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ORIGINAL ARTICLES

The Location of the Chickpea Cell Wall ßV-Galactosidase Suggests Involvement in the Transition between Cell Proliferation and Cell Elongation

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Abstract We report the generation of antibodies against a ß-galactosidase (ßV-Gal) from *Cicer arietinum* and the subsequent immunolocalization of the protein in different parts and developmental stages of the plant. ßV-Gal is a cell wall protein encoded by the *CanBGal-5* gene, which belongs to a family of at least four ß-galactosidase genes in chickpea. We have previously reported that *CanBGal-5* transcripts are located in organs with high elongation and cell division rates, such as meristematic hooks, very young epicotyls, and apical internodes. ßV-Gal protein is the only studied chickpea ß-galactosidase widely present in meristematic hooks, mainly in the meristematic apical zone. These results agree with the previously reported transcription pattern of *CanBGal-5* and may reflect its involvement in cell wall modifications during the final stages of cell proliferation, leading to the establishment of an expanding cell wall. The location of ßV-Gal in the cell wall of procambium cells and in pericycle cells of the developing lateral roots also supports the involvement of ßV-Gal in this process. During seedling and plant growth, the highest levels of *β*V-Gal protein were detected in the youngest actively growing epicotyls and in the apical growing internodes. Thus, protein levels pointed to a relationship between *β*V-Gal and the events occurring in the cell wall during the early stages of development. Immunolocalization studies in different zones of epicotyls and radicles suggest a role for ßV-Gal in cell elongation.

Keywords Cell wall · Chickpea · ß-galactosidase · Elongation · Meristem

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Introduction

ß-D-galactosidases (EC 3.2.1.23) hydrolyze the glycosidic bond between a nonreducing galactose residue and another sugar or alcohol. These enzymes have been described in different organisms, including microorganisms, plants, and animals.

In plants, ß-galactosidases have been described mainly in growth, development, senescence, and fruit-ripening processes, where they are typically thought to act on cell wall carbohydrates (Dopico and others 1990a; Konno and Katoh 1992; Buckeridge and Reid 1994; Ross and others 1994). Although the cell wall ß-galactosidases that are active on cell wall polysaccharides typically act on ß-(1-4)-linked galactosyl residues, there is another set of enzymes that hydrolyze *p*-nitrophenyl-ß-galactoside and ß-(1-3)- and ß-(1-6)-linked galactosyl residues, some of which have been shown to act on the carbohydrate chains of arabinogalactan proteins (Kotake and others 2005). Moreover, ß-galactosidases also exert an important effect on the side chains of xyloglucan (Tiné and others 2000), mainly in cotyledons involved in storage mobilization (Edwards and others 1988; De Alcantara and others 1999, 2006). Thus, it appears that although at least some of these enzymes are involved in cell wall remodeling, the large number expressed and the different activities suggest they may play a variety of roles.

ß-Galactosidase cDNA sequences from higher plants have been cloned and it is currently known that ß-galactosidases are encoded by gene families and have a high degree of similarity. ß-Galactosidase multigene families have been described in several plants, such as *Lycopersicon esculentum* with seven members (Smith and Gross 2000) and *Carica papaya* (Ali and others 1998) with three members. In fully sequenced plant genomes, such as that of *Arabidopsis thaliana*, there are 18 genes whose products have been predicted to be ß-galactosidases of the glycosyl hydrolase family 35, 12 of which are probably secreted to the wall (Ahn and others 2007). In *Oryza sativa*, seven ß-galactosidases appear in the databases, some of them having been recently characterized (Chantarangsee and others 2007). 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). The presence of a multigene family of ß-galactosidase in plants suggests that the different gene products would be active in different aspects of cell wall metabolism and raises the possibility of different functions for individual members of the ß-galactosidases family in the same plant.

In *Cicer arietinun* a ß-galactosidase multigene family with at least four members, named *CanBGal-1*, *-3*, *-4*, and *-5*, was described and the transcription pattern of these genes reported (Esteban and others 2003, 2005). *CanBGal-3* encoded the ßIII-Gal protein, whose function in pectin degradation was supported by its *in vivo* expression in a potato system (Esteban and others 2003; Martín and others 2005). ßIII-Gal is associated with cell wall loosening during epicotyl elongation (Esteban and others 2003) and acts on the ß-(1-4)-galactan side chain of rhamnogalacturonan I (Martín and others 2005). The ßIV-Gal protein was immunodetected in the cell walls of seedling epicotyls and plant stems, mostly in aged epicotyls and basal stem internodes, both undergoing low rates of elongation, in agreement with the trend of the *CanBGal-4* transcript (Martín and others 2008). This immunodetection indicates a relationship of this cell wall protein with the end of cell elongation. The specific immunolocation of the ßIV-Gal in the vascular tissue of epicotyls and stems and in a layer of sclerenchymatic cells surrounding the vascular cylinder (perivascular fibers) allowed us to postulate a function of this ß-galactosidase in the modification of cell wall polymers during the development of cells of the vascular system (Martín and others 2008).

