Limited Correlation between Expansin Gene Expression and Elongation Growth Rate[1]

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The aim of this work was to study the role of the cell wall protein expansin in elongation growth. Expansins increase cell wall extensibility in vitro and are thought to be involved in cell elongation. Here, we studied the regulation of two tomato (*Lycopersicon esculentum* cv Moneymaker) expansin genes, *LeExp2* and *LeExp18*, in rapidly expanding tissues. *LeExp2* was strongly expressed in the elongation zone of hypocotyls and in the faster growing stem part during gravitropic stimulation. *LeExp18* expression did not correlate with elongation growth. Exogenous application of hormones showed a substantial auxin-stimulation of *LeExp2* mRNA in etiolated hypocotyls and a weaker auxin-stimulation of *LeExp18* mRNA in stem tissue. Analysis of transcript accumulation revealed higher levels of *LeExp2* and *LeExp18* in light-treated, slow-growing tissue than in dark-treated, rapidly elongating tissue. Expansin protein levels and cell wall extension activities were similar in light- and dark-grown hypocotyl extracts. The results show a strong correlation between expansin gene expression and growth rate, but this correlation is not absolute. We conclude that elongation growth is likely to be controlled by expansin acting in concert with other factors that may limit growth under some physiological conditions.

Hypocotyls provide a model system for studying the basic processes of cell elongation because they grow rapidly in length, and this growth is under tight control of well-defined developmental and environmental signals (Gendreau et al., 1997). Light is one of the stimuli that reduce the growth rate of hypocotyls. All of the information available suggests that growth-modulation by the photomorphogenic pathway is repressed through photoreceptor-mediated action (for review, see Von Arnim and Deng, 1996; Chory, 1997). However, the downstream targets of photoreceptors leading to cell elongation in different light conditions are largely unknown.

It has been known for a long time that plant hormones are implicated in the control of hypocotyl elongation in some way (for review, see Davies, 1995). Brassinosteroids, auxin, and gibberellins stimulate hypocotyl growth, whereas cytokinins and abscisic acid have a growth inhibitory effect (Chaudhury et al., 1993; Jacobsen and Olszewski, 1993; Koornneef and Karssen, 1994; Zurek et al., 1994; Romano et al., 1995). Ethylene regulates hypocotyl elongation negatively in dark-grown seedlings (Smalle et al., 1997). There is good evidence that auxin is involved in mediating the effects of light and other environmental stimuli. In the tropic responses, lateral redistribution of auxin results in a differential growth rate leading to a curvature of the growing organ. After phototropic stimulation of etiolated coleoptiles, the reorientation toward the light correlates with an asymmetrical distribution of auxin (Kaufman et al., 1995). After gravitropic stimulation of soybean hypocotyls, McClure and Guilfoyle (1989) showed expression of small auxin up-regulated RNA (SAUR) genes on the lower side of the tissue.

A number of data suggest that auxin is involved in the light regulation of plant development, but the exact nature of this interaction is not well understood (Kraepiel and Miginiac, 1997). Behringer and Davies (1992) proposed that phytochrome regulation of stem elongation is partly the result of changes in auxin levels. It was observed by Tillberg (1974) and by Scott and Briggs (1963) that auxin levels in light-grown plants are higher than in dark-grown plants. But the opposite has been described as well (Fletscher and Zalik, 1964; Jones et al., 1991). Iino (1982) demonstrated a strong inhibitory effect of red light (R) on auxin biosynthesis in maize coleoptiles, associated with a decrease in mesocotyl elongation rate. Jensen et al. (1998) reported on the requirement of auxin transport for hypocotyl elongation in light-grown but not in dark-grown seedlings, suggesting that auxin has a more important role in elongation processes in the light. Light regulation of brassinolide (BL) levels or sensitivity clearly is an important player in light-regulated development, because mutants with defects in BL biosynthesis and response are severe dwarfs in both light and dark conditions (Bishop et al., 1996; Li et al., 1996; Szekeres et al., 1996).

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How are these developmental and environmental signals translated into molecular programs, leading to elongation growth? The cell wall is a key control point, as biophysical studies point out (Cosgrove, 1997). Its complex structure must withstand turgor pressure and, at the same time, allow cell expansion. For cell wall expansion to happen, three conditions must be met: (a) adequate turgor must exist inside the cell; (b) extensibility must be achieved through rearrangement or loosening of the existing cell wall; and (c) synthesis and deposition of newly formed wall components must occur. The acid growth theory suggests that the wall-loosening factor is hydrogen ions. Secreted protons decrease the apoplastic pH, and wall-loosening processes are thereby activated (for review, see Rayle and Cleland, 1992). The walls of expanding vegetative tissues possess numerous enzyme activities that may contribute to a modification of cell wall mechanical properties, including xyloglucan endotransglycosylases (XET, Fry et al., 1992), endo-1,4-β-glucanases (EGase, Hayashi et al., 1984), and expansins (McQueen-Mason et al., 1992). However, expansins are the only proteins known to date that promote cell wall extension in an in vitro assay (McQueen-Mason et al., 1993; Cosgrove and Durachko, 1994). They are thought to act by disrupting the hydrogen bonds between the cellulose microfibrils and the matrix polymers (McQueen-Mason and Cosgrove, 1994). The expansin proteins isolated from cucumber hypocotyls can account for most, if not all, of the acid-growth behavior of isolated walls (McQueen-Mason et al., 1992).

At present, the only data on expansin activity on living tissues was presented by Fleming et al. (1997, 1999) who placed expansin protein locally on the shoot apical meristem of tomato (*Lycopersicon esculentum* cv Moneymaker) and thereby induced a leaf-like structure. Expansin genes have been cloned from many different plant species and tissues (a detailed list is constantly updated by the Cosgrove laboratory under http://www.bio.psu.edu/expansins). Several classes of expansin genes exist. In the largest class, expansin gene expression or activity was found to be associated with the growing parts of the tissue or organ (McQueen-Mason et al., 1992; Keller and Cosgrove, 1995; Wu et al., 1996; Cho and Kende, 1997a, 1997b; Brummell et al., 1999). These data support a role of expansins in wall relaxation and in mediating extension growth. A different class of gene products appears to be involved in cell wall breakdown. One of these genes is the *LeExp1*, which was expressed during tomato fruit ripening rather than during fruit growth (Rose et al., 1997). Another class includes the *LeExp18* gene. It was shown to be locally up-regulated in the meristem at the incipient primordium position (Reinhardt et al., 1998). Together with the in vivo data on the expansin-induced leaf structures (Fleming et al., 1997, 1999), these studies suggest that wall loosening is important for organogenesis (Green, 1997).

In this paper we examine the expression of tomato *LeExp2*, the major expansin gene expressed in stems and hypocotyls, under a number of conditions that modulate growth. We find that *LeExp2,* but not *LeExp18*, mRNA levels correlate with growth rate in rapidly expanding hypocotyl tissue and during gravistimulation. We further find strong auxin-regulation of *LeExp2* mRNA levels. However, illumination of dark-grown hypocotyls, a treatment that inhibits hypocotyl growth, stimulates *LeExp2* expression. Expansin protein and extension activity was found to be equal in light- and dark-grown tissues. We conclude that expansins cannot be the sole regulators of extension growth.

RESULTS

Cloning of Genomic *LeExp2*

To obtain more information about possible mechanisms that regulate the expression of *LeExp2,* a genomic fragment was isolated. It contained approximately 2 kb of 5′-non-coding sequence, including a putative TATA box. The coding information of the *LeExp2* gene was contained within three exons and was identical to the *LeExp2* cDNA (Catalá et al., 2000). Figure 1A shows a schematic overview of the genomic clone, also depicting putative functional domains of expansin proteins as described by Cosgrove (1997). *LeExp2* is highly related to previously isolated tomato expansin genes (Fig. 1C).

Several putative auxin responsive elements and light responsive elements as well as a target site for a putative auxin-regulating factor, called Dof protein, were identified on the promoter of *LeExp2* based on homologies to functionally characterized elements (Fig. 1, A and D).

