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Analysis of expansin-induced morphogenesis on the apical meristem of tomato

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Abstract. Our previous work has shown that localised activity of the cell-wall-loosening protein expansin is sufficient to induce primordia on the apical meristem of tomato, consistent with the hypothesis that tissue expansion plays a key role in leaf initiation. In this paper we describe the earliest morphogenic events visible on the surface of the apical meristem of tomato (*Lycopersicon esculentum Mill.*) following treatment with expansin and report on the spectrum of final structures formed. Our observations are consistent with a proposed primary function of expansin effecting morphogenesis via altered biophysical stress patterns in the meristem. The primordia induced by expansin do not complete the full program of leaf development. We present data indicating that one reason for this might be the inability of exogenous expansin to mimic the endogenous pattern of expansin activity in the meristem. These data provide the first detailed analysis at the cellular level of expansin action on living tissue, the first description of the spectrum of structures induced by expansin on the apical meristem, and give an insight into a potentially fundamental mechanism in plant development.

Key words: Cell wall – Expansin – Leaf development – *Lycopersicon* (morphogenesis) – Morphogenesis – Shoot apex

Introduction

Over the last decade, tremendous advances have been made in our understanding of the role of spatial and temporal regulation of transcription-factor activity in controlling development. However, our understanding of how the information content defined by the patterns of transcription factors is transduced into change in form is still incomplete. Although the regulation of local cellular proliferation may be of pre-eminent importance in animal systems (Johnson and Tabin 1997), the situation in plants might be different. Thus, although classical theory predicts a major role for the regulated pattern of cell division in defining plant form (reviewed in Steeves and Sussex 1989), plants appear to be quite resilient to alterations in expression of genes regulating the cell cycle (Hemerley et al. 1995; Doermer et al. 1996) and can undergo relatively normal morphogenesis despite major changes in cytoskeletal organisation thought to determine cell division dynamics (Traas et al. 1995; Smith et al. 1996). The answer to this paradox might lie in the plant cell wall, and, in particular, in the regulation of its extensibility.

As shown in Fig. 1A, each plant cell generates an internal force (turgor pressure) which acts outwards in all directions. This force is counter-balanced by the rigidity of the surrounding cell wall as well as the force acting on the cell by its neighbours, leading to a stable equilibrium. Cells in the outermost layer (epidermis or L1 layer of the meristem) lack neighboring cells on one side. It is thought that a tensile force must be established in the outer epidermal cell wall to counterbalance the overall compressive forces acting radially outwards on the epidermal cells (Selker et al. 1992). If this is the case, then a local increase in cell wall extensibility in the outer epidermal cell layers (Fig. 1B) should automatically lead to bulging outwards of the tissue until a new physical equilibrium is achieved between epidermal cell wall tension and inner cell compression, i.e., morphogenesis occurs (Green 1997).

In the context of the apical meristem, the outermost cell layer represents an enclosed system with an epidermis under tension surrounding a corpus of cells under compression (Fig. 1C). The apical meristem re-iteratively generates new leaf primordia in a precise pattern so that if a meristem has generated two primordia (P1 and

This paper is dedicated to the memory of Paul Green (1931–1998) in recognition of his seminal contribution to our understanding of the biophysical basis of plant development.

Abbreviation: RBCS = small subunit of ribulose bisphosphate carboxylase

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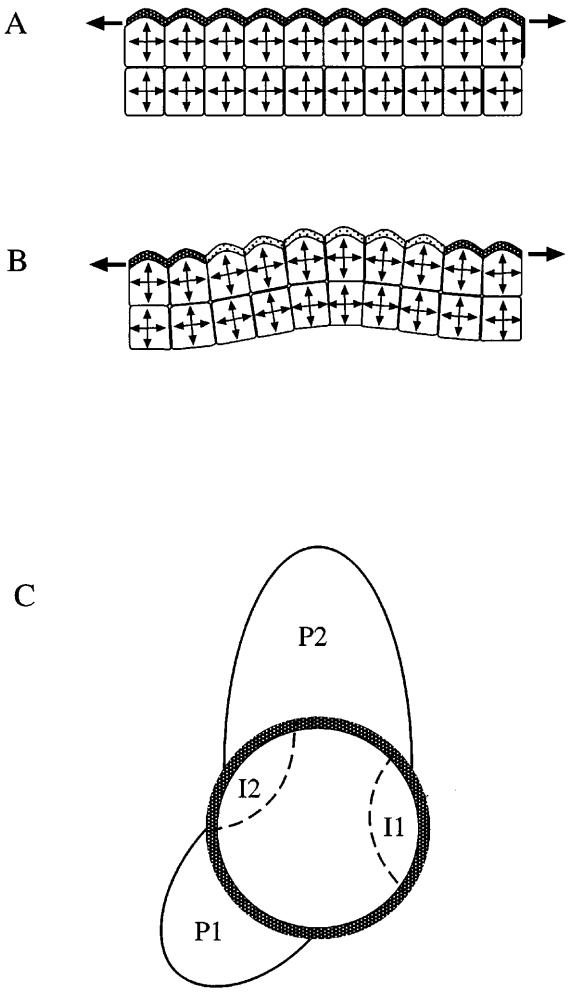


Fig. 1A–C. Biophysical theory and leaf formation. **A** Each plant cell generates an internal turgor pressure which acts outwards in all directions (*small arrows*). To counteract the internal compressive forces, a tensile force (*large arrows*) develops in the outermost epidermal cell wall (*speckled*) leading to a stable physical equilibrium. **B** With all forces remaining constant, local loosening of the outer cell wall (*light speckling*) leads automatically to tissue bulging until a new physical equilibrium is established. **C** The apical meristem consists of an outer tunica layer (*dark speckled*) under tension surrounding a central corpus of cells under compression. If a meristem has generated two leaf primordia, P2 and P1, the next sites of leaf initiation can be predicted as I1 and, subsequently, I2. Local loosening of the outer cell wall in the I2 tunica should lead to bulging outwards of the tissue (morphogenesis)

