

α -Expansins in the semiaquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum*: evolutionary aspects and physiological role in rachis elongation

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Received: 7 March 2000 / Accepted: 29 April 2000

Abstract. To investigate the evolutionary history of expansins and their role in cell elongation in early land plants, we isolated two α -expansin genes, *Mq-EXPI* and *Rd-EXPI*, respectively, from the semiaquatic ferns *Marsilea quadrifolia* L. and *Regnellidium diphyllum* Lindm. The deduced amino acid sequences of the fern expansins exhibit a high degree of identity to those of seed plants, showing that expansin genes were conserved during the evolution of vascular plants. Gel-blot analysis of *M. quadrifolia* and *R. diphyllum* genomic DNA indicated that, in both ferns, α -expansins are encoded by multigene families. Expression of α -expansin genes probed with *Mq-EXPI* was confined to the elongating region of the *Marsilea* rachis. Cell-wall proteins of *M. quadrifolia* induced in-vitro extension of acidified cucumber cell walls. In *R. diphyllum*, expression of *Rd-EXPI* increased when elongation of the rachis was enhanced by submergence or ethylene. These results indicate that α -expansins act as wall-loosening proteins in ferns, as has been proposed for angiosperms. In addition, *Rd-EXPI* may play a role in mediating elongation of the rachis in submerged plants.

Key words: Rachis elongation – Ethylene – Expansin – *Marsilea* – *Regnellidium* – Submergence

Introduction

Expansins are cell-wall proteins that mediate in vitro cell-wall extension (for a review, see Cosgrove 1999). Because the pH dependence of expansin-enhanced wall

extension resembles that of acid-induced growth and because expansin activity and acid growth are affected similarly by the same factors, e.g. metal ions, expansins are thought to be primary mediators of acid-induced growth (McQueen-Mason et al. 1992). However, the precise biochemical mechanism by which expansins loosen the cell wall has not yet been elucidated. Expansins do not appear to act by hydrolyzing any of the major polysaccharide components of the wall (Cosgrove 1999). Evidence indicates that expansins disrupt hydrogen bonds between cell-wall polymers, e.g. between cellulose and matrix polysaccharides, resulting in cell-wall creep (McQueen-Mason and Cosgrove 1994, 1995).

Since the first cloning of expansin cDNAs from cucumber (Shcherban et al. 1995), many expansin genes have been identified in a wide range of seed plants, including a gymnosperm (Cosgrove 1999; Hutchison et al. 1999). Based on amino acid sequence analysis, expansins have been divided into two subfamilies, the α - and β -expansins, which have been highly conserved during the evolution of seed plants (Cosgrove 1998, 1999; Hutchison et al. 1999). It has been suggested that expansins would be found in all groups of land plants because acid-induced growth has also been observed in mosses and ferns (Shcherban et al. 1995). It is, therefore, important to establish whether bryophytes and pteridophytes possess expansins, what the sequence relationship between the expansins of mosses, ferns and seed plants is, and whether expansins play a role in growth of mosses and ferns.

Ferns constitute a major group of the pteridophytes that evolved shortly after emergence of land plants (Qiu and Palmer 1999). To investigate the evolution of expansin structure and function, we set out to determine whether the semiaquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum* express expansin genes, whether expansin transcript levels are correlated with growth, and whether expansin-like proteins are present in the cell wall of ferns. We chose *M. quadrifolia* and *R. diphyllum* because both possess the capacity for rapid elongation. Under greenhouse conditions, the rachis of *M. quadri-*

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Abbreviations: RACE = rapid amplification of cDNA ends; RT-PCR = reverse transcriptase-polymerase chain reaction

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folia grows at a rate of up to 3 cm a day. Also, *R. diphyllum* and some semiaquatic angiosperms share a remarkable growth response to flooding. When such plants become submerged, elongation of the rachis, the petiole, or the stem is enhanced. The initial signal for this growth response is ethylene. In submerged *R. diphyllum*, ethylene accumulates in the air spaces of the rachis, increasing cell-wall extensibility and promoting rapid elongation of the rachis (Musgrave and Walters 1974; Cookson and Osborne 1979; Ridge 1985). In deepwater rice, a semiaquatic angiosperm, ethylene exerts its effect via gibberellin, which increases cell-wall extensibility and induces growth (Kende et al. 1998).

Adaptation to flooding in the semiaquatic fern *R. diphyllum* and semiaquatic angiosperms may represent an interesting case of convergent evolution. Both *R. diphyllum* and semiaquatic angiosperms recognize ethylene as signal of submergence and respond to it by greatly enhanced growth. However, the pathway of ethylene synthesis in ferns differs from that of angiosperms (Chernys and Kende 1996; Osborne et al. 1996). Recently, it has been shown that both submergence and gibberellin induce the expression of expansin genes in deepwater rice, indicating that expansins mediate, at least in part, submergence-induced growth in rice (Cho and Kende 1997b). Because of the similarity in the growth response of *R. diphyllum* and semiaquatic angiosperms, we became interested in finding out whether expansins occur in ferns and whether they may play a role in submergence-induced growth of *R. diphyllum*.

