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Molecules and Cells

Root Hair-Specific EXPANSIN A7 Is Required for Root Hair Elongation in *Arabidopsis*

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Expansins are non-hydrolytic cell wall-loosening proteins that are involved in the cell wall modifications that underlie many plant developmental processes. Root hair growth requires the accumulation of cell wall materials and dynamic cell wall modification at the tip region. Although several lines of indirect evidence support the idea that expansin-mediated wall modification occurs during root hair growth, the involvement of these proteins remains to be demonstrated *in vivo*. In this study, we used RNA interference (RNAi) to examine the biological function of *Arabidopsis thaliana EXPANSIN A7* (*AtEXPA7*), which is expressed specifically in the root hair cell. The root hair-specific *AtEXPA7* promoter was used to drive RNAi expression, which targeted two independent regions in the *AtEXPA7* transcript. Quantitative reverse transcriptase-PCR analyses were used to examine *AtEXPA7* transcript levels. In four independent RNAi transformant lines, RNAi expression reduced *AtEXPA7* transcript levels by 25-58% compared to controls. Accordingly, the root hairs of RNAi transformant lines were 25-48% shorter than control plants and exhibited a broader range of lengths than the controls. Our results provide *in vivo* evidence that expansins are required for root hair tip growth.

INTRODUCTION

In vascular plants, root hairs protrude from root epidermal cells, greatly increasing the root surface area. Root hair development can be divided into three major stages: (1) 'fate determination', in which the hair or non-hair cell fate of root epidermal cells becomes determined; (2) 'initiation', where the hair cell begins to show distinctive cytoplasmic characteristics and the hair bulge swells out from a specific position; and (3) 'tip growth', in which the hair elongates to its final size (Grierson and Schiefelbein, 2002).

Plant cell growth and morphogenesis occur through cell wall modifications such as loosening and reassembly. These processes are particularly evident at sites where the initial root hair bulge emerges from the root hair cell surface. Sustained hair tip growth requires the concentration of cell wall materials at the hair tip, with wall-related activities being carried out by a variety of enzymes and proteins (Galway, 2006). Several root hair-specific genes have been implicated in root hair growth in Arabidopsis. For example, defects in hair elongation and morphogenesis are caused by the loss of *Arabidopsis thaliana LEUCINE RICH REPEAT/EXTENSIN 1* (*AtLRX1*) and *AtLRX2* (Baumberger et al., 2001; 2003), and root hair length is reduced by the overexpression of a putative cell wall peroxidase, *ROOT HAIR SPECIFIC 18* (*RHS18*; Won et al., 2009).

Expansins (EXPs) are cell wall-loosening proteins that exhibit no apparent hydrolytic activity (Choi et al., 2006; Cosgrove, 2000). Expansin genes comprise a multigene family with members found in most plant species. Expansin genes can be divided into two subgroups, *EXPA*s and *EXPB*s, which share limited similarity and encode proteins with different substrate specificities. Promoter::reporter analyses of *Arabidopsis EXP*s revealed that two *EXPA*s (*AtEXPA7* and *18*) are expressed specifically in the root hair cell (Cho and Cosgrove, 2002; Fig. 2A). Many functional orthologs of *AtEXPA7* (i.e., *EXPA*-like genes exhibiting root hair-specific expression) have been identified experimentally among monocots and eudicots (Kim et al., 2006). Recently, we identified two root hair-specific *EXPB*s in rice and barley (Won et al., 2010). These genes encode proteins that may be specific for root hair growth in the Poaceae. Thus far, all root hair-specific *EXP*s carry root hair-specific cis-elements (RHEs) in the proximal promoter region, indicating that these genes are subject to regulation by orthologous transcriptional components (Kim et al., 2006; Won et al., 2009; 2010). However, the biological functions of these genes remain to be characterized *in vivo*. Here, we used RNA interference (RNAi) to demonstrate that *AtEXPA7* is required for normal root hair elongation in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

RNAi constructs were transformed into *Arabidopsis thaliana* (Columbia ecotype). *Arabidopsis* seeds were cold-treated at 4°C for 3 days prior to germination in the dark. *Arabidopsis* plants were transformed using *Agrobacterium tumefaciens* strain C58C1 (Bechtold and Pelletier, 1998) and transformants were selected on hygromycin-containing (10 μg ml^{-1}) phytoagar plates. Four-day-old T2 transgenic seedling roots were

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taken for the measurement of root hair length and RNA extraction.

