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Molecules and Cells

Root Hair-Specific EXPANSIN B Genes Have Been Selected for Graminaceae Root Hairs

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Cell differentiation ultimately relies on the regulation of cell type-specific genes. For a root hair cell to undergo morphogenesis, diverse cellular processes including cell-wall loosening must occur in a root hair cell-specific manner. Previously, we identified and characterized root hair-specific *cis*-elements (RHE) from the genes encoding the cell wall-loosening protein EXPANSIN A (EXPA) which functions preferentially on dicot cell walls. This study reports two root hair-specific grass *EXPB* genes that contain RHEs. These genes are thought to encode proteins that function more efficiently on grass cell walls. The proximal promoter regions of two orthologous *EXPB* genes from rice (*Oryza sativa*; *OsEXPB5*) and barley (*Hordeum vulgare*; *HvEXPB1*) included RHE motifs. These promoters could direct root hair-specific expression of green fluorescent protein (GFP) in the roots of rice and Arabidopsis (*Arabidopsis thaliana*). Promoter deletion analyses demonstrated that the RHE motifs are necessary for root hair-specific expression of these *EXPB* promoters. Phylogenetic analysis of EXP protein sequences indicated that grass EXPBs are the only orthologs to these root hair-specific EXPBs, separating dicot EXPBs to distal branches of the tree. These results suggest that RHE-containing root hair-specific *EXPB* genes have evolved for grass-specific cell wall modification during root hair morphogenesis.

INTRODUCTION

In tracheophytes, the root hair develops as a tubular protrusion from the root epidermal cell and, depending upon taxa, three types of root hair-forming cells are present (Clowes, 2000). In type 1, the root hair forms from any root epidermal cell and, thus, hair cells are distributed randomly. In type 2, the root hair is formed by the shorter cell following asymmetric cell division. In type 3, hairs can only be formed by epidermal cells in contact with two underlying cortical cells. The mechanism determining the hair/nonhair fate of type 2 appears to depend upon the unequal distribution of fate determinants between short and long cells, whereas positional cues and lateral inhibition are necessary for type 3 (Schiefelbein et al., 2009). Type 1 may represent either an ancestral or a degenerate form of other hair types. Type 2 are found in monocots, basal angiosperms, ferns and lycopodiophytes, whereas type 3 may be of more recent origin, since they are only found in eudicots and may have coevolved with this group of flowering plants (Cho, 2007; Clowes, 2000; Kim et al., 2006).

The type 3 mechanism for hair/nonhair cell fate determination has been well studied using Arabidopsis as a model system. This process requires the position-dependent activities of a receptor-like protein kinase, a WD40 protein/basic-helix-loop-helix (bHLH) transcription factor/MYB transcription factor complex and a MYB-like protein (Schiefelbein and Lee, 2006; Schiefelbein et al., 2009). In the nonhair cell position, the WD40/bHLH/MYB complex positively regulates the homeobox transcription factor GLABRA2 (GL2), which functions as a repressor of root hair morphogenetic machinery. The gene encoding ROOT HAIR DEVELOPMENT6 (*RHD6*, a bHLH), which is located downstream of *GL2*, appears to be a major transcription factor for the initiation of root hair morphogenesis (Masucci and Schiefelbein, 1996). Root hair morphogenetic processes include initiation, bulge formation and sustained tip growth, which is accompanied by diverse cellular phenomena such as polar localization of GTP-binding proteins, apoplastic and cytoplasmic pH changes, and cytoplasmic Ca^{2+} oscillations, as well as cell-wall loosening and synthesis (Grierson and Schiefelbein, 2000).

Although the mechanisms by which upstream determiners control downstream morphogenetic processes remain poorly understood, it is clear that upstream factors must express key morphogenetic genes in a root hair-specific manner. In Arabidopsis, several genes encode cell-wall proteins such as *Arabidopsis thaliana* (*At*) *PROLINE RICH PROTEIN 3* (*AtPRP3*), *LEUCINE RICH REPEAT/EXTENSIN 1* (*AtLRX1*), *LRX2*, *EAXPANSIN A7* (*AtEXPA7*) and *AtEXPA18*, which exhibit root hair-specific expression immediately prior to hair bulge formation in the trichoblast cell file (Baumberger et al., 2001; 2003; Bernhardt and Tierney, 2000; Cho and Cosgrove, 2002). Preliminary promoter analyses of the expansin genes *AtEXPA7* and *AtEXPA18* narrowed down the approximate region of root hair-specific expression (Cho and Cosgrove, 2002), and single-nucleotide-level substitution analyses of the promoters identified a 16-17 bp root hair-specific *cis*-element (RHE, Kim et al., 2006). Additional promoter analyses of *AtEXPA7* orthologs

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from other eudicots and monocots (Graminaceae), as well as other root hair-specific Arabidopsis genes, have indicated conservation of the RHE consensus within the angiosperm lineage. Cross transformations of eudicot and Graminaceae RHE-containing promoter:reporter constructs were performed between Arabidopsis and rice plants; these studies revealed that the RHE motif, and thus the cognate transcription factor, could operate universally between monocots and eudicots (Cho, 2007; Kim et al., 2006). Since the RHE consensus showed strong conservation, it was used as a screening tool for identifying additional root hair-specific genes from the genome database. Following *in silico* screening for genes containing an RHE in the proximal promoter region and further screening of root- and root hair-specific transcriptome data, a promoter:reporter assay confirmed the presence of 19 novel root hair-specific genes in Arabidopsis, many of which were cell wall- and signaling-related genes (Won et al., 2009).

