

## C 4 . Expansins as Agents in Hormone Action

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### INTRODUCTION

Expansins are wall-loosening proteins implicated in plant responses to most of the major plant hormones. These include cell enlargement, cell proliferation, fruit softening, abscission, senescence, and adaptation to water stress - all hormone-controlled responses in which the cell wall is modified so as to make it more extensible or softer or more easily separated from other walls.

In this chapter<sup>1</sup> we summarize the current evidence implicating expansins in the action of various hormones, beginning with a short summary of the characteristics of this protein and its likely mechanism of action.

### EXPANSIN DISCOVERY, STRUCTURE AND MODE OF ACTION

Expansins were originally identified in studies of acid-induced extension of isolated plant cell walls. When cell walls are taken from the growing region of plant tissues and clamped at constant tension in an extensometer, they extend in a pH-dependent manner, with low pH inducing faster extension. This pH-dependent extension is eliminated with a brief heat treatment and can be restored by addition of a crude mixture of wall proteins (Fig. 1). Upon purification, the active proteins in this mixture proved to be ~27 kD proteins, that are now called  $\alpha$ -expansins (12, 15).

From the fully-sequenced genomes of Arabidopsis and rice, we now know that the  $\alpha$ -expansin (EXP) gene family is relatively large, with 26 genes in Arabidopsis and 32 genes in rice. A second family of expansins,

Abbreviations: 1-MCP, 1-methylcyclopropene; ACC, Aminocyclopropan-1-carboxylic acid; BR, Brassinolide; NPA, Naphthylphthalamic acid; RHE, Root hair element, the *cis*-element for root hair specificity; RHF, Root hair factor, the putative transcription factor for root hair specificity; SAM, Shoot apical meristem.

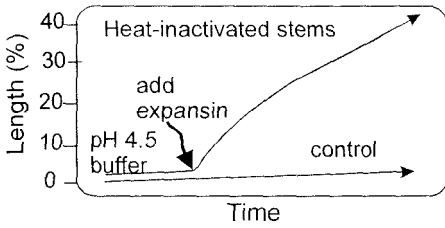


Fig. 1. Scheme for reconstituting acid-induced extension of cell walls. Hypocotyl walls are heated to inactivate the endogenous wall-loosening proteins, then protein extracted from other walls is added back and extension is measured in a constant-load extensometer.

called  $\beta$ -expansins (EXPB), was later identified on the basis of sequence similarity and expansin-like loosening effects on grass cell walls (14). This family consists of 6 genes in Arabidopsis and 19 genes in rice (see web site at <http://www.bio.psu.edu/expansins/>). The rice and Arabidopsis genomes also contain a third group of related genes called expansin-like (EXPL) and expansin-related (EXPR). This group is only

known from its sequence and from expression (microarray) data; the proteins have not yet been isolated nor have their activities been characterized, so it is premature to call them expansins.

Although  $\alpha$ - and  $\beta$ -expansins share only ~20% identity in protein sequence, they are structurally homologous to each other, each consisting of two domains (Fig. 2). Domain 1 is distantly related to the glycosyl hydrolase family-45 (GH45, see the CAZY web site at <http://afmb.cnrs-mrs.fr/CAZY/>). Much, but not all, of the active site for GH45 enzymes is conserved in domain 1 of expansins. Domain 2 may be a polysaccharide binding module, but this notion is still very speculative.

The two bona fide families of expansins ( $\alpha$ - and  $\beta$ -) have similar effects on cell walls, namely rapid induction of wall extension and stress relaxation, but they act on different polysaccharides in the cell wall. This conclusion is

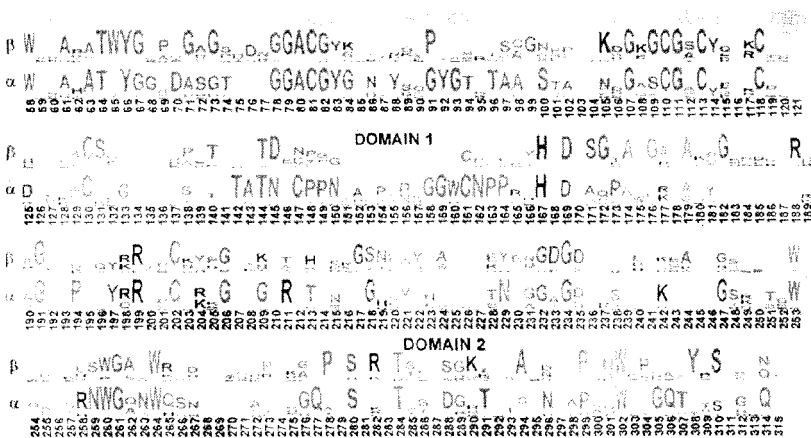


Figure 2 (Color plate page CP7). Alignment of sequence logos for  $\alpha$ -expansin and  $\beta$ -expansin. Sequence logos show the magnitude of conservation by the size of the letters (on a log scale) and this alignment shows the conserved residues shared by both families of expansins. Each logo was made from an alignment of 16 sequences (15).

based on the stronger effect of  $\alpha$ -expansins on dicot walls in extension assays, as compared with their action on grass walls, whereas the reverse is true for  $\beta$ -expansins, whose action is more marked on grass walls. Grass walls are notable for their relatively high content of arabinoxylan and reduced amounts of xyloglucan and pectin (3), and this difference in wall composition presumably accounts for the different sensitivities of dicot and grass cell walls to  $\alpha$ - and  $\beta$ -expansins.

