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A family of β-galactosidase cDNAs related to development of vegetative tissue in *Cicer arietinum*

Rocío Esteban, Emilia Labrador, Berta Dopico*

Dpto. de Fisiología Vegetal, Facultad de Biología, Universidad de Salamanca, Centro Hispano-Luso de Investigaciones Agrarias, Pza Doctores de la Reina s/n, Salamanca 37007, Spain

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Abstract

In the cell wall of *Cicer arietinum* epicotyls, there are a family of β-galactosidases, one of them named βIII-Gal is a β-galactosidase able to degrade cell wall pectins. The role of the remainder β-galactosidases has not been established. In this paper, we describe the cloning and expression pattern of a family of three *C. arietinum* β-galactosidase cDNAs (named CanBGal-1, CanBGal-4 and CanBGal-5) and we compare these results with the previously characterized CanBGal-3 cDNA clone, which encode the βIII-Gal. The shared amino acid sequence identity among the four β-galactosidase deduced proteins (named β-Gal, βIII-Gal, βIV-Gal and βV-Gal) ranged from 63% to 81%. All display the putative active site of family 35 of the glycosyl hydrolases. An unusual characteristic of one of the chickpea β-galactosidases (βI-Gal) is the presence at the C-terminus of the enzyme of a galactose binding lectin domain.

The CanBGals gene expression along seedlings and adult plant could suggests different roles of their corresponding protein throughout the chickpea plant. The expression of CanBGal-5 is related to young and meristematic stages with high cell division rate, such as the meristematic hook, very young epicotyls, and apical internodes. By contrast, CanBGal-1 and -4 seem to be more strongly related to advanced stages of epicotyl growth, increasing their expression along epicotyl age, and also in basal non-elongating stem internodes. In adult plants, CanBGal-1 shows its highest expression levels in leaves, while CanBGal-4 seems to be better represented in adult roots. This is the first report about several members of the genomic family of β-galactosidases acting during development of vegetative organs.

Keywords: Cell wall; *Cicer arietinum*; Development; Epicotyl; β-Galactosidase

1. Introduction

β-Galactosidases (EC 3.2.1.23), a widespread family of glycosyl hydrolases, are characterized by their ability to hydrolyze terminal, non-reducing β-D-galactosyl residues from β-D-galactosides. Several studies have shown that β-galactosidases catalyze the hydrolysis of terminal galactosyl residues from carbohydrates, glycoproteins and galactolipids. β-Galactosidase action has been reported to release free galactose during normal metabolic recycling of galactolipids, glycoproteins and cell wall components, and to degrade cell wall components during cell wall expansion, cell senescence and fruit ripening [1–5]. Furthermore, under favorable thermodynamic in vitro conditions many β-galactosidases have specific biosynthetic activities through both transglycosylation and reverse hydrolysis [6,7].

In higher plants, softening-related β-galactosidases have been purified from tomato [8,9], apple [3], mango [10] and papaya [11] among other fruits, and their biochemical properties have been characterized. More recently, these physiological and biochemical studies have been supported by the characterization of β-galactosidase genes, such as those described in tomato [12,13] or in Japanese pear [14].

Besides the role of pectins in fruit ripening, there are also several reports indicating that the loss of pectic galactose seems to play an important role in cell wall extensibility and hence pectic polymers, which are highly complex compounds, must be involved in the metabolism of expanding cell walls [15,16]. Fujino and Itoh [17] described a clear

* Corresponding author. Tel.: +34 9 2324500x1951; fax: +34 9 23294682.
E-mail address: bdr@usal.es (B. Dopico).

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difference in cell wall architecture between elongating and non-elongating regions, suggesting a modification in the molecular form of pectic polysaccharides during the elongation of epidermal cells. Those authors suggested that neutral sugar side-chains could be involved in the interaction with other cell wall components. β-Galactosidase is one of the enzymes that seems to play a role in the process of modification of the pectin structure, removing β-galactosyl linkages on the neutral sugar side-chains [18–20]. Several studies have focused on the biochemistry of β-galactosidases during cell wall modification during growth [5,21–24], but as far as we know, few studies have been carried out on the molecular characterization of β-galactosidase genes in vegetative elongating organs.

The purification of a family of chickpea β-galactosidases in *Cicer arietinum* epicotyls was first reported by Dopico et al. [25]. One of them, βIII-Gal, was characterized as a protein involved in the cell wall autolytic process [18,23,26]. It was proposed that βIII-Gal might be important in the cell wall changes taking place during elongation. The cDNA corresponding to βIII-Gal, named CanBGal-3, was subsequently cloned [27] and its function in pectin degradation was supported by its expression in a potato system. The role of the remainder β-galactosidases has not been yet established. In this paper, we described the cloning and expression pattern of three *C. arietinum* β-galactosidase cDNAs (named CanBGal-1, CanBGal-4 and CanBGal-5) and we compared these results with the previously characterized CanBGal-3 cDNA clone, which encode the βIII-Gal. The members of this family of β-galactosidase genes, may be involved in galactose metabolism during cell wall turnover, elongation processes, or architectural pectin changes in *C. arietinum* plants.