Expansins are non-hydrolytic cell wall-loosening proteins that are implicated in cell enlargement, fruit softening, abscission, germination, parasitism and pollen tube penetration (for reviews see: Choi et al., 2006; Cosgrove, 2000). The expansin family can be divided into two major subfamilies, EXPA and EXPB, which show a limited (~20%) amino acid identity but similar molecular mass (~27 kDa), as well as several conserved motifs. Although members of both EXP families loosen cell walls with a similar rheology, they differ in terms of gene abundance (in different taxa), solubility and substrate specificity (Cosgrove, 1999). In eudicots such as Arabidopsis and poplar, *EXPA* is better represented than *EXPB* (26-27 *EXPA* genes in comparison to 2-6 *EXPB*s), whereas monocots such as rice contain a relatively high number of *EXPB*s (34 *EXPA*s vs. 19 *EXPB*s; Sampedro and Cosgrove, 2005). EXPA proteins function more efficiently on dicot cell walls and are less soluble than EXPBs; EXPB proteins are very soluble and show specificity for grass cell walls, regardless of their dicot or grass origin (Cho and Kende, 1997; Li et al., 1993; 2003; McQueen-Mason et al., 1992).

Although expansins have not been shown to play a role in root hair development *in vivo*, several lines of evidence support their role in root hair emergence and tip growth. *A priori* local wall acidification occurs at the hair initiation point, potentially activating expansins to loosen the cell wall at that site (Bibikova et al., 1998). In addition, expansin proteins localize to the root hair tip (Baluška et al., 2000) and two RHE-containing expansin genes (At*EXPA7* and At*EXPA18*) are expressed specifically in the root hair cell files immediately prior to root hair initiation (Cho and Cosgrove, 2002). Exogenous application of expansin proteins causes swelling (at low doses) or bursting (at high doses) of the root hair tip (Cosgrove et al., 2002). Thus, root hair-specific expansins may respond to acidic apoplastic conditions by loosening cell walls, a process that mediates hair bulge formation and hair elongation, by maintaining wall plasticity at the hair tip.

A barley *EXPB* (*HvEXPB1*) was recently identified using transcriptome analysis of a root hair-defective barley mutant (Kwasniewski and Szarejko, 2006). Although the phenotype was linked with another locus, expression of *HvEXPB1* was root specific and co-segregated with the root hair phenotype, which suggests that HvEXPB1 might function in barley root hair growth. Following our identification of RHE-like motifs in the *HvEXPB1* promoter region, the ability of this promoter to drive root hair-specific gene expression was examined. In this study, the *HvEXPB1* and *OsEXPB5* (a rice ortholog) promoters, which contain putative RHE sequences, were shown to direct root hair-specific gene expression in both Arabidopsis and rice roots. These are the first root hair-specific *EXPB* genes to be identified. In addition, we discuss the possible grass-specific roles that these EXPB proteins may play during root hair formation.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (Columbia ecotype) and rice (*Oryza sativa japonica* cv. Hwa Young) were used for transformation of *promoter:GFP* (a gene for green fluorescent protein) constructs and observations of root hair-specific GFP expression. Arabidopsis seeds were cold-treated before germination at 23°C under a 16 h light/ 8 h dark photoperiod. Arabidopsis plants were transformed using *Agrobacterium tumefaciens* strain C58C1 (Bechtold and Pelletier, 1998) and the transformants were selected on hygromycin-containing plates (10 μg ml^{-1}). GFP fluorescence was observed in 5- to 6-day-old T1 transgenic seedling roots.

Rice transformation was performed essentially as described previously (Choi et al., 2000; Jeon et al., 2008; Hiei et al., 1994). The promoter constructs were transformed into *A. tumefaciens* strain AgL1. For co-cultivation with rice embryogenic calli, *Agrobacterium* was spread onto AB medium and grown for 3 days. For generation of embryogenic calli, rice caryopses were plated onto N6 medium supplemented with 2 mg L^{-1} 2,4-dichlorophenoxy acetic acid and grown in the dark. After 3 weeks, embryogenic calli were co-cultivated with *Agrobacterium* containing vector for 3 days in the presence of 10 mg ml^{-1} acetosyringone. The embryonic calli were washed with sterile water and then selected on N6 medium containing 50 mg L^{-1} hygromycin and 150 mg L^{-1} cefotaxime. After 4 weeks of growth, calli were transferred to MS medium containing 0.5 mg L^{-1} α-naphthalene acetic acid and kinetin, and grown in a controlled-environment growth chamber under an 11 h, 27°C day/13 h, 22°C night regime. Transgenic rice plants (~15 cm in height) were cultivated further in greenhouses. T1 rice plants were examined for GFP expression.