In this paper, we report that α -expansin genes have been identified in both *M. quadrifolia* and *R. diphyllum* and that they are encoded by multigene families. The deduced amino acid sequences of fern expansins are closely similar to those of seed plants. The expression patterns of α -expansin genes in both ferns are correlated with growth, and cell-wall proteins extracted from *M. quadrifolia* induce 'creep' in cucumber cell walls. Our results indicate that evolution of expansin structure and function predates the divergence of ferns and seed plants.

Materials and methods

Plant material and growth conditions

Marsilea quadrifolia L. was obtained from the greenhouse collection of the Department of Botany and Plant Pathology at Michigan State University. *Regnellidium diphyllum* Lindm. was purchased from Maryland Aquatic Nurseries (Jarrettsville, Md., USA). The plants were grown in soil covered with 2 cm of water in a greenhouse as described by Chernys and Kende (1996).

Growth measurements and treatments

The rachises of *M. quadrifolia* were marked with India ink at 2-cm intervals starting at the base of the leaflets. After 24 h, the distance between the marks was determined. The fronds whose fiddle heads had just opened but whose leaflets were still closed were designated

as young, and those whose leaflets had just opened as mature. *R. diphyllum* plants with young fronds of 7–10 cm length were excised with a 1-cm piece of rhizome attached. The fronds were marked 3 cm below the base of leaflets at the start of the experiments, and the increase in length was measured after 24 h of submergence or ethylene treatment. For submergence, fronds of *R. diphyllum*, with a weight attached to the rhizoid, were completely immersed for 24 h in Plexiglas cylinders containing distilled water. Control fronds were placed in a cylinder containing 50 ml distilled water that covered the basal 2 cm of the frond and the rhizome. For ethylene treatment, young fronds were placed into beakers containing distilled water and were enclosed in desiccators into which ethylene was injected to yield a concentration of 50 μ l/l. Control fronds were placed in desiccators without ethylene application.

Isolation of RNA

Total RNA was extracted with a combination of the hot borate (Wilkins and Smart 1996) and the guanidium thiocyanate methods (Puissant and Houdebine 1990). Briefly, 1 g of rachises was pulverized with liquid nitrogen and homogenized at 90 °C in 7 ml borate buffer [0.2 M Na-tetraborate, 30 mM EGTA, 1% (w/v) SDS, 1% (w/v) deoxycholate, 10 mM dithiothreitol, 1% (v/v) Nonidet P-40, 2% (w/v) polyvinylpyrrolidone]. The homogenate was incubated with 13 mg proteinase K (Gibco-BRL, Gaithersburg, Md., USA) at 42 °C for 1.5 h, and then with 0.9 ml 2 M KCl on ice for 1 h. After centrifugation at 17,000 *g* for 20 min, 10 M LiCl was added to the supernatant to a final concentration of 3 M. The precipitate was collected by centrifugation at 17,000 *g* for 20 min, and the pellets were dissolved in 2.5 ml of guanidium thiocyanate buffer [4 M guanidium thiocyanate, 25 mM Na-citrate, 0.5% (w/v) Sarkosyl, 0.1 M β -mercaptoethanol] and extracted with phenol/chloroform. The RNA in the supernatant was precipitated with 0.7 volume of isopropanol and collected by centrifugation at 17,000 *g* for 20 min. The RNA pellets were washed with 70% ethanol.

Isolation of cDNAs and sequencing

First-strand cDNA synthesis for reverse transcriptase-coupled polymerase chain reactions (RT-PCR) was performed with 5 μ g of total RNA from young rachises of *M. quadrifolia* or *R. diphyllum*, 0.5 μ g oligo-dT primers (Promega, Madison, Wis., USA), and 200 units of reverse transcriptase (Superscript II; Gibco-BRL) according to the protocols provided by the companies. Using degenerate primers for expansins (Rose et al. 1997), the cDNAs were amplified with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA) for 35 cycles (94 °C for 1 min, 52 °C for 2 min, and 70 °C for 1 min). The resulting DNA fragments were purified on agarose gels and subcloned into pGEM-T vector (Promega). A cDNA clone, *MqPCR1* (531 bp) was obtained from *M. quadrifolia* RNA, and two cDNA clones, *RdPCR1* (538 bp) and *RdPCR2* (529 bp) were generated from *R. diphyllum* RNA. To obtain full-length cDNAs corresponding to *MqPCR1* and *RdPCR1*, 5' and 3' rapid amplification of cDNA ends (RACE) was performed with kits from Gibco-BRL according to manufacturer's instructions. First-strand cDNAs for 5'-RACE were prepared with the following gene-specific primers: 5'-CCTTGGAACAGGGCACTTCTCTAT-3' for *MqPCR1* and 5'-CCAGTTTTGGCCCCAGTT-3' for *RdPCR1*. The gene-specific primers for 3'-RACE were 5'-GCACTTAGCACAGCCCTCTT-CAAT-3' for *MqPCR1* and 5'-TAACGGTAACCCCAACTT-3' for *RdPCR1*. The PCR reactions were performed with the above gene-specific primers and primers provided in the kits under the same conditions as RT-PCR. The resulting DNA fragments for each PCR reaction were purified, subcloned into the pGEM-T vector and sequenced with an Applied Biosystems model-377 sequencer (Perkin-Elmer, Foster City, Calif., USA) at the Michigan

State University DNA Sequencing Facility. The 274-bp overlapping sequences of *MqPCR1* and its corresponding 5'- and 3'-RACE sequences were combined to produce the full-length sequence of *Mq-EXPI*. The combined sequences of *RdPCR1* and its corresponding 5'- and 3'-RACE sequences (129-bp overlap) yielded the full-length sequence of *Rd-EXPI*. The partial cDNA clone *RdPCR2* was named as *Rd-EXP2*.