2. Materials and methods

2.1. Plant material

Chickpea seeds (*C. arietinum* L. cv. Pedrosillano) previously sterilized in 0.1% (w/v) sodium hypochlorite were germinated in water in the dark at 25 °C and 80% relative humidity on glass plates covered with filter paper. The growth period ranged from 2 to 8 days, after which the epicotyls were collected.

Different plant tissues were collected for studies of gene expression: hooks, epicotyls, mesocotyls (root–epicotyl junction zone), cotyledons and roots from 4-day-old etiolated seedlings. Stems of 11-day-old plants were divided into 5 internodes, numbered 1–5 from base to apex. Roots from 11-day-old plants and folioles, pods, flowers and seeds from adult plants were also collected. Immature folioles (L1) were those coiled up and smaller than 4 mm whereas mature folioles (L2) were those fully expanded and larger than 4 mm. Immature pods (P1) were 1 cm green pods whereas mature pods (P2) were full-grown green pods (about 2.5 cm). Immature seeds (S1) were green seeds from mature pods. Finally, mature dry seeds (S2) were also studied.

2.2. RNA extraction

Five grams of frozen material were ground in liquid nitrogen and resuspended in 10 ml of extraction buffer [10 mM Tris–HCl, pH 8.0, containing 1% (w/v) triisopropyl naphthalene sulphonic acid, 6% (w/v) 4-amino salicylic acid and 5% (v/v) phenol mixture (500 g phenol, 70 ml meta-cresol, 0.5 g 8-hydroxyquinoline, 150 ml distilled water)]. Samples were extracted as described in Muñoz et al. [28].

2.3. Screening for β-galactosidases in a chickpea cDNA library

The cDNA library used was constructed from poly (A)+ RNA extracted from epicotyls of 5-day-old etiolated chickpea seedlings grown in water as described by Muñoz et al. [28].

The screening of the library was performed as described in [27]. A heterologous screening of the library was performed using a *Malus domestica* β-galactosidase clone (pABG1, accession no. L29451, kindly provided by Dr. Ross, New Zealand) as probe. The heterologous screening afforded two different chickpea β-galactosidase cDNAs. A mixture of these two chickpea β-galactosidase clones was used as probe in further homologous screenings in order to isolate as many chickpea β-galactosidase cDNAs as possible. In these screenings two new β-galactosidase cDNAs were isolated. Thus, we finally collected four different chickpea β-galactosidase cDNAs, named CanBGal-1, CanBGal-3, CanBGal-4 and CanBGal-5.

2.4. DNA and deduced protein sequence analysis

Plasmid DNA was prepared using the Wizard plus SV miniprep kit (Promega, Madison, WI, USA) based on the alkaline lysis method [29]. Sequence analysis was performed automatically using the dRhodamine terminator cycle sequencing ready reaction kit on an ABI Prism 377 genetic analyzer (both from PE Biosystems, Norwalk, CT, USA). The DNA and deduced protein sequences were compared with other sequences in the EMBL/GenBank and Swall databases, respectively, using the FASTA algorithm [30]. Alignment and the phylogenetic tree of the chickpea and other plant β-galactosidases was done using the Clustal method from the MegAlign program: part of the Lasergene sequence analysis software available from DNASTAR. Putative signal peptides were analyzed based on the SignalP 2.0 program [31] and putative N-glycosylation sites and the β-galactosidase active site [32] were analyzed at the Prosite web page (http://www.expasy.ch/prosite). Conserved domains of the proteins were analyzed using the NCBI

Conserved Domain Search (http://www.ncbi.nlm.nuh.gov/) [33].

2.5. Northern analysis

Northern experiments were performed as described in Muñoz et al. [28]. cDNA fragments from the 3′ non-coding end of the clones were used as specific probes to minimize any potential cross-hybridization. cDNA specific probes were amplified by PCR in a GeneAmp PCR system 9700 thermocycler (PE Biosystems, Norwalk, CT, USA), cleaned by the High Pure PCR product purification kit (Roche, Germany), and labelled with ^{32}P using the Random primed kit (Roche, Germany). Total RNA (10 μg/lane) was electrophoresed, transferred onto Hybond N nylon membranes (Amersham Pharmacia Biotech, UK), and hybridized using the radiolabelled specific probes. After hybridization, membranes were washed twice with 2× SSC, 0.1% SDS at 42 °C for 5 min each and twice with 0.1× SSC, 0.1% SDS at 42 °C for 20 min each. The autoradiographs were analyzed on a Bioimage 60S Image Analyzer (Millipore, Bedford, MA, USA, Visage 4.6 K Software). Subsequent hybridization of the blots using a 500 bp fragment from 18S rRNA as probe was used as internal control to determine the relative amounts of RNA per lane. Northern experiments were performed at least two times each.

2.6. Genomic DNA extraction and Southern analysis

Genomic DNA was isolated using the Plant DNA isolation kit (Roche, Germany) from 14-day-old chickpea plant folioles. DNA (10 μg) was digested using *Hin*dIII and *Eco*RI restriction enzymes, separated on agarose gel, and transferred onto a Hybond N nylon membrane (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Blots were hybridized at 65 °C for 12 h in 1% (w/v) SDS, 5× SSPE, 5× Denhards' solution using the [^{32}P]-labelled specific probe, constructed as described above. Membranes were washed twice with 2× SSC, 0.1% SDS at 65 °C for 5 min each, twice with 0.5× SSC, 0.1% SDS at 65 °C for 20 min each and twice with 0.1× SSC, 0.1% SDS at 65 °C for 20 min each.