Promoter constructs

Deletions in promoters (-*, where * is a number) were generated by polymerase chain reaction (PCR) using the primer sets listed in Table 1 and genomic DNA samples from rice and barley seedlings as templates. The amplified fragments were digested with restriction enzymes and inserted into the HindIII/XbaI sites of the *pGPTV-HYG* vector (Becker et al., 1992), in which the *uidA* gene was replaced by *GFP*, as described previously (Kim et al., 2006). All promoter constructs were confirmed by nucleotide sequencing, and genetic integrity in transgenic plants was also confirmed by PCR analysis.

GFP fluorescence and evaluation of promoter activity

Observations of GFP fluorescence in seedling roots was performed using either an epi-fluorescence stereomicroscope (MZ FLIII, Leica, Switzerland) or a confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany). To outline the cell boundaries, some root samples were stained with propidium iodide (10 μg ml^{-1}). Green fluorescence was detected by excitation at 488 nm and emission at 543 nm. Red fluorescence from propidium iodide was detected by excitation at 568 nm and emission at 617 nm. Fluorescence images from the confocal microscope were digitized with the Zeiss LSM Image Browser Version 2.80.1123. Promoter activity was eva-luated by quantifying the GFP fluorescence using the histogram function of Adobe Photoshop (Adobe Systems Inc., USA), as described in Cho and Cosgrove (2002).

Table 1. Primers used for PCR amplification of promoter deletion constructs

Subject	Name[1]	Sequence (5′ to 3′)[2]
Deletion constructs for $P_{HvEXPB1}$		
-54	HvExBHd619F	TCC CAA GCT TAT AAC ACC TAG ACC TT
	HvExBXb763R	AGT CTC TAG ACA GAA AGC CAA CGG GCT A
-193	HvExBHd478F	GCC AAA GCT TCG TCA TCA AAG GAT
	HvExBXb763R	AGT CTC TAG ACA GAA AGC CAA CGG GCT A
-492	HvExBHd182F	AGG CAA GCT TCG ATG GCC GAC GAC TT
	HvExBXb763R	AGT CTC TAG ACA GAA AGC CAA CGG GCT A
Deletion constructs for $P_{OsEXPB5}$		
-63	OsExBHd1905F	CGC TAA GCT TCC CTA TAA ATA CCA ACC AA
	OsExBXb2020R	ACC ATC TAG ATT TTC TTG TTG GGC AAT C
-344	OsExBHd1623F	GTA GAA GCT TGA CAA ACG TAC GGA TGA
	OsExBXb2020R	ACC ATC TAG ATT TTC TTG TTG GGC AAT C

[1]In the degenerate primers: Y, C + T; R, A + G [2]Hd, *Hin*dIII; Xb, *Xba*I

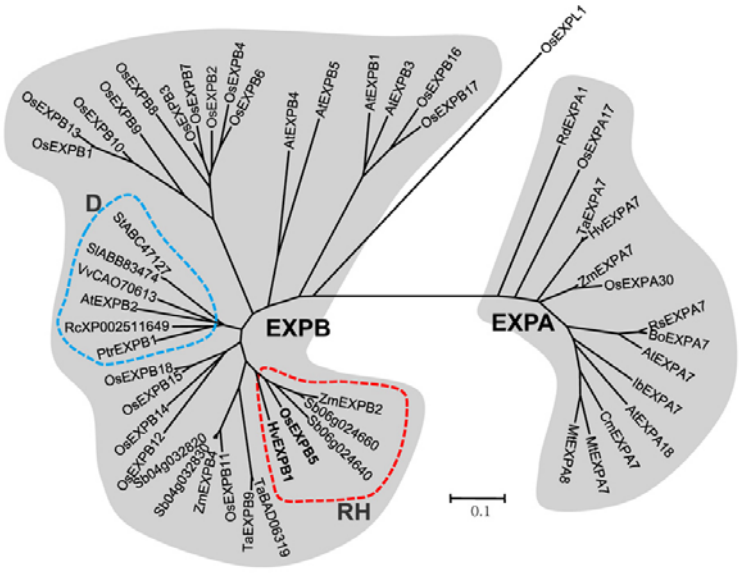


Fig. 1. Neighbor-Joining phylogenetic tree of EXPANSIN A and B protein sequences. The unrooted tree was constructed using MEGA 4.0 and demonstrates the tight evolutionary relationship between HvEXPB1 orthologs in the expansin family (the 'RH' cluster is marked by the broken line in the EXPB branch). The tree includes 32 EXPBs from Arabidopsis (At), rice (Os), sorghum (Sb), grape (Vv), maize (Zm), poplar (Ptr) and barley (Hv), as well as 13 EXPA7 paralogs and orthologs and a fern (Rd) EXPA. OsEXPL1 represents an outgroup that was added after CLUSTAL W alignment of sequences between amino acids 55 (Gly) and 181 (Ile) of AtEXPA7, and the equivalent regions of other proteins. Bootstrap values (1000 replicates) for this tree are shown in Supplemental Fig. 1. Multiple alignments for this tree are shown in Supplementary Fig. 2. Scale bar = substitutions/site. Cluster 'D' is marked by the broken line in the EXPB branch and indicates the closest dicot homologs to HvEXPB1. At, *Arabidopsis thaliana*; Bo, *Brassica oleracea*; Cm, *Cheli donium majus*; Hv, *Hordeum vulgare*; Ib, *Impatiens balsamina*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Ptr, *Populus trichocarpa*; Rd, *Regnellidium diphyllum*; Rs, *Raphanus sativus*; Sb, *Sorghum bicolor*; Ta, *Triticum aestivum*; Vv, *Vitis vinifera*; Zm, *Zea mays*.

