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Immunolocalization of a Cell Wall ß-Galactosidase Reveals its Developmentally Regulated Expression in *Cicer arietinum* and its Relationship to Vascular Tissue

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Abstract We report the generation of antibodies against a ß-galactosidase from *Cicer arietinum*, ßIV-Gal, and the subsequent immunolocalization of the protein in different parts and developmental stages of the plant. The ßIV-Gal protein is encoded by the *CanBGal-4* gene, which belongs to a family of at least four ß-galactosidase genes, transcripts of which were previously reported to be mainly present in seedling epicotyls and plant stem, its transcription pattern being inversely related to elongation rate of these organs. ßIV-Gal protein was detected in the cell walls of seedling epicotyls and plant stems. The immunodetection of ßIV-Gal protein in the cell wall protein extracts from aged epicotyls and basal stem internodes, both undergoing low rates of elongation, is in agreement with the trend of the *CanBGal-4* transcript and indicates a relationship of this cell wall protein with the end of cell elongation. The specific main location of the ßIV-Gal protein in vascular tissue of epicotyls and stems and in a layer of sclerenchymatic cells surrounding the vascular cylinder (perivascular fibers) allows us to postulate a function for this ß-galactosidase in the modification of cell wall polymers during the development of cells of the vascular system. The localization of the ßIV-Gal protein also in the cell walls of collenchyma cells in internodes is consistent with the involvement of ßIV-Gal in cell wall modifications that lead to thick cell walls, such as in vascular cells.

Keywords Cell wall · Chickpea · Collenchyma · Development · ß-Galactosidase · Vascular tissue

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Introduction

ß-Galactosidases (EC 3.2.1.23) catalyze the hydrolysis of terminal galactosyl residues from carbohydrates, glycoproteins, and galactolipids. These enzymes are widely distributed in higher plants and can be found in several plant tissues (Singh and Knox 1985; Dopico and others 1990; Raghothama and others 1991; Ross and others 1994; Buckeridge and Reid 1994). ß-Galactosidases have been involved in the degradation of cell wall components during cell wall expansion, cell senescence, fruit ripening, and storage mobilization (Singh and Knox 1985; Dopico and others 1990; Raghothama and others 1991; De Veau and others 1993; Buckeridge and Reid 1994; Ross and others 1994; Buckeridge and others 2005).

The functions of cell wall ß-galactosidases have been discussed with respect to their action on pectic galactans, arabinogalactan proteins, or xyloglucans. Most studies have focused on the degradation of pectic ß(1–4)-galactan, because the structure of this polymer is spatially regulated during the development of plant tissues, and pectin therefore plays an important role in the architecture of the cell wall and intercellular attachment (Sørensen and others 2000; McCartney and others 2003). Also, a ß-galactosidase from *Raphanus sativus* with high specificity toward ß(1–3)- and ß(1–6)-galactosyl residues has recently been reported (Kotake and others 2005). It splits off about 90% of the carbohydrate moieties of an arabinogalactan protein extract

from radish roots in a concerted action with microbial α-L-arabinofuranosidase and ß-glucuronidase. ß-Galactosidases also exert an important effect on the side chains of xyloglucans. For the complete enzymatic digestion of xyloglucan oligosaccharides, α-fucosidase, α-xylosidase, ß-glucosidase, and ß-galactosidase must be present in the apoplast (Tiné and others 2000). The sequential action of the different glycanases mentioned above may be able to degrade xyloglucan oligosaccharides and may alter the growth-modulating capability of such oligosaccharides. Few plant ß-galactosidases have been shown to be active against xyloglucan, mainly in cotyledons involved in storage mobilization (Edwards and others 1988; De Alcantara and others 1999, 2006). In nasturtium cotyledons, a ß-galactosidase able to catalyze the rapid hydrolysis of the terminal ß-galactopyranosyl units from intact xyloglucan has been reported (Edwards and others 1988). Iglesias and others (2006) reported three *Arabidopsis* genes as the best candidates for encoding xyloglucan-specific ß-galactosidase, although this hypothesis has not yet been tested.

ß-Galactosidase multigene families have been described in several plants, such as *Lycopersicom esculentum* with seven members (Smith and Gross 2000) or *Carica papaya* (Ali and others 1998) with three members. In fully sequenced plant genomes such as that of *Arabidopsis thaliana* there are 18 genes the products of which have been predicted to be ß-galactosidases of the 35-glycosyl hydrolase family; 12 of them are probably secreted to the wall. In *Oryza sativa*, seven ß-galactosidases appear in the databases. It is therefore not surprising to find considerable variations in expression patterns among cell wall ß-galactosidases (Ali and others 1998; Smith and Gross 2000; Esteban and others 2005).

We have previously described a ß-galactosidase multigene family in *Cicer arietinum* with at least four members named *CanBGal-1*, *-3*, *-4*, and *-5*, and their transcription patterns have been reported (Esteban and others 2003, 2005). *CanBGal-3* encodes the cell wall ßIII-Gal protein (Esteban and others 2003), which was previously characterized as a protein involved in the cell wall autolytic process (Dopico and others 1989) and related to cell wall changes taking place during elongation processes. Its function in the degradation of ß-(1–4)-galactan chains was confirmed after the transformation of potato plants with the chickpea *CanBGal-3* (Esteban and others 2003; Martín and others 2005). The role of the other chickpea ß-galactosidases has not yet been completely elucidated, although the transcription pattern of the *CanBGal* genes in different organs from seedlings and plants suggests different roles for their corresponding proteins along development (Esteban and others 2005). Here we focus on the study of one of these ß-galactosidases, encoded by *CanBGal-4*, transcripts of which have been reported to be strongly related to epicotyls and stem internodes, its expression increasing in aged epicotyls and in basal nonelongating stem internodes (Esteban and others 2005).