3. Results

3.1. Chickpea β-galactosidase clones

Screening of the *C. arietinum* cDNA library revealed the presence of at least four different cDNAs encoding β-galactosidases: CanBGal-1, CanBGal-3, CanBGal-4 and CanBGal-5.

CanBGal-3, CanBGal-4 and CanBGal-5 are full-length clones whose sizes were 2600, 2900 and 2700 bp, respectively. CanBGal-1, whose size was 2656, is not a full-length clone. The mRNA length estimated for this clone by Northern blot was 3200 nt.

The proteins deduced from the chickpea CanBGal clones, named βI-Gal, βIII-Gal, βIV-Gal and βV-Gal, had a significant level of shared amino acid sequence identity with one another and also with other published plant β-galactosidases. βIII-Gal and βIV-Gal are the closest at amino acid sequence level (81% identity). βI-Gal and βV-Gal show a lower degree of identity: about 63% between each other and with respect to the other two β-galactosidases.

The proteins βIII-Gal, βIV-Gal and βV-Gal present a predicted N-terminal hydrophobic signal peptide, whose putative cleavage site is indicated by an arrow in Fig. 1, as determined by Signal P [31]. The hydrophobic signal peptides are 29, 23 and 26 amino acids long for βIII-Gal, βIV-Gal and βV-Gal, respectively. After the release of the signal peptide, the mature proteins have a molecular mass of 77.9, 78 and 80 kDa for the βIII-Gal, βIV-Gal and βV-Gal, respectively. βIII-Gal and βV-Gal show a high predicted isoelectric point (8.06 and 8.45), whereas the predicted isoelectric point observed for the βIV-Gal was 5.79. No conclusive data could be obtained for βI-Gal, owing to the lack of approximately 140 amino acids at the N terminus of this protein.

The four chickpea β-galactosidase proteins display the active site of family 35 of the glycosyl hydrolases described by Henrissat [32] (marked by asterisks in Fig. 1). Position 6 of this active site was different in the four proteins, βIII-Gal and βIV-Gal have Met whereas βI-Gal and βV-Gal have Leu. According to Henrissat [32], Met and Leu are possible conserved susbstitutions.

A characteristic of the chickpea β-galactosidases is that they have different length. In particular, βI-Gal is 115 amino acids longer at the C-terminal end than the other chickpea β-galactosidases (Fig. 1). It is interesting to note that this extra peptide of the βI-Gal comprises an unusual galactose binding lectin domain, as point out the analysis of the conserved domains of the proteins using the NCBI Conserved Domain Search [33]. No other chickpea β-galactosidases present this domain.

All chickpea β-galactosidases show at least one putative N-glycosylation site. βI-Gal and βIII-Gal have only one, whereas βIV-Gal and βV-Gal have four and two, respectively. The putative N-glycosylation sites are highlighted in Fig. 1.

3.2. Phylogenetic tree

The differences among chickpea β-galactosidases are clearly seen in the phylogenetic tree, compiled using the four deduced chickpea proteins and other plant β-galactosidases, based on protein sequence alignment (Fig. 2). The distribution of chickpea β-galactosidases in this tree suggests that there are subgroups of β-galactosidases distinguishable by their sequence. *C. arietinum* β-galactosidases appear

