Journal of Plant Biology, September 2005, 48(3) : 304-310

Two Expansins, EXP1 and EXPB2, Are Correlated with the Growth and Development of Maize Roots

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Expansins are proteins that can confer extensibility in plant cells by modifying the cross-links between cellulose microfibrils and polysacchrides. Because they are present as multi-gene families, their various patterns of differential expression suggest that each expansin plays a specific role in growth and development. Here, we describe the expression of expansin genes in maize roots in response to stage of growth, hormone treatment, or environmental stimuli. *ExpB2* was the most strongly detected, with its transcript level being much higher than any other expansin in the regions undergoing cell division and elongation. Indole-3-acetic acid, which generally inhibits root elongation, induced expression of *Exp1*, but repressed that of *ExpB2*. This auxin-induced alteration was negated by treatment with 1-aminoethoxyvinylglysin (AVG), indicating that transcript levels may be modified by auxin-induced ethylene biosynthesis. *ExpB2* expression was also induced by wounding and gravistimulus treatments. Therefore, our results imply that *ExpB2* plays a role in the elongation of maize roots, and may be also involved in plant responses to environmental stimuli.

Keywords: auxin, elongation, ethylene, expansin, gravistimulation, wounding

Plant cell walls are rigid structures that consist primarily of cellulose microfibrils and hemicellulosic and pectic polysaccharides (Carpita and Gibeaut, 1993). Because of their hardness, these walls must be loosened and extended during cell growth and division. Many kinds of cell wall-modifying enzymes are involved in the regulation of extensibility, including the protein expansin, which was initially identified in actively growing cucumber seedlings (McQueen-Mason et al., 1992). Expansins disrupt the hydrogen bonds between cellulose microfibrils and adjacent matrix polysaccharides (McQueen-Mason and Cosgrove, 1994, 1995). In contrast, the function of xyloglucan endotransglucosylase/hydrolases (XTHs) is to cleave and rejoin those cross-links (Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992).

Expansins are encoded by a large multigene family. For example, *Arabidopsis* possesses 38 open reading frames (ORFs), including two pseudo-genes, that encode expansins or expansin-like proteins (Li et al., 2002). Based on their sequence homologies, expansins are classified into four subgroups (Li et al., 2003). Two such groups -- α- and β-expansins -- share common features, including signal peptides at the amino-termini as well as conserved cysteine and tryptophan residues (Cosgrove et al., 2002). However, they differ in their N-linked glycosylation motifs; these are present only in the β-expansins (Downes et al., 2001; Cosgrove et al., 2002). The remaining two groups are the γ- and δ-expansins, which encode shorter proteins and show high similarity to the amino-terminal and carboxyl-terminal halves of α- and β-expansins, respectively (Ceccardi et al., 1998; Li et al., 2003).

The biochemical mechanism of expansin action is still uncertain. Some evidence suggests that α-expansins disrupt hydrogen bonding between cellulose microfibrils and associated glucans without causing the hydrolysis of major structural polysaccharides (McQueen-Mason et al., 1993; McQueen-Mason and Cosgrove, 1994, 1995; Whitney et al., 2000). Although α-expansins may have activity similar to that of the β-expansins, they differ from each other in their substrate specificity (Cosgrove et al., 1997). γ-Expansins function as peptide hormones in regulating water balance (Maryani et al., 2001; Ludidi et al., 2002). Finally, the δ-expansins, which apparently are present only in monocots, seem to interact with cellulose, based on their sequence similarity (Shcherban et

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al., 1995; Li et al., 2003).

Despite the paucity of knowledge about the biochemical mechanism for expansin, several studies have provided preliminary insights into their biological functions. For example, expansins are differentially expressed among tissue types and developmental stages. Furthermore, they vary in their response to stimuli, e.g., hormones, pollination, and light (Cho and Kende, 1997; Cosgrove et al., 1997; Rose et al., 1997; Im et al., 2000; Vriezen et al., 2000; Wu and Cosgrove, 2000; Lee and Kende, 2001; Reidy et al., 2001; Ruan et al., 2001; Harmer et al., 2002; Pezzotti et al., 2002). This indicates that expansins are involved in several aspects of plant growth and development and that each may have a specific role. Application of expansin proteins stimulates early growth in tomato leaf primordia (Fleming et al., 1997), development of tobacco BY2 cell cultures (Link and Cosgrove, 1998), elongation of excised hypocotyls, and radial swelling of root hairs in *Arabidopsis* (Cosgrove et al., 2002). Such evidence suggests that the role of expansins may be to loosen the cell wall in the context of the orientation of cellulose microfibrils (Cosgrove et al., 2002).

