Expression of β-Expansins Is Correlated with Internodal Elongation in Deepwater Rice[1]

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Fourteen putative rice (*Oryza sativa*) β-expansin genes, *Os-EXPB1* through *Os-EXPB14*, were identified in the expressed sequence tag and genomic databases. The DNA and deduced amino acid sequences are highly conserved in all 14 β-expansins. They have a series of conserved C (cysteine) residues in the N-terminal half of the protein, an HFD (histidine-phenylalanine-aspartate) motif in the central region, and a series of W (tryptophan) residues near the carboxyl terminus. Five β-expansin genes are expressed in deepwater rice internodes, with especially high transcript levels in the growing region. Expression of four β-expansin genes in the internode was induced by treatment with gibberellin and by wounding. The wound response resulted from excising stem sections or from piercing pinholes into the stem of intact plants. The level of wound-induced β-expansin transcripts declined rapidly 5 h after cutting of stem sections. We conclude that the expression of β-expansin genes is correlated with rapid elongation of deepwater rice internodes, it is induced by gibberellin and wounding, and wound-induced β-expansin mRNA appears to turn over rapidly.

Deepwater rice (*Oryza sativa*) is a subsistence crop in regions of Southeast Asia that are flooded during the monsoon season (Catling, 1992). To avoid drowning, deepwater rice has evolved the capacity to elongate very rapidly when it becomes submerged. This adaptation permits deepwater rice to keep part of its foliage above the rising flood waters. In the flood plains of Bangladesh, elongation rates of up to 25 cm d^{-1} have been reported (Vergara et al., 1976); in our laboratory, we have measured growth rates of up to 5 mm h^{-1} (Stünzi and Kende, 1989). These unusually high growth rates, which are under environmental and hormonal control, magnify growth-related cellular, physiological, biochemical, and molecular processes. Analyzing these processes may help to understand basic aspects of plant growth (Kende et al., 1998). As rice has become the monocot model plant for molecular-genetic studies, a wealth of information is becoming available through the expressed sequence tag (EST) and genome sequencing projects.

Expansins are proteins that mediate long-term extension of isolated cell walls. They are grouped into two related families, the α- and β-expansins (for review, see Cosgrove, 2000). Although α- and β-expansins share, on average, only about 20% to 25% overall amino acid identity, their predicted secondary structures are up to 75% identical (Cosgrove et al., 1997). Both types of expansins also share conserved C (Cys) and W (Trp) residues, as well as a conserved HFD (His-Phe-Asp) motif. The β-expansins originally were represented by group I allergens of grass pollen. Cosgrove et al. (1997) showed that the maize (*Zea mays*) pollen allergen can loosen the cell wall of maize silk and of wheat (*Triticum aestivum*) coleoptiles, but to a much lesser extent the cell wall of cucumber (*Cucumis sativus*) hypocotyls. It was suggested that group I pollen allergens serve to facilitate penetration of the pollen tube through the stigma and style. Further searches of the database showed that proteins related to group I allergens also occur in vegetative tissues and that these β-expansins are more abundant in grasses than in dicots (Cosgrove et al., 1997; Cosgrove, 2000). The role of β-expansins in vegetative growth has not been established. α-Expansins show lower activity on grass cell walls than on dicot cell walls (McQueen-Mason et al., 1992; Cho and Kende, 1997a), and grasses such as maize and rice contain far greater numbers of putative β-expansin genes than does, for example, Arabidopsis (Cosgrove, 2000). It is conceivable, therefore, that β-expansins will turn out to be the primary wall-loosening proteins in grasses, whose cell wall composition differs significantly from that of dicots (Carpita, 1996). Five α- and eight β-expansins were identified recently in maize and shown to be differentially expressed at the juvenile and adult stages of the plant, as well as in various organs (Wu et al., 2001a). Expression of one of these β-expansins and of two α-expansins is promoted in maize roots at low water potential (Wu et al., 2001b). Under these conditions, elongation of the root is maintained and cell wall extensibility increased.

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Four α-expansins have been studied in deepwater rice (Cho and Kende, 1997b). The expression of the corresponding genes was found to be organ specific and was correlated with acid-inducible cell wall extensibility. Expression of two α-expansin genes, *Os-EXP2* and *Os-EXP4*, was induced by submergence and treatment with gibberellin (GA; Cho and Kende, 1997b). We report here on the identification of 14 putative β-expansin genes in the rice EST and genomic databases and on the induction of their expression by GA and wounding. This is the first step in determining the function of β-expansins in the elongation of deepwater rice and of other grass internodes and in elucidating the respective roles of α- and β-expansins in this process.

RESULTS

Identification of β-Expansin Transcripts in Deepwater Rice Internodes

A search of the rice EST and genomic databases yielded 14 putative β-expansin genes. Based on analysis by the PSORT program (Nakai and Kanehisa, 1992), their deduced protein products have a signal peptide for entry into the secretory pathway and secretion to the cell wall. The molecular masses of the mature β-expansin proteins range from 26.6 to 31.3 kD. To identify which of the β-expansins are expressed in deepwater rice internodes, gene-specific probes were prepared consisting mainly of the 3′ untranslated regions of the respective cDNAs (Table I). When no cDNA was available, the gene-specific regions were amplified by reverse-transcription (RT)-PCR from mRNA or by PCR from genomic DNA. Five β-expansin genes were found to be expressed in deepwater rice internodes (*Os-EXPB3*, *Os-EXPB4*, *Os-EXPB6*, *Os-EXPB11*, and *Os-EXPB12*). DNA dot-blot and DNA gel-blot analyses performed under the same stringency conditions as used for the RNA gel blots showed no detectable cross-hybridization between the gene-specific probes corresponding to the transcripts of these five expansin genes (Fig. 1, A and B). *Os-EXPB13* mRNA was also expressed in GA-treated internodes at a level comparable to that of *Os-EXPB12*. However, the gene-specific probe of *Os-EXPB13* contained many repeated AT sequences, and the DNA gel blot analyzed with this probe showed, besides a major band, several minor bands (results not shown). Because of the very low *Os-EXPB13* mRNA levels and the limited usefulness of the corresponding probe, the expression pattern of *Os-EXPB13* is not included in our analysis.

