A corm-specific gene encodes tarin, a major globulin of taro (*Colocasia esculenta* L. Schott)

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Abstract

A gene encoding a globulin from a major taro (*Colocasia esculenta* L. Schott) corm protein family, tarin (G1, ca. 28 kDa) was isolated from a λ Charon 35 library, using a cDNA derived from a highly abundant corm-specific mRNA, as probe. The gene, named *tar1*, and the corresponding cDNA were characterized and compared. No introns were found. The major transcription start site was determined by primer extension analysis. The gene has an open reading frame (ORF) of 765 bp, and the deduced amino acid sequence indicated a precursor polypeptide of 255 residues that is post-translationally processed into two subunits of about 12.5 kDa each. The deduced protein is 45% homologous to curculin, a sweet-tasting protein found in the fruit pulp of *Curculigo latifolia* and 40% homologous to a mannose-binding lectin from *Galanthus nivalis*. Significant similarity was also found at the nucleic acid sequence level with genes encoding lectins from plant species of the Amaryllidaceae and Lilliaceae families.

Introduction

Storage proteins offer an interesting model system to study the regulation of gene expression during development and with respect to organ specificity. They are present in storage organs and sometimes can be detected in small amounts in other plant organs and tissues [9, 10]. In several instances it was found that storage proteins of plants have other functions, in addition to their storage role. These proteins may function as proteinase inhibitors, and have antifungal or antibacterial activity [6]. Tuber storage proteins have been correlated with acyl lipoyl enzymatic activity, like patatin in potato [31, 32], and with defense responses, like sporamin in sweet potato [26].

The major corm proteins from taro (*Colocasia esculenta* L.), an important edible aroid crop that propagates vegetatively, have been characterized

[3, 4]. Taro corms have four major protein families, two albumins (A1 and A2) and two globulins (G1 and G2), that are expressed from the primordial until the latest developmental stages of corms. It has been reported that G1 and G2 globulins share homologies with proteinase inhibitors and taste-modifying proteins [4]. Immunocytochemical studies indicate that the major globulins are present in parenchyma cells, especially in those containing starch granules (Monte-Neshich et al., unpublished). A cDNA, TC1, corresponding to one of the major corm mRNAs (1.0 kb) was identified in a cDNA library from taro corms [4].

In this paper we report the isolation of a gene, tar1, corresponding to the TC1 clone, from a λ Charon 35 genomic library and the molecular characterization of both the cDNA and genomic sequence. We show that this gene encodes a protein from the G1 globulin family, which we named tarin, as a precursor polypeptide that is post-translationally processed into two subunits. We found that the deduced protein is homologous to a mannose-binding lectin from Galanthus nivalis and to curculin, a sweet protein found in the fruit pulp of Curculigo latifolia. Moreover, we show that this gene is homologous to lectin genes from other monocotyledonous species belonging to families closely related to the Araceae family.

Materials and methods

Plant material

Taro corms of 20–30 mm diameter (stages C1 to C5 of corm development, as described by De Castro et al. [4]) were harvested and stored at −80 °C until nucleic acid or protein extraction.

DNA isolation

Total genomic DNA was isolated from taro corms. Plasmid DNA isolation was carried out using a modified procedure of Birnboim and Doly [1].

Genomic library and screening

Genomic DNA was partially digested with Sau 3AI. Fragments of 16 to 20 kb were purified in a 40% sucrose gradient and ligated to λ Charon 35-Bam HI-digested arms. About 200 000 plaques from the unamplified library were screened using a radiolabelled TC1 cDNA clone [4]. A partial restriction map was made (Fig. 1) and the gene orientation determined by Southern blot analysis using as probes DNA fragments obtained from the 3′ region and the TC1 cDNA clone (data not shown).