Wu et al. (2001) have reported the differential expression of 13 genes for α- and β-expansins in various maize (*Zea mays*) tissues. Based on that research, we have now examined the expression patterns of two expansin genes -- *Exp1* and *ExpB2* -- as representatives of α- and β-expansins respectively, to better understand their roles in maize root growth.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L. cv. Golden Cross Bantam) seeds were washed with tap water and soaked in distilled water for 24 h. Afterward, they were placed between layers of water-saturated paper towels on trays (27 × 20 × 2.5 cm). The trays were positioned vertically in the dark at 28 ± 1°C in 70% relative humidity. After germinating for 2.5 d, seedlings with 1.5- to 2.0- cm -long straight-grown primary roots were selected and used for experiments.

Hormone and Stress Treatments, and Measurement of Root Elongation

Root caps were immersed (with aeration) for 2 h at 25 ± 1°C in an MES [2-(*N*-morpholino)-ethanesulfonic acid]-Tris [tris(hydroxymethyl)-aminomethane] buffer (5 mM, pH 6.8). To investigate the effect of various concentrations of indole-3-acetic acid (IAA), some root caps were immersed in an MES buffer supplemented with IAA. For ethylene treatment, seedlings were placed in a closed chamber and ethylene was injected with a syringe through a rubber septum. To measure their primary roots, seedlings were positioned vertically against the wall of a lucent chamber and exposed to a closed circuit digital camera. For gravistimulation treatment, the seedlings were positioned horizontally under the same condition as described above. For wounding treatment, we pierced the roots with a needle two or three times. Their root images were then magnified 70 times on a computer monitor with the SECANT computer program (Yongma, Korea) and their lengths recorded, as previously described (Kim et al., 2000).

DNA and RNA Gel Blot Analyses

Following the hormone treatments, total RNAs were extracted from the roots by the phenol-SDS method (Sambrook et al., 1989). These RNAs (20 μg) or the DNAs obtained by PCR were fractionated on 1% (w/v) formaldehyde-agarose gels and blotted on Hybond N⁻nylon membranes (Amersham Pharmacia Biotech, Sweden). After PCR with specific primers (Table 1), the blots were hybridized at 42°C with ^{32}P-labeled probes of expansins in a hybridization solution containing 50% (v/v) formamide, 6× SSPE, 0.5% (w/v) SDS, 5% (v/v) Irish cream, and 100 $\mu g \cdot mL^{-1}$ denatured salmon sperm DNA. The blots were washed three times at 65°C with 2× SSPE and 0.1% (w/v) SDS, then analyzed with BAS-2500 Fuji Film.

Table 1. Primers used for synthesis of gene-specific probes.

Probe name	Used primer sequence (5 '→ 3')	Probe length(bp)
Exp1	S[a]-CTACTACTATCCATCGACG AS[b]-AATAAGTTGCACGACACC	259
Exp5	S-AGGATGAACAGCTAGCGC AS-TAGTACTAGGAGCTGGATCG	305
ExpB2	S-AGCTAGCTGGTTTGCGCC AS-AAGCAACAGTGGGCGCGGG	296
ExpB6	S-TAATGATCGAGCTAGCT AS-TTAGTATGAAATTATATAACC	216
ExpB7	S-GGCCGGATAATAATATACA AS-ATCTTAATCGCACTGGTAC	240
ExpB8	S-GGATCCGTGCCCTGCCCG AS-AGCAACAGTGGGCGGGAG	312

[a]S, oligonucleotide used as a sense primer; [b]AS, oligonucleotide used as an antisense primer.

RESULTS AND DISCUSSION

Exp1 and ExpB2 Expansins Are Differentially Expressed in Maize Roots

Six expansin genes are expressed in maize roots, including *Exp1*, *Exp5*, *ExpB2*, *ExpB6*, *ExpB7*, and *ExpB8* (Wu et al., 2001). Here, to test the compatibility of the probes, we first performed RNA gel blot analysis (with probes obtained by PCR), using primers previously reported (Table 1; Wu et al., 2001). DNA gel blot analysis showed that two probes recognize *ExpB2* and *ExpB8* (Fig. 1; Wu et al., 2001), indicating high homology between both genes. However, the reactivity of these two probes with their own genes was stronger than with other genes represented by *ExpB8* to *ExpB2* (Fig. 1; Wu et al., 2001). *Exp1* and *ExpB2* also exhibited greater levels of expression than did other genes in our maize roots (Fig. 1, right panel). Based on these results, we chose *Exp1* and *ExpB2* as representatives of the α- and β-expansin gene products, respectively, for the experiments that followed.