Protein BLAST searches

BLAST searches for HvEXPB1 homologs were performed using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The entire HvEXPB1 protein sequence was used for BLAST searches against the non-redundant protein sequence database. The search gave rise to an E-value of 4e-163 for the cognate protein, i.e., HvEXPB1, and values from 1e-98 to 4e-92 for OsEXPB5, Sb06g024660, ZmEXPB2, and Sb06g024640. Only grass EXPBs were present among the top 30 sequences identified by BLAST, with EXPBs from potato and tomato representing the 31st (E = 2e-74) and 32nd (E = 3e-74) positions, respectively. Grass EXPBs were identified in the 33rd-44th places, with poplar and grape EXPBs arising in the 45th (E = 2e-68) and 46th (E = 9e-68) positions, respectively. The top five unique protein sequences, which all belonged to grass EXPBs and the top four dicot proteins, as well as the rice and Arabidopsis EXPBs, were used for the construction of the phylogenetic tree.

Sequence alignments and the phylogenetic tree

Nucleotide sequences were aligned using CLUSTAL W (DNASTAR, USA). A Neighbor-Joining phylogenetic tree was generated using MEGA version 4.0 (Tamura et al., 2007), adopting the Poisson correction distance for amino acid sequence and complete deletion for gaps. The number of bootstrap replicates was 1000.

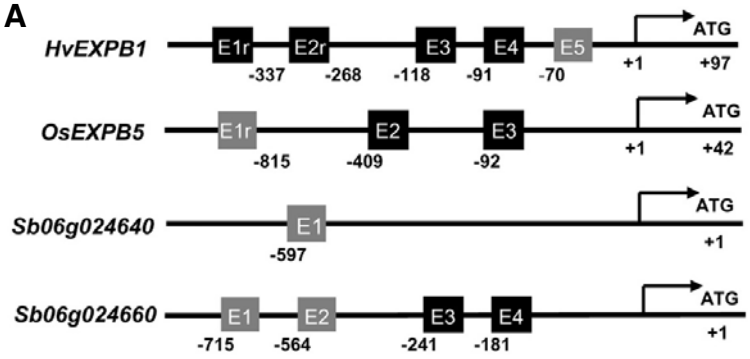
Accession number

The GenBank accession numbers for the genes analyzed in this study are AY351785 (*HvEXPB1*) and AF261273 (*OsEXPB5*).

RESULTS

HvEXPB1 orthologs are specific to the Graminaceae

To determine the phylogenetic relationship between HvEXPB1 and other expansin protein sequences, a Neighbor-Joining tree was constructed using the EXPB protein sequences from Arabidopsis and rice, as well as closely-related HvEXPB1 ho-



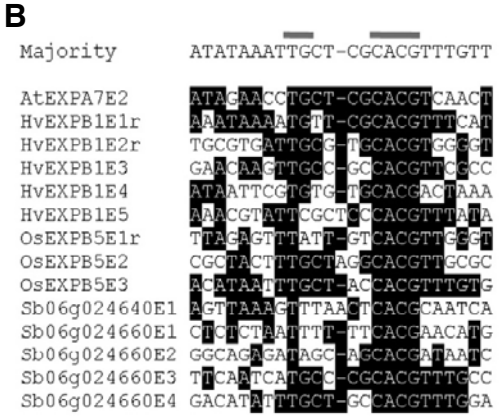


Fig. 2. RHEs in *HvEXPB1* orthologs. (A) Relative positions of RHEs in the promoter regions. Numbers below the sequence represent the nucleotide positions of the RHE motifs (black and gray boxes) relative to the putative transcription initiation site or start codon (ATG) (+1). 'r' indicates that the RHE is in the reverse direction. RHEs with major- and minor-type consensus sequences are boxed in black and gray, respectively. (B) Alignment of putative RHE sequences in *HvEXPB1* orthologs. Nucleotides corresponding to the major consensus are shaded. Highly-conserved RHE signature nucleotides are marked by gray bars above the sequence.

