Functional Conservation of a Root Hair Cell-Specific cis-Element in Angiosperms with Different Root Hair Distribution Patterns

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Vascular plants develop distinctive root hair distribution patterns in the root epidermis, depending on the taxon. The three patterns, random (Type 1), asymmetrical cell division (Type 2), and positionally cued (Type 3), are controlled by different upstream fate-determining factors that mediate expression of root hair cell-specific genes for hair morphogenesis. Here, we address whether these root hair genes possess a common transcriptional regulatory module (cis-element) determining cell-type specificity despite differences in the final root hair pattern. We identified Arabidopsis thaliana expansinA7 (At EXPA7) orthologous (and paralogous) genes from diverse angiosperm species with different hair distribution patterns. The promoters of these genes contain conserved root hair-specific cis-elements (RHEs) that were functionally verified in the Type-3 Arabidopsis root. The promoter of At EXPA7 (Type-3 pattern) also showed hair cell-specific expression in the Type 2 rice (Oryza sativa) root. Root hair-specific genes other than EXPAs also carry functionally homologous RHEs in their promoters. The RHE core consensus was established by a multiple alignment of functionally characterized RHEs from different species and by high-resolution analysis of At EXPA7 RHE1. Our results suggest that this regulatory module of root hair-specific genes has been conserved across angiosperms despite the divergence of upstream fate-determining machinery.

INTRODUCTION

Root hairs are tubular outgrowths of certain root epidermal cells. Because they constitute the majority of the root surface area, root hairs are thought to contribute to the primary root functions of absorption, anchorage, and soil microbe interactions (Grierson and Schiefelbein, 2002). The fate determination and distribution pattern of hair and non-hair cells has been intensively studied in Arabidopsis. Root hair cells are located over two underlying cortical cells (the H cell position), whereas non-hair cells are positioned over a single cortical cell (the N cell position) (Dolan et al., 1993; Galway et al., 1994). This position-dependent hair cell specification results in rows of hair cells along the longitudinal root axis and has been found in Brassicaceae and other eudicot families (Cormack, 1947; Clowes, 2000; Dolan and Costa, 2001; Figure 1). This striped pattern (Type 3) is one of three types of hair cell distribution patterns (Leavitt, 1904; Cormack, 1937, 1947; Cutter and Feldmann, 1970; Cutter and Hung, 1972; Dolan, 1996; Clowes, 2000; Dolan and Costa, 2001; Figure 1A). In the Type 1 pattern, root hair cells can differentiate from any epidermal cell. Most dicots, many monocots, and most ferns show this pattern. In the Type 2 pattern, the root epidermis consists of two sizes of cells, long and short (along the root longitudinal axis), and only the short cells differentiate into hair cells. Type 2 patterning occurs in lower ferns, monocots, and basal angiosperm families (Figure 1B). In addition to these basic root hair patterns, root hairs originating from the cortex (Pinkerton, 1936) and a Type 3 variant, in which long hairs arise from the H position and short hairs from the N position (Tsi et al., 2003), have been described.

Differences in hair cell distribution patterns indicate that different cell fate determination systems operate in the different pattern types. In Arabidopsis thaliana, when a hair cell was laser ablated and the H position was then occupied by a neighboring non-hair cell, the non-hair cell could differentiate a root hair (Berger et al., 1998), demonstrating that a positional cue determines the Type 3 cell fate. The transcriptional regulatory network underpinning Type 3 fate determination has been characterized in Arabidopsis. Briefly, the expression of GLABRA2 (GL2; a homeodomain transcription factor) is modulated by the MYB/bHLH/WD40 complex (for recent review, see Schiefelbein and Lee, 2006) or by chromatin remodeling (Costa and Shaw, 2006) in a position-dependent manner. GL2 suppresses genes required for hair differentiation in the N position (Ohashi et al., 2003). In contrast with position-dependent Type 3 patterning, Type 2 patterns depend on asymmetrical cell division. Hair-forming cells (trichoblasts) in Type 2 roots derive from a late, unequal transverse cell division in the epidermal stem cell (Cormack, 1944; Clowes, 2000). The shorter of the two daughter cells becomes a trichoblast, which is noticeably rich in cytoplasm and deeply stained by dyes. Unequal cell division may asymmetrically partition cell fate determinants as observed in such diverse organisms as bacteria, yeast, worm, insects, and plants (Scheres and Benfey, 1999; Betschinger and Knoblich, 2004;
Haecker et al., 2004). The Type 1 pattern may be either an ancestral pattern or a degenerate pattern derived from Type 2 or Type 3 (Dolan and Costa, 2001).