The aim of this article is to gain insight into the function of another of the *C. arietinum* ß-galactosidases, the ßV-Gal protein encoded by *CanBGal-5*. Previously, we had established that *CanBGal-5* transcripts are located in organs with high elongation and cell division rates, such as meristematic hooks, very young epicotyls, and apical internodes (Esteban and others 2005). These results suggested a possible role for ßV-Gal in modifying the cell wall during the early development stages of seedlings and plants. The generation of specific antibodies against this protein and the subsequent immunolocalization of the protein in different parts and development stages of the plant allow us to postulate a function for this ß-galactosidase in the final stage of cell proliferation and the subsequent establishment of a cell wall with elongation capacity.

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 the 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 these were harvested and epicotyls were collected. The epicotyl length was measured in seedlings from the first to the ninth 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 from 1 to 5 from base to apex. The internodes' lengths were measured in plants from the 6th to the 12th day.

Four-day-old hooks, epicotyls, and radicles as well as eight-day-old epicotyls and 11-day-old-stem internodes were used for immunolocalization studies.

Expression and Purification of the ßV-Gal Fusion Protein

The coding sequence of *CanBGal-5* (minus the signal sequence) was PCR-amplified from the *CanBGal-5* plasmid clone. The two oligonucleotide primers used were 5′-GGATCCAGTGTGACATATGATAGAAAAGC-3′ and 5′-CTCGAGTTATACTTGTGTTGTATTAGTTCTT-3′, adding a *Bam*HI restriction site at the 5′ end and a *Xho*I site at the 3′ end, respectively (underlined in the sequences). The PCR product was subcloned into the *Bam*HI/*Xho*I restriction site of the pET-32a(+) (Novagen, EMD Chemicals, Inc., San Diego, CA, USA) expression vector and moved into 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 (containing 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-5* deduced amino acid sequence. For the peptide design several considerations were taken into account, such as the hydrophyllicity and the antigenicity profiles as well as the probability of the peptide surface exposure in the protein. Among the chickpea ß-galactosidase sequences, an alignment was performed to avoid areas of high similarity. All these analyses were carried out using the DNAStar (DNASTAR, Inc., Wisconsin, USA) sequence analysis software. Finally, once the peptide was designed, a search using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul and others 1990) was done to establish the degree of peptide identity with protein sequences in databases other than ß-galactosidases. Synthesis of the peptide and its conjugation with the KLH (Keyhole Limpet Hemocyanin) 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 with 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 anti-ßV-Gal antibody and the peroxidase-conjugated secondary antibody were applied at 1:10,000 and 1:150,000 dilution, respectively.

Cell Wall Protein Extraction and Western Blotting

Cell walls were prepared according to Dopico and others (1989). Protein was extracted 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 3 K 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 electrotransferred onto PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). Immunoblots were prepared essentially according to the procedure of Harlow and Lane (1988) using the anti-ßV-Gal antibody at a 1:10,000 dilution and a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, Bio-Rad) at a 1:100,000. Blots were developed by chemiluminescence using the ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Immunocytochemical Labeling

Hooks, epicotyls, and radicles from 4-day-old etiolated seedlings, epicotyls from 8-day-old etiolated 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 slides (Sigma). 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 for 45 min with 5% (w/v) BSA and 3% (v/v) normal swine serum in TBS. Anti-ßV-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 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 ßV-Gal Antibodies

To clarify the function of the ß-galactosidase encoded by *CanBGal-5* in cell wall metabolism, we raised polyclonal antibodies against this protein. Thus, we designed a peptide of the *CanBGal-5*-deduced protein sequence according to the characteristics indicated in the Materials and Methods section, to ensure its specificity. This peptide was synthesized and used to immunize the rabbits.

Because in *C. 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, it was necessary to check the specificity of the antiserum raised against ßV-Gal. To carry out 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 ßV-Gal produced by *E. coli*. The expression of *CanBGal-5*, without the signal peptide, in the pET32a (+) vector led to the presence of a protein band of 100 kDa in the transformed *E. coli* cellular extracts, which was absent in the control. This molecular weight agrees with that estimated for the recombinant ßV-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 expressed ß-galactosidases were exposed to anti-ßV-Gal antibodies, the antibodies specifically recognized a single polypeptide band in the lane corresponding to the recombinant ßV-Gal protein (Fig. 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 ßV-Gal Protein in Cell Wall Protein Extracts

The anti-ßV-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 different etiolated seedling organs were separated by SDS-PAGE, the anti-ßV-Gal IgGs recognized only an 80-kDa polypeptide, coinciding with the estimated molecular weight of mature ßV-Gal, and no other protein band was detected. It was observed that

