Molecular characterization and cell cycle-regulated expression of a cDNA clone from *Arabidopsis thaliana* homologous to the small subunit of ribonucleotide reductase

Gabriel Philipps, Bernadette Clément, Claude Gigot

Institut de Biologie Moléculaire des Plantes du CNRS, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France

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Abstract **A cDNA clone isolated from an *Arabidopsis thaliana* cell suspension library showed highly significant homology to the small subunit of ribonucleotide reductase (R2) from different species. The 340 amino acid-long deduced putative protein contains all the residues that are important for the enzyme activity and structure. In *A. thaliana* this enzyme is encoded by a single-copy gene. In synchronized tobacco BY2 cells the corresponding mRNAs specifically accumulate during the S phase of the cell cycle.**

Key words: *Arabidopsis thaliana*; cDNA; Ribonucleotide reductase; Sequence; Cell-cycle regulated expression

1. Introduction

Ribonucleotide reductase (RNR) catalyses the reduction of ribonucleotides diphosphate (NDP) into deoxyribonucleotides diphosphate (dNDP) thus supplying the cells with the precursors needed for DNA synthesis. RNR is a heterodimeric iron-containing enzyme of the $\alpha 2\beta 2$ type in which the two subunits R1 and R2 are both essential for activity. The large subunit R1, which contains the sulfhydryl groups responsible for the reduction of substrate, binds substrates and allosteric effectors. The small subunit R2 contains two ferric ions and a tyrosyl free radical essential for activity. In mammals, where this enzyme has been extensively studied, the molecular mass of proteins M1 and M2 (the mammalian equivalents of proteins R1 and R2) are 90 and 45 kDa, respectively [1,2]. Both the enzyme activity and the expression of the corresponding genes are highly cell cycle regulated in both yeast and mammals (reviewed in [3,4]).

In yeast, two genes RNR1 and RNR3 encoding the large subunit have been cloned [5], and the expression of RNR1 was shown to be strictly cell cycle regulated whereas that of both RNR1 and RNR3 is induced by DNA damage [5]. The small subunit is encoded by a unique gene (RNR2) the expression of which is cell cycle regulated and induced by DNA repair processes [6,7].

In mouse, the enzyme activity of RNR was shown to be maximal during S phase [8], resulting from complex regulatory mechanisms. In synchronized cells the level of the M1 subunit was found to be constant throughout the cell cycle [9,10], the activity of the holoenzyme therefore depending on de novo synthesis and breakdown of the M2 subunit at the beginning and the end of S phase, respectively [11]. cDNA clones encoding M1 and M2 subunits have been sequenced [12,13] and used as probes to study transcriptional regulation of the corresponding genes. The levels of M1 and M2 mRNA increase abruptly upon entry of cells into S phase and then decrease when cells enter G_2 phase [13]. Interestingly, in addition to classical promoter-dependent regulation of both genes' expression, it was shown that S phase-specific expression of M2 mRNA was regulated by release from a transcriptional block located in the first intron of the M2 gene [14,15].

Surprisingly, identification and sequence analysis of cDNAs or genes corresponding to this enzyme of major importance for DNA replication were never reported in higher plants. Here we present the sequence of a cDNA from *A. thaliana* showing extensive homology at the protein level with the small subunit of RNR from both mammals and yeast. This cDNA was used as a probe to demonstrate S phase-specific accumulation of the corresponding mRNAs in a synchronized tobacco BY2 cell suspension [16].

2. Materials and methods

The cDNA (clone TAI 251) was sequenced as part of a systematic sequencing program of the transcribed genome of *A. thaliana*. It was isolated from a λZAPII cDNA library established using RNA extracted from an *Arabidopsis* (ecotype Columbia) cell suspension, 16 h after subculture (library constructed by M. Axelos and D. Trémousaygue, INRA, Toulouse). The cDNA of 1361 nucleotides was completely sequenced by generating a series of overlapping deletions using exonuclease III digestion. The adequate generated DNA fragments were sequenced using the dideoxy chain termination method [17]. Computer sequence analyses were performed with the UW-GCG facilities [18] and BLAST database research program [19]. DNA and RNA purification and Southern and Northern blot analyses were performed as previously described [20].