DNA sequencing and analysis

DNA sequencing was carried out by the chain termination method [34], using Sequenase Version 2.0 (USB Corporation). Overlapping unidirectional deletions of the cDNA insert were done using Exonuclease III/S1 nuclease digestion according to Henikoff [13]. For the genomic sequence, the Exonuclease III/Mung Bean Nuclease sequential deletion kit (Stratagene) was used. Higher reaction temperatures (42 °C) and gels containing formamide were used to resolve compressions found in GC-rich regions. The complementary DNA strands were sequenced using, as primers, synthetic oligonucleotides derived from the first-strand nucleotide sequence.

Search for DNA sequence homology of tar1 was performed against EMBL database (EMBL 38, March 1994; SwissProt 28, March 1994; GenBank 81, February 1994) using the FASTA algorithm of the GCG package. Also used were the algorithms TFASTA, BTFASTA, PILEUP for multiple sequence alignment, BESTFIT and REPEAT.

RNA isolation and primer extension analysis

Polysomal RNA from taro corms was extracted according to Kamalay and Goldberg [20]. Poly(A)$^+$ RNA was isolated using Poly(A)Tract mRNA Isolation System (Promega) according to

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                                    *   ▼*
-  32  TATAAATGGACGGGAAGACGATATTGCTAGCCAGCATCACGGCGAAGAAGAAGATCTAG

   28  CTAGGGTTTTGCATTTAGCGGCAACCCCACTCCTC ATG GCC AAG CTT CTC CTC
                                            M   A   K   L   L   L
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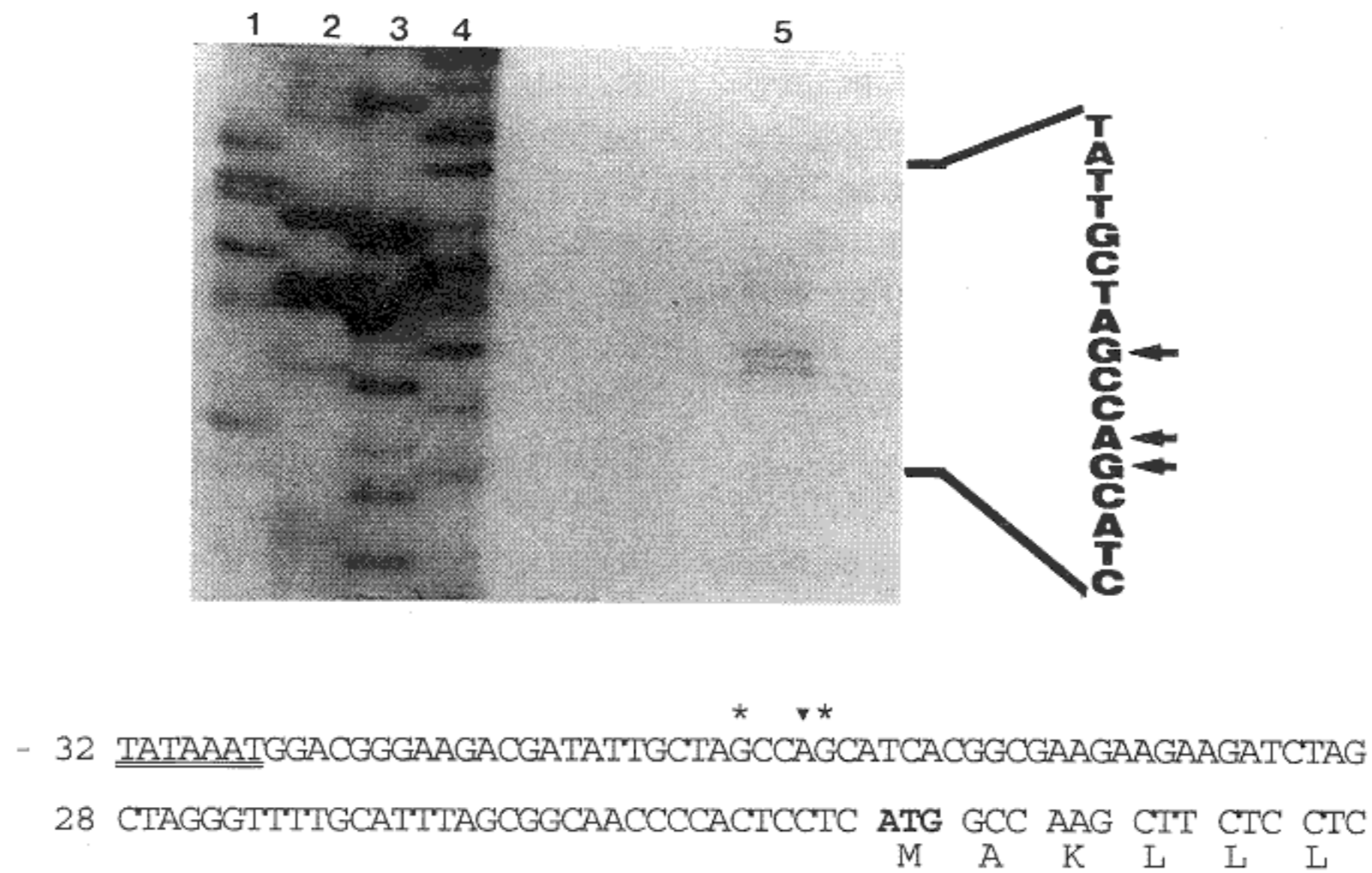
Fig. 1. Identification of the putative transcription start site. Reverse transcription products of taro mRNA are shown in lane 5. Sequencing reactions of pCEG5 are shown in lanes 1–4 (T, G, C and A, respectively). The TATA box is underlined; ATG is shown in bold; the major transcription start site is indicated by ▼, and minor ones by *.