LeExp2 Transcript Levels Correlate with Growth Rates in Hypocotyls

Because expansins are thought to be involved in cell wall relaxation and thereby to promote hypocotyl elongation, we tested whether expansin gene expression correlated with the growth rate of the tissue. The growth rate of etiolated tomato seedlings was highest at the top of the hypocotyl and decreased rapidly toward the bottom or basal part (Fig. 2A). RNA gel-blot analysis showed that *LeExp2* mRNA levels were highest in the top segment, corresponding to the zone of elongation, lower in the middle segment, and even lower in the bottom segment. In the hook region, very little transcript was found (Fig. 2B). *LeExp18* mRNA abundance was very low throughout the hypocotyl with slightly higher levels in the top segment. To test if the differences in transcript abundance along the hypocotyl reflect differences in overall transcriptional activity or are specific to *LeExp2* we studied the ex-

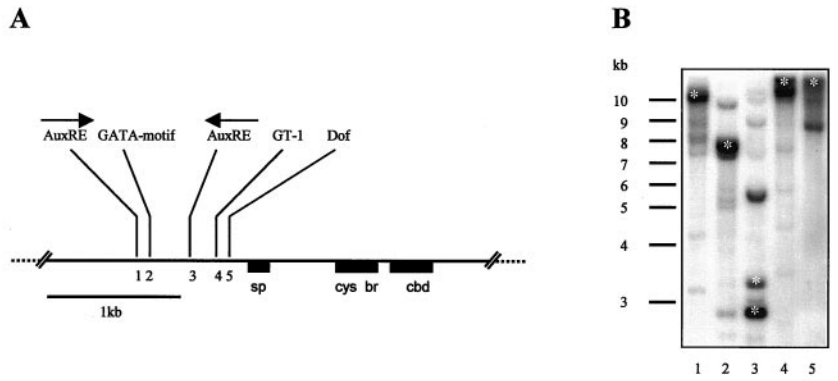

Figure 1. Schematic overview of the genomic *LeExp2* sequence. A, Numbers 1 to 5 represent putative cis-acting elements on the promoter. The exons are depicted as black rectangles, the arrows on the putative auxin-response element (AuxREs) depict the orientation of the elements with respect to the ATG. sp, Signal peptide; cys, Cys-rich region; br, basic region; cbd, cellulose-binding domain (according to Cosgrove, 1997). B, DNA gel-blot analysis of genomic DNA with a probe of 476 bp from the coding region of *LeExp2*. Genomic DNA was digested with *Eco*RI (lane 1), *Eco*RV (lane 2), *Hind*III (lane 3), *Xba*I (lane 4), or *Bam*HI (lane 5), respectively. When the blot was stripped and hybridized with a 3′ gene-specific probe, only the bands marked with an asterisk were visible (data not shown). C, The analysis of amino acid similarity was performed with the PAM-table program from WebGenetics (available at http://www.webgenetics.com). The expression patterns of these genes were analyzed either by northern blot or in situ hybridization. D, Numbers 1 to 5 correspond to the diagram in A. The upper DNA strand of the regulatory element shows its core sequence, the lower strand shows the sequence of *LeExp2*.

C

Gene	ORF (aa)	aa-similarity %	Expression pattern	References
LeExp2	**247**		**hypocotyl / stem**	**this article; Catalá et al., 2000**
Le Exp 1	261	86,8	late fruit	Rose et al., 1997
Le Exp 3	267	78,3	early fruit	Brummell et al., 1999
Le Exp 4	263	87,1	flower / fruit	Brummell et al., 1999
Le Exp 5	239	89,5	stem / flower / fruit	Brummell et al., 1999
Le Exp 18	260	84,2	I_1 in shoot meristem	Reinhardt et al., 1998

D

Relative position	Element	Core sequence LeExp2 genomic sequence	Position from ATG (+1)	Gene	References
1	AuxRE	5' TGTCAC 3' 5' ACGTTGTCACCTAC 3'	- 765	*PS/IAA 4/5*	Ballas N. et al., 1993
2	GATA-motif	5' ATGATAAGG 3' 5' TGAATGA--AAGGAAA 3'	- 703	pea *rbcS 3A*	Gilmartin P.M. et al., 1990
3	AuxRE (inverse complement)	5' GTGACA 3' 5' AGGGTGACACTG 3'	- 470	*PS/IAA 4/5*	Ballas N. et al., 1993
4	GT-1	5' GGTTAA 3' 5'TTATGGTTAATAAT 3'	- 230	tomato *rbcS*	Carrasco P. et al., 1993
5	Dof target site	5' ACTTTA 3' 5' TGGACACTTTAAACTTG 3'	- 165	*rolB*	Baumann K. et al., 1999

pression of the *rpl2* gene. We showed previously that this gene is a good indicator of general cellular activity (Fleming et al., 1993). *Rpl2* mRNA level was somewhat enhanced in the top segment, indicating an elevated total cellular activity in this part of the stem. Ribosomal RNA showed equal loading. Taken together, the *LeExp2* expression pattern correlates with the growth rate in hypocotyl tissue.

LeExp2 Transcript Levels Correlate with Growth Rates during Gravistimulation

To extend the experiments described in the previous section, we made use of another system, which depends on differential growth rates. During gravitropic stimulation the lower side of the stem tissue grows faster than the upper side, resulting in upward curvature. Four-week-old tomato plants were placed horizontally and after 30, 150, and 300 min, young stem tissue was split longitudinally in upper and lower halves and prepared for northern blotting using *LeExp2* as a probe (Fig. 3). In control stem sections, no difference in mRNA levels was apparent (Fig. 3, 0 min). However, a substantial decrease in the upper stem signal was observed after gravitropic treatment. The asymmetric distribution was most pronounced after 30 min of treatment when bending was barely visible in the very tip of the plant, compared with after 300 min when the youngest stem tissue was upright again. Hybridization with *LeExp18* did not show such a correlation of RNA level and growth rate. The transcript level of *LeExp18* generally decreased after gravitropic stimulation but no difference between the upper, slower growing tissue and the lower, faster growing tissue could be observed.

Thus, we conclude that the *LeExp2* transcript level correlates with growth rate, both in etiolated hypocotyls and in young stem tissue subjected to grav-

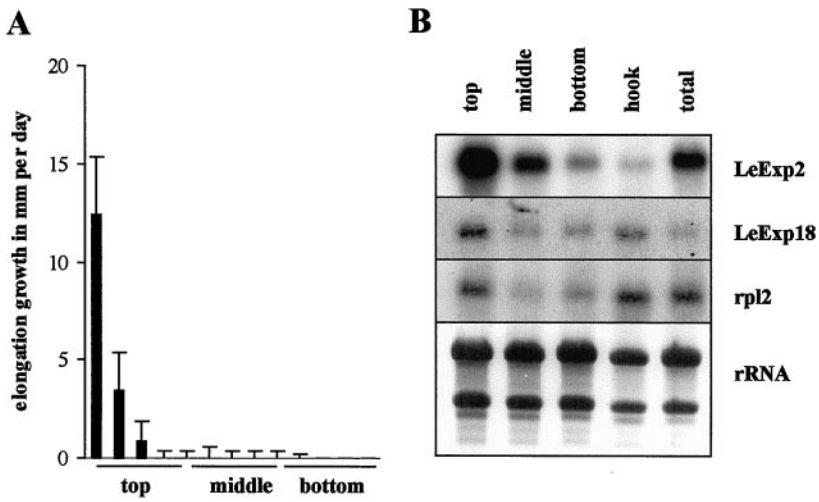

Figure 2. Expansin mRNA accumulation in the hypocotyl. A, Tomato hypocotyls were labeled with paint marks every 2 mm, and length increase was measured 24 h later. B, Lanes from left to right contain hypocotyl segments (1 cm) cut from the top, middle, and bottom regions, the hook region, or the total hypocotyl. Five micrograms of total RNA was separated per lane and hybridized with cDNA probes indicated on the right side. The lower panel represents an ethidium bromide gel and visualizes the ribosomal RNAs as a control for equal loading and intactness of the RNA.

itropic stimulation, as expected from the suggested role of expansins in wall relaxation. It is interesting to note that relative to non-gravistimulated controls, decreased mRNA levels in the upper side are observed rather than an increase in the lower part.

Hormonal Regulation of *LeExp2*

Phytohormones have been implicated in the control of hypocotyl elongation (for review, see Davies, 1995). Especially the ability of exogenous auxin to promote cell elongation in excised stem and hypocotyl segments has been studied extensively (Cleland, 1995). Therefore, we examined the effect of different hormones on the increase in hypocotyl segment length and a possible correlation with the expression pattern of expansins. Apical segments from etiolated tomato hypocotyls were pre-incubated for 2 to 3 h, followed by an incubation in buffer alone or buffer plus hormones for 16 h before the segment length was measured (Fig. 4A). The presence of BL resulted in the largest increase (21%), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) led to a decreased growth (−5%), gibberellic acid (GA_3) gave no growth stimulation, and treatment with 2,4-dichlorophenoxy-acetic acid (2,4-D), which is a synthetic auxin, resulted in an 11% length increase compared with the control. The growth changes measured were statistically significant in all of the cases except after GA_3 treatment, as determined by a Student's *t* test. This same system was used to analyze expansin transcript levels by RNA gel-blot analysis using apical segments of dark-grown hypocotyls (Fig. 4B). *LeExp2* transcript was present in untreated hypocotyls but was up to 15-fold more abundant in hypocotyls incubated with 2,4-D (see Fig. 8B). *LeExp2* mRNA expression was also enhanced following treatment with gibberellins, BL, and was slightly reduced by ACC. The same results were obtained using whole hypocotyls (data not shown).

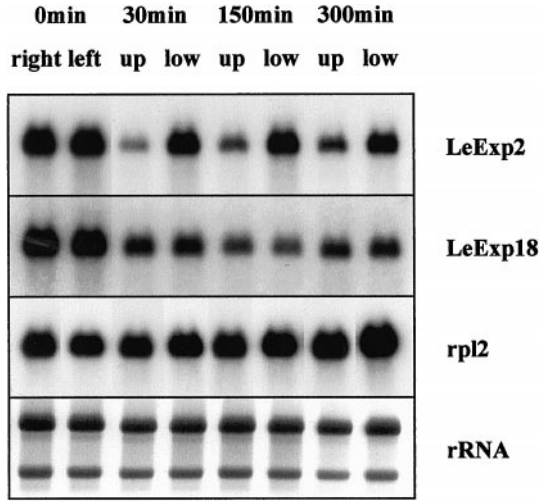

Figure 3. Expansin mRNA accumulation during gravitropic stimulation. Control stem segments were split in right and left halves before gravitropic stimulation (0 min). Gravistimulated plants were placed horizontally, and after 30, 150, and 300 min, stem segments were cut in upper (up) and lower (low) halves. Total RNA was isolated, and 10 μg was separated per lane. Hybridizations and controls were the same as in Figure 2.