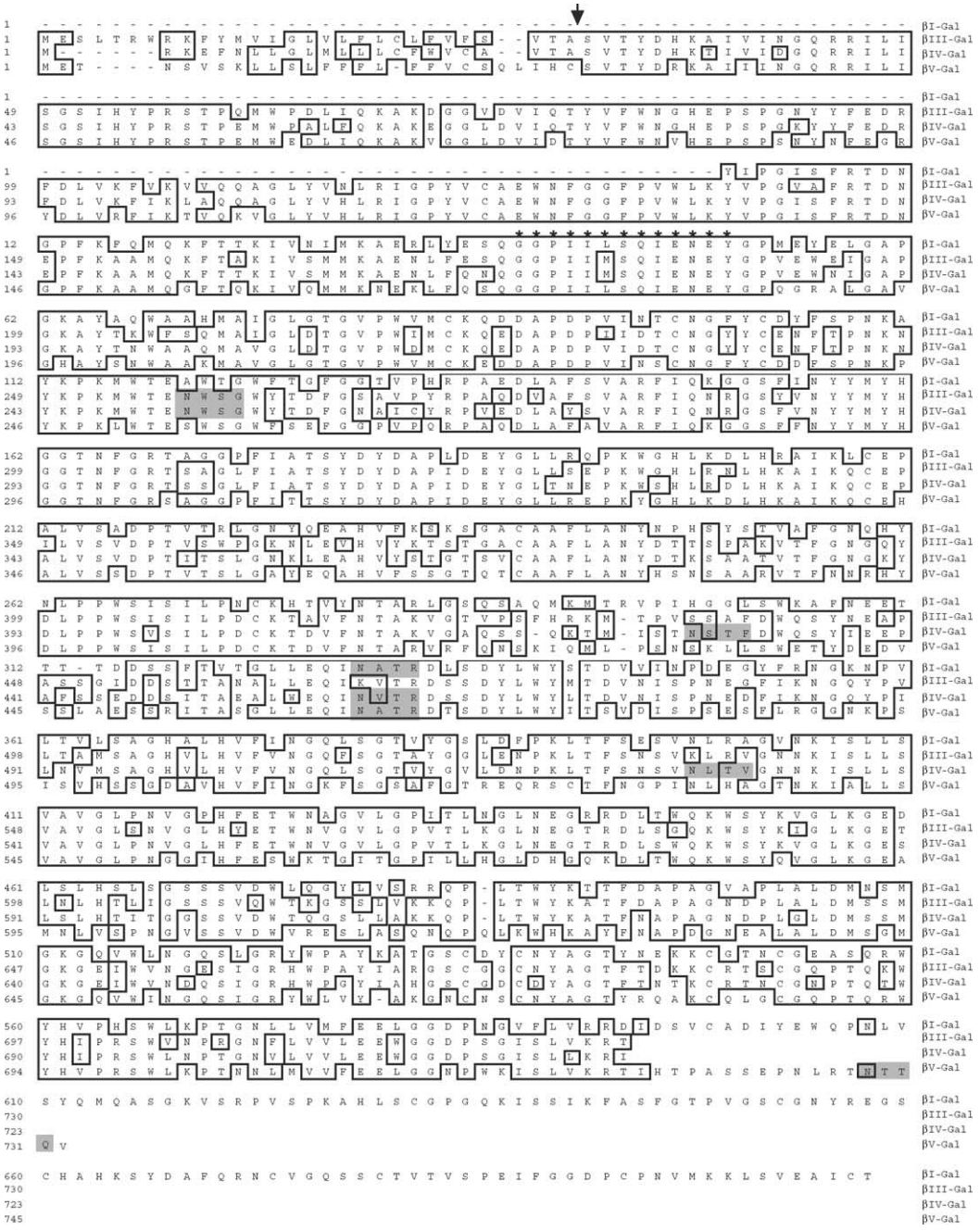

Fig. 1. Alignment of the four deduced β-galactosidase amino acid sequences of the CanBGal clones. Sequences were aligned using the Clustal method in the MegAlign program, part of the Lasergene sequence analysis software program available from DNASTAR (Madison, WI). Boxes indicate amino acid identity with respect to the majority. An arrow indicates the putative signal sequence cleavage site. The active site of family 35 of glycosyl hydrolases is marked by asterisks. Putative N-glycosylation sites are highlighted.

separated in three different phylogenetic branches. βI-Gal appears in group I, together with proteins with a molecular mass ranging from 92.1 to 93.6 kDa and 832 to 847 aminoacid residues. The larger size of those proteins was primarily due to an addition of approximately 100 amino acids at their carboxyl termini, as we have described above in βI-Gal (Fig. 1). All the proteins included in this group I present a galactose binding lectin domain.

The βV-Gal protein appears in branch II, together with β-galactosidases from *Phaseolus vulgaris*, *Vitis vinifera* and *Lycopersicon esculentum* TBG6. This group is heterogeneous in their molecular mass, but shows several conserved regions

