

Expression of α -expansin genes in young seedlings of rice (*Oryza sativa* L.)

Jirong Huang¹, Tetsuo Takano², Shigemi Akita¹

¹Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

²Asian Natural Environmental Science Center, The University of Tokyo, Midori-cho, Tanashi, Tokyo 188-0002, Japan

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Abstract. Rice is the only cereal in which germination and coleoptile elongation occur in hypoxia or anoxia. Little is known of the molecular basis directly underlying coleoptile cell extension. In this paper, we describe the expression of α -expansin genes in embryos during seed development and young seedlings grown under various oxygen concentrations. The genes *Os-EXP2* and *Os-EXP1* were predominantly expressed in the developing seeds, mainly in newly developed leaves, coleoptiles, and seminal roots. These expansins expressed in the developing seeds may give cells the potential to expand after seed imbibition begins. In coleoptiles, *Os-EXP4* and *Os-EXP2* mRNAs were greatly induced by submergence, while they were weakly detected in aerobic or anoxic conditions. Under submerged soil conditions, the signals hybridized with probes *Os-EXP4* and *Os-EXP2* in coleoptiles were strongest when coleoptiles elongated in the water layer. These data show that expansin gene expression is highly correlated with coleoptile elongation in response to oxygen concentrations. The *Os-EXP4* gene was also expressed in leaves, mesocotyls, and coleorhizas of young seedlings. The growth of these tissues was also correlated with the presence of expansins. Therefore, the evidence derived from this study clearly demonstrates that expansins are indispensable for the growing tissues of rice seedlings.

Key words: Cell extension – Environmental stress – α -Expansin – *Oryza* (expansin)

Introduction

Coleoptile elongation is a prerequisite for seedling establishment in direct-seeding rice cultivation in submerged conditions. The environmental factor that

induces coleoptile elongation is believed to be the reduced partial pressure of oxygen (Alpi and Beevers 1983). The molecular bases for coleoptile elongation induced by anoxia or hypoxia have been studied mainly with respect to anaerobic metabolism, such as the avoidance of self-poisoning and cytoplasmic acidosis, and the maintenance of adequate supplies of energy, sugar, amino acids, and cell turgor pressure (Atwell et al. 1982; Rivoal et al. 1989; Kutschera et al. 1990; Fox et al. 1995; Kawai and Uchimiya 1995; Reggiani et al. 1995; Huang et al. 1999, 2000). Because cell division rarely occurs, the elongation of rice coleoptiles is primarily attributed to cell expansion. Recent evidence has shown that cell wall extensibility is the major factor limiting cell extension (reviewed by Cosgrove 1999). In Cosgrove's model of cell wall extension, expansin, which weakens glucan-glucan binding, is the primary agent of wall extension, whereas endoglucanases, xyloglucan endotransglycosylase, and other enzymes act as secondary agents.

Expansins are encoded by a large superfamily with at least two major divisions: α -expansins and β -expansins (Cosgrove 1998). It is thought that they act differently as wall polymers and have different wall-binding properties. McQueen-Mason and Cosgrove (1994) reported that expansins bind at the interface between the polymer groups of cellulose and hemicellulose in cell walls, and induce extension by disrupting the hydrogen bonds between them. The role of α -expansins in cell extension has been reported in many plants (reviewed by McQueen-Mason and Rochange 1999). In cucumber hypocotyls, expansin activity could be found in rapidly growing cell walls, but not in the walls of non-growing cells (McQueen-Mason and Cosgrove 1992). The correlation of expansin activity with growth rates was also observed in oat coleoptiles (Cosgrove and Li 1993). In rice, four expansin genes have been cloned and their expression attributed to the internode growth of deep-water rice when submerged (Cho and Kende 1997a,b). Rice is the only cereal in which the coleoptile is able to elongate in hypoxia or anoxia. The molecular basis directly underlying cell extension of the coleoptile is still

Correspondence to: S. Akita;
E-mail: akita@mail.ecc.u-tokyo.ac.jp; Fax: +81-3-5841-5070

under investigation. In this paper, four α -expansin genes were studied in the developing seeds and young seedlings of rice grown under different conditions.

Materials and methods

Plant materials. Rice (*Oryza sativa* L. cv. Nipponbare) grains selected by 10% salt solution were surface-sterilized by soaking in 1% sodium hypochlorite for 30 min and then rinsed with distilled water three times. The grains were then soaked in distilled water for 2 d at 8 °C and germinated at 18 °C under three oxygen treatments: aerobic, on wet filter paper; hypoxic, in 2 cm of water; and anoxic, in 1 cm of soil flooded with 1.5 cm of water. The top 0.5–1.0 cm of the coleoptiles was harvested on day 8 for RNA isolation and cell wall extension measurement. The coleoptiles were then frozen in liquid nitrogen and stored at –70 °C until use. For sampling seeds in the course of maturation, the spikelets were marked on the flowering day, and then harvested on days 11, 23, and 29.