the manufacturer's instructions. Primer extension was performed using a synthetic oligodeoxynucleotide (5'-AGAGGAGAAGCTTGGCCATG-3') complementary to positions +46 to +65 (Fig. 2) including the first ATG codon of the *tar1* gene. The primer was labelled at the 5' end using
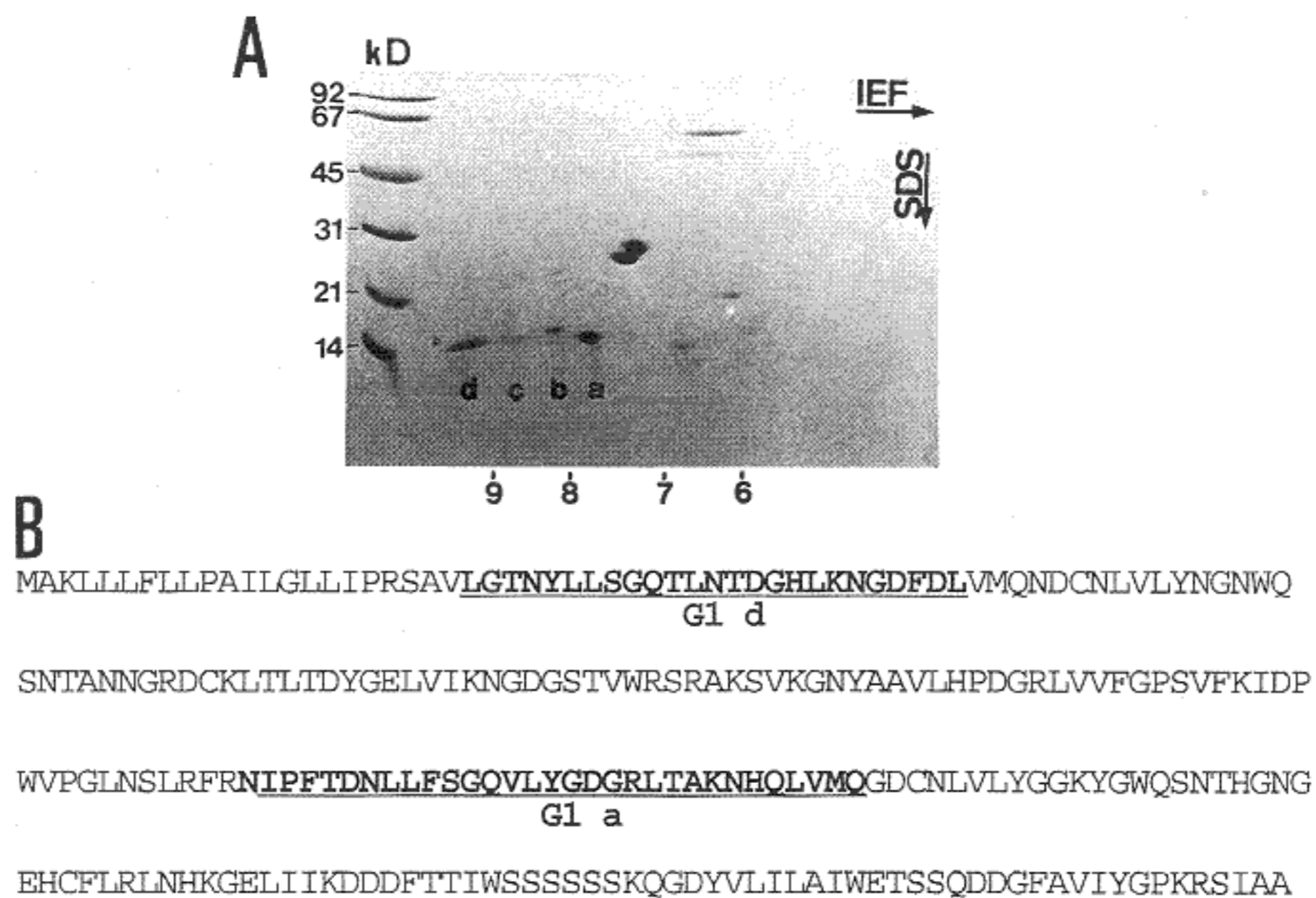