mologs and AtEXPA7 orthologs. Closely-related HvEXPB1 homologs were identified using a BLAST search of the non-redundant NCBI protein sequence database. Among the HvEXPB1 homologs represented were EXPBs from the Graminaceae (maize, wheat, sorghum and rice), which exhibited the highest BLAST scores, and dicots (potato, tomato, castor bean, grape, poplar and Arabidopsis). Phylogenetic analysis clearly divides the expansins into two subfamilies, EXPBs and EXPAs (Fig. 1; Supplementary Fig. 1). HvEXPB1 clusters tightly with grass EXPBs from rice (OsEXPB5), maize (ZmEXPB2), and sorghum (Sb06g024640 and Sb06g024660; the 'RH' cluster in Fig. 1), whereas OsEXPB5 shares the highest amino acid sequence identity (65.8% when whole protein sequences were aligned; Supplementary Figs. 2 and 3) with HvEXPB1. This phylogenetic analysis implies that EXPBs in the RH cluster could be HvEXPB1 orthologs. In Fig. 1, cluster 'D' includes the dicot EXPB homologs that showed the highest scores to HvEXPB1. However, this cluster forms an independent group that is separated from the 'RH' cluster by other Graminaceae EXPBs, including five OsEXPBs. Thus, the dicot EXPBs in cluster 'D' are unlikely to be HvEXPB1 orthologs. Comparison of percent amino acid identity between EXPBs also supports the suggestion that the 'RH' and 'D' clusters represent two separate EXPB groups (Supplementary Fig. 4).

Promoters in the proximal region of *HvEXPB1* orthologs contain RHE-like *cis*-elements

HvEXPB1 expression is impaired in the root hair-defective barley *rh11.a* mutant, and the gene transcript is found at least in root hair RNA preparation of the wild type plant (Kwasmiewski and Szarejko, 2006). This led us to examine whether *HvEXPB1* expression is root hair specific. Our previous study demonstrated that the proximal promoter regions of *EXPA7* orthologs contained multiple root hair-specific *cis*-elements (RHEs), which could direct gene expression in a root hair cell-specific manner (Kim et al., 2006). In this study, four gene promoters were identified from five of the *HvEXPB1* orthologs. Examination of the 1000 bp region upstream from the start codon (ATG) indicated the presence of multiple RHE-like motifs (Fig. 2A). These RHEs all included at least the minimum essential RHE consensus ['NNNN**T**NNN(N)NN**CACG**NN'; Kim et al., 2006], where N is any nucleotide and the nucleotides in bold must appear at those positions (Fig. 2B). However, the RHE consensus 'NNNN **TG**NN(N)NN**CACGT(/A)**N' has been found more often in functionally-verified RHEs (Kim et al., 2006). Thus, the latter consensus was considered to represent the 'major' elements, whereas sequences falling into the former consensus only (i.e., the remainder of the sequences) were classed as the 'minor' RHEs (Fig. 2). The *HvEXPB1* promoter contains four major- and one minor-type RHE-like elements within 350 bp of the putative transcription initiation site, and two of the elements were in the reverse direction. The 845-bp-long *OsEXPB5* promoter includes 3 RHEs, two of which belong to the major RHE group. RHE motifs were also identified in the promoters of the two sorghum orthologs; one minor-type RHE in the Sb06g 024640 promoter and two of each type of RHE element in the Sb06g024660 promoter. The presence of RHE-like elements in the proximal regions of *HvEXPB1* orthologs suggests that these promoters may operate specifically in root hair cells, in a similar manner to that demonstrated for *EXPA7* orthologs.

HvEXPB1 and *OsEXPB5* promoters operate specifically in the root hair cells of rice

To determine whether *HvEXPB1* and *OsEXPB5* promoters operate specifically in rice root hair cells, *Agrobacterium* was used to transform rice plants with reporter constructs containing