One of the strategies used to gain insight into the function of a given protein is the generation of specific antibodies that provide knowledge about cellular localization and the presence of the protein in different organs during plant development. Raising antibodies against ß-galactosidases is very difficult due to a high degree of similarity among the different ß-galactosidases and hence few, if any, antibodies against ß-galactosidase have been described. In this work we report the generation of specific antibodies against the ß-galactosidase encoded by *CanBGal-4* and the subsequent immunolocalization of the protein in different parts and developmental stages of the plant. The results allow us to postulate a function for this ß-galactosidase in the processes of development of cells of the vascular system.

Materials and Methods

Plant Material and Growth Conditions

Chickpea seeds (*Cicer arietinum* L. cv. *pedrosillano*) previously sterilized in 0.1% (v/v) sodium hypochlorite were germinated in water in darkness at 25°C and 80% relative humidity on glass plates covered with filter paper. The growth period studied in the etiolated seedlings ranged from 3 to 8 days, after which they were harvested and epicotyls collected. The epicotyl length was measured in seedlings from the 1st to the 9th day. Hooks, epicotyls, mesocotyls (hypocotyl–root junction zone), cotyledons, and radicles from 4-day-old etiolated seedlings were collected for subsequent Western blot studies.

Chickpea plants were grown in vermiculite at 25°C and 80% relative humidity for 11 days, after which the stems and roots were harvested. Stems were divided into five internodes, numbered 1 to 5 from base to apex. The internode length was measured in plants from the 6th to the 12th day.

Four- and 8-day-old epicotyls as well as stem internodes were also used for immunolocalization studies. The increases in length of 5-mm sections from apical, central, and basal regions of 4- and 8-day-old epicotyls was measured after 24 h.

Expression and Purification of the ßIV-Gal Fusion Protein

The coding sequence of *CanBGal-4* (minus the signal sequence) was PCR-amplified from the *CanBGal-4* plasmid clone. The two oligonucleotide primers used were

5′-GAATTCTCAGTGACTTATGATCACAAAAC-3′ and 5′-AAGCTTTCATATTCTTTTGAGCAAAGAAATT-3′, adding an *Eco*RI restriction site at the 5′ end and a *Hin*dIII site at the 3′ end, respectively (underlined in the sequences). The PCR product was subcloned into the *Eco*RI/*Hin*dIII restriction site of the pET-32a(+) (Novagen, Madison, WI, USA) expression vector and transferred to the *E. coli* strain Rosetta-gami B(DE3) (Novagen). As a control of expression, we used the plasmid pET-32a(+) without any insert.

The cells were first cultured overnight in 10 ml of Luria-Bertani (LB) medium (50 μg ml^{-1} ampicillin and kanamicin) at 37°C. One milliliter of the overnight medium was transferred to 200 ml of LB medium and was allowed to continue to grow until A_{600} reached 0.4-0.6. IPTG was added at 1 mM and was allowed to induce protein production for 3 h. Inclusion bodies were separated from the soluble fraction using the BugBuster Protein Extraction Reagent (Novagen), and the subcellular location of the recombinant protein was determined by SDS-PAGE. The fusion protein was not soluble and hence the inclusion bodies were harvested.

Antibody Production and Purification

For polyclonal antibody production, a peptide was designed from the *CanBGal-4* deduced amino acid sequence. Several considerations were taken into account for the peptide design, such as hydrophilicity and antigenicity profiles, as well as the probability of peptide surface exposure in the protein. Among the chickpea ß-galactosidase sequences, an alignment was performed to avoid areas of high similarity. All such analyses were carried out using the DNAStar sequence analysis software. Finally, once the peptide had been designed, a search using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul and others 1990) was carried out to establish the degree of peptide identity with protein sequences in databases other than those of ß-galactosidases.

Synthesis of the peptide and its conjugation with the KLH carrier protein were carried out by Sigma Genosys (UK). Two female New Zealand white rabbits were immunized by multiple subcutaneous injections with 200 μg of the KLH-peptide conjugate in Freund's complete adjuvant (Sigma, St. Louis, MO, USA) for the first injection and 100 μg in Freund's incomplete adjuvant (Sigma) for subsequent immunizations, carried out at 2-week intervals. Preimmune and immune sera were collected, and IgGs were purified with an affinity column (HiTrap protein A HP, Amersham Biosciences, Buckinghamshire, UK).

The specificity of the antibody preparation was verified by immunoblot analysis of the four known chickpea ß-galactosidases overexpressed in *E. coli* as His-tagged fusion proteins. Western blotting was carried out as described below. In this case 1.5 μg of each protein was used and the anti-ßIV-Gal antibody and the peroxidase-conjugated secondary antibody were applied at 1:2500 and 1:150,000 dilutions, respectively.

Cell Wall Protein Extraction and Western Blotting

Cell walls were prepared according to Dopico and others (1989). Protein was extracted as indicated Jiménez and others (2006) from freshly isolated cell walls with 1 M NaCl in 10 mM Na-citrate/phosphate, pH 5.5, at 4°C for 48 h. The cell wall suspension was filtered through Miracloth (Calbiochem, Darmstadt, Germany), and the protein extract was dialyzed against 20 mM Na-acetate, pH 5.0. The dialyzed protein was centrifuged for 25 min at 6500 *g* and concentrated in an Amicon device using a 3K Pall Filtron membrane (Pall Filtron, Cortland, NY, USA). The total amount of protein was assayed according to Bradford (1976) with the Protein Assay kit (Bio-Rad, Baltimore, MD, USA).