The stimulation of *LeExp2* mRNA accumulation by auxin was examined in more detail. Maximal stimulation was obtained with 5 μM 2,4-D (data not shown). Time course experiments showed an increase in mRNA levels within 1 h and a maximum between 6 to 15 h (Fig. 5A). These data suggest that *LeExp2* mRNA increase is not only required for rapid growth responses but also participates in the cell wall changes involved in sustained cell elongation.

Additional auxins were tested for their ability to induce *LeExp2* mRNA. The response shown with 2,4-D could be mimicked by α-naphthalene acetic acid (α-NAA) but not with its non-functional analog β-NAA. No increase of *LeExp2* mRNA was found with indole-3-acetic acid, presumably due to the instability of this natural auxin. *LeExp18* mRNA was

found to be slightly induced by α-NAA (results not shown). The effect of extracellular pH changes on *LeExp2* transcript level was investigated. Activators (fusicoccin) and inhibitors (vanadate) of the plasma membrane H^+-ATPase, leading either to acidification or alkalinization of the extracellular medium, were used (Schaller and Oecking, 1999). We were further interested in a possible connection of cellulose synthesis and expansin action. Therefore, a herbicide (isoxaben) reducing cellulose synthesis was tested (Fisher and Cyr, 1998). For all three of the treatments, a drastic decline of *LeExp2* transcript level was observed (Fig. 5B). The fusicoccin application was repeated, using different concentrations. An inhibitory effect was seen with 10 and 1 μM but not with 100 or 10 nM (data not shown). Transcripts deriving from the control gene *rpl2* accumulated to a small degree after 2,4-D and α-NAA stimulation. This is likely to be a genuine induction, because the rRNA levels are constant in all of the lanes. It also suggests that

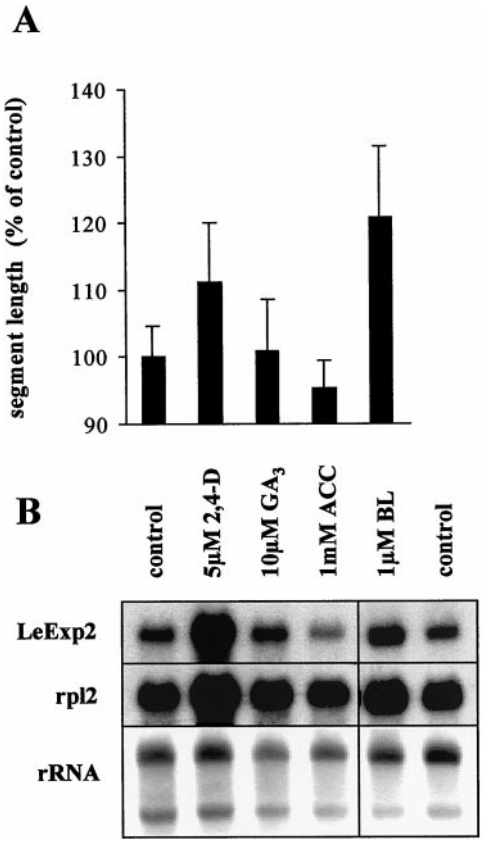

Figure 4. Effect of plant hormones on hypocotyl segment length elongation and on *LeExp2* mRNA accumulation. A, Segments (1 cm) were cut from the apical (top) region of etiolated tomato hypocotyls and incubated in buffer (control) or buffer plus 2,4-D, GA_3, ACC, or BL for 16 h. Segment length was measured after hormone treatment. The error bars represent the SDs. B, Northern-blot analysis of apical segments was performed after hormone treatment. Five micrograms of total RNA was separated per lane and hybridized with cDNA probes indicated on the left side. Controls were the same as in Figure 2.

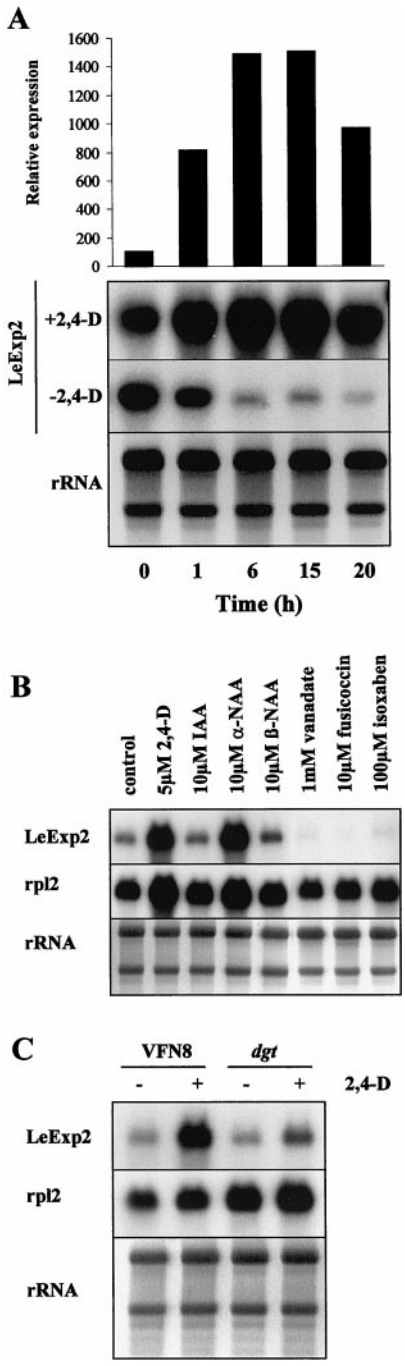

Figure 5. Time course analysis of 2,4-D and effect of auxin and other effectors on *LeExp2* mRNA accumulation in wild type and in the *dgt* mutant. A, Northern-blot analysis was performed after incubation of whole hypocotyls in buffer plus 5 μM 2,4-D for the indicated times. Bars in the top panel represent the 2,4-D-stimulated relative expression of *LeExp2* corrected by control levels (−2,4-D). B, Northern-blot analysis was performed after treatments. Whole hypocotyls were incubated in buffer (control) or buffer plus the indicated hormones or effectors. IAA, Indole-3-acetic acid; NAA, naphthalene-acetic acid. C, Northern-blot analysis was performed after auxin treatment. Stem segments from the auxin-insensitive mutant *dgt* and its corresponding wild type (cv VFN8) were incubated in buffer alone (−) or buffer plus 5 μM 2,4-D (+). Total RNA was isolated, and 10 μg was separated per lane. Hybridizations and controls were the same as in Figure 2.

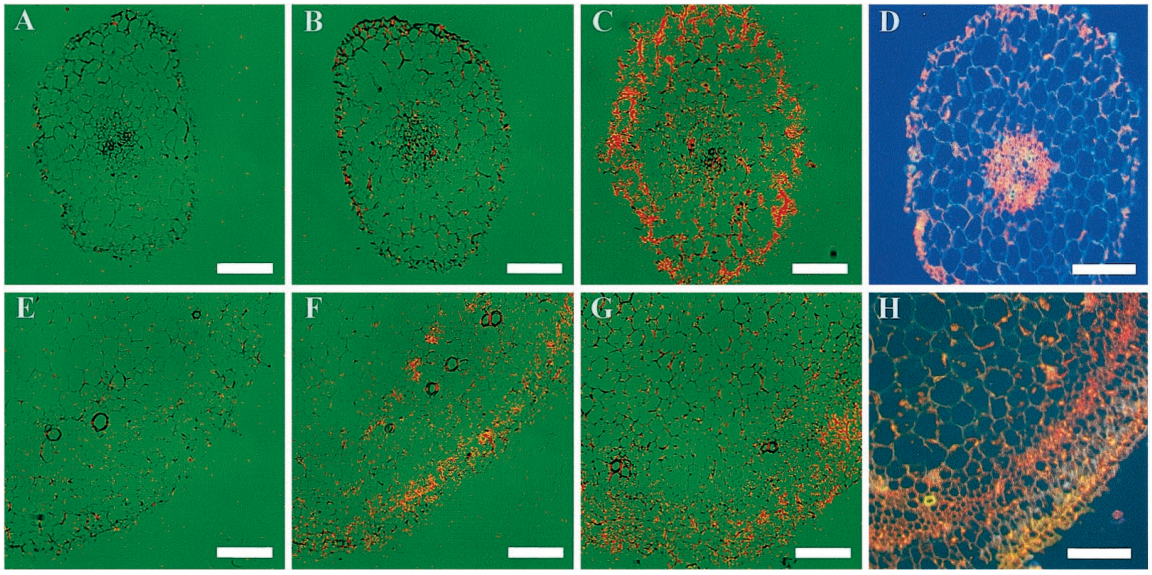

Figure 6. Localization of *LeExp2* mRNA transcript by in situ hybridization. Cross sections of apical segments from dark-grown hypocotyls are shown in A through D, cross sections of apical segments from light-grown stems in E through H. A and E represent control hybridizations with sense probes. B and F, Sections from untreated tissue were hybridized with the antisense probe; C and G, antisense hybridization of 2,4-D-treated tissue. Acridine orange staining is shown in D and H. Bar = 100 μm.

theeffects of the inhibitors are not due to a general transcriptional breakdown.

