Characterization of an *Arabidopsis thaliana* cDNA homologue to animal poly(ADP-ribose) polymerase

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Abstract **A full-length *Arabidopsis thaliana* cDNA (*app*) encoding a protein with high similarity (about 60%) to the catalytic domain of vertebrate poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) has been cloned. The N-terminal extension of the *Arabidopsis* protein shows similarities with domains of different nuclear and DNA binding proteins in agreement with nuclear localization and putative function of a plant PARP. APP is encoded by a single gene mapped at the top of chromosome 4 of the *Arabidopsis* genome and mRNA is abundant in cell suspension culture compared to its accumulation in whole plant.**

Key words: *Arabidopsis thaliana*; Evolution; Poly(ADP-ribose) polymerase

1. Introduction

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is generally described as a nuclear enzyme found in most eukaryotes, including vertebrates, arthropods, molluscs, slime moulds, dinoflagellates, fungi and other lower eukaryotes with the exception of yeast, and corresponding cDNAs have been isolated from several species of mammals, chicken, *Xenopus*, and insects (for review, see [1–3] and Fig. 5). Structure–function studies have shown that animal PARPs may be divided into at least three subdomains [4]. The N-terminal part (46 kDa) contains two zinc fingers and has a high affinity for nicked, V-shaped DNA. Interaction of PARP with nicked DNA strongly enhances the activity of the catalytic domain (54 kDa), which is very well conserved among all PARPs and located in the carboxyl-terminus of the protein. Automodification of the central domain (22 kDa) apparently serves as negative feedback regulation of the PARP.

PARP catalyses both the transfer of ADP-ribose from NAD^+, mainly to the carboxyl group of a glutamic acid residue on target proteins, and subsequent ADP-ribose polymerization (for review, see [4–7]). PARP itself, histones, high mobility group chromosomal proteins, a topoisomerase, endonucleases and DNA polymerases have been shown to be modified by PARP (for a review see [7]). The high negative charge of poly(ADP-ribosyl) chains attached to the protein decreases its affinity for DNA. These observations are in agreement with association of the PARP with chromatin and its involvement in DNA repair, replication and recombination and in cell division and differentiation [7–12]. PARP exerts other complex effects on cell metabolism due to the depletion of cellular NAD^+ [13]. Lastly, it has been suggested that nicotinamide resulting from NAD^+ hydrolysis by PARP, may be one of the early stress response signal in eukaryotes, including plants [14].

The first evidence of plant poly-ADP-ribosylation was obtained from investigations on germinating seeds, and cytological work with onion tissues. PARP activity has been further demonstrated in isolated nuclei from cultured tobacco cells, wheat, and pea [14–18]. Plant PARP is mostly associated with chromatin [19] and may have a similar role in DNA repair as in mammalian cells [20].

Here we report the cloning and characterization of the first plant cDNA encoding a PARP homologue. Its structure and expression are studied and compared to those of animal PARP. Finally, the putative function of PARP in plants is discussed.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana ecotypes Landsberg erecta and Columbia were grown in soil under 16-h light conditions at 22°C. The cell suspension culture was grown in a liquid MS medium supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid on a gyratory shaker at 80 rpm.

2.2. Isolation of PARP cDNA

Several cDNAs of *A. thaliana* have been isolated during a specific screening in yeast cells (data in preparation). Among them, the clone yb55 (1720 bp) encodes a protein showing a high similarity with the catalytic domain of animal PARPs (Fig. 1). The cloned fragment was used to screen 10^6 recombinant phages of an *Arabidopsis* cDNA library made from cell suspension culture mRNA in λZAP vector [21]. Nine positive clones were purified, and cDNA containing pBluescript SK phagemids were excised in vivo from the λ vector using the helper phage VSC-M13 (Stratagene, La Jolla, CA, USA). The sequences of the longest cDNA inserts were determined on both strands by the dideoxy nucleotide chain termination method according to the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Amplification of the 5′ end of the mRNA has been done by the anchored-PCR approach as described by Troutt et al. [22]. The cDNA template was synthesized on 5 μg of total leaf RNA using the preamplification kit (Gibco/BRL, Gaitherburg, MD, USA). The sequence of the gene specific oligonucleotide was 5′-CTGATCTAGCACCGCTGCACCC-3′ (nucleotide numbers 574–553) and the sequences of the anchored primer and the primer complementary to it were described by Troutt et al. [22].