Several root hair cell-specific genes have been identified in *Arabidopsis*: At *PRP3* (encoding a Pro-rich protein; Bernhardt and Tierney, 2000), At *LRX1* and At *LRX2* (encoding Leu-rich extensins; Baumberger et al., 2001, 2003), and At *EXPA7* and At *EXPA18* (encoding expansins; Cho and Cosgrove, 2002). These genes show almost identical spatio-temporal expression patterns in differentiating root hair cells and are thought to induce alterations in cell wall assembly during hair morphogenesis. These downstream genes are controlled by upstream fate determinants and other cues in the Type 3 pattern in *Arabidopsis* (Bernhardt and Tierney, 2000; Cho and Cosgrove, 2002). Root hairs occur in all groups of vascular plants, and common cellular and molecular behaviors are observed across different species during hair development (Peterson and Farquhar, 1996; Ridge and Emons, 2000), implying that root hair–specific genes are functionally conserved in plants. Here, we asked whether root hair cell-specific genes in different hair distribution types are modulated by independent or convergent regulatory pathways (that is, by different common cis- and trans-elements). To address this issue, we have specifically investigated the following questions: (1) Are orthologs of root hair–specific genes present in species having different hair distribution types? (2) Are those orthologous genes also root hair specific? (3) If they are root hair specific, do their promoters include a common or a type-specific cis-element for root hair specificity? (4) If those genes use a common cis-element for root hair specificity, is this cis-element functionally compatible between root hair patterning types?

In addition, this study was also designed to demonstrate experimentally the conservation of a cell type–specific cis-element over angiosperm evolution. A plethora of genomic data have enabled computational predictions of conserved regulatory noncoding elements from orthologous and paralogous genes (Gumucio et al., 1992; Jareborg et al., 1999; Wasserman et al., 2000; Kaplinsky et al., 2002; Guo and Moose, 2003; Hong et al., 2003; Inada et al., 2003; Berman et al., 2004; Haberer et al., 2004). However, the computational method can be effective only when combined with experimental analysis, which has rarely been conducted in plants (Kellogg, 2004).

In this study, we have identified genes orthologous and paralogous to *At EXPA7* from angiosperm species with different hair distribution patterns, functionally defined a root hair–specific cis-element, and demonstrated the ability of this common cis-element to drive root hair cell-specific expression of genes in roots with different distribution patterns. The results indicate that the gene regulatory module (cis- and trans-elements) for root hair specificity has been conserved despite the divergence of upstream fate-determining machinery in angiosperms.

**RESULTS**

**High-Resolution in Vivo Analyses of the Root Hair–Specific cis-Element in Representative Promoters**

Previously defined promoter regions conferring root hair specificity in *At EXPA7* and *At EXPA18* (Cho and Cosgrove, 2002) were further dissected to determine the structure of the core cis-element (RHE for root hair element). The presence of additional enhancing elements and the function of multiple RHEs were investigated in the promoters of the two paralogous root

**Figure 1.** Three Root Hair Patterning Types and Their Phylogenetic Relationship in Tracheophytes.

(A) Schematic diagram of the three root hair patterning types. Densely stainable trichoblast cells are colored in Type 2 and Type 3. Small circles indicate root hairs.

(B) The phylogenetic relationship of root hair distribution types. The taxon (order) including Type 2 is in red and Type 3 in blue. The phylogenetic tree of tracheophytes was modified from Judd et al. (1999). Root hair distribution types of the taxa were referred to the survey by Clowes (2000).
Figure 2. Analyses of \( P_{\text{AtEXPA7}} \), \( P_{\text{AtEXPA18}} \), and Their Root Hair Cell-Specific cis-Elements (RHEs) in Arabidopsis.

(A) Nucleotide sequence of the \( P_{\text{AtEXPA7}} \) RHE1 (E1 in Figure S) region and substituted nucleotides (lowercase) in 4- and 1-bp mutations. Numbers above the wild-type sequence indicate relative distance from the transcription initiation site. The TATA box is shown in bold.

(B) A 10-bp deletion analysis of the proximal upstream region of RHE1. Inset: fluorescence image of the -134:GFP root.

(C) GOF analysis of RHEs of \( P_{\text{AtEXPA7}} \). Fluorescence images of this GOF analysis are shown in Supplemental Figure 1 online.
hair–specific expansin genes. Fine base-substitution analyses of the promoters of At EXPA7 (P_{AXP7}) and At EXPA18 (P_{AXP18}) at 1- to 10-bp resolution (Figures 2A and 2F) revealed that a similar sequence motif was essential for RHE function in both promoters, regions M1 to M5 in P_{AXP7} and E18M5 to E18M7 in P_{AXP18} (Figures 2D, 2E, and 2G). A 4-bp substitution analysis of the P_{AXP7} RHE1 region sharply divided the sequence into two functional parts centering around M3; mutation of M3 did not significantly affect the RHE function (Figure 2D). A 1-bp substitution analysis identified individual nucleotides required for the function of P_{AXP7} RHE1 (Figure 2E) and revealed the strictly conserved CACG motif in the RHE. Multiple RHEs sharing this common sequence function additively in promoter activity in both genes (Figures 2C and 2H). Regions -110/-90 and -241/-196 (relative to the transcription start site) of P_{AXP7} and P_{AXP18}, respectively, may include some elements that enhance RHE activity (Figures 2B, 2G, and 2H). The full description of promoter and RHE analyses of At EXPA7 and At EXPA18 is provided in Supplemental Text 1 online.