AtEXPA7-RNAi constructs

RNAi target regions in *AtEXPA7* cDNA were amplified using the polymerase chain reaction (PCR) and the primer sets listed in Table 1. cDNA template was obtained from the roots of Arabidopsis seedlings. The two RNAi target regions, RNAi-1 and RNAi-2, are indicated in Fig. 1A. The RNAi-1 and RNAi-2 target regions were inserted into the *Xho*I/*Eco*RI and *Bam*HI/*Xba*I sites of the pHannibal vector to generate sense and antisense fragments, respectively. Next, the *Xho*I/*Xba*I fragments from the cloned pHannibal vector were transferred into the *Sal*I/*Xba*I sites of the binary vector pE7p13M (Lee et al., 2010). pE7p13M was modified from pCAMBIA1300 (Hyg+) and carries the *AtEXPA7* promoter at -480 bp from the transcription initiation site (Kim et al., 2006) (Fig. 1B). All transgenic constructs were confirmed by nucleotide sequencing and the genetic integrity of transgenic plants was confirmed by PCR amplification of genomic DNA.

Observation of root hairs

Observation of root hairs and estimations of root hair length were performed as described previously (Ganguly et al., 2010). For estimation of root hair length, digital photographs of roots were taken using a stereomicroscope (Leica MZ FLIII) at 40X to 50X magnifications. Hairs in the hair maturation region (approximately 0.78 mm from the tip) were counted and measurements were performed on 20 hairs protruding perpendicularly from each side of the root.

Quantitative reverse transcriptase PCR (qRT-PCR) analysis

Total RNA was isolated from the roots of 4-day-old seedlings (25 for each line) using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized as described previously (Lee and Cho, 2006). qRT-PCR analyses were performed using an EvaGreen Master mix (Applied Biological Materials Inc.) and a Chromo4™ Four-Color Real-Time Detector (Bio-Rad). Gene-specific signals were normalized relative to *Actin7*. Each reaction was performed in triplicate and each experiment was repeated three times using independent preparations of RNA.

Table 1. Primers used for PCR amplification of RNAi target regions in *AtEXPA7* cDNA

Type	Name[1]	Sequence (5′ to 3′)
RNAi-1 primers	E7-Ri1a-XhF1	CTT CCT CGA GAA CGT TGG TGG CGC CGG A
	E7-Ri1a-EcR1	AGC AGA ATT CCA AGC ATA GAT GGT TTC AC
	E7-Ri1b-XbF2	TTC GTC TAG AAC GTT GGT GGC GCC GGA
	E7-Ri1b-BaR3	GCAAGGATC CAA GCA TAG ATG GTT TCA C
RNAi-2 primers	E7-Ri2a-XhF1	CTA ACT CGA GCC AAG ACT CCA ACG CTG GTG
	E7-Ri2a-EcR1	CAC TGA ATT CGC ATG GCA CTC TTC GGT ATG
	E7-Ri2b-XbF2	CTA ATC TAG ACC AAG ACT CCA ACG CTG GTG
	E7-Ri2b-HdR2	TCC AAA GCT TTG GCA TGG CAC TCT TCG GT

[1]Xh, *Xho*I; Ec, *Eco*RI; Xb, *Xba*I; Ba, *Bam*HI; Hd, *Hin*dIII.

