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Original article

Brassinolides and IAA induce the transcription of four α-expansin genes related to development in *Cicer arietinum* ☆

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Abstract

Four different cDNAs encoding α-expansins have been identified in *Cicer arietinum* (*Ca-EXPA1*, *Ca-EXPA2*, *Ca-EXPA3* and *Ca-EXPA4*). The shared amino acid sequence similarity among the four α-expansin proteins ranged from 67 to 89%. All of them display common characteristics such as molecular mass (around 24 kDa), amino acid numbers, and also the presence of a signal peptide. The transcription pattern of chickpea α-expansin genes in seedlings and plants suggests a specific role for each of the four α-expansins in different phases of development or in different plant organs. High levels of *Ca-EXPA2* transcripts coincide with maximum epicotyl and stem growth, indicating an important involvement of this particular α-expansin in elongating tissues. *Ca-EXPA3* would be related to radicle development, while *Ca-EXPA4* seems to be involved in pod development. A considerable increase in the level of all *Ca-EXPA* transcripts accompanied the indole acetic acid (IAA) plus brassinolide (BR)-induced elongation of excised epicotyl segments. This IAA + BR induction was seen even for the chickpea expansin genes whose transcription was not affected by IAA or BR alone.

Keywords: Brassinolides; Cell wall; *Cicer arietinum*; Epicotyl; Expansin; Growth

1. Introduction

Plant cell growth is accompanied by the modification and expansion of cell walls, which are composed of cellulose microfibrils tethered together with hemicelluloses and embedded in a matrix of pectins and structural proteins [2].

A class of proteins called expansins promote cell wall loosening in vitro and catalyze wall extension and stress relaxation in a pH-dependent manner [31]. Biochemical and biophysical data indicate that expansins bind to the surface of cellulose microfibrils, thereby disrupting the hydrogen bonds formed with xyloglucan molecules and allowing the cell wall to extend (for a review, see [12,15,27]).

Expansin genes have been identified from many species and are highly conserved in gymnosperms and, among the angiosperms, in both monocots and dicots [11,21,26]. Their pattern of expression indicates that they are closely related to cell growth and tissue differentiation (for a review, see [5]). The occurrence of multigene families of expansins suggests that different expansins play unique developmental or tissue-specific roles [6,7,10,19,44]. It has been suggested that most of the expansin genes characterized could be involved in cell expansion during tissue growth [1,8,16,21,38]. Expansins are also expressed in tissues where cell wall disassembly is occurring instead of cell growth [4,9,40].

The regulation of expansin activity by plant hormones has been well documented. In this sense, auxin-induced acid growth response relies on the activity of expansins for wall loosening and cell elongation [28,31,32]. In addition, plant hormones regulate α-expansin gene expression. Examples are the tomato α-expansins *Le-EXPA1* and *Le-EXPA2*; the

Abbreviations: ABA, abscisic acid; BR, brassinolides; IAA, indole acetic acid; GA, gibberellin.

☆ The nucleotide sequences reported in this paper have been submitted to EMBL/GenBank database under accession numbers: *Ca-EXPA1*, AJ291816; *Ca-EXPA2*, AJ291817; *Ca-EXPA3*, AJ489608; *Ca-EXPA4*, AJ489609.

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deepwater rice α-expansin *Os-EXPA4*, and the soybean (*Glycine max*) ß-expansin *Cim1*, which accumulate in response to ethylene, auxin, gibberellin and cytokinin, respectively [8,14,39]. The regulation of expansin genes by brassinolides (BR) has been less studied, although some α-expansin genes, such as *At-EXPA5* and *At-EXPA8* from *Arabidopsis* have been recently referred as BR-inducible genes using DNA microarray analysis [17,35].

The present paper reports the isolation and identification of four complete cDNAs—*Ca-EXPA1*, *Ca-EXPA2*, *Ca-EXPA-3* and *Ca-EXPA4*—encoding four different α-expansin proteins from chickpea. Their transcription was investigated during epicotyl and root development as well as in several plant tissues. In order to elucidate the relationship between the *Ca-EXPA* genes and hormone-regulated growth, their transcription was studied in epicotyl sections under treatment with gibberellin (GA), abscisic acid (ABA), indole acetic acid (IAA) and BR.

2. Results

2.1. Chickpea α-expansin clones

Four clones, named *Ca-EXPA1*, *Ca-EXPA2*, *Ca-EXPA3* and *Ca-EXPA4*, with high sequence similarity to several α-expansins were found in a chickpea cDNA library constructed with RNA from 5-day-old chickpea epicotyls and screened as described in Section 4.