Measurement of cell wall extension. Acid-induced cell wall extension was carried out according to the method of Cosgrove (1989). Coleoptiles were abraded with carborundum, and then washed with water. The most apical 8 mm of the coleoptiles was cut and squeezed between two glass slides to remove cell sap. The segments were fixed on an extensometer between two clamps 4 mm apart under a constant load of 8 g. The wall sections were first incubated in a neutral-pH buffer (50 mM Hepes, pH 6.8) for 15 min, and then in an acidic buffer (50 mM sodium acetate, pH 4.5) for 75 min to measure wall extension.

In-situ hybridization. The samples were fixed in FAA solution [0.5% (v/v) formaldehyde, 0.5% (v/v) acetic acid, 50% (v/v) ethanol] overnight at 4 °C, dehydrated through an ethanol-2-methyl-2-propanol series, and finally embedded in Paraplast Plus (Oxford, St. Louis, Mo., USA). Ten-micrometer-thick sections were placed on glass slides (Fisher Biotech, Pittsburgh, Pa., USA) and dried on a heated platform at 50 °C overnight. Gene-specific 3' untranslated regions were prepared as described by Cho and Kende (1998) and confirmed by sequencing. The GenBank accession number for *Os-EXP1* is Y07782; *Os-EXP2*, U30477; *Os-EXP3*, U30479 and *Os-EXP4*, U85246. Digoxigenin-labeled antisense and sense RNA probes were synthesized using T₇ and T₃ RNA polymerase, respectively. The RNA probes were partially digested in alkali at 60 °C. The procedures for in-situ hybridization were based on those described by Kouchi and Hata (1993).

Northern hybridization. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Ten micrograms of total RNA was loaded in each lane, separated on formaldehyde gels, and then stained with ethidium bromide to ensure equal loading of RNA. Digoxigenin-labeled RNA probes were used in the hybridization solution. Prehybridization was in 50% (v/v) formamide, 0.02% (v/v) SDS, 0.1% (w/v) N-lauroylsarcosine sodium salt, 5× SSC (1× SSC = 0.15 M NaCl, and 0.015 M trisodium citrate dihydrate, pH 7), 2% (w/v) blocking reagent for nucleic acid hybridization and detection, 150 µg/ml sheared salmon sperm DNA and 150 µg/ml *Escherichia coli* tRNA at 68 °C for 2 h. Hybridization was in the same solution at 68 °C overnight. The membrane was washed twice in 2× SSC, 0.1% (v/v) SDS for 5 min at room temperature and twice in 1× SSC, 0.1% (v/v) SDS for 30 min at 68 °C. For immunological detection, the membrane was blocked with 1% (w/v) blocking reagent in buffer 1 (0.1 M maleic acid-NaOH, pH 7.5; 0.15 M NaCl) for 30 min at room temperature. Then it was treated with anti-digoxigenin-alkaline phosphatase conjugate in the same blocking solution for 30 min at room temperature, and washed twice with 0.3% (v/v) Tween-20 in buffer 1. The membrane was exposed to autoradiography hyperfilm (Amersham) for 1 h after applying CSPD (disodium 3-(4-meth-

oxySpiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate), ready-to-use solution (Boehringer Mannheim, Germany).

Results

Elongation rates of coleoptiles under different conditions and acid-induced cell wall extension. Coleoptile elongation was significantly promoted in hypoxia (water) (Fig. 1). There was no obvious difference between the lengths of coleoptiles grown in aerobic (on wet filter paper) and anoxic (in submerged soil) conditions until 8 d after seeding. However, elongation of the coleoptile accelerated after it emerged from the submerged soil and subsequently entered the water layer (Fig. 1). This result is consistent with those previously reported by Alpi and Beevers (1983).

Figure 2A shows three representative traces of acid-induced extension in the cell walls of plants grown under different conditions. Cell walls from anoxia-induced coleoptiles exhibited the highest extensibility, and extended about 2-fold more than those grown in aerobic conditions after 75 min extension (Fig. 2B). The wall extension of hypoxia-grown coleoptiles was intermediate between those grown in aerobiosis and anoxia. Although the rate of coleoptile elongation of air- and anoxia-grown coleoptiles was almost the same in vivo, the extension of the cell walls in vitro was completely different. Thus, the anoxic conditions maintain high extensibility of cell walls, and this allows the coleoptile to elongate rapidly under hypoxic conditions.

