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*Emerging issues*

# Domains of expansin gene expression define growth regions in the shoot apex of tomato

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## Abstract

Expansins are members of a multigene family of extracellular proteins, which increase cell wall extensibility *in vitro* and thus are thought to be involved in cell expansion. The major significance of the presence of this large gene family may be that distinctly expressed genes can independently regulate cell expansion in place and time. Here we report on *LeExp9*, a new expansin gene from tomato, and compare its expression in the shoot tip with that of *LeExp2* and *LeExp18*. *LeExp18* gene is expressed in very young tissues of the tomato shoot apex and the transcript levels are upregulated in the incipient primordium. *LeExp2* mRNA accumulated in more mature tissues and transcript levels correlated with cell elongation in the elongation zone. *In situ* hybridization experiments showed a uniform distribution of *LeExp9* mRNA in submeristematic tissues. When gibberellin-deficient mutant tomatoes that lacked elongation of the internodes were treated with gibberellin, the phenotypic rescue was correlated with an increase in *LeExp9* and *LeExp2*, but not *LeExp18* levels. We propose that the three expansins define three distinct growing zones in the shoot tip. In the meristem proper, gibberellin-independent *LeExp18* mediates the cell expansion that accompanies cell division. In the submeristematic zone, *LeExp9* mediates cell expansion at a time that cell division comes to a halt. *LeExp9* expression requires gibberellin but the hormone is not normally limiting. Finally, *LeExp2* mediates cell elongation in young stem tissue. *LeExp2* expression is limited by the available gibberellin. These data suggest that regulation of cell wall extensibility is controlled, at least in part, by differential regulation of expansin genes.

*Abbreviations*: GA, gibberellin; $GA_3$ gibberellic acid

## Introduction

Growth, mediated by cell division and cell expansion, occurs in the meristem proper, in the submeristematic region, as well as in elongating organs, such as stems and expanding leaves. The shoot apical meristem can be subdivided both in layers and in zones (Steeves and Sussex, 1989). In the layer model, L1, the outermost layer, gives rise to the epidermis, while L2 and L3 produce the internal tissues. Anticlinal cell division in L1 and L2 leads to a structure of well-defined cell layers, the tunica. The internal cells of L3 can divide in all planes and form the so-called corpus (Satina *et al*., 1940). In the zonal model, radial organization of the shoot apical meristem consists of a central zone composed of relatively infrequently dividing cells and of a peripheral zone composed of cells dividing more rapidly (Steeves and Sussex, 1989). The initial cells below the apical dome constitute the rib-zone, also called the pith-rib meristem. The task of the shoot apical meristem in vegetative development is the initiation of leaves, the proliferation of cells for the stem tissue and the maintenance of itself. Since the shoot apical meristem provides the plants with new cells it can be considered as the first growing region of the plant.

The second growing region is found in the submeristematic tissues. This tissue consists of a central pith, formed by the cells of the rib zone, and the surrounding layers, generated from the peripheral zone. Growth in height is achieved by growth of internodes. The youngest internodes are located in this submeristematic zone. This very early stem growth is characterized by increase in width and only after several plastochrons rapid elongation begins (Sunderland and Brown, 1956). In the second growing zone, the transition from the shoot apical meristem, with a small shoot axis diameter, to the stem tissue, with a much larger diameter takes place, and both, cell division and cell expansion, are necessary to achieve growth (Lyndon, 1998).

The third growing zone starts when the shoot axis has reached a certain thickness. Growth of the young stem tissue is characterized by cell elongation primarily in the apical-basal dimension. The impact of cell division on growth rate is negligible in this growing zone, especially when compared with the first two zones. Environmental signals, such as light as well as developmental signals, such as hormones have an impact on the regulation of cell elongation, but it has not been solved yet, how these signals actually change growth rate. Cell expansion is thought to be controlled by the flexibility of the primary cell wall. Cell wall loosening is necessary in order to allow turgor-driven cell enlargement (Cosgrove, 1997). The walls of expanding tissues possess numerous protein activities that may contribute to a modification of cell wall mechanical properties, including endo-1,4-$\beta$-glucanase (Hayashi *et al.*, 1984), xyloglucan endotransglycosylase (Fry *et al.*, 1992), yieldins (Okamoto and Okamoto, 1995) and expansins (McQueen-Mason *et al.*, 1992).