Sequence Analysis of β-Expansins Expressed in Deepwater Rice Internodes

The deduced amino acid sequences of the five β-expansins expressed in the internode and of *Os-EXPB13* are shown in Figure 2A. They possess the same motifs that are also characteristic of α-expansins, namely conserved C (Cys) residues in the N-terminal region of the protein, a putative catalytic domain, the HFD (His-Phe-Asp) motif in the central portion of the protein, and conserved W (Trp) residues in the putative cellulose-binding domain in the C-terminal region. In addition, rice β-expansins have one to four NXT/S (Asn-X-Thr/Ser) motifs, which may represent N-linked glycosylation sites. Rice β-expansins are somewhat more divergent from each other (51% average amino acid identity between mature proteins) than are rice α-expansins (55% average amino acid identity between mature proteins; Y. Lee and H. Kende, unpublished data). The phylogenetic analysis shows that the α- and β-expansins belong to different protein families (Fig. 2B; Cosgrove, 2000). There was no significant relationship between the phylogenetic relatedness of β-expansins and the site and level of expression of their genes.

Expression of β-Expansin Genes in Deepwater Rice Internodes

In rice, as in other grasses, stem elongation occurs mainly at the base of the highest internode, just above the second-highest node. Four β-expansin genes, *Os-EXPB3*, *Os-EXPB4*, *Os-EXPB6*, and *Os-EXPB11*, were expressed in the highest internode and in the subtending node of intact plants that had neither been treated with GA nor had been wounded (Fig. 3). The transcript level of three β-expansins was highest in the basal region of the internode 0 to 1 cm above the node. This region contains the intercalary meristem, the elongation zone, and the lower part of the differentiation zone (Kende et al., 1998). The fourth β-expansin gene, *Os-EXPB3,* was expressed at the highest level in the second-highest node. Except for *Os-EXPB3*, β-expansin mRNA was not detected 3 cm above the node in the differentiated regions that had stopped growing. *Os-EXPB12* was also expressed in the internode but at very low level. However, *Os-EXPB12* transcripts accumulated following GA treatment or wounding (see below).

Accumulation of β-Expansin Transcripts in Response to GA Treatment

We tested the effect of GA on the expression of five β-expansin genes that are expressed in internodes (Fig. 4, A and B). The time course and magnitude of induction by GA varied between β-expansin genes. *Os-EXPB3* mRNA accumulated rapidly after 3 h of GA application, and its level decreased after 12 h. The expression of *Os-EXPB4* rose gradually during 24 h of treatment with GA. In a more detailed time course experiment, we found that the level of *Os-EXPB4* transcripts had increased by 54% after 1 h and by 250% after 2 h of GA treatment (results not shown). These values do not permit us to decide

Table I. *Primer sets used for the PCR amplifications of gene-specific probes of β-expansins of rice*

Gene Name	Primer Name	Primer Sequence 5′ to 3′	Template	Product Size	Accession No.	
					cDNA	Genomic DNA
				bp		
Os-EXPB1	hk368	GTCCAGGCCAAGTGAGCATTTTA	RT product of mRNA	251	AF261270	AY039023
	hk369	GGGAGAGCAGACGCCATTAT				
Os-EXPB2	hk357	CTACGGCTCCAAAGTCCAGT	RT product of mRNA	150	U95968	AC037426
	hk358	CACACAGAATCGTAGTACAGTA				
Os-EXPB3	hk361	CCCTCGGGTATTGTATGGA	S1104[a]	223	AF261271	AC037426
	hk362	TAATTTATGACCTTTGTTCTG				
Os-EXPB4	hk353	TACCGCTCCTTCGTCCAGT	C51142[a]	249	AF261272	AC069300
	hk354	TTTTCTTTGCCTACCTCCTCCATT				
Os-EXPB5	hk359	GCAGAAGCTCGCCTCGTC	R2872[a]	198	AF261273	AY039024
	hk360	GCAAATCGCAACGCAGAAT				
Os-EXPB6	hk363	ATTTGCGTGGGATTGAG	C51730[a]	194	AF261274	AC037426
	hk364	CTGTAGCCTTAAGATTTGGTT				
Os-EXPB7	hk372	AGCTAATTACTACTACTCCACTCC	E31457[a]	177	AF261275	NA[b]
	hk373	TCCGACCATTGATTGATTC				
Os-EXPB8	hk365	CGTCATCCCCCTCAACT	S3505[a]	176	AF261276	NA
	hk328	TTTTTTTTTTTTTTTTTTTTTTTA				
Os-EXPB9	hk370	TCATCCCGGTCAACTGG	E2136[a]	246	AF261277	AC020666
	hk371	ATTCATAGTGTTCTTCAGCATCTT				
Os-EXPB10	hk440	GGAAGGCCAACGCTCTC	E1347[a]	272	AF261278	AF391111
	hk441	CACGTTGACAATCCCTTAGAAT				
Os-EXPB11						
Set 1	hk521	CCGCTCCCTGGTGAACTACTCCTA	Rice genomic DNA	–	AY046927	AF391103
	hk524	CCAAACACAGCCTAAAAGCGAGAA				
Set 2	hk522	CTCCCTGGTGAACTACTCCTAAAT	Product of first PCR	483	–	–
	hk523	AGCGAGAATTGAACTTGAGACAG				
Os-EXPB12						
Set 1	hk525	TGGCTTGTGTCACCTTCTACTG	Rice genomic DNA	–	AY046928	AF391104 (5′ region)
	hk528	CATCATTTGTTTTAGGACCAGAGT				
Set 2	hk526	TGCTGCTGTTGTTAATGTTGTTCG	Product of first PCR	317	–	AF391105 (3′ region)
	hk527	ATCAACAACGCATCTTCAAACTTA				
Os-EXPB13						
Set 1	hk533	CGTCCAAGCCAAGTGAGCATTTTA	Rice genomic DNA	–	NA	AF391106
	hk536	CGGATGGGGGCTAGTTTTTGAAGT				
Set 2	hk534	AGTGAGCATTTTAAGCAAGGAAGA	Product of first PCR	570	–	–
	hk535	TGTTATTCACGCCACCTCTTGTA				
Os-EXPB14						
Set 1	hk529	CAGGAGGGAGGATTGATGAGATG	Rice genomic DNA	–	NA	AF391107
	hk532	TGGCCGTCATGATTTCCTTC				
Set 2	hk530	ATGTAGGGGATATGTAGGGTGGTG	Product of first PCR	495	–	–
	hk531	AAAAACCGTTACCTAAATCACTCG				