All *Ca-EXPAs* were full-length clones with sizes of 1291, 1034, 1497 and 1270 bp, respectively, the non-coding regions being those that mainly contributed to such differences. The proteins deduced from the chickpea *Ca-EXPA* clones, named α-expansin 1, 2, 3 and 4 (EXPA1, EXPA2, EXPA3 and EXPA4), had a significant level of shared amino acid sequence similarity with one another and also with other published plant α-expansins. Fig. 1 shows the alignment of the four amino acid sequences, where the high degree of similarity can be observed. The four chickpea α-expansin proteins had the characteristic expansin motifs; namely, conserved Cys residues in the N-terminal region of the protein (marked with an asterisk in the figure), conserved sequences (marked with a line in the figure) including a putative catalytic domain with the His-Phe-Asp (HFD) motif in the central portion of the protein, and conserved Trp residues in the putative cellulose-binding domain in the C-terminal region (marked by "+" in the figure). EXPA3 and EXPA4 are the closest at amino acid sequence level (89% similarity). EXPA1 and EXPA2 show a lower degree of similarity: about 67% between each other and around 70–75% with respect to the other chickpea α-expansins. The most differentiated region was found at the N-terminal region, where the predicted N-terminal hydrophobic signal peptide is located, as determined by Signal P [36]. After release of the signal peptide (marked by an arrow in Fig. 1), the four mature proteins were

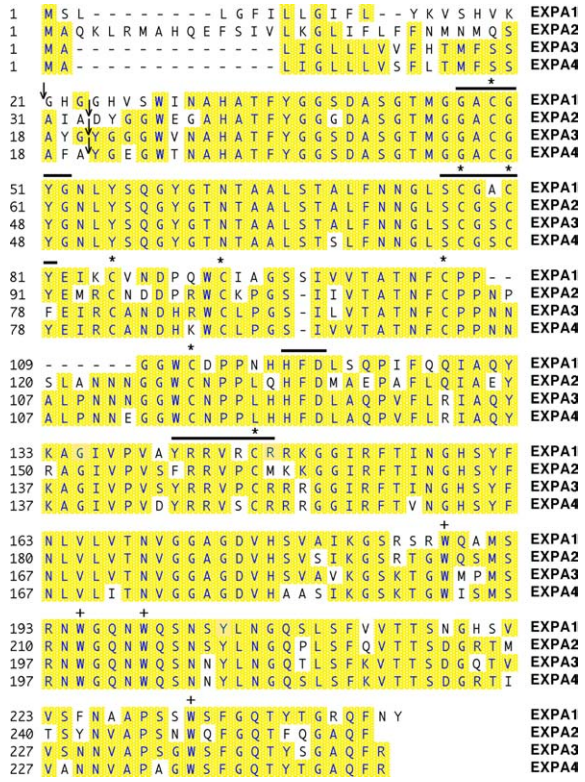

Fig. 1. Alignment of the four deduced α-expansin amino acid sequences of the *Ca-EXPA* clones. Sequences were aligned using the Clustal method in the MegAlign program, part of the Lasergene sequence analysis software program available from DNASTAR (Madison, WI). Shading indicates amino acid identity with respect to the majority. An arrow indicates the putative signal sequence cleavage site. Conserved Cys residues are marked by an asterisk. Conserved sequences are marked by a line. Conserved Trp residues are marked by "+".

quite similar in their molecular mass (24.14–24.51 kDa) and in their amino acids number (224–228). A basic predicted isoelectric point (9.13, 7.89, 9.15 and 8.80 for EXPA1, EXPA2, EXPA3 and EXPA4 respectively) was another common characteristic of the four chickpea α-expansins.

Chickpea EXPA2, EXPA3 and EXPA4 did not present any N-glycosylation site. Although the lack of N-glycosylation site is a charasteristic of most α-expansins described, EXPA1 had a putative N-glycosylation site at amino acid 202 (asn-ser-thr-leu).

2.2. Phylogenetic tree

The differences among chickpea α-expansins are clearly seen in the phylogenetic tree compiled using the four deduced proteins and other plant α-expansins based on protein sequence alignment (Fig. 2). α-Expansins appear separated in four different phylogenetic branches named A, B, C and D according to Link and Cosgrove [28]; chickpea *Ca-EXPA1*, *Ca-EXPA3* and *Ca-EXPA4* appear in group C. This clade contains α-expansin genes with heterogeneous expression patterns. Some members are transcribed in multiple tissues, such as *Le-EXPA5* [1] and *At-EXPA5* [42], while others appear to have more specialized roles, as *At-EXPA10* [12].

