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Methodology

Isolation and analysis of α-expansin genes in the tree *Anthocephalus chinensis* (Rubiaceae)

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ABSTRACT. Expansins are cell wall-associated proteins that induce wall extension and relax stress by disrupting noncovalent bonds between cellulose microfibrils and cross-linking glycan chains, thereby promoting wall creep. *Anthocephalus chinensis* is a very fast-growing economically important tree found mainly in South Asia. Sixteen cDNAs, designated *AcEXPA1* to *AcEXPA16* (GenBank accession Nos. FJ417847, JF922686-JF922700) with corresponding genomic DNA sequences (GenBank accession Nos. GQ228823, JF922701-JF922715), were isolated by amplifying conserved domain binding with genomic walking and RACE techniques from four differential growth tissues in *A. chinensis*. These α-expansin homologues were highly conserved in size and sequence; they had the same sequence structures as an N-terminal signal peptide, three exons and two introns. Their amino acid alignment showed that *A. chinensis* expansin genes are divided into three subgroups: A, B and C. This study is the first report on expansin

genes from *A. chinensis*. It will be used for a tissue-specific expression model and for studying the relationship between expansin genes, growth rate and wood quality of the xylem in this fast-growing tree.

Key words: α-expansin; Phylogenetic tree; *Anthocephalus chinensis*; Gene cloning

INTRODUCTION

All plant cells are surrounded by a cell wall that determines their shape, attaches cells together, provides essential mechanical support and rigidity, and also acts as a critical barrier against invading pathogens (O'Malley and Lynn, 2000; Cosgrove, 2000). However, it simultaneously limits cell growth and increase of cell protoplasm. Expansins (EXP) are cell wall proteins that are essential for cell wall extension and stress relaxation (Cosgrove et al., 2002). Although the exact mechanism by which expansins cause loosening and extension of cell walls is still poorly understood, a hypothesis states that expansins break noncovalent bonding, hydrogen bonding between cell wall polysaccharides, thereby allowing turgor-driven polymer creep and stimulating plant growth (McQueen-Mason and Cosgrove, 1994; Hutchison et al., 1999; Cosgrove, 2000; Cosgrove et al., 2002; Lee and Kende, 2002; Gray-Mitsumune et al., 2004, 2008; Wang et al., 2011).

Since the first report of the presence of two expansins in a crude protein extract from the cell walls of cucumber seedling hypocotyls (McQueen-Mason et al., 1992), an increasing number of expansin genes, along with their complete gene sequences, have been isolated from various plants, such as *Arabidopsis thaliana*, rice (*Oryza sativa*), poplar (*Populus trichocarpa*), papaya (*Carica papaya*), maize (*Zea mays*), and tobacco (*Nicotiana tabacum*) (Table 1). These known expansins are divided into four known classes: α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXPLA), and expansin-like B (EXPLB) (Shcherban et al., 1995; Cosgrove, 2000; Lee et al., 2001; Cosgrove et al., 2002; Kende et al., 2004; Sampedro and Cosgrove, 2005) (http://homes.bio.psu.edu/expansins/genes.htm). Research has mainly focused on the two former expansin classes, whereas expansin-like A and expansin-like B proteins are not well investigated (Lee et al., 2001; Sampedro and Cosgrove, 2005). The data in Table 1 indicate that there are more β-expansin genes in rice than in dicots, but fewer α-expansin genes in maize.

Table 1. Number of expansin genes in six model plants.

Gene style	Plant species					
	Arabidopsis	Rice	Poplar	Papaya	Maize	Tobacco
EXPA	26	33	27	15	5	6
EXPB	6	18	3	3	11	0
EXPLA	3	4	2	1	0	0
EXPLB	1	1	4	0	0	0

EXPA = α-expansin; EXPB = β-expansin; EXPLA = expansin-like A; EXPLB = expansin-like B. [http://homes.bio.psu.edu/expansins/genes.htm].