A

caaaaacaaaccctaagaataaagaaaaagaggctagaATGGGTCCAATCTCAAGTTCTTGGAGCTTTAACA
AATTCTTCTCAATAGTTTTCGTTGTTTTCGCCATCTCCGGCGAGTTCGTCGCTGGATACTATCGACCAGGCC
CATGGAGATATGCTCACGCCACTTTCTACGGTGACGAGACCGGTGGTGAAACCATGGGTGGTGCATGTGGGT
ACGGAAACCTTTTTAACAGCGGCTACGGACTGTCCACGGCGGCGCTAAGCACGACATTGTTCAATGATGGTT
ACGGATGCGGCCAATGTTTTCAGATAACATGTTCGAAATCACCGCATTGTTACTCTGGAAAATCAACAGTGG
TCACAGCCACCAATCTTTGCCCTCCTAATTGGTA**CCAAGACTCCAACGCTGGTGGTTGGTGCAATCCTCCTA**
GAACCCATTTCGATATGGCTAAACCAGCTTTCATGAAACTCGCTTACTGGAGGGCCGGTATCATCCCAGTTG
CATACCGAAGAGTGCCATGCCAAAGGAGTGGAGGTATGAGGTTTCAATTCCAAGGTAATTCTTATTGGCTTC
TTATCTTCGTCAT**GAACGTTGGTGGCGCCGGAGACATCAAGAGCATGGCCGTTAAAGGTAGCCGGACGAATT**
GGATAAGCATGAGCCACAATTGGGGAGCCTCTTACCAAGCTTTTTCCTCTCTCTATGGTCAATCTCTCTCTT
TCCGGGTCACTTCTTACACCACCGGTGAAACCATCTATGCTTGGAACGTTGCTCCGGCTAACTGGAGCGGCG
GTAAGACTTACAAGAGCACCGCTAATTTCCGTTAAaactaggcttttgccctaccaaacgaaaacggagttt
tctcttctccttctttttttgttaagagtttcggtggccttttgttgtggtggcccggctttatgttataca
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cacttcttgatcaagagaattcgtt

B

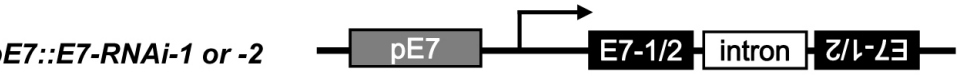

Fig. 1. RNAi target regions in *AtEXPA7* cDNA. (A) *AtEXPA7* cDNA sequence showing the coding and untranslated regions in upper and lower case, respectively. The start (ATG) and stop (TAA) codons are boxed. RNAi target regions are underlined by solid (RNAi-1) and broken (RNAi-2) lines. (B) Expression of RNAi-1 or -2 constructs is driven by the *AtEXPA7* promoter (*pE7*). The arrow indicates the transcription initiation site.