Expression of α -expansin genes in rice coleoptiles. Since expansins mediate acid-induced wall extension, we investigated the expression of four α -expansin genes in coleoptiles grown under aerobic, hypoxic, and anoxic conditions (Fig. 3). Transcripts of *Os-Exp4* and *Os-EXP2* were strongly expressed in hypoxic conditions, in which coleoptiles usually elongate markedly, but low levels were detected under both aerobic and anoxic conditions. Transcripts of *Os-EXP1* and *Os-EXP3* were almost undetectable in coleoptiles that had elongated

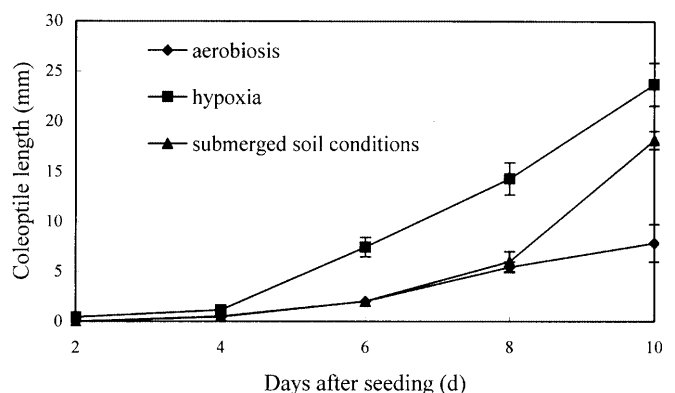


Fig. 1. Changes in coleoptile length of rice grown under different germination conditions at 18 °C. *Aerobiosis*, on wet filter paper; *hypoxia*, in 2 cm of water; *anoxia*, in 1 cm of soil beneath 1.5 cm of water. Data are mean \pm SE ($n = 20$)

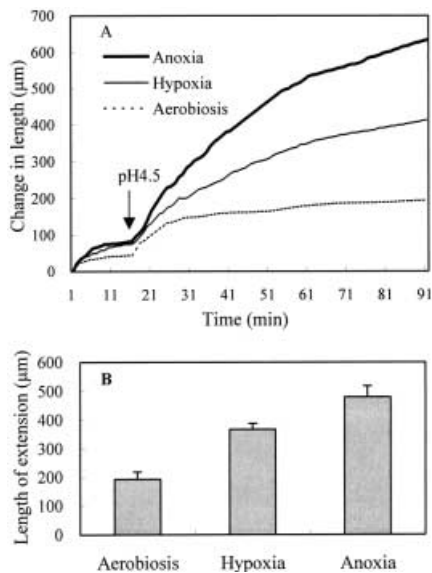


Fig. 2A,B. Acid-induced extension of cell walls from coleoptiles of rice grown for 8 d under different conditions at 18 °C. To measure wall extensibility, the top 0.8 cm of coleoptiles was clamped under a constant load of 8 g in 50 mM Hepes (pH 6.8) for 15 min. Then the bathing solution was replaced by 50 mM sodium acetate, pH 4.5 (arrow) for 75 min. **A** Representative traces of acid-induced extension from walls of coleoptiles grown under aerobic, hypoxic and anoxic conditions. **B** Increase in length after incubation in acid buffer pH 4.5 for 75 min. Data are mean \pm SE ($n = 20$)

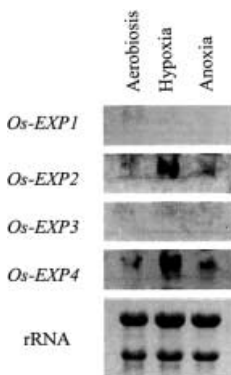


Fig. 3. Effect of different oxygen concentrations on the accumulation of rice α -expansin transcripts. Each lane contains 10 μ g of total RNA isolated from 8-d-old coleoptiles. Ethidium bromide-stained gels show that the same amount of rRNA had been loaded per lane

under any of the conditions. Our results partly contrast with those of Cho and Kende (1997a), who found that all of the expansin genes except *Os-EXP3* were expressed in coleoptiles grown on wet filter paper. This is probably due to the different kinds of rice used; they used deepwater rice in their experiment, while we used *Japonica* rice Nipponbare in ours.