A. chinensis, commonly known as kalempayan (Malaysia), laran (Sabah), kaatoan bangkal (Philippines), kalempajan (Indonesia), mau-lettan-she (Burma), and kadam (India), is

a member of Neolamarckia tribe belonging to the Rubiaceae family and is distributed widely in South Asia (Hsienshui et al., 1999). It has received high praise in the Philippines, where it has been described as "a gem of a tree" (Fox, 1971), and was universally accepted as "a miraculous tree" by the World Forestry Congress in 1972 because of its fast growth. In this context, cloning growth-related genes, *AcEXPA*, from *A. chinensis* is aimed at understanding the relationship between plant growth and *AcEXPA* expression.

MATERIAL AND METHODS

Plant material and tissue sampling

For RNA extraction, tender leaves and buds were collected from lateral branches, and cambium scrapings were collected from the diameter at breast height of a 4-year-old *A. chinensis* tree according to the method described by Gray-Mitsumune et al. (2004). Young roots were collected from 6-month-old trees cultivated in a greenhouse. All excised samples were immediately frozen in liquid nitrogen, and were then stored at -80°C in a refrigerator until RNA extraction.

DNA extraction, PCR amplification of the central conservative region of α-expansin genes and gene walking in genomic DNA

Total genomic DNA was extracted from the leaves using a DNeasy Plant Kit (Qiagen, China) according to the manufacturer protocol. Comparing the *EXPA* gene sequences in other plants from NCBI, the degenerate primers [forward: 5'-GG(AGCT)GG(AGCT)GC(AGT)TGTGG(AGT)TA(CT)GG-3', reverse: 5'-TGCCA(AG)TT(CT)TG(AGCT)CCCCA(AG)TT-3'] were designed to amplify the central conservative region of *EXPA* genes, using genomic DNA as the template. PCR was performed in a final reaction volume of 25 μL containing 20 ng genomic DNA, 1.25 U DNA polymerase (Takara, Japan), 50 ng forward primer, 50 ng reverse primer, 1X PCR buffer (Takara), and 5 mM each of all four dNTPs (Takara). Touchdown PCR conditions were as follows: 94°C hot start for 5 min; 94°C denaturation for 30 s, 62°C annealing for 30 s with 1°C decrease each cycle, and 72°C extension for 1.5 min for 13 cycles; followed by 94°C denaturation for 30 s, 50°C annealing for 30 s, and 72°C extension for 1.5 min for 30 cycles, and a final extension at 72°C for 10 min.

Genomic walking upstream and downstream PCR primers were subsequently based on the central conservative region sequences as described by the GenomeWalker Kit user manual (www.clontech.com, Takara). Seven restriction endonucleases, *Alu*I, *Dra*I, *Eco*RI, *Hae*III, *Rsa*I, *Ssp*I, and *Stu*I, were used for genomic DNA digestion and seven walking libraries were constructed according to the kit.

Total RNA isolation and cDNA synthesis

Total RNA was extracted using a RNeasy Plant Kit (Qiagen) according to manufacturer instructions. Additional on-column DNase digestions were performed three times during the RNA purification using the RNase-Free DNase Set (Qiagen). RNA was then quantified based on agarose gel electrophoresis and absorption at 260 nm. Quantified RNA was reverse-

transcribed into cDNA utilizing the PrimeScript II 1st-strand cDNA Synthesis Kit and the supplied polythymine primers (Takara).

Subcloning and sequencing

All PCR products were resolved by agarose gel electrophoresis, excised, and purified using Ultrafree-DA (Millipore, USA) centrifugal filter units. The purified DNA was ligated into a pMD19-T vector and transformed into DH5α competent cells. A positive clone for each individual was selected for further sequencing with RV-M and M13-47 primers.

Sequence assembly and open reading frame (ORF) prediction

The central conservative region sequence, with 5'-upstream and 3'-downstream of *EXPA* sequences, was assembled with DNAMAN 6.0 (Lynnon BioSoft, Canada). The ORF, intron and exon were predicted by the online tool (http://linux1.softberry.com/berry.phtml?topic=fgenesh &group=programs&subgroup=gfind, http://www.cbs.dtu.dk/services/NetGene2). Then, the full-length *EXPA* were amplified utilizing a high-fidelity DNA polymerase, PrimeSTAR HS DNA Polymerase (Takara), to confirm the accuracy of the sequences, and the 3'-untranslated region (UTR) sequence was obtained by 3'-Full RACE Core Set Ver.2.0 (Takara).