Phylogenetic analysis

The deduced full-length amino acid sequences of *Mq-EXPI*, *Rd-EXPI*, and seed plant α -expansins obtained from GenBank were aligned by the Clustal method with the MegAlign program (DNASTAR, Madison, Wis., USA). Parsimony, as implemented in PAUP*4.0 (Swofford 1998), was used to infer phylogenies based on amino acid substitutions in aligned sequences. Parsimony analyses were performed by heuristic search with TBR branch swapping, MULPARS option, ACCTRAN optimization, and 1,000 random additions. Bootstrap analyses were carried out with 1,000 replications of heuristic search with simple taxon addition while all trees were saved. *Phlp1*, a member of the β -expansins from *Phleum pratense*, was used as an outgroup.

Analysis of DNA and RNA gel blots

DNA was extracted from rachises of *M. quadrifolia* and *R. diphyllum* according to Doyle and Doyle (1990). Twenty micrograms of DNA from each fern was digested with *Bam*HI, *Eco*RI, or *Hind*III, separated in a 0.7% (w/v) agarose gel, and transferred to a nylon membrane. The membrane was hybridized with *MqPCR1* or *RdPCR1*, which correspond to the most conserved region of *Mq-EXPI* and *Rd-EXPI*, respectively. Prehybridization and hybridization were carried out in Church buffer (Church and Gilbert 1984) at 60 °C for 16 h, and the filter was washed twice at 60 °C with $2 \times$ SSC and 0.1% (w/v) SDS ($1 \times$ SSC is 0.15 M NaCl, 0.015 M Na-citrate).

For RNA gel blot analysis, 20 μ g of total RNA isolated from *M. quadrifolia* and 10 μ g from *R. diphyllum* were subjected to electrophoresis in formaldehyde gels and transferred to nylon membranes as described in Sambrook et al. (1989). *MqPCR1*, *RdPCR1*, and *RdPCR2* were used as probes for the detection of *Mq-EXPI*, *Rd-EXPI*, and *Rd-EXP2* transcripts, respectively. The hybridization was performed in the same buffer as above at 60 °C (*Mq-EXPI*) and 65 °C (*Rd-EXPI* and *Rd-EXP2*), and the membranes were washed twice with $0.1 \times$ SSC, 0.1% SDS at 65 °C (*Mq-EXPI*) and 70 °C (*Rd-EXPI* and *Rd-EXP2*). Autoradiography was performed using Hyperfilm-MP (Amersham-Pharmacia, Piscataway, N.J., USA) with an intensifying screen at -80 °C.

Extraction of cell-wall proteins

Extraction of cell-wall proteins from the upper 2–3 cm of the rachis of *M. quadrifolia* was performed according to Cho and Kende (1997a). Ammonium sulfate precipitates were resuspended in Na-acetate buffer (pH 4.5), and used for in-vitro cell-wall extension experiments.

Measurement of in vitro cell-wall extension

Cell-wall extension elicited by wall proteins from *M. quadrifolia* was measured according to the method of Cho and Kende (1997a). Four-day-old dark-grown cucumber seedlings were harvested and stored frozen for up to 5 d at -20 °C. The frozen hypocotyls were abraded with carborundum slurry (300 mesh; Fisher Scientific, Pittsburgh, Pa., USA), thawed, and boiled for

1 min in distilled water. Subapical 1-cm-long segments were excised, placed between one layer of filter paper, and pressed between two glass slides under 400 g weight for 1 min. One segment was fixed between two clamps spaced 0.5 cm apart, placed into an extensometer equipped with an angular position transducer (Kutschera and Briggs 1987), and subjected to constant tension using a weight of 20 g. The segment was immersed first in 50 mM Na-acetate buffer (pH 4.5) for 20 min, after which the buffer solution was replaced with the same buffer containing 1 mg of cell-wall proteins.

Acid-induced cell-wall extension of *R. diphyllum*

Fifteen-millimeter sections of *R. diphyllum* rachises, which had been submerged or treated with ethylene, were excised from the base of the leaflets, frozen, abraded, thawed, and pressed as described above. The native wall specimens were clamped in the extensometer (distance between clamps, 1 cm) under constant tension using a weight of 10 g. The wall sections were first immersed in 50 mM Hepes/Tris buffer (pH 6.8), for 20 min, after which the solution was changed to 50 mM Na-acetate buffer (pH 4.5).