Expansins are highly conserved, extracellular proteins that appear to mediate wall extension and relaxation during cell expansion. They are encoded by large multigene families in all plant species studied thus far (Li *et al.*, 2002). The individual genes can be expressed in different tissues and expression can be modulated by different stimuli. This suggests that regulation of cell wall extensibility could be controlled at least in part by differential regulation of expansin genes. During vegetative development, the *LeExp18* mRNA was found in the shoot apical meristem, with highest levels at the site of incipient primordium formation (Reinhardt *et al.*, 1998). *LeExp2* expression was shown to correlate with elongation growth rate in hypocotyls and during gravitropic stimulation of stem tissue (Caderas *et al.*, 2000; Català *et al.*, 2000).

Most of the expansion studies were performed in hypocotyls, stems and leaves of dicotyledonous plants or roots, coleoptiles and internodes of monocots. However, to our knowledge, the site of the primary shoot growth, directly below the shoot apical meristem has not been analysed in dicots so far.

Here, we compare the expression patterns of *LeExp18*, *LeExp2* and a new tomato expansin cDNA, *LeExp9*. *LeExp9* is highly expressed in the submeristematic tissue of the shoot apex. The up-regulation correlates with the second growing zone in the submeristematic tissue. Considering the *in vitro* and *in vivo* activities of expansin, *LeExp9* may play a crucial role in cell expansion within the youngest internodes.

We present a conceptual model in which differential regulation of expansins results in specific growth events in different developmental zones of the tomato shoot apex.

## Materials and methods

### *Plant material and growth conditions*

Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were grown in a growth room at 25±2 °C under a 16 h/8 h light/dark cycle or in the dark. Apices were dissected from plants that were grown for 3 weeks on soil and used for *in situ* hybridization. Vegetative tissues were collected and frozen for RNA extraction. 'Vegetative meristems' included P1 and P2, sometimes P3. 'Sub-meristems' contained 2 mm of the youngest stem tissue, located directly beneath the meristem with the primordia removed. The stem tissue contained the 2 cm directly below.

The *gib* mutants were kindly provided by the Tomato Genetics Resource Center at the University of California, Davis. All three *gib* mutants are in the cv. Moneymaker background. The accession numbers are: *gib1*, LA2893; *gib2*, LA2894; *gib3*, LA2895.

The *gib* mutants were germinated in petri dishes on a double layer of Whatman paper soaked with 1 mM $GA_3$. After two weeks the seedlings were transplanted on soil where they were allowed to recover for three weeks without treatment. Spraying with 100 $\mu$M $GA_3$ was done twice a week and plants were harvested after two weeks. Apices (5 mm) including P1–P6 were dissected and used for RNA extraction.

*Cloning and analysis of an expansin cDNA*

Total RNA was isolated from tomato vegetative apices, leaf primordia (P4, P5 and P6) and floral meristems of the sepal stage, as described by Caderas *et al.* (2000). After reverse transcription, using a poly(T) primer with an anchor sequence, PCR amplification was performed with one primer having a sequence homology to the anchor and a second degenerate primer (5′-CGGGAATCCAACAATGGWYTRASTTGTGG-3′) designed from amino acid domains conserved among *LeExp1*, *LeExp2* and *LeExp18*. A specific reverse primer (5′-GGGAAGCTTCAAGTACTACAAAACATTATACTG-3′) at the 3′ end of the resulting 900 bp fragment was designed and used together with the 5′ λTriplEx2 sequencing primer (Clontech) to perform a PCR on a tomato leaf primordia cDNA library generated with the SMART cDNA library construction kit (Clontech). The resulting product, designated *LeExp9*, was cloned into pBluescript vector. The EMBL database accession number of *LeExp9* is AJ243340.

*Hormone treatments*

Shoot tips consisted of the 5 mm apical region including P1–P6. The samples were dissected from 3-weeks-old plants and incubated in 2.5 mM potassium phosphate buffer pH 6.0, 2% w/v sucrose for 2–3 h. The buffer was replaced with fresh buffer (control) or with buffer containing $GA_3$. The samples were incubated with gentle agitation for 16 h overnight in a growth room. After the treatments, the tissue was frozen and stored at −80 °C for RNA analysis.

*RNA and DNA gel blot analysis*

Total RNA extraction and gel blot analysis were performed essentially as described by Caderas *et al.* (2000). RNA was quantified spectrophotometrically at 260 nm, and aliquots of 5 $\mu$g were run on 1% agarose after glyoxylation. The RNA was transferred onto nylon membrane (Nytran, Schleicher & Schuell, Dassel, Germany). Blotting and hybridization with gene specific probes were carried out under standard conditions. The signal intensities were quantified on a PhosphorImager (model GS525, BioRad, Glattbrugg, Switzerland) and the blots were exposed to X-ray film.