The  $\beta$ -expansins that have been studied in most detail make up a subgroup known in the immunological literature as group-1 allergens from grass pollen. These proteins are very abundant in grass pollen and they probably loosen the cell walls of the grass stigma and style, thereby promoting pollen tube penetration of the stigma and growth towards the ovule. They might also function in the separation of the pollen grains in the anther. The pollen allergen subgroup is distinctive in sequence from the remainder of the p-expansins, which have sometimes been called "vegetative  $\beta$ -expansins", to distinguish them from the pollen allergen class. The  $\beta$ -expansins from maize pollen have a pH optimum of  $\sim 5.5$ , which is notably different from the acidic optimum (pH  $< 4.5$ ) found for  $\alpha$ -expansins. These pollen allergens therefore do not appear to function in acid-induced growth. Whether this is also generally true for the vegetative  $\beta$ -expansins has not yet been tested, but we suspect that they have an optimum more compatible with acid growth, based on the wall extension behavior of oat and maize coleoptiles (29).

The large number of genes in the two families implies redundancy or specialization of the biological function for the different genes. In *Arabidopsis* most of the  $\alpha$ -expansin genes are expressed in specific, distinctive cell types. This observation fits well with the idea that the different genes within a family differ principally in where and when they are expressed (that is, their promoters have differentiated from each other), but not in their loosening effect on the cell wall. However, this hypothesis still needs to be tested directly.

Expansins have a characteristic loosening action on the cell wall. In extension tests under constant load ("creep" tests), expansins rapidly induce wall extension, i.e., the wall begins to extend rapidly within 1-2 min. Expansins also enhance wall stress relaxation over a time range of 100 ms to  $>100$  s. However,  $\alpha$ -expansins do not change the plasticity or elasticity of the cell wall (41). These effects on wall mechanics appear to be unique to expansin.

The precise mechanism of wall loosening by expansin is still a bit mysterious. The major matrix polymers of the cell wall are not hydrolyzed or otherwise modified, as far as we can detect. Despite a report that pollen-allergen-type  $\beta$ -expansins have protease activity, this could not be confirmed by further work and seems to be an experimental artifact (22). In our current

model, expansin disrupts the non-covalent adhesion of matrix polysaccharides to cellulose or to other scaffolding elements in the cell wall, thereby freeing the wall polymers to move in response to the mechanical forces generated by cell turgor (13).

## EXPANSINS IN AUXIN ACTION

The earliest studies of auxin made a connection between this hormone and control of cell enlargement. How auxin induces cell enlargement has long been a vexing problem in the field of plant hormones. From the biophysical perspective, the increase of plant cell volume depends on turgor pressure and cell-wall extensibility. It is now widely accepted that auxin induces cell expansion mainly by increasing the ability of the cell wall to extend, at least for its short-term action.

### Acid Growth and Expansins

About 35 years ago, research was focused on an auxin-induced factor hypothesized to enhance cell wall extensibility, a so-called *wall-loosening factor*. The *acid growth hypothesis* proposed protons ( $H^+$ ) as a mediator between auxin and cell-wall loosening (see Chapter C1). A study of the underlying molecular mechanism of this response led to the discovery of expansins.

Cosgrove (11) characterized acid-induced extension of cucumber hypocotyl walls and concluded that one or more wall proteins were required for acid-induced wall extension. Subsequent investigations showed that a novel class of proteins, eventually named *expansins*, were the principal proteins with this activity (24, 25). In line with the acid growth hypothesis, cell wall extension activity by  $\alpha$ -expansins has an acidic optima (pH 3-5.5) in both dicot stems and grass coleoptiles.

A relationship between expansin, acid growth, and auxin has been suggested by several experiments (13). Tobacco suspension cells grew three times faster in response to 0.5  $\mu$ M fusicoccin, a fungal toxin that stimulates  $H^+$  excretion from the plant cell, thereby inducing "acid growth". Partially purified  $\alpha$ -expansins at 1  $\mu$ g/mL elicited a similar level of growth stimulation as did fusicoccin. Exogenous  $\alpha$ -expansins also induced growth of excised Arabidopsis hypocotyls comparable to that induced by optimal auxin concentrations (Fig. 3), and the effects by auxin and expansin were not additive. These results suggest that auxin and expansins stimulate cell expansion through a common pathway (15). Thus, if we accept that auxin stimulates cell wall acidification, at least part of the resulting growth response should be mediated by the expansins residing in the cell wall. This might account for the early stages of auxin-induced growth, but does not exclude the possibility of other responses for later stages.

### Auxin-Regulated Expansin Genes during Cell Growth

Studies have shown that auxin regulates expression of some, but not all, expansin genes. For example, expression of an expansin gene (*LeEXP2*) cloned from the hypocotyl of tomato seedlings showed good correlation with auxin-induced growth. The hypocotyl has been a popular model system for study of auxin-induced cell growth. In the hypocotyl, the growth rate is highest at the top and gradually decreases towards the basal region. The expression pattern of *LeEXP2* reflects nicely these spatial differences in growth rate of the hypocotyl (Fig. 4A).

Auxin treatment markedly increased *LeEXP2* expression in the hypocotyl (Fig. 4B). The *LeEXP2* transcript level increased to 8 fold within 1 h after auxin treatment and reached about 15 fold in 6 h (Fig. 4D). In contrast, gibberellin (GA) had little effect on hypocotyl elongation and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had an inhibitory effect on tomato hypocotyl elongation. Consistent with these growth effects, *LeEXP2* expression level was little changed by GA and decreased by ACC treatment (Fig. 4C). Another growth hormone, brassinolide (BR), showed even stronger growth effect on tomato hypocotyls than did auxin. However, *LeEXP2* gene induction by BR was not significant, suggesting that *LeEXP2* is more intimately regulated by the auxin pathway during cell growth. It is possible that another  $\alpha$ -expansin gene is up-regulated by BR, or BR might operate via a separate pathway that does not entail enhancement of expansin gene expression. The *LeEXP2* promoter region contains putative auxin regulatory *cis*-elements (TGTCAC) that exist in *PS-IAA4* and 5, the well known auxin-regulated genes.