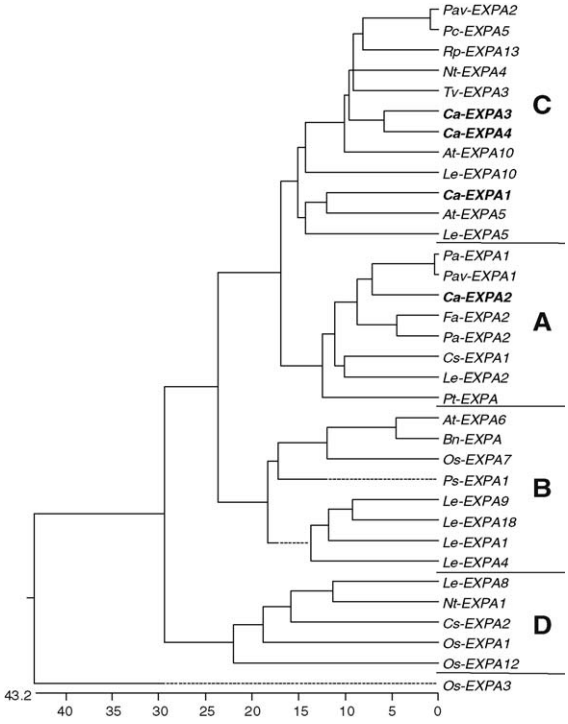

Fig. 2. Phylogenetic tree of the deduced amino acid sequences encoded by plant α-expansin genes. Alignments was made using the Clustal method. Sources of the α-expansin sequences are as follows: *At-EXPA5*, *At-EXPA6* and *At-EXPA10* from *Arabidopsis thaliana*; *Bn-EXPA* from *Brassica napus*; *Ca-EXPA1*, *Ca-EXPA2*, *Ca-EXPA3* and *Ca-EXPA4* from *C. arietinum*; *Cs-EXPA1* and *Cs-EXPA2* from *Cucumis sativus*; *Fa-EXPA2* from *Fragaria ananassa*; *Le-EXPA1*, *Le-EXPA2*, *Le-EXPA4*, *Le-EXPA5*, *Le-EXPA8*, *Le-EXPA9*, *Le-EXPA10* and *Le-EXPA18* from *L. esculentum*; *Nt-EXPA1* and *Nt-EXPA4* from *Nicotiana tabacum*; *Os-EXPA1*, *Os-EXPA3*, *Os-EXPA7* and *Os-EXPA12* from *Oryza sativa*; *Pt-EXPA* from *Pinus taeda*; *Ps-EXPA1* from *Pisum sativum*; *Pa-EXPA1* and *Pa-EXPA2* from *Prunus armeniaca*; *Pav-EXPA1* and *Pav-EXPA2* from *Prunus avium*; *Pc-EXPA5* from *Prunus cerasus*; *Rp-EXPA13* from *R. palustris* and *Tv-EXPA3* from *T. versicolor*. The branches were named A, B, C, and D according to Link and Cosgrove [28].

Ca-EXPA2 appears in branch A, together with many α-expansin genes that are expressed in rapidly growing tissues (Fig. 2). Thus, *Cs-EXPA1* and *Le-EXPA2* are expressed in elongating hypocotyl in cucumber or tomato. Other α-expansins within the A clade are expressed in expanding fruit tissue in species such as cherry, apricot, strawberry and tomato.

2.3. Tissue specificity in seedlings and adult plants

In order to determine the transcription level of each chickpea expansin gene, specific probes consisting mainly of the 3′ untranslated regions were prepared for each cDNA with a view to minimizing any potential cross-hybridization, according to Southern blot analysis (data not shown). Study of expansin gene transcription in seedling organs disclosed a specific pattern of transcription for each gene (Fig. 3). *Ca-EXPA1* transcript level was very low in all seedling organs. A similar low transcription was found for *Ca-EXPA4*, although unlike *Ca-EXPA1* no transcript was detected in hooks. *Ca-EXPA2* and *Ca-EXPA3* transcript were mainly detected in epicotyls and radicles, respectively. None of the expansin genes showed transcription in cotyledons, except for a very low level of *Ca-EXPA3* mRNA. It should be noted that the level of transcription of *Ca-EXPA1* and *Ca-EXPA4* was low in seedlings as compared with transcription in adult plants, while *Ca-EXPA3* mRNA levels in radicles reached the highest value among all the plant organs studied.

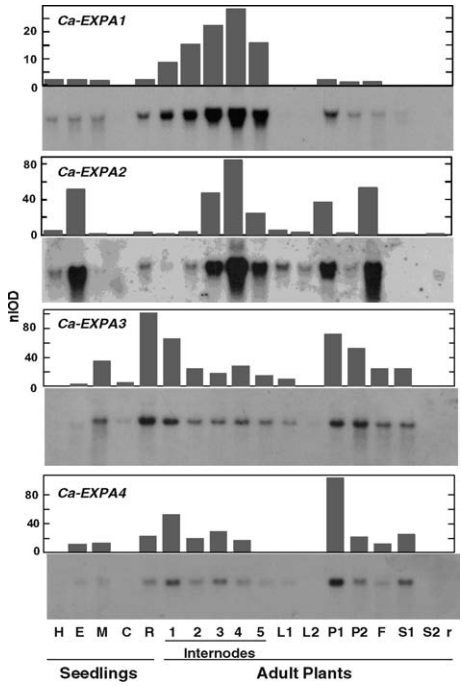

Fig. 3. Northern analysis of *Ca-EXPAs* mRNA transcription levels in different parts of *C. arietinum* seedlings and adult plants. H, hook; E, epicotyl; M, mesocotyl; C, cotyledon; R, radicle. Internodes are numbered 1–5 from the basal to the apical one. L1, young leaves; L2, mature leaves; P1, immature pods; P2, mature pods; F, flowers; S1, immature seed; S2, dry mature seed; r, adult roots. Signals were quantified and normalized according to 18S rRNA hybridization. Integrated optical density (IOD).

Ca-EXPA1 and *2* transcription along the stem internodes from 11-day-old plants showed a correlation with the internode elongation rate, the highest mRNA levels being observed at the 4th internode, just below the apical one, thereafter decreasing towards the basal one (Fig. 3). By contrast, the mRNA levels of *Ca-EXPA3* and *4* followed the opposite pattern, decreasing from the basal internode (1st) to the apical young one (5th).

