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Abstract Eukaryotic DNA replication requires an ordered and regulated machinery to control G1/S transition. The formation of the pre-replicative complex (pre-RC) is a key step involved in licensing DNA for replication. Here, we identify all putative components of the full pre-RC in the genome of the model plant *Arabidopsis thaliana*. Different from the other eukaryotes, *Arabidopsis* houses in its genome two putative homologs of *ORC1*, *CDC6* and *CDT1*. Two mRNA variants of *AtORC4* subunit, with different temporal expression patterns, were also identified. Two-hybrid binary interaction assays suggest a primary architectural organization of the *Arabidopsis* ORC, in which AtORC3 plays a central role in maintaining the complex associations. Expression profiles differ among pre-RC components suggesting the existence of various forms of the complex, possibly playing different roles during development. In addition, the expression of the putative pre-RC genes in non-proliferating plant tissues suggests that they might have roles in processes other than DNA replication licensing.

Keywords: Pre-replication complex; DNA replication; ORC; CDT1; CDC6; *Arabidopsis thaliana*

1. Introduction

Chromosomal DNA replication is a key event for an equal division of genetic material into two daughter cells. In this process, cells make one complete copy of their genetic material before cell division, restricting DNA replication to once per cell cycle. In eukaryotes, DNA replication involves ordered and regulated steps, with many proteins controlling the G1/S phase transition. Licensing DNA for replication requires binding of the origin recognition complex (ORC) – a complex of six conserved subunits ORC1–ORC6 – to replication origins during the cell cycle [1–3]. In an early step of G1, a pre-replicative complex (pre-RC) is formed as other factors bind to ORC, which functions as a landing platform. The recruitment of CDC6/CDC18 by ORC is the next step in pre-RC assembly, followed by the recruitment of CDT1/DUP onto pre-RC [4]. In addition, CDC6/CDC18 and CDT1/DUP proteins act synergistically to load a complex formed by six proteins – the MCM (MCM2–7) complex (Mini Chromosome Maintenance) – and catalyze the assembly of pre-RC which licenses DNA to replicate [4].

In plants, the process of cell division is essential for growth and is nearly a synonym of development, since together with cell expansion and cellular differentiation, it leads to the formation of organs with different shapes and different cell types. As in other eukaryotic cells, the basic plant cell cycle seems to progress in an ordered and highly regulated fashion controlled by a conserved molecular machinery [5,6]. However, little is known about the specific controls governing initiation of DNA replication in plants. The identification of components of the pre-RC in some plant species suggests that the complex might function in a conserved way among other eukaryotes, although a full replication complex has not yet been reported for plants [7–12]. Since plants adopt particular strategies of development, distinct from those of other eukaryotes, external controls that regulate individual steps of the basic cell cycle may be different [13,14]. Indeed, it is already well known that controls coupling DNA replication with mitosis are quite flexible in plants. During development, plant cells often modify their classical cell cycle and undergo endoreduplication events that allow them to increase their ploidy level [15]. However, the consequences of this modified cell cycle for plant development are not completely understood.

Comprehension of plant developmental strategies requires knowledge of the basic molecular machinery controlling initiation of DNA replication. This report is the first that identifies genes from all components of the pre-RC in the model dicotyledonous plant *Arabidopsis thaliana*. Overall, our data support the idea of a pre-RC that has been conserved in the course of evolution. Nevertheless, differing from other eukaryotes studied so far, *Arabidopsis* houses in its genome two putative homologs of *ORC1*, *CDT1* and *CDC6*. To investigate possible physical interactions among the *Arabidopsis* ORC subunits, yeast two hybrid assays were performed. The results indicate that AtORC2 interacts with AtORC3 and AtORC4b, and that AtORC3 interacts with all the other AtORCs, except AtOR-

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C1a and itself. The expression patterns of the *ORC* genes and of the two homologs of *CDT1* and *CDC6* were analyzed by RT-PCR and mRNA in situ hybridization. Interestingly, the expression of individual components of the pre-RC is differentially modulated, suggesting that different forms of the complex might coexist in plants, possibly performing different functions. In addition, the expression patterns of the putative pre-RC genes in *Arabidopsis* suggest that they might have roles in processes other than DNA replication licensing.

2. Materials and methods

2.1. Plant material

Seeds of *A. thaliana* ecotype Columbia were germinated in Murashige and Skoog (MS) medium supplemented with 1% sucrose at pH 5.7 under a 12 h photoperiod at 20 °C. Seedlings were harvested 20 days after sowing to collect roots and leaves for RNA expression assay. Inflorescence stems, flower buds, open flowers and siliques were harvested from a pool of 15 plants grown in soil and vermiculite (2:1) cultivated in an in vivo growth chamber. Sterile root cultures were established by culturing 3-week-old *Arabidopsis* seedlings (grown in vitro) in liquid MS medium containing 3% sucrose, at 20 °C with constant agitation. *Arabidopsis* ecotype Landsberg erecta L-MM1 suspension cells were cultivated in Murashige and Skoog minimal organic (MSMO) medium supplemented with 3% sucrose, 500 μg/l 1-naphthaleneacetic acid (NAA) and 50 μg/l kinetin at 20 °C with constant agitation. For the cell cycle blockers assay, root cultures were treated with 30 μM oryzalin or 10 mM hydroxyurea in the same medium and conditions for 48 h. Alternatively, L-MM1 suspension cells were treated with 15 μM oryzalin, or 10 mM hydroxyurea in the same medium and conditions for 8 h. To test the sucrose response, suspension cultures were transferred to fresh medium depleted of sucrose for three days and then transferred again to fresh medium with or without sucrose for 6 or 12 h.

2.2. In silico analysis: annotation of Arabidopsis thaliana pre-RC genes

The genome of *A. thaliana* (www.arabidopsis.org) was searched for homologs of pre-RC complex genes using BLAST [16]. Alignment of pre-RC gene sequences from *Arabidopsis* and its homologs in monocot plants and in other eukaryotic organisms was carried out using the ClustalW algorithm [17]. Phylogenetic analysis was performed with the Mega2 program [18] and a bootstrap analysis using the neighbor joining method with 1000 replications was employed. The DNA sequences were translated into hypothetical proteins, whose theoretical characteristics were obtained using several programs in the ExPASy (Expert Protein Analysis System) server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/). Protein sequences were entered into MotifScan (pattern searches), ProDOM (protein domain identification), Interpro (protein domain and pattern search identification), NetPhos (prediction sites for phosphorylation) and PESTfind (identification of PEST sequences). Putative regulatory promoter elements of pre-RC genes were searched by computer analysis of the putative promoter region (1500 bp upstream of the translation start codon) with the program PLACE (plant *cis*-acting regulatory domain identification). Gene duplications were predicted using "Paralagons in *A. thaliana*" (http://wolfe.gen.tcd.ie/athal/dup).