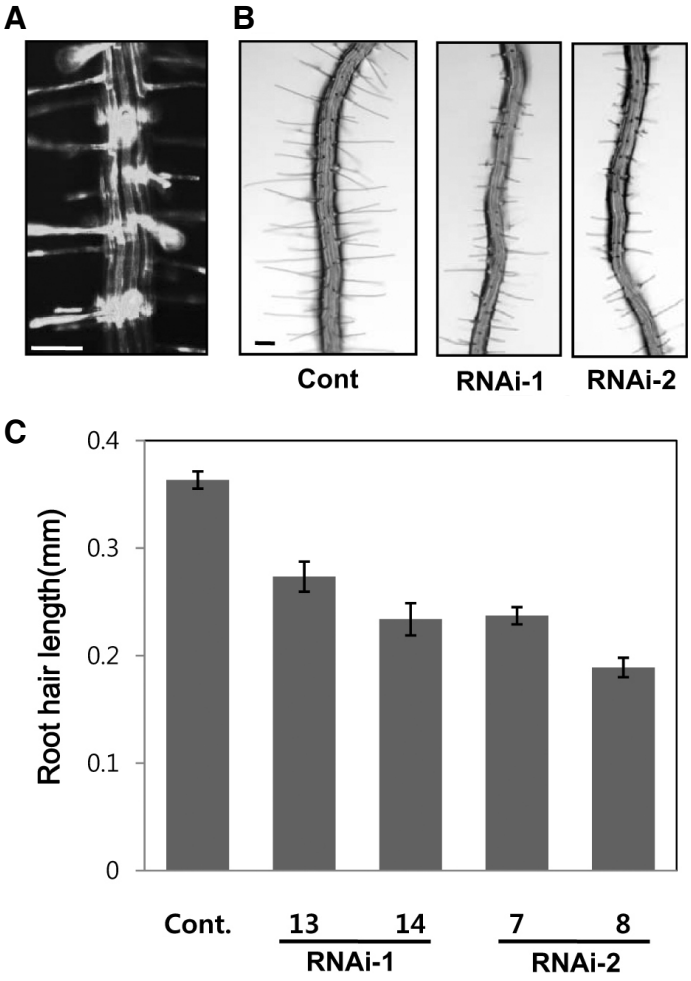

Fig. 2. RNAi against *AtEXPA7* inhibited root hair elongation. (A) Fluorescence microscopy of a root from a pE7:YFP transformant. The *AtEXPA7* promoter (*pE7*) directs root hair-specific expression of YFP. Bar = 100 μm. (B) Root images of control (Cont, *pE7:YFP*) and *AtEXPA7*-RNAi (*pE7:E7-RNAi*) lines (RNAi-1 and RNAi-2). Bar = 100 μm. (C) Root hair lengths in control and *AtEXPA7*-RNAi transformants. Numbers indicate independent RNAi transgenic lines. Data represent means ± S.E. (N = 495 [control], 540 [#13], 521 [#14], 597 [#7], and 556 [#8]).

Accession numbers

The accession numbers for the genes analyzed in this study are AT1G12560 (At*EXPA7*) and AT1G62980 (*AtEXPA18*).

RESULTS AND DISCUSSION

We generated two RNAi constructs to knock-down transcript levels of *AtEXPA7* in root hair cells. The RNAi constructs were 130 bp (357-486 bp from the start codon) and 177 bp (552-728 bp) in length (Fig. 1A). RNAi transcript expression was driven by the *AtEXPA7* promoter (*pE7*) to obtain root hair cell-specific RNAi expression during the root hair initiation and tip growth stages (Fig. 1B).

Twenty six RNAi-1 and 25 RNAi-2 transformants were obtained in the T1 generation, all of which had shorter root hairs than controls, and two independent lines for each RNAi construct were chosen for further analysis in the T2 generation. Measurements were taken for 521-597 root hairs per independent line. The root hairs of RNAi lines were 25-48% shorter than in the control line *pE7::YFP* (YFP, yellow fluorescent protein; Cho et al., 2007), with RNAi-2 lines exhibiting slightly shorter root hairs than RNAi-1 lines (Figs. 2B and 2C).

We analyzed the distribution of root hair lengths in the transgenic lines. Root hair number was counted using 0.05 mm intervals. The relative number of roots found within each root hair length interval was presented as a percentage of total root hairs. In control plants, the majority of root hairs (with > 10% of all root hairs in each hair length interval) were distributed between 0.25 and 0.50 mm, i.e., five 0.05 mm intervals (Fig. 3A), whereas they were distributed through 7 intervals (0.10-0.45 mm) in RNAi-1 plants (Fig. 3B) and 5 intervals (0.10-0.35 mm) in RNAi-2 transformants (Fig. 3C). In both RNAi lines, the root hair distribution pattern also showed a decrease in long hairs and a proportionate increase in short hairs. This overall reduction in root hair length suggests that RNAi lines have a lower growth capability and an earlier cessation of root hair tip growth, compared to the controls. In addition, these results suggest that AtEXPA7 is required for sustained root hair tip growth.