The actual intron and exon regions were obtained by directly comparing the genomic and transcriptional sequences. The N-terminal signal peptide cleavage sites were predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP2.0).

Phylogenetic analysis of α-expansins

Amino acid sequences deduced from the cDNA sequences were aligned by ClustalW with its default setting for protein multiple alignment [weight matrix, Gonnet; gap opening penalty, 10; gap extension penalty, 0.1; residue-specific penalties, ON; hydrophilic penalties, ON; gap separation distance, 4; end gap separation, OFF; delay divergent cutoff (%), 30] and viewed with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The corresponding alignment of the nucleotide sequences between *A. chinensis* α-expansin genes and that of other plants was performed with ClustalW using its default setting for DNA multiple alignment [weight matrix, IUB; gap opening penalty, 15; gap extension penalty, 6.66; use negative matrix, OFF; delay divergent cutoff (%), 30]. A phylogenetic tree was generated from this alignment using the MEGA 4 program with the neighbor-joining method, maximum composite likelihood, bootstrap method, 100 bootstrap replications, and the complete deletion option (Gray-Mitsumune et al., 2004; Tamura et al., 2007). The phylogenetic tree was unrooted.

RESULTS

Amplification of the conserved domain of α-expansin genes

To establish methods for isolating *A. chinensis* α-expansin genes, we BLASTed most α-expansin genes in plants. As an example, the four expansin amino acid sequences, AtEXPA6 (AAB38072), PaEXPA1 (AAC33529), NtEXPA4 (AAC96080), and PtEXPA1 (AY675563),

demonstrated alignment for conservative regions (Figure 1) using ClustalW. The forward and reverse primers were designed as 5'-GG(AGCT)GG(AGCT)GC(AGT)TGTGG(AGT)TA(CT)GG-3' and 5'-TGCCA(AG)TT(CT)TG(AGCT)CCCCA(AG)TT-3' from "GGACGYG" and "NWGQNWQ", respectively, which are indicated as F and R, respectively, in Figure 1. Then, we used the primers to amplify the conservative region of *A. chinensis* α-expansin genes. PCR amplification produced three electrophoretic bands, shown in Figure 2. Thirty positive clones from the PCR products of each vector-ligated band were sequenced, and 16 different sequences were obtained and identified as conservative regions in α-expansin genes by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