2.3. Real time PCR

Total RNA was extracted from the frozen materials according to [19]. After treatment with RNase-free DNaseI (0.5 u/μg RNA), total RNA (7.5 μg) was transcribed using the primer dT of the First-Strand cDNA Synthesis Kit according to the manufacturer's protocol (Amersham Biosciences). Oligonucleotides used for real time RT-PCR were designed in gene specific regions of each gene (*AtORC1a*, *AtORC1b*, *AtORC2*, *AtORC3*, *AtORC4a*, *AtORC4b*, *AtORC5*, *AtORC6*, *AtCDT1a*, *AtCDT1b*, *AtCDC6a* and *AtCDC6b*), using Primer Express 2.0 (Perkin–Elmer Applied Biosystems, Foster City, CA) or Primer3 softwares: GGTTGGCCAATTACTTGTGA (orc1a.fwr), AATCAAACTGTGTTACATTATCAATCTC (orc1a.rev), TCACTTCGGATGCAATTCGA (orc2.fwr), CATATCAGAAATACAATTTCAGGCTACTG (orc2.rev), GCCGATCCTCCGCAATG (orc3.fwr), TCTGCTGTGTCGCTGGAATT (orc3.rev), GGCATTTGAGCACCTGAGAGA (orc4a.fwr), CTCCTGTCTGAGACTGTCCTCTATTCT (orc4a.rev), TGCCTAAGGGAGAATAGAGGA (orc4b.fwr), GGTCCAATAACTTGGGAAGG (orc4b.rev), GCGGAAGCTGCCCATTAG (orc5.fwr), TGAGCCAGATCTTCAGAGACCAT (orc5.rev), AGAATCCAGAGAAAGGAACTAAGAGAGT (orc6.fwr), GGTCTCAGATTTCTTTGGGAAA (orc6.rev), AAATGTCGACTGCCGAAACAG (Cdt1a.fwr), AAGTGAAATGTCATGTGAAGTTGCTT (Cdt1a.rev), AATCCGATCACGTCTTGAAGAAG (Cdt1b.fwr), GAACCACGATCTCAAGAAAGCA (Cdt1b.rev), GATTCTTCCGCAA-CTGTCTTCTATG (cdc6a.fwr), GAATGAAGGAACCAACCCATCTA (cdc6a.rev), TTGCTCTGCAGGTAAACAGC (cdc6b.fwr), CACAGTAGACAGTTGCGGAAA (cdc6b.rev), TCACTGGAAAGACCATTACTCTTGAA (ubi14.fwr), AGCTGTTTTCCAGCGAAGATG (ubi14.rev), CGAAGAAGCTGAAGAACCAA (cycB1;1.fwr), ATGCAGTGTTTGGGAATGAA (cycB1;1.rev). cDNA was amplified using the SYBR-Green® PCR Master kit (Perkin–Elmer Applied Biosystem) on the GeneAmp 9600 thermocycler (Perkin–Elmer Applied Biosystems) under standard conditions.

2.4. mRNA in situ hybridization

In situ hybridization was performed essentially as previously described [20]. Seedlings of *Arabidopsis* and its close relative *Raphanus sativus* (radish) were hybridized with ^{35}S-labeled *AtORC1a*, *AtORC2*, *AtORC3*, *AtORC4a*, *AtORC5*, *AtORC6* and digoxigenin-labeled *AtCDT1a* and *AtCDT1b* gene specific antisense and sense RNA as probes (as controls). After hybridization, slides were washed for 2 h in 2XSSC at RT and 2 h in 0.1XSSC containing 50% formamide at 45 °C. Slides were dipped in photographic emulsion and developed when hybridization signal was detected. For the CDT genes, stringent washing conditions were applied.

2.5. Yeast two-hybrid assay

The open reading frames of the putative *Arabidopsis* ORC subunits, CDC6a, CDT1a and CDT1b, were cloned in-frame in both the Gateway binding domain (BD) cloning vector and in the Gateway activation domain (AD) cloning vector (Invitrogen) to act as "bait" and "prey", respectively, in the Mating Type Yeast Two Hybrid assay. The BD cloning vector constructs were used to transform yeast strain PJ694 α type and the AD cloning vector constructs were used to transform the A type. Both A and α types were plated in SD agar media lacking leucine and tryptophan, respectively. After two days at 28 °C, plates were stamped to an YPD agar plate, to form a grade between A and α yeast types. After one day of incubation at 28 °C, the plates were stamped on selective plates, lacking leucine, tryptophan and histidine and incubated for 3 days at 28 °C.

3. Results

3.1. Identification of putative homologs of the pre-RC genes in Arabidopsis

Putative homologs of all components of the eukaryotic pre-RC were identified in the *Arabidopsis* genome using bioinformatic tools (Table 1, Fig. 1). cDNAs of all *Arabidopsis* pre-RC genes identified were cloned and sequenced, except for *AtORC2*, *AtCDC6a* and *AtCDC6b*, which have already been characterized [7,9,10]. The MCM complex was not investigated further in this work. Comparison of the coding sequences of *Arabidopsis* genes with the Genebank database showed that the pre-RC proteins share extensive sequence similarity with the corresponding proteins of other eukaryotes, suggesting that they were conserved during the course of evolution.

Origin recognition complex. The pre-RC genes, *AtORC1a*, *AtORC1b*, *AtORC3*, *AtORC4*, *AtORC5* and *AtORC6*, were identified in the *Arabidopsis* genome using BLAST. It is interesting to note that two *Arabidopsis* homologs of *ORC1* were identified – named *AtORC1a* and *AtORC1b*, a feature that has not been reported in any other eukaryotic species so far. Both *AtORC1a* and *AtORC1b* are located on chromosome 4

Table 1
Putative pre-RC proteins coded by the *Arabidopsis* genome

Protein	Acc no.	Length aa	Molecular weight (kDa)	Chromosome no.
AtOrc1a	At4g14700	809	89.8	4
AtOrc1b	At4g12620	813	90.2	4
AtOrc2	At2g37560	363	40.0	2
AtOrc3	At5g16690	734	82.7	5
At Orc4	At2g01120	403	45.2	2
AtOrc5	At4g29910	534	60.6	4
AtOrc6	At1g26840	284	31.7	1
AtCdc6a	At2g29680	539	59.9	2
AtCdc6b	At1g07270	473	52.0	1
AtCdt1a	At3g54710	486	54.0	3
AtCdt1b	At2g31270	571	63.4	2
AtMcm2	At3g09660	777	85.9	3
AtMcm3	At5g46280	776	86.3	5
AtMcm4	At2g16440	720	80.9	2
AtMcm5	At2g07690	727	81.0	2
AtMcm6	At5g44635	831	92.8	5
AtMcm7	At4g02060	716	80.3	4

(Fig. 1), and although they are very similar to each other (79% identity and 83% sequence similarity), no duplication event is predicted to have occurred for either of the two *AtORC1* genes. Sequencing of the isolated cDNAs of *Arabidopsis* ORC subunits revealed some minor differences from the coding sequences predicted in the genome annotation, mainly related to exon/intron predictions. Nevertheless, *AtORC3* and *AtORC4* genes were considerably misannotated. In the *AtORC3* gene, only 12 exons out of 17 were predicted in the genome. In the case of *AtORC4*, only the last four exons are present in a predicted ORF encoding an unknown protein. Comparing EST databases from plant species and using bioinformatic tools, it was possible to identify the complete gene upstream to the predicted truncated ORF, comprising 16 exons in all. Interestingly, two classes of *AtORC4* cDNAs were cloned, representing two splicing variants of *AtORC4*. The longer transcript, which contains 14 extra amino acids in the 5′ region of the 15th exon, was called *AtORC4a* and the shorter one was called *AtORC4b*. Sequence analyses were performed using *AtORC4a* predicted amino acid sequence.