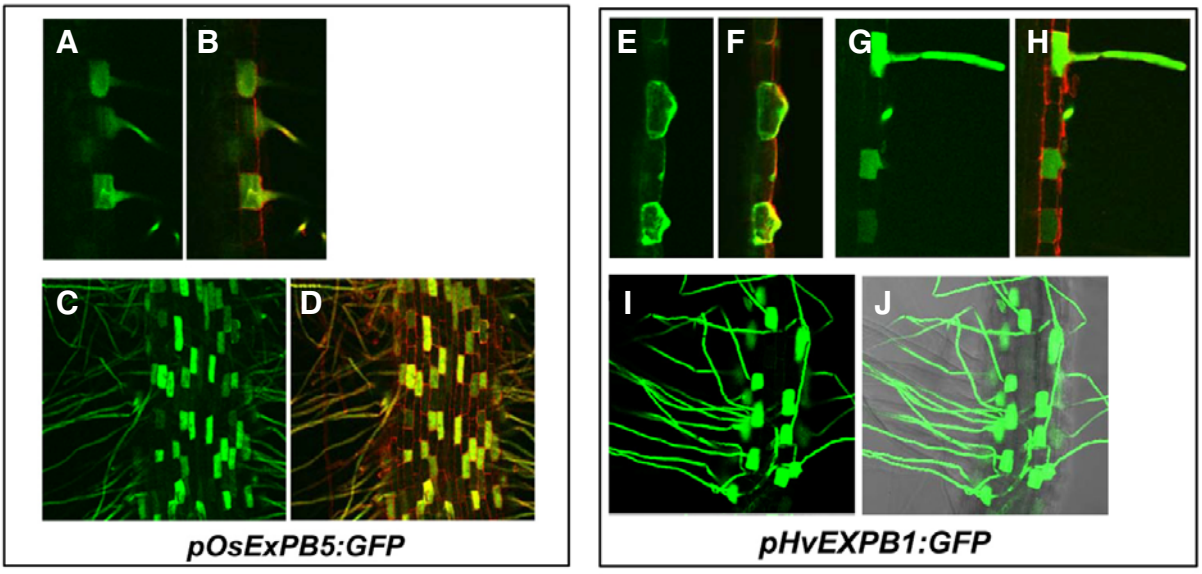


Fig. 3. RHE-containing *OsEXPB5* and *HvEXPB1* promoters function specifically in the rice root hair cell. (A-D) Confocal microscopy images showing GFP expression from the *OsEXPB5* promoter (*pOsEXPB5*; -344):*GFP* construct in a transgenic rice root. GFP signals (A and C) are merged with red signals from propidium iodide in (B) and (D), respectively. (E-J) Confocal microscopy images showing GFP expression from the *pHvEXPB5*(-492):*GFP* construct in a transgenic rice root. GFP signals (E and G) are merged with the red signals from propidium iodide in (F) and (H), respectively. The GFP signal in (I) is merged with the bright field image (J).

deletions at various positions upstream of the translational start site. The regions deleted in the *HvEXPB1 promoter:GFP* and *OsEXPB5 promoter:GFP* constructs were sequences upstream of -492 and -344 (relative to the putative translational start site +1), respectively. A confocal microscope was used to visualize GFP fluorescence in the regenerated T1 transgenic rice root. Green fluorescence was observed predominantly in root hairs and root hair-forming cells containing the *OsEXPB5* and *HvEXPB1 promoter:GFP* constructs (*pOsEXPB5:GFP* and *pHvEXPB5:GFP*, respectively; Fig. 3). GFP expression appeared to begin in a few cells prior to the emergence of a root hair bulge. In the rice root epidermis, root hair cells typically show a type 2 distribution pattern (Kim et al., 2006). Thus, after asymmetrical division, the hair is grown by the shorter cell, which results in an alternating hair distribution pattern along the long axis of the root (Clowes, 2000). In this study, the type 2 alternating pattern of GFP expression was observed in both transgenic lines examined, although the expression in some hair cell positions was missing or observed in the neighboring cell (Fig. 3D).

HvEXPB1 and *OsEXPB5* promoters direct root hair cell-specific gene expression in Arabidopsis

Previously, we isolated RHE-containing promoters from *EXPA7* orthologs in the Graminaceae and showed that they directed root hair cell-specific expression in both rice and Arabidopsis. This finding suggests the conservation of RHE and RHE-binding transcription factors between monocots and dicots. To determine whether or not the RHEs in *HvEXPB1* and *OsEXPB5* promoters can also function in Arabidopsis root hair cells, truncated *promoter:GFP* constructs were introduced into Arabidopsis plants.

For *HvEXPB1* promoter analysis, three deleted *promoter:GFP* constructs were generated, where the -492 deletion includes 5 RHEs, the -193 deletion includes 3 RHEs, and the -54 deletion exclude all RHEs (Fig. 4A). Then, GFP fluorescence levels from multiple independent transgenic lines (10-15) for each promoter-deletion construct were quantitatively estimated. -492 and -193 promoter deletions showed a similar level of GFP fluorescence from the transgenic roots where the fluorescence was specific to the root hair cell (Figs. 4B-4D). However, the -54 deletion, which includes no RHE, almost completely eliminated the GFP expression in the root.

For the *OsEXPB5* promoter, 2 deleted *promoter:GFP* constructs were introduced into Arabidopsis. The -344 deletion includes only one RHE (E3) and the -63 deletion has no RHE (Fig. 5A). The -344 deletion showed a root hair-specific expression of GFP in the Arabidopsis root (Fig. 5D) which was however much weaker than the -492 deletion of the *HvEXPB1* promoter probably due to the fewer number of RHE in the -344 deletion *OsEXPB5* promoter. When E3 was excluded by the -63 deletion, the *OsEXPB5* promoter completely lost GFP expression in the root (Figs. 5B and 5C). The promoter analyses of RHE-containing two 'RH' group *EXPB* promoters suggest that RHEs on those promoters practically direct root hair-specific gene expression in both monocot (grass) and dicot.

DISCUSSION

In this study, we identified several grass *EXPB* genes that carry putative RHE motifs in the proximal promoter regions. In addition, two of those gene promoters were shown to direct gene expression in a root hair cell-specific manner in both Arabidopsis and rice plants. Phylogenetic analysis suggests that these root hair-specific EXPBs may be specific to the Graminaceae family and not present in dicots (Fig. 1). The grass EXPB cluster 'RH' does not include any dicot members and the closest dicot group (cluster 'D') is separated from the 'RH' cluster by other grass EXPB clusters. It is unlikely that members of the dicot 'D' and monocot 'RH' clusters are orthologous and, thus, the 'RH' cluster could be unique to the grass family. Spatial expression patterns of 'D' cluster members support the suggestion that these groups are not orthologous. For example, mRNA