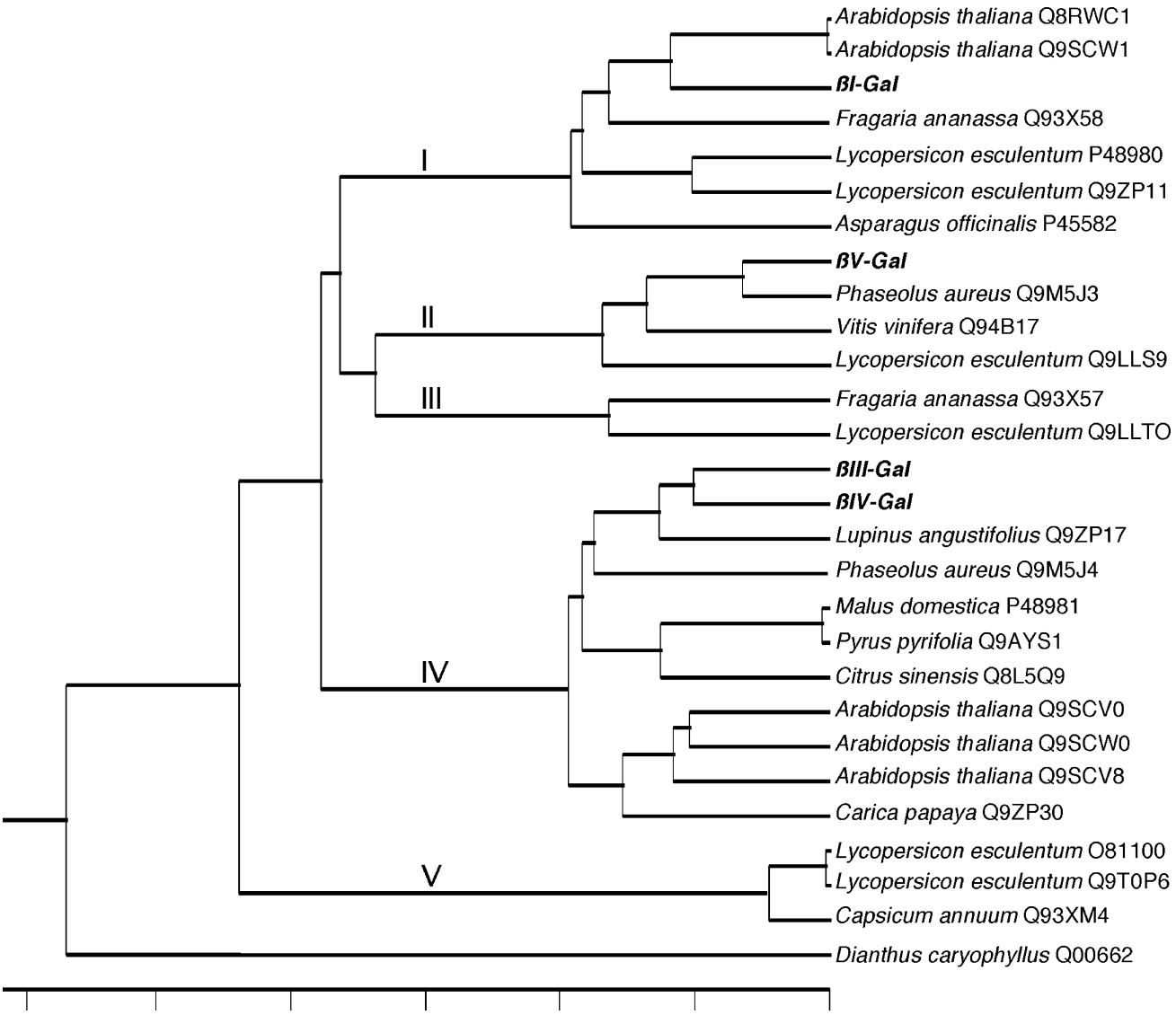

Fig. 2. Phylogenetic tree representing the evolutionary relationship among the four deduced proteins from the CanBGal cDNAs of *Cicer arietinum* and several plant β-galactosidases.

within the group (data not shown), such as Gln at the positions 272 and 361, whereas are Glu in the other groups. The proteins analyzed in this group have been related to early development processes in grape [34,35] and tomato [13] fruits.

The βIII-Gal and βIV-Gal, together with β-galactosidases from other leguminosae such as *Vigna radiata* and *Lupinus angustifolius* are clustered in group IV. All the galactosidases in this group IV are between 721 and 731 amino acids long and have molecular weights ranging from 79 to 82.1. Most of them have been related to cell wall pectin degradation.

Although sharing a molecular weight similar to group IV, the proteins clustered in group V present a clear difference in the active site of family 35 of the glycosyl hydrolases described by Henrissat [32]. All of them have Met and Ala at positions 6 and 7, respectively, instead of Leu and Ser, present in most of the plant β-galactosidases described to date. Some β-galactosidases have Met or Ala at those positions but never both together as in group V.

The β-galactosidase from *Dianthus caryophyllus*, detectable in senescing carnation petals and under strict regulation by ethylene [1], appears on a separate branch.

3.3. Expression pattern during growth

The temporal expression pattern in epicotyls of the four CanBGal was examined using RNA extracted from epicotyls ranging in age from 2 days (when the epicotyl emergence starts) to 8 days (when the rate of growth starts to fall) (Fig. 3). (Data previously published referred to CanBGal-3 [27] were included to a better discussion of the results). Specific probes were used for each cDNA in order to minimize any potential cross-hybridization. CanBGal-1 and CanBGal-4 mRNAs were detected throughout the growth of

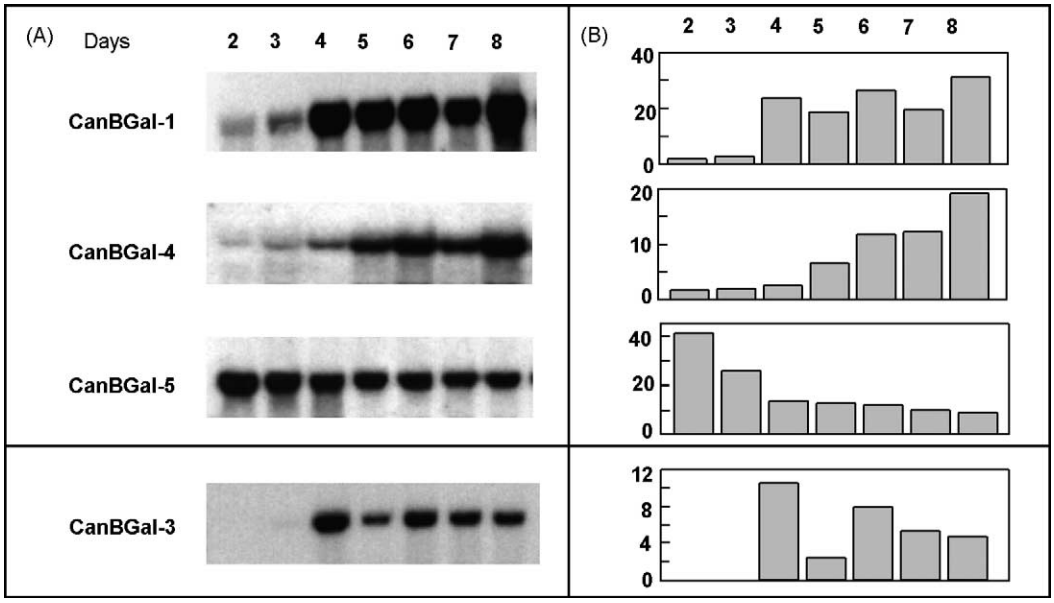

Fig. 3. Northern analysis of CanBGal mRNA expression levels in seedling epicotyls during elongation (A). Signals were quantified and normalized according to 18S rRNA hybridization and are presented as integrated optical density (B). Numbers refer to days after sowing.