2.3. DNA and RNA blot hybridization analyses

Total *Arabidopsis* DNA and RNA were extracted as described by Shirzadegan et al. [23]. DNA and RNA gel blot analyses and construc-

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The nucleotide sequence reported in this paper has been deposited in the EMBL database with Accession Number Z48243.

Abbreviations: APP, *Arabidopsis thaliana* homologue of PARP; EDTA, ethylenediaminetetraacetic acid; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; 5′ RACE, rapid amplification of 5′ cDNA ends; RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate.

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tion of recombinant plasmids were performed according to standard procedures [24]. Prehybridization and hybridization of DNA or RNA blots were done in 0.25 M Na-phosphate, pH 7.2, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin [25]. Prehybridizations were done at 65°C for 2 h, hybridization at 65°C overnight. Membranes were washed three times for 15–20 min with 50–200 mM of Na-phosphate buffer, pH 7.2, 1% SDS and 1 mM EDTA at 60–65°C.

Restriction fragment length polymorphism (RFLP) mapping analyses have been performed on 99 recombinant inbred (RI) lines provided by C. Lister and C. Dean (John Innes Institute, Norwich, UK; [26]). The marker generated by *Dra*I restriction was used for the mapping of the *Arabidopsis* chromosome. Computing of the hybridization patterns was carried out by C. Lister (Norwich). Mapping data are available via AAtDB.

A

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A.t.   1 MANKLKVDELRLKLAERGLSTTGVKAVLVERLEEAIAEDTKKEESKSKRK
                 _______________"A1"_____________ ------"B"-----

A.t.  50 RNSSNDTYESNKLIAIGEFRGMIVKELREEAIKRGLDTTGTKKDLLERLC
         ------"B"---------- _______________"A2"___________

                                  NAD-binding domain ➟
M.m. 525                                          TLKGGAAVDP-
S.p. 511                                          KIKDGLAVDP-
A.t. 101 NDANNVSNAPVKSSNGTDEAEDDNNGFEEEKKEEKIVTATKKGAAVLDQW
         -----------------"C"-----------------    *     *