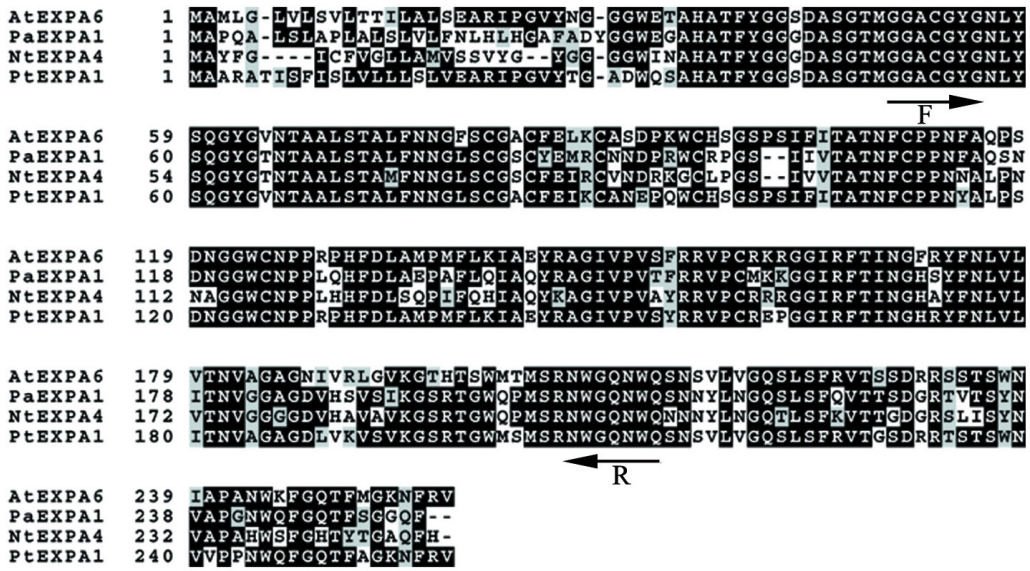

Figure 1. Alignment of AtEXPA6 (AAB38072), PaEXPA1 (AAC33529), NtEXPA4 (AAC96080), and PtEXPA1 (AY675563). The numbers shown at the left of each sequence are the positions of amino acid residues in the corresponding proteins. Identical and similar amino acid residues are shaded with black and gray, respectively. The location and directions of primers are indicated by arrows.

Isolation of *AcEXPA1-16* full-length cDNA sequences

In order to amplify the full-length of the genes, genomic walking and RACE (rapid amplification of cDNA ends) were adopted based on the fragment sequences. We eventually isolated 16 *A. chinensis* α-expansin genes designating them *AcEXPA1-16* according to the nomenclature of Kende et al. (2004). Detailed information of *AcEXPA1-16*, including full-length base number, ORF base number, length of predicted amino acid, and signal peptide length, is provided in Table 2. The total amino acid length of *AcEXPA1-16* ranged from 240 to 258, whereas their signal peptide length ranged from 19 to 28 amino acids. All *AcEXPA1-16* introns had the nucleotides GT at the 5'-splice site and AG at the 3'-splice site, except intron 2 in *AcEXPA4*, which had TA at the 5'-splice site and GA at the 3'-splice site and neither belonged to the U2-type

intron (GT-AG) (Simpson and Filipowicz, 1996) nor the U12-type intron (AT-AC) (Levine and Durbin, 2001; Patel and Steitz, 2003). Additionally, α-expansin sequences from several different tissues were isolated and showed that most sequences obtained were completely identical except for two, two, and one silent mutations in the *AcEXPA1*, *AcEXPA5*, and *AcEXPA10* ORF, respectively (Figure 3A-C), one sense mutation in the *AcEXPA5* ORF with a C-to-G substitution, which results in the amino acid proline changing to alanine (Figure 3B). Although several reports have shown that mutations might lead to phenotypic alteration as well as change in the molecular function of the genes (Strauss, 2000; Petry and Loos, 2005; Yang and Nielsen, 2008), in the case of our findings, we do not yet know what the consequences of these mutations are.

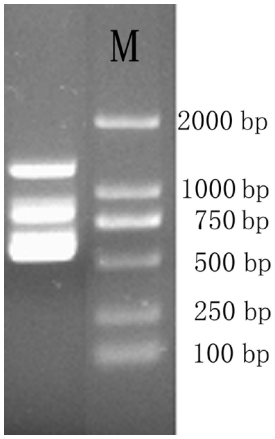

Figure 2. PCR product with the degenerated primers. Left column = PCR products with three bands; right column = molecular marker with number labeled at the right.

Table 2. Detailed information of *AcEXPA1-16*, including ORF length, length of the predicted protein, signal peptide length, and intron splice site base model.

Gene name	ORF length (bp)	Amino acid length (AA)	Signal peptide (AA) (http://www.cbs.dtu.dk/services/SignalP/)	Intron in ORF			
				Intron 1		Intron 2	
				5'-terminal	3'-terminal	5'-terminal	3'-terminal
AcEXPA1	777	258	21	GT	AG	GT	AG
AcEXPA2	768	255	28	GT	AG	GT	AG
AcEXPA3	768	255	28	GT	AG	GT	AG
AcEXPA4	759	252	25	GT	AG	TA	GA
AcEXPA5	777	258	21	GT	AG	GT	AG
AcEXPA6	762	253	26	GT	AG	GT	AG
AcEXPA7	747	248	20	GT	AG	GT	AG
AcEXPA8	771	256	19	GT	AG	GT	AG
AcEXPA9	777	258	22	GT	AG	GT	AG
AcEXPA10	723	240	20	GT	AG	GT	AG
AcEXPA11	753	250	22	GT	AG	GT	AG
AcEXPA12	747	248	20	GT	AG	GT	AG
AcEXPA13	747	248	20	GT	AG	GT	AG
AcEXPA14	741	246	20	GT	AG	GT	AG
AcEXPA15	744	247	20	GT	AG	GT	AG
AcEXPA16	768	255	23	GT	AG	GT	AG
Eukaryote				GT	AG	GT	AG