Results

Isolation and sequence analysis of α -expansin cDNAs of ferns

A 531-bp cDNA fragment (*MqPCR1*) from *M. quadrifolia* and two different cDNA fragments of 538 bp (*RdPCR1*) and 529 bp (*RdPCR2*) from *R. diphyllum* were obtained by RT-PCR as described above. Sequence analysis showed that these three DNA fragments were highly similar to the corresponding regions of α -expansins. The nucleotide sequences of *RdPCR1* and *RdPCR2* were 65% identical to each other. Full-length cDNA sequences were constructed from the overlapping DNA fragments of *MqPCR1* and *RdPCR1* and their corresponding 5'- and 3'-RACE products. They were designated, respectively, as *Mq-EXPI* (*Marsilea quadrifolia-expansin1*; Accession No. AF202119) and *Rd-EXPI* (*Regnellidium diphyllum-expansin1*; Accession No. AF202120). The *Mq-EXPI* cDNA is 1,374 bp in length and contains an open reading frame of 771 bp encoding a polypeptide of 257 amino acids. The *Rd-EXPI* cDNA is 1,115 bp in length with an open reading frame of 756 bp encoding a polypeptide of 252 amino acids. The amino acid identity between *Mq-EXPI* and *Rd-EXPI* is 72% over the entire coding region. The highest level of identity, 73%, between *Mq-EXPI* and an expansin of a seed plant, is to Pt-EXP of loblolly pine. *Rd-EXPI* shows the highest level of identity to Pt-EXP (71%) and Cs-EXPI of cucumber (70%). The deduced amino acid sequences of *Mq-EXPI* and *Rd-EXPI* were aligned with three representative α -expansins from seed plants (Fig. 1). α -Expansins of all seed plants have a signal peptide of around 23 amino acids and possess eight conserved cysteines in their N-terminal regions and four conserved tryptophan residues in their C-terminal regions (Cosgrove 1999). According to the PSORT program (Nakai and Kanehisa 1992), the deduced protein sequences of *Mq-EXPI* and *Rd-EXPI* were predicted to contain N-terminal signal peptides of

Mq-EXP1	1	<i>massttswgfvllwlsllllsfflapgya</i> GGSGWRDAHATFYGGSDASG
Rd-EXP1	1	<i>massat</i> ---- <i>lswafvls</i> <i>lfsfsgls</i> --ygSSGWNDAHATFYGGSDASG
Pt-EXP	1	---- <i>mrmelvkisialaslltftiwl</i> ltgahgYGGWESAHAATFYGGSDASG
Os-EXP4	1	----- <i>maiagvllflflarqasaa</i> YGCGWQSAHAATFYGGSDASG
Cs-EXP1	1	----- <i>mafsyspfsslfl</i> lpffffvftfaDYGGWQSGHATFYGGSDASG
Mq-EXP1	51	TMGGACGYDLYSSGYCIHTAALSTALFNGCLSCGACFELKCSGGSRWCL
Rd-EXP1	44	TMGGACGYDLYTSGYCTNTAALSTALFNGCLSCGACFAVKCSGGNTRWCL
Pt-EXP	47	TMGGACGYGNLYSQGYCTNTAALSTALFNDCLSCGACYEMRONDPPQWCL
Os-EXP4	40	TMGGACGYGNLYSQGYCTNTAALSTALFNDCAACGS CYELRCDNAGSSCL
Cs-EXP1	44	TMGGACGYGNLYSQGYCTNTVALSTALFNNCLSCGACFEMTCTNDPKWCL
Mq-EXP1	101	SCFTVTISATNFCPPNNALSSSTNGGCNPPLEHFDMAQPAYQIAYQGGI
Rd-EXP1	94	PCITVEVTATNFCPPNWAEASDNGGCNPPRKHFDMAQPAFEQIAYRGGI
Pt-EXP	97	PCITVTVTATNFCPPNNALPNDNGGCNPPLOHFDMAEPAFLKIAKYRGGI
Os-EXP4	90	PCISITVTATNFCPPNYGLPSDDGGGCNPPRPHFDMAEPAFLHIAQYRAGI
Cs-EXP1	94	PCITIRVTATNFCPPNFALPNNNGGCNPPLOHFDMAEPAFLQIAQYRAGI
Mq-EXP1	151	VFVQYRVRVPCSKEGGVHFTINGHAFNVLVLTINVCAGDVHAVSVKGSRT
Rd-EXP1	144	VFVKYRVRVPCQKKGVRFTINGNPNFNLVLTINVCAGDVVAVSVKGSRT
Pt-EXP	147	VFLLYTRVPCLRKGGIRFTVNGHSYFNLVLTINVCAGDVHAVSVKGSRT
Os-EXP4	140	VFVFSFRVPCVKKGGIRFTVNGHSYFNLVLTINVCAGDVRSVSVKGSRT
Cs-EXP1	144	VFVFSFRVPCMKKGGVRFTINGHYSYFNLVLTINVCAGDVHVSVKGSRT
Mq-EXP1	201	GWQPMTRNWGNWQSNANLCCGALSFMVTTSDGRTLTSFNAAAPSSWQFGQ
Rd-EXP1	194	GWIPMTRNWGNWQCGTTLTGOALSFAVTLDNCSVLTITVNVANADWRFQ
Pt-EXP	197	GWQPMSTRNWGNWQSNAYLNGQSLSFQVTTSDGRTVVSNNVAPSNWQFGQ
Os-EXP4	190	GWQPMSTRNWGNWQSNAFLDGQSLSFQVTASDGRVTTSNNVAHPGWQFGQ
Cs-EXP1	194	GWQSMSTRNWGNWQSNNYLNGQSLSFQVTLSDGRTLTAYNLVPSNWQFGQ
Mq-EXP1	251	TFVGEQL
Rd-EXP1	244	TFEGDQFNT
Pt-EXP	297	TFEGSQV
Os-EXP4	240	TFEGGQF
Cs-EXP1	294	TFEGPQF