To ascertain the relationship between root development and gene expression, we measured time-dependent increases in growth. The etiolated maize roots lengthened quickly, especially from Days 2 to 4 after germination (Fig. 2A). Interestingly, both *Exp1* and *ExpB2* were most highly expressed at Day 2, just before exponential root growth began; expression then declined after this time point (Fig. 2B). The expression level of *ExpB2* was greater than that of *EXP1*, with transcript being detectable until Day 4, at which time the root growth rate also started to decrease (Fig. 2A).

To determine their patterns of elongation more precisely, we divided the lower portions of the roots into three regions (1, 2, and 3), beginning at 4-mm intervals from the root tips (Fig. 3A). Region 1, nearest the apex, showed the greatest increase in length, indicating that this was the locus for most active cell division and elongation (Fig. 3B). In contrast, Regions 2 and 3 showed no growth increment (Fig. 3B). To examine the relationship between root growth and gene expression, we performed RNA gel blot analysis using total RNAs extracted from tissues derived from these three regions. The greatest level of *ExpB2* transcript was found in Region 1 (Fig. 3C). Although a significant amount of *ExpB2* mRNA was also observed in Region 2, *Exp1* mRNA was more abundant.

In deepwater rice, gene expression of expansins is closely related to internodal elongation (Cho and Kende, 1997). Most mRNAs are distributed in the epidermis of the elongation zone (Cho and Kende, 1998). This implies that expansins function at the

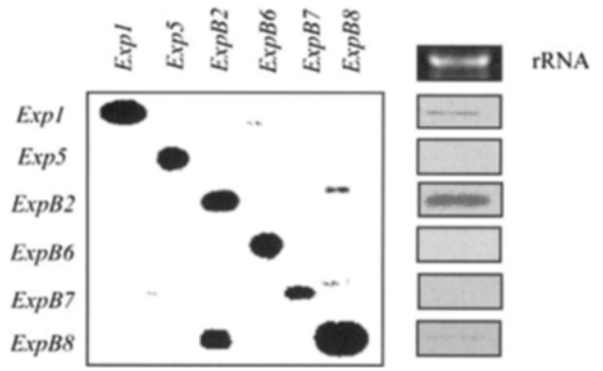

Figure 1. Specificity of expansin probes. Test of cross-hybridization (left panel). One ng of DNA was loaded and separated on 1.5% agarose gels. DNA was transferred, blotted onto Hybond N^+ nylon membranes, and hybridized with ^{32}P-labeled expansin probes indicated on left side. Expression pattern of expansin genes in maize (right panel). Twenty μg of total RNA extracted from roots of 2.5-d-old seedlings was used for gel blot analysis. Blots were hybridized with same probes used for cross-hybridization in left panel.

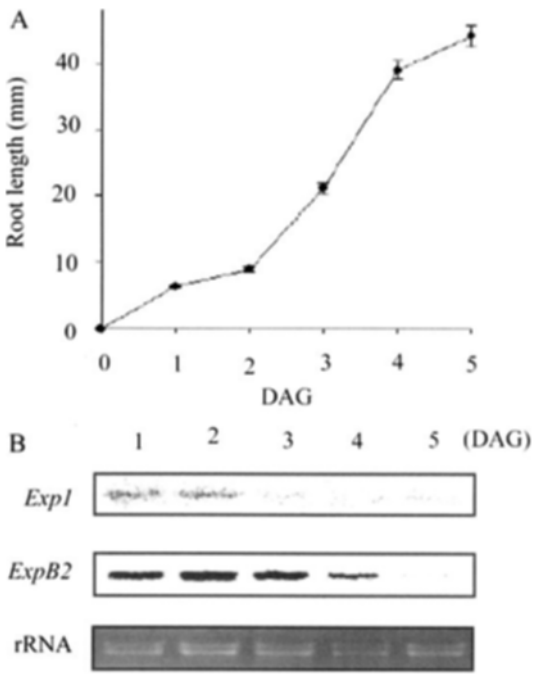

Figure 2. Expression of expansin genes. **A.** Elongation of maize primary roots over time. Values represent means ± SE of three replicates. DAG, days after germination. **B.** Expression of *Exp1* and *ExpB2*. Twenty μg of total RNA extracted from roots grown for days indicated was used for gel blot analysis.

