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Expression analysis of a *Cucurbita* cDNA encoding endonuclease*

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The nuclear matrices of plant cell nuclei display intrinsic nuclease activity which consists in nicking supercoiled DNA. A cDNA encoding a 32 kDa endonuclease has been cloned and sequenced. The nucleotide and deduced amino-acid sequences show high homology to known 14-3-3 protein sequences from other sources. The amino-acid sequence shows agreement with consensus sequences for potential phosphorylation by protein kinase A and C and for calcium, lipid and membrane-binding sites. The nucleotide-binding site is also present within the conserved part of the sequence. By Northern blot analysis, the differential expression of the corresponding mRNA was detected; it was the strongest in sink tissues. The endonuclease activity found on DNA-polyacrylamide gel electrophoresis coincided with mRNA content and was the highest in tuber.

It was shown previously that in growth-stimulated cells, nuclear matrices are enriched in 32 kDa endonuclease [1]. The purified enzyme cleaved DNA showing no sequence specificity. The enzyme associated with the nuclear matrix introduced a single nick into one DNA strand, and this specificity dependent on a 65 kDa protein present in the nuclear matrix. It was also found that only supercoiled DNA was a target for the endonuclease attack. We have suggested that the 65 kDa protein modulates the nuclease function [2, 3].

To determine the molecular and functional features of the 32 kDa polypeptide we undertook the molecular cloning and expression analysis of its gene from a *Cucurbita pepo* cDNA library. The gel purified recombinant 32 kDa protein was as active as that purified from *Cucurbita* seedlings in a DNA-polyacrylamide system for assaying nuclease activity [4]. Comparison of sequence homology revealed that the 32 kDa polypeptide is a member of the multifunctional 14-3-3 protein family; members of this family are known to show several different activities, including activation of the phosphorylation-dependent monoamine synthesis [5], stimulation of calcium-dependent exocytosis [6] and potential transactivation [7], which seems to be of special importance for the cell functioning.

MATERIALS AND METHODS

Plant material. The seeds of *Cucurbita pepo* var. *patissonina* were obtained from Wrocław seed center (Centrala Nasienna Wrocław) and grown in a plant growth chamber under a light/dark regime of 16 h light and 8 h dark.

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cDNA library construction. mRNA was isolated from seedlings, and cDNA was synthesised and inserted into a λ-ZAP II vector according to the Stratagene protocol. The cDNA was ligated into the *Eco*RI-*Xho*I site of the vector. 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 on a lawn of *E. coli* XL 1-Blue host cells.

Sequencing of endonuclease cDNA. Nucleotide sequencing of cDNA subclones was carried out on single stranded templates according to the dideoxy chain termination method using the Pharmacia protocol. The amino-acid sequence was deduced from the nucleotide sequence using a computer program. Sequence analysis was performed using the programs of the University of Wisconsin Genetic Computer Group (GCG Package, Version 7). Sequence comparisons on cDNA and deduced amino-acid sequences were performed in GenBank, EMBL and SwissProt databases using the FASTA search program [8].

Northern blot analysis. Total RNA was prepared from different *Cucurbita pepo* tissues using the guanidinium isothiocyanate procedure [9]. Following electrophoresis RNA was transferred to a Hybond filter, which was prehybridized in 250 mM phosphate buffer, pH 7.2, containing 1 mM EDTA and 1% bovine serum albumin, and hybridized in the same solution containing 10^8 d.p.m. of the radiolabelled (multiprime labelling kit — Boehringer Mannheim) cDNA fragment. The probe consisted of a 1200 bp *Xba*I — *Xho*I cDNA insert of plasmid A 215. Hybridization was carried out at 65°C. Membranes were washed three times, for 30 min each, with 0.1 × SSC (saline-sodium citrate, pH 7.0) containing 0.5% SDS at 65°C.

Southern hybridization. Genomic DNA was extracted from fresh tissue by the cetyltrimethylammonium bromide method of Rogers and Bendich [10]. DNA aliquots (0.5 μg/ml) were digested with appropriate restriction endonucleases (10 units/μg) to completion as indicated by the restriction enzyme manufacturer (Boehringer). Approximately 10 μg of each DNA sample was separated on 1% agarose gel and transferred onto Pall B (Biodyne) membrane. Hybridization was carried out at 65°C in 250 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, 1% bovine serum albumin and 10^8 d.p.m. of ^{32}P-labelled A 215 cDNA fragment. Filters were washed with 0.1 × SSC containing 0.1% SDS at 65°C.