MAKLLLFLLPAILGLLIPRSAV**LGTNYLLSGQTLNTDGHLKNGDFDL**VMQNDCNLVLYNGNWQ
                      G1 d

SNTANNGRDCKLTLTDYGELVIKNGDGSTVWRSRAKSVKGNYAAVLHPDGRLVVFGPSVFKIDP

WVPGLNSLRFR**NIPFTDNLLFSGQVLYGDGRLTAKNHQLVMQ**GDCNLVLYGGKYGWQSNTHGNG
           G1 a

EHCFLRLNHKGELIIKDDDFTTIWSSSSSSSKQGDYVLILAIWETSSQDDGFAVIYGPKRSIAA

Fig. 2. Identification of *tar1* gene products. A. Coomassie blue staining of total corm soluble protein separated by 2D PAGE. B. Comparison of the deduced protein sequence of the product of *tar1* gene with the NH$_2$-terminal sequences of the globulins G1d and G1a (underlined).

T4 polynucleotide kinase and $(\gamma\text{-}^{32}P)$-ATP, hybridized with polysomal poly(A)$^+$ RNA from taro corms and extended with reverse transcriptase. The extended products were analyzed on an 8% polyacrylamide sequencing gel. The transcription start site was localized by comparison with the sequence of the genomic clone using the same end-labelled oligonucleotide as primer.

Protein isolation, sequencing and western blot analysis

Total proteins were extracted according to Carneiro et al. [3] from taro corms at five stages of development, C1 to C5, as described by De Castro et al. [4]. Proteins for sequencing were fractionated by two-dimensional electrophoresis and transferred to PVDF (Millipore) membranes. Microsequencing was performed by an Applied Biosystem 4758 Gas Phase Protein Sequencer, using 500 pmol per sample. For western blot proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF (Millipore) or nitrocellulose (Amersham) membranes. Transfers were carried out as described by Hirano and Watanabe [15]. Antiserum production and western blot analysis were carried out as described in Grossi de Sa et al. [11].