The transcription pattern of *Ca-EXPA* clones in chickpea plants was broad. *Ca-EXPA1* did not show significant transcription in any part except the internodes. The other three expansin genes showed high transcript levels in the reproductive organs, the most significant being the flowers for *Ca-EXPA2* and pods for *Ca-EXPA3* and *4*. The immature pod was the organ with the highest mRNA levels for *Ca-EXPA4*. The higher transcription in immature pods compared with mature ones should be noted. A very low signal or even no signal at all was detected for all the genes studied in other plant organs, including the leaves, dry seeds and roots. *Ca-EXPA3*

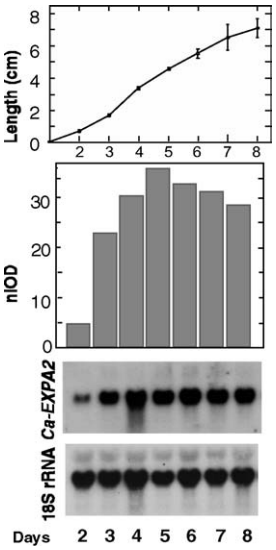

Fig. 4. Northern analysis of *Ca-EXPA2* mRNA transcription levels in epicotyls from seedlings grown in water. Numbers refer to days after sowing. Signals were quantified and normalized according to 18S rRNA hybridization. IOD.

and *4*, unlike *Ca-EXPA1* and *2*, were expressed in immature seeds (Fig. 3).

2.4. Transcription pattern during epicotyl growth

Since *Ca-EXPA2* transcript level was high in epicotyls, the temporal transcription pattern through epicotyl growth was examined. RNA was extracted from epicotyls ranging in age from 2-day-old (when epicotyl emergence starts) to 8-day-old (when the rate of growth starts to fall) (Fig. 4). The *Ca-EXPA2* transcript level increased with the epicotyl elongation rate, being lower in 2-day-old epicotyls and reaching the highest value in 5-day-old epicotyl (Fig. 4). The *Ca-EXPA1*, *Ca-EXPA3* and *Ca-EXPA4* mRNAs levels were very low throughout epicotyl growth and no variations were observed with epicotyl age (data not shown).

2.5. Transcription pattern during radicle growth

Because of the high *Ca-EXPA3* transcription found in 4-day-old chickpea roots, we decided to evaluate the changes in *Ca-EXPA* mRNA levels in radicles at different developmental stages in order to determine whether one of these expansins might be involved in radicle elongation. Total RNAs were extracted from radicles of 2, 3, 4 and 5-day-old seedlings and were subjected to RNA Northern blot analysis. Older roots were not studied since secondary roots were initiated on day 5 after germination. As shown in Fig. 5, *Ca-EXPA3* mRNAs were detected at each developmental stage studied, with no significant changes along radicle growth. *Ca-EXPA1* and *Ca-EXPA4* transcript levels, although low, increased with radicle age; contrariwise, *Ca-EXPA2* transcripts decreased in that period. Owing to the low values found in *Ca-EXPA1*, *2* and *4* transcription in radicles, mRNA

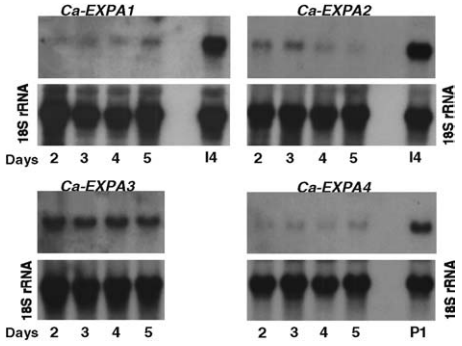

Fig. 5. Northern analysis of *Ca-EXPA* mRNA transcription levels in radicles from seedlings grown in water. Numbers refer to days after sowing. RNA control from the fourth internode (4I) or immature pod (P1) were used to check the correct hybridization for *Ca-EXPA1* and *2*, and for *Ca-EXPA4*, respectively. Blots were hybridized with 18S rRNA as a loading control.

controls from the fourth internode (for *Ca-EXPA1* and *2*) or immature pods (for *Ca-EXPA4*) were used to check that efficient hybridization had taken place.

2.6. Effect of hormone-induced elongation on α-expansin gene transcription

To determine the relationship between growth-regulating hormones and chickpea α-expansin transcript accumulation, excised subapical epicotyl segments (10 mm long) were allowed to elongate in vitro, in either the presence or absence of IAA, BR, GA_3 and ABA. The growth of excised chickpea epicotyl segments was seen to be significantly stimulated by exogenous application of IAA and BR. Growth promotion reached 11.7% with IAA after 8 h of treatment. BR induced the elongation of epicotyl segments by itself (8.8%) and also enhanced IAA-induced elongation (17.9%), although its effect on elongation was more pronounced after 8 h of treatment. Neither GA_3 nor ABA caused changes in the rate of chickpea epicotyl elongation [34].

IAA treatment of epicotyl segments elicited a significant increase of *Ca-EXPA2* and *Ca-EXPA4* transcript levels at 4 and 8 h of treatment (Fig. 6). Little or no effect was detected for *Ca-EXPA1* and *Ca-EXPA3*, except for *Ca-EXPA3* at 8 h. Neither GA_3 nor ABA affected the levels of chickpea α-expansin transcripts. We also investigated the effect of BR and the combination of IAA and BR. Like IAA, treatment with BR alone induced the transcription of the corresponding *Ca-EXPA2* and *Ca-EXPA4* genes but had little effect on *Ca-EXPA1* and *Ca-EXPA3* gene transcription. Despite this, it is important to note that a considerable increase in the level of all *Ca-EXPA* transcripts accompanied the IAA plus BR-induced elongation of excised epicotyl segments (after both 4 and 8 h of treatment). This induction was observed even for chickpea α-expansin genes whose expression was not affected by IAA or BR alone (Fig. 6).