Localization of α -expansin gene expression in young seedlings. To determine the spatial and temporal expression of α -expansin genes in young seedlings, digoxigenin-labeled antisense or sense RNA probes were used for hybridization. Since no signals were detected with

the sense probe in the tissues examined, we present only the results of in-situ hybridization with the antisense probes. The expression of α -expansin genes in coleoptiles under the three germination conditions is shown in Fig. 4A–J. The results of gene expression from in-situ hybridization were basically consistent with those from northern hybridization. No signal appeared in the slides hybridized with the *Os-EXP1* and *Os-EXP3* probes (data not shown).

No signals were detected with the *Os-EXP2* and *Os-EXP4* probes in coleoptiles elongating under aerobic and anoxic conditions (Fig. 4A,F,C,H), although northern blotting results showed weak expression in 8-d-old coleoptiles (Fig. 3). This difference is probably caused by the different precision of the two analytic methods. In hypoxia-grown coleoptiles, the expression of α -expansin genes *Os-EXP2* and *Os-EXP4* was first detected on day 7 (data not shown), and became very strong on day 8 when the inner hollow of the coleoptile cylinder had not yet formed at the top part of coleoptiles (Fig. 4B,G). The signals were detected in both the mesophyll and the exterior epidermal cells. Under submerged soil conditions, the signals hybridized with *Os-EXP2* and *Os-EXP4* probes were detected in the mesophyll cells on day 10 when the coleoptiles had elongated enough to enter the water layer (Fig. 4D,I), and then weakened on day 12 (Fig. 4E,J) when the coleoptiles had grown into the air. These patterns of α -expansin expression in the coleoptile are highly correlated with the elongation rates of the coleoptile under the submerged soil conditions.

The expression of the *Os-EXP4* gene was also studied in the other tissues of young seedlings by in-situ hybridization (Fig. 5). In aerobic conditions, the signal first appeared in the tip of the first leaf and the middle part of the scutellum in 8-d-old seedlings (Fig. 5B), and then spread to the other leaves, mesocotyls and the coleorhiza in 9-d-old seedlings (Fig. 5C). The signal in the mesocotyl was mainly localized in the vascular bundle. It seems that α -expansin transcripts occur when the leaves and mesocotyls begin to grow rapidly. In contrast, the *Os-EXP4* gene was strongly expressed in the coleorhiza under hypoxia, during which moderate elongation of the coleorhiza was observed, and weakly expressed in the first leaf (Fig. 5A). Thus, the expression of the *Os-EXP4* gene seems to correlate with the growth of leaves, mesocotyls and the coleorhiza in young seedlings.

Expression of α -expansin genes in developing seeds. Since the expression of the four α -expansin genes was not detected by in-situ hybridization in any tissues of the young seedling during the early stage of growth (data not shown), we postulated that α -expansins are synthesized in the course of seed maturation. Hence, we examined the expression of α -expansin genes in developing seeds.

Of the four α -expansin genes, predominantly *Os-EXP2* was expressed in the embryo of developing seeds (Fig. 6). Its signal mainly appeared in young leaves and the seminal root. In the coleoptile, the signal was strong in the vascular bundle. A weak signal was observable in