Results

Gene isolation, sequence and structure

Three genomic clones were isolated after library screening, and all yielded Eco RI fragments of 5.7 kb (λTC1-1), 17.0 kb (λTC1-2) and of 7.5 kb (λTC1-3). The 5.7 kb fragment of clone λTC1-1 was subcloned into pGEM 7Zf(+) (Stratagene) as pCEG5, and partially mapped by restriction endonucleases. Genomic DNA unidirectional deletions were obtained from an internal 1.2 kb Bam HI fragment of subclone pCEG5. Comparison between the cDNA and the genomic DNA showed total identity between the two sequences (data not show) and no introns in the genomic

sequence. The *tar1* gene has an open reading frame (ORF) of 765 bp (data not shown). Two potential translation start sites [5] are present in the 5' region of the sequence, the first of which, located at position +63, fits best the criteria of Kozak [22] and Lutcke et al. [24] and was therefore considered to be the translation start site. A typical structure of a gene encoding storage protein was found, including two stop codons and two putative polyadenylation motifs, AATAAA and AATAAT (data not shown).

Three products were synthesized during primer extension analysis. Two major potential transcription start sites were found located in two adjacent bases A and G. The first base A was assumed to be the transcription start site (+1), based on the remarkable conservation of A at the transcription initiation site of several genes found by Joshi [18]. A fainter signal was detected that corresponds to base at position −3 (Fig. 1). A TATA box is found 32 bp upstream of the transcription start site.

The gene encodes two proteins of the G1 globulin family

The deduced amino acid sequence of the gene indicated a polypeptide of 255 residues. The hydropathic profile shows a highly hydrophobic region at the NH$_2$ terminus of the protein (data not shown). The gene products were identified by comparison of the deduced protein sequence and the NH$_2$ terminal sequence of the most abundant proteins from taro corms (Fig. 2A, B). This comparison shows that the polypeptide has a peptide-signal of 23 residues and two domains of 116 amino acids each, corresponding to two proteins of ca. 12.5 kDa, observed as isoforms G1a and G1d, in 2D-PAGE analysis of total soluble corm proteins. These isoforms are part of the G1 family of globulins, with estimated native molecular mass of ca. 26–28 kDa, that corresponds to c. 40% of total proteins found in taro corms. The first domain starts on residue 24 and it is identical to globulin G1d. The second domain starts on residue 140 and corresponds to G1a (Fig. 2B). Com-

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              1                                                    50
Tarin-G1d  .......MAK LLLFLLPAIL GLLIPRSAVA LGTNYLLSGQ TLNTDGHLKN
Tarin-G1a  .......... .......NIP FTDNLLFSGQ VLYGDGRLTA

Lec-Galan  .......... .TILLILATI FLGVIT.PSC LSENVLYSGE TLPTGGFLSS
Curc-Curla .......... .......... .......... ..DNVLLSGQ TLHADHSLQA

              51                                                   100
Tarin-G1d  GDFDLVMQND CNLVLYNGNW QSNTANNG.. .RDCKLTLT DYGELVIKNGD
Tarin-G1a  KNHQLVMQGD CNLVLYGGKY GWQSNTHGNG .EHCFLRLN HKGELIIKDDD

Lec-Galan  GSFVFIMQED CNLVLYNVDK PIWATNTGGL SSDCTLSMQ TDGNLVVYTPS
Curc-Curla GAYTLTIQNN CNLVKYQNGR QIWASNTDRR GSGCRLTLL SDGNLVIYDHN

              101                                                  150
Tarin-G1d  GSTVWRSRAK SVKGNYAAVL HPDGRLVVFG PSVFKIDPWV PGLNSLR...
Tarin-G1a  FKTIWSSSSS SKQGDYVLIL QDDGFAVIYG PAIWETSSKR SIAA......

Lec-Galan  NKPIWASNTD SQNGHYVCIL QKDRNVVIYG TDRWATGTYT GAVG......
Curc-Curla NNDVNGSACC GDAGKYALVL QKDGRFVIYG PVLWSLGP.. ..........