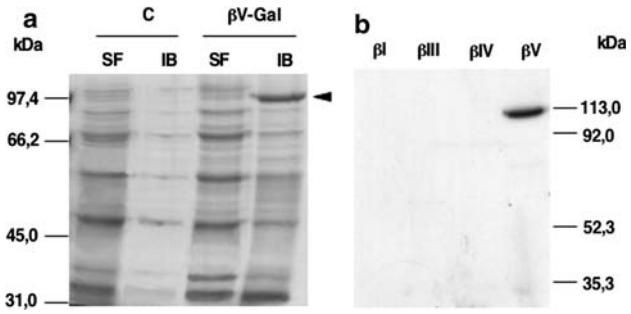

Fig. 1 **a** Overexpression of ßV-Gal. Coomassie-stained SDS-PAGE gel showing bacterial overexpression of recombinant ßV-Gal protein. ßV-Gal was overexpressed in *E. coli* as described in Materials and Methods. Crude lysates of bacterial cultures transformed with pET-32a(+) vector used as negative control (C) or pET-32a(+)/*CanBGal-5* (ßV-Gal) 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 anti-ßV-Gal antibodies

the ßV-Gal showed high levels in meristematic hooks (Fig. 2a). This protein was also detected in epicotyls, mesocotyls, and radicles (Fig. 2a).

When the Western blots were carried out with cell wall protein extracts from etiolated epicotyls, the chickpea ßV-

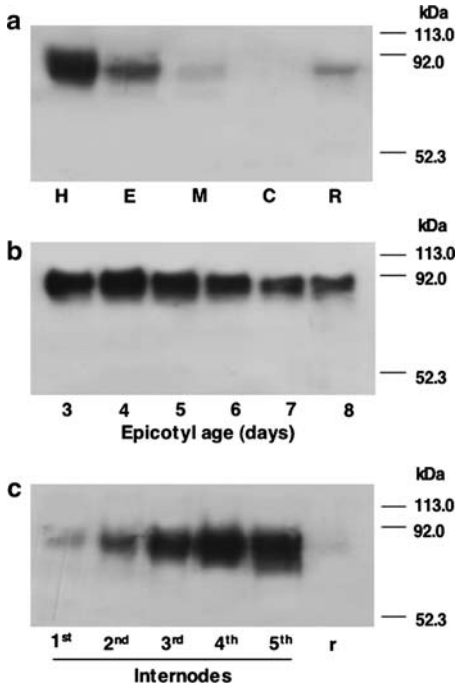

Fig. 2 Immunodetection by Western blot of the ßV-Gal protein in different cell wall protein extracts using anti-ßV-Gal antibodies. **a** Cell wall proteins from several parts of 4-day-old etiolated seedlings. **b** Cell wall proteins from seedling epicotyls along growth. **c** Cell wall proteins from 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

Gal protein was detected along epicotyl growth from days 3 to 8. Figure 2b shows that the protein levels decreased with the age of epicotyls; the highest levels of ßV-Gal were detected in the youngest and most actively growing epicotyls (Fig. 2b). The growth curve of epicotyls shows that the growth rate of epicotyls decreased with age (Martín and others 2008).

In 11-day-old plants, the ßV-Gal protein was detected by Western blot in cell wall protein extracts from the different stem internodes (Fig. 2c); the level of protein decreased from the apical to the basal internode. The maximum recognition was observed in the apical fifth internode, which was undergoing active growth on the 11th day (Jiménez and others 2006). Almost no protein was detected in the basal first internode that at that moment was not undergoing any growth. No protein was detected in roots (Fig. 2c).

Immunolocalization of the ßV-Gal Protein

To determine the tissue and cellular location of the ßV-Gal protein in meristematic hooks, epicotyls, radicles, and stem internodes, immunocytochemical studies were conducted using the anti-ßV-Gal antibodies.

Immunocytochemical analysis of 4-day-old meristematic hooks was performed in three longitudinal sections of the hooks. The ßV-Gal was located all over the organ (Fig. 3), including the apical meristem cells, epidermis, parenchyma cells, and vascular tissue cells. As an exception, it was not possible to detect the protein in the cells of the primary xylem (Fig. 3c, c1).

In apical, central, and basal cross sections of 4-day-old epicotyls, the ßV-Gal showed wide localization and was detected in the cell walls of cortex and pith and also in the vascular cells (Fig. 4a, b, c). The labeling in the cell wall of