P2 in Fig. 1C) then the positions at which the successive leaf primordia will be produced can be predicted as being I1 and then I2 (Fig. 1C). According to the model described in Fig. 1, a localised alteration in epidermal cell wall extensibility at the I1 or I2 position should be sufficient to induce the initial step of leaf morphogenesis. Recently, we reported on experiments supportive of such a mechanism (Fleming et al. 1997). To summarise, beads loaded with the protein expansin (an effector of cell wall extensibility; reviewed by Cosgrove 1997) and placed on the I2 position of tomato meristems led to the formation of bulges on the apical meristem, i.e., morphogenesis. Moreover, some of these bulges were able to develop into leaf-like primordia, as assessed by morphology and marker-gene expression, suggesting that the initiation of a simple bulge on the meristem was in itself sufficient to initiate at least part of the leaf development program. In addition, in-situ hybridisation analysis has indicated that expansin genes are specifically expressed at the site of primordium initiation in both tomato (Fleming et al. 1997; Reinhardt et al. 1998) and rice (Cho and Kende 1998).

Our previous analysis was limited to events observable by light and confocal microscopy 5 d after expansin action, i.e., some time after the initial induction. In this paper using cryo-scanning electron microscopy, we describe the earliest visible changes in surface structure and cell division pattern on the meristem following expansin treament. In addition, we report on the spectrum of structures observed following localised expansin activity and the developmental fates of these structures. The results are consistent with expansin acting primarily to modulate cell wall extensibility, and with cell wall extensibility playing a key role in plant morphogenesis.

Materials and methods

Purification of expansin and meristem manipulation. Expansin was purified based on the method previously described (McQueen-Mason et al. 1992). Briefly, cucumber hypocotyls were homogenised in 25 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and the cell wall material collected. The extractable cell wall proteins were precipitated with 60% (w/v) ammonium acetate and collected by centrifugation. The proteins were then desalted on a Sephadex G-25 column and loaded in 15 mM Mes (pH 6.5), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnSO_4$, 100 mM NaCl onto a Concanavalin A column (Vector Laboratories, Peterborough, UK). After elution with 400 mM methyl α-D-gluco-pyranoside, 200 mM NaCl, 15 mM Mes (pH 6.5), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnSO_4$, the proteins were concentrated on a Centricon-30 (Amicon, Stonehouse, Gloucestershire, UK) before separation on a Mono S PC 1.6/5 cation exchange column (Pharmacia) using a gradient of 0.0 to 0.4 M NaCl in 20 min at a flow rate of 100 µl/min. Fractions containing expansin activity (assayed as described in McQueen-Mason et al. 1992) were collected and the pH was adjusted to 4.5 with acetic acid. These fractions, which were used in the experiments described below, induced extension rates of between 0.93%/h and 4.21%/h in the expansin extension assay. The protein concentration of the fractions was approximately 50 ng/µl with the 29-kDa expansin protein being the predominant band visualised on silver-stained SDS-PAGE gels. Aliquots (10 µl volume) of Sepharose S-300 beads (Pharmacia) pre-equilibrated in 50 mM sodium acetate (pH 5.2) were incubated overnight at 4 °C with an equal volume of the purified expansin fractions, the beads briefly centrifuged, washed briefly with 50 mM sodium acetate (pH 5.2), then manually manipulated onto meristems of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) apices using pulled capillary tubes under a stereomicroscope. In each set of experiments with expansin, apices were treated in parallel with beads loaded with buffer only. Apices were prepared by dissection under a stereomicroscope to reveal the apical meristem leaving one to three primordia intact along with a subapical region of approx. 3–5 mm. Excised apices were placed on 1 × MS medium (Serva) containing 2% sucrose, 0.8% agarose, 10 nM gibberellic acid and 10 nM kinetin (Hussey 1971) in multiwell slides (Life Technologies, Basel, Switzerland). After manipulation of the beads onto the meristem, apices were incubated under lights with a 16/8 h light/dark regime for periods of 1–14 d at 22 °C before analysis. Analysis of

morphogenesis was by use of the stereomicroscope or cryo-scanning electron microscopy (Cryo-SEM).

Cryo-scanning electron microscopy analysis of the shoot apex. Apices were frozen by plunging into liquid nitrogen, then the frozen samples mounted onto a Gatan (München, Germany) cryo-holder and cryo-sputter coated in a MED 020 (Bal-Tec, Liechtenstein) with 7 nm of platinum at a temperature of 140 K. The samples were then cryo-transferred in liquid nitrogen to a Hitachi S-900 in-lens field emission microscope. Images of the frozen hydrated samples were obtained at a temperature of 140 K and an accelerating voltage of 10 kV using the secondary electron signal. Images were recorded digitally with a Gatan Digi-Scan interface.

Histology and in-situ hybridisation. Tissue samples were prepared for histological analysis and in-situ hybridisation as previously described (Fleming et al. 1993). Briefly, samples were fixed in 4% formaldehyde, 0.1% glutaraldehyde in sodium phosphate buffer (pH 7.2), dehydrated in ethanol, exchanged with xylene, then embedded in paraffin. Sections (7 μm) were mounted on poly-L-lysine-coated slides, treated with proteinase K followed by acetic anhydride, then hybridised overnight at 42 °C with ^{35}S-labelled riboprobes (sense or antisense) for the small submit of tomato ribulose bisphosphate carboxylase (RBCS). After washing to a stringency of 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7) at 37 °C for 20 min, sections were dehydrated and coated with emulsion. After exposure for 3 to 5 weeks, slides were developed, stained with toluidine blue, then signal (silver grains) visualised using a polarising filter block. Images were captured using a Hamamatsu charge-coupled device and imported into Photoshop (Adobe). Silver grains were then rendered red to aid visualisation. No significant signal above background was observed with sense probes (data not shown).