              151                                                  200
Lec-Galan  ....IPESPP SE.KYPTAGK IKQVTEK... .......... ..........
Curc-Curla .......... .NGCRRVNG. .......... .......... ..........
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Fig. 3. Multiple sequence alignment of proteins. Lec-Galan, lectin from *Galanthus nivalis*; Curc-Curla, curculin; Tarin-G1d and Tarin-G1a, taro globulin subunits G1d and G1a, respectively.

puter analysis of the two domains shows that the two G1 subunits, G1d and G1a, share 60% homology (Fig. 3). The calculated pI of the two subunits is 8.7 and 6.5, respectively, which is close to the pI of G1d and G1a, estimated by iso-electric focusing (Fig. 2A).
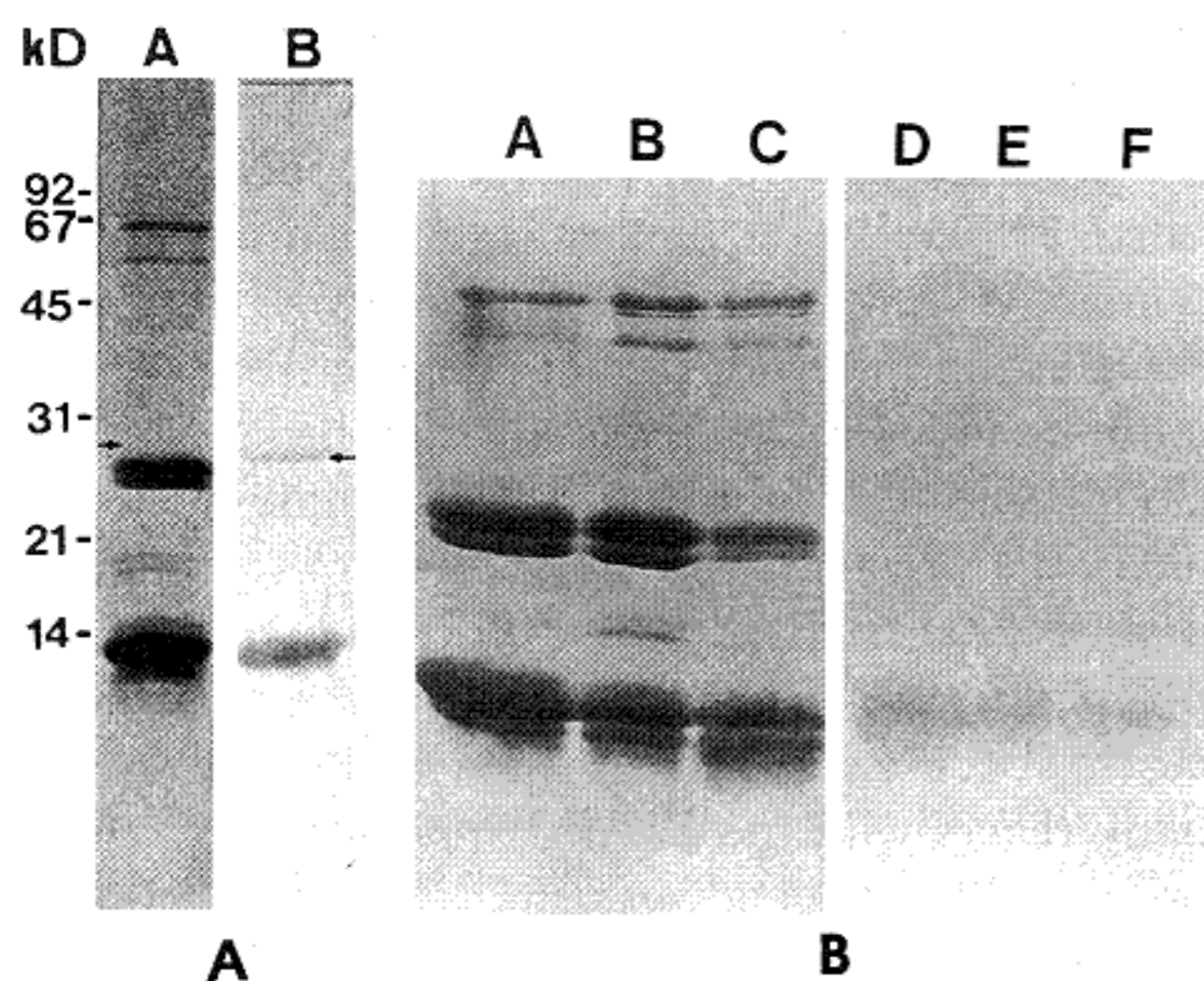