**EXPA7 Orthologs from Diverse Angiosperms with Different Root Hair Patterns**

Our next question was whether genes orthologous to At EXPA7 (or At EXPA18) exist in species having different root hair distribution types. A BLAST search using At EXPA7 to query the databases identified closely related sequences from cabbage (Brassica oleracea) (Type 3; Bo EXPA7), rice (Oryza sativa) (Type 2; Os EXPA17 and Os EXPA30), and Medicago truncatula (Type 1; Mt EXPA7 and Mt EXPA8). The root hair types were determined by direct observation. EXPA7 orthologs from other species were obtained by PCR using primers from conserved regions of the above sequences (see Supplemental Table 1 online). Coding sequences were cloned from barley (Hordeum vulgare) (Type 1; Hv EXPA7), wheat (Triticum aestivum) (Type 2; Ta EXPA7), maize (Zea mays) (Type 1; Zm EXPA7), radish (Raphanus sativus) (Type 3; Rs EXPA7), celadine (Chelidonium majus) (Type 1; Cm EXPA7), and balsam (Impatiens balsamina) (Type 3; Ib EXPA7). The root hair types are as described by Clohes (2000).

The amino acid sequence between 55 (Gly) and 181 (Ile) of At EXPA7 and the equivalent regions of each expansin protein sequence were aligned by CLUSTAL W. A neighbor-joining phylogenetic tree was generated from this alignment (Figure 3). The tree shows that these EXPA7-related expansins (EXPA7s) are a monophyletic group. Of 1000 bootstrap replicates, 67% support the establishment of the EXPA7 clade (see Supplemental Figure 3 online). Gramineaceae EXPA7s, except for Os EXPA17, form a tight subclade distinct from the eudicot EXPA7s. Os

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**Figure 2.** (continued).

(D) A 4-bp substitution analysis (M1 to M8 as shown in [A]) of the RHE1 region.

(E) A 1-bp substitution analysis (1 to 16 as shown in [A]) of the RHE1 core.

(F) Nucleotide sequence of the P_{AXP18} RHE1 (E1 in Figure 5) region and substituted nucleotides (lowercase) in promoter mutations (E18M1 to E18M8). Numbers above the wild-type sequence indicate the relative distance from the transcription initiation site.

(G) Substitution analysis of the region surrounding RHE1.

(H) GOF analysis of RHE1. Fluorescence images of this GOF analysis are shown in Supplemental Figure 2 online.

*Cont* in (B) to (E), (G), and (H) shows the promoter activity of P_{AXP7} GFP transformants. Error bars indicate SE; number of transgenics is indicated in Methods.
Figure 4. RHEs Are Functionally Conserved in EXPA7 Orthologs from Species with Different Root Hair Pattern Types and in Arabidopsis Root Hair Genes Other Than Expansins.

Deleted (-), substituted (-mE), or GOF (−/−) promoter fragments of EXPA7 orthologs from rice ([A] and [B]), wheat (C), maize (D), M. truncatula (E), balsam (F), and cabbage (G) were fused to GFP and analyzed in Type 3 Arabidopsis roots. The promoter fragments of other Arabidopsis root hair genes (At LRX1 [H] and At PRP3 [I]) also were fused to GFP and analyzed in Arabidopsis. Insets are fluorescence images of the Arabidopsis root containing the designated promoter constructs. Error bars indicate SE; number of transgenics is indicated in Methods.
At EXPA7 (3) & At EXPA18 (3) & Bo EXPA7 (3) & Mt EXPA7 (1) & Ib EXPA7 (3) & Os EXPA30 (2) & Ta EXPA7 (2) & Zm EXPA7 (1) & Os EXPA17 (2) & At LRX1 (3) & Bo LRX1 (3) & At LRX2 (3) & At PRP3 (3) & Bo PRP3 (3)

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**EXPA17** is located at the base of the EXPA7 clade, indicating its early divergence from the clade.

**RHEs of EXPA7 Orthologs Are Functionally Conserved**

Promoter sequences of EXPA7 orthologs were obtained either from databases or by PCR-based cloning. The MEME algorithm (http://meme.sdsc.edu/meme/meme.html) was used to locate putative RHE regions in the promoters. Deletion, base substitution, and gain-of-function (GOF) constructs of the orthologous promoters fused to the green fluorescent protein (GFP) gene were introduced into Arabidopsis plants (Type 3) by Agrobacterium tumefaciens–mediated transformation, and root hair–specific promoter activity was analyzed in vivo (Figure 4).

