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Expression of Expansin Genes Is Correlated with Growth in Deepwater Rice

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Expansins are a family of proteins that catalyze long-term extension of isolated cell walls. Previously, two expansin proteins have been isolated from internodes of deepwater rice, and three rice expansin genes, *Os-EXP1*, *Os-EXP2*, and *Os-EXP3*, have been identified. We report here on the identification of a fourth rice expansin gene, *Os-EXP4*, and on the expression pattern of the rice expansin gene family in deepwater rice. Rice expansin genes show organ-specific differential expression in the coleoptile, root, leaf, and internode. In these organs, there is increased expression of *Os-EXP1*, *Os-EXP3*, and *Os-EXP4* in developmental regions where elongation occurs. This pattern of gene expression is also correlated with acid-induced in vitro cell wall extensibility. Submergence and treatment with gibberellin, both of which promote rapid internodal elongation, induced accumulation of *Os-EXP4* mRNA before the rate of growth started to increase. Our results indicate that the expression of expansin genes in deepwater rice is differentially regulated by developmental, hormonal, and environmental signals and is correlated with cell elongation.

INTRODUCTION

Because plants are sessile organisms, it is an adaptive advantage that their growth and development are subject to control by environmental conditions. Regulatory effects of the environment are often mediated by hormonal signals and can therefore be mimicked by application of the appropriate plant growth regulator(s). Greatly accelerated internodal elongation of deepwater rice in response to submergence exemplifies how an environmental stimulus induces growth via a series of hormonal interactions (Raskin and Kende, 1984a, 1984b; Hoffmann-Benning and Kende, 1992). Deepwater rice is a subsistence crop in large areas of Southeast Asia that are inundated by flood waters during the monsoon season. The livelihood of >100 million people depends on the successful cultivation of this rice (Mannan, 1988). The remarkably high growth rates of deepwater rice—up to 25 cm a day (Vergara et al., 1976)—permit this rice to emerge from the rising flood waters and to avoid drowning. The yield of deepwater rice is far below that of improved rice cultivars, which lack elongation capacity (Mannan, 1988). Understanding the molecular basis of the growth response of deepwater rice is necessary for the eventual introduction of increased elongation capacity into high-yielding modern rice cultivars.

The primary signal for enhanced elongation is the reduced partial pressure of O_2 in the submerged tissue. Under low O_2 tension, ethylene synthesis is enhanced; ethylene renders the internode more responsive to gibberellin (GA) by lowering the level of endogenous abscisic acid (Raskin and Kende, 1984a, 1984b; Hoffmann-Benning and Kende, 1992). GA is the immediate growth-promoting hormone and acts by enhancing cell elongation and cell division activity in the intercalary meristem (Sauter and Kende, 1992; Sauter et al., 1993).

Hormonal and environmental stimuli that promote growth of plant cells act by inducing stress relaxation of the primary cell wall, which is thought to be principally a network of cellulose microfibrils interconnected by hemicelluloses (Carpita and Gibeaut, 1993). On the basis of this cell wall model, two wall-loosening processes can be envisioned: breakage of covalent bonds within cross-linking polymers or disruption of noncovalent bonds, such as hydrogen or ionic bonds, between wall polymers. It has been proposed that cleavage of covalent bonds is catalyzed by wall hydrolases or endotransglycosylases (Fry, 1989; Fry et al., 1992; Nishitani and Tominaga, 1992). Current evidence does not support the notion that these enzymes are able to cause long-term extension of isolated cell walls (McQueen-Mason et al., 1993; Cosgrove and Durachko, 1994). Recently, a new group of wall proteins, the expansins, has been identified as wall-loosening factors that can promote long-term extension of isolated cell walls (reviewed in McQueen-Mason, 1995; Cosgrove, 1996). Evidence indicates that expansins cause cell wall loosening by disrupting hydrogen bonds between cellulose microfibrils and matrix polymers (McQueen-Mason and Cosgrove, 1994).

Two expansin proteins have been isolated from deepwater rice internodes (Cho and Kende, 1997a), and three expansin

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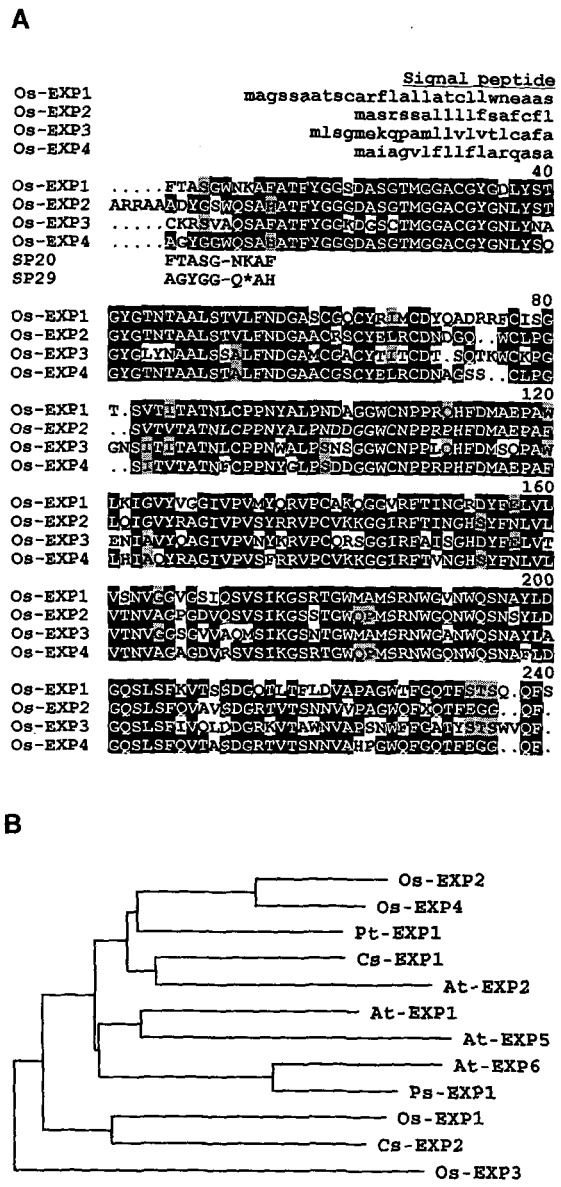

Figure 1. Comparison of the Deduced Amino Acid Sequences of Rice Expansin Proteins.