Fig. 4. Identification of precursor polypeptide *in vivo*. A. Coomassie blue staining of SDS-PAGE of total soluble protein from taro corms in stage C1 of development (lane A) and western blot analysis using antibody raised against G1 globulins (lane B). The precursor polypeptide is indicated by an arrow. B. Coomassie blue staining of SDS-PAGE and corresponding western blot of total soluble protein from taro corms in stage C2 (lanes A, D), stage C3 (lanes B, E) and C5 (lanes C, F) of corm development.

In order to confirm the existence of a precursor polypeptide *in vivo*, western blot analysis of crude protein extract from corms in various stages of corm development (stages C1 to C5) was carried out, using SDS-PAGE. A band of 28 kDa as well as a band of 12.5 kDa, reacted with the antibody raised against the G1 globulin family in protein from stage C1 (Fig. 4A). Only the 12.5 kDa band reacted with the antibody in stages C2, C3 and C5, suggesting the presence of a precursor molecule in the younger cormels.

Tarin may have additional properties besides its storage function

The nucleotide sequence of *tar1* is 54–58% homologous to lectin genes from *Galanthus* sp., *Narcissus* sp., *Hippeastrum* sp. and *Allium sativum* (data not shown). The alignment of the deduced protein sequences of taro and related protein genes is shown in Fig. 3.

The deduced protein sequence shows 45% identity to curculin, a taste-modifying protein found in the pulp of the small fruits of *Curculigo latifolia* described by Yamashita *et al.* [39]. It also shows 40% identity with a mannose-specific lectin precursor of *Galanthus nivalis*.

Discussion

Storage proteins genes have been described for few tuber and root storage organs. The best characterized are patatin and proteinase inhibitors from potato [31, 21] and sporamin from sweet potato [26]. In taro corms two major globulin families (G1 and G2) have been reported [3, 4]. These proteins accumulate very early and throughout most stages of corm development and are degraded in old corms, when new cormels bud off. They are found in the corm parenchymal tissue, in vacuole-like structures, mostly in the cells that contain starch granules (Monte-Neshich et al., unpublished). In this paper we report the isolation of a taro corm-specific gene, tar1, and the characterization of both the cDNA and genomic DNA sequence. It is transcribed into a highly abundant mRNAs of taro, of 1.0 kb. The gene encodes two subunits of a protein that belongs to one of the most abundant protein families in taro corms, the G1 globulin, tarin. This family comprises about 40% of total corm protein (Monte-Neshich et al., unpublished).

The tar1 gene has structural features similar to those of other storage protein genes [12, 27]. The TATA box sequence (TATAAAT), at −32 bp, is similar to the consensus described for dicot and cereal storage proteins, and for lectins [18]. The presence of two ATG codons has also been observed in other storage protein genes, such as the genes encoding the 1.7S albumin of Brassica napus [17] and avenin, the major globulin from Avena sativa [35]. The presence of two termination codons is also commom in storage proteins, examples of which are found in the genes coding for legumin from pea [25] and the 2S albumin from Brazil nut [8]. The two polyadenylation sites, are in an AT-rich region that fits well with the classical consensus described for this region by Joshi [19].

Three products were observed in the primer extension analysis, which may correspond to three copies of these gene as previously verified by genomic Southern blot [4]. Considering the occurrence of at least five major isoforms in the G1 globulin family (Fig. 2A) it may be that this gene belongs to a multigene family. Generally, storage proteins are encoded by multigene families highly homologous at the nucleotide and protein levels [27]. Several multigene families have been reported in plants, as in the case of storage proteins of tubers [29], cereals [28], legumes [7], of lectins [37] and of enzymes, such as α-amylase [16, 36].