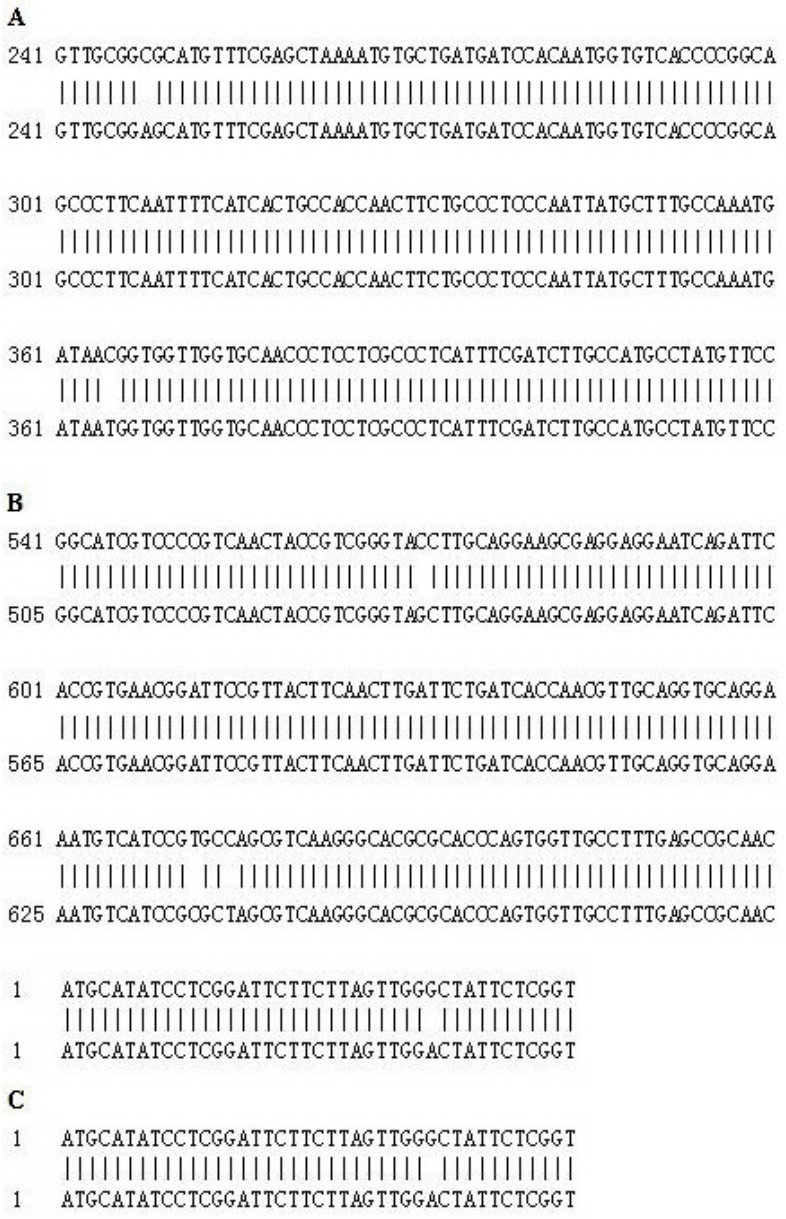

Figure 3. Mutation in *AcEXP1*, *AcEXP5* and *AcEXPA10* ORF. **A.** *AcEXP1*, **B.** *AcEXP5*, **C.** *AcEXPA10*.