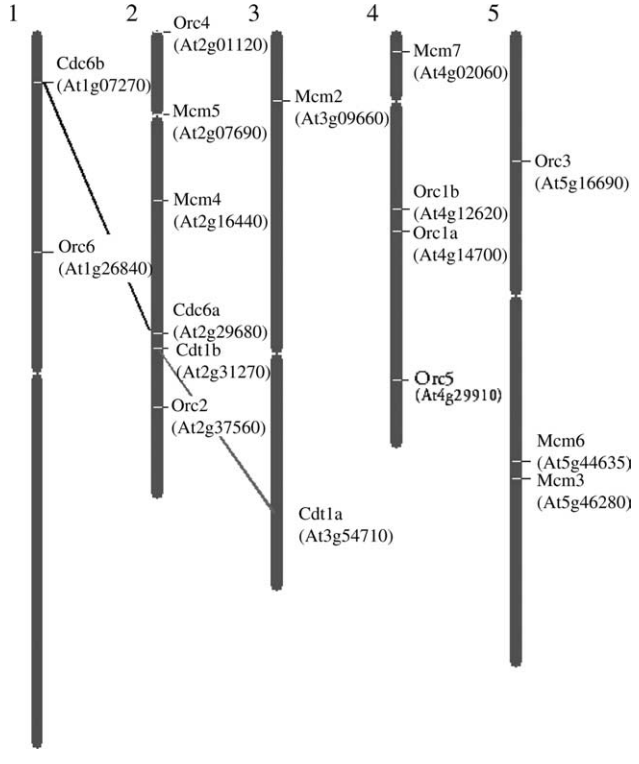

Fig. 1. Chromosome map of pre-RC components identified in the *A. thaliana* genome. Black bars represent the chromosome and horizontal lines define the *loci* positions in each chromosome according to the chromosome map program (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). Predicted regions of duplications of *AtCDT1* and *AtCDC6* genes are indicated as diagonal lines in the map.

To better understand ORC function and regulation in the *Arabidopsis* cell cycle, in silico sequence analysis of all *Arabidopsis* ORCs and their homologs from other eukaryotes were performed to identify phosphorylation sites and possible domains important to DNA replication control or to some other essential protein function. A summary of the results is presented in Table 2.

The N-terminal portion of AtORC1a and AtORC1b contains a BAH (Bromo-Adjacent Homology) domain (positions 135–341 and 200–344, respectively) with seven conserved regions. Associated with this domain, a PHD (Plant Homeodomain) zinc finger motif with a Cys_4–His–Cys_3 pattern, spanning 47 residues (Table 2), is found in both AtORC1a and AtORC1b (starting at positions 163 and 168, respectively). BAH domains have been implicated in linking DNA methylation, replication, and transcriptional regulation in mammals [21]. The PHD motif is conserved in nuclear proteins thought to be involved in chromatin-mediated transcriptional regulation and has been described in different organisms, from humans to plants [22]. Interestingly, only plant ORC1 proteins exhibit the PHD zinc finger motif.

AtORC1a, AtORC1b, AtORC4 and AtORC5 belong to the AAA^+ ATPase protein family. AAA^+ ATPase domains were identified at positions 461–670 in AtORC1a, 466–675 in AtORC1b, 51–208 in AtORC4 and 75–293 in AtORC5. Their primary sequences contain a putative purine nucleoside triphosphate-binding site (CDC-NTP) that includes the consensus motifs Walker A (P-loop) and Walker B (A-loop) [23,24]. The Walker A and Walker B motifs are implicated in binding and hydrolysis of ATP, respectively, which are necessary during pre-RC assembly. Nevertheless, only a putative Walker A motif (P-loop) was identified in ORC4 and ORC5 subunits from other species (Table 2). An exception was the maize protein ZmORC5 where no ATPase domain was detected probably because the N-terminal region is missing [11].

Interestingly, the deduced amino acid sequence of AtORC3 shared homology with domain 1 of cullins, between residues 87 and 111. This protein family is a component of a series of ubiquitin ligases that organize the degradation of a wide range of proteins, in which the SCF (Skp1/Cullin/F-box) complex takes part [25]. The significance of the presence of this domain for AtORC3 function remains to be demonstrated. AtORC2 and AtORC6 amino acid sequences were devoid of any characteristic domain (Table 2).

The presence of Cdk phosphorylation sites in subunits of *Arabidopsis* ORC was investigated. Four consensus sites were found in the AtORC1a and in AtORC1b N-terminal regions (starting at positions 14, 18, 45 and 101, and positions 12, 42, 100 and 114, respectively), and one consensus site was identi-

Table 2
Domains of *A. thaliana* pre-RC complex proteins compared with other eukaryotes

Protein	*A. thaliana*	*O. sativa*	*Z. mays*	*H. sapiens*	*M. musculus*	*X. laevis*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
ORC1	BAH domain[a] PHD zinc finger[a] AAA ATPase[a]	BAH domain PHD zinc finger AAA ATPase Pro-rich	BAH domain PHD zinc finger AAA ATPase	BAH domain AAA ATPase	BAH domain AAA ATPase	BAH domain AAA ATPase	BAH domain AAA ATPase	AAA ATPase	BAH domain AAA ATPase
ORC2	No hits	No hits	No hits	No hits	No hits	No hits	No hits	No hits	AT hook
ORC3	Domain 1 cullins	No hits	No hits	No hits	No hits	ND	AAA ATPase (P-loop)	ND	No hits
ORC4	AAA ATPase	AAA ATPase (P-loop)	AAA ATPase (P-loop)	AAA ATPase (P-loop)	AAA ATPase (P-loop)	AAA ATPase (P-loop)	AAA ATPase (P-loop)	ND	No hits
ORC5	AAA ATPase	AAA ATPase (P-loop)	No hits	AAA ATPase (P-loop)	AAA ATPase (P-loop)	ND	AAA ATPase (P-loop)	AAA ATPase (P-loop)	AAA ATPase (P-loop)
ORC6	No hits	No hits	ND	No hits	No hits	ND	No hits	ND	No hits
CDC6	AAA ATPase[a]	AAA ATPase	ND	AAA ATPase	AAA ATPase	AAA ATPase[a]	ND	AAA ATPase AT-hook	AAA ATPase
CDT1	No hits[b]	ND	ND	No hits	No hits	No hits	No hits	No hits	ND

ND – not determined, the gene was not described yet.
[a] The domain was identified in both homologs.
[b] No characteristic domain was identified in both homologs.

fied in the C-terminal portion of AtORC3 protein (starting at position 483). Cdk phosphorylation sites were not found in AtORC2, AtORC4, AtORC5 or AtORC6.

The anaphase promoting complex (APC) dependent degradation pathway has been implicated in degradation of several pre-RC components of the eukaryotic cell cycle [26]. A search for KEN-, D- and A-box and GXEN amino acid motifs, found in substrates of the APC-dependent degradation pathway, was performed in the *Arabidopsis* ORC subunits. Putative D-boxes were found in AtORC1a (positions 474 and 527), AtORC1b (positions 479 and 532), AtORC2 (position 141), AtORC3 (position 421), AtORC5 (position 116) and AtORC6 (positions 53, 108 and 254). On the other hand, none of the searched proteins exhibited the KEN- or A-box amino acid motif. AtORC1a was the only subunit that exhibited a putative GXEN (GKEN) motif at position 544. PEST sequences, a region rich in proline, glutamate, serine and threonine which is target for rapid proteolysis, were also searched in ORC complex proteins from *Arabidopsis* using PEST-find algorithm. In the AtORC1a amino acid sequence, two PEST regions starting at positions 25 and 147 were found. In contrast, AtORC1b protein has only one PEST sequence, starting at position 153.