Fig. 1. Multiple alignment of the deduced amino acid sequences of fern expansin cDNAs with three representative α -expansins from seed plants. The deduced amino acid sequences of Mq-EXP1, Rd-EXP1, Cs-EXP1 (*Cucumis sativus*), Os-EXP4 (*Oryza sativa*), and Pt-EXP (*Pinus taeda*) were aligned with the Clustal-W program (Thomson et al. 1994). The predicted signal peptide sequences are indicated in italic script. Amino acid residues identical among all five expansins are shown with reverse contrast. Conserved cysteine (C) and tryptophan (W) residues are marked by asterisks and circles, respectively

30 and 24 amino acid residues, respectively (Fig. 1). The spacing of the invariable cysteine and tryptophan residues was strictly conserved in both fern expansins (Fig. 1). Our sequence analysis indicates that both fern cDNAs encode α -expansins.

Phylogenetic analysis of α -expansins

To assess the evolutionary relationships between the α -expansin genes of ferns and seed plants, a phylogenetic tree was generated with the 29 currently available, full-length amino acid sequences of α -expansins (Fig. 2). *Phlp1*, a member of the β -expansins from *Phleum pratense*, was used as an outgroup. The two fern expansins, Mq-EXP1 and Rd-EXP1, form their own branch rather than being a member of any other previously identified expansin subfamily of seed plants. This is also true for the tree constructed with the truncated DNA sequences corresponding to mature proteins (data not shown).

Genomic analysis of *M. quadrifolia* and *R. diphyllum*

We employed DNA gel blot analysis to estimate the genomic complexity of α -expansin genes in both ferns

(Fig. 3). *MqPCR1* and *RdPCR1*, which correspond to the most conserved region of *Mq-EXP1* and *Rd-EXP1*, respectively, were used as probes at moderate stringency. Three restriction digests of *M. quadrifolia* DNA produced two to four prominent fragments hybridizing with *MqPCR1*, with additional weak bands in lanes E and H (Fig. 3A). Similarly digested DNA of *R. diphyllum* showed multiple DNA fragments that hybridized to *RdPCR1* (Fig. 3B). Using PCR, we identified three *R. diphyllum* expansin cDNAs, namely *RdPCR1*, *RdPCR2*, and a 3'-RACE product (data not shown). These results indicate that, in both *M. quadrifolia* and *R. diphyllum*, α -expansins are encoded by multiple genes.

Correlation of growth and expansin mRNA levels along the Marsilea rachis

The growth rate and α -expansin mRNA levels were determined in the growing and non-growing regions of the rachis of *M. quadrifolia*. The elongation zone, located in the top 2 cm of the rachis just under the base of the leaflets, grew at a rate of 2 cm a day (Fig. 4A). The 2-cm region of the rachis just below the elongation zone grew very little during the same period. The level of α -expansin mRNA detected with *Mq-EXP1* was high in the elongating, upper region, but very low in the non-

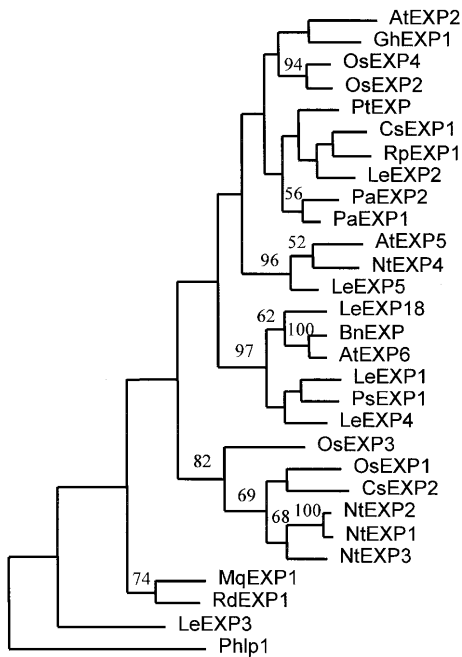


Fig. 2. Phylogenetic tree of expansin proteins. The full-length amino acid sequences of Mq-EXP1 (AF202119), Rd-EXP1 (AF202120), and α -expansins of seed plants available in the GenBank database were aligned by the Clustal method of MegAlign (DNASTAR). The tree was constructed with the PAUP program, using a β -expansin, Phlp1 (X78813), as an outgroup. One of sixteen equally most parsimonious trees was randomly chosen with bootstrap values placed above the lines. The accession numbers are given in parentheses. At-EXP2 (U30481), At-EXP5 (U30478), and At-EXP6 (U30480) from *Arabidopsis thaliana*; Bn-EXP (AJ000885) from *Brassica napus*; Cs-EXP1 (U30382) and Cs-EXP2 (U30460) from *Cucumis sativus*; Gh-EXP1 (AF043284) from *Gossypium hirsutum*; Le-EXP1 (U82123), Le-EXP2 (AF096776), Le-EXP3 (AF059487), Le-EXP4 (AF059488), Le-EXP5 (AF059489), and Le-EXP18 (AJ004997) from *Lycopersicon esculentum*; Nt-EXP1 (AF049350), Nt-EXP2 (AF049351), Nt-EXP3 (AF049352), and Nt-EXP4 (AF049353) from *Nicotiana tabacum*; Os-EXP1 (Y07782), Os-EXP2 (U30477), Os-EXP3 (U30479), and Os-EXP4 (U85246) from *Oryza sativa*; Pa-EXP1 (U93167) and Pa-EXP2 (AF038815) from *Prunus armeniaca*; Ps-EXP1 (X85187) from *Pisum sativum*. Pt-EXP (AF085330) from *Pinus taeda*; Rp-EXP1 (AF167360) from *Rumex palustris*