For Western blotting, proteins (3 μg per lane) were separated by SDS-PAGE (Laemmli 1970) and electro-transferred onto PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). Immunoblots were prepared essentially according to the procedure of Harlow and Lane (1988), using the anti-ßIV-Gal antibody at a 1:2500 dilution and a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, Bio-Rad, Baltimore, MD, USA) at a dilution of 1:100,000. Blots were developed by chemiluminescence using the ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Immunocytochemical Labeling

Epicotyls from 4- and 8-day-old seedlings and the five internodes from 11-day-old plants were fixed in FAA fixation solution (formalin:acetic acid:50% ethanol, 5:5:90) for 24 h, followed by a rinse in wash solution [50% (v/v) ethanol] for 2 h. Samples were then dehydrated in increasing concentrations of ethanol and xylene:ethanol and embedded in paraffin (Paraplast Plus, Sigma, St Louis, MO, USA). Sections (12 μm thick) were cut and mounted onto high-molecular-weight poly-L-lysine (Sigma, St. Louis, MO, USA). The samples were then deparaffinized with xylene and rehydrated through a graded ethanol series. The sections were incubated for 5 min in 10 mM citrate buffer, pH 6.0, at 100°C to inactivate endogenous alkaline phosphatase activity, because an alkaline phosphatase-conjugated secondary antibody was used to develop the reaction. The samples were then washed twice in Tris-buffered saline (TBS: 0.1 M Tris, 0.1 M NaCl, pH 7.4). Free binding sites were blocked over 45 min with 5% (w/v) BSA and 3% (v/v) normal swine serum in TBS. Anti-ßIV-Gal antibody (1:100 dilution in TBS with 3% BSA) was

applied to the sections for 2 h at room temperature. Excess antibody was removed with extensive washing in 0.5% (w/v) Tween 20, 1% (v/v) BSA in TBS. After a second blocking, the secondary antibody [goat anti-rabbit IgG conjugated with alkaline phosphatase, at 1:300 dilution in TBS with 3% (w/v) BSA] was applied and the preparation was extensively washed as above. The color reaction carried out to visualize the antigen-antibody complexes was performed in TBS supplemented with 50 mM $MgCl_2$, pH 9.5, containing 5-bromo-4-chloro-3-indolyl-phosphate (0.075 mg/ml) and 4-nitroblue tetrazolium chloride (0.1 mg/ml) according Wolf and others (1973). The sections were then dehydrated in a graded ethanol series, dipped in xylene, and mounted in Entellan (Merck, Darmstadt, Germany).

Results

Raising and Specificity of the ßIV-Gal Antibodies

To clarify the function of the ß-galactosidase encoded by *CanBGal-4* in cell wall metabolism, we decided to raise polyclonal antibodies against this protein. Thus, we designed a peptide of the *CanBGal-4*-deduced protein sequence, according to the characteristics indicated in Materials and Methods, to ensure its specificity. This peptide was synthesized and used to immunize the rabbits.

Because in *Cicer arietinum* the ß-galactosidase multigene family has at least four members (*CanBGal-1*, *CanBGal-3*, *CanBGal-4*, and *CanBGal-5*) and the proteins encoded by *CanBGal* clones, designated ßI-Gal, ßIII-Gal, ßIV-Gal, and ßV-Gal, have a high level of shared amino acid sequence identity with one another, especially ßIII-Gal and ßIV-Gal (81% identity), an attempt to check the specificity of the antiserum raised against ßIV-Gal was necessary to evaluate further results.

To perform this analysis, we overexpressed the four chickpea ß-galactosidases in *E. coli* as His-tagged fusion proteins and used them in immunoblot assays to rule out any potential cross-reaction. Figure 1A shows the ßIV-Gal produced by *E. coli*. The expression of *CanBGal-4*, except the signal peptide, in the pET32a (+) vector led to the presence of a protein band of 98 kDa in the transformed *E. coli* cellular extracts, which was absent in the control. This molecular weight agrees with that estimated for the recombinant ßIV-Gal protein. Study of the subcellular location of the recombinant protein obtained 3 h after IPTG induction indicated that the protein appeared as inclusion bodies. When the four overexpressed ß-galactosidases were exposed to anti-ßIV-Gal antibodies, the antibodies specifically recognized a single polypeptide band in the lane corresponding to the recombinant ßIV-Gal protein (Figure 1B) but did not recognize any band in the lanes corresponding to the other chickpea ß-galactosidases, thus establishing their specificity.

Western Blot Immunodetection of the ßIV-Gal Protein in Cell Wall Protein Extracts

The anti-ßIV-Gal IgGs were used for Western blot analysis in cell wall protein extracts from different chickpea seedling and plant organs and developmental stages. When total cell wall protein extracts of etiolated seedling epicotyls were separated by SDS-PAGE, the anti-ßIV-Gal IgGs recognized only an 80-kDa polypeptide, coinciding with

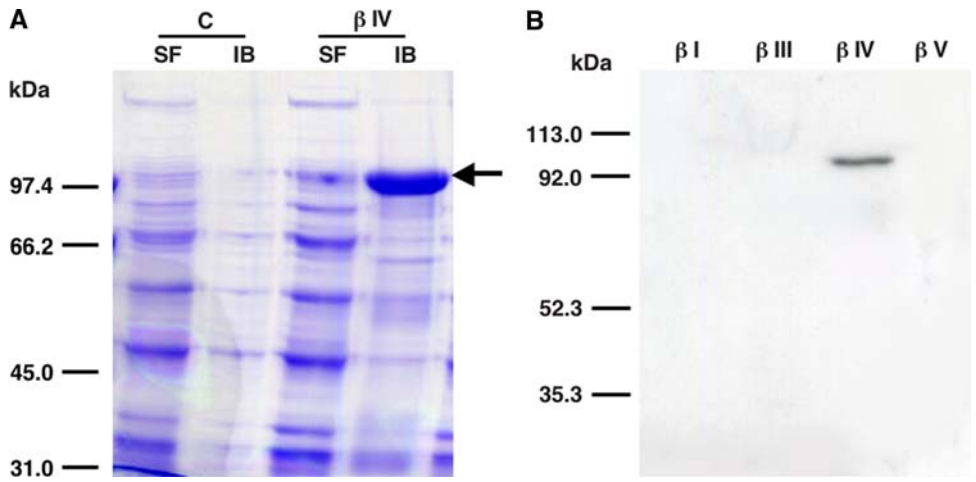

Fig. 1 (**A**) Overexpression of ßIV-Gal. Coomassie-stained SDS-PAGE gel showing bacterial overexpression and purification of recombinant ßIV-Gal protein. ßIV-Gal was overexpressed in *E. coli* and purified as described in Materials and Methods. Crude lysates of bacterial cultures transformed with pET-32a(+) vector used as negative control (C) or pET-32a(+)/CanBGal-4 (ßIV) construct were harvested 3 h after induction with IPTG and separated into a soluble fraction (SF) and inclusion bodies (IB). (**B**) Western blot analysis carried out with recombinant ßI-Gal, ßIII-Gal, ßIV-Gal, and ßV-Gal proteins and with anti-ßIV-Gal antibodies