3. Discussion

Screening of a *Cicer arietinum* cDNA library revealed the presence of at least four different cDNAs encoding

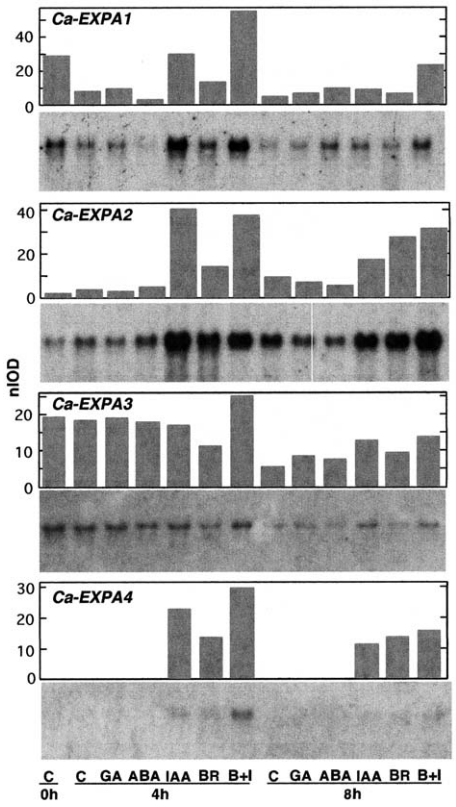

Fig. 6. Northern analysis of *Ca-EXPA* mRNA transcription levels in subapical epicotyl sections of *C. arietinum* after 4 and 8 h of treatment with plant growth regulators, as described in Section 4. C, untreated sections used as control. B + I, Brassinolides plus IAA. Signals were quantified and normalized according to 18S rRNA hybridization. IOD.

α-expansins: *Ca-EXPA1*, *Ca-EXPA2*, *Ca-EXPA3* and *Ca-EXPA4*. The presence of multigene families of expansins has been reported in several plant species. The large number of expansins poses intriguing questions regarding the function of these proteins and the significance of their redundancy, and determining the pattern and control of each expansin gene expression is the first step in elucidating the function of individual expansins. Several studies on α-expansin gene expression have been published recently; the results suggest that expansin genes are expressed differently, depending on the tissue used and the stimuli imposed, and that they are mainly associated with growth. Thus, Wrobel and Yoder [43] reported the differential transcription of three α-expansins in *Triphysaria versicolor*. Likewise, several α-expansins have been identified in cotton [18] and in tomato, strawberry and pear fruits, with a different pattern of transcription during fruit growth and ripening [1,19,20].

The four α-expansins analyzed in *C. arietinum* show common characteristics. In fact, most plant expansins are very homogeneous as regards both length and weight [44]. However, it should be noted that unlike most α-expansins, which are not glycosylated proteins, EXPA1 from *C. arietinum* have a putative N-glycosylation site. EXPA-3 from *Lycopersicon esculentum* has also been reported to have an N-glycosylation site [1]. To date, we do not know whether they are glycosylated, and the role of glycosylation, if indeed it occurs, is not clear as regards the function of α-expansins [13].

From our results in transcription studies, it seems clear that the corresponding chickpea EXPA2 gene is the most closely related to epicotyl growth, as seen from its high level of transcript in this organ (Fig. 3). Additionally, a relationship between *Ca-EXPA2* mRNA level and epicotyl growth was observed (Fig. 4). In agreement with the putative function of EXPA2 in epicotyl growth, it should be noted that it is the only chickpea α-expansin that is included within phylogenetic group B (Fig. 2), according to the classification of Link and Cosgrove [28]. This group contains α-expansins related to fast-growing tissues, such as *Cs-EXPA1*, *Le-EXPA2* and *Pt-EXPA*, which are expressed in elongating hypocotyls of cucumber, tomato and pine, respectively [3,21,42].

The transcription pattern of chickpea α-expansin genes in seedlings and plants could indicate a specific role for each of the four α-expansins in different phases of development or in different plant organs. Thus, in seedlings these α-expansins would be related to elongating organs but not to meristematic or reserve tissues, and while *Ca-EXPA2* shows the highest transcription in epicotyls, *Ca-EXPA3* is highly expressed in radicles (Fig. 3). In plants, an important role of EXPA1 in internode growth could be speculated since no significant hybridization was detected in any other organ studied, either from seedlings or from adult plants (Fig. 3). Finally, stress should be placed on the strong transcription of some of the chickpea *Ca-EXPA* in reproductive organs (Fig. 3). The high level of *Ca-EXPA4* transcription in immature pods, together with the difference in transcript levels between mature (P2) and immature pods (P1), indicates the possible involvement of EXPA4 in pod development.