growing, lower region of the rachis. The upper region ceases to grow as the fronds mature; at that time, no expansin transcript could be detected in that region (Fig. 4B). Also, expansin mRNA was only found in actively expanding young leaflets, but not in mature ones (Fig. 4C). These results indicate that there is a close correlation between the growth of the rachis and the leaflets in *M. quadrifolia* and the pattern of expansin gene expression.

In-vitro wall-extension activity of *M. quadrifolia* cell-wall proteins

To determine whether cell-wall proteins of *M. quadrifolia* have wall-loosening activity, wall-protein fractions were extracted from the growing region of rachises and added in acidic buffer to boiled cucumber wall specimens that had been fixed between clamps in an extensometer.

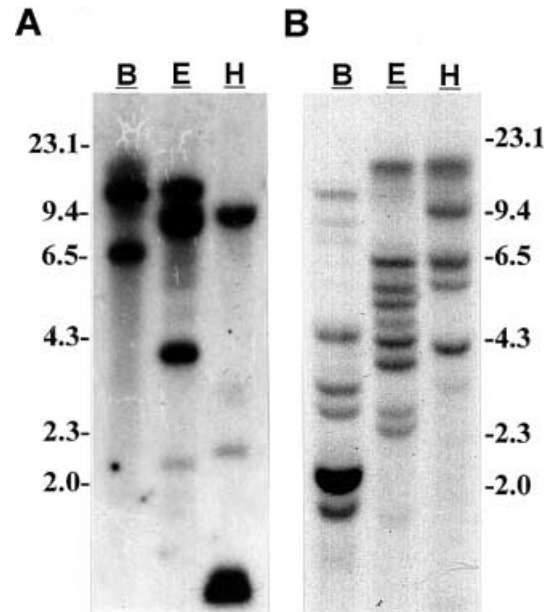
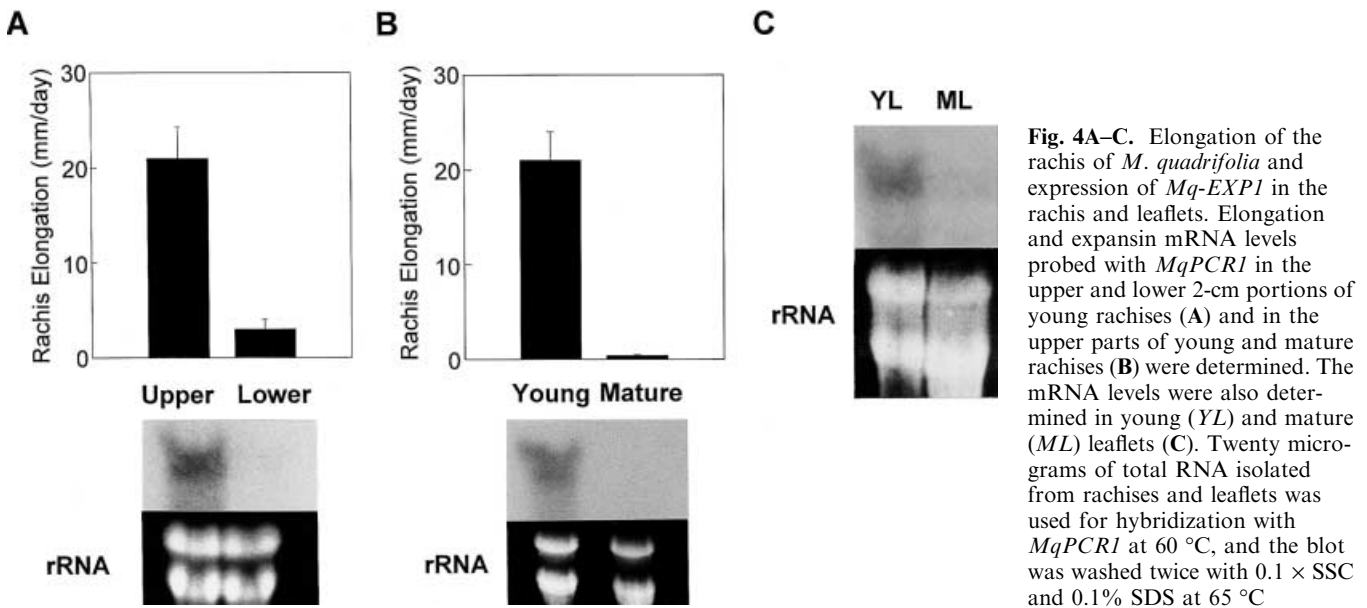


Fig. 3. Genomic DNA gel blot analysis. Genomic DNA (20 μ g per lane) of *M. quadrifolia* (A) or *R. diphyllum* (B) was digested with *Bam*H1 (B), *Eco*R1 (E), or *Hind*III (H). The nylon membrane was hybridized at 60 $^{\circ}$ C with *MqPCR1* (A) and *RdPCR1* (B), respectively, and washed twice with $2 \times$ SSC and 0.1% SDS at the same temperature. The numbers indicate the size of markers in kbp

The crude *M. quadrifolia* wall proteins induced extension of cucumber cell walls, whereas boiled *M. quadrifolia* cell-wall proteins did not (Fig. 5). These results are indicative for expansin activity in preparations of cell-wall proteins (McQueen-Mason et al. 1992). The cell walls of the *M. quadrifolia* rachis were still fully extensible in acid buffer after boiling for 1 min or heating at 80 $^{\circ}$ C for 10 min. In such cell-wall preparations, added cell-wall proteins did not cause further extension (results not shown).