All the EXPA7 orthologous promoters included multiple RHEs in the proximal promoter region and were similar to RHEs from *P* _AEXPA7_ and *P* _AEXPA8_, including the hallmark CACG motif (Figure 5). Despite the different root hair types from which the promoters originated, the orthologous promoters showed root hair cell-specific expression of GFP in the Type 3 Arabidopsis root (Figures 4A to 4G). Deletion or base substitution of RHEs greatly decreased the activity of the orthologous promoters. GOF analyses of representative orthologous promoters demonstrated that the RHE fragments were sufficient to drive root hair–specific gene expression (Figures 4A, 4E, and 4F; see Supplemental Figure 5 online). These results collectively suggest that RHE-mediated regulation of root hair–specific genes has been conserved, even though the upstream fate-determining components have diverged.

**Figure 5.** RHEs from EXPA7 Orthologs and Several Root Hair–Specific Genes.

Numbers above each sequence indicate the deletion constructs used for promoter analyses. Numbers below the sequence are starting nucleotide positions of the RHE core (black box), and the larger numbers above the RHE core indicate the spacer length (two or three nucleotides). The gray boxes indicate the conserved RHE extensions in Graminaceae *P* _EXPA5_. Nucleotide positions are numbered relative to the putative transcription initiation site or start codon (ATG). The root hair cell distribution type is designated in parentheses after the gene name. An “r” after the RHE number indicates a reverse orientation of the RHE, and a question mark indicates that the RHE has not been experimentally verified.

**Figure 6.** The RHE-Containing Promoters from Type 2 and Type 3 Pattern Genes Are Also Root Hair Specific in the Type 2 Rice Root.

Confocal microscopy images of transgenic rice roots harboring *P* _OEXPA7(-318):GFP* [A] to [C] and *P* _OEXPA8(-386):GFP* [D].

(A) Green fluorescence is shown in differentiating hair cells (trichoblasts). Red fluorescence shows the propidium iodide–stained cell wall.

(B) Green fluorescence from mature root hairs.

(C) Reconstituted confocal cross-sectional view of the rice root expressing *P* _OEXPA8:GFP*. The GFP-expressing hair cells are contacting either a single or two underlying cortical cells (asterisks).

(D) Arabidopsis EXPA7 promoter is also active in the rice root hair cells but follows the Type 2 pattern. Green fluorescence is found only in the root hair cells. Bars = 20 μm.
RHEs of Other Arabidopsis Root Hair Genes Are Functionally Conserved

Three additional root hair–specific genes have been reported in Arabidopsis, At LRX1, At LRX2, and At PRP3 (Bernhardt and Tieme, 2000; Baumberger et al., 2001, 2003). These genes are expressed only in root hair cells; genes that are expressed both in hair cells and other cell types of the root are not included in this designation. These root hair–specific genes show the same expression pattern as At EXPA7 and At EXPA18, and we therefore analyzed the root hair–specific cis-element of these genes. Promoters of these additional root hair genes contained RHE(s) in the proximal promoter region: two reverse RHEs in P_{ALRX1}, one reverse RHE in P_{ALRX2}, and one reverse RHE in P_{APRP3} (Figure 5). Deletion and base substitution analyses of RHEs in P_{ALRX1} and P_{APRP3} demonstrated that the RHE motif is required for the root hair specificity of these promoters (Figures 4H and 4I). These data indicate that other root hair–specific genes in Arabidopsis exploit the same cis-element to drive cell type–specific expression.

A full description of the promoter analyses of EXPA7 orthologs and other Arabidopsis root hair genes is presented in Supplemental Text 2 online.

P_{OSEXPA30} and P_{AEXPA7} Are Also Root Hair Specific in the Type 2 Rice Root

To determine whether the rice EXPA7 ortholog promoter is operative in rice root hair cells and whether the Arabidopsis (Type 3) EXPA7 promoter retains root hair cell specificity in Type 2 rice roots, P_{OSEXPA30(-318)}:GFP and P_{AEXPA7(-386)}:GFP were introduced into rice embryonic calli using Agrobacterium-mediated transformation, and the transgenic seedling roots were examined (Figure 6). P_{OSEXPA30} driven GFP expression was detectable specifically in the hair cells and in a few cells before the hair bulge started to emerge (Figure 6A). The expression pattern was consistent with the Type 2 hair cell distribution, where long and short epidermal cells alternate along the long axis of the root, and the shorter cells become hair cells. Moreover, the rice Type 2 hair cells were located on either a single or two underlying cortical cells (Figure 6C), clearly distinct from the position-dependent Type 3 pattern. This Type 2 pattern in rice appears to be slightly disturbed in the upper hair maturation zone, where consecutive hair cells were occasionally observed (our unpublished data). The P_{OSEXPA30} activity was limited to root hair cells, at least in seedlings. This result indicates that Os EXPA30 is a functional equivalent to At EXPA7 in rice. The At EXPA7 promoter also was root hair cell specific in rice (Figure 6D). The results from transgenic rice plants suggest that the cis-element for root hair specificity is compatible between Type 2 and Type 3 species despite differences in upstream fate-determining mechanisms between the two types.