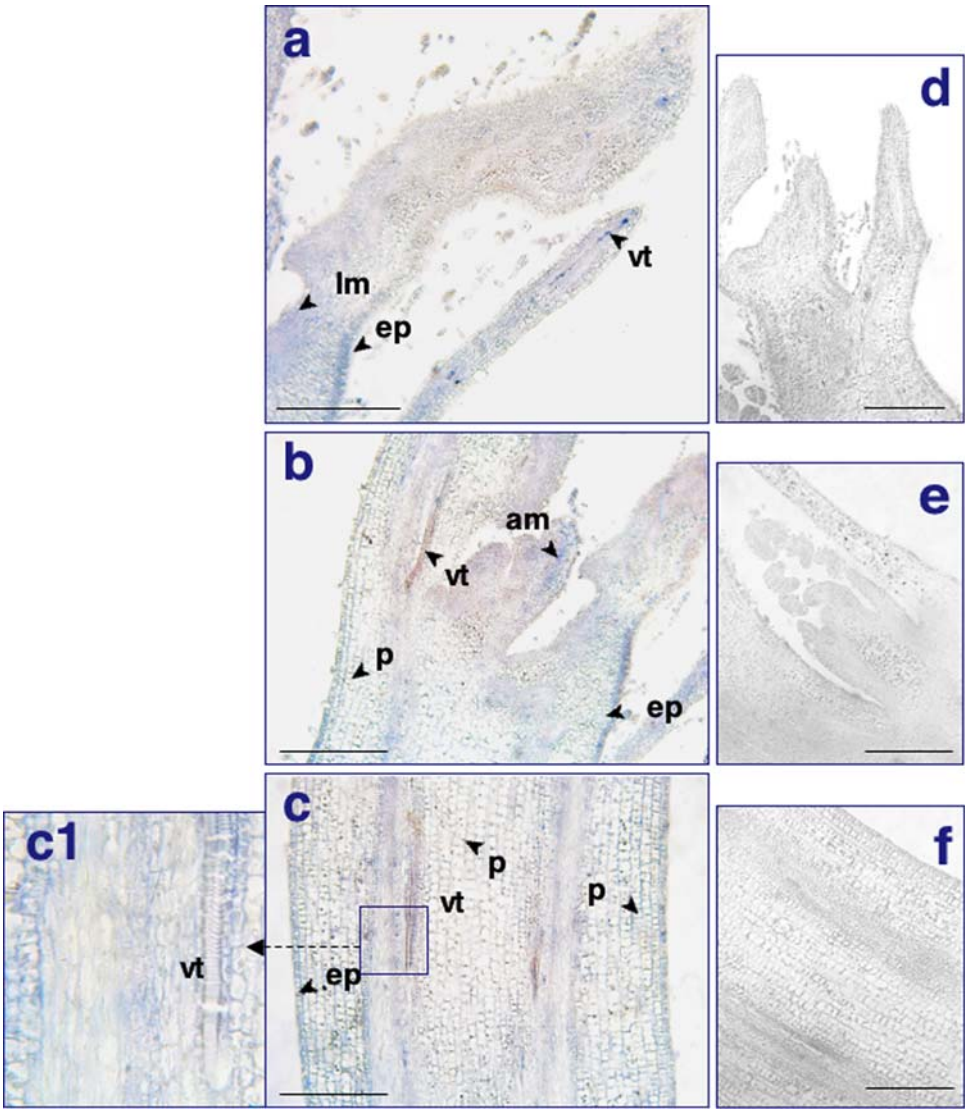

Fig. 3 Immunolocalization of ßV-Gal in meristematic hooks from 4-day-old *C. arietinum* etiolated seedlings. Longitudinal sections were taken from the upper (**a**, **d**), central (**b**, **e**), and lower (**c**, **f**) zones of hooks and were immunostained with anti-ßV-Gal antibodies (**a**, **b**, **c**) or with preimmune serum (**d**, **e**, **f**). (**c**1) A higher magnification of the boxed region in c. am, apical meristem; ep, epidermis; lm, primary leaf mesophyl; p, parenchyma; vt, vascular tissue. Arrowheads indicate the labeled tissue. Scale bars = 100 µm

