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cDNA cloning and expression of an *Arabidopsis* GTP-binding protein of the ARF family

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A cDNA clone encoding a small GTP-binding protein, the ADP-ribosylation factor (ARF) was isolated from a cDNA library of *Arabidopsis thaliana* cultured cells. The predicted amino acid sequence was highly homologous to the known yeast, bovine and human ARF sequences. Southern analysis of *Arabidopsis* genomic DNA suggested the existence of at least two copies of ARF genes. The level of ARF mRNA was found to be nearly constant during all cell growth stages in suspension cultures.

ADP-ribosylation factor; Small GTP-binding protein; Suspension cell culture; *Arabidopsis thaliana*

1. INTRODUCTION

Specific targeting of a protein to its functional site is a complex mechanism which requires precise information encoded by the amino acid sequence, and is also mediated by an array of molecules controlling the membrane traffic [1]. A number of GTP-binding proteins are known to be involved in the regulation of vesicle-mediated protein transport through the secretory pathway of eukaryotic cells [2,3]. Both heterotrimeric and monomeric GTP-binding proteins are implicated in intracellular transport and secretion [4,5]. Structural features of the sequences of low molecular weight monomeric proteins permits them to be divided into two main classes: (i) the rab family in mammals and its homologs in yeast, YPT1 and SEC4, and (ii) the ARF family. The ARF protein, i.e. the ADP-ribosylation factor, was first identified in mammalian cells as a cofactor required for ADP-ribosylation by the cholera toxin of the adenylate cyclase $G_{s\alpha}$ subunit [6]. Subsequently, ARF was localized to the Golgi apparatus of mammalian cells [7] and the same protein and corresponding genes were identified in yeast [8,9]. These results suggested a more generalized role for this factor. According to a recent study, ARF proteins may modulate vesicle budding and uncoating within the Golgi apparatus through a GTP-driven cycle [10]. Here, we report the first characterization of a cDNA encoding a GTP-binding protein of the ARF family from higher plants and we study its expression in *Arabidopsis thaliana* cell suspension cultures.

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2. MATERIALS AND METHODS

2.1. *Plant material*

Cell suspensions (line T87) derived from *Arabidopsis thaliana* L. (Heynh.), ecotype Columbia, were isolated and maintained in JPL medium as previously reported [11].

2.2. *Construction of an* Arabidopsis *cDNA library*

All routine DNA and RNA manipulations were carried out following procedures described by Sambrook et al. [12]. Total RNA was isolated from 18 g of fresh cells harvested 16 h after their transfer into a new culture medium. RNA isolation was performed using a phenol–chloroform extraction procedure. Poly(A)$^+$ RNA (34 μg) was obtained by fractionation on oligo(dT)-cellulose (Sigma). The cDNA library was constructed into the lambda-ZAPII vector by unidirectional cloning using a synthesis kit (Stratagene): 3×10^6 clones were obtained from 5 μg poly(A)$^+$RNA. From an aliquot of the amplified library, a recombinant pBluescript SK$^-$ phagemid stock was rescued by in vivo excision. Recombinant clones were isolated by plating with XL1-Blue *E. coli* host cells.

2.3. *DNA sequencing and data analysis of ARF1 cDNA*

Nucleotide sequencing of randomly selected cDNA clones and the isolated ARF1 cDNA was carried out on double-stranded templates according to the dideoxy chain termination method [13], using Sequenase (USB). Sequence comparisons were performed in GenBank or EMBL databases on partial nucleotide sequences of the cDNA inserts using the search program FASTA [14]. Analysis of the ARF1 cDNA deduced amino acid sequence was performed using programs of the University of Wisconsin Genetic Computer Group [15].

2.4. *Preparation of ARF1 and EF-1α probes*

An ARF1 probe was prepared by PCR amplification from the ARF1 cDNA clone using M13 oligonucleotide primers and Super Taq DNA polymerase (Stehelin) in the conditions described in [16]. The EF-1α probe corresponded to the *BglI–BglII* fragment of the 3'-coding region of the *Arabidopsis* A1 EF-1α gene [17]. The PCR-generated ARF1 fragment (1027 bp) and the EF-1α fragment (404 bp) were purified by agarose gel electrophoresis and labelled with [α-^{32}P]dCTP (NEN) by oligo-labelling [12].