Protein extraction and nuclease assay. The proteins from plant organs were extracted with the Laemmli sample buffer and subjected to SDS/12% polyacrylamide gel electrophoresis. For the nuclease assay [4], salmon sperm DNA (10 μg/ml) was included in the SDS/12% acrylamide gel prior to polymerization. Following electrophoresis, the gel was washed four times (20 min each) with 15 volumes of 40 mM Tris/Cl, pH 7.4, and thereafter incubated in 10 volumes of the same buffer containing 2.5 mM $MgCl_2/CaCl_2$ for 12–24 h at room temperature. Then the gel was stained with ethidium bromide and photographed under UV light. To quantitate the enzyme activity, the picture was scanned with a laser densitometer.

RESULTS

Nucleotide sequence of the cDNA encoding the 32 kDa polypeptide and characteristics of the recombinant protein

Screening the λ-ZAP II expression library derived from *Cucurbita* mRNA with a polyclonal antibody, anti-32 kDa endonuclease [1], resulted in finding of seven positive clones. Three of them having the expected length, were selected for subcloning into pBluescript and sequencing. They were identical, each containing a 5′ non-translated leader sequence of 98 bp, a 825 bp open reading frame and a 3′ non-translated sequence of 246 bp. The 3′-end contained a consensus for polyadenylation site (AATAAA) and a poly-(A) tail of 18 bp. The 825 bp open reading frame codes for a predicted protein of 275 amino-acid residues having a molecular mass of 31111 Da and an isoelectric point of 4.77. The cDNA sequence was deposited in the EMBL/GenBank under accession number X76086. In the *Cucurbita* genome the predicted protein is represented by a single copy gene (Fig. 1). The smallest DNA fragment (1.9 kb) containing the intact nuclease gene was found among the *Xba*I/*Bgl*II DNA digestion products (Fig. 1).

For further investigations the clone was expressed in *E. coli* DH 5 α cells with the use of the β-galactosidase promoter in the presence of 0.7 mM IPTG (isopropyl-β-D-thiogalactopy-

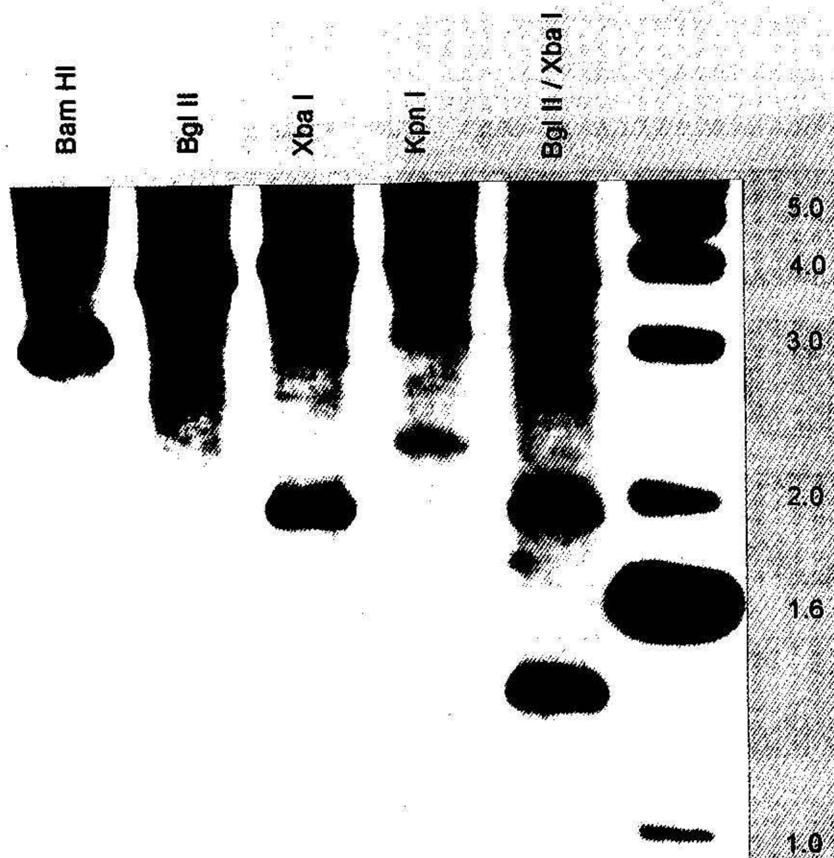

Fig. 1. Southern blot analysis of BamHI, BglII, XbaI, KpnI and BglII/XbaI — digested DNA from Cucurbita cotyledons hybridized to ^{32}*P-labelled cDNA encoding the 32 kDa endonuclease.*
The first lane on the right side corresponds to a DNA (1 kb ladder — Bio-Rad) length marker (in kb).