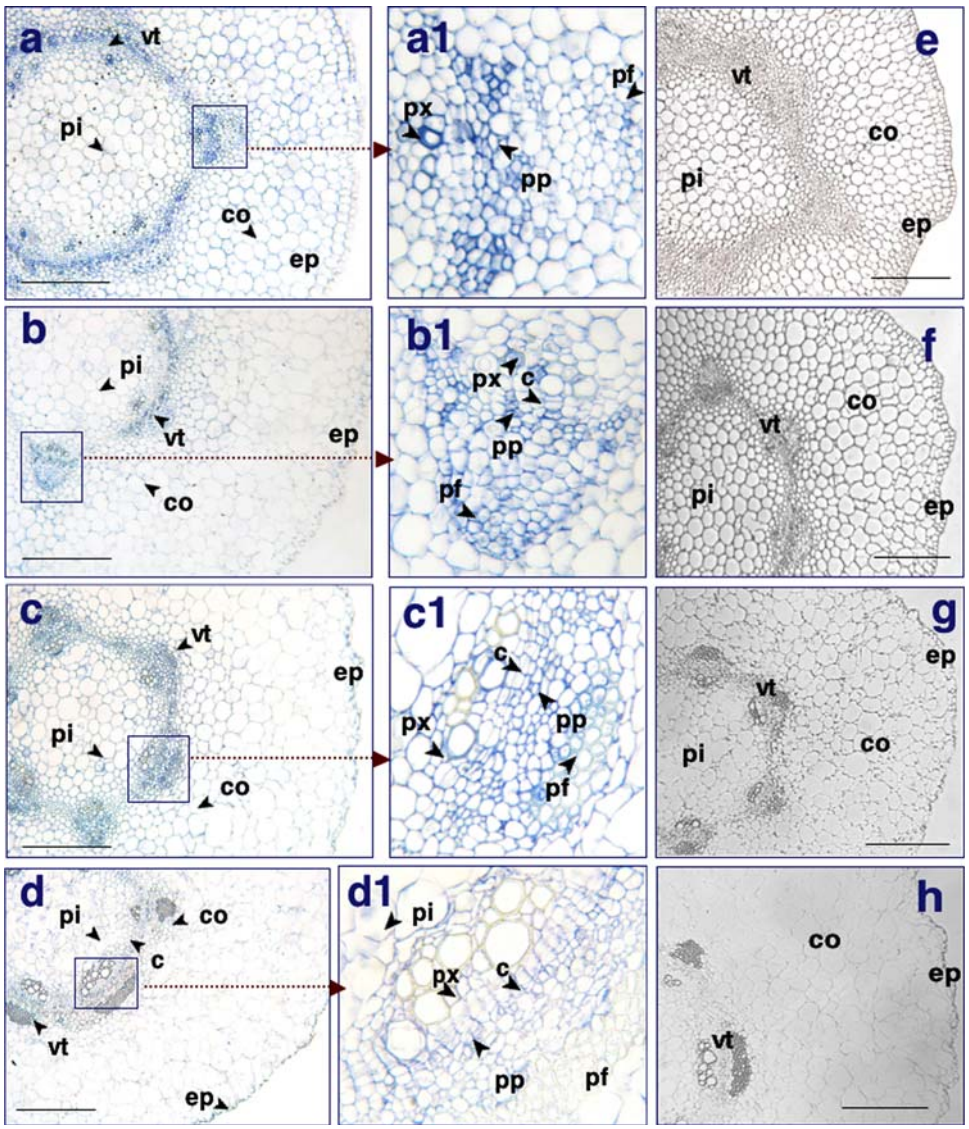

Fig. 4 Immunolocalization of ßV-Gal protein in epicotyls of *Cicer arietinum* etiolated seedlings. Cross-sections were taken from apical (**a**, **e**), central (**b**, **f**), and basal (**c**, **g**) regions of 4-day old epicotyls and from the basal region of 8-day-old epictoyls (**d**, **h**). **a**, **b**, **c**, **d** Epicotyl sections treated with anti-ßV-Gal antibodies. **e**, **f**, **g**, **h** Epicotyl sections treated with preimmune serum. **a1**, **b1**, **c1**, **d1** Higher magnifications of the boxed regions in a, b, c, and d, respectively. c, procambium; ep, epidermis; co, cortex; pi, pith; pf, phloem fiber; pp, primary phloem; px, primary xylem; vt, vascular tissue. Arrowheads indicate the labeled tissue. Scale bars = 100 µm

parenchyma cells, both cortex and pith cells, was more intense in apical sections, with a high elongation rate (Fig. 4a). The labeling was progressively less intense in central and basal zones of epicotyls, whose elongation rate was lower. In these basal regions the protein was located in the cell wall of cortex cells close to the vascular cylinder (Fig. 4b, c). In the vascular tissue, the ßV-Gal was located in the different cell types—primary xylem, primary phloem, procambium, and phloem fibers—although the labeling was less intense or even absent in the xylem cells close to the pith (Fig. 4a1, b1, c1). In the basal section of the epicotyls, the labeling in the phloem fibers was restricted to the cell wall of the fibers closest to the primary phloem (Fig. 4c1). In this basal section the strong labeling of the procambium cells was not present in the cell division plate.

The decrease in the labeling in the basal section of 4-day-old epicotyls is more evident in basal sections of older epicotyls (8-day-old) (Fig. 4d), where it is clear that ß-Gal is not located in the xylem cells close to the pith in the phloem cells either; the labeling is strong in the companions cells and in the cells close to the procambial zone (Fig. 4d1). No label could be observed in the phloem fibers. Also, in this section it is possible to see clearly that the labels do not appear in the cell walls of the division plate cell (Fig. 4d1).

No recognition was observed when the analyses were carried out with preimmune serum in apical, central, or basal sections of 4-day-epicotyls (Fig. 4e, f, g, respectively) or in the basal section of 8-day-old epicotyls (Fig. 4h).

In 4-day-old radicles the location of ßV-Gal followed the same pattern as in epicotyls, including the decrease from the apical to the basal section of the labeling in parenchymatic cells, its restriction to the cells close to the vascular tissue, and the decrease of labeling in the inner xylem cells (Fig. 5). Unlike in epicotyls, the protein appeared located in the epidermis all over the radicle