Finally, we tested the auxin-response of *LeExp2* in the *diageotropica (dgt)* mutant. It was shown previously that exogenously applied auxin leads to increased hypocotyl length in cv VFN8 (wild type) but not in *dgt* tomatoes (Kelly and Bradford, 1986). Furthermore, known auxin-inducible genes only show very low transcript expression after auxin-stimulation in the mutant compared with the control (Zurek et al., 1994; Mito and Bennett, 1995). Based on the finding that auxin enhanced expansin expression (Figs. 4B and 5A), we would expect a reduced stimulation of *LeExp2* RNA accumulation in the mutant tissue. Our results are consistent with this hypothesis. Auxin-dependent *LeExp2* transcript accumulation could be observed in the *dgt* mutant, but the induction was far less pronounced than in the isogenic wild-type line (Fig. 5C). These results indicate that a correlation exists between hormone-dependent elongation growth and *LeExp2* expression. However, the correlation is not absolute because although auxin treatment was the stronger inducer of expansin expression, the elongation growth of hypocotyl segments was highest following BL treatment.

To determine which cells within the stem and hypocotyl expressed expansin mRNA in situ hybridization using LeExp2 as a probe was carried out (Fig. 6). These experiments showed an apparently higher expression in the cortex and other small cells (Fig. 6, A, B, E, and F). Application of auxin led to a signal increase (Fig. 6, C and G). The distribution of the mRNA closely followed the distribution of the fluorescence after staining with acridine orange, a general stain for nucleic acids (Fig. 6, D and H; Fleming et al., 1993). From this we conclude that LeExp2 is expressed in all of the cells of the growing hypocotyls and stems with no obvious quantitative differences between cell types.

Light Regulation of Expansin Expression

It is well known that when dark-grown seedlings are exposed to light, the extension rate decreases drastically. However, the effect of light on expansin gene expression has not been studied so far. To determine the growth rate, we measured the total length of dark- and light-grown hypocotyls every 2nd d (Fig. 7A). The rate of extension between d 5 and 13, as calculated from the slopes of these growth curves, was 6-fold higher in the dark than in the light. The scanning electron microscopy (SEM) data (Fig. 7, B and C) clearly picture the morphological differences of epidermal tissue. Short cells with a hairy appearance were found in light-grown hypocotyls, whereas the etiolated seedlings consisted of extended cells with only a few trichomes. If this dark-induced growth were mediated solely by expansin, one would expect expansin gene expression to be dark-induced.

Northern-blot analysis using *LeExp2* as a probe was performed on dark-adapted and control hypocotyls and, contrary to expectation, this resulted in a higher level of mRNA in the light-grown control tissue (Fig. 8A). This repressive effect of dark treatment was also seen with *LeExp18* in hypocotyls (data not shown) and in stem tissue (see Fig. 10). When cDNAs encoding other cell wall proteins such as XET (*Xet*) and EGase (*Cel*) were used as probes, no dark induction

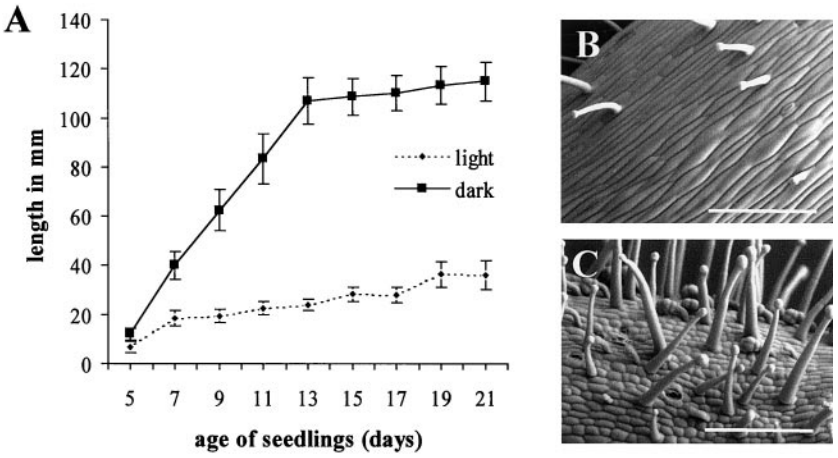

Figure 7. Hypocotyl elongation kinetics and morphology of light- and dark-grown seedlings. A, Hypocotyl length was measured from d 5 to 21 in light- and dark-grown tomato seedlings. The calculated growth rate was six times higher in dark-grown seedlings than in light-grown ones. B, SEM of the apical region in a dark-grown hypocotyl. Bar = 200 μm. C, SEM of the apical region in a light-grown hypocotyl. Bar = 200 μm.

was observed, and the mRNA levels were similar in both of the conditions. In the experiment of Figure 8A, seedlings were dark-adapted. The reverse experiment where dark-grown seedlings were exposed to light and RNA was extracted from total hypocotyls also showed higher *LeExp2* mRNA accumulation in the light (Fig. 8B, lanes 1 and 4). When light-grown and dark-grown hypocotyl extracts were compared by northern analysis, similar data were obtained (data not shown).

It was recent that two distinct genetic pathways controlling hypocotyl cell elongation in light- and dark-grown Arabidopsis seedlings were identified (Desnos et al., 1996). The fact that expansin mRNA accumulation is higher in the light could indicate its importance as a cell wall modifier, regulated by a light-specific pathway. In Figure 4B we found that auxin and to some extent BLs enhance *LeExp2* expression in the dark, but we do not know if this regulation is dependent on the light conditions. This does not seem to be the case. In etiolated hypocotyl tissue, the *LeExp2* expression was enhanced strongly by 2,4-D, and minimally with BL (Fig. 8B, lanes 5 and 6) as seen already in Figure 4B. In light-induced hypocotyl tissue, the *LeExp2* expression was increased after auxin treatment but not after BL treatment (Fig. 8B, lanes 2 and 3). The influence of these two hormones on *LeExp2* expression is very similar in either light condition, giving no evidence for separate light-dependent pathways of expansin regulation. In addition, auxin-treatment led to equal transcript induction of a tomato *gh3* homolog, a known auxin-responsive gene (Hagen et al., 1991) in light- and dark-grown seedlings.

To specify the light quality necessary for expansin transcript induction, R and far red-light (FR) treatments were performed on dark-grown seedlings, and total RNA was subsequently isolated from hypocotyls. Hybridization with *LeExp2* fragment resulted in a typical phytochrome response (Fig. 9A) where transcript level is induced after a 5-min R treatment and partly reversed by an additional 15-min FR light. Fifteen minutes of FR on its own led to some induction already. Five-minute R stimulated expansin transcript level to the same extent if not higher as a 24-h light treatment.

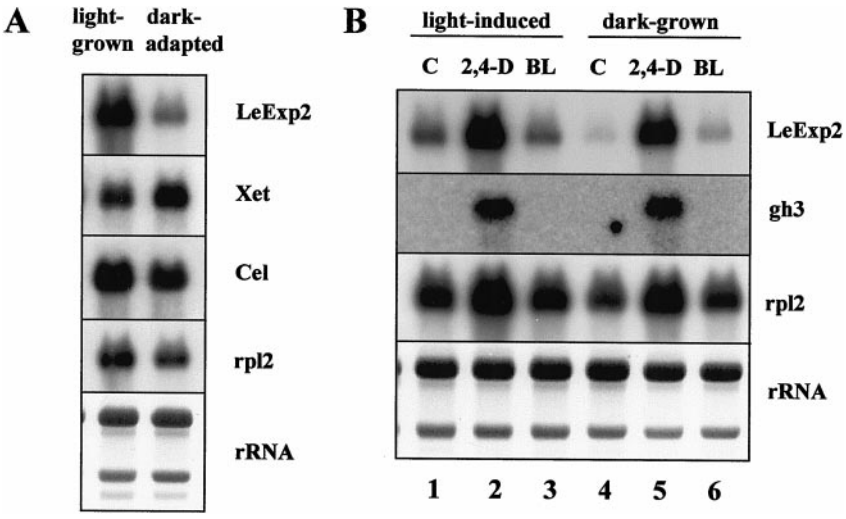

Figure 8. Effect of dark-adaptation and light-induction on *LeExp2*, *Xet*, *Cel*, or *gh3* mRNA accumulation in whole hypocotyls. A, One-half of light-grown seedlings remained in a 16- to 8-h light to dark cycle (light grown), and the other one-half was transferred to darkness for 3 d (dark adapted). B, One-half of dark-grown seedlings remained in the dark (dark grown), and the other one-half was light-induced for 24 h (light induced). Hypocotyls were incubated in buffer (C) or buffer plus 5 μM 2,4-D or 1 μM BL, respectively. Total RNA was isolated from hypocotyls, and 10 μg was separated per lane. Hybridizations and controls were the same as in Figure 2.