The lrx1 Mutant Could Be Rescued by P_{AEXPA7}–Driven Expression of At LRX1

We tested whether the P_{AEXPA7} promoter is functionally compatible with the promoters of other root hair genes. The Arabidopsis lrx1 mutant has frequently aborted, swollen, or branched root hairs (Baumberger et al., 2001). The P_{AEXPA7(-134)}:LRX1 construct was introduced into lrx1 mutant plants. The P_{AEXPA7}–driven expression of wild-type At LRX1 successfully rescued the root hair defects in lrx1 plants (Figure 7). This result suggests that RHEs are functionally compatible between different root hair genes.

Construction of the RHE Consensus

The consensus RHE was established both by a multiple alignment of RHEs from EXPA7 genes and other root hair genes and by functional analyses of P_{AEXPA7} RHE1 (At EXPA7E1). The RHE core consists of 16 to 17 bp, which is divided into two conserved parts joined by a short linker region that includes one additional nucleotide in some RHEs. Conservation of the 16- or 17-bp RHE core, relative to flanking regions, is such that fewer than three of the four nucleotide species or predominant nucleotide species occur in a given position, with the exception of the linker region (Figure 8A). The two conserved parts of the RHE core appear to form an incomplete palindrome of each other (Figure 8C). The sequence conservation is stronger in the right part (RP) than in the left part (LP). RP is typically represented by the signature G/T(CAGGT)/A, in which the CACG motif is strictly conserved in all the functionally verified RHEs (Figure 8A). Any single base substitution in this CACG motif abrogated the promoter activity almost completely (Figure 2E). In LP, only the TG motif is highly conserved, and some partial conservations are found in different RHE subgroups. T in the TG motif is conserved in all RHEs, and its replacement was fatal to promoter activity (Figure 2E). Although the G of this TG is conserved in most RHEs, it can be functionally replaced with A or C, as shown in At EXPA7E3 and Mt EXPA7E3r, respectively (Figure 8A). The promoters with these minor variant RHEs were still root hair specific (Figures 2C, -386/-200, and 4E, -78; see Supplemental Figure 1 online, -386/-200). A point mutation at this position (no. 8 in 1-bp mutations; Figure 2A) in At EXPA7E1 affected the promoter activity much less than did a mutation in the T position (no. 7) (Figure 2E).

The linker between LP and RP varies in length, containing either two or three nucleotides, and we refer to these linkers as two-nucleotide or three-nucleotide spacers (Figure 8C). To test the influence of the spacers on RHE function, we made a 1-bp deletion from the three-nucleotide spacer in At EXPA7E1 and a

![Figure 7](image-url). The At EXPA7 Promoter Can Functionally Substitute for the At LRX1 Promoter.

(A) Aberrant root hair phenotype of the Arabidopsis lrx1 mutant. (B) Introduction of P_{AEXPA7(-134)}:LRX1 into the lrx1 mutant rescues the root hair phenotype. Bar = 100 μm for (A) and (B).
1-bp insertion into the two-nucleotide spacer in AtEXPA18E1. The 1-bp insertion or deletion was placed in the nonconserved RHE linker region of the -241 and -134 deletion promoters of $P_{AtEXPA18}$ and $P_{AtEXPA7}$, respectively (Figures 9A and 9B). A base pair addition in the At EXPB18E1 linker greatly decreased the promoter activity, and a 1-bp deletion in the At EXPB7E1 linker caused a decrease of ~60% in promoter activity. These data indicate that the linker length is critical to RHE function, even though nucleotide substitution of the linker is not.

**Orientation of RHE**

In many enhancer elements, a forward or reverse orientation is equally functional (Lewin, 2004). Twelve of 29 putative or functionally identified RHEs are in the reverse direction (Figure 5). Although both forward and reverse RHEs are found together in $P_{AtEXPA7}$, the same direction for multiple RHEs in a given promoter is noticeably preferred.

**DISCUSSION**

Studies of gene regulation at the transcriptional level require the characterization of cis-regulatory elements, trans-acting factors, and their interactions in regulating transcription. The prerequisite to such studies is the identification of functional cis-regulatory sequences that are located in noncoding DNA regions at varying distances from the transcription start site. A cis-element provides the ultimate information controlling where, when, and at what level a gene is expressed. Although there are many computational predictions and a few experimental tests of whole promoters, we currently have a paucity of examples of well-characterized cis-regulatory elements from either plants or...
animals, particularly in elements characterized in an evolutionary context (Berman et al., 2004; Kellogg, 2004).

In this study, we experimentally dissected root hair–specific promoters from diverse angiosperm species, characterized a root hair–specific cis-element (RHE) in a high-resolution in vivo analysis, and demonstrated the structural and functional conservation of RHEs across angiosperms. In addition to phylogenetic conservation of RHEs, the ontogenetic conservation of RHEs, despite diverse upstream regulatory manners among different angiosperm taxa, is intriguing.