In situ *hybridization*

In situ hybridization experiments were performed according to the protocol described by Fleming *et al.* (1993), with minor modifications. From embedded tomato apices, longitudinal sections (7 $\mu$m) were cut and used for hybridization. Before exposure, slides were treated with a solution of 50 $\mu$g/ml RNase and finally washed in 2× SSC at room temperature and in 0.1× SSC at 42 °C, each for 30 min. After development, the slides were stained in toluidine blue and viewed on a LSM 410 microscope (Carl Zeiss, Oberkochen, Germany). Images were taken under bright-field light (shown in false green colour) and overlaid with epifluorescence images taken under polarized light exhibiting the silver grain signal (shown in false red colour). In all cases, control hybridizations were performed with the corresponding sense probes, and in all instances, the signal obtained was negligible compared with that obtained using the antisense probe. All probes mentioned are antisense probes unless indicated else.

## Results and discussion

*Expression of expansin genes in the shoot apex*

Expansin gene expression patterns during vegetative shoot growth were compared. *LeExp18* and *LeExp2* were included in our studies because both genes were previously shown to be expressed in the vegetative phase of development; in the apex or in growing hypocotyls and stem tissue, respectively (Reinhardt *et al.*, 1998; Caderas *et al.*, 2000).

The transcript levels of the *LeExp18*, *LeExp9* and *LeExp2* expansin genes were examined in young stems, submeristematic regions and vegetative meristems (including the two or three youngest leaf primordia). The expression patterns of *LeExp18* and *LeExp9* were very similar and showed high transcript accumulation in young organs of the growing shoot. One striking difference, however, was detected in the submeristematic region, representing the youngest stem tissue. In this zone *LeExp9* was up-regulated (Figure 1D). The submeristematic tissue sample contained the 2 mm of tissue directly underneath the vegetative meristem, from which P4 to P6 were excised. The expression pattern of *LeExp2* was different and showed high mRNA levels in the stem. As a measure of overall transcriptional activity, we analysed the expression of the *rpl2* gene. Taken together, *LeExp2* expression is