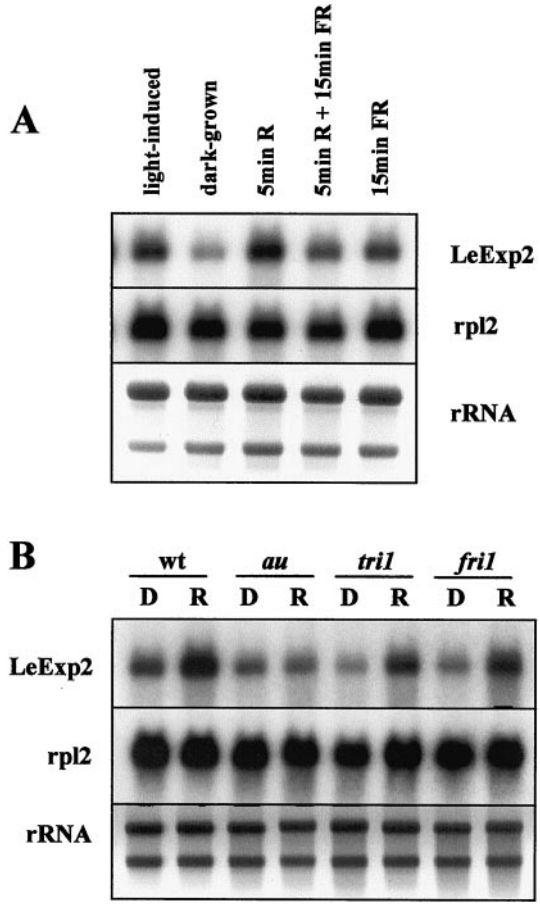

Figure 9. Effect of light quality on *LeExp2* mRNA accumulation in wild type and in phytochrome mutants. A, Dark-grown wild-type hypocotyls were 24-h light-induced or treated with 5-min R, 5-min R and 15-min FR treatment, or 15-min FR only and returned to darkness for 16 h. B, Dark-grown hypocotyls (D) were treated for 5 min with R in wild type (wt), and in the phytochrome mutants *aurea (au)*, *tri1*, and *fri1*. The R-treated seedlings were returned to darkness for 16 h. Total RNA was isolated, and 10 μg was separated per lane. Hybridizations and controls were the same as in Figure 2.

Mutants provide a useful tool for a complementary approach to understand the mechanism of the expansin light sensitivity. Three tomato phytochrome photoreceptor mutants were tested for their ability to induce *LeExp2* mRNA after R treatment (Fig. 9B): the chromophore mutant *aurea* (Terry and Kendrick, 1996), the B-like phytochrome mutant *tri* (Van Tuinen et al., 1995a), and the Phy A mutant *fri* (Van Tuinen et al., 1995b). Expansin transcript induction after R illumination was observed in wild-type hypocotyl tissue and in the Phy A- and Phy B-deficient mutants, but no response in the *aurea* mutant was detected. Taken together, these data indicate that the light induction of expansin expression is under phytochrome control. Phy A does not appear to be involved, but the situation with Phy B is more complex due to the presence of multiple *phyB* genes in tomato (Hauser et al., 1995).

Because we found that elongation growth correlates with expansin transcript level in hypocotyl tissue and also in stem tissue during gravitropism, we investigated the light-inducibility of expansins in stems (Fig. 10). Dark adaptation of young stem tissue resulted in a decrease of mRNA level of *LeExp2* and of *LeExp18* below detection limit (lanes 1 and 4). In Figure 8B we showed that expansin induction by hormones was very similar in light-induced and dark-grown seedlings. Strong auxin stimulation together with a slight BL stimulation for *LeExp2* mRNA was observed in light-induced and control seedlings. A comparable hormone induction was found for *LeExp2* in stem tissue grown in the light (Fig. 10, lanes 2 and 3). *LeExp18* expression was enhanced weakly following 2,4-D treatment and was decreased after BL treatment. However, in all of the dark-adapted samples, no signal was detected with *LeExp2* or *LeExp18* as a probe (Fig. 10, lanes 4–6). Ribosomal RNA and *rpl2* mRNA verified the presence of nucleic acids although with lower levels in the dark-adapted plants as seen with *rpl2* hybridization. A decrease of expansin expression level after dark adaptation does not only occur in hypocotyls but also in stem tissue. The hormonal induction of expansin expression in stem tissue could only be found in light-grown plants but not in dark-adapted plants.

From the above experiments it is clear that *LeExp2* mRNA levels in dark-grown and light-grown plants do not correlate with growth rate. However, due to post-transcriptional regulation of gene expression the

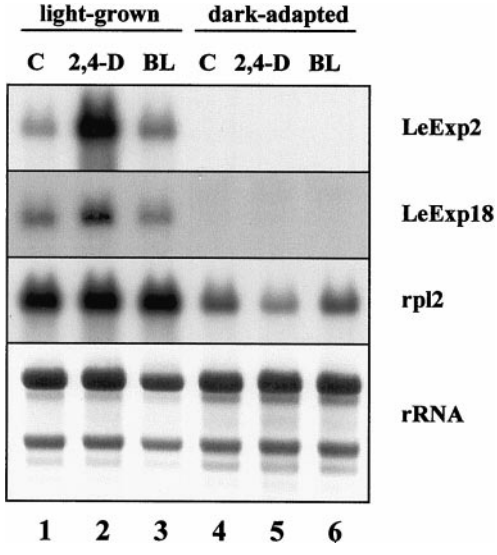

Figure 10. Effect of dark-adaptation on *LeExp2* and *LeExp18* mRNA accumulation in stem segments. One-half of 4-week-old plants remained in a 16- to 8-h light to dark cycle (light grown), and the other one-half was transferred to darkness for 3 d (dark adapted) prior to hormone treatments. Segments of 2-cm length were isolated from the growing region and were incubated in buffer (C), or buffer plus 5 μM 2,4-D or 1 μM BL, respectively. Total RNA was isolated, and 10 μg was separated per lane. Hybridizations and controls were the same as in Figure 2.

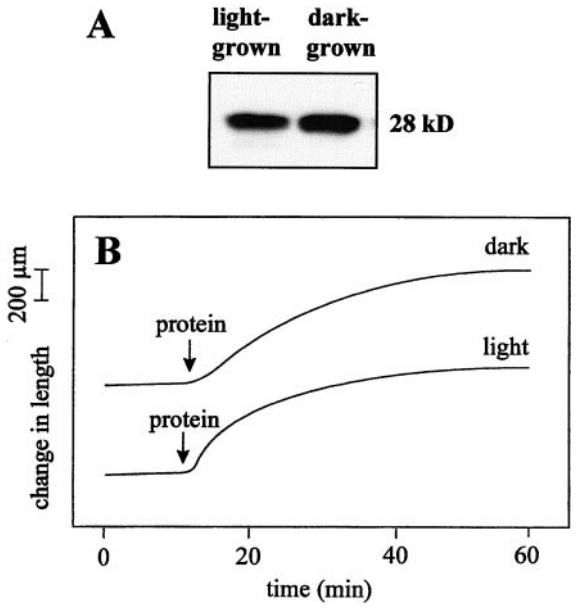

Figure 11. Expansin expression and cell wall extensibility of protein extracts from light- and dark-grown whole hypocotyls. A, Expansin protein expression in light- and dark-grown tomato hypocotyls. Ten micrograms of crude cell wall protein extract was loaded per lane and probed with anti-LeExp1 antibody. B, In vitro extension activity induced with light- and dark-grown protein extracts. Equal concentrations of crude cell wall proteins, isolated from light- and dark-grown tomato hypocotyls, were added to the extending material, as indicated, and length increase was recorded. The average expansin activity was 21.5 μm/min (SE 3.8 for dark-grown protein extract) and 25.5 μm/min (SE 4.9 for light-grown extracts). Values are the means ± SE of six measurements.

transcript levels might not correspond with expansin protein levels or expansin activities. Alternatively, the probe used in the northern blots might fail to detect a yet unidentified dark-induced expansin gene. A western blot using an antiserum generated against LeEXP1 gene product was performed (Fig. 11A). Extraction of crude cell wall proteins from hypocotyls of light- and dark-grown tomato seedlings and separation of equal amounts by SDS-PAGE resulted in a cross-reaction with hypocotyl expansins in both of the extracts. The antibody specifically recognized a 28-kD expansin protein band with similar intensities. Preimmune serum did not label the protein under the same conditions (data not shown). Crude cell wall proteins from hypocotyls were used for extension activity assays (Fig. 11B). The average expansin activity for dark-grown protein extracts was 21.5 μm/min (3.8 μm/min SE). For light-grown extracts the average expansin activity was 25 μm/min (4.9 SE). This showed that the extension was similar in extracts from light- and dark-grown seedlings, corresponding with the protein data. Despite the fact that a higher growth rate is detected in dark-grown hypocotyls, expansin protein levels and extension activities are similar under both light and dark conditions.

DISCUSSION

Expansins were discovered as proteins that have the ability to induce cell wall extension in vitro (McQueen-Mason et al., 1992). It is now well established that expansins are encoded by large multigene families. The individual gene members display distinctly different expression patterns, suggesting that they are involved in distinct processes. The fruit LeEXP1, for instance, is likely to be involved in the ripening-associated breakdown of cell walls (Rose et al., 1997). Most expansin genes, however, are thought to encode proteins that mediate cell expansion and tissue growth (Cho and Kende, 1997a). In a number of plant species, expansin activity was found to be associated with the growing parts of the tissues (McQueen-Mason et al., 1992; Keller and Cosgrove, 1995; Wu et al., 1996). The currently available data show a qualitative correlation between expansin expression and growth rate. In this study we performed a detailed analysis of the expression of the tomato *LeExp2* gene, the major expressed gene in hypocotyl and stem.