(A) Alignment of the rice expansin amino acid sequences deduced from cDNA sequences. SP20 and SP29 are the N-terminal amino acid sequences of two expansins isolated from air-grown rice internodes (Cho and Kende, 1997a). The amino acid at position 6 could not be determined, and position 8 of SP29 (*) may be either a T or an S residue. Gaps are shown as dots; letters on black and gray background indicate identical amino acids. Signal peptide sequences were predicted using the PSORT program (Nakai and Kanehisa, 1992).

(B) Phylogenetic tree of expansins. Mature protein regions without signal peptides were compared using the clustal method of DNASTAR Megalign (DNASTAR Inc., Madison, WI). GenBank accession numbers are U30382 (Cs-*EXP1*), U30460 (*Cs-EXP2*), U30476 (*At-EXP1*), U30477 (*Os-EXP2*), U30478 (*At-EXP5*), U30479 (*Os-EXP3*), U30480 (*At-EXP6*), U30481 (*At-EXP2*), U64892 (*Pt-EXP1*), U85246 (*Os-EXP4*), X85187 (*Ps-EXP1*), and Y07782 (*Os-EXP1*).

genes have been identified from rice (Shcherban *et al.*, *1995*; Cho and Kende, 1997a). Expansin gene families have also been found in cucumber and Arabidopsis (Shcherban et al., 1995). The redundancy of expansin genes indicates that expression of individual expansin genes may be differentially regulated at various developmental stages and by diverse environmental stimuli. This would permit the plant to fine-tune its response to developmental signals and changes in the environment. We report here on the identification of a fourth rice expansin gene, *Os-EXP4*, whose expression is highly correlated with growth, and show tissue-specific expression of expansin genes and their developmental, hormonal, and environmental regulation. Our results support the hypothesis that expansins may at least in part determine the growth rate of plant organs. Therefore, increasing the expression of the proper expansin genes may raise the growth potential of high-yielding rice cultivars.

RESULTS

Cloning of *Os-EXP4* and Sequence Analysis of the Rice Expansin Gene Family

We have previously isolated two expansin proteins, SP20 and SP29, from rice internodes and have sequenced their N-terminal amino acids (Cho and Kende, 1997a). The N-terminal amino acid sequence of SP20 is identical to that encoded by *Os-EXP1*. Because six of eight N-terminal amino acids of SP29 match an amino acid sequence deduced from the nucleotide sequence of *Os-EXP2*, we concluded tentatively that SP29 is encoded by a rice expansin gene that is different from yet very similar to *Os-EXP2*. To isolate the cDNA coding for SP29, we screened a cDNA library prepared from deepwater rice internodes with a probe from the conserved coding region of *Os-EXP2*. Positive clones were further screened with gene-specific probes of *Os-EXP1* and *Os-EXP2*. One cDNA clone that did not hybridize with the gene-specific probes was sequenced and shown to represent the new rice expansin gene *Os-EXP4* (GenBank accession number U85246).

Os-EXP4 consists of 1229 bp, with an open reading frame of 738 bp, 54 bp of the 5′ flanking sequence, and 437 bp of the 3′ flanking sequence. The open reading frame encodes a protein of 246 amino acids, with a signal peptide predicted by the PSORT program (Nakai and Kanehisa, 1992) (Figure 1A). The mature protein of 228 amino acids has a calculated molecular mass of 24 kD, which agrees well with the molecular mass of SP29 as determined by SDS-PAGE (Cho and

A

	Os-EXP1	Os-EXP2	Os-EXP3
Os-EXP2	24.5		
Os-EXP3	26.2	25.5	
Os-EXP4	25.9	26.4	21.4

B

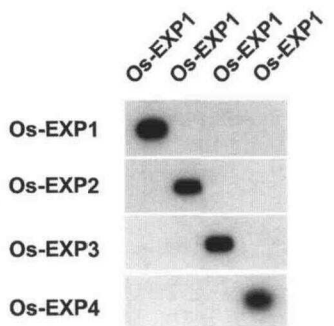

Figure 2. Gene-Specific Rice Expansin cDNA Probes.

(A) The percentage of nucleotide identity of the gene-specific rice expansin cDNA probes.

(B) DNA gel blot analysis showing the specificity of the gene-specific probes. Plasmid DNAs given above the blots were digested with restriction enzymes, subjected to electrophoresis, blotted, and hybridized under stringent conditions with the gene-specific probes indicated at left.

Kende, 1997a). The deduced amino acid sequence of *Os-EXP4* indicates that it indeed encodes SP29 (Figure 1A). Eight N-terminal amino acids of SP29 match those encoded by *Os-EXP4*. At position 8, amino acid sequencing yielded both T and S residues; the amino acid deduced from *Os-EXP4* is serine. The cleavage sites of the signal peptides of Os-EXP1 and Os-EXP4, predicted by the PSORT program, are consistent with the N-terminal amino acid sequences of SP20 and SP29. The deduced amino acid sequences of the rice expansin family show that *Os-EXP2* and *Os-EXP4* are most closely related to each other, with an amino acid identity of 85.8%; *Os-EXP3* is least related to the other rice expansin genes and also to all other known plant expansin genes (Figure 1B).

To investigate the differential expression of each rice expansin gene in various rice tissues, gene-specific probes were prepared consisting mainly of the 3′ untranslated regions of the respective cDNAs. These regions have 21.4 to 26.4% nucleotide identity to each other (Figure 2A). DNA gel blot analyses performed under the same conditions as used for RNA gel blotting (65°C washing temperature) showed that all four probes were indeed gene specific (Figure 2B).