the estimated molecular weight of mature ßIV-Gal; no other protein band was detected. The chickpea ßIV-Gal protein was detected during epicotyl growth from days 3 to 8. Figure 2A shows that the protein levels increased with the age of epicotyls, the highest levels being observed in epicotyls from 7- and 8-day-old-seedlings. The growth curve of epicotyls shows that the growth of epicotyls decreased with age (Figure 3A). In the youngest actively growing 3-, 4-, and 5-day-old epicotyls (Figure 3A), only very low levels of protein were detected (Figure 2A).

When the Western blots were carried out with cell wall protein extracts from different seedling and plant organs, the ßIV-Gal protein was detected mainly in organs such as epicotyls and mesocotyls in etiolated seedlings (Figure 2B) and stem internodes in 11-day-old plants (Figure 2C). The maximum recognition was observed in the basal first

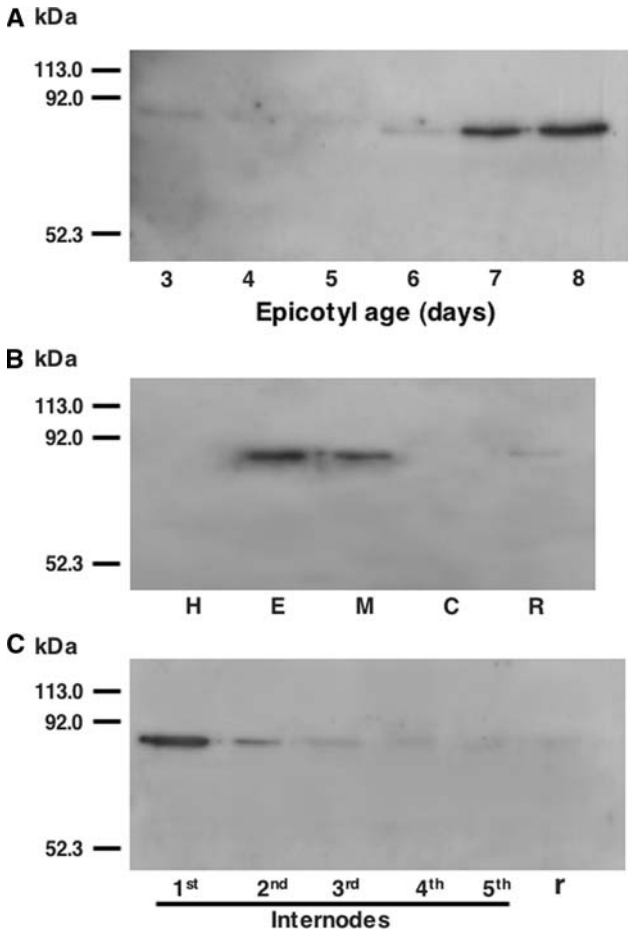

Fig. 2 Immunodetection by Western blot of ßIV-Gal protein in different cell wall protein extracts using anti-ßIV-Gal antibodies. (**A**) Cell wall proteins from seedling epicotyls along growth; numbers refer to days after sowing. (**B**) Cell wall proteins from several parts of 4-day-old seedlings. (**C**) Cell wall proteins from stem internodes and roots from 11-day-old plants; internodes are numbered 1st to 5th from the base to apex. H, hook; E, epicotyl; M, mesocotyl; C, cotyledon; R, radicle; r, root

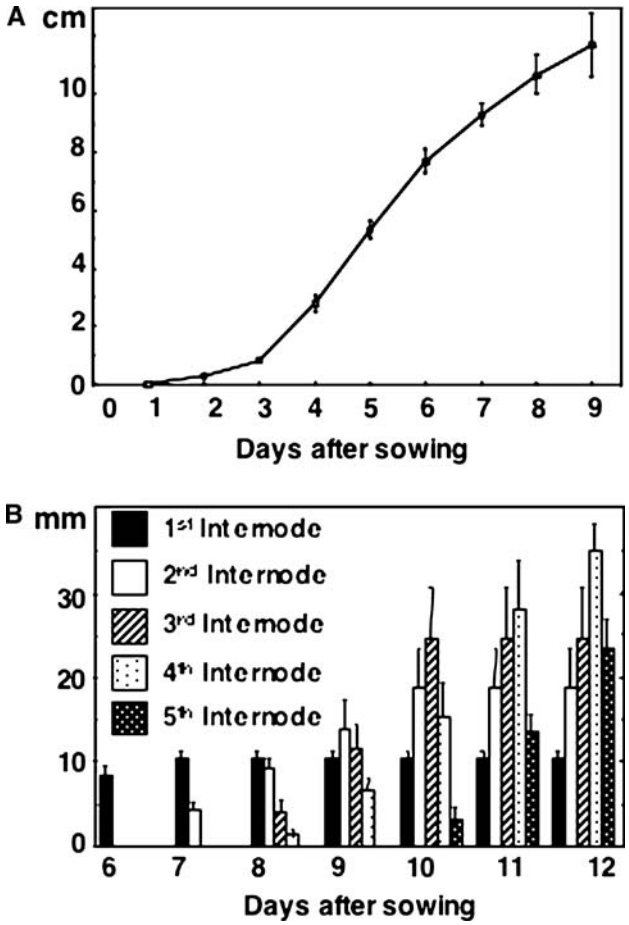

Fig. 3 Growth of seedling epicotyls and plant stems of *C. arietinum*. (**A**) Total accumulated growth of epicotyls from etiolated seedlings. (**B**) Internode length of plants grown from the 6th to the 12th day. Internodes were numbered 1st to 5th from base to apex. Numbers refer to days after sowing. The data are means of three experiments ± SE

internode, which was not undergoing any growth on the 11th day (Figure 3B; Jiménez and others 2006). Almost no protein was detected in apical internodes (fourth and fifth internodes), which at that moment were undergoing a very active growth as shown in Figure 3B. No protein was detected in roots (Figure 2C), although a weak signal was detected in radicles (Figure 2B).