Although it has long been speculated that cell elongation in roots is caused by expansins, as far as we know there is only one report addressing a root-specific α-expansin gene in soybean *Gm-EXPA1* [25] that could be responsible for root elongation. In chickpea, although no α-expansin transcript was detected in adult roots, *Ca-EXPA3* showed a high level of transcripts in radicles (Fig. 3). Also, EXPA3 shows a high degree of similarity with expansins involved in root growth such as EXPA13 from *Rumex palustris* (Q8L5S1, 83.6% similarity) or EXPA3 from *T. versicolor* (Q9M517, 83.5% similarity) [43]. No variations were detected in *Ca-EXPA3* transcription along radicle growth (Fig. 5), indicating that this α-expansin gene may be necessary for root development, although not directly related to root elongation.

An interesting result was the response of chickpea α-expansin genes to treatment with growth-regulating hormones. The effect of hormones on expansins have been studied in different plant materials [28,31,32], and a relationship of some hormones with the induction of expansin genes has been reported [8,14,39]. In chickpea, BR induces epicotyl subapical segment elongation and also enhances auxin-induced elongation [33], as has been reported for other

materials such as azuki bean epicotyls [29] or cucumber hypocotyls [22]. BR growth induction in chickpea was well correlated with the induction of *Ca-EXPA* transcription, this induction being higher when BR and IAA were present simultaneously (Fig. 6), suggesting that expansin genes are involved in BR-induced elongation. This effect was common to all four chickpea α-expansins cDNAs, even though some of them had low levels of transcription in epicotyl sections. Although some α-expansin genes, such as *At-EXPA5* and *At-EXPA8* from *Arabidopsis* have been described as BR-inducible genes using DNA microarray analysis [17,35], there are few reports of the induction of α-expansin gene transcription by BR in relation to organ elongation. By contrast, treatment of tomato hypocotyl segments with BR, which promotes tissue elongation, causes no increase in *Le-EXPA2* mRNA levels [3]. Further studies are necessary to establish whether other α-expansin gene(s) may be related to brassinosteroid-induced elongation.

Chickpea α-expansin genes were also induced by IAA in epicotyl segments, the most significant effect being observed in the case of *Ca-EXPA2*; this supports the involvement of chickpea EXPA2 in epicotyl growth. The induction of expansin expression by IAA seems to be common for most expansins, such as in tomato [3] or cucumber [30]. By contrast, there is no general effect of GA_3 on plant α-expansins. Thus, the expression of specific expansin genes in rice is correlated with the induction of rapid internode elongation by giberellins [7,23,24]. However, in chickpea, GA_3 has no effect on epicotyl elongation [33] and does not affect *Ca-EXPA* transcript levels (Fig. 6), showing that probably GAs induce expansin gene expression only in systems where GAs induce elongation.

In summary, the transcription pattern of chickpea expansin genes suggests a specific role for each of the four expansins in different phases of development or in different plant organs. The accumulation of *Ca-EXPA2* mRNA indicated an important involvement of this particular expansin in epicotyl and stem elongation. *Ca-EXPA3* would be related to radicle development, while *Ca-EXPA4* seems to be involved in pod development. The considerable increase in the level of all *Ca-EXPA* transcripts that accompanied the IAA plus BR-induced elongation of excised epicotyl segments suggests regulation of chickpea expansins by these hormones.

4. Methods

4.1. Plant material

Chickpea seeds (*C. arietinum* L. cv. Castellana) 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-d. Epicotyls and radicles were collected from 2- to 8-day-old and from 2- to 5-day-old seedlings, respectively. Different plant tissues were collected for studies of gene transcription: hooks, epicotyls, mesocotyls (root–epicotyl junction zone), cotyledons and roots from 4-day-old seedlings. Stems of 11-day-old plants, grown in the light, were divided into five 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 who were fully expanded and larger than 4 mm. Immature pods (P1) were 1-cm long 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.

4.2. Plant growth regulator treatments

After 4 d of growth in water, subapical 10 mm epicotyl-sections were excised. After rinsing in distilled water for 1 h, subapical sections were immersed in a 10 mM potassium-citrate buffer (pH 6.0) in the presence of 10 μM IAA, 10 μM GA_3, 10 μM ABA or 0.2 μM BR for 4 or 8 h. Control sections were immersed in 10 mM potassium-citrate buffer (pH 6.0). Three different sets of 50 segments each were used in each treatment.

4.3. RNA and genomic DNA extraction

For RNA extraction, 5 g of frozen material was 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 sulfonic 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. [33]. Genomic DNA was isolated using the Plant DNA isolation kit (Roche, Germany) from 1 g of 14-day-old chickpea plant folioles.

4.4. Isolation of α-expansins in a chickpea cDNA library

RT-PCR and genomic DNA PCR amplification were used for the isolation of several chickpea α-expansin fragments. These fragments were used for the isolation of full-length α-expansins by screening a chickpea cDNA library. Total RNA extracted from 5-day-old etiolated epicotyl was used to synthesize first-strand cDNAs, using the Superscript II preamplification system for first-strand cDNA synthesis (Gibco BRL, USA) using oligo dT as primer, and was subsequently used as a template for PCR amplification. Genomic DNA isolated from leaves was also used as a template for PCR amplification. Degenerated PCR primers used in PCR amplification of both cDNA and genomic DNA were designed with reference to the conserved amino acid sequences of 34 expansins. The sequence of the upstream primer was 5′