To determine whether or not AtEXPA7 is essential during the early stage of root hair morphogenesis, i.e., hair initiation, root hair numbers were compared between control and RNAi lines. However, RNAi targeting of *AtEXPA7* expression did not appear to have a significant effect on root hair numbers. Although this result may indicate that AtEXPA7 functions primarily in tip growth rather than hair initiation, it is also possible that the partial suppression of *AtEXPA7* transcript levels was insufficient for the inhibition of hair initiation. Although not currently available, an *AtEXPA7* knock-out mutant or an *AtEXPA7*/*AtEXPA18* double mutant would be helpful for characterizing the roles played by EXPs during the hair initiation stage.

The RNAi transformants were examined by qRT-PCR to confirm interference with the *AtEXPA7* transcript. Target transcripts were reduced by 45 and 25% in RNAi-1 lines #13 and 14, respectively, and by 49 and 58% in RNAi-2 lines #7 and 8, re

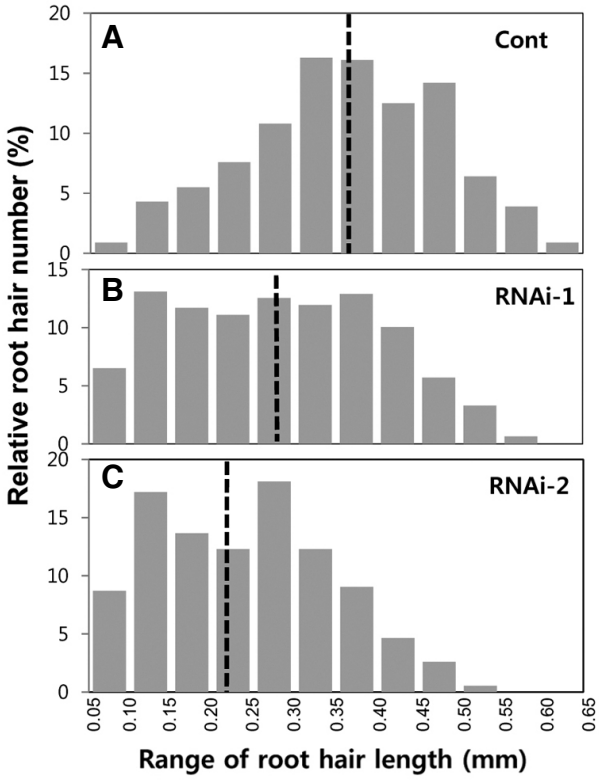

Fig. 3. Root hair length distribution. (A) Distribution of root hair length in control plants (Cont, *pE7:YFP*). (B) Distribution of root hair length in *AtEXPA7*-RNAi-1 transformants. (C) Distribution of root hair length in *AtEXPA7*-RNAi-2 transformants. The numbers of root hairs observed were 540 (Cont), 913 (RNAi-1) and 791 (RNAi-2). The vertical dash lines represent means.

Table 2. Primers for qRT-PCR

Type	Name	Sequence (5′ to 3′)
E7-RT-primers	E7-rt-F	GAT ATG CTC ACG CCA CTT
	E7-rt-R	GTC CGT AGC CGC TGT TA
E18-RT-primers	E18-rt-F	TCG TCA GAC CAT TTA CGC TTA
	E18-rt-F	CTC AAC AAC AAA TGG CAA AAC C
Actin primers	act_rtF2	TCC CGC TAT GTA TGT CGC CAT C
	act_rtR1	CTG GAA CAA GAC TTC TGG GCA TCT