[a] EST clone nos. [b] NA, Not available.

whether or not a significant increase in the expression of *Os-EXPB4* precedes the onset of accelerated growth at around 40 min after application of GA (Sauter and Kende, 1992). *Os-EXPB6* and *Os-EXPB11* mRNA started to accumulate between 3 and 6 h after start of GA treatment and leveled off after 12 h. The expression of *Os-EXPB12* was not significantly enhanced by GA above the value at time 0. Because the expression of this β-expansin gene declined in control internodes, its expression level in GA-treated internodes was, at 24 h, about 4-fold higher than that in control internodes. This pattern of gene expression was similar to that observed for *Os-EXP1* (Cho and Kende, 1997b). The effect of GA on the expression of individual β-expansin genes was confirmed in at least four independent experiments.

The Time Course of β-Expansin Gene Expression in Internodes during Incubation of Stem Sections in Water

The expression of β-expansin genes increased in internodes following excision of stem sections. We determined the time course of this increase during incubation in water and found that the expression pattern differed among the five genes that are expressed in the internode following isolation of the sections (Fig. 5, A and B). There was a decrease in

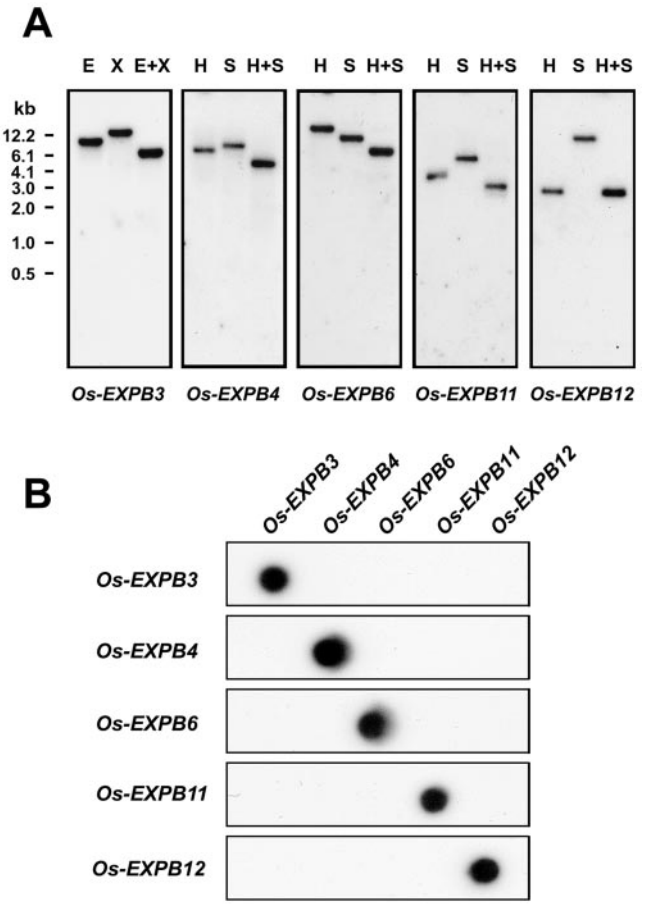

Figure 1. DNA gel- and dot-blot analyses showing the specificity of the gene-specific probes. A, Genomic DNA was digested with *Eco*RI (E), *Xba*I (X), *Hin*dIII (H), *Sac*I (S), *Eco*RI and *Xba*I (E + X), or with *Hin*dIII and *Sac*I (H + S). The digested DNA was separated by gel electrophoresis, blotted onto Hybond-N+ membrane, and hybridized under the same conditions as described for RNA gel-blot analysis, using the gene-specific probes indicated under each blot. B, Gene-specific DNA fragments cloned in the pGEM-T Easy vector were digested with *Eco*RI or *Not*I and purified by gel electrophoresis. The inserts were blotted onto Hybond-N+ membrane and hybridized under the same conditions as employed for RNA gel-blot analysis, using the gene-specific probes indicated at left.