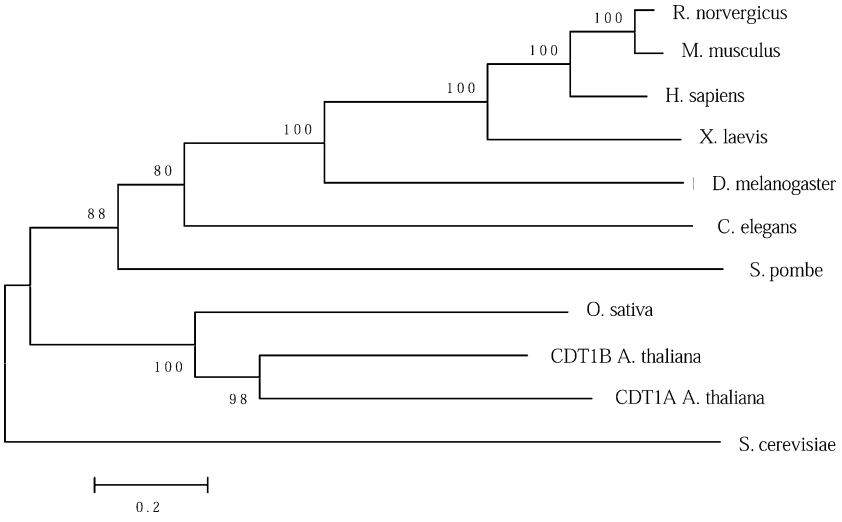

Fig. 2. Phylogenetic analysis of CDT1 proteins from eukaryotes. Protein sequences obtained from GeneBank were aligned with *CDT1a* and *CDT1b* protein sequences from *Arabidopsis*. Alignment was obtained using the CLUSTALW algorithm and the unrooted phylogenetic tree was generated using Mega2 program (Molecular Evolutionary Genetics Analysis, version 2). The bootstrap values based on 1000 replicates are on the tree nodes (*Arabidopsis CDT1a*, At3g54710 and *CDT1b*, At2g31270; *S. pombe*, P40382; *Mus musculus*, AF477481.1; *Homo sapiens*, ABO53172.1; *X. laevis*, AJ250122.1; *Drosophila melanogaster*, AF279146.1; *Rattus novergicus*, XM226545.1, *Oryza sativa*, CAE02517, *S. cerevisiae*, NP012580, *Caenorhabditis elegans*, NP491126).

The AtORC2, AtORC3 and AtORC5 subunits also have PEST regions in their amino acid sequences starting at positions 293, 1 and 16, respectively. Altogether, the data indicate that ORC proteins may be target for a rapid destruction by proteolysis. Interestingly, no potential proteolysis signal sequences were found in AtORC4.

CDT1. The search for Cdt1 homologs in the *Arabidopsis* genome identified coding sequences for two putative *CDT1* genes, named *AtCDT1a* and *AtCDT1b*. This is the first time that two CDT1 homologs have been found in any eukaryotic species. The two genes are apparently located on chromosome segments that were duplicated in ancient or pseudo-ancestral genomes according to the tool Paralogons in *A. thaliana* [27] (Fig. 1). This would explain the low percentage of identity between the two copies (36% identity and 56% similarity). Analysis of these two sequences showed that both genes have seven exons at similar sizes. No KEN-, D- and A-box or GXEN motifs were identified in AtCDT1b and two PEST sequences were found, initiating at positions 310 and 379. On the other hand, only two non-canonical D-box (positions 110 and 471) were found in AtCDT1a. The data suggest that these proteins might also be target for rapid destruction by proteolysis.

Phylogenetic relationships among CDT1 homologs were analyzed by carrying out amino acid alignments using ClustalW [17] and generating unrooted phylogenetic trees using the Mega2 program [18] (Section 2). The cladogram obtained (Fig. 2) shows the *Arabidopsis* CDT1 homologs in the same branch, located in the same group as putative rice CDT1 and set apart from metazoans and yeast. Interestingly, after duplication, the two *Arabidopsis* copies might have diverged, because apart from the close plant homologs, AtCDT1a shares a greater similarity with human CDT1 (24% identity and 50% similarity), while AtCDT1b is closer to the *Drosophila* CDT1 homolog (34% identity and 53% similarity), called DUP [28]. The analysis also suggests that metazoan CDT1 homologs have a greater similarity with plant proteins than to the yeast