Genomic Analysis

DNA gel blot analysis was used to estimate the complexity of the expansin gene family in the rice genome (Figure 3). Each gene-specific probe detected a single band, indicating that the four known rice expansin genes exist as single copies (Figures 3A to 3D). Because some probes contained short stretches from the coding region and because relatively low-stringency conditions were used (55°C washing temperature), some blots showed additional faint bands whose sizes corresponded to known expansin bands. *Os-EXP3* has a BamHI site in the middle of the coding region. Therefore, a band (~1 kb) shown in lane B+H in Figure 3C corresponds to the 3′ half of the gene. A probe prepared from the 5′ flanking and coding region of *Os-EXP3* detected the 5′ half (~1.3 kb) of the gene (data not shown). The DNA gel blot analysis performed with a probe from the coding region of *Os-EXP1* showed the four expansin genes and several unidentified bands (Figure 3E, lane E). The bands of ~10.3 and 6.3 kb (Figure 3E, lane E) could be detected in other gel blot analyses that were performed with probes from the coding regions of other expansin cDNAs (results not shown). These additional bands may represent other expansin genes or related genes, such as those encoding pollen

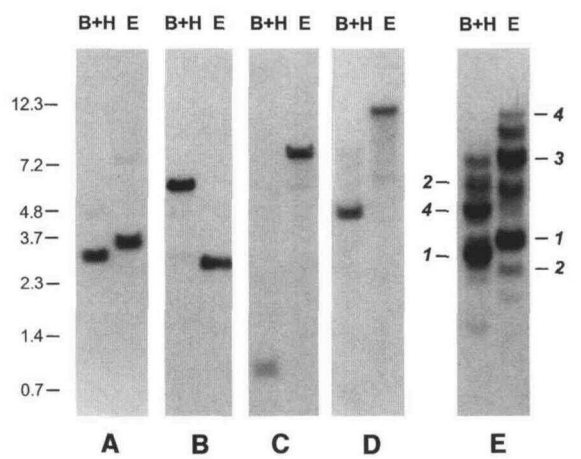

Figure 3. DNA Gel Blot Analysis of the Expansin Gene Family in the Rice Genome.

Five micrograms of rice genomic DNA was digested either with EcoRI (E) or with BamHI and HindIII (B+H) and subjected to gel blot hybridization. Gene-specific probes were used.

(A) *Os-EXP1*.

(B) *Os-EXP2*.

(C) *Os-EXP3*.

(D) *Os-EXP4*.

(E) Probe from the coding region of *Os-EXP1*. Each identified expansin band is labeled with its respective number. The length of the DNA markers is indicated at left in kilobases.

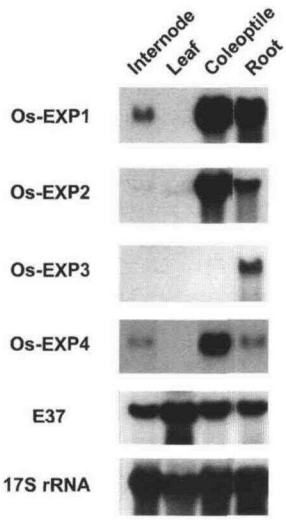

Figure 4. Tissue-Specific Expression Pattern of Rice Expansin Genes.

Each lane contains 20 μg of total RNA isolated from the basal 1-cm region of uppermost internodes, expanding leaves of mature plants, and the apical region of coleoptiles and roots. The transcript levels of *E37* encoding a plastid inner envelop protein and 17S rRNA served as internal loading controls.

allergens and their homologs, which are considered to be a subfamily of the expansins (Cosgrove et al., 1997).

Organ-Specific Expression of Expansin Genes

The transcript levels of the four expansin genes were examined in the expanding region of young leaves from 11- to 13-week-old air-grown plants; in the basal 1-cm region of the uppermost internode, which contains the intercalary meristem and elongation zone from the same adult plants; and in the apical region of coleoptiles and roots from 3-day-old seedlings (Figure 4). Air-grown mature plants grow slowly, whereas the coleoptile and root of seedlings elongate at a fast rate. In the coleoptile and internode, *Os-EXP1*, *Os-EXP2*, and *Os-EXP4* were expressed, but no detectable *Os-EXP3* signal was found in either tissue. In the root, all expansin genes were expressed. This was the only tissue in which *Os-EXP3* mRNA was found. Only *Os-EXP2* transcript could be detected at low abundance in the growing zone of leaves. Expansin mRNAs accumulated to higher levels in the apical regions of coleoptiles and roots than in the more slowly expanding tissues of internodes and leaves. The transcript level of *E37*, which encodes a major 37-kD protein of the inner plastid envelope (Teyssier et al., 1996), served as control for the quantity of total RNA loaded because its expression remains constant in rice internodes induced to grow rapidly by GA (Van der Knaap and Kende, 1995); it is also of similar magnitude in root and coleoptile apices. In leaves, which contain more plastids than do other organs, the abundance of *E37* mRNA was increased. Therefore, 17S rRNA was used as an additional internal loading control.

Expression of Expansin Genes in Different Developmental Regions of Coleoptiles, Roots, and Internodes

Os-EXP1 and *Os-EXP4* were expressed at higher levels in the apical 1-cm region of the coleoptile, where growth occurs, than in the adjacent basal region; the transcript level of *Os-EXP2* was similar in both regions (Figures 5B and 5C). In the root, expression of *Os-EXP1*, *Os-EXP3*, and *Os-EXP4*

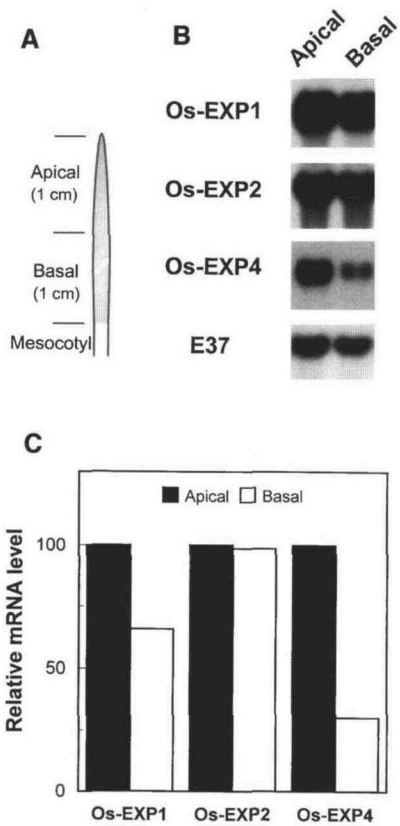

Figure 5. Differential Expression of Rice Expansin Genes in the Apical and Basal Regions of the Coleoptile.

(A) The apical and basal regions of the coleoptile from dark-grown 3-day-old rice seedlings.
(B) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from each region. *E37* was used as the internal loading control.
(C) Quantification of mRNA levels of each expansin gene. Blots shown in **(B)** were quantified with a PhosphorImager, and the relative mRNA levels were calculated by setting the value for the apical region to 100.