Gravitropic responses of the stem and the root provide additional evidence for a role of expansin in auxin-regulated cell. In response to gravity, a horizontally laid stem bends upward as a result of differential growth by the top and bottom halves of the horizontal stem. It is generally accepted that this differential growth results from auxin redistribution between the upper and lower parts of the stem (or root). The expression of tomato expansin *LeEXP2* decreased markedly in the top half of the stem within 30 min after

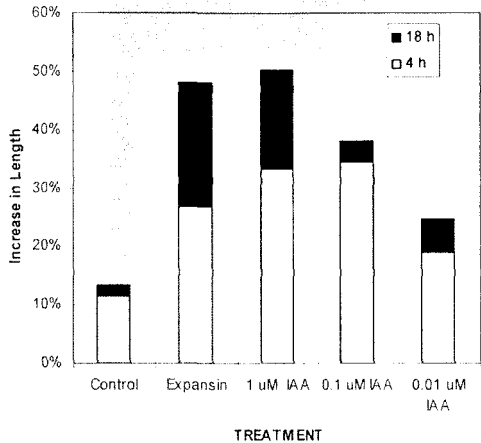


Figure 3. Elongation of Arabidopsis hypocotyl segments by expansin or auxin. The growing regions of etiolated Arabidopsis hypocotyls were excised and floated on a solution supplemented with partially purified cucumber  $\alpha$ -expansin (10  $\mu$ g/mL) or with IAA. Length was measured 4 h and 18 h after treatment (15)

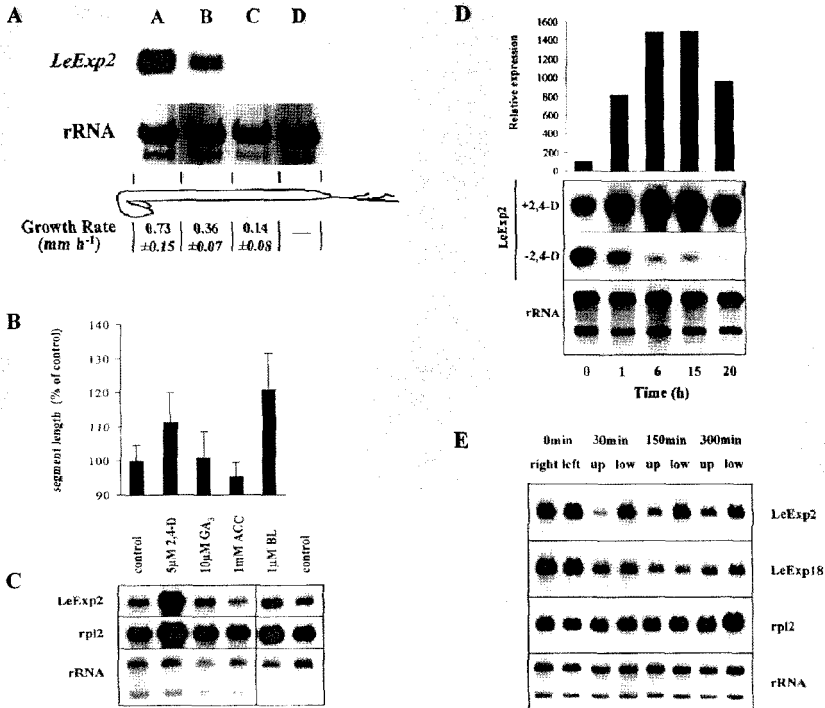


Figure 4. Regulation of expansin genes in the tomato hypocotyl and stem. A, Growth rates and *LeEXP2* mRNA levels in different hypocotyl regions. B, Hypocotyl growth in response to hormones. C, Changes of expansin mRNA levels in hormone treatments. D, Time course of the *LeEXP2* mRNA change after 2,4-D treatment. E, Changes of expansin mRNA levels after gravitropic stimulation of the stem. Total RNA was prepared from the upper or lower halves of the stem after stimulation. A, From (4). B-E, From. (2).

gravity stimulation, while not changing in the bottom half (Fig. 4E) (2). Reduced *LeEXP2* expression in the upper part of the stem could reduce elongation in the upper part, thereby resulting in upward bending. Another study examined gravitropism of the maize root, which bends downward in response to gravitropic stimulus. More expansin protein was detected by immunolocalization in the upper half of the root than in the lower half (Fig. 5). Moreover, naphthylphthalamic acid (NPA), an auxin transport inhibitor, considerably delayed both gravitropism and the asymmetrical distribution of expansin proteins (42). This indicates that auxin is required for differential expansin expression across the gravistimulated root.

With respect to the evolution of cell growth mechanisms, "acid growth" of cell walls appears to be universal in the plant kingdom. Consistent with this, expansin genes have been found in angiosperms, gymnosperms, ferns and bryophytes (23). It seems likely that expansin-mediated wall loosening

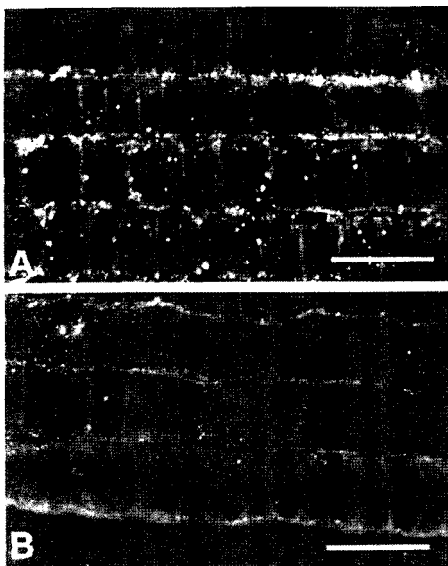


Figure 5. Distribution of expansin proteins during gravitropic stimulation in the maize root. After 30 min of horizontal orientation, the upper part (A) showed stronger expansin signals, remarkably in the periclinal walls, than did the lower part (B). Anti-CsEXPI (a cucumber expansin) antiserum was used for the primary antibody reaction, and fluorescent secondary antibody was used for visualization. Bars = 50 nm. (42)

and cell expansion evolved early in the origin of land plants and that these processes are often regulated by auxin (and other hormones too; see below).