Inmunolocalization of the ßIV-Gal Protein

To determine the tissue and cellular location of the ßIV-Gal protein in epicotyls and stem internodes, immunocytochemical studies were conducted using the anti-ßIV-Gal antibodies, as described in the Materials and Methods.

Because one of the aims of this work was to establish a putative relationship between the detection of protein and the elongation rate, 4- and 8-day-old epicotyls with high and low elongation rates, respectively (Figure 3A; Muñoz

Table 1 Increase in length of 0.5-cm sections from apical, central, and basal regions of 4- and 8-day-old chickpea epicotyls over 24 h

	Length increase ± SE (cm)		
	Apical section	Central section	Basal section
4-day-old epicotyls	1.71 ± 0.15	0.25 ± 0.10	0.04 ± 0.02
8-day-old epicotyls	1.2 ± 0.20	0 ± 0	0 ± 0

The data are means of three experiments ± SE

and others 1993), were used for immunolocalization studies. Also, because chickpea epicotyls show a growth gradient that decreases from the apical to the basal zone (Table 1; Seara and others 1988), three sections with different growth activities in the apical, central, and basal zones of 4- and 8-day-old epicotyls were separated and used for these studies.

When slices from apical, central, and basal sections of 4-day-old epicotyls were immunolabeled with anti-ßIV-Gal, the ßIV-Gal protein showed a very specific labeling (Figure 4). In apical sections, with a high elongation rate (Table 1), the protein appears clearly located in the cell wall of a layer of sclerenchymatic cells, as a sheath surrounding the vascular cylinder (perivascular fibers), and in the cell wall of some primary xylem cells (Figure 4A, and at higher magnification, 4B). In the central and basal zones of the epicotyls (Figure 4D, E and G, H, respectively), where the elongation rate was lower (Table 1), immunolabeling was observed in vascular cells, both primary xylem and phloem cells, and a signal was also detected in phloem fibers. No recognition was observed in apical, central, or basal sections (Figure 4C, F, and I, respectively) when the analyses were performed with preimmune serum.

In 8-day-old epicotyls, with a lower elongation rate (Figure 3A, Table 1), the ß-IV-Gal protein was detected in apical, central, and basal sections (Figure 5). In apical sections, the protein was located in the cell wall of some primary xylem cells and in primary phloem cells (Figure 5A and at higher magnification 5B). In the central and basal sections (Figure 5D, E and G, H, respectively), the protein was detected in the cell wall of primary phloem cells and in the xylem fibers associated with tracheary cells. The protein was also weakly detectable in the cell wall of phloem fibers in central epicotyl sections, and more intensely in the basal sections. No recognition was