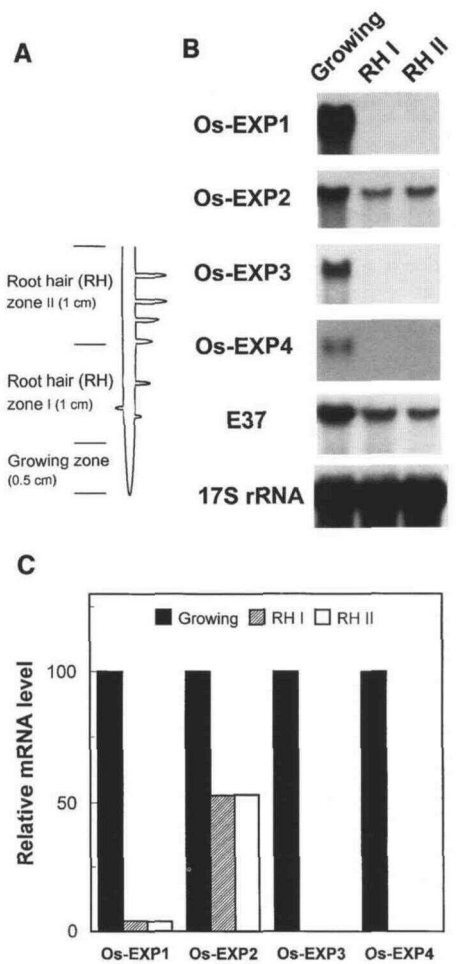

Figure 6. Differential Expression of Rice Expansin Genes in Different Developmental Regions of the Root.

(A) Different developmental regions of the primary root from dark-grown 3-day-old rice seedlings.

(B) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from each region. *E37* and 17S rRNA were used as internal loading controls.

(C) Quantification of mRNA levels of each expansin gene. Blots shown in **(B)** were quantified with a PhosphorImager, and the relative mRNA levels were calculated by setting the value for the growing region to 100.

transcripts was confined to the apical 5-mm region, which corresponds to the growing zone (Figures 6B and 6C). *Os-EXP2* mRNA also accumulated mainly in the apical region but was expressed in the root hair zone as well. The level of *E37* mRNA was lower in the root hair zone than in the apical region, but the abundance of 17S rRNA remained constant along the root, confirming equal loading of total RNA. In air-grown internodes, *Os-EXP1* and *Os-EXP4* were expressed at the highest levels in the basal growing zone, with transcript levels declining in the nongrowing upper regions (Figures 7B and 7C). The mRNA abundance of *Os-EXP2* showed the opposite tendency; it was much lower in the basal growing zone than in the upper nongrowing regions.

Differential Cell Wall Extensibility along the Developmental Regions of the Coleoptile, Root, and Internode

To correlate expansin action and expression of expansin genes, we examined acid-induced extensibility of isolated cell walls from the same developmental regions that were

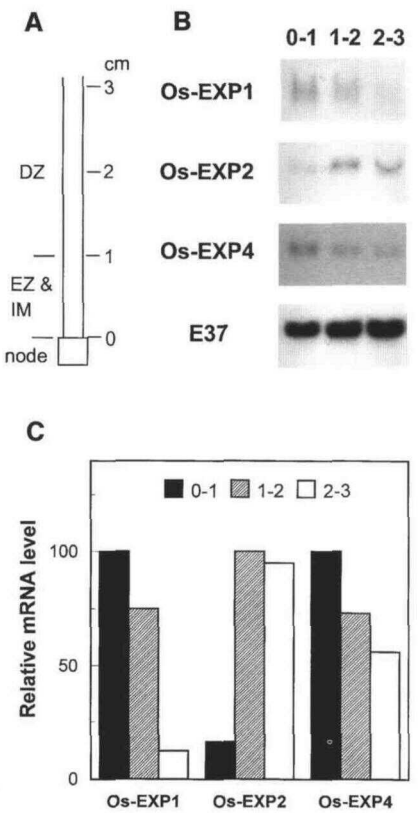

Figure 7. Differential Expression of Rice Expansin Genes along the Different Developmental Regions of the Uppermost Internode.

(A) Different developmental regions of the uppermost internode from 11- to 13-week-old rice plants. DZ, differentiation zone; EZ, elongation zone; IM, intercalary meristem.

(B) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from each region. *E37* was used as the internal loading control. The numerals 0-1, 1-2, and 2-3 indicate distance from the node in centimeters.

(C) Quantification of the message levels of each expansin gene. Blots shown in **(B)** were quantified with a PhosphorImager, and the relative mRNA levels were calculated by setting the highest value for each blot to 100.

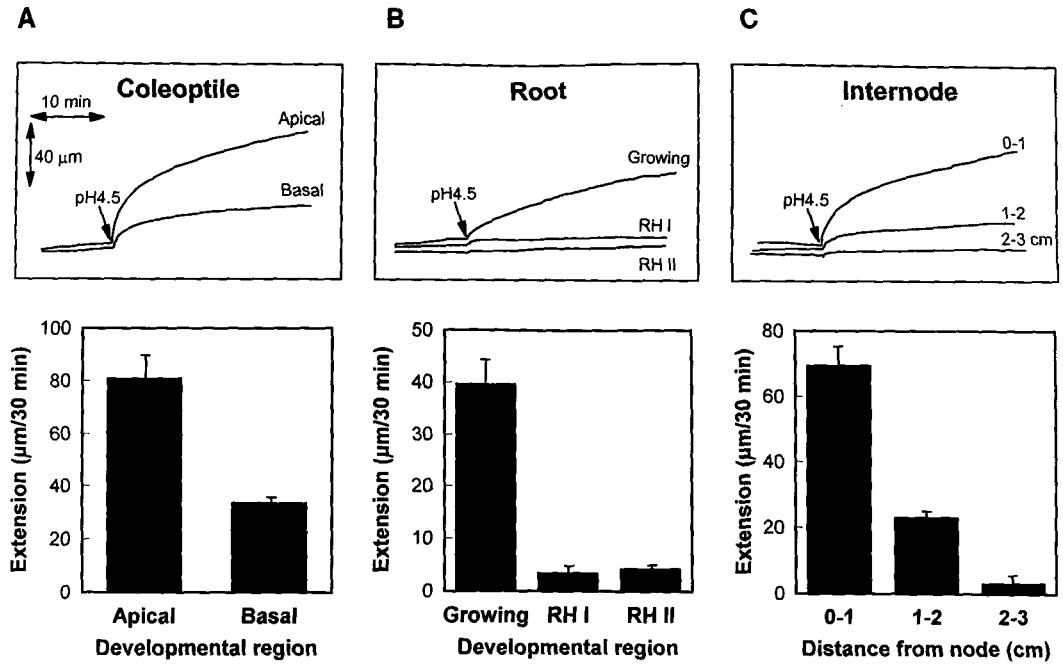

Figure 8. Acid-Induced Extension of the Cell Walls from Different Developmental Regions of Coleoptiles, Roots, and Internodes.