epicotyls and their mRNA levels increased with the length of the epicotyls. On the contrary, the CanBGal-5 mRNA levels decreased with the age of epicotyls. CanBGal-3 started to be expressed on 4 days and no transcript was observed in very young epicotyls (2 and 3 days) [27].

Expression along the stem internodes from 11-day-old plants (Fig. 4) showed a similar correlation to growth as that found along epicotyl age (Fig. 3). The mRNA levels of CanBGal-1 and CanBGal-4 increased from the apical young internode (5th) to the basal one (1st), whereas CanBGal-5 expression followed the opposite pattern, showing the highest mRNA levels in apical internodes and decreasing towards the basal ones, where almost no expression was detected. CanBGal-3 showed the highest level of transcripts in the 3rd internode and almost no expression was found in the youngest (5th) internode; i.e., the one closest to the meristematic tissue (Fig. 4).

3.4. Tissue specificity in seedlings and adult plants

As expected, according to the screening of the library, all the CanBGals were expressed in epicotyls, showing their

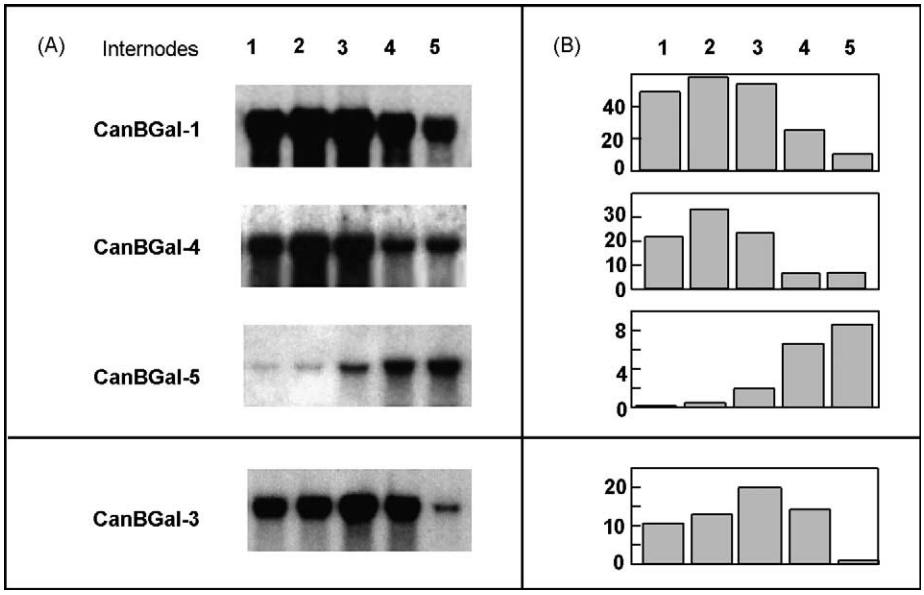

Fig. 4. Differential expression of CanBGals in the five internodes of 11-day-old plants (A). Signals were quantified and normalized according to 18S rRNA hybridization and are presented as integrated optical density (B). Internodes are numbered 1–5 from the basal to the apical one.

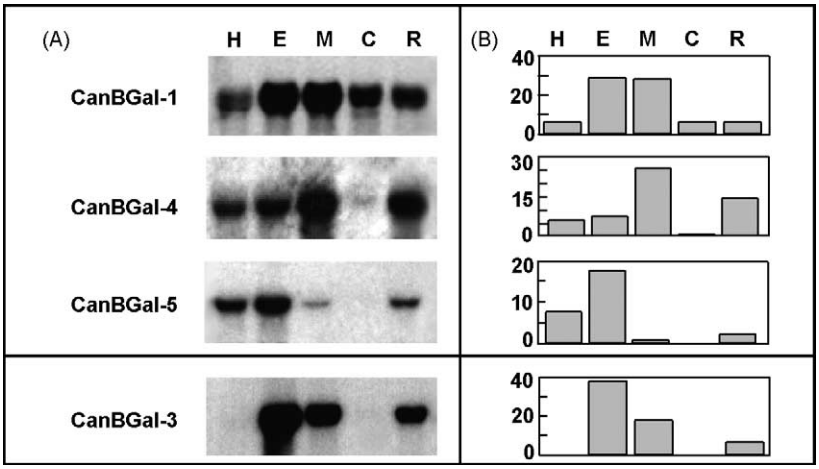

Fig. 5. Differential expression of CanBGals in different parts of *C. arietinum* 4-day-old seedlings (A). Signals were quantified and normalized according to 18S rRNA hybridization and are presented as integrated optical density (B). H: hook; E: epicotyl; M: mesocotyl; C: cotyledon; R: radicle.

highest level of expression in these seedling organs, except for CanBGal-4 (Fig. 5). CanBGals transcripts were also detected in other elongating seedlings organs, such as mesocotyls and radicles, but at lower levels than in epicotyls, except for CanBGal-4 where the highest transcript level was found in both mesocotyls and radicles. Only CanBGal-1 was significantly expressed in cotyledons, although a very low mRNA level of CanBGal-4 was also detected in those organs. Transcripts of CanBGal-1, -4 and -5 were found in apical hooks (meristematic tissue) but always at lower levels than in epicotyls. CanBGal-5 expression in hooks represented the second highest transcript level, after that of epicotyls. No CanBGal-3 transcripts were detected in hooks [27].