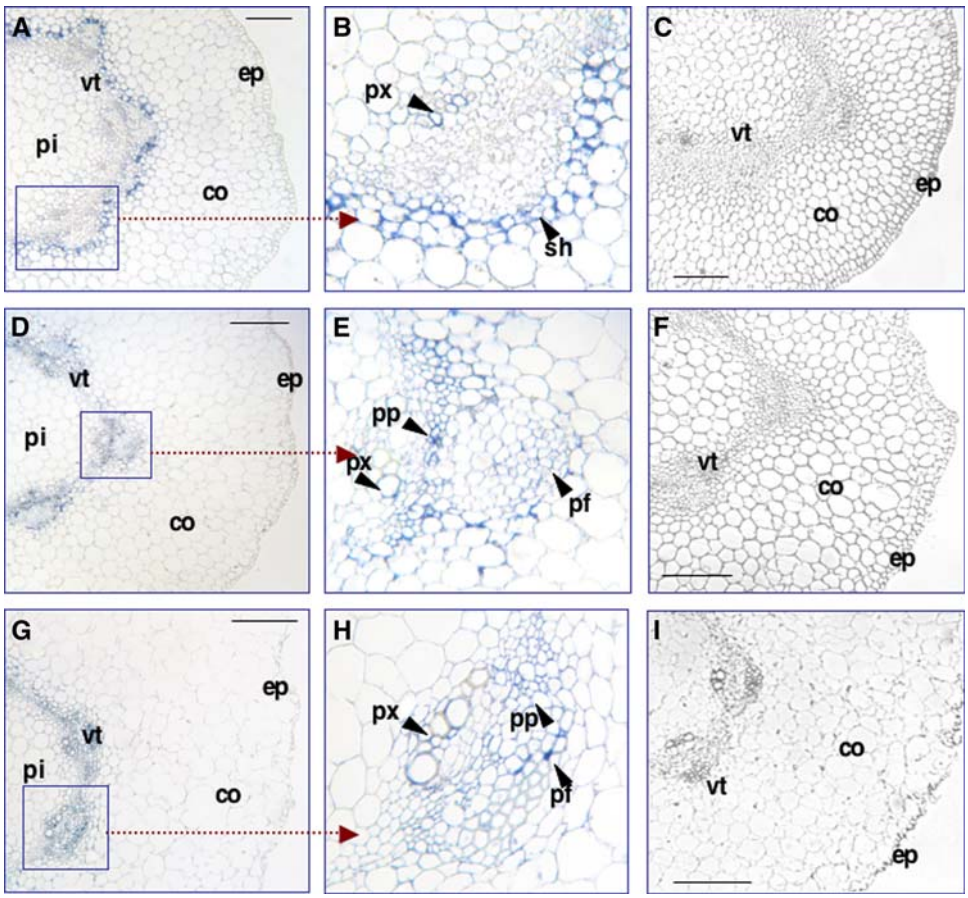

Fig. 4 Immunolocalization of ßIV-Gal protein in epicotyls from 4-day-old *Cicer arietinum* seedlings. Cross-sections were taken from apical (**A, B, C**), central (**D, E, F**) and basal (**G, H, I**) regions of epicotyls. (**A, D, G**) Epicotyl sections treated with anti-ßIV-Gal antibodies. (**B, E, H**) Higher magnification of the boxed regions in **A**, **D**, and **G**, respectively. (**C, F, I**) Epicotyl sections treated with preimmune serum. ep, epidermis; co, cortex; pi, pith; pf, phloem fiber; pp, primary phloem; px, primary xylem; sh, sheath of perivascular fibers; vt, vascular tissue. Scale bars = 100 µm

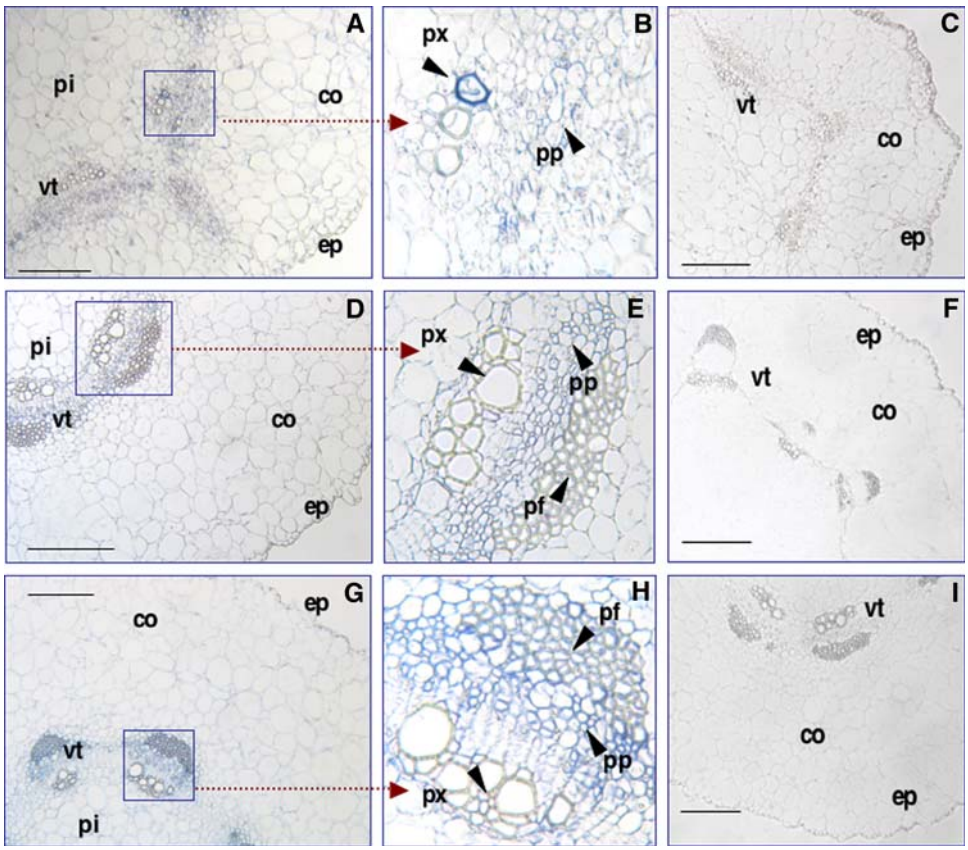

Fig. 5 Immunolocalization of ßIV-Gal protein in epicotyls from 8-day-old *Cicer arietinum* seedlings. Cross-sections were taken from apical (**A, B, C**), central (**D, E, F**), and basal (**G, H, I**) regions of epicotyls. (**A, D, G**) Epicotyl sections treated with anti-ßIV-Gal antibodies. (**B, E, H**) Higher magnification of the boxed regions in **A, D** and **G**, respectively. (**C, F, I**) Epicotyl sections treated with preimmune serum. ep, epidermis; co, cortex; pi, pith; pf, phloem fiber; pp, primary phloem; px, primary xylem; vt, vascular tissue. Scale bars = 100 µm

observed in apical, central, or basal sections (Figure 5C, F, I, respectively) when the analyses were performed with preimmune serum.

The location of ßIV-Gal in chickpea stem was determined by studying its immunolocalization in the five internodes of an 11-day-old plant (Figure 6). At this particular chickpea plant age, only the apical fourth and fifth internodes exhibit active growth (Figure 3B; Jiménez and others 2006), whereas the first, second, and third internodes have already ended their elongation (Figure 3B). Immunocytochemistry of transversal sections of the five different internodes revealed a growth-related pattern of location. Thus, immunodetection was low in the younger growing fifth and fourth internodes (Figure 6), whereas a strong degree of labeling was detected in the older internodes (basal first and second ones) with no elongation.

In the fifth apical internode, ßIV-Gal protein was located in only several primary xylem cells (Figure 6A), similar to the labeled cells found in the apical region of 4- and 8-day-old epicotyls. In the fourth and third internodes, a low degree of antibody recognition was detected in the cell walls of primary phloem cells (Figure 6B, C, G). In the third internode, it was also possible to detect ßIV-Gal protein in the phloem fibers (Figure 6C) and in the epidermis (Figure 6G). In the two basal second and first internodes, the primary phloem cells appeared clearly labeled and a strong degree of labeling was detected in the cell walls of the phloem fibers (Figure 6D, E), the signal being stronger in the inner phloem fibers. ßIV-Gal protein was also detected in the cell wall of the epidermal cells and subepidermal collenchyma cells in the second and first internodes (Figure 6H, I). Labeling in the collenchyma cells was detected at the inner region of the primary cell wall and not at the thickened areas of cell junctions. Labeling was also restricted mainly to the tangential walls of the collenchyma cells, with little labeling in the radial walls.