The tobacco BY2 cell suspension was cultivated and synchronized as described by Nagata et al. [16].

3. Results and discussion

3.1. Primary structure of the Arabidopsis R2

The 1361 nt-long cDNA insert of clone TAI 251 (accession number X77336) contained an open reading frame of 1020 nt starting with an ATG at position +133 nt and ending at an in-frame stop codon at +1153. The start ATG codon is preceded by an in-frame stop codon at position +40. As in many plant genes the 206 bp-long 3′ non-coding region ending with a 17 nt poly(A) tail does not contain a typical polyadenylation signal. A putative polyadenylation sequence AATAATA was found 32 bp upstream of the 3′ end. The encoded putative protein is 340 amino acids long with a molecular mass of 39,200 Da and showed 64% sequence identity with the human, mouse

*Corresponding author. Fax: (33) 88 61 44 42.

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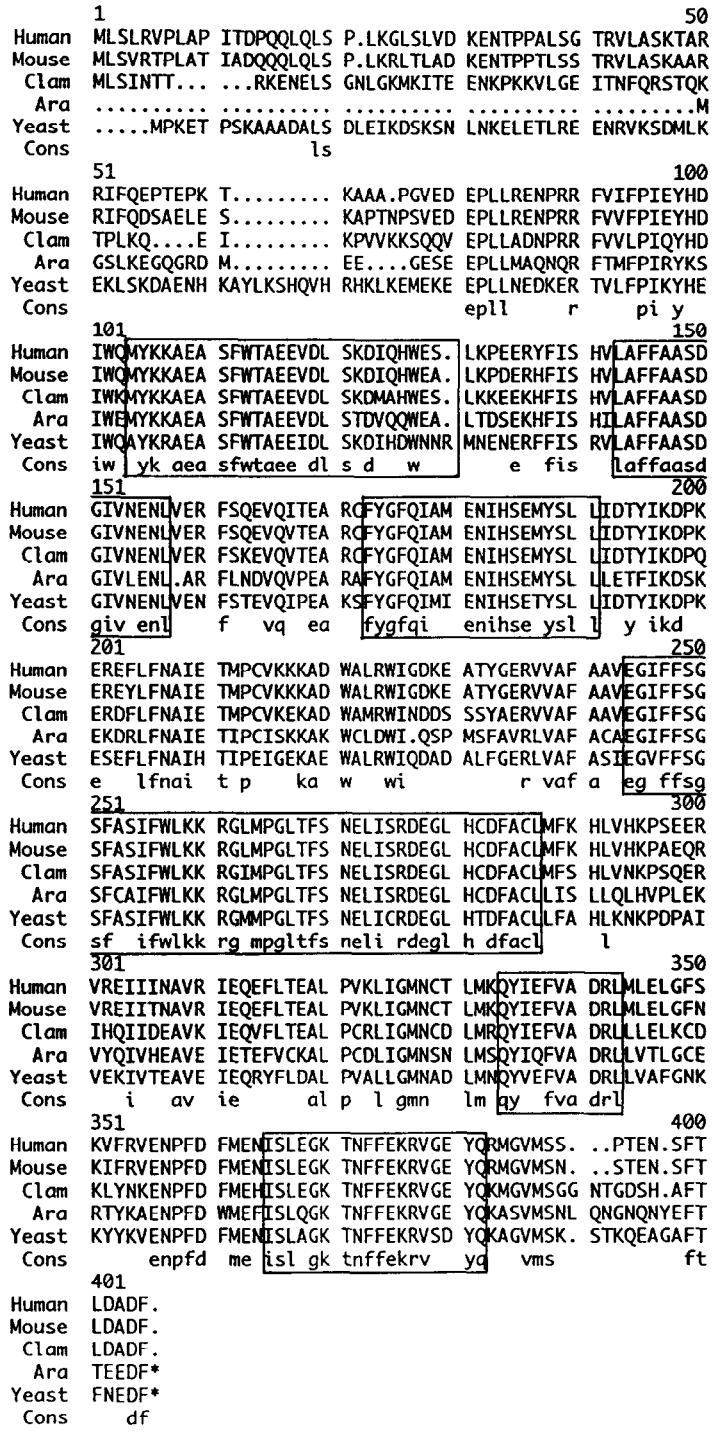