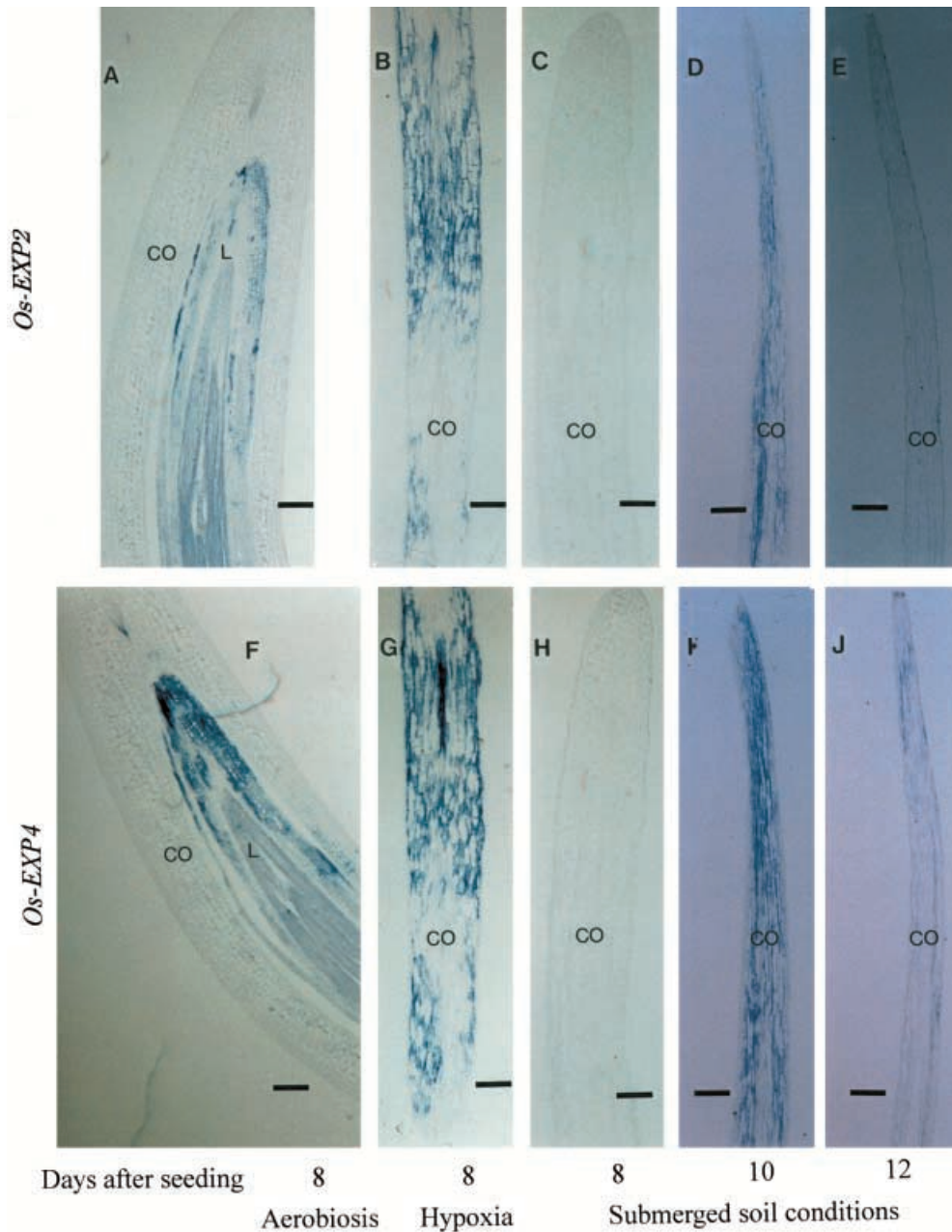


Fig. 4A–J. Localization of α -expansin gene expression in sections cut parallel to the rice coleoptile surface as detected by in-situ hybridization with anti-sense RNA probes. Apart from A and F, the pictures show the top parts of coleoptiles in which the inner hollow has not yet formed. **A–E** Hybridization with the *Os-EXP2* probe; **F–J** Hybridization with the *Os-EXP4* probe. **A, F** Shoots grown in air; the stained tissue is the leaf. **B, G** Coleoptiles grown in hypoxia; mesophyll and epidermal cells of the coleoptile are stained. **C, H; D, I; E, J** Coleoptiles grown in submerged soil conditions for 8, 10, and 12 d, respectively. Coleoptiles of **C, H** elongated in the soil, while those of **D, I** and **E, J** elongated enough to emerge into the water layer and air, respectively. **C, H** No staining in any cells. **D, I** Staining mainly in mesophyll cells. **E, J** Weak staining in a few mesophyll cells. *CO*, coleoptile; *L*, leaf. Bars = 125 μ m (**A–C, F–H**); 250 μ m (**D, E, I, J**)

the coleorhiza and a relatively strong signal at the epidermis of the coleorhiza. The *Os-EXP1* gene was also moderately expressed in the developing seeds. The pattern of its expression was the same as that of the *Os-EXP2* gene. However, the hybridization signals with probes *Os-EXP3* and *Os-EXP4* were very weak. Therefore, expansin gene expression is also tissue-specifically regulated in the developing seeds. The expression of these expansin genes in the developing seeds may be critical for the extension of the primary cell walls.