A good correlation of *LeExp2* mRNA levels was found in the growing zones of hypocotyls and during gravitropic stimulation. In the hypocotyls, the largest growth increase was seen at the top, little in the middle, and none in the bottom part. The transcript levels followed the same pattern, however, we still detected a signal in the bottom part where no growth at all occurred, indicating a slight disagreement (Fig. 2). During gravitropism, *LeExp2* transcript accumulation strongly correlated with the differential growth of stem tissue (Fig. 3). *LeExp18* mRNA levels did not correlate with growth, neither in the hypocotyl, where it was of very low abundance, nor during gravistimulation. This suggests that *LeExp18* is not a regulator of extension growth in stems and hypocotyls.

The growth-promoting hormones auxin and BL increased *LeExp2* transcript levels in the hypocotyl, whereas the precursor of the growth inhibitory hormone ethylene decreased *LeExp2* transcript level (Fig. 4). However, a closer examination of hormonal regulation of growth rate and *LeExp2* transcript level showed that the correlation is not quantitative. Auxin led to the strongest expansin induction, but segment length increased most after BL treatment. It has been shown previously that exogenously applied auxin and BL stimulated tomato hypocotyl growth with the latter having a stronger effect (Zurek et al., 1994). These data suggest that a different set of signaling events occurs in response to these growth regulators. Elongation growth might not only be promoted by auxin-induced expansins, XETs and EGases (Catalá et al., 1997), but by other factors under the control of brassinosteroids.

Light has a dramatic effect on elongation growth. We calculated the growth rate of hypocotyls and

found a 6 times higher rate in dark-grown tissue than in light-grown tissue. One would expect this enormous growth to be associated with higher levels of cell wall-loosening enzymes. A tomato EGase, expressed at high levels in rapidly expanding tissues, accumulated in etiolated hypocotyls but not in green hypocotyls (Brummell et al., 1997) as would be expected. Our results with tomato expansins, however, show exactly the opposite, a negative correlation with growth rate. We find more *LeExp2* mRNA in light-treated hypocotyls and stem tissue compared with dark-treated ones (Figs. 8 and 10). The two light responsive elements identified in the promoter may mediate this light induction. The unexpected data on *LeExp2* transcript levels could be explained by yet undiscovered expansin genes highly expressed in the dark, which would be responsible for the promotion of this higher growth rate in darkness. However, our efforts to identify dark-induced expansin genes remained unsuccessful, and we are confident that *LeExp2* is the major gene expressed in stems and hypocotyls. Analysis of protein levels revealed similar amounts of expansin protein in crude cell wall extracts from light- and dark-grown hypocotyls. The discrepancy between the mRNA and protein data could be explained by post-transcriptional regulation of *LeExp2* gene expression or by more efficient extraction of expansin proteins from light-grown tissue. Higher amounts of reactive oxygen species could be present in the light (Polle, 1997), which may reduce the extractable protein level in the light either through cross-linking to other wall components or by protein degradation. On the western blots, we could identify a lower band, possibly a degradation product, in the light-grown but not in the dark-grown protein extract when overexposing the x-ray films.

How could equal levels of expansin protein and activity give a 6-fold higher growth rate in the dark? We envisage three possibilities. First, additional cell wall proteins account for growth in the dark. Second, light-grown cell walls might be more rigid or less susceptible, thus requiring relatively more expansin. Third, other growth processes might replace extension growth in the light. In the first scenario, dark-induced elongation growth would not only be promoted by expansins but also by additional factors. The previously identified XETs and EGases are active on wall polymers, but they were shown not to have cell wall extension activity in vitro (McQueen-Mason et al., 1993; Cosgrove and Durachko, 1994). These and other proteins may therefore act in concert with expansins. According to the second scenario, cell walls from light-grown tissue would have different physical-chemical properties. Cell structure is obviously different in light and dark (Fig. 7, B and C; Cosgrove and Li, 1993), and it may be that light-grown walls have a thicker, more condensed or more highly cross-linked cell wall structure. Such a more rigid network would have a larger requirement for the growth-inducing expansins to achieve growth. Not only the cell wall composition could be slightly different in light- and dark-grown seedlings but also the sensitivity of the extracellular matrix toward expansin action. If the conditions in the light-grown cell walls were suboptimal (e.g. pH) or the walls less sensitive, relatively more expansins would be needed. The third possibility is that in the light other growth processes are more active, and thus, the reduction in extension growth rate may not be accompanied by a corresponding decrease in cell wall synthesis. These three possibilities are not mutually exclusive. In conclusion, our work shows that the expression of expansin genes in many cases does not correlate with observed rates of extension growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (*Lycopersicon esculentum* cv Moneymaker) plants were grown in a growth room at 25°C ± 2°C under various light conditions described below. The phytochrome mutants (*au*, *tri1*, and *fri1*) provided by M. Koornneef (Wageningen Agricultural University, The Netherlands) were in a cv Moneymaker background. The *dgt* mutant provided by R. Chetelat (Tomato Genetics Resource Center, University of California, Davis) was in a cv VFN8 background.

Seedlings were grown in covered plastic trays containing a moist irrigation mat used in gardening (Standard Bewässerungsmatte, Gartenbau Hack, Worb, Switzerland). Whole hypocotyls, 1-cm-long apical segments or 1-cm-long consecutive top middle and bottom segments, were dissected from 6- to 10-d-old seedlings by cutting directly below the apical hook in dark-grown seedlings or directly below the apical region in light-grown seedlings. Stem segments were dissected from the growing region of the main shoot (cv Moneymaker) of 4-week-old or from the side shoot (cv VFN8) of flowering plants grown on soil.

Plants were grown under a 16- to 8-h light to dark cycle "light-grown" or in the dark "dark-grown." For the dark adaptation experiments, light-grown material was transferred to the dark 3 d prior to the experiments. For the light-induction experiments, dark-grown material was illuminated for 24 h with constant white light (144 μmol m^{-2} s^{-1}) in a growth chamber prior to the experiments. For phytochrome experiments, dark-grown seedlings were illuminated with R (21 μmol m^{-2} s^{-1}) for 5 min and/or FR (4 μmol m^{-2} s^{-1}) for 15 min and returned to the dark for 16 h before hypocotyls were dissected and frozen for RNA isolation. The spectral distribution for R showed a single peak at 655 nm with one-half maxima at 648 and 662 nm. The FR spectrum peaked at 750 nm with one-half maxima at 710 and 780 nm. Control seedlings were grown in the dark.

Hypocotyl Measurements

For Figure 2A, dark-grown seedlings were carefully removed from the tray, and the hypocotyls were labeled

every 2 mm with a waterproof marker before they were placed back under dark conditions. This procedure was carried out under green safelight. After a 24-h growth period, the spacing between the original marks was measured. Fifty-six hypocotyls with an initial length ranging from 13 to 36 mm were analyzed in three independent experiments. Representative data of the average length increase from seven seedlings of 25-mm long hypocotyls is shown.

For the elongation kinetics of Figure 6A, the average values of light- and dark-grown seedlings were calculated from 51 to 129 total hypocotyl length measurements.

Hormone Treatments

Whole hypocotyls, 1-cm-long apical hypocotyl segments, and 2-cm-long stem segments cut 3 mm below the apical meristem were treated with hormones or other substances essentially as described by Catalá et al. (1997). The samples were incubated in a buffer containing 2.5 mM potassium phosphate (pH 6.0) and 2% (w/v) Suc for 2 to 3 h. The buffer was replaced with fresh buffer (control) or with buffer containing different hormones or alternatively with sodium-orthovanadate (Na_3VO_4, Sigma, St. Louis), fusicoccin (Sigma), or isoxaben (kindly provided by Dow-Elanco, Indianapolis). The samples were incubated with gentle agitation for 16 h overnight. All of the experiments using dark-grown or -adapted plants were carried out under green safelight, and all of the experiments using light-grown or light-induced plants were carried out under 16- to 8-h light to dark conditions. After the treatments, the tissue for RNA analysis was frozen in liquid nitrogen and stored at −80°C.

The length of 24 1-cm hypocotyl apical segments was measured after hormone treatment using a dissecting microscope with a stage micrometer. The data represent the average values from two independent experiments.

Gravitropism Experiment

At 0 min, stem segments of 5 cm were cut 5 mm below the apex of 4-week-old plants, soil-grown in square-shaped pots. The leaves were removed, and the stems were split longitudinally in two halves (left and right, controls) and frozen in liquid nitrogen for RNA isolation. The remaining plants were then placed horizontally, and after 30, 150, and 300 min, stem segments were split longitudinally, separated into upper and lower halves, and frozen in liquid nitrogen.

SEM

Freshly cut etiolated and light-grown hypocotyls were placed on a cool stage (−20°C) and observed in low vacuum (50 Pa) at 20 kV with an S-3500 N scanning electron microscope (Hitachi, Tokyo).

Genomic DNA Isolation and Analysis

The genomic clone was isolated by screening a library from tomato (cv VFN8) in λEMBL-3 at high stringency (65°C) with a partial *LeExp2* cDNA. Screening was performed according to Sambrook et al. (1989).

Sequence analysis and comparison were performed with the programs at WebGenetics (http://www.webgenetics.com). The total length of the genomic fragment is 4,919 bp, with 2,074 bp 5′ of the ATG and 1,529 bp 3′ of the stop codon. The sequence is available at the EMBL sequence database (accession no. AJ239068).