Os-EXPB3 transcript level during the first 2 h after cutting, followed by an increase until 5 h. The level of *Os-EXPB3* mRNA decreased rapidly after 5 h of incubation, as did the transcript levels of *Os-EXPB4*, *Os-EXPB6*, and *Os-EXPB11*. The expression of *Os-EXPB4*, *Os-EXPB6*, and *Os-EXPB12* was enhanced within 30 min of excision, whereas expression of *Os-EXPB11* started to increase after 2 h. *Os-EXPB6* showed a second peak of expression at 5 h of treatment. *Os-EXPB11* mRNA accumulated from 2 h onwards and reached a peak at 3 h. We found this pattern of *Os-EXPB* expression in excised stem sections in two independent experiments. The stem sections used to investigate the effect of GA on *Os-EXPB* transcript accumulation were first incubated in water for 8 h to dissipate the effect of excision before GA was applied.

The Time Course of β-Expansin Expression in Internodes of Whole Plants in Response to Wounding

To determine whether excision of stem sections enhanced expression of *Os-EXPB* genes because of a wound effect, we determined the level of *Os-EXPB* transcripts in internodes of whole plants that had been wounded by piercing six pinholes into the stem 2 cm below the second-highest node where the stem section would have been excised. Transcripts of all five *Os-EXPB* genes accumulated as a result of wounding, but again with varying time courses (Fig. 6, A and B). The expression of the *Os-EXPB* genes declined after 9 h but at a slower rate than in stem sections. Wound-induced induction of each of the five *EXPB* genes was observed in at least three independent experiments.

DISCUSSION

Cosgrove et al. (1997) recognized structural similarities between α-expansins and group I allergens of grass pollen and showed that maize pollen extract and Zea mI allergen increase the extensibility and stress relaxation of maize silk and wheat coleoptile cell walls. Extensibility of cucumber hypocotyl cell walls was barely enhanced, if at all, by this new type of expansin, now called β-expansin. Thus, β-expansins may act selectively on cell walls of monocots, whereas α-expansins have been shown to loosen more effectively the cell walls of dicots than those of monocots (McQueen-Mason et al., 1992; Cho and Kende, 1997a). β-Expansins have also been found in the EST collections derived from vegetative organs of rice and maize (Cosgrove et al., 1997; Cosgrove, 2000). Because grass cell walls differ in their composition from the cell walls of dicots (Carpita, 1996), β-expansins may interact with grass-specific cell wall polysaccharides such as (1→3),(1→4)-β-D-glucans and glucuronoarabinoxylans. The significance of β-expansins in grasses is also indicated by the fact that we found 14 putative β-expansin genes in the rice database, which is still incomplete (Table I), whereas only five are present in the Arabidopsis genome.

The role of β-expansins in vegetative tissues is as yet unknown. In the present study, we established the expression pattern of β-expansin genes in the growing internode of deepwater rice. We found that five β-expansin genes are expressed in internodes that had been induced to grow rapidly by GA (Fig. 4). Except for *Os-EXPB3*, whose transcript level was highest in the node just below the growing internode, the accumulation of the most abundant internodal β-expansin mRNAs correlated well with elongation (Fig. 3). It was highest just above the node in the 1-cm

region of the internode that contains the intercalary meristem and the elongation zone. The relevance of *Os-EXPB3* expression in the second-highest node is not known. This node does not elongate but is still expanding. It also contains adventitious root initials, which express expansin genes (Cho and Kende, 1998).

The expression of β-expansin genes in the internode is enhanced by GA (Fig. 4, A and B) and, surprisingly, by excision of stem sections (Fig. 5, A and B) and by wounding of whole plants (Fig. 6, A and B). The accumulation of expansin mRNA in GA-treated tissue is consistent with the notion that expansins are involved in mediating GA-induced rapid internodal elongation in deepwater rice (Cho and Kende, 1997b), but the significance of wound-induced expression of β-expansin genes is not clear at all. The rapid disappearance of β-expansin transcripts 5 h after excision of stem sections (Fig. 5, A and B) indicates that β-expansin mRNA is unstable and is turning over rapidly. Whereas GA is capable of inducing expression of β-expansin genes after the wound effect has dissipated, wounding for a second time causes only a small increase in β-expansin mRNA levels, if at all (Y. Lee and H. Kende, unpublished data).

In earlier work, we studied the expression pattern of four α-expansin genes in deepwater rice (Cho and Kende, 1997b). Three of these are expressed in the growing internode, and the transcript levels of two, *Os-EXP2* and *Os-EXP4*, increased upon treatment with GA. We recently have identified 23 additional α-expansin genes in the EST and genomic databases (Y. Lee and H. Kende, unpublished data). The number of α-expansin genes in rice is very likely higher than 27 and will have to be reassessed when the complete genome of rice will be available. Of the newly identified α-expansin genes, seven are expressed in the internode (Y. Lee and H. Kende, unpublished data), bringing the total number to 10.

The nucleotide and derived amino acid sequences in the genomic and EST databases are derived from the Japonica rice cv Nipponbare, whereas our work is performed with the Indica deepwater rice cv Pin Gaew 56. Both rice cultivars belong to the same species, *Oryza sativa*. To what extent are their protein and corresponding nucleic acid sequences comparable? The genetic differences between deepwater and lowland rices appear to be confined to a few genes (see Kende et al., 1998). In three genes of Pin Gaew 56 (including *Os-EXP4*), whose open reading frames we have compared with those of Nipponbare, there was, on average, one base substitution per 165 bases. In the untranslated regions of seven genes (including five expansin genes) we found, on average, one base substitution per 316 bases. Thus, the nucleotide and derived amino acid sequences of different rice cultivars are, probably, nearly identical for most genes.