ranoside) and tested for binding polyclonal antibodies raised against *Cucurbita* endonuclease, as well as for enzyme activity. It has been shown that these antibodies recognize the product with molecular mass of 32 kDa. The product when analysed on DNA-acrylamide gel showed nuclease activity also at the 32 kDa protein band (Fig. 2).

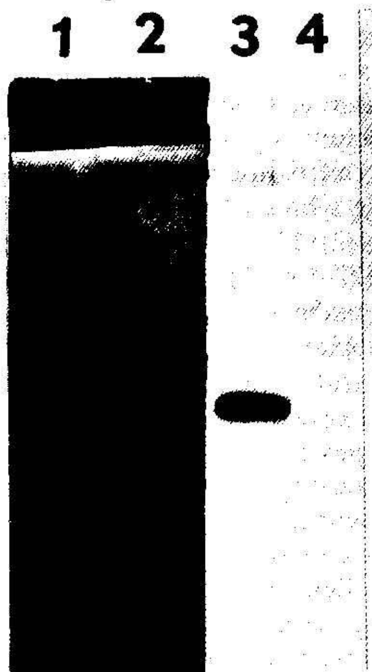

Fig. 2. Nuclease activity and immunoblot of extract of E. coli cells transformed with cDNA encoding the 32 kDa endonuclease.
Ten μg protein was loaded in each lane. Lanes 1 and 2: the 0.1% SDS extract, resolved by DNA-polyacrylamide gel electrophoresis; the nuclease activity was visualized by the decrease in ethidium bromide staining. The proteins resolved by polyacrylamide gel electrophoresis were blotted onto a nitrocellulose filter and probed with antibodies against the 32 kDa endonuclease. Lanes 1 and 3: cells transformed with the vector containing the cDNA; lanes 2 and 4: cells transformed with the vector (pBluescript) without insert.

Similarity to other cDNAs and proteins

A search through the EMBL database revealed high homology of the cloned cDNA to the 14-3-3 protein family. The highest homology (over 80% identity) was detected to protein from *Arabidopsis thaliana, Hordeum vulgare, Oenothera hookeri, Zea mays* and *Spinacia oleracea.* There was also a substantial similarity to human, sheep, bovine, yeast and *Xenopus laevis* 14-3-3 proteins (over 60% identity). Limited similarity to the cellular membrane protein (VP5), protein kinase C-like (PKC), nuclear migration protein (NUM1), AMP nucleosidase and *src* tyrosine kinase was also detected. Figure 3 presents the aligned amino-acid sequences of 14-3-3 pro-

Fig. 3. Sequence alignment of the Cucurbita pepo derived 32 kDa protein: A215 with Oenothera hookeri: Oenho [21], Arabidopsis thaliana: Arath [12], Spinacia oleracea: Spio [21], Hordeum vulgare: Horvu [22] and human [15] 14-3-3 protein. Numbers indicate amino acid positions. Residues identical to A215 are boxed.

teins from *Oenothera*, *Arabidopsis*, spinach, barley and *Cucurbita* as well as of human origin, showing highly conserved regions (boxed) with less conserved amino-acid residues in between. Large differences in the aligned sequences concern the N- and C-terminus. The largest molecule so far within the 14-3-3 protein family is that from *Cucurbita*.