Effects of submergence and ethylene on elongation of the rachis and on expression of expansin genes in *R. diphyllum*

Elongation of the rachis in *R. diphyllum* is greatly stimulated by submergence. This growth response is mediated by ethylene, which becomes trapped in the submerged parts of the plant (Cookson and Osborne 1979). We examined the level of expansin transcripts in air-grown (control), submerged, and ethylene-treated fronds (Fig. 6). When the fronds were immersed completely in distilled water for 24 h, elongation of the rachis was stimulated about 5-fold, and the level of *Rd-EXP1* mRNA increased in submerged rachises. Treatment with ethylene also increased elongation of the rachis and the level of *Rd-EXP1* mRNA. These results show a clear correlation between elongation of the rachis and expression of *Rd-EXP1*. In contrast, the level of *Rd-EXP2* transcripts did not change in response to submergence and ethylene treatment (Fig. 6).



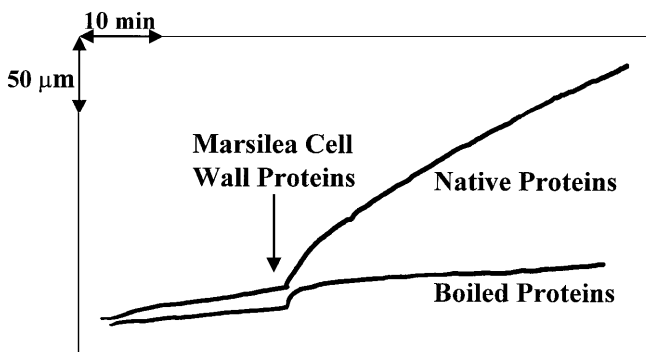
Effect of submergence and ethylene on acid-induced extensibility of the *Regnellidium* rachis

As a measure of endogenous expansin activity, we examined the acid-induced extensibility of native cell walls from submerged and ethylene-treated rachises. Submergence and ethylene treatment increased cell wall extensibility 1.7- and 2.4-fold over controls (Fig. 7A, B), which correlated well with the accumulation of *Rd-EXP1* mRNA and the promotion of growth under the same conditions (Fig. 6).

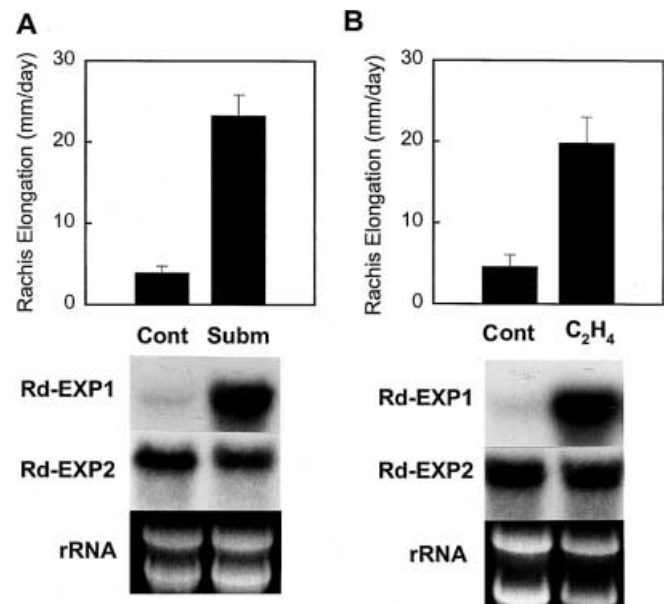
Discussion

We identified α -expansin genes in the semiaquatic ferns *M. quadrifolia* and *R. diphyllum*. The deduced expansin proteins of both ferns show all the characteristic features

of α -expansins, and their amino acid sequences are closely similar to expansins of seed plants, including a gymnosperm (Fig. 1). This relatedness indicates that α -expansin genes have been highly conserved during the evolution of vascular plants. The phylogenetic tree shows a subfamily made up of only the fern α -expansins with moderate bootstrap support (74%; Fig. 2), indicating that the two expansin genes are most closely related. Our phylogenetic analysis confirms that the α -



of *M. quadrifolia* on in-vitro cell-wall extensibility of cucumber hypocotyl segments. Boiled cucumber hypocotyl segments were immersed in 50 mM Na-acetate buffer (pH 4.5) for 20 min following which the solution was replaced with the same buffer containing 1 mg of cell-wall proteins from rachises of *M. quadrifolia*. As a control, the cell-wall proteins were boiled for 5 min. Cell-wall extensibility was analyzed with an extensometer