M.m. 535 -DSGLEHSAHV-LEKGGKVFSATLGLVDIVKGTNSYYKLQLLEDDKENRY
S.p. 521 -DSGLEDVAHVYVSRNKEKYNVVLGITDIQKNKNSFYKLQLLESDMKNRF
A.t. 151 IPDEIKSQYHV-LQRGDDVYDAILNQTNVRDNNNKFFVLQVLESDSKKTY
                  **          *          *    ** ** *
M.m. 583 WIFRSWGRVGTVIGSNKLEQMPSKEDAIEHFMKLYEEKTGNAWHSK-NFT
S.p. 570 WVFRSWGRIGTTIGGNKLDNFSNLVDAIVQFKELYLEKSGNHFENRENFV
A.t. 200 MVYTRWGRVGVKGQSKLDGPYDSWDRAIEIFTNKFNDKTKNYWSDRKEFI
             *** *                ** *      *  *        *
M.m. 632 KYPKKFYPLEIDYGQDE-------EAVKKLTVNPGTKSKLPKPVQDLIKM
S.p. 620 KVAGRMYPIDIDYAEDS-------KID--LSAEHDIKSKLPLSVQDIIKL
A.t. 250 PHPKSYTWLEMDYGKEENDSPVNNDIPSSSSEVKPEQSKLDTRVAKFISL
                    **                          ***   *   *
M.m. 675 IFDVESMKKAMVEYEIDLQKMPLGKLSKRQIQAAYSILSEVQQAVSQGSS
S.p. 661 MFDVDSMKRTMMEFDLDMEKMPLGKLSQKQIQSAYKVLTEIYELIQGGGT
A.t. 300 ICNVSMMAQHMMEIGYNANKLPLGKISKSTISKGYEVLKRISEVIDR-YD
           *  *   * *       * **** *   *   *  *
M.m. 725 DSQILDLSNRFYTLIPHDFGMKKPP--LLNNADSVQAKVEMLDNLLDIEV
S.p. 711 NAKFIDATNRFYTLIPHNFGTQSPP--LLDTTEQVEQLRQMLDSLIEIEC
A.t. 349 RTRLEELSGEFYTVIPHDFGFKKMSQFVIDTPQKLKQKIEMVEALGEIEL
                  *** *** **                    *   *  **
M.m. 773 AYSLLRGGSDDSSKDPIDVNYEKLKTDIKVVDRDSEEAEIIRKYVKNTHA
S.p. 759 AYSLLQTEDSKADINPIDKHYEQLKTKLEPLDKNSEEYILLQKYVKNTHA
A.t. 399 ATKLLSVDPGLQD-DPLYYHYQQLNCGLTPVGNDSEEFSMVANYMENTHA
         *  **          *    *  *          ***       *  ****
M.m. 823 TTHNAYDLEVIDIFKIEREGECQRYKPFKQLHNRRLLWHGSRTTNFAGIL
S.p. 809 ETHKLYDLEVVDIFKVARQGEARRYKPFKKLHNRRLLWHGSRLTNFAGIL
A.t. 448 KTHSGYTVEIAQLFRASRAVEADRFQQPSSSKNRMLLWHGSRLTNWAGIL
          **  *  *    *  *  *  *   *    ** ******* ** ****
                       "Lys-893"
M.m. 873 SQGLRIAPPEAPVTGYMFGKGIYFADMVSKSANYCHTSQGDPIGLILLGE
S.p. 859 SHGLKIAPPEAPVTGYMFGKGIYFADMVSKSANYCCTSHHNSTGLMLLSE
A.t. 498 SQGLRIAPPEAPVTGYMFGKGVYFADMFSKSANYCYANTGANDGVLLLCE
         * ** ***************** ***** *******        *  ** *
M.m. 923 VALGNMYELKHASH-ISKLPKGKHSVKGLGKTTPDPSAN-ISLDGVDVPL
S.p. 909 VALGDMMECTAAKY-VTKLPNDKHSCFGRGRTMPNPSESIIREDGVEIPL
A.t. 548 VALGDMNELLYSDYNADNLPPGKLSTKGVGKTAPNPSEAQTLEDGVVVPL
         **** * *           **  * *  * * * * **       ***  **
M.m. 971 GTGISSGVNDTSLLYNEYIVYDIAQVNLKYLLKLKFNFKTSLW
S.p. 958 GKPITNDSLKSSLLYNEFIIYDIAQVNIQYMLRMNFKYK----
A.t. 598 GKPVERSCSKGMLLYNEYIVYNVEQIKMRYVIQVKFNYKH---
         *           ***** * *   *    *     *  *
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B

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Domain "A"

APP "A1"   4  KLKVDELRLKLAERGLSTTGVKAVLVERLEEA
hnRNP     10  KLKVSELKEELKKRRLSDKGLKAELMERLQAA
APP "A2"  74  GMIVKELREEAIKRGLDTTGTKKDLLERLCND
Consensus     klkV ELreel kRgLsttG Ka L ERL  a

              _____Helix____  -Loop-  __Helix__


Domain "B", basic (K/R)-rich

APP          38  EDTKKEESKSKR-KRNSSNDTYESNKLIAIGE
H.s 90      104  EEEKKEEKKKK--KRKVTNVTREWEML
RNA Hel.    387  KALEQSGSKVPDELRK-LNDTYE-KKR
TransF       85  RRKNNEAAKRSREKRR-LNDLVLENKLIALGE
Consensus        e  kkEesK kreKRk lNDTyE nKLia ge