In yet another study linking expansins to auxin action, expression of a gymnosperm  $\alpha$ -expansin (from loblolly pine) increased by as much as 100 fold in response to auxin during adventitious rooting of the hypocotyl, in which active cell growth and differentiation occur (19). Expression of a  $\beta$ -expansin gene from the moss *Physcomitrella patens* also increased within 2 h after auxin treatment of the chloronemal tissue (35). It was proposed that this auxin-regulated  $\beta$ -expansin plays a role in the developmental transition from chloronemata to caulonemata. However, knock-out mutations of the moss expansins did not show marked phenotypes, perhaps due to overlapping expression and a common function of multiple expansin genes expressed in the few cell types in the moss.

### Auxin, Lateral Organ Formation, and Expansins

Another auxin-related function of expansins is in leaf primordium initiation on the flanks of the shoot apical meristem (SAM). Initiation of lateral organs such as leaves and flowers on the SAM follows a regular pattern, called *phyllotaxy*. Recent work has implicated auxin as a major regulator of lateral organ formation and patterning in the SAM (31), and also expansin involvement in primordium initiation on the SAM.

Loss-of-function mutation of the Arabidopsis auxin efflux carrier PIN1 or treatment of the tomato shoot apices with NPA (naphthylphthalamic acid, an auxin transporter inhibitor) caused defects in lateral organ formation, but did not affect other SAM processes such as cell division and meristem

identity (30). These treatments resulted in a leafless naked stem (resembling a pin, ergo the name of the gene). However, localized application of auxin (IAA) to the naked meristem restored leaf formation (Fig. 6).

This experiment supports the idea that local auxin concentrations set up by the auxin transporter modulate the initiation of lateral

organs and their spatial patterning. Furthermore, it was proposed that auxin affects the spatial organ patterning by regulating the spatial expression pattern of the very early organ identity genes such as *LFY*, *ANT*, and *CUC2*.

Other studies indicate the regulation of lateral organ formation directly by expansin. In-situ hybridization showed that an  $\alpha$ -expansin gene was expressed specifically at the location of future leaf primordia initiation on the tomato SAM (32) (Fig. 7). Previous work showed that Sphacryl beads loaded with expansin protein induced leaf-like organs on the tomato SAM and altered leaf phyllotaxis. This result indicates that expansin can induce premature enlargement of incipient leaf primordia, and this then changes the subsequent pattern of phyllotaxy. These conclusions were reinforced by use of tobacco plants transformed with a  $\alpha$ -expansin gene whose expression was controlled by the tetracycline-inducible promoter (28). Micro-application of tetracycline to the site of

incipient leaf initiation (12), which normally forms the primordium after II, induced a leaf primordium, skipping the normal leaf initiation at II and reversing the phyllotaxis (Fig. 8A). In contrast to the previous study using exogenous expansin proteins, the leaf primordia stimulated by this technique developed into a normal leaf (Fig. 8C).

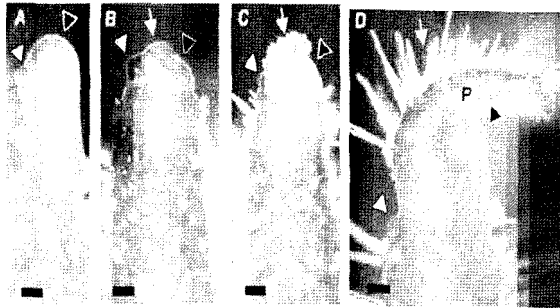


Figure 6. Induction of the leaf by IAA in the NPA-treated tomato apical meristem. A, Four days after treatment with control paste (white arrowhead). B-D, Induction of leaf primordium (white arrows) with lanolin paste containing 10 mM IAA (white arrowheads); 1 day (B), 2 days (C), and 4 days (D) after auxin treatment. Black arrowheads point the meristem. Bars=100  $\mu$ m. (30)

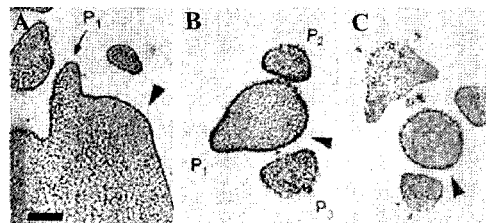


Figure 7 (Color plate page CP7). In situ localization of *LeEXP18* mRNA in the tomato shoot apical meristem. A-B, Localized expression of *LeEXP18* mRNA (in red). A, Longitudinal section. B, Cross section. C, Distribution of histone H4 transcript as a control (in red). Arrowheads indicate the site of incipient leaf initiation. Bar=100  $\mu$ m. (32)



Figure 8. Endogenous expansin-induced leaf morphogenesis in tobacco. A, Scanning electron micrograph (SEM) of a shoot apex where tetracycline-loaded lanolin was placed onto the 12 position of the meristem (m). After 3 days, a leaf primordium bulge (arrow) has formed at this position. B, SEM of an apex treated with buffer at 12 position. No bulge has formed. C, A leaf normally developed from endogenous expansin-induced primordium. D, Altered leaf morphology (arrow) by local induction of endogenous expansin. Tetracycline was applied onto one flank of the leaf primordium (P2 stage) and it was allowed to grow 2-4 weeks. Bars=150  $\mu$ m (A and B), 0.5 cm (C), and 1 cm (D). (28)

These results show that localized expansin expression can prematurely advance development of leaf primordia. However, they do not demonstrate that expansin expression *alone* is sufficient for leaf initiation (only distal regions containing incipient leaf primordia were shown to development into leaves, other regions of the SAM evidently did not respond in the same way). Interestingly, localized induction of expansin in the young leaf primordium changed the leafshape, resulting in a lobe (Fig. 8D).