2.5. *Southern and Northern analyses*

A. thaliana (Columbia) plant DNA was isolated according to [18]. Minipreparations of total RNA were obtained from *Arabidopsis* cell suspensions (0.5 g fresh cells) using a guanidine hydrochloride/phenol–chloroform procedure [19]. Southern and Northern blots were performed using Hybond N^+ membranes (Amersham). Hybridizations with ^{32}P-labelled ARF1 and EF-1α probes (6.5×10^5 cpm · ml^{-1}) were carried out at 65°C in 5 × SSPE/5 × Denhardt's solution/0.5% w/v SDS in the presence of 10 $\mu g \cdot ml^{-1}$ poly(U) and 33 $\mu g/ml^{-1}$ denaturated salmon sperm DNA. Membranes were washed at 65°C in 2 × SSPE/ 0.1% w/v SDS, 1 × SSPE/0.1% w/v SDS and 0.1 × SSPE/0.1% w/v SDS, successively.

3. RESULTS

In order to identify genes specifically expressed in dividing cells, a cDNA library was constructed in lambda-ZAP phage from mRNA isolated from exponentially growing cells of *A. thaliana*. A number of cDNAs were randomly selected and sequenced. Among these clones, we characterized a cDNA exhibiting a high homology (70% to 77%) to the nucleotidic sequences of yeast ARF genes and bovine or human ARF cDNAs. The *Arabidopsis* clone (ARF1) contained an 820 bp insert including an open reading frame of 543 nucleotides encoding a protein of 181 amino acids with a calculated M_r of 20 580 Da. The accession number of the *Arabidopsis* cDNA sequence deposited in the EMBL/GenBank database is M95166.

Comparison of the *Arabidopsis* ARF1 amino acid sequence with ARF from other species [9,20–23], including an 'ARF-like' sequence from *Drosophila* [24] is presented in Fig. 1. Except for their amino- and carboxy-termini, the sequences are highly conserved. Human ARF1 and bovine ARF 2 show the highest homologies to the plant sequence, corresponding to 88% identity and 95–96% similarity accounting for the conservative substitutions. The degree of similarity is lower for the protozoan *G. lamblia* protein (68% identity) while the 'ARF-like' sequence from *Drosophila* is still more divergent (56% identity). Different conserved motifs can be recognized in the *Arabidopsis* sequence, in common with the other ARF proteins (Fig. 1). Three of these motifs are identical to the proposed consensus sequences involved in GTP binding [25]. The P motif, GLDAAGKT for ARF, constitutes the phosphate binding loop (consensus sequence GXXXXGKS/T). The G′ motif DXXGQ, part of the sequence TVWDVGGQD, characteristic for all ARF except for *Drosophila* ARFL, interacts with the gamma-phosphate of GTP, while the G motif NKXD, here NKQD, is supposed to be specific for the guaninyl binding. The *Arabidopsis* ARF1 sequence also contains homologies to sequences important for other functions. A glycine-myristoylation site is recognized at position 2. The *N*-myristoylation, probably involved in the protein binding to the membranes, has been demonstrated for bovine brain ARF [26]. A potential ASN glycosylation site

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      1▼                    P                                  50
A.tha MGLSFGKLFS RLFAKKEMRI LMVGLDAAGK TTILYKLKLG EIVTTIPTIG
bovin ..NV.E...K S..G..... ..... ..... .....
human ..NI.AN..K G..G..... ..... ..... .....
C.alb ...TIS...A S.LGRR.... ..... ..... .....
S.cer ...YAS.... N..GN..... .....G... ..V..... .VI.....
G.lam ..QGAS.I.G K..S...V.. ..... .....M.. .V...V....
D.mel ..GVLS Y.R G.LGSR.... .IL...G... .....R.QV. .V.....