Fluorescent labelling and visualisation of protein. Chymotrypsinogen A (Pharmacia) and bovine serum albumen (Sigma) were labelled with the fluorescent label, Cy3.5 dye (Amersham) following the manufacturer's instructions. Briefly, protein was dissolved in 0.1 M sodium carbonate buffer (pH 9.3) and then mixed with the Cy3.5 dye at room temperature for 45 min. Labelled protein was then separated from unconjugated dye using a Sephadex G-50 column (Pharmacia). The labelled protein was eluted with phosphate-buffered saline (10 mM K_3PO_4, 150 mM NaCl, pH 7.2). The eluate was then concentrated using a Centricon TM microconcentrator (exclusion limit 10 kDa or 30 kDa; Amicon) and the conjugated protein was stored at 4 °C before use.

The labelled protein was loaded onto Sepharose S-300 beads (as described above), then beads were manipulated onto the apical meristem. Fluorescence was visualised using a 515–560-nm excitation and 590-nm barrier filter at various time points after manipulation of the beads onto the meristems and images captured using software provided by Zeiss. Optical sections for confocal microscopy were taken as previously described (Fleming et al. 1996).

Results

Scanning electron microscopy analysis of expansin-induced morphogenesis. To analyse the early events following localised expansin activity on the meristem, we used cryo-SEM. The results of this analysis are shown in Fig. 2. Immediately following manipulation of beads onto the meristem there is no apparent damage to the meristem surface (Fig. 2A,B). The beads cover a large area of the I2 region with a very fine contact area between individual beads and the meristem surface. The earliest morphogenic events were observed after 2 d (Fig. 2C,D). In Fig. 2C, a rippling or undulation of the tissue can be seen producing two slight protrusions on the meristem surface. In the furrow produced there is a lack of cellular detail due to the accumulation of an amorphous substance on the meristem surface, probably due to the expansin solution diffusing out of the beads. The debris on the surface of the tissue is probably derived from the beads and crystallisation of the fluid contained within them. Figure 2D shows an example of an early expansin-induced morphogenic event in which a group of cells internal to the base of the P2 primordium appear to be folding upwards in a small hump. The apex of the bulge seems not to be intact, a common feature of expansin-induced primordia (see also Fig. 2H). A bead can also be seen trapped within the axil of the P2 primordium. Of the 42 apices analysed at this early stage of reaction to expansin treatment, 5 (12%) showed clear alteration of surface structure or shape compared to apices treated with beads loaded with buffer alone. In no case (n = 34) was there any evidence of buffer alone inducing the structural surface changes observed after treatment with expansin (cf. Fig. 2C and D). The surface structure of such control apices was comparable to those shown in Fig. 2A,B and E, i.e., smooth. The early alteration in surface structure induced by expansin varied between gentle undulations (as in Fig. 2C) and distinct protrusions (Fig. 2D). Physical damage can be excluded as a cause of these protrusions since, firstly, the beads have only a slight contact with the meristem surface, and, secondly, because in samples which were mishandled during preparation the meristem damage observed was massive and obvious and qualitatively different from the changes induced by expansin. Samples showing such handling damage were excluded from the analysis. It should be noted that at these early stages of expansin-induced morphogenesis there is no overt massive increase in cell size on the meristem surface, suggesting that any expansin-induced expansion is coupled with cell division.

The initial undulation and bulge formation observed following expansin treatment is distinct from the initial morphological changes apparent during natural leaf initiation. This is shown in Fig. 2E in which a broad bulging of the meristem surface is generating the leaf primordium P1. Subsequently, naturally formed leaves undergo stereotypical morphogenesis to generate a classical peg structure appending the apical meristem (Fig. 2F). In contrast, expansin-induced bulges displayed a wide spectrum of structures after 4–5 d of growth. These structures ranged from small protrusions consisting of only a few cells (Fig. 2G) to large primordium-like structures with axial polarity (Fig. 2H). These larger primordia were often observed to be indented at the tip, creating a hollow terminal structure (Fig. 2H) distinct from natural leaf primordia (Fig. 2F). Similar to naturally formed primordia, the cells on the surface of the larger expansin-induced structures were arranged in longitudinal arrays parallel with the main axis of the leaf (Fig. 2F,H), as opposed to the more random arrangement seen on the meristem surface. Within this spectrum of forms, morphologically apparently normal primordia were also generated following