How does expansin modulate organogenesis in the SAM? Expansin itself is not likely to function as a ligand to start a signaling cascade nor is it likely to be a direct regulator for gene expression. Expansin loosens the cell wall and stimulates cell enlargement, which will change the pattern of compressive and tensile forces in the SAM. Paul Green (18) proposed the hypothesis that the pattern of such physical forces within the SAM is part of the mechanism that establishes and maintains the stable pattern of leaf initiation. The reversal of phyllotaxy upon localized expansin expression is consistent with this idea. A stress-sensing mechanism, such as mechano-sensitive ion channels, may be activated by expansin-induced cell growth, or cell wall modification by expansin may stimulate some wall-associated signaling molecules that are sensitive to wall shear. These stress-sensing mechanism could then initiate a signaling cascade regulating cell identity and gene expression.

It is plausible that auxin operates upstream of expansin action in the SAM. As mentioned earlier, local application of auxin induces leaf organogenesis in the SAM as did expansin. Localized auxin molecules may elevate local expression of certain expansin genes. Consistent with this idea, *LeEXP18*, the tomato expansin gene expressed at the site of incipient leaf initiation, is up-regulated by auxin (2). In summary, a tenable, if speculative,

model for lateral organ formation in the SAM is as follows: (a) pre-patterning of auxin distribution, which likely is affected by previous local auxin concentration (29) and activity of auxin transporters; (b) local elevation of expansin gene expression via local auxin, (c) changes in the pattern of physical forces between cells as a result of cell enlargement, and consequent mechano-sensitive signaling into the cell from the extracellular matrix, and finally (d) activation of gene expression required for organ formation (division, cell specification, and so on).

### **Other Wall-Loosening Factors in Auxin-induced Growth**

In addition to expansin-mediated acid growth, hydroxyl radicals ( $\bullet\text{OH}$ ) have also been proposed as potential wall-loosening factors (37). Hydroxyl radicals are very aggressive reductants that can react and cleave essentially all biological polymers, including cell wall polysaccharides. Under appropriate conditions they may be generated by reaction of superoxide radicals or other reactive oxygen species with copper or iron ions or by iron-containing peroxidase, which is commonly found in cell walls. Reactive oxygen species, including hydroxyl radicals, are implicated in cell death associated with the hypersensitive response, in cross-linking phenolic polymers in the cell wall, and, more recently, in various signaling functions. The hypothesis advanced by Schopfer and coworkers is that controlled release of  $\bullet\text{OH}$  in the cell wall may contribute to the cell-wall loosening.

A recent study demonstrated that  $\bullet\text{OH}$  could induce the irreversible extension (creep) of isolated cell walls, similarly as shown with acid solutions or expansins (36). The creep activity induced by  $\bullet\text{OH}$  has an acidic optimum and artificially generated  $\bullet\text{OH}$  also induced elongation of living coleoptile and hypocotyl segments. Relevant to this section, auxin could induce the formation of superoxide radicals ( $\text{O}_2\bullet^-$ ), a potential precursor of  $\bullet\text{OH}$ , in the epidermis, and auxin-induced stem growth was inhibited by  $\bullet\text{OH}$  scavengers (which, it must be said, are relatively nonspecific in action). This work indicates that  $\bullet\text{OH}$  can be a potent wall-loosening factor *in vitro*; it remains to be seen whether  $\bullet\text{OH}$  is generated endogenously in the necessary concentration and in the specific location required for it to play a significant role in auxin-mediate cell growth.

### **EXPANSINS IN GIBBERELLIN ACTION**

The role of expansins in gibberellin (GA) action has been mainly studied in two subjects; cell growth and seed germination, which are also the most actively researched topics in the study of GA. In this section, we describe the regulation of expansin gene expression by GA and their likely role in these two physiological processes.

### Expansins in GA-Induced Cell Growth

GA was first discovered by Japanese scientists in 1926 due to its growth-stimulating activity. Many insightful studies of GA-mediated growth have been done using dwarf cultivars defective in GA biosynthesis. The dwarf rice cultivar Tanginbozu shows an extreme sensitivity of growth to GA as low as 3.5 picogram of GA3. Another model system, deepwater rice, can grow at rates of 25 cm per day upon submergence, mostly through stem internodal growth (20). The marked growth response of the stem takes place via a chain of hormonal interactions upon submergence: (1) increase in ethylene level, (2) lowered abscisic acid (ABA) level, (3) increased ratio of GA to ABA, and (4) elongation of the stem. Thus, direct application of GA to the stem can also induce the growth response. GA-induced and submergence-induced stem growth resulted in more cells and longer cells. In physical terms, GA resulted in greater extensibility of the internodal cell wall. The remarkable growth potential of deepwater rice stems and the GA effect on cell-wall properties led to study of the role of expansins in this growth process (20, 21). Two  $\alpha$ -expansin proteins were purified from the growing stems of deepwater rice, and the transcript level of one (*OsEXP4*) of these expansin genes was induced within 30 min after treatment with GA or submergence, preceding the growth response by these stimuli (Fig. 9). Further investigation showed that 5  $\alpha$ -expansin, 4  $\beta$ -expansin and one expansin-like genes were up-regulated by GA in the deepwater rice stem. Submerged rice stem sections had greater amounts of expansin protein per unit cell-wall material, and the cell wall from the submerged stem showed 2 fold higher acid extension than did that from the unsubmerged stem. These results support the hypothesis that a major part of the GA-induced elongation in deepwater rice is mediated by increased expression and activity of expansins.