Discussion

The role of cell wall ß-galactosidases in the metabolism of different cell wall polymers is still under debate. The presence of a multigene family of ß-galactosidases in plants (Ali and others 1998; Smith and Gross 2000) suggests that the different gene products would be active in different aspects of cell wall metabolism.

Chickpea ßIV-galactosidase is a cell wall protein encoded by the *CanBGal-4* gene, which belongs to a family of at least four ß-galactosidase genes (Esteban and others 2005). In a previous report we established that *CanBGal-4* transcripts are present mainly in elongating organs such as epicotyls, radicles, and stems, although its transcription

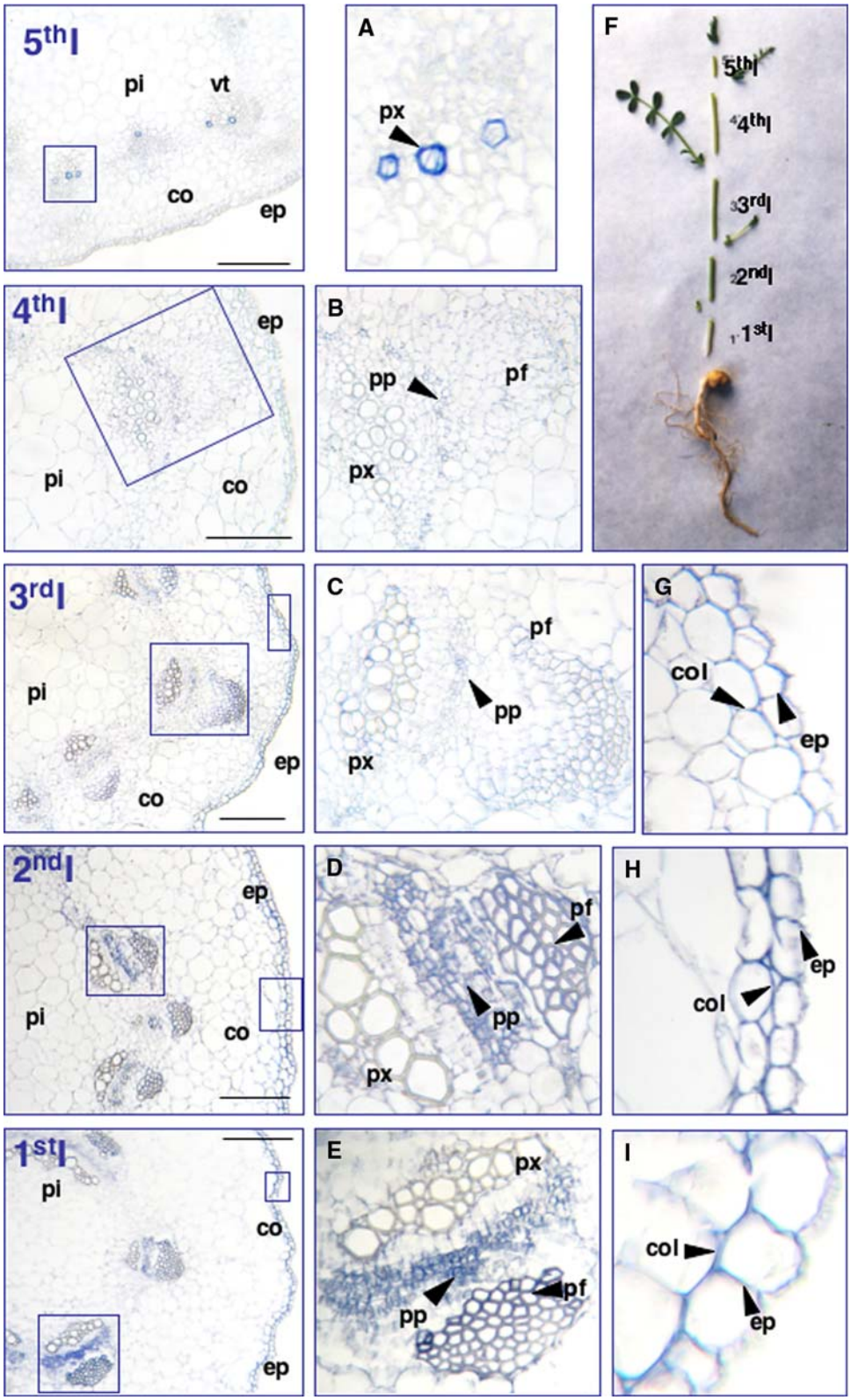

Fig. 6 Immunolocalization of ßIV-Gal protein in internodes from 11-day-old *Cicer arietinum* stems. Cross-sections from stem internodes numbered 1st to 5th from basal to apical were treated with anti-ßIV-Gal antibodies. Cross-sections were taken from 5th, 4th, 3rd, 2nd, and 1st internodes. (**A, B, C, D, E**) Higher magnification of the boxed regions of vascular tissue in 5th, 4th, 3rd, 2nd, and 1st internodes, respectively. (**G, H, I**) Higher magnification of the boxed regions of epidermal region in 3rd, 2nd, and 1st internodes, respectively. (**F**) Photograph of an 11-day-old plant; the stem was divided into five internodes. co, cortex; col, collenchyma; ep, epidermis; pf, phloem fibers, pi, pith; pp, primary phloem; px, primary xylem. Scale bars = 100 µm

pattern seems to be inversely related to the elongation rate of these organs (Esteban and others 2005). Thus, young epicotyls undergoing exponential growth as well as younger upper internodes showed low *CanBGal-4* transcript levels, whereas *CanBGal-4* transcripts increased as the epicotyls aged (7- and 8-day-old epicotyls showed the

highest values) and in basal internodes (Esteban and others 2005), where elongation rates were practically zero (Jiménez and others 2006).