RNA and DNA Gel-Blot Analysis

Total RNA was isolated from tomato hypocotyls or stem tissue. In brief, plant material was ground in liquid nitrogen and extracted using hot extraction buffer (Phenol: 100 mM LiCl, 100 mM Tris [tris(hydroxymethyl)aminomethane]/HCl, pH 8.0, 10 mM EDTA, and 1% (w/v) SDS; 1:1, v/v). Chloroform:isoamylalcohol (0.5 volume, 24:1, v/v) was added. After centrifugation, the supernatant was precipitated in a first step with 1 volume of 4 M LiCl and in a second step with 3 M sodium-acetate, pH 5.2, in cold 100% ethanol. Aliquots of 5 or 10 μg were glyoxylated and then run on 1% (w/v) agarose gels before transfer to nylon membranes (Nytran, Schleicher & Schuell, Dassel, Germany). After deglyoxylation and fixation by baking and UV-light treatment, blotting and hybridization were carried out under standard conditions (Sambrook et al., 1989). Blots were hybridized at 65°C with randomly labeled probes as described below. The washing steps included 2× SSC, 0.1% (w/v) SDS at 65°C for 10 min followed by two washes in 0.1× SSC, 0.1% (w/v) SDS at 65°C for 10 min each. The blots were exposed to x-ray film. To measure the relative *LeExp2* expression shown in Figure 5A, the signal intensities were quantified on a molecular imager (model GS525, Bio-Rad Laboratories, Glattbrugg, Switzerland). Equal loading and integrity of the ribosomal RNA were confirmed by ethidium bromide stained Tris-borate/EDTA gels made in parallel to those used in the hybridizations. All of the treatments, RNA extractions, and northern blots were repeated at least once, and representative data are shown.

High M_r total genomic DNA was isolated from young tomato plants, as described by Reinhardt et al. (1998). Aliquots of 10 μg were digested with *Eco*RI, *Eco*RV, *Hind*III, *Xba*I, or *Bam*HI, run on a 0.75% (w/v) agarose gel, and transferred to a nylon membrane (Nytran N, Schleicher & Schuell). Blotting and hybridization procedures were performed under standard conditions (Sambrook et al., 1989). Blots were hybridized at 65°C and washed in 6× SSC, 0.1% (w/v) SDS for 7 min, then in 2× SSC, 0.1% (w/v) SDS for 7 min, and finally in 1× SSC, 0.1% (w/v) SDS for 5 min. All of the washing steps were carried out at 65°C.

cDNA Clones Used for Northern and Southern Blotting

Two fragments of the *LeExp2* gene were used as probes. The first probe was generated by PCR using the primers 5′-GGTGGAGCTTGTGGGTAT and 5′-TGGCCCCAATTTCTAGAC. This probe covers 476 bp of the coding region and, to a large extent, overlaps with the probe used by

Catalá et al. (2000). Since our probe reacts with multiple bands in a genomic Southern blot (Fig. 1B), a gene-specific probe from the 3′-untranslated region of *LeExp2* was generated. A 248-bp fragment was amplified by PCR using the oligonucleotides 5′-GGCATAAAAGGGGTGAGTA and 5′-CATGATGACATTAAGTTGCCC as primers. This probe hybridizes to unique bands in four of five digests (asterisks in Fig. 1B). All of the northern blots with the exception of Figures 2 and 5B were hybridized sequentially with both of the probes with essentially identical results. Results obtained with the probe from the coding region are shown. An extensive search by RT-PCR and library screening did not uncover additional expansin cDNAs expressed in stems or hypocotyls, indicating that no other genes are expressed at appreciable levels in these tissues. The cDNA of *LeExp18* consisted of the 3′-untranslated region and was described by Reinhardt et al. (1998). Fleming et al. (1993) described the cDNA of *rpl2*. RT-PCR on RNA isolated from etiolated tomato hypocotyl treated with 2,4-D was used to generate *Xet*, *Cel*, and *gh3* probes. Degenerate PCR primers were designed from conserved regions of tomato *Xet* sequences: *LeXET* (D16456), *Le-tXET-B1* (X82685), and *Le-tXET-B2* (X82684) and comprised 5′-GCGGATCCGCAGRGCAYGAYGARATWGATT-3′ and 5′-GCAAGCTTGTAYTTYTGMYGAACCCAWCG-3′. A 530-bp cDNA fragment was amplified and subcloned in pBluescript SK^- (Strata-gene, La Jolla, CA). Degenerate PCR primers for *Cel* were designed from tomato *Cel1* (U13054), *Cel2* (U13055), and *Cel4* ((U20590) and comprised 5′-GCGGATCCWCWMARAATGTCATAYATGG-3′ and 5′-GCAAGCTTCATTMAYRTAWGTBGYRGGYTC-3′. A 260-bp cDNA fragment was amplified and subcloned in pBluescript SK^- (Stratagene). Degenerate PCR primers for *gh3* were designed from tobacco *Nt-gh3* (Roux and Perrot-Rechenmann, 1997) and soybean *Gm-gh3* (Hagen et al., 1991) and comprised 5′-GCGGATCCARAGYATGTAYACYCAAATG-3′ and 5′ GCAAGCTTCATGTTTGGCATGATKGTG-3′. A 470-bp cDNA fragment was amplified and subcloned in pBluescript SK^- (Stratagene). All of the probes were randomly labeled using a Rediprime I or II kit (Amersham, Buckinghamshire, UK).

In Situ Hybridization Analysis of *LeExp2* Gene Expression

In situ hybridization experiments were performed according to the protocol described by Fleming et al. (1993) but with minor modifications. From dark-grown hypocotyls and light-grown stems, treated either with or without hormones, we cut transverse sections (7 μm) through the growing region and used them for in situ hybridization. Hybridization was performed overnight at 50°C with sense and anti-sense probes of LeExp2, labeled with [α-^{33}P]rUTP (Hartmann Analytic GmbH, Braunschweig, Germany). The slides were exposed in autoradiography emulsion (NTB-2, Eastman-Kodak, Rochester, NY) for 7 weeks prior to processing in developing (D-19, Eastman-Kodak) and fixation solutions (x-ray fixer AL-4, Eastman-Kodak).

After development, the slides were stained with toluidine blue and viewed on an LSM 310 microscope (Carl Zeiss AG, Jena, Germany). Images were taken under bright-field light (shown in false green color) and overlaid with images taken by side illumination with white light, exhibiting the silver-grain signal (shown in false red color). Some slides were not subjected to hybridization but were stained with a 0.01% solution of acridine orange for 1 min to assess the amount of total RNA in the tissues.

Protein Extraction and Immunoblotting

Crude cell wall proteins were isolated from light-grown and dark-grown tomato hypocotyl tissue. The isolation procedure is modified from McQueen-Mason et al. (1992). Whole hypocotyls were dissected and placed into 25 mM Tris/HCl, pH 7.5, 0.1% (v/v) Triton X-100 kept on ice either on the bench or under green safelight depending on the experimental light conditions. The material was homogenized with a blender, wall fragments were retained by filtration through 50-μm mesh-nylon membranes and thoroughly washed in 25 mM Tris/HCl, pH 7.5. Proteins were solubilized from washed wall fragments in 25 mM Tris/HCl, pH 7.5, 1 M NaCl and precipitated by addition of 390 g/L of $(NH_4)_2SO_4$. The precipitate was resuspended in 15 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.5, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnSO_4$, and desalted on a Sephadex G-25 column in the same buffer.

Crude cell wall protein concentration was determined using the Bradford assay (Bio-Rad Laboratories), and equal amounts were separated on a 12% SDS-PAGE gel. Similar amounts of total cell wall protein were extracted per cell wall mass, based on fresh weight. After transfer to Nitrocellulose (Schleicher & Schuell) and exposure to 1:1,500 diluted rabbit anti-LeEXP1 antiserum and 1:20,000 diluted anti-rabbit HRP-conjugated antibody, the signal was detected using a chemiluminescent system (Super Signal Substrate, Pierce Chemical, Rockford, IL). The antiserum to LeEXP1 was generated in rabbits and was raised to the deduced mature LeEXP1 polypeptide expressed in *Escherichia coli* as a fusion protein with a His tag at the N terminus. Two-dimensional gel electrophoresis was performed on both of the light- and dark-grown cell wall hypocotyl protein extracts. It was blotted onto nitrocellulose and was probed with the anti-LeEXP1 antiserum. One major spot on each blot suggests that the antibody is likely to recognize one protein isoform (J.K.C. Rose, unpublished data).

Extension Assay

Expansin assays were carried out using a custom-made extensometer as described by McQueen-Mason et al. (1992). Extension experiments were carried out on a composite material derived from the pellicle produced by growing *Acetobacter xylinum* in the presence of soluble tamarind xyloglucan, as described by Whitney et al. (1995). Pieces of pellicle, 2 mm wide and 10 mm long, were cut by hand using a new razor blade. Slices of pellicle were pressed between two microscope slides coated with laboratory tissue under a weight of 300 g for 5 min to remove

excess liquid and assist in handling the material. These partially dried slices were then hung in the extensometer and extended under a constant load of 11 g. The extending material was initially bathed in 50 mM sodium acetate, pH 4.5. After about 10 min of extension, the bathing solution was replaced with one containing equal amounts of protein from either dark- or light-grown whole tomato hypocotyls in the same buffer and extension monitored for a further 50 min. Expansin activity was found to be concentration dependent on this substrate. The total cell wall protein concentration used varied between 2 to 3 μg/μL, and this was well below saturation level. XETs and EGases have no extension activity on this substrate (S. McQueen-Mason, unpublished data). Expansin activity was calculated as the rate of extension of the material in the 10-minute period after protein addition, minus the rate of extension before protein addition. Figure 10B shows representative extension traces for proteins isolated from dark- and light-grown plants. Six replicate measurements were made for both of the treatments with 100-μL volume each. Over-all experiments were repeated three times.