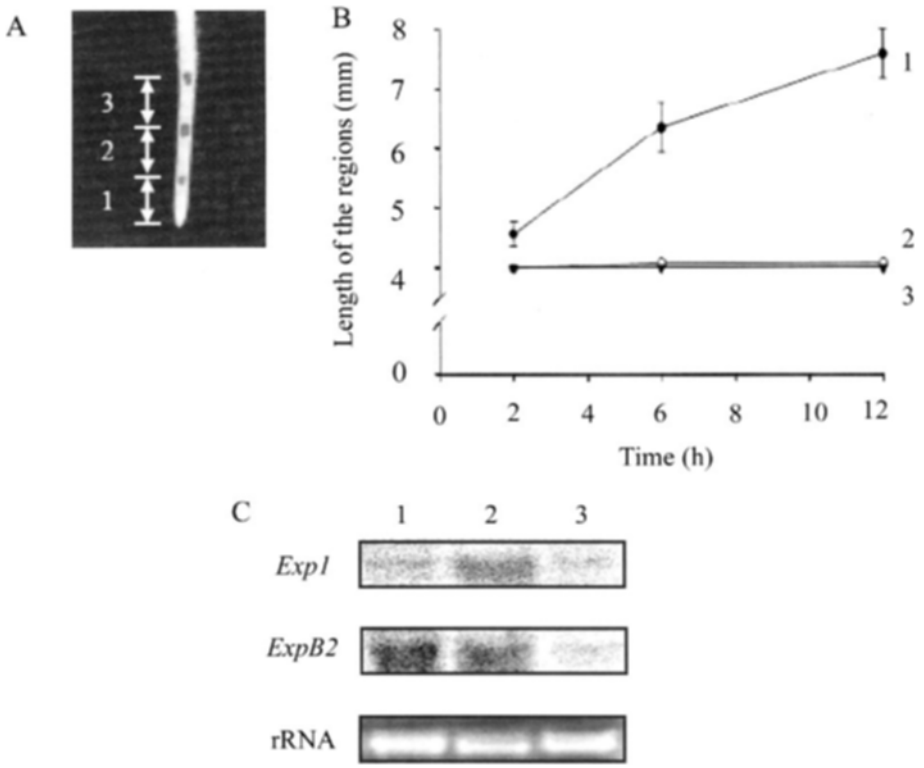

Figure 3. Elongation and expression of expansins in different regions of maize roots. **A.** Three zones from 2.5-d-old roots were delineated into 4 mm-long sections. **B.** Elongation over time, by regions shown in **A.** Values represent means ± SE of three replicates. **C.** Expression of *Exp1*and *ExpB2* in three regions shown in **A.** Twenty μg of total RNA extracted from each was used for gel blot analysis.