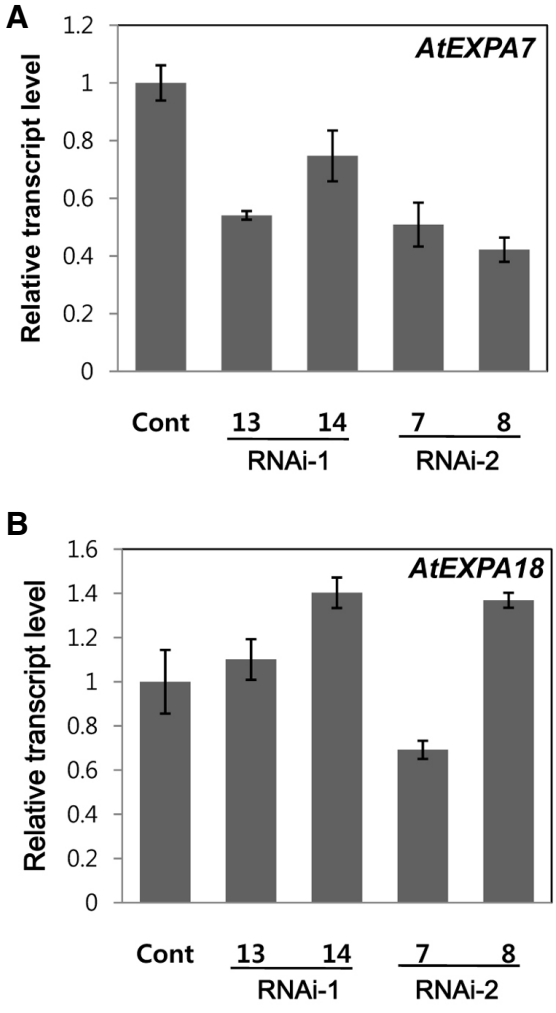

Fig. 4. Quantitative RT-PCR analysis of *AtEXPA7* and *AtEXPA18* transcripts. (A) Relative levels of *AtEXPA7* transcripts in the roots of control (Cont, *pE7:YFP*), *AtEXPA7*-RNAi-1 and *AtEXPA7*-RNAi-2 transformants. (B) Relative levels of *AtEXPA18* transcripts in the roots of control (Cont, *pE7:YFP*), *AtEXPA7*-RNAi-1 and *AtEXPA7*-RNAi-2 transformants. Data represent means ± S.E. from three independent experiments.

spectively (Fig. 4A). On the other hand, the RNAi lines showed no significant decrease in transcription of *AtEXPA18*, which is a closest paralog of *AtEXPA7* (Fig. 4B). Therefore, it is likely that a reduction in the *AtEXPA7* transcript level resulted in the inhibition of root hair elongation observed in *AtEXPA7*-RNAi transformants. Moreover, although AtEXPA18 is expressed in a root hair-specific manner and may perform similar functions to AtEXPA7, it is not able to compensate completely for reduced AtEXPA7 levels during root hair elongation. Thus, it is unlikely that other root-specific *AtEXP*s could complement *AtEXPA7*.

Expansins perform a wide range of biological activities during plant development and they are particularly associated with processes requiring dynamic cell wall modifications. Transgenic and genetic studies have demonstrated that expansins play roles in cell enlargement, fruit softening, abscission, germination, pathogenesis and pollen tube growth (reviewed in Cho and Cosgrove, 2004; Choi et al., 2006; Cosgrove, 2000). Thus far, we have only had circumstantial evidence to suggest that expansins might be involved in root hair growth. For example, expansins require acidic pH levels for optimum wall-loosening activity (Cosgrove, 2000) and, prior to hair bulge formation, sites of hair emergence exhibit local acidification (Bibikova et al., 1998). Moreover, expansin proteins localize at the root hair tip (Baluška et al., 2000) in *Arabidopsis*, and exogenous application of purified expansin proteins will cause the *Arabidopsis* root hair tip to burst (Cosgrove et al., 2002). Previously, we identified two Arabidopsis expansin genes (*AtEXPA7* and *AtEX-PA18*) that contain RHE consensus motifs in their promoter regions (Cho and Cosgrove, 2002; Kim et al., 2006). Here, we demonstrated that the reduction of the levels of the *AtEXPA7* transcript suppresses root hair elongation. This finding indicates that expansins play a positive role in root hair growth and likely perform active cell wall modification activities during the elongation of the hair tip.

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