Domain "C", hydrophilic and acidic (E/D)-rich

APP         105  NVSNAPV-KSS-NGTDEAEDDNNGFEEEKKEEKIVTAT-KK
hnRNP        75  NLGGNSAGRSGA-GL-EQEAAAGGDEEEEEEEEEEGISAL
CPD1 D.m.   226  NA--APVEKHEDNDDD-QDDENSGEEEHSPPEKTVVAP-KK
B23 Rat     168  KLDEDDDEDDEDDEDDEDDDDDDFDEEET-EEK-V--PVKK
Consensus        Nl  apveksedngdDEqeDdn GdEEE  EEK V  p KK
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Fig. 1. Deduced amino acid sequence of the *Arabidopsis app* cDNA. (A) The amino acid sequence of the open reading frame encoded by the longest cDNA is presented (A.t. is *Arabidopsis thaliana*). Comparison with mouse (M.m.) and *Sarcophaga peregrina* (S.p.) PARP is shown from the beginning of the catalytic (NAD binding) domain indicated by an arrow. The accession number of the protein sequences in databases are P09874 (mouse), D16482 (*S. peregrina*) and Z48243 (*Arabidopsis*). Gaps introduced to obtain maximal homology are indicated by horizontal lines. The Lys-893 that has been shown to be involved in the active site is indicated above the sequences. The repeated sequences (A1 and A2), basic region (B) and putative automodification domain (C) as described in the text are underlined. (B) Similarity of the N-terminus of *Arabidopsis* APP with different nuclear proteins; a heterogenous nuclear ribonucleoprotein (hnRNP, S22765) from human, a RNA helicase from *Dictyostelium* (RNA Hel., X81823), a human transcription factor (TransF, X64318), a nuclear heat-shock protein (HSP90, P24724), a chromosomal protein from *Drosophila* (CDP1, P22058) and a rat nuclear phosphoprotein (B23, P13084). Capitals indicate amino acids conserved in all sequences but one and bold capitals conserved in all sequences aligned.

2.4. Computer analyses

Computing was carried out using GCG (Genetics Computer Group Inc., Madison, WI, USA) software. Amino acid sequences were first aligned using the PILEUP program. Predictions of the secondary structure were obtained using Blocks Searcher software [27]. Pair-wise amino acid similarity and identity were calculated using the Bestfit program. Phylogenetic analyses have been carried out using Clustal V software [28] and its Neighbor Joining analysis (NJ) or using PAUP version 3 (for MacIntosh computer [29]).

3. Results

3.1. Isolation and characterization of Arabidopsis app *cDNA*

During experiments carried out to characterize *Arabidopsis* cDNAs encoding proteins that allow yeast cells to grow under different stress conditions (data in preparation), a cDNA (yb55) homologous to the catalytic domain of animal PARP (62% similarity with human PARP) was identified. The cDNA insert was used to screen an *Arabidopsis* oligo(dT)-primed cDNA library of *Arabidopsis thaliana* cell culture. Nine positive clones were isolated, and the nucleotide sequences of the 3 longest cDNAs (B, C and Q; Fig. 2B) were determined. The longest cDNA (C) is 2137 bp and contains one open reading frame, which encodes a putative protein of 637 amino acids (Fig. 1A) with a calculated molecular mass of 72 kDa and an isoelectric point of 6.2. The 5′ end of *app* cDNA was amplified by anchored-PCR. The PCR product is 10 bp longer at the 5′ end than the longest cDNA (2147 bp) and contains an in-frame stop codon, 127 bp upstream from the first ATG codon. Furthermore, using the yb55 cDNA as probe, a single 2.3 kb mRNA was detected by Northern analysis (Fig. 3). Taken together these data demonstrate that a full-length *app* cDNA was cloned. Northern blot analysis also shows that in unstressed *Arabidopsis*, the *app* mRNA steady-state level is low compared to the mRNA abundance in cell suspension culture (Fig. 3).

To determine the copy number of the *app* gene and to find a possible RFLP marker, a Southern blot analysis was performed on genomic DNA isolated from *Arabidopsis* ecotypes Columbia and Landsberg erecta. A single hybridizing fragment was detected when genomic DNAs were digested with *Sac*II, *Xho*I, and *Xba*I enzymes and hybridized with the yb55 probe (Fig. 4). RFLP polymorphism has been found using *Dra*I (Fig. 4). Using markers generated by this enzyme, the *app* gene was mapped to the top of chromosome 4 between markers g3843 and m448a. From Southern and mapping analyses, we can conclude that *app* is most probably a single-copy gene in the *Arabidopsis* genome.