      51      ★  o  G'                                        100
A.tha FNVETVEYKN ISFTVWDVGG QDKIRPLWRH YFQNTQGLIF VVDSNDRD  RV
bovin ..... ..... ..... ..... .....E  ..
human ..... ..... ..... ..... .....E  ..
C.alb ..... ..... .....Y ..... .....I.. ..... .....  .I
S.cer .....Q... ..... ..R..S.... .YR..E.V.. .I.....S  I
G.lam ..... .N..... ..S..... .Y...DA..Y .I..A.IEPK.I
D.mel ....Q.T... LK.Q...L.. .TS...Y..C .YS..DAI.Y ....A...  .I

      101                    G                    ●        ●150
A.tha VEARDELHRM LNEDELRDAV LLVFANKQDL PNAMNAAEIT DKLGLHSLRQ
bovin N...E..T.. .A..... ....V..... ..... .....
human N...E..M.. .A..... ..... ..... .....H
C.alb N...E..QL. .....K... ...L..... ..... E.M....IMN
S.cer G...EVMQ.. .....N.. W..... .E..S..... E.....I.N
G.lam ED..N...TI .G.....A ..... .K..STTD.. E....QE.KK
D.mel GISK...LY. .R.E..AG.I .V.L.....M DGC.TV..VH HA...EN.KN

      151          o                  181
A.tha RHWYIQSTCA TSGEGLYEGL DWLSNNIASK A
bovin .N....A... ...D..... .....QLKNQ K
human .N....A... ...D..... .....QLRNQ K
C.alb .P.F..A... .T.D..... E....QVGK
S.cer .P.F..... ..... E.....LKNQ S
G.lam .D....P... R..D...Q.. ....DY.FD. KNKKKGKKR
D.mel .TFQ.FK.S. .K....DQAM .....TLQ.R K
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Fig. 1. Alignment of ARF1 DNA deduced amino acid sequences from *A. thaliana* and other species. Identical amino acids are indicated by dots. References for sequences are: bovine ARF2 [20], human ARF1 [21], *C. albicans* ARF1 [22], *S. cerevisiae* ARF2 [9], *G. lamblia* ARF H7 [23] and *D. melanogaster* ARFL [24]. Putative motifs involved in GTP-binding are overlined. Potential myristoylation sites are indicated by arrows and the ASN-glycosylation site by a star. Potential phosphorylation sites are indicated by a closed circle for protein kinase C and by an open circle for caseine kinase 2.

is found at position 60. Putative serine/threonine phosphorylation sites (i) by protein kinase C, are present at positions 140 and 147 and (ii) by caseine kinase 2, at positions 64 and 161.

The number of *A. thaliana* ARF genes was investigated by Southern analysis (Fig. 2). The pattern of the restriction fragments hybridized to the ARF1 cDNA probe is complex, showing several bands of weak or strong signal intensities. These results suggest that ARF is encoded by a multigene family and are consistent with the recent isolation of two distinct genomic clones from *Arabidopsis* (data not shown).

The estimation of the ARF gene transcript levels during the growth cycle of a cell culture was carried out by Northern hybridization using the *Arabidopsis* ARF1 cDNA probe. A single band of about 0.9 kb was detected under high stringency conditions (Fig. 3). The ARF mRNA level is rather high, compared to the high transcript level of the translation elongation factor EF-1α, an abundant protein of the cell, used as control. In

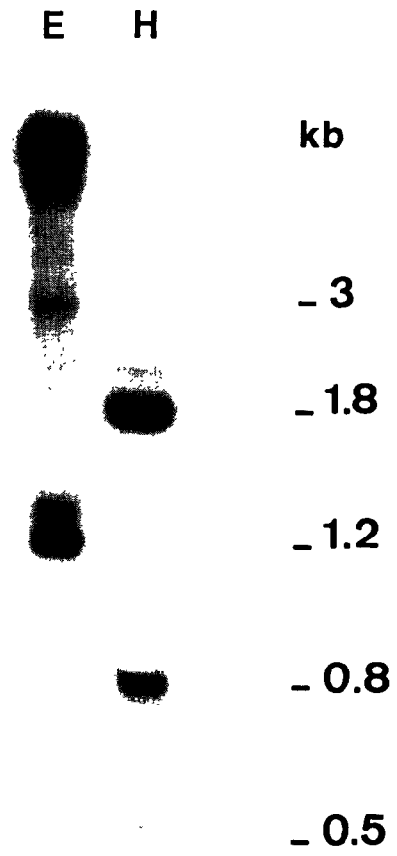

Fig. 2. Southern blot analysis of *Arabidopsis* DNA. Eight μg of DNA were digested with *Eco*RI (line E) or *Hin*dIII (line H) and fractionated by agarose gel electrophoresis. Hybridization was performed using the ^{32}P-labelled ARF1 cDNA probe (see section 2).

contrast to EF-1α expression, the ARF mRNA level (relative to total RNA) is nearly constant throughout the cell growth cycle, including in stationary phase cells.