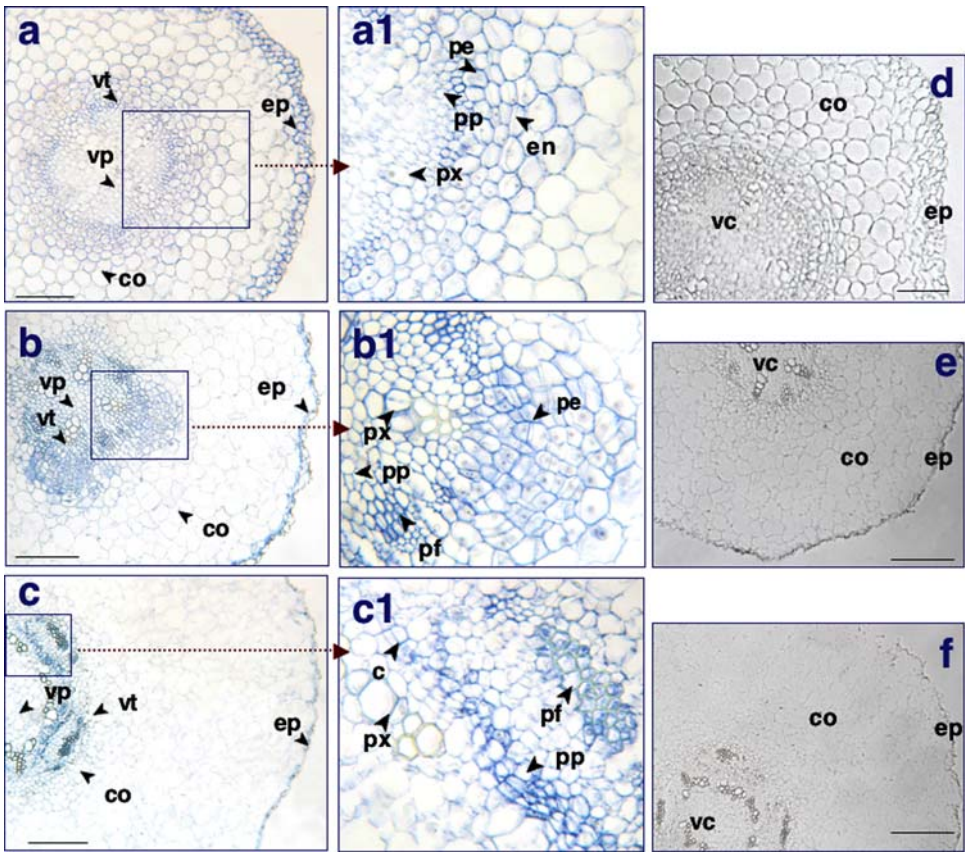

Fig. 5 Immunolocalization of ßV-Gal protein in radicles from 4-day-old *Cicer arietinum* etiolated seedlings. Cross-sections were taken from apical (**a**, **d**), central (**b**, **e**), and basal (**c**, **f**) regions of radicles. **a**, **b**, **c** Radicle sections treated with anti-ßV-Gal antibodies. **d**, **e**, **f** Radicle sections treated with preimmune serum. **a1**, **b1**, **c1** Higher magnification of the boxed regions in a, b, and c, respectively. c, procambium; en, endodermis; ep, epidermis; co, cortex; pe, pericycle; pf, phloem fiber; pp, primary phloem; px, primary xylem; vc, vascular cylinder; vp, vascular parenchyma; vt, vascular tissue. Arrowheads indicate the labeled tissue. Scale bars = 100 µm

sections. Also, it is worth noting the intense labeling observed in the pericycle cells that are developing lateral roots (Fig. 5b1).

Immunolocation analysis was also carried out on the five internodes of the 11-day-old chickpea plants, with different elongation rates (Fig. 6). By day 11, the first (1st), second (2nd), and third (3rd) internodes had completely ceased their elongation, whereas the fourth (4th) and fifth (5th) internodes continued to grow on the following day (Jimenez and others 2006) (graphic included in Fig. 6). Immunocytochemistry of transversal sections of the five different internodes revealed a growth-related pattern of localization. Thus, immunodetection was high in the younger 5th and 4th internodes, those with elongation capacity, and decreased in older basal 3rd, 2nd, and 1st internodes. In young internodes, the protein ßV-Gal was located in all types of cells—parenchyma, collenchyma, vascular tissue and epidermis—with the exception of the primary xylem cells close to the pith (Fig. 6 5tha). In older internodes, the labeling was less intense in the vascular tissue (Fig. 6 3rda, 2nda) and in the parenchyma and epidermal cells (Fig. 6 3rdb, 2ndb). In the vascular tissue, the protein was limited to the cambium cells and the xylem and phloem cells closest to the cambium, but was not present around the xylem cells close to the pith or in the phloem fibers (Fig. 6 2nda).

Discussion

Chickpea ßV-galactosidase is a cell wall protein encoded by the *CanBGal-5* gene, which belongs to a family of at least four ß-galactosidase genes (Esteban and others 2005). We previously reported that *CanBGal-5* transcripts are located in organs with high elongation and cell division rates, such as meristematic hooks, very young epicotyls, and apical internodes (Esteban and others 2005). These results suggested a possible role for the ßV-Gal in modifying the cell wall during the early developmental stages of seedlings and plants.

The generation of antibodies against ßV-Gal protein and later confirmation that these anti-ßV-Gal antibodies did not elicit cross-reaction with any of the remaining three ß-galactosidases identified in chickpea epicotyls (Fig. 1b) allowed us to conduct Western blot and immunolocation studies that confirm that the ßV-Gal protein is indeed located in the cell wall and allowed us to give insight about the putative function of this protein.