Fig. 1. Comparison of the predicted amino acid sequence of clone TAI 251 and the R2 sequences of man, mouse, clam and yeast. Gaps were introduced to optimize alignment. Small letters of the lower line (cons.) represent amino acids identical in all five proteins. Regions of high level of homology and containing residues that are essential for enzyme activity and structure are boxed. The amino acid numbering resulting from alignment is arbitrary.

and vaccinia virus R2, and 61% with the corresponding protein from yeast. Identities are lower in the N-terminal part of the protein which is shortened by 50 residues as compared to the mammals and yeast counterparts (Fig. 1). All of the residues which were described to be important for the catalytic activity and the structure of the enzyme are conserved [21,22]. They include Asp-150, Glu-181, His-184, Glu-244, Glu-278 and His-281 for iron ligands, Tyr-188, Phe-248, Phe-252, Ile-274 and Glu-278 for the tyrosyl radical environment, Trp-113, Glu-117, Asp-123, Arg-276, Tyr-335, Ser-366, Glu-375 and Tyr-381 involved in the binding to the R1. The amino acid numbering is arbitrary and results from the sequence alignment data.

The high level of sequence identities with R2 from several other organisms and the highly conserved residues responsible for the enzyme structure and catalytic activity allowed us to unambiguously identify the TAI 251 clone as a cDNA corre-

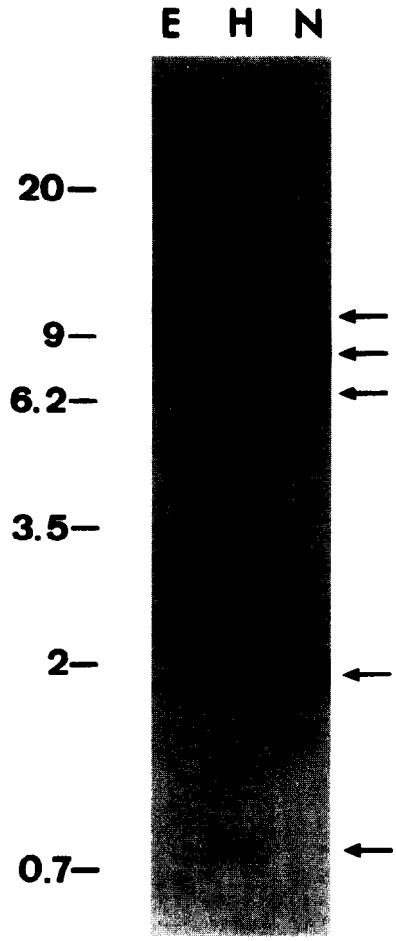

Fig. 2. Genomic blot of *A. thaliana* DNA digested with *Eco*RV (E), *Hin*dIII (H) or *Nco*I (N) and hybridized to the insert of the clone TAI 251. The numbers indicate the position of size markers.

sponding to the small subunit of the ribonucleotide reductase from *Arabidopsis thaliana*, and therefore represents the first sequence of this enzyme being published in plants.