TG GG(A/G/T) GG(A/G/T) GCT TGT GG(A/G) TA(C/T)G 3′ and that of the downstream primer was 5′ (C/G)(A/T) (C/T)TG CCA GTT (C/T)TG (C/G/T)CC CA 3′. The two oligonucleotides corresponded to the amino acid sequences of MGGACGYG and NWGQNWQ. The PCR reactions were subjected to 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The amplified cDNA fragments were cloned into pGEM-T easy vector (pGEM Vector System from Promega) following the manufacturer's instructions. The fragments were sequenced as described below. Three different α-expansin fragments were obtained: Expan 8 (from genomic DNA PCR amplification) and Expan 30 and Expan 36 (from RT-PCR of 5-day-old epicotyl total RNA). The cDNA library used was constructed in the Uni-ZAP XR vector from poly $(A)^+$ RNA extracted from epicotyls of 5-day-old etiolated chickpea seedlings grown in water as described by Muñoz et al. [33]. A mixture of Expan 8, Expan 30 and Expan 36 fragments was used as probe for the screening of this cDNA library. The probe was labeled with ^{32}P using the Random primed kit (Roche, Germany).

4.5. DNA sequencing 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 [41]. 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 [37]. Alignment and the phylogenetic tree of the four chickpea and other plant α-expansins were obtained 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 [36] and putative N-glycosylation was analyzed at the Prosite web page (http://www.expasy.ch/prosite).

4.6. Northern analysis

Northern experiments were performed as described in Muñoz et al. [33]. cDNA fragments from the 3′ non-coding end of the clones were used as specific probes to minimize any potential cross-hybridization among α-expansin mRNAs. cDNA specific probes were amplified by PCR in a GeneAmp PCR system 9700 thermocycler (PE Biosystems), cleaned by the High Pure PCR product purification kit (Roche, Germany), and labeled with ^{32}P using the Random primed kit (Roche, Germany). Total RNA (10 μg per lane) was electrophoresed, transferred onto Hybond N nylon membranes (Amersham Pharmacia Biotech, UK), and hybridized using the radiolabeled 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 4.6K 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.

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References

[1] D.A. Brummell, M.H. Harpster, P. Dunsmuir, Differential expression of expansin gene family members during growth and ripening of tomato fruit, Plant Mol. Biol. 39 (1999) 161–169.

[2] N.C. Carpita, D.M. Gibeaut, Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth, Plant J. 3 (1993) 1–30.

[3] C. Catalá, J.K.C. Rose, A.B. Bennett, Auxin-regulated genes encoding cell wall-modifying proteins are expressed during early tomato fruit growth, Plant Physiol. 122 (2000) 527–534.

[4] F. Chen, K.J. Bradford, Expression of an expansin is associated with endosperm weakening during tomato seed germination, Plant Physiol. 124 (2000) 1265–1274.

[5] H.T. Cho, Modulation of plant organ growth by expansins, the cell wall loosening proteins, AgBiotechNet 3, ABN 069, 2001 (http://www.agbiotechnet.com/reviews/availabe.asp).

[6] H.T. Cho, H. Kende, Expansins in deepwater rice internodes, Plant Physiol. 113 (1997) 1137–1143.

[7] H.T. Cho, H. Kende, Expansins and internodal growth of deepwater rice, Plant Physiol. 113 (1997) 1145–1151.

[8] H.T. Cho, H. Kende, Expression of expansin genes is correlated with growth in deepwater rice, Plant Cell 9 (1997) 1661–1671.

[9] P.M. Civello, A.L.T. Powell, A. Sabehat, A.B. Bennett, An expansin gene expressed in ripening strawberry fruit, Plant. Physiol. 121 (1999) 1273–1279.

[10] D.J. Cosgrove, Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement, Plant Cell 9 (1997) 1031–1041.

[11] D.J. Cosgrove, Cell wall loosening by expansins, Plant Physiol. 118 (1998) 333–339.

[12] D.J. Cosgrove, Expansive growth of plant cell walls, Plant Physiol. Biochem. 38 (2000) 109–124.

[13] D.J. Cosgrove, Z.C. Li, H.-T. Cho, S. Hoffmann-Benning, R.C. Moore, D. Blecker, The growing world of expansins, Plant Cell Physiol. 43 (2002) 1436–1444.

[14] D.N. Crowell, A.T. Kadlecek, R.M. Amasino, Cytokinin-induced mRNAs in cultured soybean cells, Proc. Natl. Acad. Sci. USA 87 (1990) 8815–8819.

[15] C.P. Darley, A.M. Forrester, S.J. McQueen-Mason, The molecular basis of plant cell wall extension, Plant Mol. Biol. 47 (2001) 179–195.

[16] A.J. Fleming, S.J. McQueen-Mason, T. Mandel, C. Kuhlemeier, Induction of leaf primordial by the cell wall protein expansin, Science 276 (1997) 1415–1418.

[17] H. Goda, Y. Shimada, T. Asami, S. Fujioka, S. Yoshida, Microarray analysis of brassinosteroid-regulated genes in Arabidopsis, Plant Physiol. 130 (2002) 1319–1334.

[18] S.E. Harmer, S.J. Orford, J.N. Timmis, Characterization of six α-expansin genes in *Gossypium hirsutum* (upland cotton), Mol. Genet. Gen. 268 (2002) 1–9.