(A) Acid extension of the coleoptile cell walls. Extension of the middle 5-mm regions from the 1-cm-long apical and basal segments (see Figure 5A) was measured.

(B) Acid extension of the root cell walls. Extension of the middle 4-mm regions from each root segment (see Figure 6A) was measured. RH, root hair.

(C) Acid extension of the internodal cell walls. Extension of the entire 1-cm segment of each region (see Figure 7A) was measured.

After the cell wall preparations were incubated in pH 6.8 buffer for 20 min, the solution was changed (arrows) to pH 4.5 buffer. Coleoptile and internodal cell walls were subjected to constant tension by using a weight of 10 g, with 5 g being used for roots. The chart recorder tracings are from a single representative experiment. Data are mean values ±SE ($n = 5$ for the coleoptile; $n = 4$ for the root and internode).

used in the gene expression studies. Extensibility of the cell wall from the apical region of the coleoptile was approximately twofold higher than that from the basal region (Figure 8A). In the root, wall extensibility was entirely confined to the apical 5-mm region (Figure 8B). In internodes, cell wall extensibility also declined sharply from the growing to the nongrowing region (Figure 8C).

Accumulation of Expansin Transcripts in Response to GA and Submergence

We compared the levels of the three internodal expansin transcripts in air-grown (control), submerged, and GA-treated internodes from 11- to 13-week-old plants (Figures 9A and 9B). *Os-EXP1* mRNA levels decreased at the beginning of incubation, probably as a result of excising the stem sections that contain the growing internode. Between 24 and 48 hr of incubation, the transcript level of *Os-EXP1* returned to its initial value in GA-treated internodes; in submerged internodes, it returned to approximately half of its original value. *Os-EXP2* mRNA accumulated gradually during 48 hr of treatment with GA but rapidly between 3 and 6 hr of submergence. In contrast, the abundance of *Os-EXP4* transcript increased rapidly within 6 hr of incubation with GA and within 3 hr of submergence.

Because the level of *Os-EXP4* transcript increased earlier than that of *Os-EXP2* in response to both treatment with GA and submergence, we determined the time course of *Os-EXP4* mRNA accumulation during the first 6 hr of submergence and incubation in GA (Figure 10). Under both experimental conditions, the transcript level of *Os-EXP4* increased within 30 min and reached a maximum after 3 hr.

DISCUSSION

Conservation of Expansins during Evolution

The rice expansin gene family does not represent a separate monocotyledonous branch in the phylogenetic tree of known

expansins (Figure 1B). The closely related rice expansins *Os-EXP2* and *Os-EXP4* form a subfamily with a *Pinus taeda* expansin (*Pt-EXP1*), a cucumber expansin (*Cs-EXP1*), and an Arabidopsis expansin (*At-EXP2*). The high similarity of expansins from a gymnosperm and from monocotyledonous and dicotyledonous angiosperms is evidence for the conservation of this family of proteins during the evolution of seed plants. *Os-EXP1 and another cucumber expansin, Cs-EXP2*, are closely related, whereas *Os-EXP3* showed least similarity to other expansins.

Acid-induced cell elongation occurs in algae, mosses, ferns, gymnosperms, and angiosperms (Taiz, 1984; Cosgrove, 1996). Because expansins mediate extension of isolated plant cell walls under acid conditions, it is conceivable that they play a role in cell enlargement of both lower and higher plants. Even though phylogenetically separate organisms have cell walls of different composition, expansins may act by a common mechanism suggested for higher plant cell walls, namely, by disrupting hydrogen bonds between load-bearing wall polymers (McQueen-Mason and Cosgrove, 1994).

Differential Expression Pattern of Expansin Genes

Expansin genes are differentially expressed in the major vegetative parts of rice plants, and their mRNAs are most

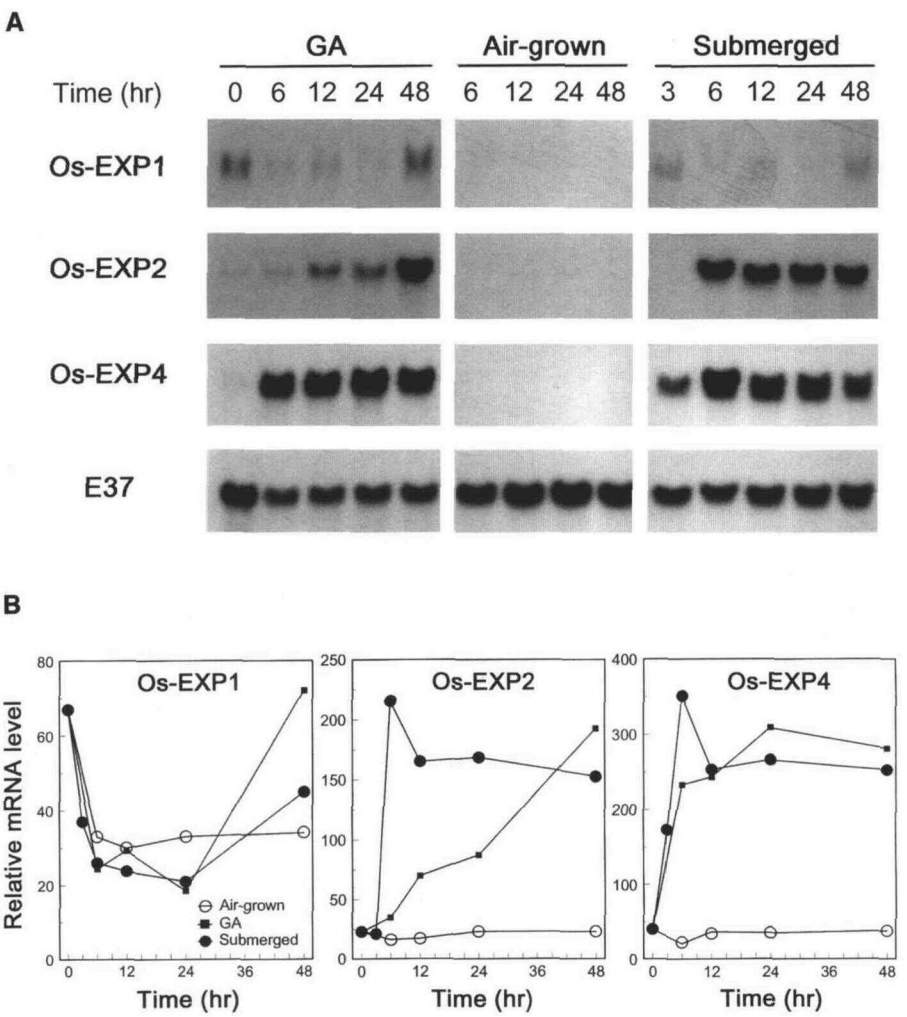

Figure 9. Accumulation of Rice Expansin Transcripts in GA-Treated and Submerged Internodes.