Discussion

Expression of α -expansin genes and coleoptile elongation. This study revealed that cell walls from anoxia- or

hypoxia-grown coleoptiles were more extensible at pH 4.5 than those from aerobiosis-grown coleoptiles. Maintenance of higher extensibility of coleoptiles grown in anoxia makes it possible for the coleoptile to elongate rapidly in hypoxia. Coleoptile elongation induced by lower O_2 concentrations is regulated mainly by the hormone ethylene (Horton 1991). However, either hormones or environmental stimuli that modulate the rate of cell expansion act to change the cell wall properties (Cosgrove 1997). Cell expansion might be affected by cell wall structure, wall-loosening enzymes and cell wall acidity (reviewed by Rayle and Cleland 1992; Cosgrove 1993). In the present study, we focused on the wall-loosening elements. Expansin, which has been identified as loosening plant cell walls in vitro, is the primary enzyme for expanding cell walls during cell

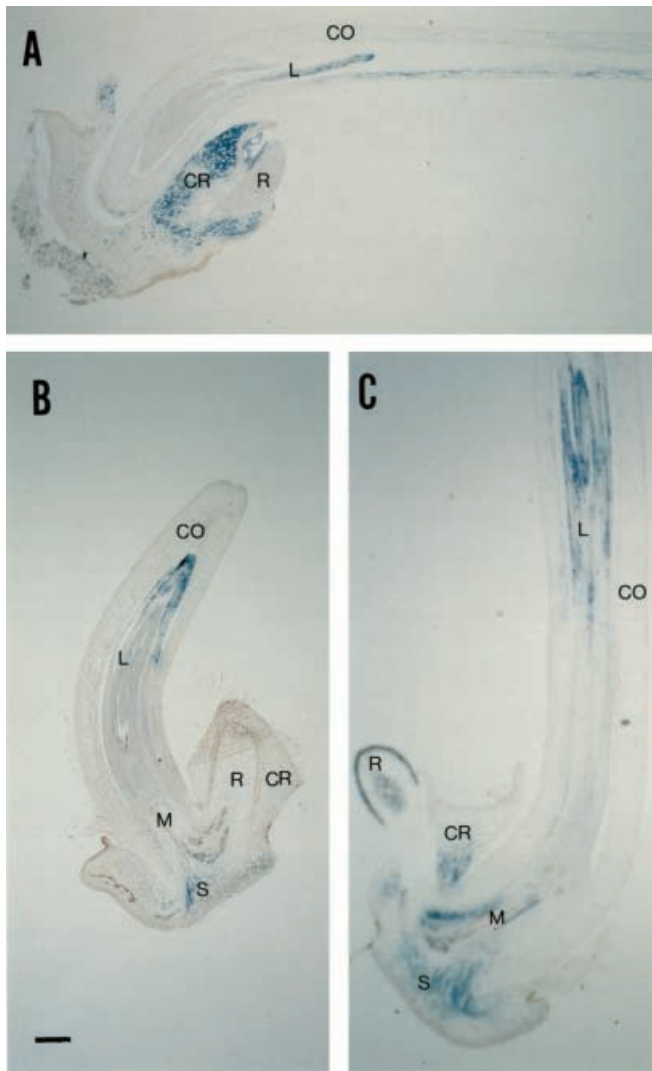


Fig. 5A–C. Expression of the expansin gene *Os-EXP4* in young rice seedlings as detected by in-situ hybridization with the antisense probe. Seedlings were grown in hypoxic (A, day 8) and aerobic (B, day 8; C, day 9) conditions at 18 °C. CO, coleoptile; L, leaf; CR, coleorhiza; R, root; M, mesocotyl; S, scutellum. Bar = 250 μ m

growth. The results both from northern blotting and in-situ hybridization showed that hypoxia greatly induced the expression of α -expansin genes *Os-EXP4* and *Os-EXP2* in the coleoptile, while there were fewer α -expansin transcripts in the air- and anoxia-grown coleoptiles. Under the submerged soil conditions, we observed that as long as coleoptiles emerged from the soil and then grew in water, α -expansin genes *Os-EXP4* and *Os-EXP2* were immediately expressed and the rate of elongation was accelerated. On the other hand, when coleoptiles emerged from the water layer and subsequently entered the air, expression of *Os-EXP4* and *Os-EXP2* was soon suppressed. These results confirm the role of α -expansins in the elongation of submerged rice coleoptiles. In deepwater rice, α -expansin gene expression is also induced in the internode by submergence and contributes to the rate of internode growth (Cho and Kende 1997b). However, the detected

α -expansin gene expression in anoxia-grown coleoptiles was not agreement with the data for acid-induced extension of cell walls in vitro. Even though expansins do mediate acid-induced extension of cell walls, it might be also true that the rate of cell wall extension is not solely determined by the activity of expansins. We observed that cell walls of the coleoptiles raised in anoxia were much weaker and thinner than those either in hypoxia or in aerobiosis. Thus, the above discrepancy might be mainly due to the fact that de-novo synthesis of cell walls is the most heavily inhibited in anoxia. In addition, it cannot be excluded that other expansin genes were expressed in anoxia-elongated coleoptiles whereas other elements, such as cell turgor and H^+ -pump activity that coordinately promote cell extension in vivo, were maintained at a lower level in anoxia, because anoxia normally suppresses the whole metabolic activity of plants.