Although gene expression studies are useful for checking the involvement of a given protein in a specific process, the location of the protein encoded by a given gene could provide more information about the actual role of the protein in the process. In this work, using polyclonal antibodies raised against a designed peptide from the *CanBGal-4*-deduced amino acid sequence, we report the location of the ßIV-Gal protein in seedlings and plants of *C. arietinun* and its visualization in specific cells of epicotyls and stems. The specificity of antibodies to ßIV-Gal was previously checked by Western blot analysis performed with the recombinant chickpea ßI-, ßIII-, ßIV-, and ßV-Gal, which confirmed that the anti-ßIV-Gal antibodies did not elicit cross-reactions with any of the other three cell wall ß-galactosidases identified in chickpea epicotyls (Figure 1B).

The detection of the ßIV-Gal protein in cell wall protein extracts from seedlings and from plant stems (Figure 2) agrees with the *CanBGal-4* gene expression pattern (Esteban and others 2005) and confirms the presence of the ßIV-Gal protein mainly in elongating organs such as epicotyls and stems, when cell elongation is very low or has ceased (Figure 3). The fact that ßIV-Gal levels increased as the epicotyls aged and consequently their growth rate decreased (Figures 2A, 3A) is also in agreement with the trend of the *CanBGal-4* transcript (Esteban and others 2005) and indicates that this cell wall protein appears with the cessation of cell elongation.

The specific main location of the ßIV-Gal protein in the vascular tissue of epicotyls and stems (Figures 4–6) and in a layer of sclerenchymatic cells surrounding the vascular cylinder (perivascular fibers) suggests the involvement of ßIV-Gal in the modification of cell wall polymers during vascular cell differentiation.

During cytodifferentiation into tracheary elements (TEs), cell walls undergo structural changes such as a localized thickening of cell walls, lignification of secondary walls, and a partial degradation of primary walls. However, although extensive research has been carried out on secondary wall formation and lignin accumulation, the primary wall degradation associated with TE differentiation has received limited attention. Most of our knowledge about this process comes from electron microscopic observations of developing TEs (O'Brien 1970; Esau and Charvat 1978; Burgess and Linstead 1984; Nakashima and others 2000). Different cell wall enzymes such as pectate lyases (Domingo and others 1998; Sterky and others 1998), pectin methyl esterases, or endopolygalacturonases (pectinases) (Torki and others 2000) have been related to the cell wall degradation that occurs during vascular tissue differentiation, although their fuction is not fully established. Because cell wall degradation associated with vascular differentiation involves neutral polymers such as ß(1-4)-galactan (Ohdaira and others 2002), ß-galactosidase enzymes might also be involved in this physiologic process. Our results showing the presence of ßIV-Gal protein in vascular cells during their differentiation, and the consequent primary cell wall degradation (Figures 4–6) may reflect this function. This explains why in young epicotyls and newly formed stem regions (apical internodes) (Figure 3, Table 1; Muñoz and others 1993; Jiménez and others 2006) with undifferentiated vascular cells, the labeling was of low intensity and the ßIV-Gal appeared around the vascular tissue.

The intense protein recognition by anti-ßIV-Gal antibodies in only several isolated protoxylem cells, observed both in apical stem internodes (Figure 6) and in the apical region of 8-day-old epicotyls (Figure 5A, B), suggests that these are the cells in which xylem differentiation starts and where primary cell wall degradation begins to occur. When vascular cell formation became apparent, the presence of the ßIV-Gal increased, immunolocalization becoming more intense and more specific to developing vascular cells, as seen in the basal zones of epicotyls (Figures 4 and 5) and in the first and second basal internodes (Figure 6). Finally, when the secondary cell wall was totally developed, as in the xylem vessels of the basal first internode and in the developed phloem fibers (Figure 5E), immunodetection decreased and no ßIV-Gal protein was detected in these cell walls. All these results lead us to propose the involvement of ßIV-galactosidase in the modification of primary cell walls during the development of the vascular system of chickpea seedlings and plants, probably by decreasing neutral galactan, as reported by Ohdaira and others (2002), for the development of tracheary elements in *Zinnia elegans*. A *β*-galactanase from lupin cotyledons, similar to ßIV-Gal from *C. arietinum* (87% similarity in amino acid sequence), is capable of attacking galactan polymers, leaving other polymers of the primary wall with some integrity (Buckeridge and others 2005). In the case of ßIV-Gal from *C. arietinum*, it is likely that a similar mechanism operates during development, modifying protoxylem walls.

The fact that the ßIV-Gal protein was strongly detected in the basal sections of the epicotyls where elongation rates were practically zero (Table 1), whereas detection was progressively lower in the central and apical zones of the epicotyls with a high growth rate (Table 1, Figures 4 and 5), again relates the presence of this protein to the end of cell elongation in epicotyls, although this probably reflects the relationship between organ elongation and the development of a vascular system.

The location of the ßIV-Gal protein in the cell walls of collenchyma cells in internodes (Figure 6G, H, I) is consistent with the above-mentioned relationship of ßIV galactosidase with cell wall modifications that lead to thick

cell walls, such as in vascular cells. The walls of collenchyma cells are much thicker than those of typical primary cell walls and are often thickened at the junction between cells (Mauseth 1988). The presence of galactan side chains in pectins decreases the ability of pectin molecules to crosslink and form a coherent gel network (Hwang and Kokini 1991). The decrease in neutral galactan side chains in epidermal cells and the thickened collenchyma cell wall may indicate that extensive crosslinking of the homogalacturonan chains occurs at these locations, promoting rigidity and strength, as has been reported by Jones and others (1997). The location of the ßIV-Gal protein at the inner region of the primary cell wall, not at the thickened areas of the cell junction, and the more intense immunolabeling at the tangential walls rather than at the radial walls of collenchyma cells are consistent with the (1-4)-ß-galactan LM5 antibodies labeling found in the collenchyma cells of tomato (Jones and others 1997).

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