Amino-acid sequence of the *Cucurbita* 32 kDa polypeptide

Analysis of this sequence reveals several motifs that could be involved in the 32 kDa protein function. There is a motif RRXS (Fig. 3, residues 62–65) for potential phosphorylation by protein kinase A and protein kinase C [11, 12]. It was shown [13, 14] that the sheep brain 14-3-3 protein is a potent inhibitor of the Ca^{2+}/phospholipid-dependent protein kinase C. The inhibition is caused by the presence of a short stretch of amino-acid residues that resembles a protein kinase C pseudosubstrate site (Fig. 3, residues 60–65). Within the conserved part of the sequences there is a stretch (Fig. 3, residues 132–146) showing a high similarity (over 60%) to the conserved C-terminal sequence of a family of calcium, lipid and membrane-binding proteins, called lipocortins or annexins, thus suggesting a broad spectrum of molecules interacting with 14-3-3 proteins [15]. Amino acid residues 177–183 (Fig. 3) resemble the proposed "B-type" nucleotide binding sequence RXGX (hydrophobic) D [16] with the exception that the motif ends with Asn instead of Asp. Interestingly, a similarity (38%) was also found to the *Xotch* protein (Fig. 3, residues 77–92) which is believed to be involved in the nervous system development in *Xenopus* [17]. This stretch of homology contains the epidermal growth factor (EGF)-like repeat. Rather high homology (63.6%, Fig. 3, residues 90–100) was found to *src* tyrosine kinase [18]; this sequence is located in

close vicinity to and partially overlaps the EGF-like repeat.

Finally it should be pointed out that rather high homology was found at the nucleotide level between the *Cucurbita* 32 kDa protein and the potato ADP ribosylation factor (ARF) protein at the nucleotide level (Fig. 4). Forty nine nucleotides of the *Cucurbita* protein (residues 926 to 969) show 84% identity to our earlier cloned (accession X74461) potato ARF protein (residues 502–550).

Interestingly the FAS protein, which is functionally identical to ARF protein, resembles closely the 14-3-3 protein [19].

Organ distribution of 32 kDa mRNA

The transcript encoding the 32 kDa protein was found to be present in significant amounts in *Cucurbita pepo* seedlings and cotyledons (Fig. 5). The transcript was undetectable in leaves and only faintly seen in root and stem cells. More detailed analysis was undertaken also of potato tissues because of their availability, like in *Cucurbita* tissues, a significant amount of the 32 kDa protein transcript was found in cotyledon, tuber and stolon. Again the transcript was almost undetectable in leaves and faintly seen in nodes and internodes.

Consistent with this observation is the result of the assay for nuclease activity by DNA-polyacrylamide gel electrophoresis (Fig. 6). The roughly estimated level of enzyme activity was about five times as high in tubers as in leaves. Thus, it appears that the 32 kDa protein transcript pattern in plants differs quantitatively from organ to organ. This may indicate that expression of this protein is organ-specific.

DISCUSSION

Our experimental approach was designed at recovering of the cDNA encoding the protein involved in DNA nicking and primarily localized in the nuclear matrix. The DNA-polyacrylamide gel electrophoresis and immunoblotting assay were used for screening recombinant proteins.

The enzyme assay on DNA-polyacrylamide gel clearly showed nuclease activity derived from DNA insertion and the molecular mass of the active fractions corresponded closely to the mass of the expected active protein. Consistently, the immunoblot analysis revealed a band which corresponded to the endonuclease molecular mass and showed no cross-reactivity with other proteins.

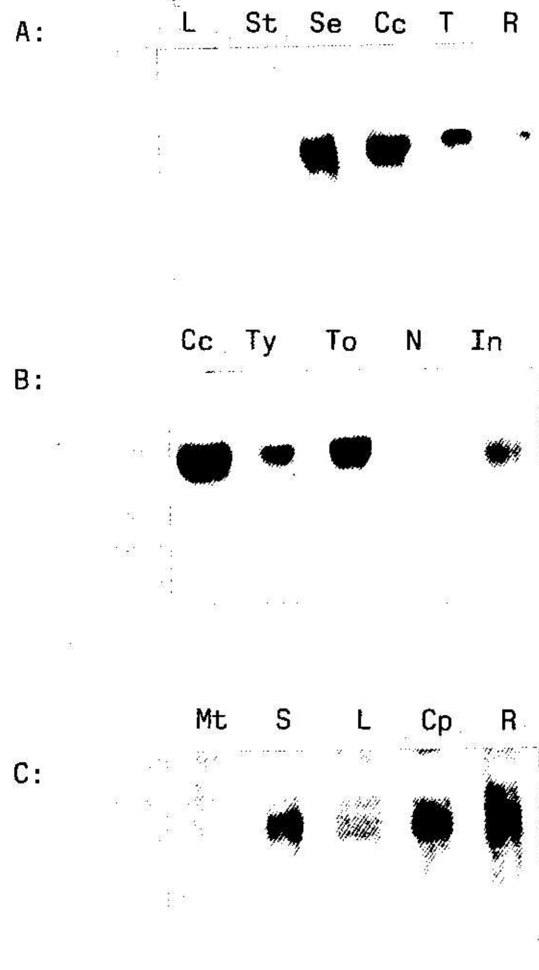

Fig. 5. Northern blot analysis of the 32 kDa protein gene expression in various tissues of Cucurbita (A) and potato plants (B, C).