The expression pattern of CanBGal clones in adult plants was broad. Besides the stem internodes, in adult plants the highest expression of CanBGal-1 and CanBGal-3 was found in mature leaves. Of note was the higher transcript level in old leaves (L2) as compared with young ones (L1). CanBGal-4 and CanBGal-5 showed maximum transcript levels in young pods (P1) and in the case of CanBGal-4, also in adult roots (Fig. 6). All the clones showed very low or no expression in dry seeds and in mature pods. It should be noted that CanBGal-3 was poorly expressed in young leaves and not expressed in adult roots. CanBGal-4 had the broadest range of expression and CanBGal-5 was poorly expressed in mature leaves and flowers (Fig. 6).

4. Discussion

Most studies on β-galactosidase genes refer to their expression in ripening fruit, where the degradation of galactan is associated with the ripening process (reviewed by

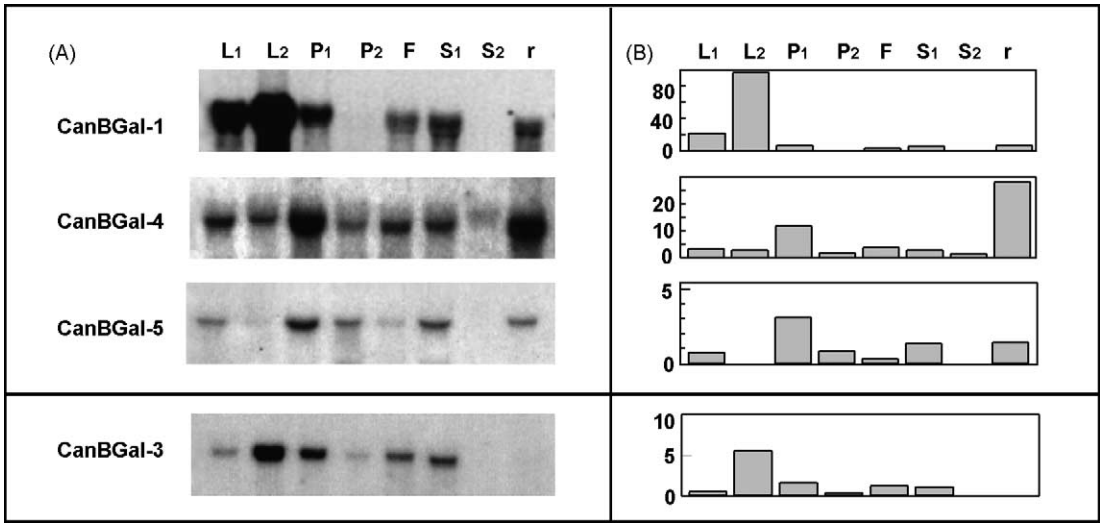

Fig. 6. Differential expression of CanBGals in different parts of *C. arietinum* adult plants (A). Signals were quantified and normalized according to 18S rRNA hybridization and are presented as integrated optical density (B). L1: young leaves; L2: mature leaves; P1: immature pods; P2: mature pods; F: flowers; S1: immature seed; S2: dry mature seed; r: adult roots.

Brummell and Harpster [36]). However, in elongating vegetative organs, the function of the different β-galactosidase genes has been studied to a lesser extent, although changes in the neutral side chains of pectin that could be mediated by galactosidases during growth have been reported [15,23,37–40]. Kikuchi et al. [41] suggested that the neutral side chains of RG-I, such as galactan and arabinan, play an important role in linkages between pectins and other components, such as cell wall hemicelluloses, enhancing the rigidity of cell walls.

In *C. arietinum*, we have previously reported several β-galactosidases purified from epicotyl cell walls [25], and one of them, βIII-Gal was proposed to be involved in the cell wall changes taking place during elongation [23]. During our search for cDNAs coding β-galactosidases, a total of four cDNA clones, named CanBGal-1, CanBGal-3, CanBGal-4 and CanBGal-5, all with a significant level of sequence identity, were found. These four genes would be members of a small family of β-galactosidase genes. All of them appear to be present in the chickpea genome as a single copy, as determined by Southern blot (data not shown). The high sequence homology of the four clones with one another and with other plant β-galactosidases, together with the presence of the glycosyl hydrolase family 35 consensus sequence [32] (Fig. 1), suggest that they may all have β-galactosidase activity. Other β-galactosidase gene families have been described in plants, such as in *L. esculentum* with seven members [13] or *Carica papaya* [11] with three members. In fully sequenced plant genomes such as *Arabidopsis thaliana* there are 43 database entries corresponding to β-galactosidases of the glycosyl hydrolase family 35, however several of them are just fragments. In *Oryza sativa*, seven β-galactosidases appear in the databases.