The key factor controlling the elongation of rice coleoptiles under hypoxic or anoxic conditions is still a matter for dispute. In the acid-growth model, both wall extensibility and apoplastic acidification are important factors affecting cell extension (Rayle and Cleland 1992). In this study, in-situ hybridization showed that expansin genes *Os-EXP2* and *Os-EXP1* are expressed during seed development and transcripts of the four α -expansin genes are not detected during the early stage of coleoptile elongation. In the process of seed germination, the embryo first swells to crack the hull, and then the coleoptile and the seminal root emerge. Cell enlargement must occur after water absorption in such swollen embryos. Thus, based on the evidence released from the present study, expansins stored in dry seeds may play an important role in seed germination and the early elongation of the coleoptile. At this stage, a change in apoplastic pH may activate and regulate the cell expansion in hypoxia or anoxia. However, de-novo synthesis of expansins is indispensable for rapid coleoptile elongation (such as in hypoxia). And furthermore, it is imperative that other expansins such as β -expansins need to be studied for better understanding of the molecular basis for elongation of rice coleoptiles.

Menegus et al. (1991) have reported that the important effect of anoxia on plant cells is cytoplasmic acidification and that rice has the ability to prevent cytoplasmic acidification in anoxia. The plant plasma-membrane H^+ -ATPase is one of the key enzymes regulating cytoplasmic pH (Palmgren 1998). Reggiani et al. (1992) indicated that anoxia induced the accumulation of putrescine, which stimulates H^+ -ATPase activity on the plasma membrane. In *Elodea densa*, a species which, like rice, is able to grow in water, the activity of the H^+ -pump in leaf membranes increases strongly when the cytoplasmic pH drops below its normal value of around 7.5 (Beffagna and Romani 1991). So, the proton pump may trigger cell elongation in rice coleoptiles subjected to anoxia. In maize coleoptiles, auxin-induced acid growth has been reported to correspond to a simultaneous change in apoplast pH (Peters et al. 1998), and the acidification results from a plasma-membrane H^+ -ATPase in the non-vascular tissue (Frias et al. 1996).