Total RNA (50 µg) was fractionated by denaturing agarose gel electrophoresis and, after transfer to nylon membranes hybridized against the ^{32}P-labelled cDNA encoding the 32 kDa endonuclease isolated from clone A215. A: L, leaf; St, stem; S, seedling; Cc, cotyledon; T, potato tuber; R, root. B, C: Cc, *Cucurbita* cotyledon; Ty, young tuber; To, older tuber; N, node; In, internode; Mt, mother tuber; S, stolon; L, leaf; Cp, cotyledon; R, root.

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926 ACATGTTTTA.TTCTGGAGAAGTGCT.TGTTTGGAACT...TTGGCTTT 969   A215 cDNA
    ||||||  || ||||||||||||| ||| | |  || |||   ||||||||
502 ACATGTGCTACTTCTGGAGAAGGGCTATATGAGGGACTGGATTGGCTTT 550   ARF cDNA
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Fig. 4. Nucleotide sequence alignment of Cucurbita pepo A215 with potato ARF cDNA fragment.

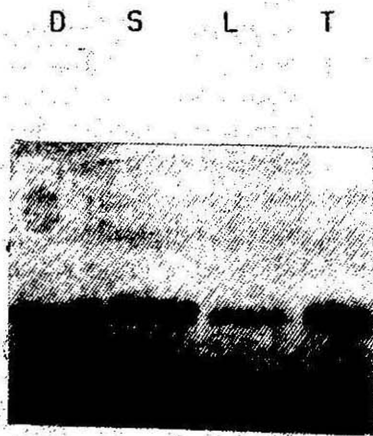

Fig. 6. DNA-SDS polyacrylamide gel electrophoresis of protein extract from potato stolon (S), leaf (C) and tuber (T). (D), DNase I (Sigma) 0.4 μg as a standard.

Onto each slot 80 μg of protein was applied. The nuclease activity was visualized as described in Materials and Methods.

Careful inspection of the amino-acid sequence of this protein pointed to the presence of several functional motifs. Among them are a type B nucleotide binding motif and an RRASWR motif (residues 62–67) identified in maize (R NLS A) as a nuclear localization signal which is responsible for protein partitioning between nucleus and cytoplasm (for review see Raikhel, [20]).

Thus we conclude that the cloned cDNA, encodes the *Cucurbita* 32 kDa protein.

A search over the database revealed that the cloned protein is highly homologous to the 14-3-3 protein family. It is well known that these proteins of molecular mass ranging from 29 kDa to 33 kDa, are localized preferentially in neurons and function as protein kinase-dependent activators of tyrosine and tryptophan hydroxylases. Both hydroxylases are known to be rate-limiting enzymes in the pathway of monoamine biosynthesis. However, this cannot be the only function of the 14-3-3 protein, as it is also present in tissues which do not synthesise these polypeptides [15].

It can be expected that, like the neuron 14-3-3 proteins, plant 14-3-3 proteins regulate protein kinase activity and activate the biosynthesis of neurotransmitters in plants.

Our earlier data suggested involvement of the 32 kDa protein in DNA metabolism regulation and its presence in the nuclear matrix. The activity of the 32 kDa polypeptide was regulated by another matrix protein with molecular mass of 65 kDa [3]. These observations are consistent with the finding that the 14-3-3 homolog from *Arabidopsis* is part of a complex which binds to G box DNA and that the binding takes place only in the presence of another protein with apparent molecular mass of 69 kDa [12].

The general role for a plant 14-3-3 protein, as we predicted in DNA metabolism, is consistent with its gene expression. It is demonstrated that mRNA encoding the 32 kDa polypeptide is not randomly distributed and its highest level in sink organs was found. It is possible that the sink tissues differ in their function from source organs as a result of the 32 kDa endonuclease gene expression.

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