3.2. Genomic organization and expression of the R2 gene

Southern blot analysis was performed with genomic DNA of *Arabidopsis thaliana* digested with *Eco*RV an enzyme that has no restriction site inside the cDNA and *Hin*dIII and *Nco*I, enzymes that have a restriction site at position 687 and 476, respectively. Hybridization with the insert of clone TAI 251 used as a probe showed one band of about 11 kb with *Eco*RV and two bands of 8 kb and 1.9 kb with *Nco*I, and 6.5 kb and 0.8 kb (weak) with *Hin*dIII (Fig. 2). These results are consistent with the existence of one copy of the R2 gene in the genome of *Arabidopsis thaliana*.

As mentioned above the expression of the R2 gene in mammals is strictly coupled to the cell cycle showing an abrupt induction at the G1/S transition [13,14] whereas in yeast there is only a modest two-fold induction at this step of the cell cycle [4]. In the absence of an appropriate synchronizable *Arabidopsis* cell suspension and considering the high level of sequence homology between the plant R2 and that of other distant species, we used the highly synchronizable Tobacco BY2 cell suspension to study the expression of the R2 gene during the cell cycle [16]. Stationary phase cells were subcultured and treated with Aphidicolin for 24 h, then washed and treated with Propyzamide, an anti-tubulin drug, for 4 h at the onset of the subsequent mitosis. After release from the Propyzamide block 90% of the cells fulfill synchronous mitosis and enter the next cell cycle [16]. Total RNA prepared from cells at different steps of the synchronization procedure (Fig. 3) were blot-hybridized to the R2 cDNA. The hybridization patterns clearly show that the level of R2 mRNA is highest during S phase (Fig. 3C) and almost undetectable during G_2. Low levels of mRNA were found in M phase, hardly detectable in metaphase (Fig. 3E), and slightly more abundant in late anaphase (Fig. 3F). This low level of expression at the end of mitosis could possibly correspond to a limited rate of dNTP synthesis necessary to re-equilibrate the pools of dNTP in the two daughter cells. Surprisingly, the amounts of R2 mRNAs are as high after the 24-h Aphidicolin treatment as in mid-S phase (Fig. 3B and C), suggesting that (i) the Aphidicolin block of DNA synthesis occurs after the induction of the R2 gene expression, and (ii) the transcription of the R2 gene is not directly coupled to the level of DNA synthesis. Very similar results were obtained recently when studying the expression of histone H3 and H4 genes under the same conditions (our unpublished results).

In order to study in more detail the onset of R2 gene expres-

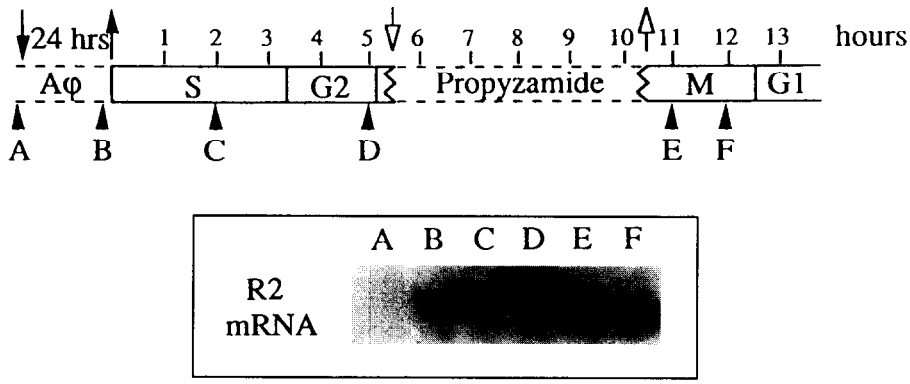

Fig. 3. R2 mRNA levels in synchronized TBY2 cells. The upper part represents the duration of the different steps of a typical synchronization experiment and that of the phases of the cell cycle. Filled and open arrows indicate the addition of Aphidicolin (A) and Propyzamide, respectively. The black arrowheads indicate the stages when RNAs were prepared from cells and blot-hybridized to the TAI 251 insert used as a probe (lower part). (A) Stationary phase cells, (B) cells subcultured for 24 h in the presence of 3 μg/ml of Aphidicolin, (C) S phase, (D) G2 phase, (E) metaphase, (F) late anaphase-to-telophase.