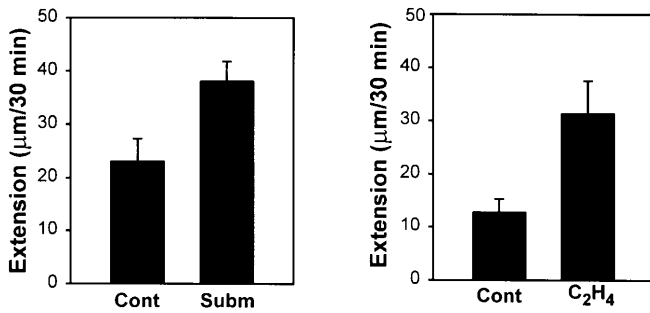


Fig. 7A,B. Submergence- and ethylene-induced cell-wall extensibility of *R. diphyllum*. *Regnellidium* fronds were submerged (A) or treated with 50 $\mu\text{l}/\text{l}$ ethylene (B) for 24 h, and the cell-wall extensibility of 1-cm rachis sections was analyzed in an extensometer. Extension of the sections in acidic buffer was determined after 30 min. The data are the means \pm SE of four to five measurements

expansin genes constitute a complex gene family in vascular plants, providing evidence for an extensive duplication of α -expansin genes before divergence of ferns and seed plants. This interpretation is compatible with the result that *M. quadrifolia* and *R. diphyllum* contain several putative expansin genes (Fig. 3, and data not shown). To clarify the origin of α -expansin genes and to establish the ancestral relationships among them, α -expansin genes and/or proteins need to be isolated from bryophytes and from charophytes, from which land plants are believed to have arisen (Qiu and Palmer 1999). Blast searches against the genome of a cyanobacterium, *Synechocystis* sp. strain PCC 6803 (www.kazusa.or.jp:8080/cyano), did not show any sequences with significant similarities to the fern expansin genes.

α -Expansin genes in deepwater rice are highly expressed in actively growing tissues (Cho and Kende 1997b). In cotton, the level of α -expansin mRNA was found to be high as long as the fiber cells were elongating (Shimizu et al. 1997). These expression patterns of expansin genes are consistent with the proposed function of expansins as cell-wall-loosening proteins mediating cell elongation. A similar conclusion can be drawn from our results with fern expansins. The expression of α -expansin genes of *M. quadrifolia* is limited to actively growing tissues, such as the elongating region of the rachis and the expanding leaflets (Fig. 4). When the rachises of *R. diphyllum* are induced to grow by submergence or ethylene treatment, the level of *Rd-EXPI* mRNA increases accompanied by increases in wall extensibility (Figs. 6 and 7). Furthermore, cell-wall proteins of *M. quadrifolia* exhibit expansin-like activity when applied to heat-inactivated cucumber cell-wall specimens. These results indicate conservation of expansin function in ferns and seed plants. Because boiling did not eliminate the extensibility of *M. quadrifolia* cell walls in acidified medium, no significant effect of *Marsilea* cell-wall proteins on the extension of boiled rachis cell walls could be demonstrated. The extensibility of boiled rachis cell walls in acidic buffer alone may reflect structural differences between the cell walls of ferns and angiosperms and may mask any response to expansins.

In *R. diphyllum*, submergence promotes elongation of the rachis, a response mediated by ethylene that accumulates in the submerged parts of the plant (Musgrave and Walters 1974; Cookson and Osborne 1978). Both submergence and treatment with ethylene increase cell-wall extensibility of the rachis, indicating that elongation is a consequence of cell-wall modifications (Fig. 7; Cookson and Osborne 1979). Submergence and treatment with ethylene also elicit an increase in the level of *Rd-EXPI* mRNA (Fig. 6). These results indicate that the effect of submergence on wall extensibility and cell elongation in *R. diphyllum* may result, at least in part, from ethylene-induced expression of expansin genes. The series of events leading to elongation of the rachis are similar to those found in deepwater rice where submergence and ethylene promote internodal growth (Kende et al. 1998). In deepwater rice, submergence also induces expression of expansin genes, and expansin proteins may be responsible for wall loosening and cell elongation (Cho and Kende 1997a,b).

The response of semiaquatic ferns and angiosperms to ethylene may represent a case of convergent evolution. Ethylene functions as the signal of submergence in both classes of plants and elicits rapid growth, probably at least in part through the wall-loosening action of expansins. However, the pathway of ethylene biosynthesis is different in ferns and angiosperms. In ferns, 1-aminocyclopropane-1-carboxylic acid (ACC) does not serve as the precursor of ethylene (Chernys and Kende 1996; Osborne et al. 1996) as it does in angiosperms (Kende 1993). Whereas the ACC-dependent ethylene biosynthetic pathway has evolved after the divergence of ferns and seed plants, the evolution of expansins predates the appearance of the ferns.

This research was supported by the U.S. Department of Energy through Grant No. DE-FG02-91ER20021. J.H.K. was the recipient of a postdoctoral fellowship from Korea Research Foundation during part of this work. We thank Drs. Alan Bennett and Jocelyn Rose (University of California, Davis, USA) for the gift of the expansin oligonucleotide primers and Dr. Tao Sang (Michigan State University, East Lansing, Mich., USA) for help in analyzing the phylogenetic relationships between expansins.

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