The characterization and analysis of the gene expression of several CanBGals could help to understand the putative role of the corresponding encoded proteins in chickpea cell wall metabolism. In a recent report, the CanBGal-3 was identified as the cDNA corresponding to βIII-Gal and its function in cell wall pectin degradation was supported by its expression in a potato system [27]. Here, we studied the gene expression at the mRNA level of the remainder CanBGals in order to find the possible role for each chickpea β-galactosidases.

From the present results we can observed that the expression of CanBGal-5 was related to young and meristematic stages with a high cell division rate, such as meristematic hooks, very young epicotyls and apical internodes (Figs. 3–5). It would appear that the protein βV-Gal is involved in the initial stages of plant development. Few examples of β-galactosidases related to high cell division stages have been described. As far as we know, only Nunan et al. [35] in grape, and Smith and Gross [13] in tomato (TBG6), have reported β-galactosidases whose transcripts were accumulated specifically in the early stages of fruit development; a stage with an intensive cell division activity followed by cell expansion. It is interesting to note that these two proteins appeared together with chickpea βV-Gal in the group II of the phylogenetic tree, indicating a possible relationship between the structure and function of these proteins.

In contrast with CanBGal-5, CanBGal-1 and -4 seem to be more expressed in advanced stages of epicotyl growth, increasing with age and also in basal, non-elongating stem internodes (Figs. 3 and 4). No β-galactosidase genes related to advanced stages of growth have been described, although the expression of several β-galactosidases has been reported to increase during senescence, as in carnation [1] and in asparragus [42,43]. However, the release of galactose in senescence is accompanied by cell wall disassembly, whereas the expression patterns of CanBGal-1 and -4 are highest when there is an increase in cell wall rigidity. We can, therefore, propose that the activity of these proteins could be to hydrolyze an unknown substrate whose modification causes cell wall rigidity, probably by compacting pectins.

Even though CanBGal-1 and CanBGal-4 seem to share the same pattern of expression at the mRNA level in epicotyls and stems, it should be noted that the predicted isoelectric points for the βI-Gal and βIV-Gal proteins are quite different. This suggests the possibility that these proteins might be bound to a different substrate located at a different site in the cell wall. Furthermore, in adult plants CanBGal-1 shows its highest expression in leaves, while CanBGal-4 seems to be more important in adult roots. It should also be noted that the CanBGal-1 transcripts are the only ones abundant in cotyledons, whose cell walls could have galactose-rich polymers different from those found in epicotyls, indicating a putative substrate specificity.

The presence of a galactose binding lectin domain in the protein βI-Gal suggests a particular role of this protein. Although this domain is far away from the putative active site, and therefore might not affect the catalytic properties of the enzyme, this domain, if proven to be functional could affect the biochemical characteristics of the enzyme containing it. The presence of this domain is not usual between β-galactosidases; according to databases only about 42 proteins among all the plant β-galactosidases described contain it [33]. Trainotti et al. [44] refer two β-galactosidases in strawberry with a similar lectin-like domain, suggesting their possible sugar-binding activity on the basis of the highly conserved structural identity between animal lectins and the C-terminal domain of these two strawberry proteins.

The expression pattern of the CanBGal cDNAs studied in the present report was quite different from the expression of CanBGal-3 encoding the βIII-Gal able to hydrolyze cell wall pectins [27]. In that case, the highest level of transcript accompanied the stages of maximum elongation, such as 4-day-old epicotyls [27] or stem internodes with high growth ability (Fig. 4). No transcript was found in the regions with high cell division rate such as apical intenodes, hooks or very young epicotyls.

The CanBGals gene transcription in seedlings and adult plant (Figs. 5 and 6) could suggests specific function and/or location of their corresponding protein throughout the chickpea plant. The idea that these four β-galactosidases from chickpea could be involved in different modifications of galactosyl residues in the cell wall in different phases of development or in different organs is based on the high number of specific natural substrates described for β-galactosidases. An example indicating such high specificity is provided by the β-galactosidase from *Copaifera langsdorffi* cotyledons, with considerable specificity towards xyloglucan oligosaccharide [45]. For hydrolysis, this β-galactosidase recognises only the galactosyl substitution of the xylose adjacent to the non-reducing end glucose residue of xyloglucan oligosaccharides.

In a similar way to *C. arietinum*, it is common to find β-galactosidase isoforms in the same species; these do not usually show the same ability to hydrolyze pectins or other cell wall substrates. In general, these isoforms present differences, indicating that they act in a different way or against different substrates with different degrees of branching [11,25,46]. The cloning of several β-galactosidase isoforms could help to clarify the role of each one in the same or different processes, although to date this point has only been studied in fruits such as tomato [13]. Several β-galactosidase clones have been described in *Fragaria ananassa* or *Phaseolus aureus*, but to our knowledge no data on these clones have been published. As far as we know, this is the first report of several members of the genomic family of β-galactosidases acting in vegetative organs. This group of β-galactosidases acts during the development and growth of vegetative organs in *C. arietinum* and could thus affect different processes and allow a high regulation of the processes involved. More work will be necessary to establish the specific substrate and action of each β-galactosidase.

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