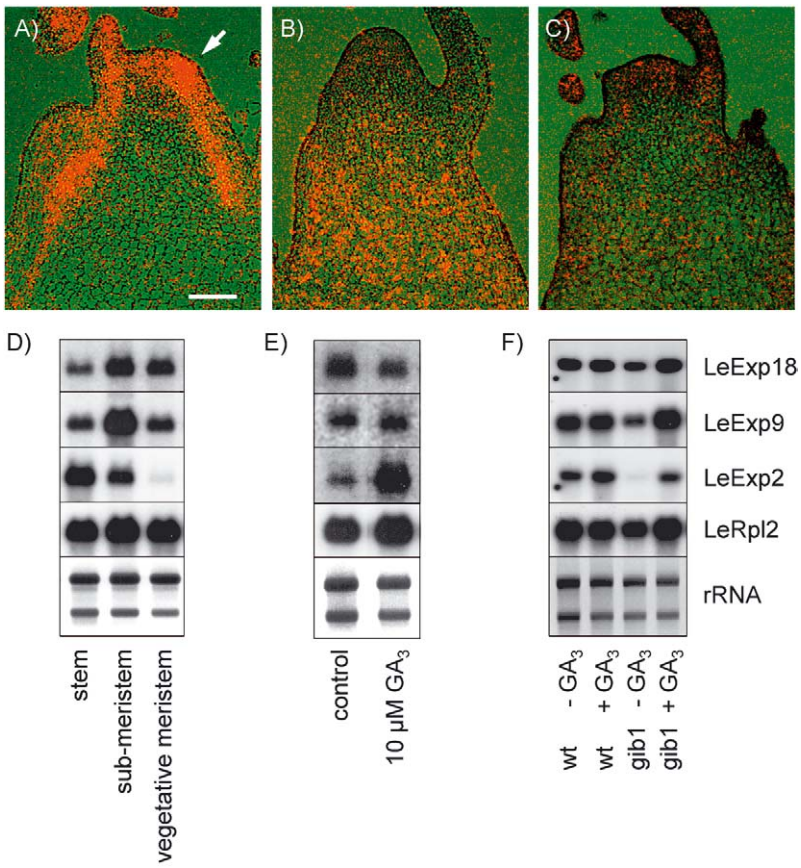


*Figure 1.* Differential expression and regulation of expansins in the shoot apex of tomato A–C) Localization of tomato *LeExp18*, *LeExp9* and *LeExp2* in the vegetative shoot apex. Longitudinal sections of three-weeks-old apices were used for *in situ* hybridizations. A. Hybridization with the *LeExp18* probe. The site of the incipient leaf is indicated by an arrowhead. B. Hybridization with the *LeExp9* probe. C. Hybridization with the *LeExp2* probe. (Bar = 160 $\mu$m) D–F. RNA gel blot analysis. D. RNA from various vegetative tissues were separated per lane, and hybridized with the gene-specific probes indicated on the right. The lower panel represents an ethidium bromide gel, and visualizes the ribosomal RNAs, as control for equal loading and intactness of the RNA. E) Gibberellin regulation of expansin mRNA levels in shoot tips. Shoot apices (5 mm) including P1 to P6 from 3-weeks-old tomato plants were dissected and incubated in buffer (control) or buffer plus $GA_3$ for 16 h. F. Differential response of expansin gene expression in the *gib1* mutant. Shoot apices (5 mm) including P1 to P6 from gibberellin treated and untreated plants, respectively, were dissected. For all northern blot analyses 5 $\mu$g of total RNA were separated per lane and hybridized with the cDNA probes indicated on the right. *Rpl2* expression is included as a control for general metabolic activity (Mandel *et al.*, 1995).

associated with more mature tissue, such as stem and leaf, whereas *LeExp18* and *LeExp9* expression coincides with young tissue in the apical region. However, although *LeExp18* and *LeExp9* transcripts were found in the same organs, *LeExp9* mRNA accumulated to a higher level in the submeristematic stem tissue.

To determine the cellular localization of expansin gene expression within the shoot apical region, *in situ* hybridization experiments were performed. Figure 1A–C show the differential expression of *LeExp18*, *LeExp9* and *LeExp2* in longitudinal sections through a vegetative apex. As reported earlier by Reinhardt *et al.* (1998), the signal derived from the *LeExp18* probe was observed in the meristem and in young primordia, as well as in subepidermal tissues of the young stem corresponding to provascular tissue. The levels of the *LeExp18* mRNA were up-regulated opposite the youngest leaf primordia, corresponding to the site of the incipient primordia (Figure 1A). The *LeExp9* transcript accumulated uniformly throughout the submeristematic region, which is the youngest stem tissue. In the meristem proper and in young primordia, the hybridization signal was significantly decreased (Figure 1B). The *LeExp9* expression pattern correlates with the growing zone between the meristem proper and the stem tissue. In this area, internode growth must occur to achieve shoot growth in width and in length (Lyndon, 1998). We propose a function for the Le-

EXP9 protein in wall loosening of the submeristematic tissues to obtain cell expansion. *LeExp2* expression in the uppermost millimeter was not significantly above background level (Figure 1C) but it is expressed in stem tissue below. Together with previous data (Caderas *et al*., 2000), this implicates a role for *LeExp2* in stem elongation. Thus, *LeExp18*, *LeExp9* and *LeExp2* are expressed in consecutive growth domains along the apical-basal axis.

### LeExp9 *and* LeExp2 *expression are correlated with stem elongation* in vivo

Phytohormones have been shown to regulate tomato expansin transcription (Rose *et al*., 1997; Caderas *et al*., 2000; Català *et al*., 2000). Since gibberellin (GA) is thought to exert its elongation effect on very young stem tissue (Sachs, 1965), we examined its effect on *LeExp9*, *LeExp18* and *LeExp2* expression in the 5 mm apical region (Figure 1E). While *LeExp18* and *LeExp9* levels were essentially unaffected by gibberellic acid ($GA_3$), *LeExp2* expression was strongly induced by $GA_3$ treatment. Because of these findings we decided to investigate the relevance of GA levels on expansin expression *in vivo* in GA-deficient (*gib1*) tomatoes that have a decreased endogenous GA level (Koornneef *et al*., 1981).

Comparable data were obtained with two other mutants, *gib2* and *gib3* (not shown). The *gib1* mutants showed a severe phenotype with strongly reduced elongation of the internodes (not shown). $GA_3$ treatment led to the elongation of the internodes and the mutants gained a wild-type appearance. Wild-type plants themselves reacted to $GA_3$ treatment with the typical GA response. They grew slender and were light green (not shown).