Intron-exon model of *AcEXPA1-16*

To learn how the intron/exon structure changed during the evolution of the expansin gene family, the genomic structure of the cDNA of each *AcEXPA* family member was com-

pared and the intron/exon organization of each gene was determined (Figure 4). A schematic representation of the genomic organization of the AcEXPA family members, depicting the number and location of introns within the *AcEXPA* transcriptional region, is shown in Figure 2. We also found that the intron-exon structure of *AcEXPA1-16* is similar to the model of *Arabidopsis* α-expansin genes, where most of the genes are disrupted by introns 1 and 2 (https://homes.bio.psu.edu/expansins/arabidopsis.htm, Figure 2) (Sampedro and Cosgrove, 2005). Although we detected extreme conservation in the coding sequences and positions of intron/exon boundaries of all *AcEXPA* genes, the sizes and sequences of the introns in the coding region and 3'-UTR were found to be significantly divergent among the 16 *AcEXPA* genes.

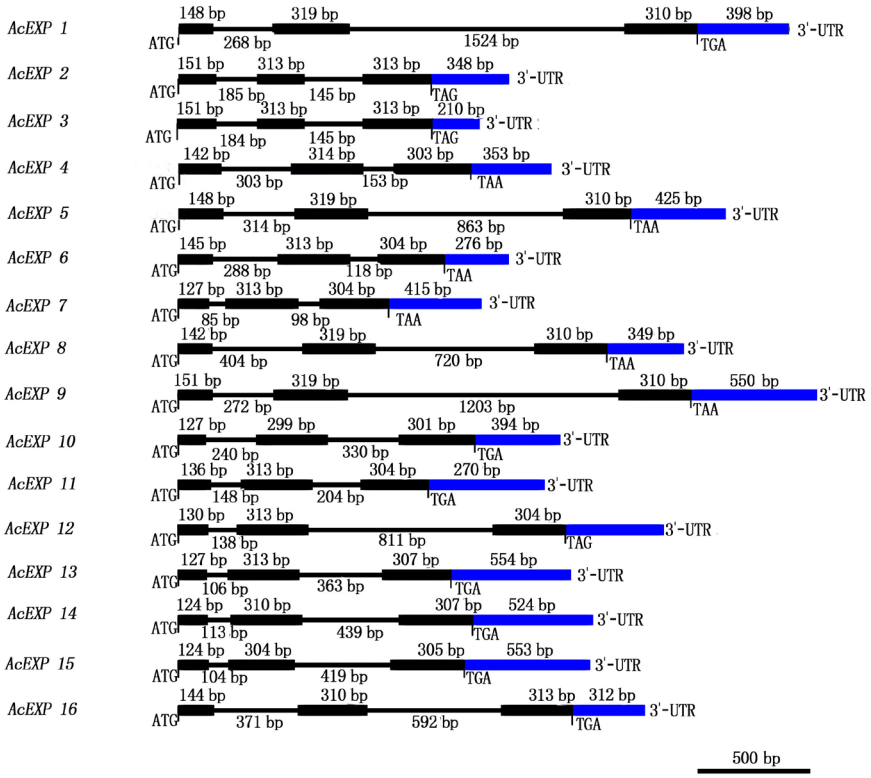

Figure 4. Schematic representation of the structure of the EXPA family in *Anthocephalus chinensis* with the corresponding gene name. Exons and UTRs are indicated as black and blue boxes, respectively, and lines between boxes are introns. The start code, stop code and length of fragment are labeled in the figure.

Amino acid sequence alignment

To investigate the similarity of *A. chinensis* α-expansin genes, the amino acids of the genes were compared. The coding sequences were deduced from cDNA sequences, and then aligned by ClustalW. Figure 5 illustrates the alignment of the 16 α-expansin genes. The genes contain key features of expansin, including N-terminal signal peptides, a series of conserved

C (cysteine, Cys) residues similar to glycoside hydrolase family 45 (GH45) proteins, an HFD (histidine-phynylalanine-aspartate, His-Phe-Asp) motif, which is speculated to form part of the active site, and four W (tryptophan, Trp) residues near the C terminus that might be involved in the interaction with cellulose (Shcherban et al., 1995; Sampedro and Cosgrove, 2005), although there is leucine (L) instead of W at the first W site in *AcEXPA12*.