(A) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from the basal 2-cm region of the uppermost internodes that had been treated with 50 μM GA_3, submerged, or kept in air for 0 to 48 hr. *E37* served as internal control.
(B) Quantification of the mRNA levels of each gene. Blots shown in **(A)** were quantified with a PhosphorImager.

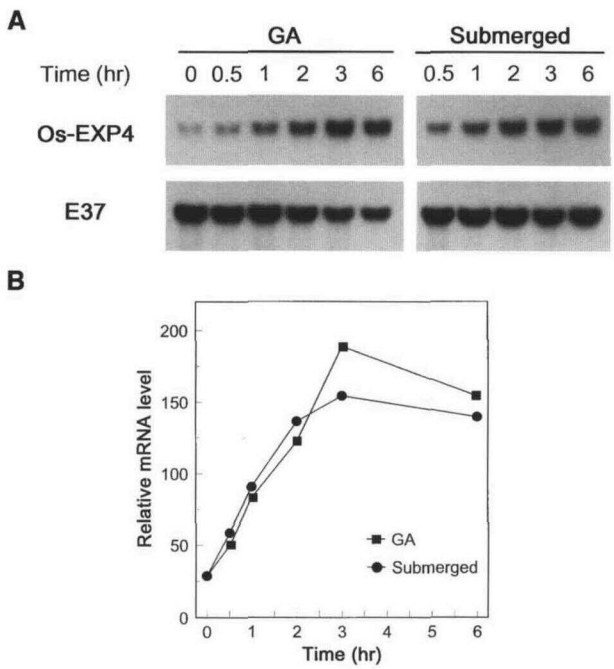

Figure 10. Accumulation of the *Os-EXP4* Transcript in GA-Treated and Submerged Internodes.

(A) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from the basal 2-cm region of the uppermost internodes that had been treated with 50 μM GA_3 or submerged for 0 to 6 hr.
(B) Quantification of *Os-EXP4* mRNA levels. Blots shown in **(A)** were quantified with a PhosphorImager.

abundant in actively growing organs, such as the coleoptile and the primary root (Figure 4). In expanding leaves, only low expression of *Os-EXP2* has been found. It is conceivable that there are leaf-specific expansins that have not yet been identified and whose transcripts are not represented in our cDNA library prepared from deepwater rice internodes. DNA gel blot analysis indicates that there may indeed be more than four expansin genes in rice (Figure 3), in addition to the allergen-type β-expanins identified by Cosgrove et al. (1997). Expression of *Os-EXP3* is confined to the very short apical region of the root.

Differential expression of expansin genes also occurs along the developmental regions of the coleoptile, root, and internode (Figures 5 to 7). Whereas mRNA abundance of *Os-EXP1*, Os-*EXP3*, and *Os-EXP4* is correlated with cell elongation, expression of *Os-EXP2* appears to be less linked to primary growth. In contrast to the expression of other expansin genes, *Os-EXP2* continues to be expressed in the root hair zones where growth of the primary root has ceased (Figures 6B and 6C). In the uppermost internode, the expression level of *Os-EXP2* is higher in the nongrowing differentiation zone than in the intercalary meristem and the elongation zone (Figures 7B and 7C). The difference in expression of *Os-EXP2* and the other expansin genes leads us to suggest a distinct role for the Os-EXP2 protein. The nongrowing regions of the internode and the root are both differentiating tissues. Therefore, *Os-EXP2* may play a role in the differentiation of the vascular system and perhaps in the differentiation and growth of root hairs. In maize roots, cell wall proteins extracted from the basal region showed higher extension activity than did cell wall proteins from the apical region (Wu et al., 1996). It is possible that an expansin with a function(s) similar to that of *Os-EXP2* is expressed at an elevated level in the basal region of the primary root of maize as well.

We have previously reported on the highly localized occurrence of expansin protein along the inner epidermal layer and around immature vascular bundles of rice internodes (Cho and Kende, 1997b). We do not know the role of expansins in those tissues, but the high concentration of expansins in specific cells further supports the notion that certain expansins, for example, those associated with the developing vasculature, may have physiological functions other than promoting cell elongation. McQueen-Mason and Cosgrove (1995) showed that the two cucumber expansins have slightly different pH dependence and cell wall rheological effects, which may indicate differences in functions, and Rose et al. (1997) have identified a family of expansins that may play a role in cell wall disassembly of ripening fruits. Thus, there is mounting evidence that besides mediating cell wall extension, expansins may also function in other processes involving cell wall modifications. However, the biochemical mechanism of expansin action, namely, disruption of hydrogen bonds between cell wall polymers, may be common to all expansin-mediated reactions.

Expansin Gene Expression Is Correlated with Rapid Internodal Growth of Deepwater Rice Induced by Submergence and GA

The response of deepwater rice internodes to submergence illustrates how a physiological process, in this case rapid internodal elongation, is controlled by the interaction between plant hormones (Raskin and Kende, 1984b; Hoffmann-Benning and Kende, 1992). Both the initial environmental signal, submergence, and the immediate growth-promoting hormone, GA, greatly increased the mRNA abundance of *Os-EXP2* and *Os-EXP4* (Figure 9). Submergence and GA promoted expression of *Os-EXP4* within 30 min (Figure 10), which is within the observed lag time of 40 min for GA-induced internodal growth (Sauter and Kende, 1992) and 3 hr and 20 min for submergence-induced elongation (Rose-John and Kende, 1985). The accumulation of *Os-EXP2* mRNA was considerably slower than that of *Os-EXP4* in both GA-treated and submerged internodes. These results support the hypothesis that Os-EXP4 plays a role in submergence- and GA-enhanced growth of deepwater rice.