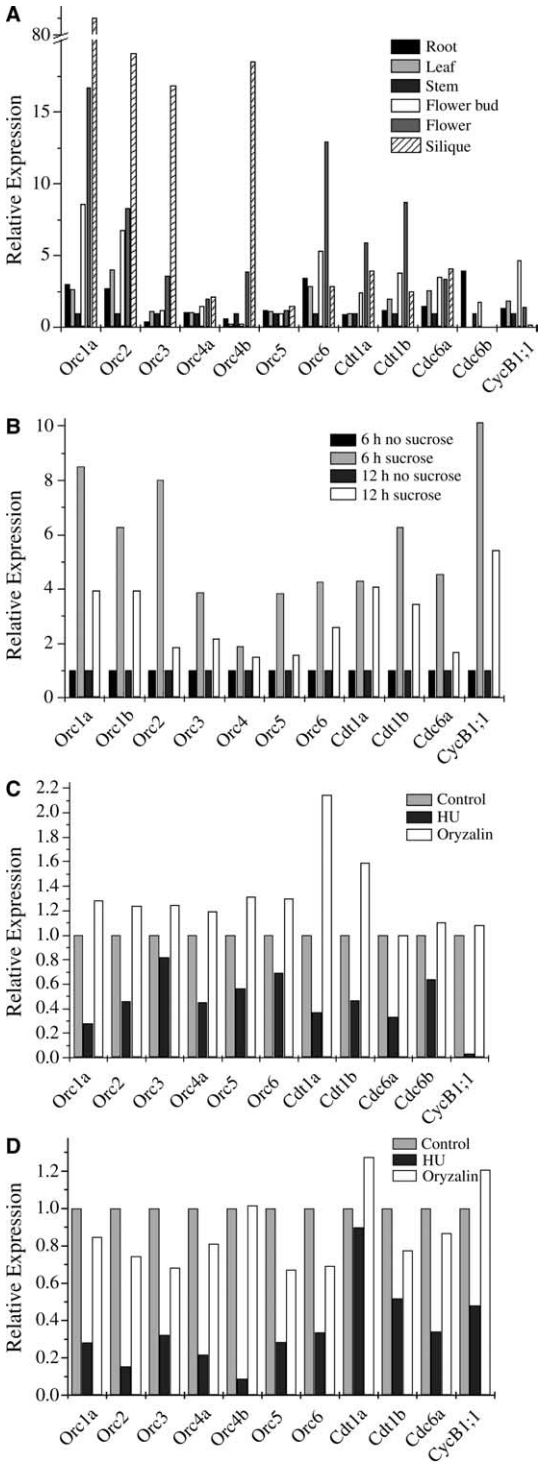

Fig. 3. Relative expression profile of *Arabidopsis ORC1–6* subunits, *CDT1a*, *CDT1b*, *CDC6a* and *CDC6b*. The transcript level is represented as a ratio of the absolute value of the studied gene to the absolute value of *AtUbi14* gene. Due to the ratio calculation, the SDS values are not represented in this figure. (A) Pre-RC genes expression profile in different plant organs. The data are normalized to the level of expression in the inflorescence stems. Color codes of the bars are indicated in the figure. Absolute values of inflorescence stems are: *ORC1a*, 4.11E–04; *ORC2*, 1.17E–03; *ORC3*, 1.66E–03; *ORC4a*, 9.54E–03; *ORC4b*, 7.35E–04; *ORC5*, 2.80E–02; *ORC6*, 2.11E–03; *CDT1a*, 3.34E–03; *CDT1b*, 7.65E–03; *CDC6a*, 2.61E–03; *CDC6b*, 6.52E–05; *CYCB1;1*, 1.79E–03. (B) Pre-RC expression profile in *Arabidopsis* cell culture following sucrose treatment. Expression levels obtained after 6- and 12-h treatments with sucrose are normalized to the corresponding controls (6- or 12-h without sucrose). 6 h without sucrose (light gray), 6 h with sucrose (black), 12 h without sucrose (white) and 12 h with sucrose (dark gray). 6 h- or 12 h- controls absolute values are, respectively: *ORC1a*, 9.92E–03 and 2.10E–02; *ORC2*, 1.28E–02 and 3.53E–02; *ORC3*, 3.46E–02 and 5.09E–02; *ORC4a*, 4.70E–01 and 4.19E–01; *ORC5*, 6.98E–02 and 1.33E–01; *ORC6*, 7.54E–02 and 9.74E–02; *CDT1a*, 1.19E–01 and 1.05E–01; *CDT1b*, 3.44E–02 and 7.36E–02; *CDC6a*, 6.27E–02 and 9.64E–02; *CYCB1;1*, 2.74E–02 and 2.26E–02. (C) Expression pattern of pre-RC genes in root cultures of *Arabidopsis* seedlings treated with 10 mM hydroxyurea (black) or 30 μM oryzalin (white) is normalized to untreated controls (gray). Absolute values of the controls are: *ORC1a*, 1.44E–03; *ORC2*, 3.89E–03; *ORC3*, 1.38E–03; *ORC4a*, 2.41E–02; *ORC5*, 2.73E–02; *ORC6*, 5.81E–03; *CDT1a*, 6.42E–03; *CDT1b*, 1.41E–02; *CDC6a*, 4.57E03; *CDC6b*, 1.45E–05; *CYCB1;1*, 3.91E–04. (D) Expression pattern of pre-RC genes in *Arabidopsis* L-MM1 cell suspension treated with 10 mM hydroxyurea (black) or 15 μM oryzalin (white) is normalized to untreated controls (gray). Because treatments using hydroxyurea and oryzalin were performed independently, each treatment was normalized to its respective control. Absolute values of the controls of hydroxyurea and oryzalin treatments are, respectively: *ORC1a*, 2.40E–02 and 3.42E–02; *ORC2*, 4.21E–02 and 2.62E–02; *ORC3*, 1.79E–01 and 1.91E–01; *ORC4a*, 1.19E–01 and 2.66E–01; *ORC4b*, 1.59E–04 and 2.06E–04; *ORC5*, 4.14E–02 and 1.81E–01; *ORC6*, 5.18E–02 and 9.61E–02; *CDT1a*, 1.07E–01 and 4.73E–01; *CDT1b*, 6.29E–02 and 1.02E–01; *CDC6a*, 4.58E–02 and 4.42E–02; *CYCB1;1*, 4.48E–02 and 2.56E–02.

Table 3
Summary of in situ hybridization results obtained with *AtORC1–6*, *AtCDT1a* and *AtCDT1bAt* antisense RNA as probe

		ORC1	*ORC2*	*ORC3*	*ORC4*	*ORC5*	*ORC6*	*CDT1a*	*CDT1b*
Root	Root meristem	+	+	+	+	+	⁎	+	±
	Collumela root cap	+	+/–	–	+	±	⁎	+/–	+/–
	Lateral root cap	+	±	±	±	±	+	±	+
	Lateral root meristem	+	+	±	+	±	+	+	++
	Elongation zone	+	*	±	±	+	⁎	±	±
	Young root	+	+	±	+	+	+	++	++
	Mature root	⁎	⁎	+	⁎	++	+	+	+
Shoot	Shoot apical meristem	+	+/–	+	±	+	+	+/–	±
Apex	Young leaf	++	+	+	+	++	++	+	+
	Maturing leaf	++	+	±	±	⁎	⁎	±	±
	Ground meristem	+	+/–	+	+	++	+	±	±
	Stem	+	±	±	±	±	±	±	±

Signal intensity: (–) not detected, (±) weak signal, (+) signal, (++) strong signal, (*) patchy signal, (⁎) sometimes patchy, (+/–) not always expressed.

Schizosaccharomyces pombe protein. Furthermore, the recently identified *Saccharomyces cerevisiae* CDT1 ortholog lies apart from the proteins of *S. pombe* and the other eukaryotes, because of the poor overall identity with these proteins [29].

Alignments of amino acid sequences from all other putative *Arabidopsis* ORC proteins and eukaryotic homologs were also carried out. Results of phylogenetic relationships were similar as that observed in the CDT1 analysis (data not shown). In general, plant proteins are found in a separate branch, closer to metazoans than to yeasts.

3.2. Relative expression profile of putative pre-RC genes of Arabidopsis

To better understand the function of *Arabidopsis* pre-RC during development, the expression pattern of its component genes – except for the genes of the MCM complex – was analyzed using cDNA real-time PCR (Fig. 3). Transcriptional regulation of the two different variants of *AtORC4* mRNA was investigated by using primers specific for each mRNA. Unfortunately, the primers designed for *AtORC1b* were not specific and could not be used for real-time PCR analysis. The data from real-time PCR are the result of at least twice two experiments and representative results are shown in Fig. 3. In all treatments, expression of *AtCYCB1;1* was used as a cell division marker [30].

A different pattern of expression of the *Arabidopsis ORC* genes was observed in the various plant organs analyzed (Fig. 3A). *AtORC1a*, *AtORC2* and *AtORC6*, similarly to *AtCYCB1;1*, exhibited lower mRNA levels in vegetative organs with low proliferation rates, such as leaves and inflorescence stems, and high mRNA levels in flower buds – organs with high numbers of dividing cells (Fig. 3A). Remarkably, *AtORC1a*, *AtORC2*, *AtORC3* and *AtORC4b* were strongly expressed in open flowers and siliques – organs with overall low frequency of diving cells; and *AtORC6* showed high levels of expression only in open flowers. Contrasting with the expression profile of the other *ORC* genes, *AtORC4a* and *AtORC5* showed relatively constant mRNA levels in the different plant organs (Fig. 3A). *AtCDT1a* and *AtCDT1b* had similar expression profiles among *Arabidopsis* organs, being more expressed in reproductive organs. In general, *AtCDT1b* exhibited higher mRNA levels than *AtCDT1a* (see absolute values in the legend) (Fig. 3A). On the other hand, the *AtCDC6a* transcript profile in different plant organs was quite different from that of *AtCDC6b*. *AtCDC6b* was strongly expressed in roots and its mRNA could not be detected in leaf, flower and silique, while *AtCDC6a* was expressed in all plant organs analyzed (Fig. 3A).