3.2. Comparison of the deduced primary structure of APP with animal PARP

The similarity of APP (from amino acid 140; Fig. 1A) with the catalytic domain of animal PARP is about 62–63% (41–45% identity) for mammals, *Xenopus*, and insects, and 54% (30% identity) for *Caenorhabditis elegans*. An important lysine residue (Lys-893 [30]) is located on a stretch of amino acids considered as the PARP signature (...TGYMFGKG...), that is absolutely conserved in all PARP sequences and in APP (Fig. 1A).

The N-terminal domain of the plant protein does not reveal any sequence similarity with the corresponding domain of PARP from vertebrates. However, this extension is composed of four stretches of amino acids (named A1, A2, B and C) showing similarity to the N-terminus of a human heterogeneous nuclear ribonucleoprotein (hnRNP [31]) and to other nuclear proteins (Fig. 1B). A1 and A2 show similarity to each other and to hnRNP. The predicted secondary structure of A1 and A2 is a helix-loop-helix (HLH). B is rich in basic amino acids (K and R) and may function as a nuclear localization signal [32]. Alternatively, B and A2 (Fig. 2A) may form a basic-HLH structure typical of some DNA binding proteins.

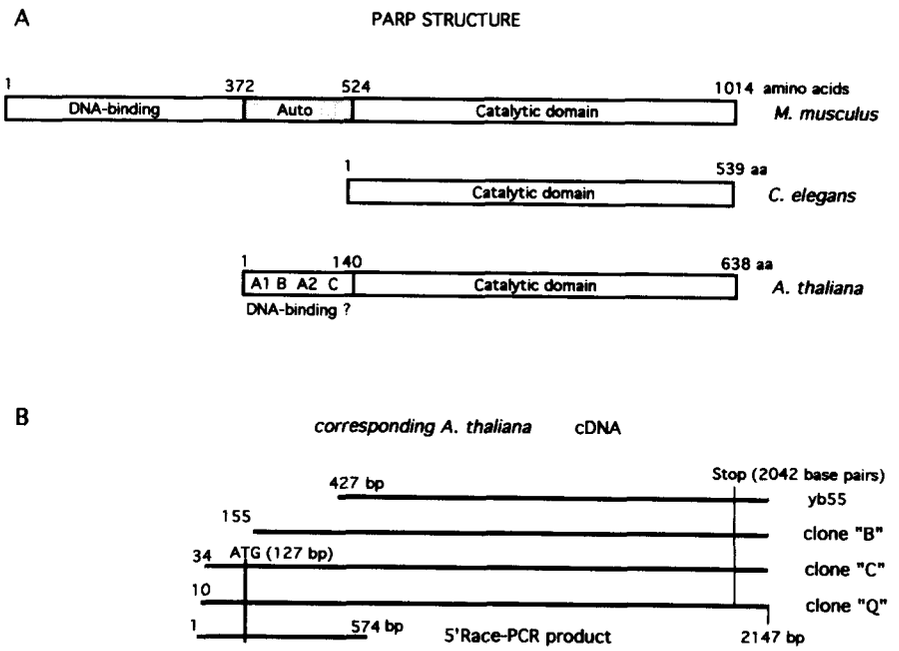

Fig. 2. Schematic presentation of the PARPs. (A) Comparison of the structure of mouse, *Arabidopsis* and *C. elegans* proteins. (B) Structure of the isolated *A. thaliana* cDNAs and of the 5′ anchored-PCR product.

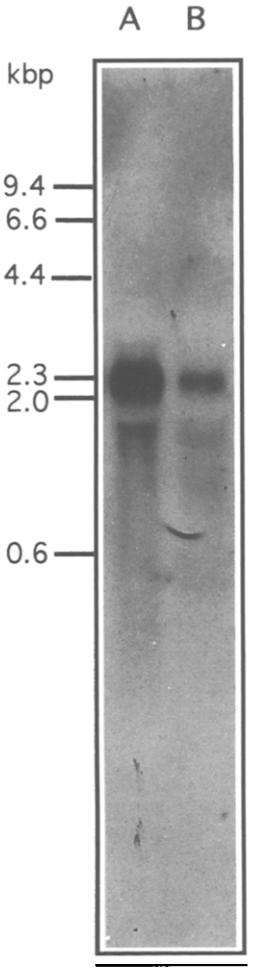

Fig. 3. Determination of *app* mRNA size. Total RNA (15 μg) was denatured with glyoxal, electrophoresed on a 1.5% agarose gel in a 40 mM ethanolamine (pH 7) buffer adjusted with phosphoric acid, and blotted on nylon membranes. Labelled and glyoxylated λ *Hin*dIII fragments were loaded to evaluate the size of the mRNA. RNA was cross-linked to the membrane with UV light, glyoxal was removed by incubation of the membranes in 40 mM NaOH for 15 s, and hybridized with ^{32}P-labeled (yb55) probe. The hybridizing band of about 2.3 kb was detected with RNA from cell suspension culture (A) and whole plant (B).