Expansin Gene Expression and Cell Wall Extensibility

Acid-induced extensibility of native cell walls is indicative for the expansin content of the wall and for the susceptibility of the cell wall to expansin action (Cosgrove, 1996). These two parameters may at least in part also determine the growth rate of the tissue. In oat coleoptiles (Cosgrove and Li, 1993) and cucumber hypocotyls (McQueen-Mason, 1995), susceptibility to expansin rather than expansin content may be a determining factor in establishing the rate of growth. In contrast, elongation of maize roots under water stress (Wu et al., 1996) and growth of deepwater rice internodes as a function of the developmental stage or as a result of submergence are correlated with both expansin content and susceptibility of the cell wall to expansin (Cho and Kende, 1997b). The pattern of acid-induced wall extensibility of different developmental regions of the coleoptile, root, and internode (Figure 8) is similar to the expression pattern of the *Os-EXP1*, *Os-EXP3*, and *Os-EXP4* genes (Figures 5 to 7). This is further evidence that growth of these three vegetative organs is determined at least in part by the level of expansin.

Conclusions

In this study, we have shown that (1) four expansin genes are differentially expressed in the coleoptile, root, and internode of deepwater rice; (2) developmental, hormonal, and environmental signals affect expression of expansin genes differentially; and (3) the expression pattern of some of the expansin genes is highly correlated with wall extensibility and cell elongation. We propose that individual expansins play different roles in cell expansion and differentiation and that expansin action contributes to the growth of the major vegetative organs of rice and ultimately to survival of deepwater rice under submergence.

METHODS

Plant Material

Rice (*Oryza sativa* cv Pin Gaew 56) seeds were germinated on two sheets of moist Whatman No. 1 filter paper in a Petri dish in darkness at 30°C for 3 days. Seedlings with coleoptiles 2- to 2.5-cm long and roots 4- to 6-cm long were selected for RNA isolation and cell wall extension measurements. For experiments with internodes, rice plants were grown as described previously (Stünzi and Kende, 1989). Twenty-centimeter-long stem sections containing the uppermost internode were excised from 11- to 13-week-old plants, according to the method of Raskin and Kende (1984a). For treatment in air and gibberellic acid (GA_3), 15 to 20 sections were incubated in distilled water or in 50 μM GA_3 such that the water level remained below the basal node. For submergence, the sections, with a weight attached, were completely immersed in 2.5-liter, 60-cm-deep plexiglass cylinders containing distilled water (Kutschera and Kende, 1988). They were incubated for up to 48 hr at 27°C under continuous light (cool-white fluorescent tubes, 53 μmol m^{-2} sec^{-1}; General Electric, Cleveland, OH).

cDNA Cloning and DNA Sequencing

The rice expansin *Os-EXP4* cDNA was cloned from a rice internode cDNA library. A 750-bp probe containing the coding region was generated from *Os-EXP2* by digestion using SalI and EagI. Plaque-forming units (100,000) of the cDNA library were screened with this probe. Single positive plaques were screened with *Os-EXP1*– and *Os-EXP2*–specific probes in the fifth screening. Hybridization of the plaque blots on nitrocellulose (Protran; Schleicher & Schuell) was performed in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, and 5 × Denhardt's solution (1 × Denhardt's solution is 0.01% Ficoll, 0.01% PVP, 0.01% BSA) at 62°C for 16 hr. The nitrocellulose membranes were washed with 0.2 × SSC and 0.1% SDS at 62°C. A clone that hybridized with the probe from the *Os-EXP2* coding region but not with *Os-EXP1*– and *Os-EXP2*–specific 3′ probes was isolated. The insert from the phage DNA was isolated and cloned into pBluescript SK− phagemid (Stratagene, La Jolla, CA).

The DNA insert was sequenced using Taq FS DNA polymerase and fluorescent-dideoxy terminators in a cycle-sequencing method. The resultant DNA fragments were separated by electrophoresis and analyzed using an automated Applied Biosystems (Foster City, CA) 373A Stretch DNA sequencer at the W.M. Keck facility of Yale University (New Haven, CT). This new rice expansin cDNA clone was named *Os-EXP4*.

Preparation of the Probes

Gene-specific probes were prepared mainly from the 3′ untranslated regions of the respective transcripts. A 440-bp fragment between the HincII and EagI restriction sites (including 142 bp from the coding region) was used for the *Os-EXP1*–specific probe, and a 580-bp fragment between two EagI restriction sites (including 72 bp from the coding region) was used for the *Os-EXP2*–specific probe. Probes were generated by polymerase chain reaction with oligonucleotide primers corresponding to 3′ gene-specific regions of *Os-EXP3* (5′-GTCGCCCCGTCCAACTGGTTC-3′ and 5′-AATTGGTGGGCAAAACATTCA-3′) and *Os-EXP4* (5′-CCAGTTCTAGCCGCCACCGACATC-3′ and 5′-ATTCCGTTGCAAGGCCATCACTCC-3′). The length of the polymerase chain reaction probes was 270 bp for *Os-EXP3* (including 35 bp from the coding region) and 457 bp for *Os-EXP4*. A probe (656 bp) used for genomic DNA gel blot analysis was prepared from the coding region of *Os-EXP1* by HincII restriction digestion.

RNA and DNA Gel Blot Analyses

Total RNA was extracted according to the method of Puissant and Houdeline (1990). Twenty micrograms of total RNA was separated on formaldehyde gels and stained with ethidium bromide to ensure equal loading of RNA. The RNA was then capillary transferred to nylon membranes (Hybond N; Amersham) with 20 × SSC for 16 hr. Radiolabeled DNA probes were prepared using a random priming DNA labeling kit (Boehringer Mannheim). The membranes were prehybridized at 42°C in 5 × SSC, 10 × Denhardt's solution, 0.1% SDS, 0.1 M potassium phosphate, pH 6.8, and 0.2 mg mL^{-1} salmon sperm DNA and hybridized at 42°C in 5 × SSC, 30% formamide, 10% dextran

sulfate, 10 × Denhardt's solution, 0.1 M potasium phosphate, pH 6.8, and 0.2 mg mL^{-1} salmon sperm DNA. The membranes were then washed twice for 40 min with 2 × SSC and 0.2% SDS and twice for 40 min with 0.2 × SSC and 0.1% SDS at 65°C. Autoradiography was performed using Hyperfilm-MP (Amersham) at −80°C with two amplification screens. The message levels were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed with the ImageQuant program (Molecular Dynamics). For the RNA gel blot analyses with *E37* and 17S rRNA, the membranes were stripped and probed again with one of the above probes.