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LITERATURE CITED

Ballas N, Wong L-M, Theologis A (1993) Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J Mol Biol **233:** 580–569

Baumann K, De Paolis A, Costantino P, Gualberti G (1999) The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the *rolB* oncogene in plants. Plant Cell **11:** 323–333

Behringer FJ, Davies PJ (1992) Indole-3-acetic acid levels after phytochrome-mediated changes in the stem elongation rate of dark- and light-grown *Pisum* seedlings. Planta **188:** 85–92

Bishop GJ, Harrison K, Jones JDG (1996) The tomato *dwarf* gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. Plant Cell **8:** 959–969

Brummell DA, Bird CR, Schuch W, Bennett AB (1997) An endo-1,4-β-glucanase expressed at high levels in rapidly expanding tissue. Plant Mol Biol **33:** 87–95

Brummell DA, Harpster MH, Dunsmuir P (1999) Differential expression of expansin gene family members during growth and ripening of tomato fruit. Plant Mol Biol **39:** 161–169

Carrasco P, Manzara T, Gruissem W (1993) Developmental and organ-specific changes in DNA-protein interactions in the tomato *rbcS3B* and *rbcS3C* promoter regions. Plant Mol Biol **21:** 1–15

Catalá C, Rose JKC, Bennett AB (1997) Auxin regulation and spatial localization of an endo-1,4-β-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. Plant J **12:** 417–426

Catalá C, Rose JKC, Bennett AB (2000) Auxin-regulated genes encoding cell wall modifying proteins are expressed during early tomato fruit growth. Plant Physiol **122:** 527–534

Chaudhury AM, Letham S, Craig S, Dennis ES (1993) *amp1*: a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J **4:** 907–916

Cho HT, Kende H (1997a) Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell **9:** 1661–1671

Cho HT, Kende H (1997b) Expansins and internodal growth of deepwater rice. Plant Physiol **113:** 1145–1151

Chory J (1997) Light modulation of vegetative development. Plant Cell **9:** 1225–1234

Cleland RE (1995) Auxin and cell elongation. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry, and Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 214–227

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

Cosgrove DJ, Durachko DM (1994) Autolysis and extension of isolated walls from growing cucumber hypocotyls. J Exp Bot **45:** 1711–1719

Cosgrove DJ, Li Z-C (1993) Role of expansin in cell enlargement of oat coleoptiles. Plant Physiol **103:** 1321–1328

Davies PJ (1995) Plant Hormones: Physiology, Biochemistry, and Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands

Desnos T, Orbovic V, Bellini C, Kronenberger J, Caboche M, Traas J, Höfte H (1996) *Procuste 1* mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. Development **122:** 683–693

Fisher DD, Cyr RJ (1998) Extending the microtubule/microfibril paradigm. Plant Physiol **116:** 1043–1051

Fleming AJ, Caderas D, Wehrli E, McQueen-Mason S, Kuhlemeier C (1999) Analysis of expansin-induced morphogenesis on the apical meristem of tomato. Planta **208:** 166–174

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

Fleming AJ, McQueen-Mason S, Mandel T, Kuhlemeier C

(1997) Induction of leaf primordia by the cell wall protein expansin. Science **276:** 1415–1418

Fletscher RA, Zalik S (1964) Effect of light quality on growth and free indoleacetic acid content in *Phaseolus vulgaris*. Plant Physiol **39:** 328–331

Fry SC, Smith RC, Renwich KF, Martin DJ, Hodge SK, Matthews KJ (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem J **282:** 821–828

Gendreau E, Traas J, Desnos T, Grandjean O, Caboche M, Höfte H (1997) Cellular basis of hypocotyl growth in *Arabidopsis thaliana*. Plant Physiol **114:** 295–305

Gilmartin PM, Sarokin L, Memelink J, Chua N-H (1990) Molecular light switches for plant genes. Plant Cell **2:** 369–378

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

Hagen G, Martin G, Li Y, Guilfoyle TJ (1991) Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. Plant Mol Biol **17:** 567–579

Hauser BA, Cordonnier-Pratt M-M, Daniel-Vedele F, Pratt LH (1995) The phytochrome gene family in tomato includes a novel subfamily. Plant Mol Biol **29:** 1143–1155

Hayashi T, Wong Y-S, Maclachlan G (1984) Pea xyloglucan cellulose: II. Hydrolysis by pea endo-1,4-β-glucanases. Plant Physiol **75:** 605–610

Iino M (1982) Action of red light on indole-3-acetic-acid status and growth in coleoptiles of etiolated maize seedlings. Planta **156:** 21–32

Jacobsen SE, Olszewski NE (1993) Mutations at the *spindly* locus of *Arabidopsis* alter gibberellin signal transduction. Plant Cell **5:** 887–896

Jensen PJ, Hangarter RP, Estelle M (1998) Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown *Arabidopsis*. Plant Physiol **116:** 455–462

Jones AM, Cochran DS, Lamerson PM, Evans ML, Cohen JD (1991) I. Changes in the abundance of indoleacetic acid and a 22-kilodalton auxin-binding protein in the maize mesocotyl. Plant Physiol **97:** 352–358

Kaufman PB, Wu L-L, Brock TG, Kim D (1995) Hormones and the orientation of growth. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry, and Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 547–570

Keller E, Cosgrove DJ (1995) Expansins in growing tomato leaves. Plant J **8:** 795–802

Kelly MO, Bradford KJ (1986) Insensitivity of the *diageotropica* tomato mutant to auxin. Plant Physiol **82:** 713–717

Koornneef M, Karssen CM (1994) Seed dormancy and germination. *In* EM Meyerowitz, CR Somerville, eds, Arabidopsis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 313–334

Kraepiel Y, Miginiac E (1997) Photomorphogenesis and phytohormones. Plant Cell Environ **20:** 807–812

Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science **272:** 398–401

McClure BA, Guilfoyle T (1989) Rapid redistribution of auxin-regulated RNAs during gravitropism. Science **243:** 91–93

McQueen-Mason S, Cosgrove D (1994) Disruption of hydrogen bonding between wall polymers by proteins that induce plant wall extension. Proc Natl Acad Sci USA **91:** 6574–6578

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

McQueen-Mason S, Fry SC, Durachko DM, Cosgrove D (1993) The relationship between xyloglucan endotransglycosylase and in vitro cell wall extension in cucumber hypocotyls. Planta **190:** 327–331

Mito N, Bennett AB (1995) The *diageotropica* mutation and synthetic auxins differentially affect the expression of auxin-regulated genes in tomato. Plant Physiol **109:** 293–297

Polle A (1997) Defense against photooxidative damage in plants. *In* JG Scandalios, ed, Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 623–666

Rayle DL, Cleland RE (1992) The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiol **99:** 1271–1274

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

Romano CP, Robson PRH, Smith H, Estelle M, Klee H (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy6-1* hypocotyl elongation and *axr1* auxin-resistant mutants. Plant Mol Biol **27:** 1071–1083

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

Roux C, Perrot-Rechenmann C (1997) Isolation by differential display and characterization of a tobacco auxin-responsive cDNA *Nt-gh3*, related to *GH3*. FEBS Lett **419:** 131–136

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schaller A, Oecking C (1999) Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. Plant Cell **11:** 263–272

Scott TK, Briggs WR (1963) Recovery of native and applied auxin from the dark-grown "Alaska" pea seedling. Am J Bot **50:** 652–657

Smalle J, Haegman M, Kurepa J, van Montagu M, van der Straeten D (1997) Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proc Natl Acad Sci USA **94:** 2756–2761

Szekeres M, Németh K, Koncz-Kàlmán Z, Mathur J, Kauschmann A, Altmann T, Rédei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of

CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell **85:** 171–182

Terry MJ, Kendrick RE (1996) The *aurea* and *yellow-green-2* mutants of tomato are deficient in phytochrome chromophore synthesis. J Biol Chem **271:** 21681–21686

Tillberg E (1974) Levels of indol-3yl-acetic acid and acid inhibitors in green and etiolated bean seedlings (*Phaseolus vulgaris*). Physiol Plant **31:** 106–111

Van Tuinen A, Kerckhoffs HJ, Nagatani A, Kendrick RE, Koornneef M (1995a) A temporarily red light-insensitive, mutant of tomato lacks a light-stable, B-like phytochrome. Plant Physiol **108:** 939–947

Van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koornneef M (1995b) Far-red light-insensitive, phytochrome A-deficient mutants of tomato. Mol Gen Genet **246:** 133–141

Von Arnim A, Deng X (1996) Light control of seedling development. Annu Rev Plant Physiol Plant Mol Biol **47:** 215–243

Whitney SEC, Brigham JE, Darke AH, Reid G, Gidley MJ (1995) *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. Plant J **8:** 491–504

Wu Y, Sharp RE, Durachko DM, Cosgrove DJ (1996) Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity, and wall susceptibility to expansins. Plant Physiol **111:** 765–772

Zurek DM, Rayle DL, McMorris TC, Clouse SD (1994) Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid-regulated stem elongation. Plant Physiol **104:** 505–513