[19] E.P. Harrison, S.J. McQueen-Mason, K. Manning, Expression of six expansin genes in relation to extension activity in developing strawberry fruit, J. Exp. Bot. 52 (2001) 1437–1446.

[20] K. Hiwasa, J.K.C. Rose, R. Nakano, A. Inaba, Y. Kubo, Differential expression of seven α-expansin genes during growth and ripening of pear fruit, Physiol. Plant 117 (2003) 564–572.

[21] K.W. Hutchinson, P.B. Singer, C. Díaz-Sala, M.S. Greenwood, Expansins are conserved in conifers and expressed in response to exogenous auxin, Plant Physiol. 120 (1999) 827–832.

[22] M. Katsumi, Interaction of a brassinosteoid with IAA and GA_3 in the elongation of cucumber hypocotyl sections, Plant Cell Physiol. 26 (1985) 615–625.

[23] Y. Lee, H. Kende, Expression of ß-expansins is correlated with internodal elongation in deepwater rice, Plant Physiol. 127 (2001) 645–654.

[24] Y. Lee, H. Kende, Expression of α-expansin and expansin-like genes in deepwater rice, Plant Physiol. 130 (2002) 1396–1405.

[25] D.K. Lee, J.H. Ahn, S.K. Song, Y.D. Choi, J.S. Lee, Expression of an expansin gene is correlated with root elongation in soybean, Plant Physiol. 131 (2003) 985–997.

[26] Y. Li, C.P. Darley, V. Ongaro, A. Fleming, O. Schipper, S.L. Baldauf, S.J. McQueen-Mason, Plant expansins are a complex multigene family with an ancient evolutionary origin, Plant Physiol. 128 (2002) 854–864.

[27] Y. Li, L. Jones, S.J. McQueen-Mason, Expansins and cell growth, Curr. Opin, Plant Biol. 6 (2003) 603–610.

[28] B.M. Link, D.J. Cosgrove, Acid-growth response and α-expansins in suspension cultures of bright yellow 2 tobacco, Plant Physiol. 118 (1998) 907–916.

[29] K. Mayumi, H. Shibaoka, A possible double role for brassinolide in the reorientation of cortical microtubules in the epidermal cells of azuki bean epicotyls, Plant Cell Physiol. 36 (1995) 173–181.

[30] S.J. McQueen-Mason, D.J. Cosgrove, Expansin mode of action on cell walls: analysis of wall hydrolysis, stress relaxation, and binding, Plant Physiol. 107 (1995) 87–100.

[31] S.J. McQueen-Mason, D.M. Durachko, D.J. Cosgrove, Two endogenous proteins that induce cell wall expansion in plants, Plant Cell 4 (1992) 1425–1433.

[32] S.J. McQueen-Mason, S.C. Fry, D.M. Durachko, D.J. Cosgrove, The relationship between xyloglucan endotransglycoxylase and in vitro cell wall extension in cucumber hypocotyls, Planta 190 (1993) 327–331.

[33] F.J. Muñoz, B. Dopico, E. Labrador, Two growth-related organ-specific cDNAs from *Cicer arietinum* epicotyls, Plant Mol. Biol. 35 (1997) 433–442.

[34] F.J. Muñoz, E. Labrador, B. Dopico, Brassinolides promote the expression of a new *Cicer arietinum* ß-tubulin gene involved in the epicotyl elongation, Plant Mol. Biol. 37 (1998) 807–817.

[35] C. Müssig, S. Fischer, T. Altmann, Brassinosteroid-regulated gene expression, Plant Physiol. 129 (2002) 1241–1251.

[36] H. Nielsen, J. Engelbrecht, S. Brunak, G. Von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of cleavage sites, Protein. Eng. 10 (1997) 1–6.

[37] W.R. Pearson, D.J. Lipman, Improved tools for biological sequence analysis, Proc. Natl. Acad. Sci. USA 85 (1988) 2444–2448.

[38] D. Reinhardt, F. Wittwer, T. Mandel, C. Kuhlemeier, Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem, Plant Cell 10 (1998) 1427–1437.

[39] J.K.C. Rose, A.B. Bennett, Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening, Trends Plant Sci. 4 (1999) 176–183.

[40] J.K.C. Rose, H.H. Lee, A.B. Bennett, Expression of a divergent expansin gene is fruit-specific and ripening-regulated, Proc. Natl. Acad. Sci. USA 94 (1997) 5955–5960.

[41] J. Sambrook, E.F. Fritsch, T. Maniatis, A Laboratory Manual, Molecular CloningCold Spring Harbor Lab Press, New York, USA, 1989.

[42] T.Y. Shcherban, J. Shi, D.M. Durachko, M.J. Guiltinan, S.J. McQueen-Mason, M. Shieh, D.J. Cosgrove, Molecular cloning and sequence analysis of expansins: a highly conserved, multigene family of proteins that mediate cell wall extension in plants, Proc. Natl. Acad. Sci. USA 92 (1995) 9245–9249.

[43] R.L. Wrobel, J.I. Yoder, Differential RNA expression of α-expansin gene family members in the parasitic angiosperm *Triphysaria versicolor* (Scrophulariaceae), Gene 266 (2001) 85–93.

[44] Y. Wu, R.B. Meeley, D.J. Cosgrove, Analysis of expression of the α-expansin and α-expansin gene families in maize, Plant. Physiol. 126 (2001) 222–232.