ßV-Gal is the only studied chickpea ß-galactosidase widely present in meristematic hooks (Fig. 2), both in parenchymatic and meristematic cells, although the labeling is more intense in the meristematic apical zone (Fig. 3). These results agree with the transcription pattern of *CanBGal-5* (Esteban and others 2005) and relate this

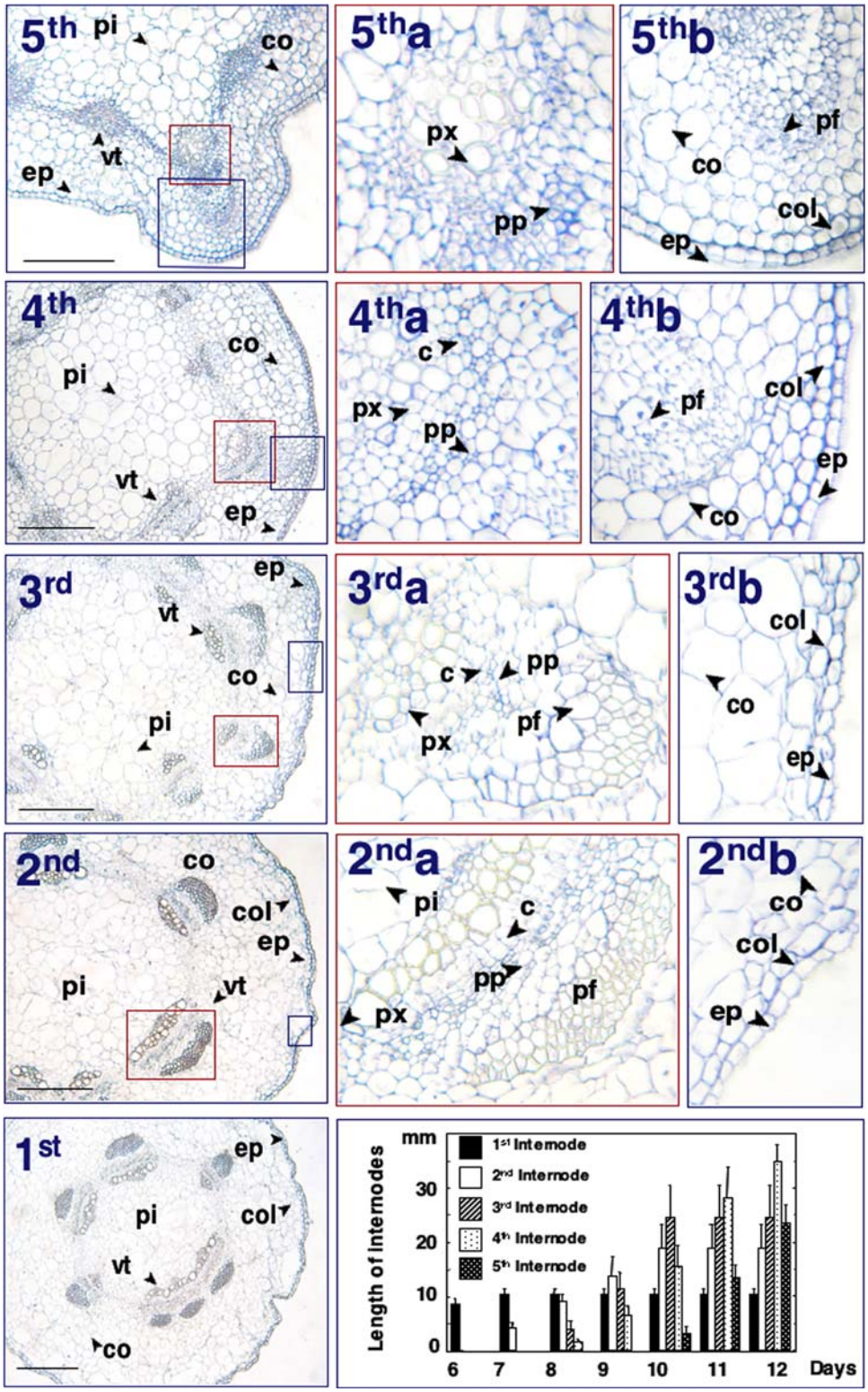

Fig. 6 Immunolocalization of ßV-Gal protein in internodes from 11-day-old *Cicer arietinum* stems. Internodes numbered 1st to 5th from base to apex were treated with anti-ßV-Gal antibodies. Cross-sections were taken from 5th, 4th, 3rd, 2nd, and 1st internodes; higher magnification of red boxed regions of vascular tissue are represented in 5tha, 4tha, 3rda, and 2nda; higher magnification of the blue boxed regions of epidermal zone are in 5thb, 4thb, 3rdb, and 2ndb. The graph represents the internode lengths of *C. arietinum* plants grown from the 6th to the 12th day in vermiculite at 25°C and 80% relative humidity. c, procambium; co, cortex; col, collenchyma; ep, epidermis; pf, phloem fibers; pi, pith; pp, primary phloem; px, primary xylem; vt, vascular tissue. Arrowheads indicate the labeled tissue. Scale bars = 100 µm