The absence of sucrose in plant cell culture growth media brings cell proliferation to a halt. In order to investigate the influence of sucrose on the expression patterns of the putative *Arabidopsis* pre-RC genes, cell cultures were grown in the presence or absence of this nutrient (Fig. 3B). Levels of mRNA for all *Arabidopsis* pre-RC genes studied decreased when cells were depleted of sucrose and were induced when sucrose was added to the growth medium. A similar result was obtained with the rice *ORC1* homolog [8], where *ORC1* expression was enhanced by sucrose.

The expression of the putative *Arabidopsis* pre-RC genes was also investigated in roots (Fig. 3C) and Arabidopsis cell suspension culture (Fig. 3D) treated with the cell cycle blockers hydroxyurea and oryzalin. These drugs arrest cells at early S phase and at the G2/M boundary, respectively [31]. The overall expression pattern of pre-RC components was similar in both root culture and cell suspension although minor differences were observed probably due to physiological differences in the *biological* systems. The expression levels of most of the pre-RC genes were reduced by hydroxyurea treatments, except for *AtCDT1a* in the cell suspension assay which showed little or no difference comparing to the control. Interestingly, *AtCDT1* homologs exhibited a small increase of expression after oryzalin treatment, and *AtORC3* expression was only weakly reduced in hydroxyurea treated root culture. A search for putative regulatory promoter elements of the *Arabidopsis* pre-RC genes revealed that, excluding *AtORC6*, all promoter sequences have a putative E2F consensus binding motif (TTTYYCGYY). Two E2F binding sites were identified in the promoters of the two *AtORC1* and *AtCDC6* homologs.

3.3. In situ hybridization analysis of putative pre-RC genes of Arabidopsis

The spatial localization of pre-RC genes expression was analyzed by mRNA in situ hybridization of different *Arabidopsis* tissues. Our expression analyses were mainly concentrated during root development, because it is a well described developmental process suitable for studies of a large number of genes [32,33]. In addition, expression in tissues of the shoot apex, inflorescence and siliques was also observed. Tissue sections of *Arabidopsis* and its close related species radish were hybridized with *AtORC1–6*, *AtCDT1a* and *AtCDT1b* sense

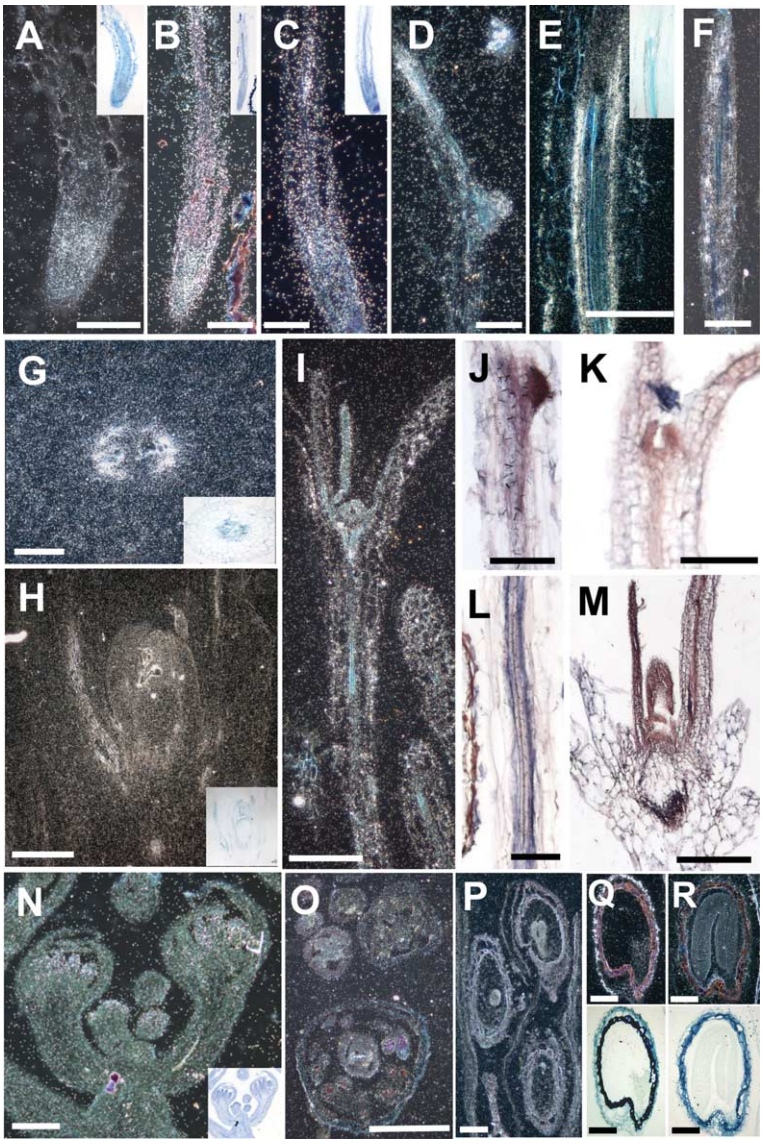

Fig. 4. *Arabidopsis* pre-RC gene expression in plant tissues. In situ localization of *AtORC1–6* and *AtCDT1a* and *AtCDT1b* in *Arabidopsis* and radish roots and shoot apex. Radioactive hybridization signals are seen as white grains under dark-field optics and non-radioactive signal as a purple stain under bright-field optics. Bright field images are included to help visualization of plant morphology. (A–C) are longitudinal sections through *Arabidopsis* root tips hybridized with *AtORC1*, *AtORC4* and *AtORC6*, respectively; (D) longitudinal section of a lateral root hybridized with *AtORC2*; (E, F) longitudinal sections of roots hybridized with *AtORC1* and *AtORC4*; (G, H) cross section of root and longitudinal section of a shoot apex of radish hybridized with *AtORC1* and *AtORC2*, respectively; (I) longitudinal section of *Arabidopsis* seedling hybridized with *AtORC1*; (J) longitudinal section of a lateral root of *Arabidopsis* hybridized with *AtCDT1b*; (L) longitudinal section of a root hybridized with *AtCDT1a*; (K, M) longitudinal sections of *Arabidopsis* shoot apex hybridized with *AtCDT1a* and *AtCDT1b*, respectively; (N) longitudinal section of *Arabidopsis* inflorescence hybridized with *AtORC6*; (O) cross section of *Arabidopsis* flowers hybridized with *AtORC1*; (P–R) longitudinal sections of *Arabidopsis* siliques hybridized with *AtORC1*. Bars = 100 μm except for: H, bar = 500 μm and N, bar = 200 μm.

and antisense RNA probes. The probes used did not allow us to distinguish *AtORC1a* from *AtORC1b* or *AtORC4a* from *AtORC4b*. For the *AtCDT1* homologs experiments, stringent conditions during washes were applied in order to avoid cross hybridization. Sense probes were used during control hybridizations and did not show any signal above background (data not shown). The expression pattern of *AtCDC6a* has been previously reported [9,10]. Little variation on the expression patterns was observed depending on the developmental stage of the seedling. Expression patterns observed for all genes analyzed are summarized in Table 3.