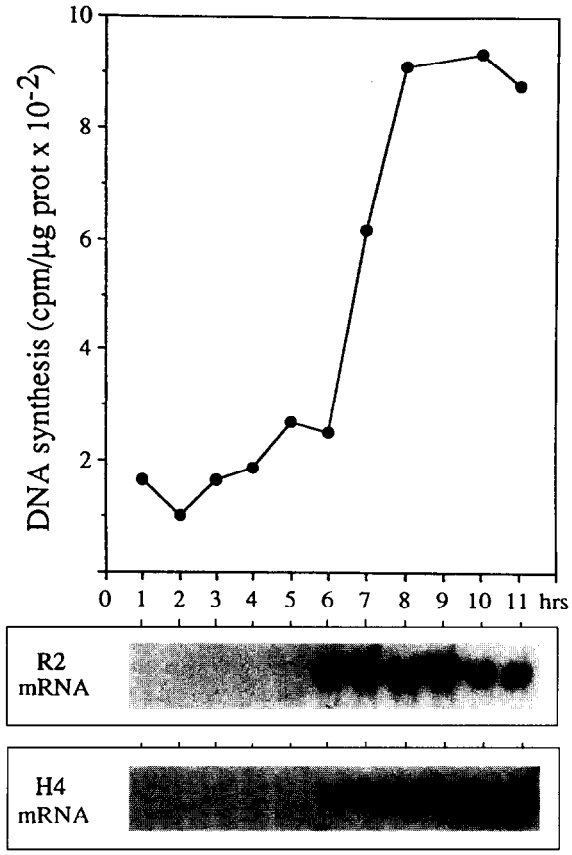

Fig. 4. Induction of R2 mRNA accumulation at the G_1/S transition. (Upper panel) DNA synthesis was monitored by pulse labeling with [^{3}H]TTP with 1 h sampling intervals after release from the Propyzamide block (see Fig. 3). The corresponding total RNAs were blot-hybridized to, successively, TAI 251 insert (panel R2) and histone H4 coding region (panel H4).

sion at the G_1/S transition, we followed the steady-state level of R2 mRNA during the G_1 and S phases following the release from the Propyzamide block. Cells were collected every hour after completion of mitosis, DNA synthesis was estimated by pulse-labeling with [^{3}H]TTP and the R2 mRNA steady-state level by Northern blot hybridization. The results presented in Fig. 4 clearly indicate that the amounts of R2 mRNA abruptly increase in parallel to DNA synthesis and stay at very high levels for at least 5 h. The histone H4 gene used as a control for S phase-specific expression showed very similar regulation.

These results show that, like in other eukaryotes, the R2 transcripts accumulate during S phase in plant cells and are absent during the rest of the cell cycle. In mouse cells, where the level of M1 protein remains constant during the cell cycle [9,10], the rise in RNR activity in early S phase results from de novo synthesis of M2 [11], itself resulting from complex transcriptional regulatory mechanisms of the M2 gene [14,15]. Studies of the protein levels and transcriptional regulation of both R1 (once cloned) and R2 genes in synchronized plant cells will show whether similar regulatory mechanisms were maintained or arose through evolution.

Alternatively, the regulation of holoenzyme activity during the cell cycle of plant cells could result from a coordinated promoter-directed transcriptional control. Such regulation of transcription has been recently shown for histone genes [23], and both functional and structural analyses of the promoter regions of these genes revealed the existence of several *cis*-acting elements [24,25], of which many are common to all histone genes sequenced up to now in plants. It will be of interest once the genes encoding R1 and R2 subunits have been cloned and sequenced in plants, to determine whether, contrary to animal systems, promoters of plant genes showing identical or very similar expression patterns share at least some common regulatory mechanisms.

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