The petiole of dicot *Rumex palustris* also elongates rapidly upon submergence, and this growth process follows a similar hormonal cascade as found in deepwater rice, except that auxin is added to GA as the final growth stimulator (38). The mRNA accumulation of a *R. palustris*  $\alpha$ -expansin gene (*RpEXPI*) upon submergence was correlated with submergence-induced petiole elongation. In *R. acetosa*, a *Rumex* species

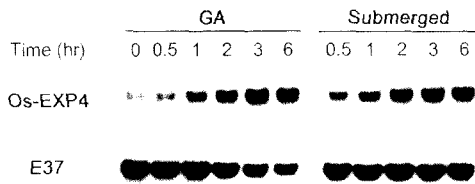


Figure 9. Accumulation of *OsEXP4* transcript in response to GA or submergence. Growing stem sections from deepwater rice, after 2-hr stabilization in water, were incubated for 0 to 6 hr in 50  $\mu$ M GA3 or submerged. Total RNA was extracted at the indicated time points from the growing basal 2 cm region. E37 is the loading control. (From 9)

that does not elongate in response to submergence, the transcription of *RpEXPI* did not increase by submergence. These results closely parallel those found in deepwater rice and further support the role of expansins in GA action.

A recent study using transgenic rice plants adds further evidence for the *in-vivo* function of expansin during cell and organ growth (10). Rice plants harboring an antisense *OsEXP4* construct had significantly reduced seedling height, mature plant height, and mesocotyl cell length (Fig. 10). Cell walls were also less extensible at low pH than were controls. Overexpression of *OsEXP4* resulted in the opposite phenotypes, although excessively high expression of *OsEXP4* suppressed plant growth. Moreover, the growth capability of seedlings upon submergence was also considerably reduced in the antisense lines, while the sense lines maintained a similar level to control. This study supports the conclusion that GA induces cell growth at least partially through expansin-mediated loosening of the cell wall.

### Expansins during Seed Germination

Both successful germination in favorable conditions and continued dormancy in unfavorable conditions are critical for a plant species to maintain its



Figure 10. Transgenic rice plants harboring sense and anti-sense constructs of *OsEXP4*. a, Antisense; b, Control; c, Sense. Expression of the sense and antisense *OsEXP4* constructs were driven by the constitutive ubiquitin 1 (Ubi-1) promoter. (From 10)

reproduction by means of seeds. Seed germination is characteristically controlled by an antagonistic rivalry between GA and ABA, where GA promotes germination and ABA inhibits it. For an endospermic seed to germinate, the storage products in the endosperm need to be mobilized. In some cases, the hydrolysis of storage macromolecules includes not only starch, protein, and oil inside the cell, but also polymers outside the cell such as cell wall polysaccharides. For emergence of the radicle, successful growth of the radicle cells and, in cases where the endosperm overlies the radicle, weakening of the endosperm cells on the path of radicle growth are required. Diverse enzymatic activities are thought to be involved in hydrolysis and weakening of the

cell walls during seed germination. A role for expansin in these processes has been hypothesized.

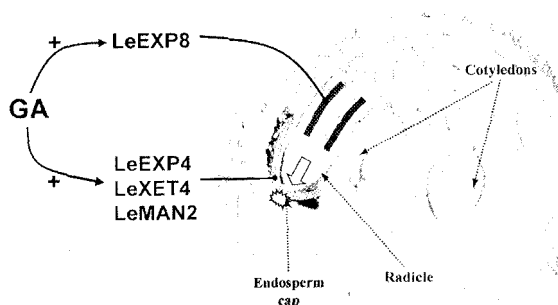
Most studies in this area have focused on the dynamics of gene expression in germinating tomato seeds. Characterization of the effect of expansins on cell walls of germinating seed has not yet been published. Expression of two  $\alpha$ -expansin genes was found to correlate with radicle growth and weakening of the endosperm barrier (5, 6). *LeEXP8* is specifically expressed in the elongating region of the radicle, and *LeEXP4* is expressed in the endosperm cap, the very endosperm portion where the radicle must penetrate for emergence. *LeEXP4* expression in the endosperm cap is also accompanied by the expression of other genes encoding wall-hydrolytic enzymes such as xyloglucan endotransglycosylase (*LeXET4*) and endo- $\beta$ -mannanase (*LeMAN2*) (Fig. 11) (7, 26). Expression of these four genes starts prior to the radicle emergence and is positively regulated by GA. Antagonists of germination, such as ABA and low water potential, partly inhibit  $\alpha$ -expansin gene expression in the tomato seed, though this varies between the two genes. These studies suggest that  $\alpha$ -expansins, together with wall-hydrolytic enzymes, play a role in growth of the radicle and weakening of the endosperm cap so as to complete the germination process.

## EXPANSINS IN ETHYLENE ACTION

### Expansins in Ethylene-Mediated Cell Growth

As described previously, ethylene is the first hormone in the hormonal cascade causing stem and leaf growth upon submergence in deepwater rice and *Rumex*. In *Rumex*, the  $\alpha$ -expansin gene *RpEXPI* was shown to be induced by ethylene treatment as well. The ethylene-mediated organ growth after submergence is conserved even in certain ferns. When submerged, the rachis of the semiaquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum* elongate rapidly.  $\alpha$ -Expansin genes, with high sequence similarity to those found in angiosperms, were identified from these ferns, and their

Figure 11. Cross section of a germinating tomato seed showing the expression locations of expansins and other cell wall degrading enzymes under the control of gibberellin



expression in the rachis showed a strong induction by submergence and ethylene. These expression patterns also were closely correlated with changes in rachis elongation and cell-wall extensibility by the two growth stimulators. These results in ferns and in angiosperms suggest that expansins are common targets of growth-inducing stimuli in vascular plants (21).