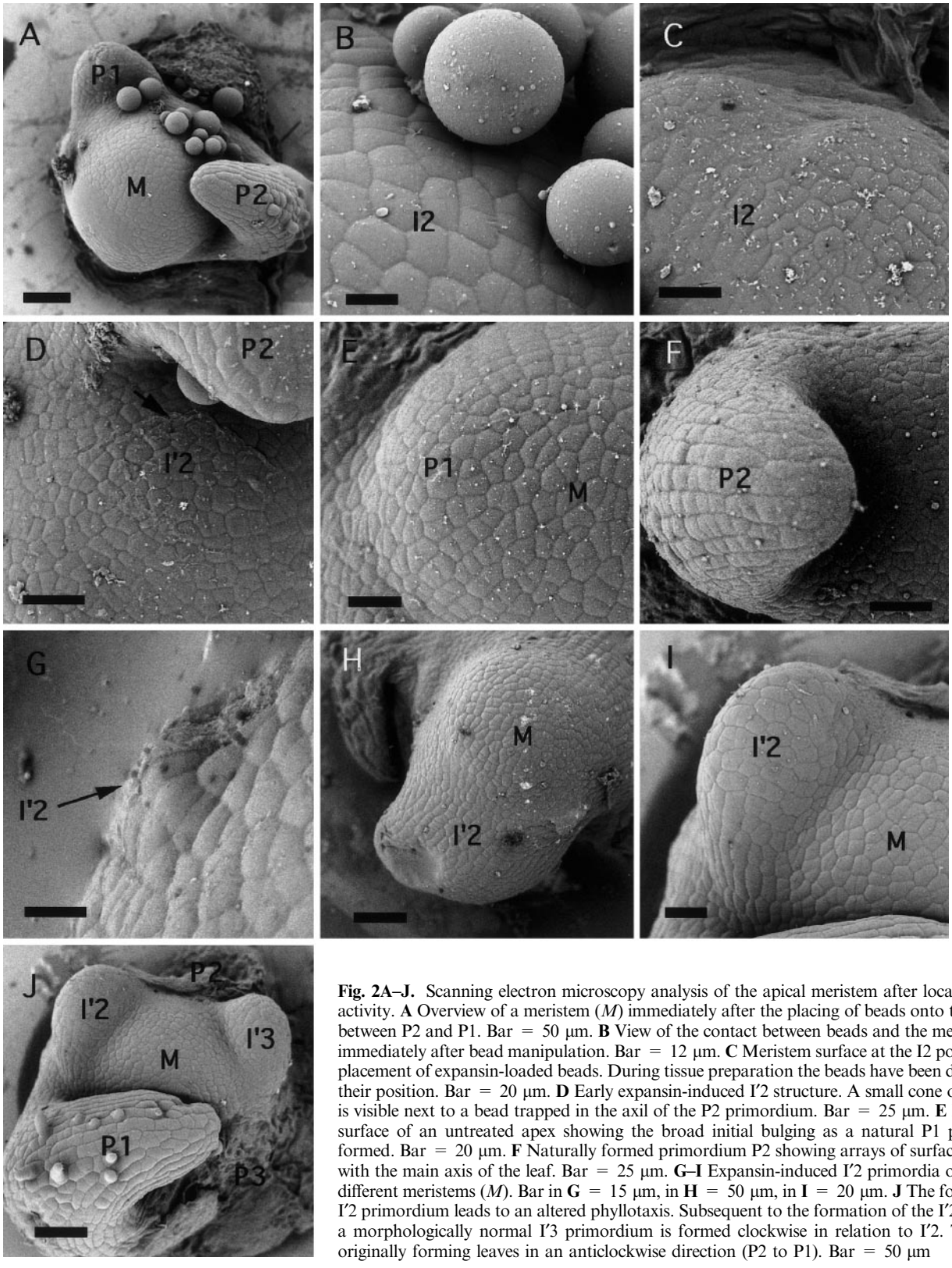


Fig. 2A–J. Scanning electron microscopy analysis of the apical meristem after localised expansin activity. **A** Overview of a meristem (*M*) immediately after the placing of beads onto the I2 position between P2 and P1. Bar = 50 μm. **B** View of the contact between beads and the meristem surface immediately after bead manipulation. Bar = 12 μm. **C** Meristem surface at the I2 position 2d after placement of expansin-loaded beads. During tissue preparation the beads have been dislodged from their position. Bar = 20 μm. **D** Early expansin-induced I′2 structure. A small cone of cells (*arrow*) is visible next to a bead trapped in the axil of the P2 primordium. Bar = 25 μm. **E** Meristem (*M*) surface of an untreated apex showing the broad initial bulging as a natural P1 primordium is formed. Bar = 20 μm. **F** Naturally formed primordium P2 showing arrays of surface cells parallel with the main axis of the leaf. Bar = 25 μm. **G–I** Expansin-induced I′2 primordia on the flank of different meristems (*M*). Bar in **G** = 15 μm, in **H** = 50 μm, in **I** = 20 μm. **J** The formation of an I′2 primordium leads to an altered phyllotaxis. Subsequent to the formation of the I′2 primordium, a morphologically normal I′3 primordium is formed clockwise in relation to I′2. The apex was originally forming leaves in an anticlockwise direction (P2 to P1). Bar = 50 μm

exogenous expansin activity at the I2 region (Fig. 2I). This similarity extended both to the shape of the organ and the pattern of surface cell divisions.

Following the formation of such expansin-induced primordia, the subsequent pattern of phyllotaxis of the meristem was reversed. Thus, as shown in Fig. 2J, the anticlockwise direction of the phyllotactic spiral from P2 to P1 is reversed to clockwise by the formation of I′2 (expansin-induced) and I′3 primordia. It should be noted that the I′3 primordium is so denoted because it inserts slightly higher on the meristem than does I′2. However, the I′3 primordium arises in approximately

the same position as the I1 primordium was expected to. Thus, at this early stage of development it is difficult to say with confidence whether the I′3 primordium has arisen de novo as a result of the influence of the I′2 primordium or whether it is a slightly delayed I1 primordium. Later in development, as the stem tissue elongates and the meristem generates new leaf primordia, this reversal in phyllotaxis is maintained (Fleming et al. 1997).