4. DISCUSSION

We have isolated a clone coding for a protein with high homology to the ADP-ribosylation factor ARF from an *Arabidopsis* cDNA library. Gene expression of ARF in plants provides additional evidence that this protein has roles other than its activity as the cholera toxin activator, identified in mammals. However, the *Arabidopsis* ARF1 amino acid sequence is highly homologous to mammalian ARFs, which are known as cofactors of in vitro ADP-ribosylation, in contrast with the *Drosophila* 'ARF-like' protein with which homology is more restricted and which is not a cofactor of ADP-ribosylation [24]. Thus, *Arabidopsis* ARF1 may be involved with the regulation of cell ADP-ribosyltransferases, although these enzymes are not yet characterized in plants.

A new role for ARF proteins has been postulated recently [7,10], indicating that ARF is involved in the Golgi vesicle-mediated secretory process. In this regard, it was interesting to estimate ARF gene expression during cell proliferation, knowing that membrane traffic is arrested during mitosis [27]. Our data showed that the ARF transcript level was the same in actively dividing cells and in quiescent cells, suggesting that the *Arabidopsis* ARF gene expression was not dependent on cell multiplication. However, we do not know if this gene expression may be modulated during the course of the cell cycle itself, since the cell divisions were not synchronized. In HeLa cells, two GTP-binding proteins of the rab family are known to be phosphorylated during M-phase by the protein kinase p34^{cdc2}, a key element involved in the control of the cell cycle [28]. *Arabidopsis* ARF activity might be regulated by a similar modification: ARF sequence contains several putative serine/threonine phosphoacceptor sites, although the absence

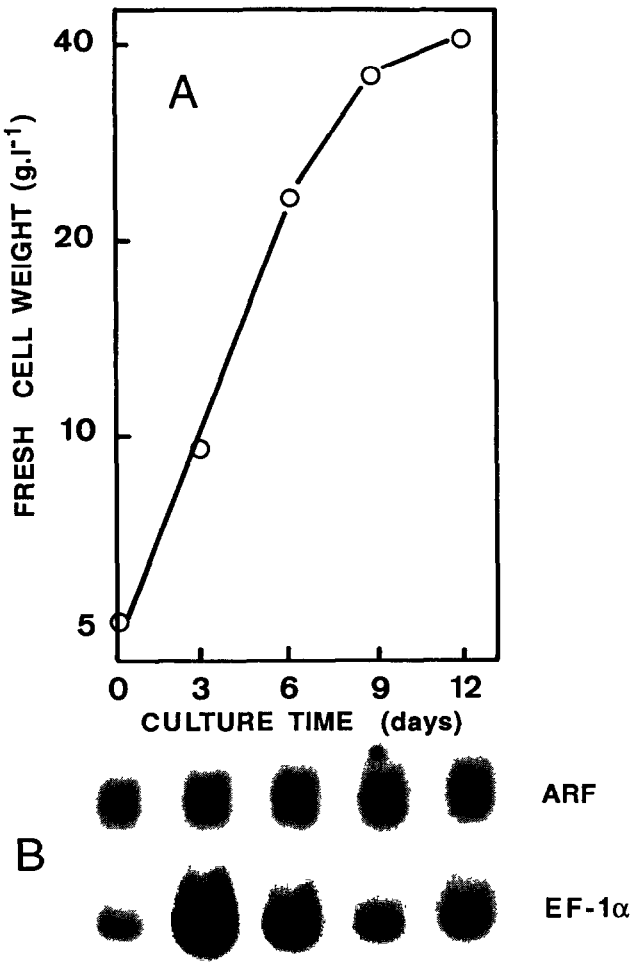

Fig. 3. ARF gene expression during the course of an *Arabidopsis* cell suspension culture. A. Growth curve of the culture. Fresh cell weight values (g per liter of medium) are plotted on a log scale. B. Northern blot analysis of ARF transcript levels. Twelve μg of total RNA isolated from 0, 3, 6, 9 and 12 day-old cells were fractionated by denaturating-gel electrophoresis [12]. Hybridizations were performed using ^{32}P-labelled ARF1 and EF-1α probes (see section 2).

of the putative consensus motif $^{T}/_{S}$ PX $^{K}/_{R}$, specific for p34 kinase, might exclude this type of processing.

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