All *AtORC* homologs showed expression in root apical meristems or emerging lateral root meristems in a homogeneous to slightly patchy expression pattern (Fig. 4A–D). Similar to that observed for *AtCDC6a* (unpublished data), during some stages of root development, a strong expression in the collumela root cap is often observed for all *AtORC* homologs except *AtORC3* (data not shown). *AtORC1*, *AtORC2*, *AtORC3*, *AtORC4* and *AtORC5* are homogeneously expressed in the elongation zone (just above the root meristem) – where tissue differentiation and endoreduplication occur, while *AtORC6* maintains a patchy expression pattern as for the root meristem (Fig. 4C). Above the elongation zone, the expression of all *AtORC* genes is mainly seen in the vascular cylinder (Fig. 4E and F), being stronger in protoxylem and also often in protophloem elements. *AtORC1* and *AtORC2* expression is

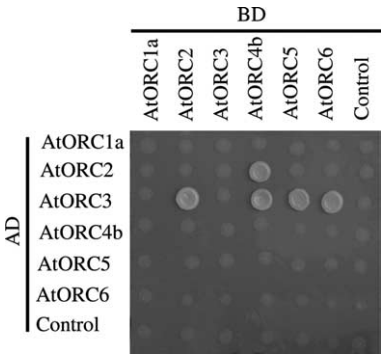

Fig. 5. Binary interactions of the AtORC subunits. The ORFs were cloned in Gateway BD and AD cloning vectors (Invitrogen) to act as "bait" and "prey", respectively. Empty vectors were used as negative controls. The ability of each individual subunit to bind each other was tested in all possible combinations using mating type yeast two hybrid assay (Section 2). Mated yeasts were inoculated in liquid SD leu/trp and saturated cultures were spotted in low stringency selective plates (SD leu/trp/his).

also often observed in pericycle cells flanking the xylem poles in maturing roots (Fig. 4G). All *AtORC* genes are strongly expressed in maturing vascular tissue undergoing secondary growth (Fig. 4E and F). *AtORC1*, *AtORC2*, *AtORC3* and *AtORC6* are more homogeneously expressed in the vascular cylinder, mainly around to forming metaxylem elements, while *AtORC4* and *AtORC5* show patchy hybridization signal (Fig. 4E and F).

During development of the aerial part of the seedling, an homogeneous expression of the six *AtORC* genes was observed in the shoot apical meristem and leaf primordia at different intensity levels (Table 3 and Fig. 4H and I). As the leaf matures, expression remains mainly in vascular bundles and in the epidermis. In the hypocotyls, expression was seen in the vascular tissue and in the epidermis mainly close to the apical hook.

The expression patterns of *AtCDT1a* and *AtCDT1b* during *Arabidopsis* development strongly overlapped among them, and with the *AtORC* genes. Both *AtCDT1* homologs were expressed in the root apical meristem, lateral root meristem (Fig. 4J) and were strongly expressed in young vascular tissues (Fig. 4L). In the shoot apex, expression of the *AtCDT1* genes was often weak in the shoot apical meristem and stronger in the leaf primordia and young leaves (Fig. 4M). In maturing leaves, expression was mainly observed in the vascular bundles.

During flower development, strong expression in flower primordia and in flowers in which the floral organs are differentiated is shown for *AtORC6* and *AtORC1* (Fig. 4N and O). Expression of *AtORC1* was also seen throughout the silique and fertilized ovules, in endosperm, embryos at globular and heart stages, but not in mature embryos (Fig. 4P–R).

3.4. Interaction of the AtORC subunits in the yeast two-hybrid system

In order to investigate the arrangement of the pre-RC components within the complex, a preliminary characterization of possible physical interactions among the *Arabidopsis* ORC subunits was performed by yeast two hybrid assays (Fig. 5). Only one member of each gene family was incorporated in the analysis. In this experiment, the ability of each individual component to bind each other was tested in all possible combinations. In addition, the two AtCDT1 homologs and AtCDC6a were included in the assays, but no interactions were observed (data not shown). The results indicate that AtORC2 interacts with AtORC3 and AtORC4b, and that AtORC3 interacts with all the other AtORCs, except AtORC1a and itself. These data suggest a primary architectural organization of the ORC complex, in which AtORC3 plays a central role in maintaining the complex associations. The lack of interactions with AtORC1a, AtCDC6a and the AtCDT1 homologs could indicate that more than one subunit or the complete assembled complex might be necessary to mediate these associations.

4. Discussion

This paper presents the first report on the identification of all putative components of the full pre-RC in plants, and the cloning and expression analysis of a group of these genes. All plant homologs were identified in the genome of the model plant *A. thaliana*, including one putative *AtORC4* homolog previously considered to be missing from the genome [11]. During the preparation of this manuscript, a report on the identification of an *ORC4* homolog and two *ORC1* homologs in the *Arabidopsis* genome was published [12]. Apart from the MCM proteins, not included in this study, the results show that all other putative *Arabidopsis* pre-RC genes are expressed at the mRNA level. Therefore, the data further support the idea that the basic molecular mechanism of DNA replication is conserved in plants and suggest that it might function similar to the other eukaryotes. However, further investigation of protein levels and functional activities related to DNA replication is still required. Studies on *AtORC1a* revealed that the plant protein partially complements a null mutant strain of *S. pombe* (unpublished results).

A unique feature of the *Arabidopsis* genome is the presence of two paralogous *ORC1*, *CDC6* and *CDT1* genes. Except for *Xenopus laevis*, that was recently shown to have two copies of *CDC6* genes [34], all eukaryotes studied until now have only one copy of each of these genes. Interestingly, both *AtCDC6* and *AtCDT1* homologs are localized in chromosome segments that were duplicated in the *Arabidopsis* genome [27]. According to the program "Paralogons in *A. thaliana*", *CDC6* duplication was a recent event, whereas *AtCDT1* duplication occurred long ago. Concurrently, the similarity between *AtCDT1a* and *AtCDT1b* amino acid sequences was lower than between *AtCDC6a* and *AtCDC6b* homologs. Nevertheless, no duplication event was predicted for either of the two *ORC1* genes found in the *Arabidopsis* genome. This is quite intriguing since their nucleotide sequences are highly conserved (79% identity between *AtORC1a* and *AtORC1b*). Regardless of how long ago they were duplicated, the question that arises is: why does *A. thaliana* have two copies of these genes? One possibility is that during evolution, these duplicated genes acquired different functions and/or involvement in different stages during development. In *X. laevis*, the two isoforms of *CDC6* are functional and are expressed in different developmental stages [34]. Similarly, *AtCDC6a* and *AtCDC6b* expression pattern is different in plant organs, suggesting different roles in development.

Another interesting event in the *Arabidopsis* pre-RC molecular machinery is the differential splicing of the *AtORC4* gene, resulting in two mRNA variants with different temporal

expression patterns. *AtORC4a* mRNA levels are relatively constant in the different plant organs analyzed, while *AtORC4b* seems to be expressed later during development. This feature is not exclusive to *Arabidopsis* genes, since an alternatively spliced variant of human *ORC5* has already been described [35]. The data on expression profile suggest that the two mRNA variants of *AtORC4* might have different functions and/or could be differentially regulated during development.