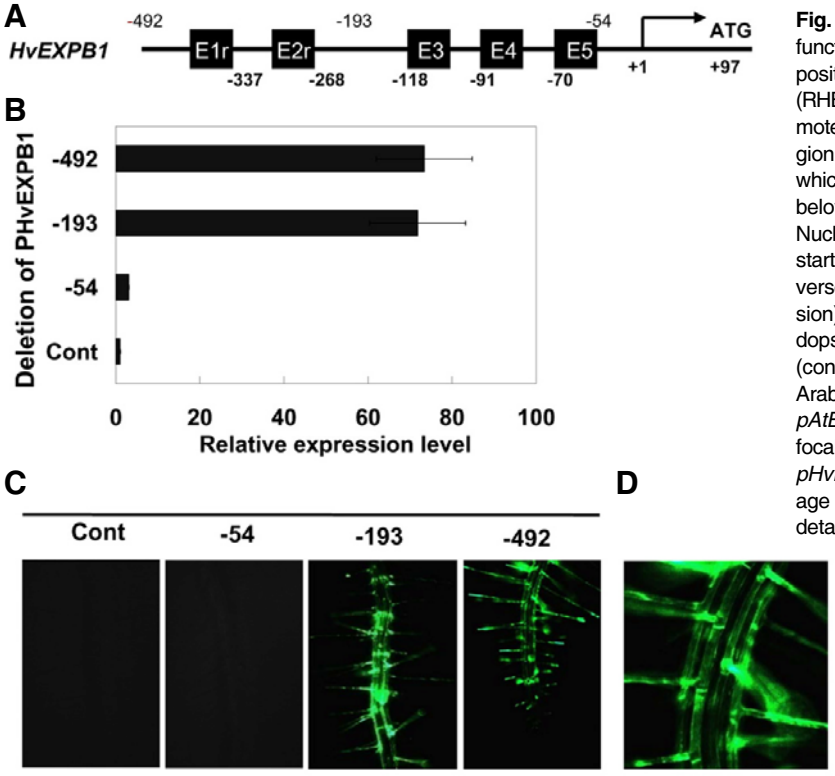


Fig. 4. The RHE-containing *HvEXPB1* promoter functions in Arabidopsis root hair cells. (A) Relative positions of putative root hair-specific *cis*-elements (RHE; E) in the *HvEXPB1* promoter (*pHvEXPB1*). Promoter fragments were fused to the *GFP* coding region. Numbers above the line indicate the positions at which upstream sequences were deleted. Numbers below the sequence indicate the positions of RHEs. Nucleotide positions are relative to the translational start site (+1). 'r' indicates that the RHE is in the reverse direction. (B) Relative activities (GFP expression) of the truncated *HvEXPB1* promoters in Arabidopsis root. Bar indicates standard errors: n = 10 (control; Cont), 15 (-54), 15 (-193), and 11 (-492). Arabidopsis transformants containing the construct *pAtEXPA7:GUS* were used as the control. (C) Confocal microscopy images of roots harboring truncated *pHvEXPB1:GFP* constructs. (D) The magnified image of *pHvEXPB1:GFP* (-492) in (C) showing the detailed root hair cell-specific expression pattern.