Based on the total identity of the cDNA and the genomic sequences, the gene has no introns. The absence of introns has also been reported for some other storage proteins, such as the zeins of maize and the 1.7S albumin from Brassica napus [27, 17].

The protein deduced from the gene sequence has 255 amino acid residues with a calculated molecular mass of 28 kDa and pI 8.2. The 23 amino acid signal peptide is typical of storage proteins, with a lysine close to the NH_2 terminus, a hydrophobic region in the center and an alanine residue at the 3′ end [27]. The occurrence of a signal-peptide, which is responsible for protein compartmentalization, agrees with the immuno-cytolocalization of the G1 globulin in vacuole-like structures (Monte-Neshich et al., unpublished).

By western blot analysis it was demonstrated the presence of a small amount of a polypeptide of ca. 28 kDa, in the earliest stage of corm development (Fig. 4A). This polypeptide was not observed in the western blot of proteins from older corms (Fig. 4B) and could not be seen in Coomassie-stained gel. This polypeptide is most likely the precursor polypeptide of this protein, that is post-translationally processed into two polypeptides of about 12.5 kDa each. The processing occurs very rapidly since most of the antibody reacts with the band corresponding to the processed 12.5 kDa proteins. The native G1 globulins have an estimated mol. mass of 26–28 kDa and seem to consist of dimers, with two subunits of 12.5 kDa each. Since the two subunits are encoded by a single gene, it is very likely that the G1 globulin is composed of the two protein domains processed from the precursor polypeptide encoded by the tar1 gene, upon removal of the signal-peptide and internal cleavage. In this model, these subunits would correspond

to the two globulins G1a and G1d, observed in 2-dimensional gels (Fig. 2A). Similar processing occurs in one of the lectins from *Allium* sp., encoded by a gene homologous to *tar1*. It is post-translationally processed into two highly homologous subunits of 12.5 and 11.5 kDa [38].

The database search for homology to other known genes shows that the *tar1* gene has significant homology to other lectin genes from *Narcissus* sp., *Hippeastrum* sp., *Galanthus* sp. and *Allium sativum* (data not shown). These species belong to Amaryllidaceae and Lilliaceae families, of the Liliidae order, and are highly homologous [37]. Taro belongs to the Araceae family, Arales order, which seems to have evolved from a liliaceous ancestry [14]. The homology found among the taro gene and genes from species of the Liliidae order further supports this hypothesis. All these genes encode proteins composed of subunits of similar sizes, in the range of 12.5 kDa. There are, however, differences in the molecular structure of these lectins. Amaryllis (*Hippeastrum* hybr.) lectins are tetramers whereas a daffodil (*Narcissus pseudonarcissus*) lectin is a dimer [37]. We have also found that these globulins have 45% identity to curculin, a taste-modifying protein from *Curculigo latifolia*, a homodimer with subunits of 12.5 kDa, and have 40% identity to a mannose-specific lectin from snowdrop (*Galanthus nivalis*), a tetramer having also subunits of 12.5 kDa.

The physiological role of storage proteins in plants is not yet precisely known; some are related to defense mechanisms, such as Kunitz trypsin inhibitors and proteinase inhibitors [2, 23], and some have enzymatic activity, such as patatin [32]. It is likely that the tarins may play another role in the cell besides its storage function. Some lectins from the Arales order have been detected in seeds and tubers, with the property to agglutinate blood cells from small animals like rabbits, rat and Guinea pig [33] as well as the heads and tails of immature spermatozoids [30], but not human blood cells.

The molecular characterization of a corm-specific gene from taro allows us to infer that this protein may be a homodimer composed of two subunits of 12.5 kDa each, having 60% identity. The molecular structure characterization of this gene and database search for homology to other reported genes and proteins gave an insight on possible additional properties of G1 globulins, besides its storage function in taro corms. The G1 globulins have now being purified by HPLC in our laboratory, and experiments are in progress to test its agglutinating properties. Preliminary results suggest that this protein may indeed belong to the family of lectins.

Acknowledgements

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