The C motif also shows some similarities with the human hnRNP and with other nuclear proteins, including a rat nuclear phosphoprotein associated to single-stranded DNA (B23) and a *Drosophila* DNA binding protein (CDP1) (Fig. 1B). The predicted secondary structure of C is a loop that separates the N-terminal extension from the catalytic domain. C is hydrophilic and rich in acidic residues (E, D); in this respect, it may correspond to the automodification domain of animal PARP.

3.3. Phylogenetic analysis

Different trees obtained using both distance and parsimonious analyses are congruent (data not shown). A consensus tree obtained after bootstrap analyses is shown in Fig. 5B. A high consistency index value obtained for parsimonious analysis (CI = 0.934) indicated the reliability of the relationships obtained (low homoplasy). Branch length values calculated by the distance method (NJ) show a rather constant evolutionary rate of the protein and suggest the use of the plant sequence as the root in the PARP tree. In addition, PARP relationships fit results obtained with other proteins, and especially emergence of nematodes early in metazoa evolution [33].

4. Discussion

In this work we have isolated an *Arabidopsis* cDNA encoding a protein with a high similarity (54–62%) to PARPs in the catalytic domain containing the PARP signature. Such a high similarity suggests that the *app* gene encodes a plant PARP. However, the plant protein is shorter than the vertebrate PARP. Interestingly, antibodies raised against the N-terminal part of the human enzyme do not recognize either the *Physarum polycephalum* nor the plant PARPs [2,34], suggesting a low similarity or absence of this domain in these two species. In addition, a sequence very similar to the PARP catalytic domain has been recently found in the *C. elegans* genome (accession number Z47075). A corresponding cDNA has been sequenced (EST database library Z14432), confirming that the gene is expressed. The ORF encodes a polypeptide of 538 amino acids matching the catalytic domain, and has no N-terminal extension (Fig. 2A). Phylogenetic analysis shows that animal proteins are monophyletic, suggesting that only one isoform was preexisting in the common animal and plant ancestor. These observations indicate that a short PARP corresponding to the catalytic domain would be the ancestral form of the protein and that acquisition of different N-termini occurred later during animal evolution (at least after nematodes emerged from metazoa).

PARP activity has been clearly demonstrated in plants, and histones have been identified as a target for poly-ADP-ribosylation [17]. However, although commonly used, in-gel or blot assays (based on PARP automodification) failed to reveal this activity in plants [1,2]. Only recently, using maize extracts, automodification of a 116 kDa protein has been obtained in a

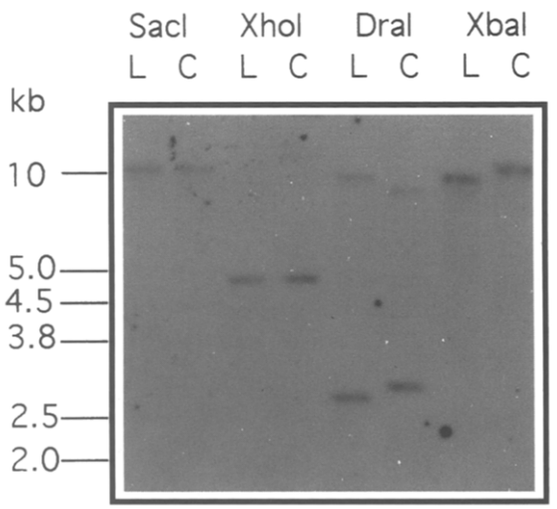

Fig. 4. Determination of the *app* gene copy number. Genomic DNA (2 μg) from *Arabidopsis thaliana* cv Lansberg erecta (L) and Columbia (C) were digested with *Sac*I, *Xho*I, *Dra*I, and *Xba*I restriction enzymes and separated by electrophoresis on a 0.7% agarose gel. After blotting, the nylon membrane was hybridized with a probe corresponding to clone yb55 (Fig. 2B).