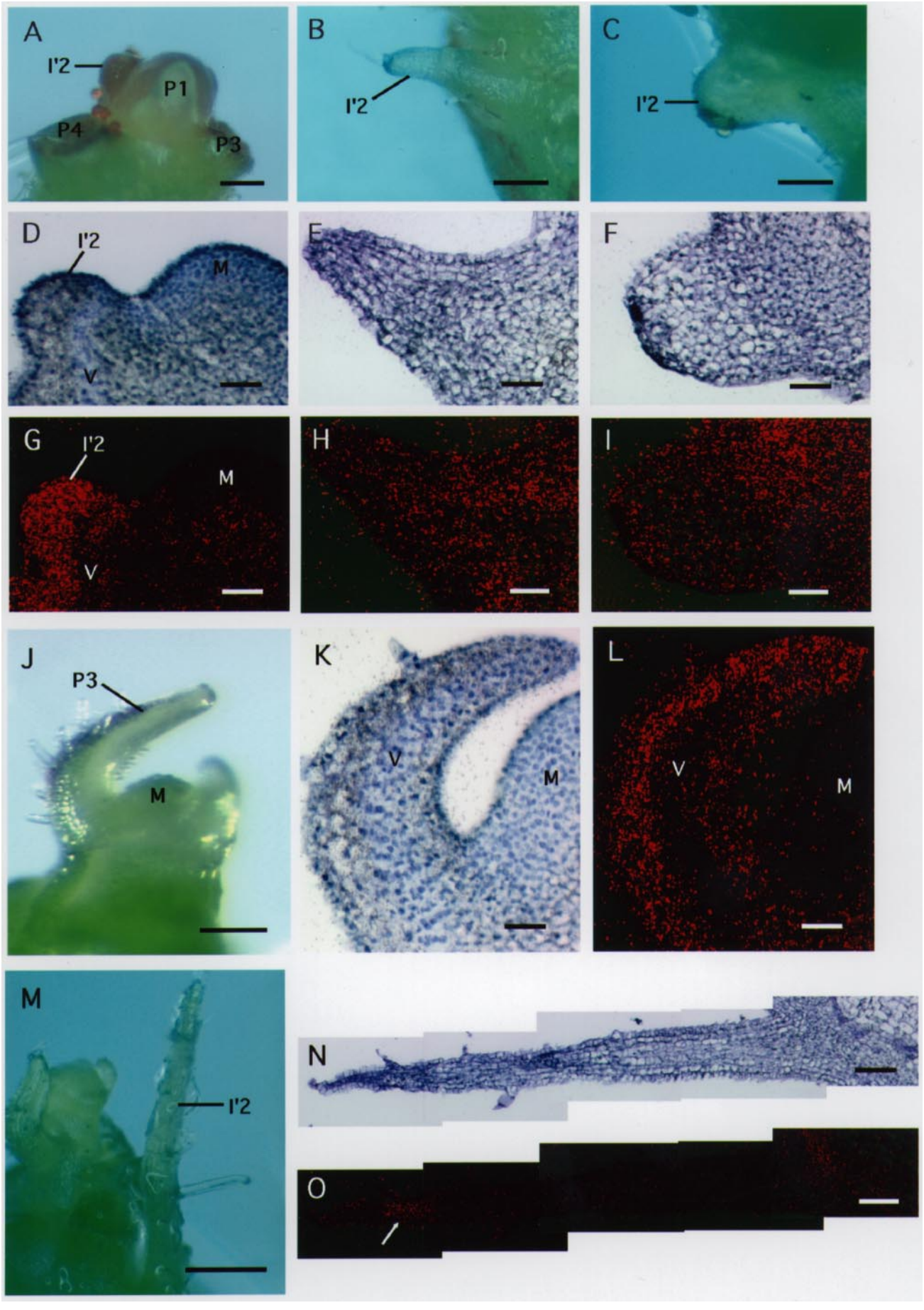
Expansion induces a variety of structures. After 5 d of growth, some expansin-induced primordia were visible at the resolution of the stereomicroscope. An example of such a structure is shown in Fig. 3A. This I′2 primordium subtends the apical meristem and has a proximal-distal axis, but the morphology of the primordium is abnormal compared with naturally formed tomato leaves (Dengler 1984), elongation being stunted. After 14 d, the cells in the I2 region exposed to expansin have been displaced onto the stem by the continued growth of the meristem. Some of these structures showed leaf-like characteristics (i.e., a proximal-distal axis, and limited dorsi-ventrality), as shown in Fig. 3B. This morphology is similar to natural leaf primordia of plastochron age P2 to P3 (Fig. 3J). However, other expansin-induced structures on the stem lacked obvious polarity and more resembled simple bulges (Fig. 3C).

Sections taken through these expansin-induced structures revealed an abnormal histology compared with naturally formed leaves. Figure 3D shows a section through the I′2 primordium shown in Fig. 3A. A loop of vascular tissue is visible at the base of the primordium but it seems to terminate abnormally at the adaxial surface and does not extend to the tip of the primordium. Sections through primordia that have undergone some axial extension (Fig. 3E) or general enlargement (Fig. 3F) do not reveal any vascular tissue. In the extended primordia, the cells tend to be arranged in linear files parallel to the main axis of the organ (Fig. 3E) whereas in the enlarged structures cell division appears to have been more random (Fig. 3F). These differences in histology were also reflected by the pattern of RBCS transcript accumulation which can be used as a negative marker for meristem and vascular tissue and a positive marker for leaf differentiation (Fleming et al. 1993). In the I′2 primordium appending the meristem (Fig. 3G) a high RBCS signal is visible trhoughout the structure with the exception of the loop of presumptive vascular tissue. No appreciable signal is apparent in the adjacent apical meristem. In the larger structures, such as those shown in Fig. 3H,I a lower level of RBCS transcript is present in the main body of the primordium with an elevated signal in the basal region where the primordium joins the stem. This pattern of RBCS transcript in the expansin-induced primordia is distinct from that seen in naturally formed leaf primordia (Fig. 3K,L). In these organs, there is a central vascular bundle lacking RBCS expression which extends to the leaf tip. This vascular tissue is surrounded by adaxial and abaxial mesophyll tissue containing high levels of the RBCS transcript.