In conclusion, five β-expansins are expressed in the elongating region of rice internodes, and their expression is enhanced by treatment with GA. This indicates that β-expansins, just like α-expansins, are involved in mediating internodal elongation. In addition, expression of β-expansins is promoted by wounding. The wound effect is propagated from the site of wounding 2 cm below the node to the 2-cm region above the node. One hypothesis to explain the wound response would stipulate a role for β-expansins in the repair of damaged cell walls. The obvious questions that arise from our work concern the function(s) of the individual α- and β-expansins in vegetative growth of grasses and the interaction, if any, between these two classes of expansins.

MATERIALS AND METHODS

Plant Material

Seeds of deepwater rice (*Oryza sativa* L. cv Pin Gaew 56) were obtained from the International Rice Research Institute (Los Baños, Philippines). Plants were grown as described by Stünzi and Kende (1989). The uppermost internodes from 11- to 13-week-old plants were used for our experiments. Twenty-centimeter-long stem sections that included the highest and second highest nodes and the growing internode were excised according to Raskin and Kende (1984). All plant material was collected around 10 AM, except for the wounding experiment (see legend, Fig. 6).

GA Treatment and Wounding

Stem sections were placed into 250-mL beakers containing 25 mL of double-distilled water within a closed cylinder through which water-saturated air was passed for 8 h to dissipate the effect of wounding. They were then transferred to 250-mL beakers containing 25 mL of 50 μM GA_3 or 25 mL of distilled water (control). Incubation was allowed to proceed for the indicated periods, after which the basal 2-cm portion of the uppermost internodes was excised, frozen immediately in liquid nitrogen, and stored at −80°C until use. To wound intact plants, six pinholes were pierced with a gauge 26 needle in a circle around the stem 2 cm below the second highest node. The basal 2-cm portion of the uppermost internodes was excised at the times indicated, frozen immediately in liquid nitrogen, and stored at −80°C until use.

Isolation of Nucleic Acids

Genomic DNA was isolated according to Dellaporta et al. (1983) and total RNA according to Verwoerd et al. (1989). The PolyATtract kit (Promega, Madison, WI) was used to enrich poly(A^+) RNA, and the enriched product is referred to as poly(A^+) RNA.

Preparation of Probes

For RT-PCR, total RNA was isolated from young plants, and poly(A^+) RNA was purified. One hundred nanograms