The correlation between internode elongation and expansin expression was tested by northern hybridization. The expression of *LeExp18*, *LeExp9* and *LeExp2* was compared in *gib1* mutants with and without $GA_3$ treatment. Neither in the wild type nor in the *gib1* mutants were *LeExp18* mRNA levels significantly affected by $GA_3$ when normalized with *rpl2* levels, which serve as a control for general metabolic activity (Figure 1F). Concerning the *LeExp9* mRNA levels, no difference was found between treated and untreated wild-type plants. However, in untreated *gib1* mutants *LeExp9* was expressed more weakly (50% after normalization with *rpl2* expression levels) than in the wild type. Exogenous application of $GA_3$ restored the expression of *LeExp9* back to wild-type level.

An even stronger effect was found with *LeExp2*. In *gib1* mutants the mRNA level was reduced by 80%. In the rescued mutant plants, *LeExp2* expression was induced and the amount of transcript reached wild-type level. Wild-type plants showed a substantial increase in *LeExp2* expression after treatment with $GA_3$. Thus, *LeExp18* was not affected by $GA_3$, *LeExp9* was not affected by exogenous application but was reduced in *gib* mutants, while *LeExp2* levels were responsive both to increases and decreases in $GA_3$ levels.

A classical function of the plant hormone gibberellin is to promote shoot elongation. Recently, however, GA has also been implicated in the switch between indeterminate and determinate cell fate (Sakamoto *et al*., 2001), which, in other words, is the switch between meristem and leaf identity. It is thought that the meristem identity gene *stm1* and the GA biosynthetic *AtGA20ox1* gene act antagonistically to establish correct patterning within the peripheral zone of the meristem (Hay *et al*., 2002). The three expansin genes studied here react in a strikingly differential manner to modulations of the GA concentration. *LeExp18* is entirely insensitive, both to exogenous application of the hormone and to reduction of its endogenous concentration by genetic means. Thus, while expansins are directly implicated in primordium induction, this switch from meristem to primordium identity does not involve modulation of *LeExp18* expression by GA. Consistent with this, we note that the *gib1* mutation is severely stunted but has no defects in leaf initiation.

Similar to *LeExp18*, *LeExp9* is not induced by exogenous $GA_3$ application. However, in contrast to *LeExp18*, its expression level is reduced in the *gib1* mutant. This suggests that *LeExp9* expression is GA-dependent but that, under normal conditions, GA is not limiting for *LeExp9* expression. *LeExp2* represents a third mode of expression. This gene is induced by exogenous $GA_3$ application and repressed in the *gib* mutants. Thus, *LeExp2* expression levels are critically dependent on ambient GA concentration. We propose a concept in which consecutive growth events in the tomato shoot apex are at least partially regulated by the proper expression of different expansins, which can be seen as markers to discriminate between the three apical growth zones (Figure 2). Taken together, our data support the hypothesis that differential expression of the expansin genes rather than differential functions of the proteins are the raison d'être for the existence of the large expansin multigene family.

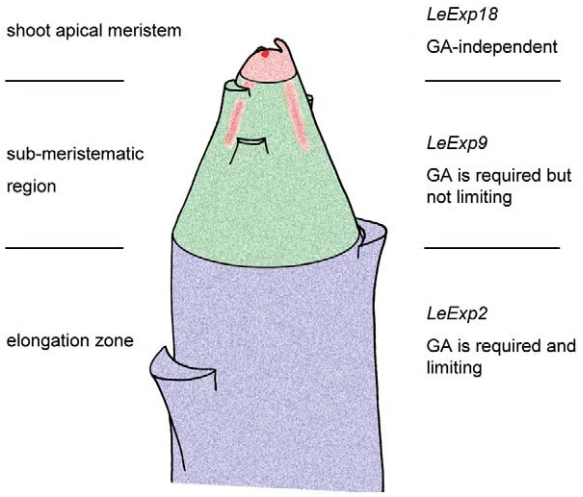


*Figure 2.* Model for the regulation of cell expansion in the shoot apex of tomato. The shoot apex of tomato can be divided into three growth regions. *LeExp18* (red) is expressed in the shoot apical meristem where it is upregulated at the position of the incipient leaf (red dot). *LeExp18* is thought to have a role in leaf initiation, which is not regulated by GA. In the submeristematic region *LeExp18* and *LeExp9* (green) expression domains partially overlap. But whereas *LeExp9* transcript accumulates uniformly across the submeristematic region *LeExp18* is only expressed in provascular tissue. *LeExp9* is involved in cell expansion in the submeristematic region. This process requires GA, but GA is not normally limiting. *LeExp2* is expressed in the elongation zone (blue). Cell elongation in this region is limited by the availability of GA.

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