Fig. 3A–O. Localised expansin activity leads to the generation of a variety of structures. **A** Light micrograph of an expansin-induced primordium (*I′2*) on an apical meristem 5 d after localised expansin activity on the 12 position. Primordia P4 and P3 have been cut back and stained with safranin red. The P1 primordium is intact. Bar = 50 μm. **B** Light micrograph of an expansin-induced primordium (*I′2*) on the stem of a plant 2 weeks after expansin activity on the apical meristem. Bar = 100 μm. **C** Light micrograph of an expansin-induced primordium (*I′2*) showing no overt polarity. Bar = 120 μm. **D** Section of the tissue shown in **A.** A loop of vascular tissue (*V*) can be seen at the base of the expansin-induced primordium (*I′2*) which has formed on the flank of the meristem (*M*). Bar = 40 μm. **E** Section of the I′2 structure shown in **B**. Bar = 40 μm. **F** Section of the I′2 primordium in **C**. Bar = 40 μm. **G** In-situ hybridisation of the section shown in **D** with an antisense probe for RBCS. Signal (*red dots*) is visible in the I′2 primordium with virtually no signal apparent either in the vascular tissue (*V*) or meristem (*M*). Bar = 40 μm. **H,I** In-situ hybridisations of the sections shown in **E** and **F**, respectively, with an antisense probe for RBCS. Bars = 40 μm. **J** Light micrograph of an untreated shoot apex showing the P3 primordium subtending the meristem (*M*). Bar = 150 μm. **K** Section taken through a naturally formed leaf primordium at the P2 stage of development. Vasculature (*V*) can be seen to extend along the length of the primordium adjacent to the meristem (*M*). Bar = 40 μm. **L** In-situ hybridisation of the section shown in **K** with an antisense probe for RBCS. A high signal is visible in the mesophyll tissue surrounding the vasculature (*V*) which, like the meristem (*M*), shows no signal. Bar = 40 μm. **M** Light micrograph of an elongated I′2 primordium. Bar = 250 μm. **N** Montage to show section of the I′2 primordium in **M**. Bar = 80 μm. **O** Montage to show in-situ hybridisation of the section shown in **N**. Virtually no signal is visible except at the very tip of the organ (*arrow*) and at the base in the stem region. Bar = 80 μm

After prolonged growth, some of the expansin-induced primordia underwent extensive axial elongation (Fig. 3M–O). However, whereas by this plastochron age natural leaves have undergone extensive lamina expansion coupled with lobing to generate the final unipinnate leaf form characteristic of tomato (Dengler 1984), expansin-induced primordia underwent only limited lamina expansion and always lacked lobes. Moreover, sections through these primordia show cells arranged primarily in arrays along the main axis of the leaf with no obvious differentiation into vascular or mesophyll tissue (Fig. 3N). This was corroborated by in-situ hybridisation with the RBCS probe (Fig. 3O) which revealed virtually no signal except in occasional small patches (Fig. 3O, arrowed).

Analysis of protein diffusion patterns on the apical meristem. Our data show that exogenous expansin does not always lead to the induction of visible morphogenesis and that even following the initiation of morphogenesis a wide variety of structures are produced. Thus, of a total of 350 meristems treated with expansin-loaded beads on the I2 position, 57 showed some type of morphogenic response (visible at either the SEM or light-microscope level) with 22 generating structures showing obvious axial polarity (leaf-like). One possible reason for this variability might lie in the technique of applying protein via beads onto the meristem. To test this possibility, we labelled protein with a fluorescent dye and visualised the diffusion of the protein in the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I'2
P1
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B
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meristem following application on loaded beads. Due to the small amounts of purified expansin obtainable, we performed these experiments using commercially available proteins, chymotrypsinogen A (25 kDa) and bovine serum albumin (60 kDa). Obviously, the physicochemical parameters of both of these proteins are unlikely to match those of expansin, but we reasoned that the pattern of protein diffusion observed should provide a general indication of how exogenous proteins penetrate the meristem. The results of a typical experiment using labelled chymotrypsinogen are shown in Fig. 4.

A single bead loaded with fluorescently labelled chymotrypsinogen was placed on an apical meristem (Fig. 4A). Fluorescence imaging of this tissue shows the loaded bead as a bright fluorescent spot (Fig. 4B). After 48 h culture of the shoot apex, the labelled bead was still visible and was surrounded by a ring of fluorescent material (Fig. 4C). The absolute diameter and shape of this ring varied from bead to bead and from experiment to experiment (data not shown) but such diffusion was repeatedly seen and led to a ring equivalent to one to three times the bead diameter after 24–48 h. At later time points the intensity of the fluorescent ring decreased and it was impossible to precisely delimit its diameter, although beads retained their fluorescence throughout the experiment suggesting that they could still act as a source of protein. At shorter time points (hours after bead manipulation) some staining of the meristem surface was always visible, suggesting a rapid initial flow of protein out of the beads (data not shown). Manipulation of meristems carrying loaded beads to investigate the penetration of the fluorescently labelled protein into the tissue revealed that the majority of the protein remained on the outer surface of the tunica (Fig. 4D).