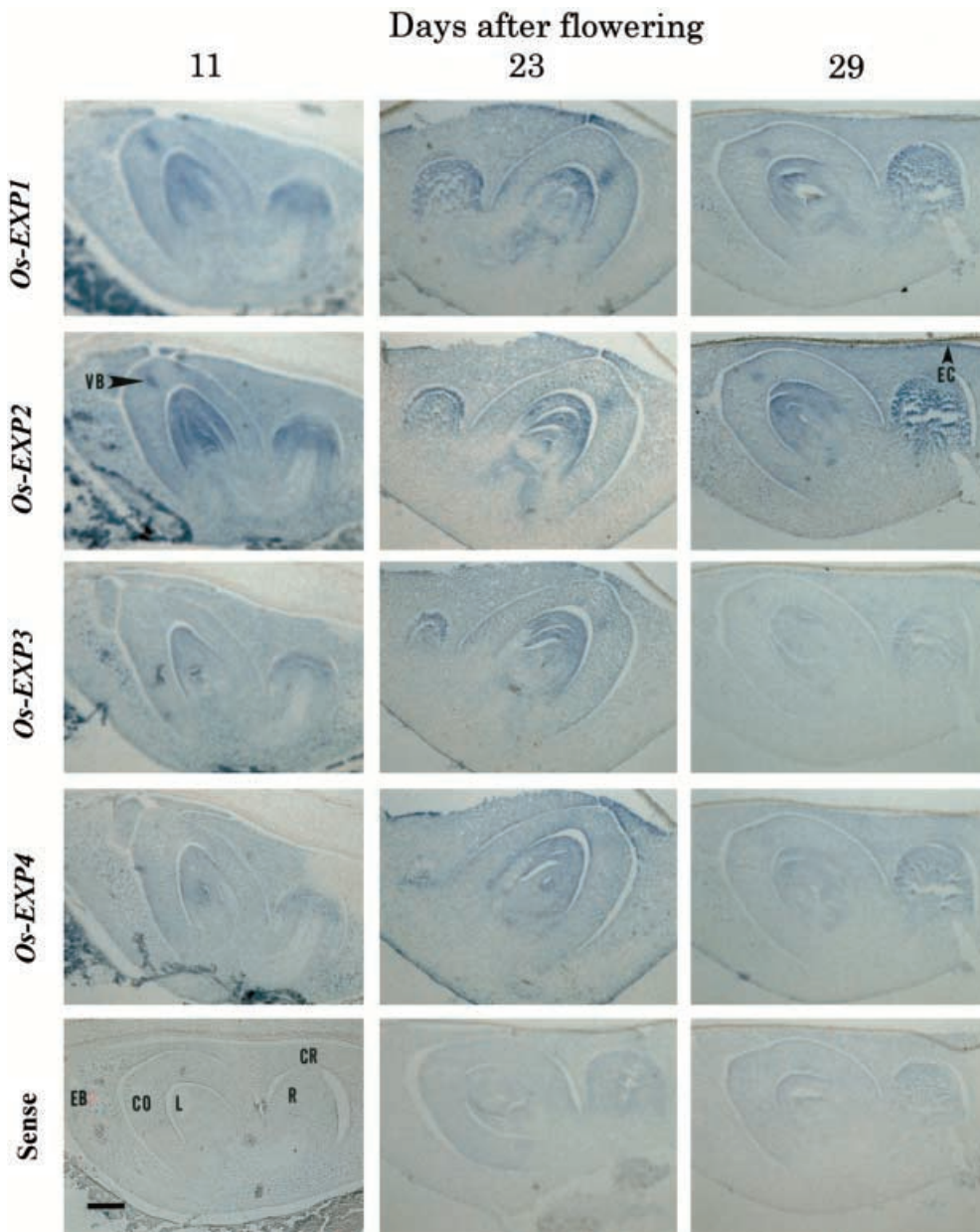


Fig. 6. Localization of mRNAs for four α -expansin genes in the course of rice seed development by in-situ hybridization with antisense and sense probes. Developing seeds were sampled on days 11, 23, and 29 after flowering. *CO*, coleoptile; *L*, leaf; *R*, root; *EP*, epiblast; *CR*, coleorhiza; *VB*, vascular bundle; *EC*, epidermis of the coleorhiza. Bar = 125 μ m

Role of α -expansin gene expression in other tissues of young seedlings. Our results revealed that α -expansin genes were developmentally expressed in different organs, as well as in response to submergence. In young seedlings raised under aerobic conditions, the *Os-EXP4* gene is obviously expressed in growing leaves and mesocotyls. In the mature seeds of rice, the primordia from the coleoptile through to the third leaf have already differentiated. With germination, these leaf primordia restart their development. Although this study has not investigated the relationship between resumption of leaf growth and α -expansins in detail, it looks likely that α -expansin gene expression is related to the leaf development after these leaves start to regrow. Cho and Kende (1997a) did not detect the expression of expansin genes in expanding leaves of deepwater rice. Using older leaves might have resulted in their failure to detect the expression of expansin genes. The develop-

ment of mesocotyls, similar to leaves, needs cell division and expansion. Expression of α -expansin genes in mesocotyls may imply that expansins are also indispensable for mesocotyl growth. In direct-sown rice, after coleoptiles emerge into the atmosphere, the growth of mesocotyls is another important trait for pushing the coleoptilar node to near the soil surface, so that the tiller can develop and grow well.

The coleorhiza is thought to protect the seminal root as it emerges from the kernel after imbibition commences (Suzuki et al. 1991). In addition, long coleorhiza hairs produced from the outer epidermal cells anchor seeds in the soil (Morita et al. 1997). In the coleorhiza, a relatively strong expression of expansin genes was detected during seed development. Expansins may be necessary for the epidermal cells to expand and form the long hairs of the coleorhiza. Noda and Hayashi (1957) reported that the coleorhiza elongated when rice seeds

were germinated in hypoxic conditions. Such elongation of the coleorhiza was also observed in this study, and the *Os-EXP4* gene was strongly induced in the coleorhiza by submergence (Fig. 5A). Hence, expansin gene expression may also contribute to coleorhiza elongation during submergence. However, it is hard to understand what the function might be of *Os-EXP4* expression in the coleorhiza of young seedlings grown under aerobic conditions since elongation has not yet occurred. An effect of the coleorhiza on seedling growth has been reported by Noda and Hayashi (1957), who showed that pruning the coleorhiza severely suppressed the elongation of seminal roots, but not coleoptile elongation. As well as loosening cell walls, some other functions of expansins are known, e.g. softening of fruits (Rose et al. 1997), induction of the leaf primordium (Reinhardt et al. 1998), and disassembly of cell walls (Cosgrove and Durachko 1994). It will be interesting to study the role of expansin further in the coleorhiza.

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