A

	1	2	3	4	5	6	7	8	9
1 Mouse	-	95	96	88	85	61	64	58	50
2 human	92	-	94	86	84	61	63	58	51
3 Bovine	90	88	-	88	83	62	63	58	51
4 Chicken	79	77	77	-	87	61	66	58	47
5 *Xenopus*	74	72	71	78	-	60	64	59	51
6 *Drosophila*	43	43	43	43	43	-	76	57	48
7 *S.peregrina*	46	44	45	47	46	61	-	57	49
8 *A.thaliana*	38	38	40	38	38	37	37	-	53
9 *C.elegans*	28	28	28	28	27	27	28	30	-

above diagonal: % similarity
below diagonal: % identity

B

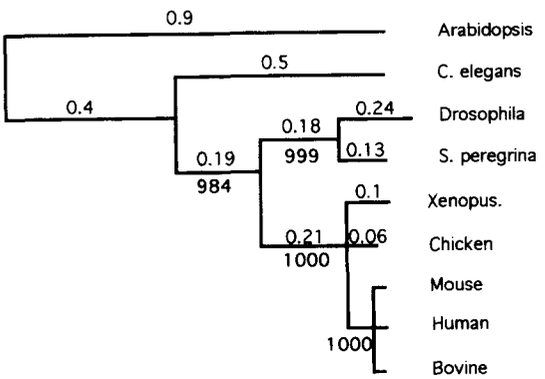

Fig. 5. Phylogenetic analyses. (A) Pair-wise comparison of the PARP proteins calculated using the Bestfit program from GCG (for accession number, see B). (B) The presented consensus tree (unrooted) was obtained using both the Neighbor Joining (NJ) method of Clustal V software (options: all sites with gaps deleted and distance corrected by Kimura's empirical method) and Parsimony analysis with PAUP 3 (options; branch-and-bound, heuristic search, with weighted matrices used in PROTPARS of the PHYLIP package [37]). Boot-strapping confidence values (n = 1000) are shown as numbers at the forks. Branch lengths, calculated by the Clustal V program as proportional divergence, are shown above the branches. Sources for sequences are P09874 (human), P27008 (rat), P11103 (mouse), P26446 (chicken), P18493 (bovine), P31669 (*Xenopus*), P35875 (*Drosophila*), D16482 (*Sarcophaga peregrina*), Z47075 (*C. elegans*), and Z48243 (*Arabidopsis thaliana*).

gel assay [18]. The discrepancy in size between the maize protein (116 kDa) and *Arabidopsis* APP (about 72 kDa) awaits molecular cloning of the maize PARP. It is to be noted that the C domain of APP is hydrophilic and rich in acidic residues (E, D) and, therefore, could be a target for automodification. The N-terminal extension of PARP has some similarities with domains of different nuclear and DNA binding proteins. Although the biological significance of these similarities has to be determined, these observations are in agreement with both the nuclear localization of plant PARP activity and the putative interaction of the protein with DNA [17,20,35]. Furthermore, the *Arabidopsis* APP may have a structure similar to that of animal PARP with an N-terminal DNA binding domain (A1, B and A2), an automodification region (C) and the carboxyl-terminal catalytic domain (Fig. 2A).

In animal, PARP is controlled at different levels including a strong induction of protein activity by DNA breaks, regulation by phosphorylation or at the level of transcription (for references, see [36]). Our results suggest that PARP activity could be regulated at the level of mRNA accumulation as shown in plant cell culture.

It has been recently suggested that plant PARP is involved in DNA repair [20] and the early response to stress (e.g. oxidative stress) [14]. Furthermore, a high activity has been found in seedling and in crown gall tumor tissue of tobacco [17,19], and we have shown higher mRNA accumulation in *Arabidopsis* cell suspension culture than in whole plant. These results suggest that PARP may also be involved in cell division and differentiation, as in mammalian cells. The availability of *app* cDNA will permit us to address the in vivo function of poly-ADP-ribosylation in transgenic plants.

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