Spatio-temporal specificity of expansin action. Another possible reason why exogenous expansin did not consistently lead to the formation of primordia is that there is a spatial/temporal regulation of the competence of the I2 region tissue to respond to expansion by the generation of primordia. To address this question we placed expansin-loaded beads at particular areas of the I2 region at particular times during the plastochron. As can be seen from Fig. 2A and the results of our analysis of protein diffusion on the meristem surface (Fig. 4), the expansin-loaded beads placed on the meristem are likely to lead to a relatively broad area of tissue being affected. Therefore, we restricted our experiments to placing the beads either nearer the P1 or the P2 primordium at a time point either early (P1 axial length less than 50 μm) or late (P1 length greater than 50 μm) in the plastochron. The results of these experiments are shown in Table 1.

Our data indicate that there appears to be no spatial difference in the responsiveness of the I2 tissue to expansin, i.e., tissue both near the P1 primordium and the P2 primordium can respond to expansin by the formation of leaf primordia.

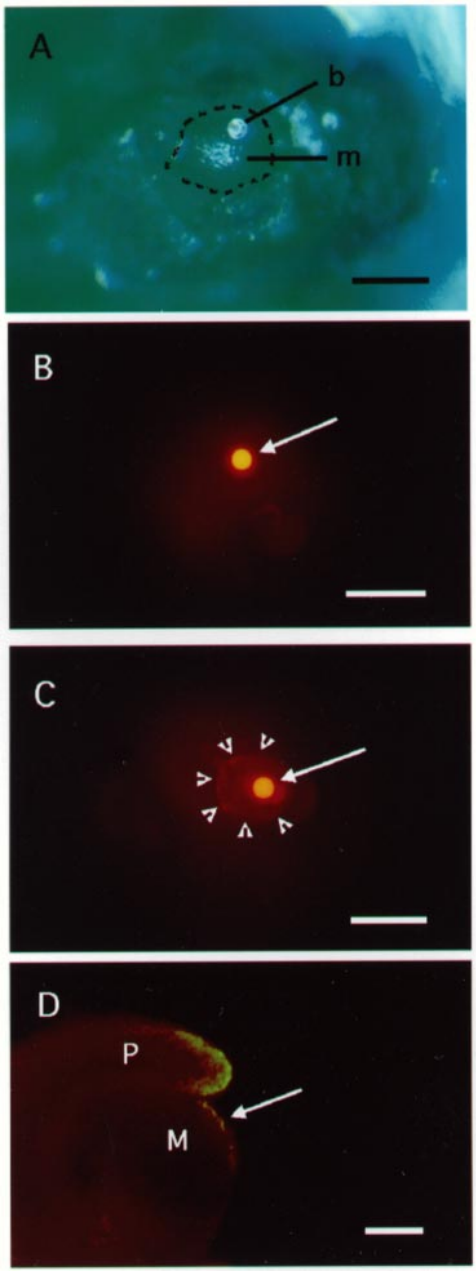

Fig. 4A–D. Analysis of protein diffusion from beads onto the apical meristem. **A** Light micrograph of a tomato shoot apex. A bead (*b*) loaded with fluorescently labelled chymotrypsinogen A has been placed on the surface (*outlined*) of the apical meristem (*m*). Bar = 80 μm. **B** Fluorescent image of an apex immediately after the placing of a bead containing fluorescently labeled chymotrypsinogen A on the meristem surface. The bead (*arrow*) is surrounded by a small halo of fluorescent material. Bar = 80 μm. **C** Fluorescent image of the apex shown in **B** 48 h after manipulation of the bead onto the meristem surface. The bead (*arrow*) is surrounded by a ring of fluorescent material (*arrowheads*) extending approximately two bead diameters. Bar = 80 μm. **D** Lateral optical section of an apex 48 h after the manipulation of a bead containing fluorescently labelled chymotrypsinogen A onto the meristem surface. The bead has been dislodged during sample preparation. A thin localised layer of fluorescent material (*arrow*) is visible on the meristem (*M*) surface. Fluorescence in the tip of the leaf primordium (*P*) and subapical region is autofluorescence from chlorophyll. Bar = 40 μm

Table 1. Spatio-temporal sensitivity of the meristem to expansin activity. Beads loaded with expansin were positioned either next to the P1 or P2 primordium. If the P1 primordium had an axial length < 50 μm, the meristem was adjudged to be in an early stage of the plastochron, whereas if the P1 primordium had an axial length > 50 μm the meristem was adjudged to be in a late stage of the plastochron (Fleming et al. 1996). The number of meristems showing altered morphogenesis after 5 d of culture was observed. Numbers in parentheses indicate number of meristems treated

Bead position	Plastochron stage of meristem at time of bead positioning		
	Early	Late	Total
P1	5 (20)	4 (25)	9 (45)
P2	7 (23)	3 (18)	10 (41)
Total	12 (43)	7 (43)	19 (96)

Discussion

Cell wall extensibility and leaf initiation. The mechanism of plant morphogenesis remains debatable. In particular, the relative role of cell division and expansion is unclear. On the one hand, the immobility of plant cells and the often precise orientation of new cell walls suggests that cell division patterns should play a key role in defining organ initiation and shape. Yet a number of observations and experimental manipulations indicate that plants can utilise cell division-independent mechanisms to generate form, with regulated cell wall extensibility being a prime suspect. This conundrum has recently been addressed in a number of reviews (Clark and Schiefelbein 1997; Jacobs 1997; Meyerowitz 1997). The SEM analysis of the earliest events on the meristem surface following expansin action reported here shows a rippling of the surface reminiscent of the buckling behaviour predicted by biophysical theory (Selker et al. 1992). Our data are thus consistent with the hypothesis that expansin acts primarily to alter cell wall extensibility (Cosgrove 1997) and that the cell wall of the meristem surface is biophysically preset to respond to such localised alteration of extensibility by morphogenesis (Green 1997). Moreover, our data are consistent with the idea that initially small undulations induced by expansin on the meristem surface are sufficient to trigger further developmental processes which lead to the formation of primordia with at least some morphological and molecular characteristics of leaves. Whether the physical bulging induced by expansin feeds back onto the cells within the bulge via, for example, stretch-activated ion channels and subsequent signal transduction cascades (Haley et al. 1995; Ingber 1997) remains open to speculation. It is also interesting to note that the induction of morphogenesis by expansin at the I2 position seems to block the initiation of the natural leaf which would normally arise later from this group of cells. This is consistent with the idea that leaf initiation requires a certain local biophysical pattern of stress and that disruption of this pattern (by, for example, expansin treatment) prevents later normal leaf initiation (Selker et al. 1992).