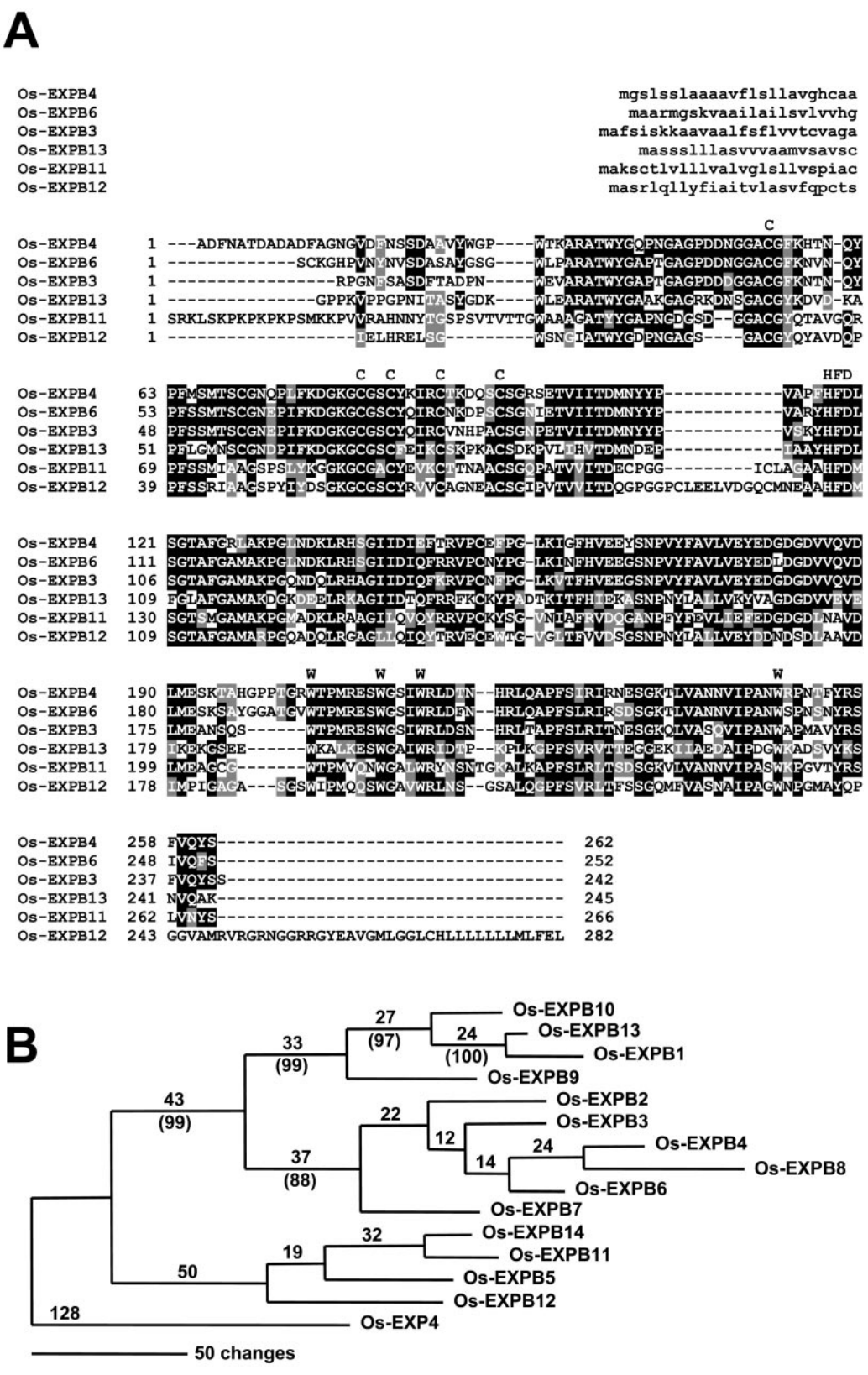

Figure 2. Comparisons of the deduced amino acid sequences of rice β-expansins. A, Alignment of the deduced amino acid sequences of rice β-expansin expressed in internodes. Positions of the highly conserved Cys residues, the HFD domain, and Trp residues are indicated. Signal peptide sequences were determined with the PSORT program (Nakai and Kanehisa, 1992) and are represented in lowercase. B, Phylogenetic tree of rice β-expansins. Amino acid sequences of mature protein regions,

(*Legend continues on next page.*)

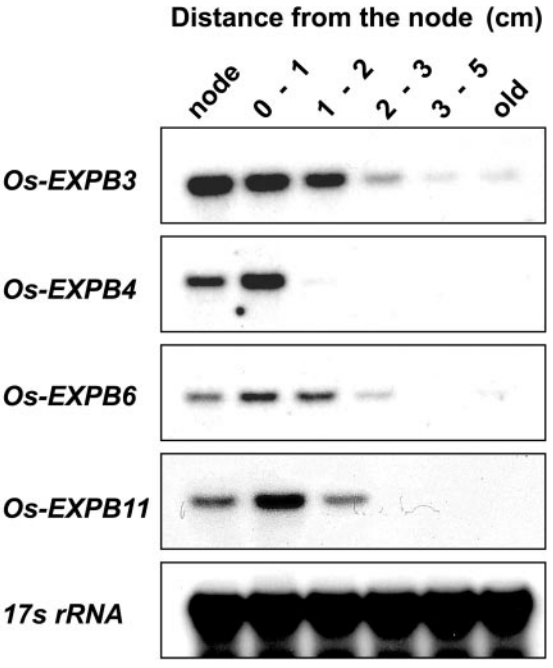

Figure 3. RNA gel-blot analysis of β-expansin gene expression in the second-highest node and in different regions of the uppermost internode. Each lane contained 20 μg of total RNA isolated from the node and the internodal regions indicated above the lanes. Node, Second highest node; 0 to 1 cm, internodal region containing the intercalary meristem and most of the elongation zone; 1 to 2 cm, internodal region containing the upper part of the elongation zone and the differentiation zone; 2 to 3 cm and 3 to 5 cm, internodal regions containing the differentiation zone; old, oldest part of the internode 5 cm above the node. 17s rRNA was used as internal loading control.

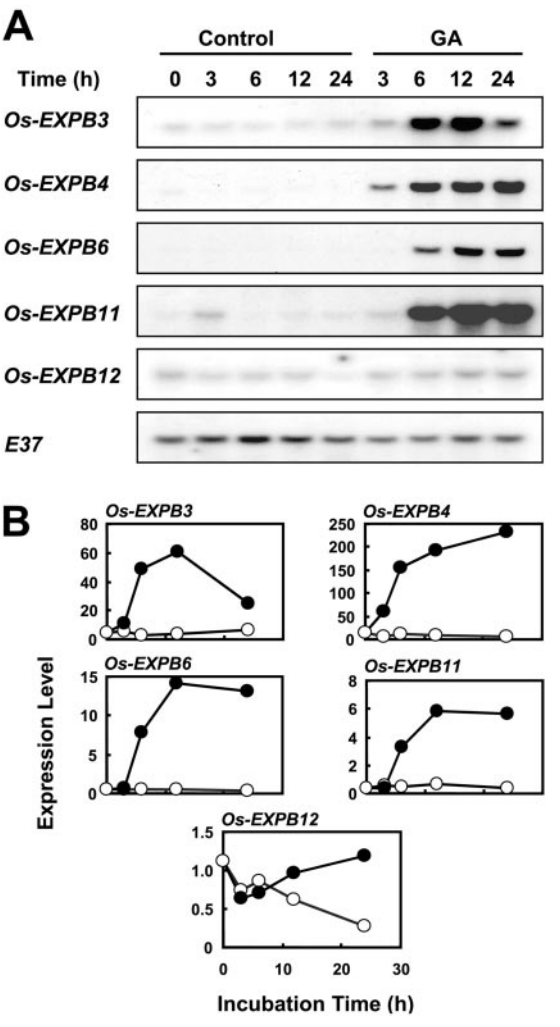

Figure 4. Expression of β-expansin genes in GA-treated stem sections. Stem sections were first incubated for 8 h in distilled water to dissipate the wound effect and then transferred to 50 μM GA_3 or distilled water (control) for the times indicated above the lanes. A, RNA gel-blot analysis. Each lane contained 20 μg of total RNA isolated from the 0- to 2-cm region above the second-highest node, which includes the intercalary meristem, the elongation zone, and the lower part of the differentiation zone. Exposure times varied according to the intensity of the signal. All blots were hybridized to *E37*, a constitutively expressed gene, as a loading control. B, Quantification of the mRNA levels of each gene. Expression levels are shown in PhosphorImager values × 10^{-3} after 24 h of exposure. Control (○), GA treatment (●).

of poly(A^+) RNA was subjected to RT-PCR using Superscript II (Life Technologies, Rockville, MD) and 1 μL of oligo$(dT)_{18}$ (500 μg mL^{-1}) as a reverse primer. After incubation at 42°C for 50 min and inactivation of the reverse transcriptase for 15 min at 70°C, the reactions were subjected to 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, in the presence of the gene-specific primer pairs as indicated in Table I. RT-PCR products were purified by gel electrophoresis before being cloned into the pGEM-T Easy vector (Promega) for sequencing.