**RHEs Can Be Divided into Two Syntactic Subgroups**

The RHE consensus consists of two parts linked by a spacer: RP and LP. While the RP of all RHEs includes the hallmark motif CACG, the LP appears less conserved. However, when RHEs were separately grouped into two- and three-nucleotide spacer RHEs, different conservation patterns were observed between the two subgroups (Figure 9C). In two-spacer RHEs, the dominant nucleotide for positions 3 and 7 is C, and position 3, in particular, is highly conserved, whereas a C in these positions occurs very rarely in three-spacer RHEs. By contrast, in three-spacer RHEs, position 4 is dominated by G, which occurs only in E2 of Gramineaeae and balsam EXPAt orthologs in two-spacer RHEs. LP and RP are in a weak inverted repeat (palindromic) relationship. Because of the biased conservation in LP between two- and three-spacer RHE subgroups, the palindrome appears to be better established in the two-spacer RHE subgroup. More than four out of six positions for a single repeat unit are palindromic in 60% of two-spacer RHEs, while fewer than three positions are palindromic in most three-spacer RHEs (Figure 9C).

Both two- and three-spacer RHEs occur together in the same promoter in many cases (Figure 5), and they are equally functional for root hair specificity as shown by diverse analyses (Figures 2 and 4). However, a single nucleotide insertion into the two-spacer RHE or deletion from the three-spacer RHE resulted in a large reduction in promoter activity (Figures 9A and 9B). These data suggest that the flexibility in linker spacing between LP and RP needs to be understood in the syntactic context of each RHE subgroup. Different linker spacings between two repeated cis-elements may allow transcription factors to dimerize in different combinations (Latchman, 2004). Subtle syntactic differences between two RHE subgroups might be distinguished by structurally and functionally related members of a transcription factor family (hereafter called RHF for root hair factor). The RHEs of At EXPAt, At EXPAt18, and At PRP3 are not only responsible for root hair specificity but also for responses to hormonal and environmental stimuli (Bernhardt and Tiemey, 2000; Cho and Cosgrove, 2002). The hormonal/environmental stimuli ultimately induce root hair–specific expression of these genes. This stimuli-mediated signaling pathway might also regulate RHF-mediated root hair gene expression.
RHE-Containing Root Hair Genes Are Likely to Be Downstream Genes Mediating Hair Morphogenesis

The RHE-containing root hair–specific genes identified thus far encode cell wall–related proteins, such as expansins, LRXs, and PRPs. Although not root hair cell specific, At CSLD3 (KOJAK), encoding a cellulose synthase-like protein, is expressed preferentially in root hair cells (Favery et al., 2001). The proximal region of the At CSLD3 promoter includes two RHE-like elements (data not shown), which do not match the RHE consensus at a few noncircular positions but could form functional elements when the 1-bp substitution results from At EXPA7E1 are considered (Figure 2E). The root hair morphogenetic process consists of hair initiation (or bulge formation) and tip growth, which is necessarily accompanied by intensive cell wall modification, synthesis, and assembly (Grierson and Schiefelbein, 2002). The above cell wall proteins are representative of the cell wall dynamics during root hair development: expansins for wall loosening (Baluška et al., 2000; Cho and Cosgrove, 2002), CSLD3 for wall synthesis (Favery et al., 2001), and LRXs (Baumberger et al., 2003) and PRPs (Bernhardt and Tierny, 2000) for wall assembly. A concerted regulation of these cell wall genes would be required to implement root hair development efficiently. All these RHE-containing promoters are active from hair initiation through tip growth (Bernhardt and Tierny, 2000; Cho and Cosgrove, 2002; Baumberger et al., 2003), suggesting that the genes they control are instrumental in root hair development.

RHE Has Been Functionally Conserved through Angiosperm Divergence of Root Epidermal Patterning

The root hair has been a basic cellular structure for 400 million years in the tracheophyte lineage, indicating its important role in the successful adaptation of land plants (Peterson, 1992). Our study identified the functional conservation of RHE in orthologous genes from both Graminaceae and eudicots. These data indicate that the occurrence of RHE predated the divergence of angiosperms (170 to 235 million years ago; Yang et al., 1999) and are consistent with the divergence time (~270 million years ago) of the RHE-containing Os EXPA17, which is estimated by adopting the rate of $9 \times 10^{-10}$ nonsynonymous substitutions-site$^{-1}$year$^{-1}$ for grass nuclear genes (Gaut, 1998; Muse, 2000). These collectively imply a strong functional constraint on RHE. Because Type 2 root hair distribution is found in the most primitive tracheophytes, such as Lycopodium and Selaginella (Dolan, 1996; Figure 1B), it will be interesting to know whether RHEs are also associated with these.

Leavitt (1904) originally distinguished two different root hair distribution types; currently, three basic types are recognized in vascular plants (Cormack, 1935, 1947; Dolan and Costa, 2001). At the cellular level, fate-determining mechanisms for the three types of patterns are well defined: Type 1 contains no distinct trichoblast cells and thus generates a random distribution, Type 2 is generated by asymmetric cell division, and Type 3 is cell position dependent (Dolan and Costa, 2001).