For cDNA gel blot analysis, 2 ng of purified plasmid DNA was restriction digested, separated by electrophoresis, and transferred to a nylon membrane. Prehybridization, hybridization, washing, and autoradiography of the blots were performed as described for RNA gel blot analysis.

Genomic DNA was isolated from rice internodes using the method described in Ausubel et al. (1987). Five micrograms of DNA was digested either with EcoRI or with BamHI and HindIII, separated in an 0.8% agarose gel, and transferred to a nylon membrane. Prehybridization, hybridization, and autoradiography of the blots was performed as described for RNA blot analysis, except that the blots were washed at 55°C.

Measurement of Cell Wall Extension

Cell wall extension (creep) was measured according to the method of Cosgrove (1989), as modified for rice tissue (Cho and Kende, 1997a). Coleoptiles, roots, and the uppermost internodes were frozen at −80°C, abraded with carborundum (300 mesh; Fisher, Fair Lawn, NJ) slurry, and thawed. One-centimeter-long sections of the coleoptiles, 5-mm-long sections of the roots, and 1.5-cm-long sections of the internodes were cut from each region (Figures 5A, 6A, and 7A) and pressed between filter paper to remove water and cell sap. The sections were inserted between two clamps spaced 5 mm apart for the coleoptile, 4 mm apart for the root, and 1 cm apart for the internode, placed into an extensometer equipped with an angular displacement transducer (Kutschera and Briggs, 1987), and subjected to constant tension using a weight of 10 g for the coleoptile and the internode and 5 g for the root. For acid-induced extension, the segments were submerged in 50 mM Hepes-Tris buffer, pH 6.8, for 20 min, after which the buffer was replaced with 50 mM sodium acetate buffer, pH 4.5.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology, 1st ed. (New York: Wiley Interscience).

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3,** 1–30.

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

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

Cosgrove, D.J. (1989). Characterization of long-term extension of isolated cell walls from growing cucumber hypocotyls. Planta **177,** 121–130.

Cosgrove, D.J. (1996). Plant cell enlargement and the action of expansins. Bioessays **18,** 533–540.

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

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

Cosgrove, D.J., Bedinger, P., and Durachko, D.M. (1997). Group I allergens of grass pollen as cell-wall loosening agents. Proc. Natl. Acad. Sci. USA **94,** 6559–6564.

Fry, S.C. (1989). Cellulases, hemicelluloses and auxin-stimulated growth: A possible relationship. Physiol. Plant. **75,** 532–536.

Fry, S.C., Smith, R.C., Renwick, K.R., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem. J. **282,** 821–828.

Hoffmann-Benning, S., and Kende, H. (1992). On the role of abscisic acid and gibberellin in the regulation of growth in rice. Plant Physiol. **99,** 1156–1161.

Kutschera, U., and Briggs, W.R. (1987). Differential effect of auxin on *in vivo* extensibility of cortical cylinder and epidermis in pea internodes. Plant Physiol. **84,** 1361–1366.

Kutschera, U., and Kende, H. (1988). The biophysical basis of elongation growth in internodes of deepwater rice. Plant Physiol. **88,** 361–366.

Mannan, M.A. (1988). Deepwater rice in perspective. In Proceedings of the 1987 International Deepwater Rice Workshop (Manila, Philippines: International Rice Research Institute), pp. 5–9.

McQueen-Mason, S.J. (1995). Expansins and cell wall expansion. J. Exp. Bot. **46,** 1639–1650.

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

McQueen-Mason, S.J., and Cosgrove, D.J. (1995). Expansin mode of action on cell walls. Plant Physiol. **107,** 87–100.

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

Nakai, K., and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14,** 897–911.

Nishitani, K., and Tominaga, R. (1992). Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. J. Biol. Chem. **267,** 21058–21064.

Puissant, C., and Houdeline, L.-M. (1990). An improvement of the single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. BioTechniques **9,** 148–149.

Raskin, I., and Kende, H. (1984a). Regulation of growth in stem sections of deep-water rice. Planta **160,** 66–72.

Raskin, I., and Kende, H. (1984b). Role of gibberellin in the growth response of submerged deep water rice. Plant Physiol. **76,** 947–950.

Rose, J.K.C., Lee, H.H., and Bennett, A.B. (1997). Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proc. Natl. Acad. Sci. USA **94,** 5955–5960.

Rose-John, S., and Kende, H. (1985). Short-term growth response of deepwater rice to submergence and ethylene. Plant Sci. **38,** 129–134.

Sauter, M., and Kende, H. (1992). Gibberellin-induced growth and regulation of the cell division cycle in deepwater rice. Planta **188,** 362–368.

Sauter, M., Seagull, R.W., and Kende, H. (1993). Internodal elongation and orientation of cellulose microfibrils and microtubules in deepwater rice. Planta **190,** 354–362.

Shcherban, T.Y., Shi, J., Durachko, D.M., Guiltinan, M.J., McQueen-Mason, S.J., Shieh, M., and Cosgrove, D.J. (1995). Molecular cloning and sequence analysis of expansins—A highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc. Natl. Acad. Sci. USA **92,** 9245–9249.

Stünzi, J.T., and Kende, H. (1989). Gas composition in the internal air spaces of deepwater rice in relation to growth induced by submergence. Plant Cell Physiol. **30,** 49–56.

Taiz, L. (1984). Plant cell expansion: Regulation of cell wall mechanical properties. Annu. Rev. Plant Physiol. **35,** 585–657.

Teyssier, E., Block, M.A., Douce, R., and Joyard, J. (1996). Is *E37*, a major polypeptide of the inner membrane from plastid envelope, an *S*-adenosyl methionine–dependent methyltransferase? Plant J. **10,** 903–912.

Van der Knaap, E., and Kende, H. (1995). Identification of a gibberellin-induced gene in deepwater rice using differential display of mRNA. Plant Mol. Biol. **28,** 589–592.

Vergara, B.S., Jackson, B., and De Datta, S.K. (1976). Deep-water rice and its response to deep-water stress. In Climate and Rice (Los Baños, Philippines: International Rice Research Institute), pp. 301–319.

Wu, Y., Sharp, R.E., Durachko, D.M., and Cosgrove, D.J. (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.