For PCR amplification from plant genomic DNA, primer set 1 (Table I) was used for the first round of PCR to amplify the fragments containing the putative 3′-untranslated regions under the following conditions: 35 cycles of 94°C for 30 s, 50 to 60°C for 30 s, and 72°C for 30 s. The second, nested round of PCR was performed using primer set 2 (Table I) under the same conditions as employed during the first round of PCR. PCR was performed with *Taq* DNA polymerase (Promega) according to the manufacturer's instructions in a PTC200 thermal cycler (MJ Research, Watertown, MA). The PCR products were purified by gel electrophoresis and cloned into the pGEM-T Easy vector for sequencing.

DNA fragments containing the inserts of gene-specific regions of β-expansin genes, of *E37*, and of 17S rDNA were

Figure 2. (*Legend continued from preceding page.*)
i.e. without signal peptides, were compared using the Clustal method of DNASTAR MegAlign. The tree was constructed with the PAUP program (Swofford, 1993), using the mature amino acid sequence of the rice α-expansin Os-EXP4 (U85264) as the outgroup. Numbers indicate the amino acid changes and the numbers in parentheses indicate the bootstrap values. The GenBank accession nos. are given in Table I.

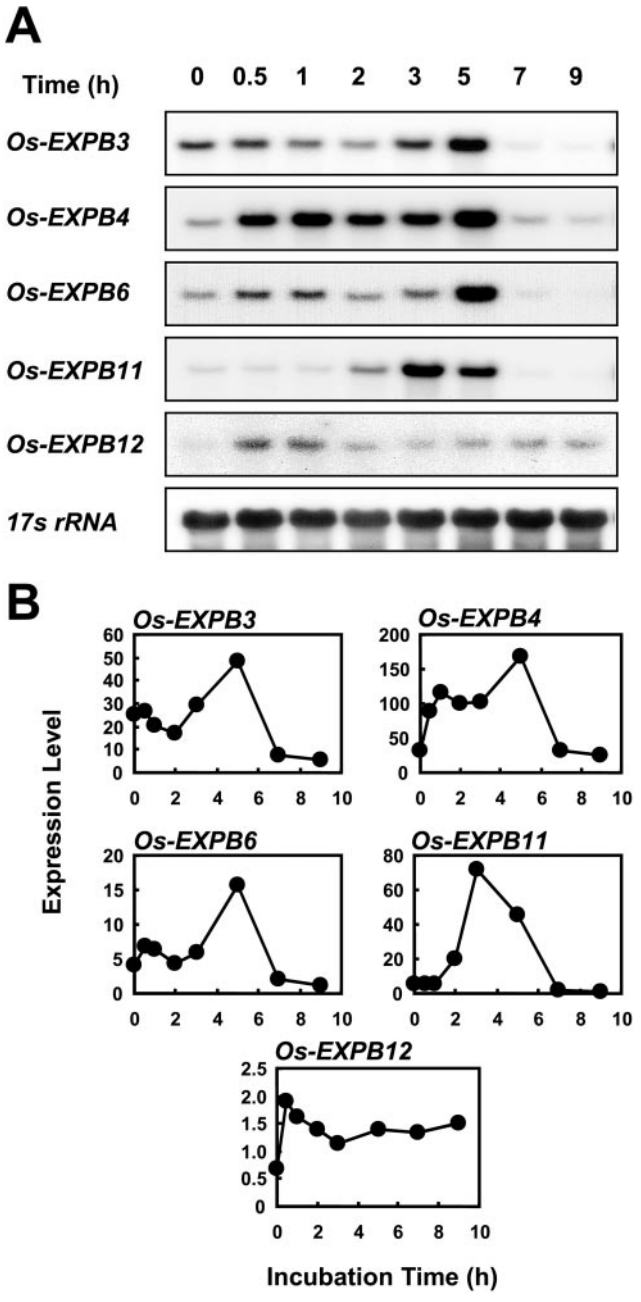

Figure 5. Expression of β-expansin genes in stem sections. Stem sections were excised and incubated in water for the times indicated above the lanes. A, RNA gel-blot analysis. Each lane contained 20 μg of total RNA isolated from the 0- to 2-cm region above the second-highest node, which includes the intercalary meristem, the elongation zone, and the lower part of the differentiation zone. Exposure times varied according to the intensity of the signal. All blots were hybridized to 17s rRNA as a loading control. B, Quantification of the mRNA levels of each gene. Expression levels are shown in PhosphorImager values $\times 10^{-3}$ after 24 h of exposure.

excised from the cloning vectors with restriction enzymes and isolated from agarose gels with a DNA purification system (Wizard PCR Preps, Promega). *E37* is a truncated cDNA encoding parts of a chloroplast inner membrane protein; the *E37* transcript is constitutively expressed (Van der Knaap and Kende, 1995). 17S rRNA (Zarembinski and Theologis, 1993) and *E37* served as loading controls.