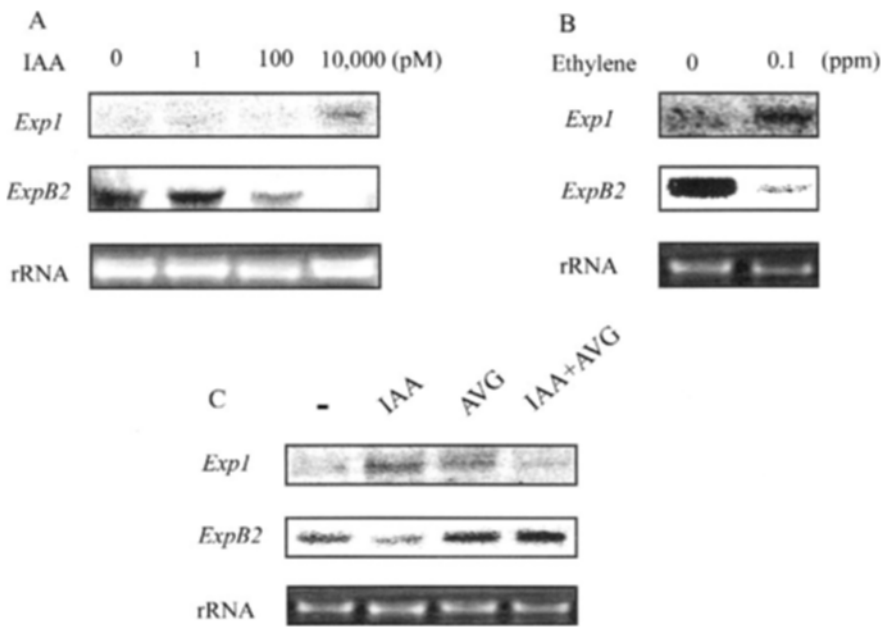

Figure 4. Influence of plant hormones on expansins. **A.** Effect of various concentrations of IAA on expression of *Exp1*and *ExpB2* in 2.5-d-old maize roots. **B.** Effect of ethylene on expression of *Exp1*and *ExpB2*. Seedlings (2.5 d old) were placed in closed chamber that was injected with indicated amount of ethylene. Total RNA was extracted from roots and used for RNA gel blot analysis. **C.** Effect of auxin-induced ethylene on expression of *Exp1*and *ExpB2*. Roots of 2.5-d-old maize seedlings were pretreated with 0.1 mM AVG for 2 h, then treated with 10 nM IAA. Twenty μg of total RNA was used for gel blot analysis.

genetic level in this species. A soybean expansin, GmEXP1, may also be involved in root-lengthening due to its expression being exclusive to the elongation region (Lee et al., 2003). That soybean expansin also accelerates the growth of transgenic tobacco plants expressing this protein. Therefore, based on those previous results and our expression data, we can conclude that the ExpB2 expansin gene is an important factor closely correlated with growth in maize roots. This is supported by the facts that 1) expression of ExpB2 is highest in Region 1, where cells actively divide and grow; and 2) expression of that gene is prolonged until growth ceases in those roots. Furthermore, both Exp1 and ExpB2 appear to be at least partially responsible for the maturation of root cells beyond this growth zone (e.g., in Region 2).

Effect of Auxin and Ethylene on Expression of Expansins in Maize Roots

Treatment with 10 nM indole-3-acetic acid (IAA) strongly inhibited the elongation of maize roots (data not shown). Using this information, we then investigated its effect on transcript levels for both expansins. Expression of *ExpB2* greatly decreased as the concentration of IAA decreased (Fig. 4A); at 10 nM, its transcript completely disappeared. This suggests that ExpB2 plays a more significant role in root growth than does Exp1. In the case of the latter, its transcript level was increased by IAA only at the highest IAA concentration, i.e., 10 nM (Fig. 4A).

In tomato, the *LeExp2* gene is strongly expressed in the hypocotyl elongation zone, being induced by IAA (Caderas et al., 2000). However, in the aerial portions, IAA inhibits root elongation via alkalinization (Evans et al., 1980), causing the stiffening of cell walls (Buntemeyer et al., 1998) or a reduction of reactive oxygen species (Liszkay et al., 2004). Therefore, all of those results, along with our current findings, raise the possibility that ExpB2 is involved in regulating auxin-inhibited root growth in maize.

Increases in ExpB2 gene expression are assumed to be due to IAA-induced ethylene because it is well known that the former promotes production of the latter (Yang and Hoffman, 1984; Abeles et al., 1992). To examine if this is true, we treated intact maize roots with exogenous ethylene, and found that, similar to our IAA results, *Exp1* transcript increased while that of *ExpB2* was repressed (Fig. 4B). Furthermore, the addition of 1-aminoethoxyvinylglycine (AVG), a specific inhibitor of ethylene biosynthesis, completely abolished this IAA effect on expansin transcript levels (Fig. 4C). This indicates that auxin-induced alterations in gene expression may be derived from the effect of ethylene produced by IAA. In tomato, the *LeExp1* gene is expressed specifically in ripening fruit as a result of ethylene treatment (Rose et al., 1997). In *Rumex palustris*, expression of *RpExp1* is also promoted by ethylene, which is accumulated and thus induces leaf elongation upon submergence (Vriezen et al., 2000). Therefore, we believe that our IAA-induced decrease in maize root growth could be ascribed to the action of IAA-induced ethylene.