### **Expansins during Ethylene-Involved Fruit Ripening**

Animals are attracted to well-ripened fruit by its smell, color and the softness. For fruit ripening species are classified as climacteric (bananas, apples, tomatoes, etc) or non-climacteric (citrus species, strawberries, etc), where the former is sensitive, but the latter is minimally- or non- sensitive to ethylene. Fruit softening is accompanied by depolymerization and solubilization of pectins and hemicelluloses. Although cell-wall hydrolases were thought to drive the softening, transgenic experiments suggest that other processes may be important (33).

A tomato  $\alpha$ -expansin gene, *LeEXPI*, was first found to be expressed in a ripening-specific manner and up-regulated by endogenous and exogenous ethylene. Subsequent studies with transgenic tomatoes demonstrated the *in vivo* role of *LeEXPI* in fruit softening. The fruits from antisense lines were slightly firmer throughout fruit development, whereas in sense lines the softening time was advanced, even to the green fruit stages, and softness also increased. These changes of physical characteristics in the transgenic fruits were also accompanied by changes in the status of wall polymers, suggesting that expansins soften tomato fruit primarily through a relaxation of the wall by direct action and through controlling the access of pectinases to the pectins in the wall.

In contrast to expansin genes from climacteric tomatoes, an  $\alpha$ -expansin gene (*FaEXP2*) from non-climacteric strawberries was insensitive to ethylene, although its pattern of gene expression was correlated with fruit ripening. Thus, the expression of certain  $\alpha$ -expansin genes suggests a role in fruit softening and this is supported by the results with sense and antisense transformed tomato lines (1). The effects noted in the latter experiments were significant, but relatively subtle. Thus, expansins are not the sole catalysts of fruit softening.

Another ethylene-controlled process that shares some similarities to fruit softening is flower petal senescence. Expansin genes are expressed in the flower petals of peas (*Pisum*) and four o'clocks (*Mirabilis*), with a likely, though untested, role in petal growth and opening.

### **Expansins in Root Hair Development**

The root hair is a protrusion from a root epidermal cell. Its growth is a highly polarized cellular process and its initiation by epidermal cells involves and probably requires localized cell-wall acidification and loosening (17).

Root hairs contribute greatly to the surface area of the root, and their initiation and elongation is regulated both by ethylene and auxin, and also by environmental stimuli.

The regulation of two root hair-specific  $\alpha$ -expansin genes (*AtEXP7* and *AtEXP18*) from Arabidopsis was studied to dissect the role of ethylene and auxin at the gene regulation level (8). The expression of these genes starts immediately before the root hair bulge appears, indicating a close relationship to hair initiation (Fig. 12A). The Arabidopsis root epidermis consists of alternative longitudinal files of hair and non-hair cells, whose fates are determined by positional information and interactions between transcription factors (34). Exogenous ethylene can induce root hairs from the non-hair cell position accompanied by the induction of the root hair expansin genes. Treatments with ethylene and auxin restored root hairs in the hairless mutant *rhd6*, and similarly restored expression of the hair-specific expansin gene (Fig. 12F-H). Similar results were found with a treatment in which the root was separated from the agar medium (probably causing water stress). Because the ethylene antagonist 1-methylcyclopropene (1-MCP) inhibits the effects of auxin and root separation, ethylene is likely to mediate the effects of these two factors on root hair formation and expansin gene expression. Promoter analyses of *AtEXP7* revealed that signals from all the root hair regulatory factors (developmental, hormonal and environmental cues) converge onto a small promoter region, most likely for binding by a single transcription factor (Fig. 13).

In contrast, treatment of wild type with 1-MCP and mutations of the ethylene signaling components did not affect root hair formation and expansin gene expression. This was interpreted to mean that the ethylene signaling cascade in wild type is not essential to affect these two processes under normal condition. This study proposed that there are two pathways that modulate root hair initiation and expression of related genes: an ethylene-dependent pathway (requiring high ethylene concentration) and a

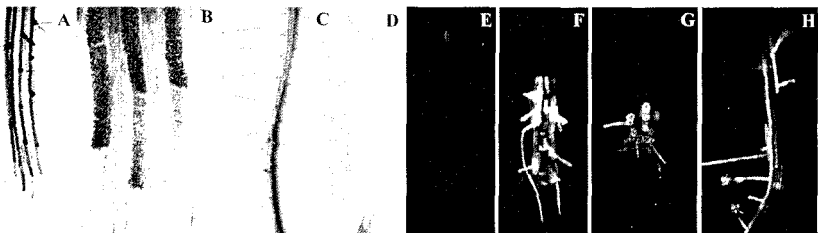


Figure 12 (Color plate page CP8). Expression pattern of *AtEXP7* in the Arabidopsis root. A-B, *AtEXP7* promoter::GUS expression. The reporter gene expressed only in the hair cell files, starting immediately before the bulge emerges. C, Wild-type root. D, Root from hairless *rhd6*. E-H, Confocal images showing the expression of *AtEXP7*::GFP (green fluorescent protein) in *rhd6*. Control (E), 5  $\mu$ M ACC (the ethylene precursor, F), 30 nM IAA (G), and the root separated from the agar medium (H) (From 8)