RNA Gel-Blot Analysis

Twenty micrograms of total RNA was separated electrophoretically in a 1.2% (w/v) formaldehyde-agarose gel

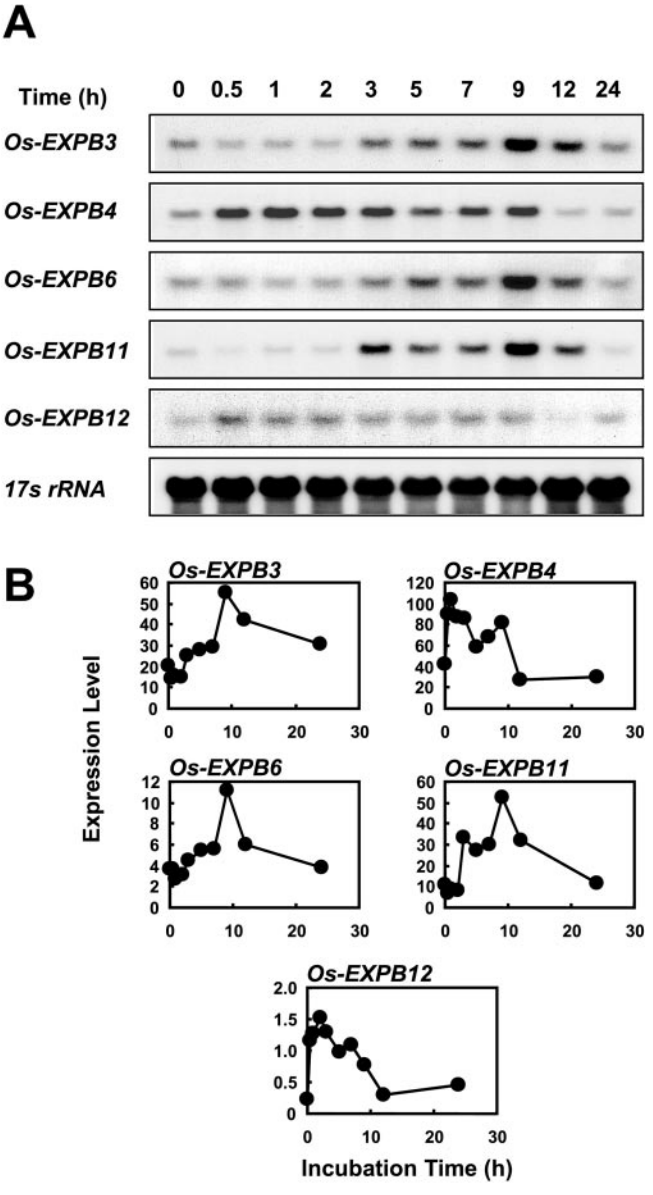

Figure 6. Expression of β-expansin genes in the internodes of wounded plants. The internodes were wounded by piercing pinholes around a circle into the stems 2 cm below the second-highest node at around 10 AM. The tissue samples were collected at the times indicated above the lanes. A, RNA gel-blot analysis. Each lane contained 20 μg of total RNA isolated from the 0- to 2-cm region above the second-highest node, which includes the intercalary meristem, the elongation zone, and the lower part of the differentiation zone. Exposure times varied according to the intensity of the signal. All blots were hybridized to 17s rRNA, as a loading control. B, Quantification of the mRNA levels of each gene. Expression levels are shown in PhosphorImager values $\times 10^{-3}$ after 24 h of exposure.

(Ausubel et al., 1987) and transferred to a Hybond-N+ membrane (Amersham Pharmacia, Piscataway, NJ). Blots were prehybridized in 5× sodium chloride sodium phosphate + EDTA, 10× Denhardt's solution, 1.5% (w/v) SDS, and 50% (v/v) formamide for 3 h at 42°C and hybridized in the same solution overnight at 42°C. Fifty nanograms of template DNA was used for the preparation of probes in the presence of [^{32}P]dCTP (3,000 Ci mmol^{-1}; New England Nuclear, Boston) using a random prime labeling kit (Boehringer Mannheim). High-stringency washes were performed twice for 30 min in 0.2× sodium chloride sodium phosphate + EDTA and 0.1% (w/v) SDS at 65°C. The radioactivity on blots was quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) after 24 h of exposure. Autoradiography was performed using Hyperfilm MP (Amersham Pharmacia).

DNA Gel-Blot Analysis

Four micrograms of genomic DNA was digested with *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I, *Eco*RI and *Xba*I, or *Hin*dIII and *Sac*I, separated in an agarose gel (0.8%, w/v), and transferred to a Hybond-N+ membrane. Fifty nanograms of template DNA was used for the preparation of probes in the presence of [^{32}P]dCTP and [^{32}P]dATP (both at 3000 Ci mmol^{-1}) using the random-primer labeling kit. DNA gel blots were hybridized and washed using the same conditions as employed for RNA gel-blot analysis.

DNA Dot-Blot Analysis

One nanogram of DNA used to prepare probes was blotted onto a Hybond-N+ membrane with a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Ten nanograms of template DNA was used for the preparation of probes in the presence of [^{32}P]dCTP (3,000 Ci mmol^{-1}) using the random-primer labeling kit. DNA dot blots were hybridized and washed using the same conditions as employed for RNA gel-blot analysis.

DNA and Amino Acid Sequence Analysis

The nucleotide and deduced amino acid sequences were analyzed with the DNASTAR program (DNASTAR, Madison, WI). Multiple sequence alignments were performed using the Clustal W Multiple Sequence Alignment program and printed using BOXSHADE 3.20 (www.ch.embnet.org). The prediction of the protein localization site was performed using the PSORT program (Nakai and Kanehisa, 1992). A phylogenetic tree was generated by heuristic parsimony analysis using PAUP (Swofford, 1993) after alignment by the Clustal method with the MegAlign program (DNASTAR). Starting trees were obtained via random stepwise addition, and bootstrap analysis was performed with 100 replicates using the tree bisection and reconnection branch swapping method.

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