Effect of Wounding and Gravistimulation on Expression of Expansins in Maize Roots

In deepwater rice, gene expression of expansins is

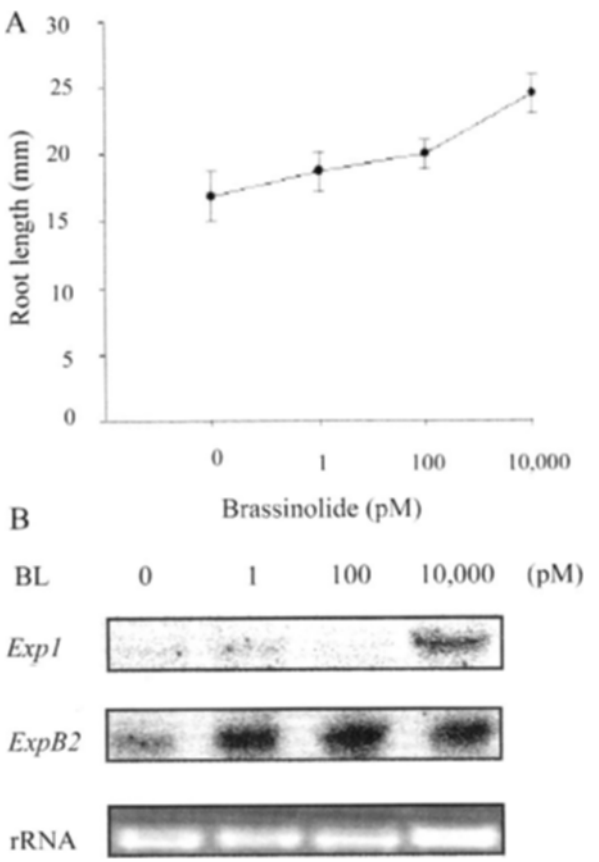

Figure 5. Effect of environmental treatments on expression of *Exp1* and *ExpB2*. **A.** Roots of 2.5-d-old maize seedlings were wounded by piercing with a needle, then incubated for 2 h. Total RNA was extracted from roots and used for RNA gel blot analysis. **B.** Effect of gravistimulation on expression of *Exp1* and *ExpB2*. Roots of 2.5-d-old maize seedlings were placed horizontally in water-saturated chamber for indicated times before RNA was extracted from roots. Twenty μg of total RNA was used for gel blot analysis.

induced by wounding -- β-expansins to a greater extent than a-expansins (Lee and Kende, 2001, 2002). However, their exact function in the wounding response is not yet known. To investigate this in maize, we pierced the roots with a needle and extracted total RNA from Region 2 to compare the expression of *Exp1*and *ExpB2*. As with the deepwater rice, wounding increased the transcript level of *ExpB2*, a β-expansin, but had little influence on *Exp1*, an α-expansin (Fig. 5A). This indicates that ExpB2 participates in the wounding response.

Zhang and Hasenstein (2000) have reported that, within the first 30 min, gravistimulation treatment causes differential distribution of expansin proteins between the upper and lower halves of maize roots. To study this at the protein level, we extracted total RNA from the bending portion of the roots, and conducted RNA gel blot analysis. Within 10 min after the initiation of treatment, the transcript level of *ExpB2* increased over that of *Exp1* (Fig. 5B). Zhang and Hasenstein (2000) have performed immunohistochemical assays of maize using antibodies raised against cucumber expansins (Li et al., 1993), and have detected only a single band from the root extracts. All these results, therefore, suggest that the detected protein band is the product of the ExpB2 gene, because among the six expansins expressed in maize roots, its transcript level is higher than that of the other genes. Furthermore, *ExpB2* expression is strongly correlated with cell elongation.

In conclusion, we have demonstrated here that expansins are differentially expressed in response to developmental cues, hormonal treatments with IAA and ethylene, or stresses caused by wounding or gravistimulation. One possible interpretation is that expansins may specifically function in plant growth and development by regulating cell-wall extensibility. In particular, the main protein involved seems to be ExpB2 because the response of its expression to growth-regulating hormones is highly correlated with root elongation. Likewise, expression of that protein is greater in the actively growing region. Further analysis of ExpB2 functioning in developing maize roots will necessitate probing physiological parameters through the use of ExpB2-deficient transgenic plants. Root extensibility analyses of wild-type and deficient plants, using Intron analysis, will also be helpful in such study.

ACKNOWLEDGEMENT

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (KRF-2001-050-D00042) to S.C. Chang.

Received March 23, 2005; accepted June 13, 2005.

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