protein to meristematic tissue. Few examples of ß-galactosidases related to rapid-division stages have been described. Barnavon and others (2000) reported a ß-galactosidase in *Vitis vinifera* whose transcripts accumulate in the green stage of fruit development, characterized by a high cell division rate stage followed by marked cell expansion. Also, high levels of ß-galactosidase activity have been observed during the mitotic stage in pollen development in *Brassica campestris* (Singh and Knox 1985). No role for the release of galactose during this

process has been established. However, several studies have shown a differential cell wall composition between meristematic and elongating cells, including changes in pectic polysaccharides, among other cell wall components (Willats and others 1999, 2000; Serpe and others 2002). The location of ßV-Gal in this zone may reflect its involvement in cell wall modifications during the final stages of cell proliferation, leading to the establishment of an expanding cell wall.

The location of ßV-Gal in the cell wall of the developing lateral roots in pericycle cells (Fig. 5b1) also supports the involvement of ßV-Gal in this process. The pericycle, a tissue that retains its meristematic characteristics, is the origin of the lateral root primordia and is a clear example of an active cell division process followed by cell elongation.

During seedling and plant growth, the highest levels of ßV-Gal protein were detected in the youngest and most actively growing epicotyls (Fig. 4a) and in the 5th internode, the most apical one (Fig. 6). Thus, protein levels were in agreement with *CanBGal-5* transcript levels (Esteban and others 2005) and pointed to a relationship between ßV-Gal and the events occurring in the cell wall during the early stages of development, exactly the opposite of ßIV-Gal proteins (Martín and others 2008). Many authors have studied the role of ß-galactosidases during growth (Masuda and others 1985; Dopico and others 1990b; Konno and Tsumuki 1993), and these proteins have been proposed as key enzymes in cell wall loosening that occurs prior to rapid cell elongation, as reported by O'Donoghue and others (1998) in asparagus stems or by Rogers and others (2001) and Hrubá and others (2005) in tobacco pollen tubes. The immunolocation studies also supported the notion that ßV-Gal could be related to cell elongation. The labeling in cortical cells obtained when using anti-βV-Gal antibodies in 4-day-old-epicotyls (Fig. 4), as well as in 4-day-old radicles (Fig. 5), was more intense in the apical zones than in the basal zones, in agreement with the different growth rates in these epicotyl and radicles zones. Similar results were obtained previously when the location of an elongation-related chickpea XTH was analyzed (Jiménez and others 2006), suggesting that ßV-Gal could act together with other cell wall-modifying enzymes to promote cell wall loosening and allow cell elongation. A decrease in anti-ßV-Gal labeling intensity from the apical and most actively growing zones toward basal zones was also seen in the epidermal cells of 4-day-old radicles (Fig. 5). This could reflect the involvement of this ß-galactosidase in cell wall modifications leading to elongation in this tissue. A differential distribution of several cell wall components such as galactans or arabinogalactan proteins (both potential ß-galactosidase substrates) has been observed in different species such as *A. thaliana* (Willats and others 2001; McCartney and others 2003) and flax (Vicré and others 1998).

In vascular tissues, both in phloem and xylem cells, ßV-Gal was always associated with the developing tracheary elements close to the cambial zone (Figs. 4, 5, and 6) and was absent from those that had completely differentiated and had ceased their elongation. In phloem cells, the labeling was also less pronounced in the basal sections and was almost absent from the basal region of 8-day-old epicotyls (Fig. 4d), where ßV-Gal was located mainly in companion cells, characterized by a later differentiation during development than vessels (Esau and Charvat 1978). The same pattern was also seen in cell walls of phloem fibers in epicotyls (Fig. 4). ßV-Gal was present only in the youngest phloem fibers (those next to the phloem) and was absent in the basal sections of 8-day-old epicotyls. During flax stem growth, fibers develop in two main steps—cell elongation and thickening of secondary walls—and fiber cell elongation is restricted to the top of the stem (Morvan and others 2003), which again suggest the relationship between ßV-Gal and the initial stages of the elongation process.

The presence of ßV-Gal in radial expanding procambial cells in 8-day-old epicotyls (Fig. 4d1) again supports its role in the cell wall changes during the late stages of cell proliferation and the onset of cell elongation. The occurrence of cell wall polysaccharides shows a differential spatial distribution in procambial cells when compared with other nonparenchymatic tissues, suggesting that structural variations of these polymers would be involved in the modulation of cell wall properties. Thus, Guillemin and others (2005) reported a reduction in galactan side chains in cambial cell walls in sugar beet root. Ermel and others (2000) have also reported a reduction in pectic polysaccharides during the end of cambial activity in aspen.

In summary, our data allow us to propose that ßV-Gal could act in a coordinated way with other degrading enzymes during the early stages of development, with high cell division and elongation rates, and that its action could give rise to a modified cell wall, thus permitting cell elongation and differentiation.

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