Since our previous data indicate that at least one specific expansin gene is up-regulated at the site of leaf initiation (Reinhardt et al. 1998), the results reported here are consistent with the hypothesis (Fleming et al. 1997; Reinhardt et al. 1998) that expansin is a target gene for transcription factors involved in patterning the meristem (Jackson et al. 1994). According to such a hypothesis, localised increase in expansin gene activity leads to local bulging of the tissue, i.e., the primary event in morphogenesis. In such a model, the altered expansin activity in the epidermis would provide the altered physical stress conditions leading to the buckling of the surface layers and the elevated level of expansin in the internal corpus cells would allow these cells to respond to the change in stress parameters by expanding and dividing to fill the space left by the tunica as it bulges outwards. Our observation that expansin-induced primordia did not develop into normal leaves may reflect the fact that exogenous expansin is unlikely to mimic the endogenous pattern of gene expression (as indicated by our experiments with the diffusion pattern of labelled proteins). Indeed, our results suggest that externally supplied proteins are likely to be restricted to the epidermal cell wall. The outcome of such an abnormal pattern of expansin activity is difficult to predict, but it is likely that such a dramatic imbalance in extensibility between the epidermis and underlying layers would lead to abnormal morphological events, as we observed.

The role of expansins in morphogenesis. The data presented here add to a growing body of evidence that expansin may play a key role in the control of plant form. Expansins are encoded by relatively large gene families (Shcherban et al. 1995), some members of which show specific patterns of expression. Thus, expression of specific expansin genes has been correlated with hypocotyl expansion (McQueen-Mason et al. 1992), internode elongation in deepwater rice (Cho and Kende 1997), tomato fruit ripening (Rose et al. 1997), and leaf formation (Cho and Kende 1998; Reinhardt et al. 1998). Our future research will be directed towards elucidating the role of expansin in leaf initiation.

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References

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

Cho H-T, Kende H (1998) Tissue localization of expansins in deepwater rice. Plant J 15: 805–812

Clark SE, Schiefelbein JW (1997) Expanding insights into the role of cell proliferation in plant development. Trends Cell Biol 7: 454–458

Cosgrove DJ (1997) Relaxation in a high stress environment: the molecular basis of extensible cell walls and cell enlargement. Plant Cell 9: 1031–1041

Dengler NG (1984) Comparison of leaf development in normal (+/+), entire (e/e), and lanceolate (La/+) plants of tomato, *Lycopersicon esculentum* 'Ailsa Craig'. Bot Gaz 145: 66–77

Doerner P, Jorgensen J-E, You R, Steppuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. Nature 380: 520–523

Fleming AJ, Mandel T, Roth I, Kuhlemeier C (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell 5: 297–309

Fleming AJ, Manzara T, Gruissem W, Kuhlemeier C (1996) Fluorescent imaging of GUS activity and RT-PCR analysis of gene expression in the shoot apical meristem. Plant J 10: 745–754

Fleming AJ, McQueen-Mason S, Mandel T, Kuhlemeier C (1997) Induction of leaf primordia by the cell wall protein expansin. Science 276: 1415–1418

Green PB (1997) Expansin and morphology: a role for biophysics. Trends Plant Sci 2: 365–366

Haley A, Russel A, Wood N, Allan A, Knight MR, Campbell AKC, Trewavas AJ (1995) Mechanical effects on cytoplasmic calcium. Proc Natl Acad Sci USA 92: 4124–4128

Hemerly A, Engler JD, Bergounioux C, Van Montagu M, Engler G, Inzé D, Ferreira P (1995) Dominant negative mutants of Cdc2 kinase uncouple cell division from iterative plant development. EMBO J 14: 3925–3936

Hussey G (1971) In vitro growth of vegetative tomato shoot apices. J Exp Bot 22: 688–701

Ingber E (1997) Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol 59: 575–599

Jackson D, Veit B, Hake S (1994) Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120: 405–413

Jacobs T (1997) Why do plant cells divide? Plant Cell 9: 1021–1029

Johnson RL, Tabin CJ (1997) Molecular models for vertebrate limb development. Cell 90: 979–990

McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4: 1425–1433

Meyerowitz EM (1997) Genetic control of cell division patterns in developing plants. Cell 88: 299–308

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

Rose JKC, Lee HH, Bennett AB (1997) Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proc Natl Acad Sci USA 94: 5955–5960

Selker JML, Steucek GL, Green PB (1992) Biophysical mechanisms for morphogenetic progressions at the shoot apex. Dev Biol 153: 29–43

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) 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: 9245–9249

Smith G, Hake S, Sylvester AW (1996) The *tangled-1* mutation alters cell division orientations throughout maize leaf development without altering leaf shape Development 122: 481–489

Steeves TA, Sussex IM (1989) Patterns in plant development. Cambridge University Press, Cambridge

Traas J, Bellini C, Nacry P, Kronenberger J, Bouchez D, Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375: 676–677