Transcriptional regulation in Type 3 fate determination results from the interaction between MYBs (WEREWOLF and CAPRICE), basic helix-loop-helixes (GL3/ENHANCER OF GL3), and a WD40 protein (TRANSPARENT TESTA GLABRA) (Grierson and Schiefelbein, 2002; Schiefelbein, 2003; Schiefelbein and Lee, 2006). In early root epidermal development, these factors are active in both the hair (H) and non-hair (N) cells. However, later in development, the output of the factors in the H position unilaterally inhibits the interaction of the factors in the H position, which releases the inhibition of root hair morphogenesis in the H position. The changes in activity are established by positional cues from underlying cortical cells (Schiefelbein, 2003), where the receptor-like kinase SCRAMBLED (SCM) is a candidate for generating or modulating the cue (Kwak et al., 2005). Another line of evidence implicates position-dependent chromatin reorganization in Type 3 patterning (Costa and Shaw, 2006).

Type 2 fate determinants mediating asymmetric cell division need to be characterized. In contrast with the well-characterized examples in animal systems, the machinery for asymmetric cell division in plant cells remains largely unknown. However, regardless of the mechanism underlying the unequal cell division, the division ultimately results in an asymmetrical distribution of cell fate determinants between the two daughter cells (Scheres and Benfey, 1999; Betschinger and Knoblich, 2004). In animal cells, the machinery for asymmetric cell division is conserved in different species and cell types, but the fate determinants are not (Betschinger and Knoblich, 2004), indicating that different cell types require a different set of fate determinants to establish the asymmetry. Known cell fate determinants are diverse, ranging from transcriptional regulators to protein degradation components (Betschinger and Knoblich, 2004).

Figure 10. Model Illustrating How Root Hair Cell Specificity of Gene Expression Is Determined in Different Hair Patterning Types. There may be orthologous RHF's, the RHE binding factors, that can interact with different signaling pathways activated by different fate-determining machineries.
Type 3 root hair patterns have been found only in eudicots and Type 2 patterns only in monocots, basal angiosperms, and basal tracheophytes (Dolan, 1996; Clowes, 2000; summarized in Figure 1B). This phylogenetic separation of Type 2 and Type 3 patterns suggests that the Type 3 fate-determining machinery coevolved with eudicots and that the Type 2 machinery is more ancient. The random Type 1 pattern occurs throughout all branches of the tracheophytes and is even found in the same family or species (e.g., Solenocampa soleirolii), where Type 2 or Type 3 patterns occur (Clowes, 2000). These observations strongly suggest that the Type 1 pattern results from inactivation of the machineries for the other patterns. For example, inactivation of SCM converts the Type 3 Arabidopsis root hair distribution to the random Type 1 pattern (Kwak et al., 2005). However, we cannot rule out the possibility that Type 1 could be the ancestral pattern.

Our study revealed that, despite different upstream fate-determining pathways, the downstream transcriptional module for root hair morphogenesis has been conserved. Functionally and structurally conserved RHE from many root hair–specific genes strongly suggests that RHF orthologs exert a common action on RHEs in different hair distribution types. Thus, the RHE and RHF from different root hair patterns have evolved to interact with different signaling pathways activated by divergent upstream fate-determining components. The functional conservation of RHE and the orthogonal relationship of RHE-containing EXPAs in species with different root hair types strongly support the hypothesis that the emergence of the RHE-RHF module predates the divergence of the fate-determining machineries. This hypothesis suggests that in the extant three root hair patterns, the emergence of different upstream regulators could generate novel regulatory networks for the RHE-RHF module (Figure 10).

METHODOLOGY

Plant Materials and Growth Conditions

Arabidopsis thaliana (Columbia ecotype) and rice (Oryza sativa cv Hwa Young; a japonica) were used for transformation of the promoter:GFP constructs and observations of root hair–specific GFP expression. The At irx1 mutant (SALK_057038) was obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). 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 (pMP90) (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-d-old T1 transgenic seedlings (n = 17 to 40 for the evaluation of promoter activities).

Rice transformation was performed essentially as described previously (Hiei et al., 1994; Choi et al., 2000). The promoter constructs were transferred to Agrobacterium strain AgL1. For cocultivation with rice embryogenic calli, Agrobacterium was spread onto AB medium and grown for 3 d. For generation of embryogenic calli, rice caryopses were initially plated onto N6 medium supplemented with 2 mg L⁻¹ 2,4-D in the dark. After 3 weeks, embryogenic calli were cocultivated with vector bearing Agrobacterium for 3 d in the presence of 10 mg mL⁻¹ acetosyringone, washed with sterile water, and then selected on N6 medium containing 50 mg L⁻¹ hygromycin and 150 mg L⁻¹ cefotaxime for 4 weeks. Calli were transferred to Murashige and Skoog 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 growth regime. Transgenic rice plants (~15 cm in height) were cultivated further in greenhouses. T1 and T2 rice plants were observed for GFP expression.