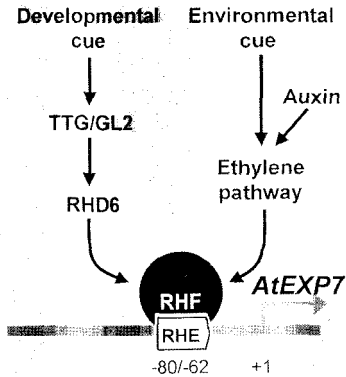


Figure 13. Regulation of root hair-specific  $\alpha$ -expansin gene *AtEXP7* by two separate pathways. Defects in TTG or GL2 raise root hairs and *AtEXP7* expression in both hair and non-hair cells, whereas the RHD6 mutation blocks root hair formation and *AtEXP7* expression. RHE, the *cis*-element rendering *AtEXP7* both specified in the hair cell and responsive to ethylene. RHF, a putative transcription factor that binds to RHE. Numbers at the bottom indicate the relative position from the transcription start (From 8)

second internal developmental pathway that is independent of ethylene (Fig. 13). Although several genes have been identified that are required for normal root hair development, none of them have yet encoded an expansin. Thus, while expression of root hair-specific expansin genes is tightly correlated with root hair initiation and elongation, it is not clear whether they are essential for either process.

## EXPANSINS IN CYTOKININ ACTION

Cell suspension cultures typically require cytokinins in the medium in order to maintain cell proliferation, which includes both cell division and cell enlargement. When deprived of cytokinin, such cells stop growing and subsequent addition of cytokinin to the medium results in greatly increased transcript levels of a  $\beta$ -expansin, called Cim1 (for cytokinin-induced message) (16). *Cim1* mRNA accumulated 20-60-fold upon cytokinin addition to cytokinin-starved soybean suspension cultures. Cytokinin accomplished this, at least in part, by increasing the stability of *Cim1* mRNA. A further study characterized the Cim1 protein and found that cytokinin acted synergistically with auxin to induce the accumulation and proteolytic processing of Cim1 protein in the culture medium. It is not clear whether such proteolytic processing is simply stepwise degradation of the protein in the medium or has other significance. These studies implicate expansins in cytokinin-induced cell proliferation.

This idea is further strengthened by results of a study of haustorial development in parasitic plants such as *Striga asiatica* and *Triphysaria versicolor* (27, 39). Haustorial formation in *Triphysaria* can be induced *in vitro* by the cytokinin 6-benzylaminopurine and this response involves localized swelling and proliferation of epidermal hairs near the root tips. The expression of two  $\alpha$ -expansin genes was increased upon cytokinin application. Haustorial development could also be induced by exudates from

maize roots and by the inducing substance 2,6-dimethoxybenzoquinone. However, these treatments did not increase expression of these  $\alpha$ -expansin genes. Thus, these two genes are linked to the cytokinin response, and not specifically to haustorial development.

## **EXPANSINS IN ABSCISIC ACID ACTION**

Three sets of studies have linked expansins to the action of abscisic acid. The first two deal with tomato seed germination and *Rumex* petiole elongation upon submergence; these were described above in the section on GA. The third set involves responses of maize roots to drought.

In many plants, shoot growth is typically inhibited more strongly by low water potential than is root growth. This differential response increases the root:shoot ratio and is considered a favorable adaptation to low water availability. In maize seedlings transplanted to relatively dry rooting medium, the apical part of the root elongation zone continues to grow, whereas the basal half of this zone, as well as the shoots, are strongly inhibited in growth. The continued growth of the root at low water potential occurs because the walls become more extensible, and this involves enhanced levels of expansin protein and transcripts in these root cells (40). Transcript levels for two  $\alpha$ -expansins and one  $\beta$ -expansin were rapidly increased upon restriction of water availability. Roots treated with the ABA biosynthesis inhibitor, fluoridone, did not exhibit this growth adaptation to low water potential. This suggests that drought-induced increases in ABA level may act as a signal to activate genes needed for the root adaptation. However, direct application of ABA to well-watered roots did not cause the level of expansin transcripts to increase in the same way that drought did. Thus, while ABA may be required to induce the root response, it does not mimic drought with respect to altered expression of expansin genes. This, in turn, implies that drought modulates expansin gene expression via an ABA-independent pathway.

## **CONCLUDING REMARKS**

The initial discovery of expansins stemmed from studies of cell wall enlargement, but in the intervening decade many unexpected aspects of expansin function have been discovered. We now know that expansins comprise a large multigene family, some of whose members are regulated by one or more of the plant hormones. This regulation is different for different gene, and moreover is specific to certain cell types. For instance, ethylene promotes expression of *EXP7* in *Arabidopsis* roots, but it does not promote expression of this expansin in other cell types (though it may modulate expression of some expansin genes in other cell types). Many effects of

plant hormones involve significant changes in the cell wall, i.e. stimulation of cell enlargement, germination, fruit softening, abscission, etc., and there is abundant evidence that such wall modifications involve the action of expansins. Alteration of expansin gene expression by transgenic methods confirms the role of expansins in wall loosening, especially during cell growth. However, until now, no one has identified an expansin knock-out mutant with a major developmental phenotype. This might be explained by overlapping expression of multiple expansin genes or by compensatory physiological changes by the plant to make up for the genetic defect. A hint of the latter explanation may be seen in transgenic plants overexpressing expansin - some of them have reduced growth despite high levels of expansin activity. Presumably such plants have modulated other properties of the wall in order to limit the effects of high, unregulated expression of expansin. Future work will need to identify the steps between expansin gene expression and the early steps in hormone signal transduction. When that is accomplished, we will have a complete link between initial hormone reception and final physiological effect - something that has eluded plant biologists up to now.

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**\*Note added after publication:** Expansin gene nomenclature has been revised. See the web site at <http://www.bio.psu.edu/expansins/> or: Kende et al. (2004) Nomenclature for members of the expansin superfamily of genes and proteins. *Plant Molecular Biology* 55: 311-314