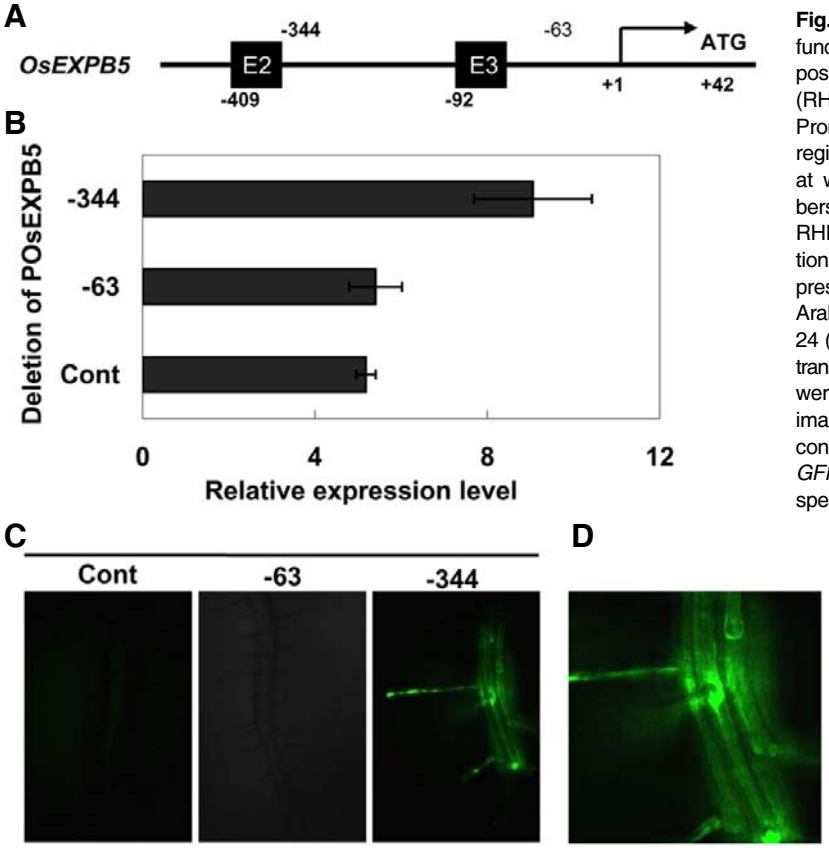


Fig. 5. The RHE-containing *OsEXPB5* promoter is functional in Arabidopsis root hair cells. (A) Relative positions of putative root hair-specific *cis*-elements (RHE; E) in the *OsEXPB5* promoter (*pOsEXPB5*). Promoter fragments were fused to the *GFP* coding region. Numbers above the line indicate the positions at which upstream sequences were deleted. Numbers below the sequence indicate the positions of RHEs. Nucleotide positions are relative to the translational start site (+1). (B) Relative activities (GFP expression) of the truncated *OsEXPB5* promoters in Arabidopsis root. Bar indicates standard errors: n = 24 (control; Cont), 10 (-63), and 24 (-344). Arabidopsis transformants containing the construct *pAtEXPA7: GUS* were used as the control. (C) Confocal microscopy images of roots harboring truncated *pHvEXPB1: GFP* constructs. (D) The magnified image of *pHvEXPB1: GFP* (-344) in (C) showing the detailed root hair cell-specific expression pattern.

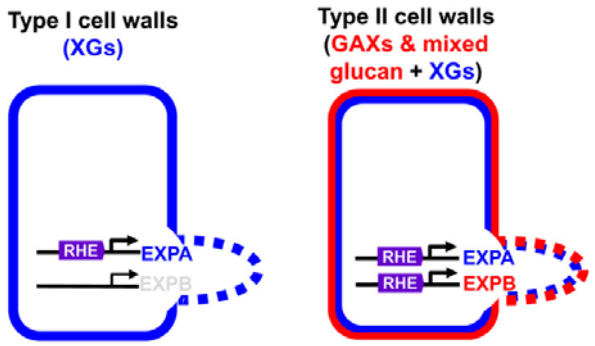


Fig. 6. A model illustrating the selection of RHE-containing root hair-specific *EXPB* genes in the grass family. Dicotyledonous root hair cell walls may only require EXPA for the loosening of the cell wall and, thus, for root hair formation and elongation. On the other hand, root hair growth in grass may require the activity of root hair-specific EXPB in addition to EXPA due to additional cell wall components specific to grasses.

expression of *AtEXPB2* (a 'D' cluster member) is highly specific to the endosperm, with no detectable expression in root tissues (https://www.genevestigator.com/), implying that this 'D' cluster member is not root hair-specific by the RHE-containing promoter. These findings prompted the question "why would RHE-containing EXPBs ('RH' cluster members) occur specifically in grasses?"

Dicots and grasses have different cell wall compositions, and EXPA and EXPB exhibit different cell wall specificities. Thus, we propose that grass root hair-specific EXPBs may be required specifically for grass root hair morphogenesis. Primary cell walls consist of cellulose microfibrils, hemicelluloses, pectins and minor structural proteins. Depending upon the hemicellulose composition, these primary cell walls are classified into two types (Carpita and Gibeaut, 1993). Xyloglucans (XGs) are the primary hemicelluloses of type 1 cell walls, which are found in gymnosperms, dicots and non-graminaceous monocots. Type 2 cell walls occur in the Graminaceae and closely-related monocot families. The major hemicelluloses are glucuronoarabinoxylans (GAXs) and (1,3)(1,4)-β-mixed glucans, in addition to some XGs. Rheological studies of expansin proteins show that EXPA and EXPB exhibit preferential wall-loosening of type 1 and type 2 cell walls, respectively (Cho and Kende, 1997; Li et al., 1993; 2003; McQueen-Mason et al., 1992). Considering the necessity for cell-wall reassembly during root hair growth (Galway, 2006), it is conceivable that substrate-specific EXPs should be recruited for modification of cell walls with different compositions. For example, EXPA may modify XG-linked cell wall structures in both type 1 and 2 cell walls, whereas EXPB may only work on GAX- or mixed glucan-linked wall organization in type 2 cell walls (Fig. 6).

Whether or not the dicotyledonous Arabidopsis root hair cell wall includes type 2 cell wall components remains unknown; however, the absence of root hair-specific EXPBs suggests that such components may not be the major factors in Arabidopsis root hair growth. Instead, growth may rely on root hair-specific EXPA-dependent modification of type 1 cell wall components. In contrast to Arabidopsis, genes encoding EXPAs and EXPBs are expressed in the root hair cells of monocotyledonous rice and barley. Moreover, the promoters of these genes carry the RHE motifs required for root hair-specific expression. This finding suggests that both type 1 and type 2 cell wall components are involved in wall modification and growth of root hairs in these monocots. This study demonstrated root hair-specific expression of RHE-containing *EXPB* genes in grass family members. We hypothesize that the RHEs were recruited to the *EXPB* gene promoters of Graminaceae plants for root hair morphogenesis, since this family have a cell wall composition that is different from other angiosperms.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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