Promoter Constructs

Various promoters, 5’-deleted (••, where •• is a number), base-substituted (M°, E°M°, mE°; or •°), or combinations of these promoter mutations were generated by PCR using the primers listed in Supplemental Table 1 online. Deleted and GOF promoters were amplified with the two primers shown as a double set in the primer list. For base-substituted promoters, a substituted megaprimer was first generated using the first and the second primers, and the substituted promoter fragment was amplified with the megaprimer and the third primer listed in the triple primer set (Ke and Madison, 1997). GOF plus base-substituted promoters were amplified using the same primer double set for the GOF but with the base-substituted promoter as template. Deleted promoters and base-substituted promoters were inserted into the HindIII-XbaI or HindIII-XmaI sites of the pGPTV-HYG vector (Becker et al., 1992), where the uidA gene was replaced by GFP (Cho and Cosgrove, 2002). GOF promoters were inserted into the HindIII-XbaI site of the same vector carrying the minimal cauliflower mosaic virus 35S promoter (p35S) upstream of GFP. All promoter constructs were confirmed by nucleotide sequencing, and transgenic plants were also confirmed by PCR analysis and nucleotide sequencing.

Cloning EXPAn Orthologs

The coding and promoter sequences of cabbage (Brassica oleracea; Bo EXPAn) and rice (Os EXPAn17 and Os EXPAn30) orthologs were obtained by a BLAST search using At EXPAn to query The Institute for Genomic Research (http://www.tigr.org/) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) databases. Coding regions of other EXPAn orthologs were obtained by PCR using the primer sets in Supplemental Table 1 online with genomic DNA as the template. Promoter regions were cloned using the GenomWalker kit (BD Biosciences) following the manufacturer’s protocol. Two consecutive PCRs were performed with two gene-specific primers and the adaptor primers (AP1 and AP2) shown in Supplemental Table 1 online. Template genomic DNAs were prepared from each plant tissue using the DNeasy plant mini kit (Qiagen).

Complementation of the irx1 Mutant

The coding region of At LRX1 was amplified by PCR using the primer set listed in Supplemental Table 1 online and inserted into the XmaI-SacI site of pGPTV-HYG, replacing the uidA gene. The At EXPAn promoter (~134 deletion) was inserted into the HindIII-XbaI site upstream of the At LRX1 coding region. This P_ExpA(+134)/LRX1 construct was introduced into root hair-defective At irx1 mutant plants, and T1 to T3 plants were observed.

Observation of GFP and Evaluation of Promoter Activity

GFP fluorescence from seedling roots was observed using either an epifluorescence stereomicroscope (MZ FLIII; Leica) or a confocal laser scanning microscope (LSM 510; Carl Zeiss). To outline the cell boundaries, roots were stained with propidium iodide (10 μg mL⁻¹) in some samples. 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 evaluated by quantifying the GFP fluorescence using the histogram function of Adobe Photoshop (Adobe Systems) as described by Cho and Cosgrove (2002).

Sequence Alignments and Phylogenetic Trees
Nucleotide and protein sequences were aligned using CLUSTAL W (DNASTAR) with the default parameters (gap penalty, 10.0; gap length penalty, 0.2; delay divergent seqs, 30%; DNA transition weight, 0.5). Neighbor-joining phylogenetic trees were generated using MEGA version 3.0 (Kumar et al., 2004), adopting the Poisson correction distance for amino acid sequence. The number of bootstrap replicates was 1000.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ899786 (lb EXPAT7), DQ899793 (Ta EXPAT7), DQ899789 (2m EXPAT7), DQ899790 (Mt EXPAT7), DQ899791 (Mt EXPAT8), DQ899788 (Hv EXPAT7), DQ899785 (Bo EXPAT7), DQ899792 (Rs EXPAT7), DQ899787 (cm EXPAT7), AP000616 (Os EXPAT17), and AC062967 (Os EXPAT30). Arabidopsis Genome Initiative locus identifiers for the Arabidopsis genes are At1G12880 (At EXPAT7), At1G62980 (At EXPAT18), At1G12040 (At LRX1), At1G62440 (At LRX2), and At3G62680 (At PRP3).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Gain-of-Function Promoter Analysis of P<sub>EXPAT</sub> in Arabidopsis Roots.

Supplemental Figure 2. Gain-of-Function Promoter Analysis of P<sub>EXPAT</sub> in Arabidopsis Roots.

Supplemental Figure 3. Neighbor-Joining Phylogenetic Tree of Expansin Protein Sequences with Bootstrap Values (from 1000 Replicates).

Supplemental Figure 4. Multiple Alignments of Expansin Protein Sequences by CLUSTAL W.

Supplemental Figure 5. Promoter Analysis of EXPAT Orthologs in Arabidopsis Roots.

Supplemental Table 1. Primer List.

Supplemental Text 1. High-Resolution Analysis of P<sub>EXPAT7</sub> and P<sub>EXPAT8</sub>.

Supplemental Text 2. Analyses of RHE-Containing Promoters from EXPAT Orthologs and Other Arabidopsis Root Hair Genes.

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