In general, the putative pre-RC genes of *Arabidopsis* exhibited a similar pattern of gene expression during cell cycle. The data show that mRNA levels of most *Arabidopsis* pre-RC genes studied are cell cycle regulated with decreased levels of expression at G1/S transition. It has already been shown previously that *AtCDC6a* mRNA is reduced in S phase [9,10]. In addition, previous experiments with *Arabidopsis* cell suspension synchronization revealed that *AtORC6*, *AtCDT1b* and *AtCDC6b* [36] and *AtORC1* and *AtCDC6a* [37] are also cell cycle regulated. Homologs in other eukaryotes followed the same pattern [38–42]. It has also been shown that human, drosophila and fission yeast CDT1 homologs are cell cycle regulated [11,43–46]. Different from the other pre-RC genes investigated, both *AtCDT1* homologs showed a small increase in the mRNA levels in oryzalin treated cells, suggesting that they are expressed in G2/M phase of the cycle, in accordance to [36]. In contrast, budding yeast CDT1 seems not to fluctuate during the cell cycle [47]. Similarly, both mRNA and protein levels of yeast *ORC1* and *ORC2* [48,49] and human *ORC2–ORC5* remained constant during the cell cycle [50–52]. Therefore, mRNA fluctuations of all *Arabidopsis ORC* subunits during the plant cell cycle contrast with what is shown in general for metazoans and yeasts. As discussed above, it is not unexpected, since it has been demonstrated that the regulation of the pre-RC genes during the cell cycle is extremely diverse among eukaryotes, in spite of the genes being highly conserved [4].

It is well documented that expression of human and *Drosophila ORC1* is dependent on E2F and its level is modulated during cell cycle [51,53]. Interestingly, *Drosophila ORC2* promoter also has an E2F motif but its regulation is not E2F dependent [53]. Notably, boxes for E2F binding sites were described in the promoters of *AtORC3* and the two *AtCDC6* and *AtORC1* genes [37,54]. In this study, E2F motifs were reported in the promoter regions of the two *AtCDT1* homologs and the other *Arabidopsis ORC* genes, except *AtORC6*. It suggests that the cell cycle regulated expression of the *Arabidopsis* pre-RC genes might be mediated by the E2F pathway. Supporting this idea, it has been already demonstrated that a retinoblastoma-related/E2F pathway also operates in plants [55,56]. In addition, *Arabidopsis* plants ectopically expressing E2Fa/DPa showed higher levels of *AtCDC6a* and *AtORC1a* mRNA [54], while overexpression of E2Fc leads to a decrease in AtCDC6a mRNA levels [57]. Also, both *AtORC1b* and *AtCDC6a* were downregulated in dominant negative mutants of *Arabidopsis* DPΔBD [37].

In general, the expression analysis correlates mRNA levels of the *Arabidopsis* pre-RC genes with cell proliferation, supporting a role for these genes in DNA replication. In plants, the absence of sucrose halts cells in a phase similar to quiescence in mammals. Analyses of cultured cells depleted of sucrose showed a drastic reduction of mRNA levels of all genes studied. Similarly, rice *ORC1* mRNA levels were also reduced when cell proliferation halted after removal of sucrose from the culture medium [8]. Consistent with the data from previous studies on pre-RC components of other plants [8–11], all *Arabidopsis* pre-RC genes studied in this report were highly expressed in proliferating tissues such as flower buds. Nevertheless, high expression levels were also observed in organs with low overall cell division rates such as mature flowers and siliques, as indicated by low *AtCYCB1;1* mRNA levels. In situ hybridization experiments showed that high *AtORC1* mRNA levels are located throughout the silique and in endosperm, a tissue where endoreduplication occurs; and in developing embryos. In situ hybridization assays also revealed that *Arabidopsis* pre-RC genes are expressed in other non-proliferating tissues, such as root elongation zone, where differentiation and endoreduplication occur [58]; in vascular tissues of maturing roots and leaves; in pericycle cells – a tissue that retains the competence to divide and to form lateral roots. The presence of both mRNA and protein of human ORC subunits in somatic non-proliferative cells was also demonstrated, except for *HORC1* which was present only in proliferating tissues [59]. Such high mRNA levels of the *Arabidopsis* pre-RC genes in non-dividing tissues may be related to endoreduplication events in these organs. High *AtCDC6a* mRNA levels were also exhibited in mature flowers and siliques, and previous work suggested that *AtCDC6a* expression is associated not only with cell division but also with endoreduplication events [9,10].

Alternatively, pre-RC genes may have some function other than controlling initiation of DNA replication. Studies in other eukaryotes have suggested different functions for some of the pre-RC proteins. ORC1 and ORC5 subunits were discovered to be associated with proteins involved in transcriptional silencing in yeast [60–63]. In metazoans, ORC was also suggested to be involved in chromosome remodeling leading to transcriptional regulation [64–66]. In particular, two domains that are present in AtORC1a and AtORC1b suggest that the plant proteins might be involved in transcriptional regulation: BAH, involved in DNA methylation and transcriptional regulation [21]; and PHD, involved in chromatin remodeling and also in transcriptional regulation [22]. Curiously, this latter motif was found solely in plant ORC1 proteins. Further studies suggested that ORC may be playing some role in ribosome biosynthesis [67] and in chromosome segregation and cytokinesis [68]. Recently, it was reported that AtORC2 could be connecting DNA replication to chromosome structure [12]. Finally, yeast CDC6 homolog seems to participate in the exit from mitosis by inactivating mitotic cyclin-dependent kinases [69].

Studies on mRNA expression profile of *Arabidopsis* pre-RC genes by real-time PCR and in situ hybridizations showed that the spatial and temporal modulation of gene expression in different plant organs and root tissues varied among the genes studied. This was particularly evident with the *Arabidopsis* ORC subunit genes, which are expected to encode proteins that act as a holo complex formed by similar ratios of each component. Comparable results were obtained in studies on the human ORC subunit genes, where different mRNA levels were observed in several human tissues [59]. It is possible that mRNA and protein stability differ among the different ORC genes. Protein levels of the *Arabidopsis* pre-RC genes remain to be determined in the different plant organs and tissues, in order to confirm a differential expression. Nevertheless, the data already suggested that *Arabidopsis* ORC subunits, and possibly

all the components of the full pre-RC, might function not only as a holo complex, but also as subcomplexes of proteins, or even act as separate subunits. In support of this idea, it was recently described that hORC could form subcomplexes in distinct phases of the cell cycle [70].

Studies in other eukaryotes have demonstrated that the ORC subunits interact in a well defined architecture, and models have been proposed for the assembly of the human and maize complexes based on binary interactions in co-immunoprecipitations, pull-down and two-hybrid assays [11,71,72]. Most of the interactions found in two-hybrid experiments with the *Arabidopsis* proteins are in agreement with the models described for humans, excluding the HsORC2–HsORC6 interaction that was not observed in our assay [71]. The assembly of the maize ORC complex has also been characterized by two hybrid experiments [11]. The same ORC interaction patterns were identified in the two plant species, except that in the present work, a physical association between AtORC3 and AtORC6 is also reported. It suggests that the architecture of ORC complexes might be conserved in plants. While data indicate possible physical interactions that might be occurring during *Arabidopsis* ORC assembly in vivo, further experiments must be conducted in order to unravel the *Arabidopsis* ORC subunit arrangement within the complex.

We conclude that the basic molecular machinery that licenses DNA replication seems to be conserved among eukaryotes. Although apparently distant organisms have evolved independently and found similar ways to regulate DNA replication, the specific controls governing this basic machinery may differ according to the developmental features of each organism. Plants have proved to be an exciting and appropriate system for developmental studies. Our data on gene structure and expression already revealed particularities in the pre-RC of *Arabidopsis*. Further characterization of these genes can provide insights into possible connections between cell cycle machinery and developmental and differentiation controls.

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