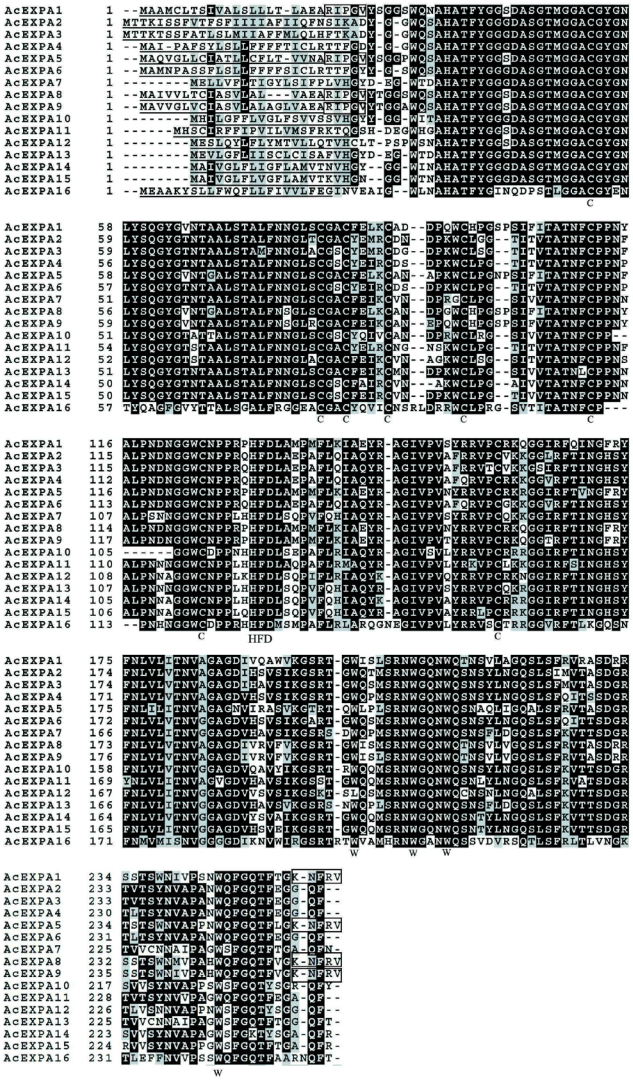

Figure 5. Alignment of the predicted protein sequences of *AcEXPA1-16*. The N-terminal signal peptides stablished by the SignalP program (www.cbs.dtu.dk/services/SignalP/) are underlined. The conserved Cys, Trp residues and His-Phe-Asp motif residues are indicated by the letters C, W, and HFD, respectively. RIPGV and KNFRV motifs at both the N and C ends of mature proteins are boxed. Identical and similar amino acids are represented by black and gray boxes, respectively. Gaps are shown as dashes. Multiple alignments were done by CLUSTAL-W and viewed with the BOXSHADE program.

Phylogenetic analysis of α-expansin genes

The α-expansin gene family splits into four major phylogenetic branches (Figure 6) (Cosgrove, 1998; Link and Cosgrove, 1998; Gray-Mitsumune et al., 2004). Four expansin genes, *AcEXPA1*, *AcEXPA5*, *AcEXPA8*, and *AcEXPA9*, aligned with subgroup A expansin genes previously described by Link and Cosgrove (1998) and Gray-Mitsumune et al. (2004). The predicted proteins from this subgroup exhibit unique sequence conservation, namely near the C terminus the amino acid sequence immediately after the predicted signal peptide cleavage site RIPGV, and the C-terminal sequence KNFRV (Figure 5). These two motifs can serve as signature sequences of subgroup A, which is abundant in secondary xylem (Gray-Mitsumune et al., 2004; Wang et al., 2011). Nevertheless, the tissue-specific expressions of these four corresponding genes in *A. chinensis* are unclear. *AcEXPA2*, *AcEXPA3*, *AcEXPA4*, *AcEXPA6*, and *AcEXPA11* belong to subgroup B, and *AcEXPA7*, *AcEXPA10*, *AcEXPA12*, *AcEXPA13*, *AcEXPA14*, and *AcEXPA15* belong to subgroup C. Phylogenetic analysis showed that *AcEXPA16* belongs neither to any of the above-mentioned subgroups nor to subgroup D in the phylogenetic tree.

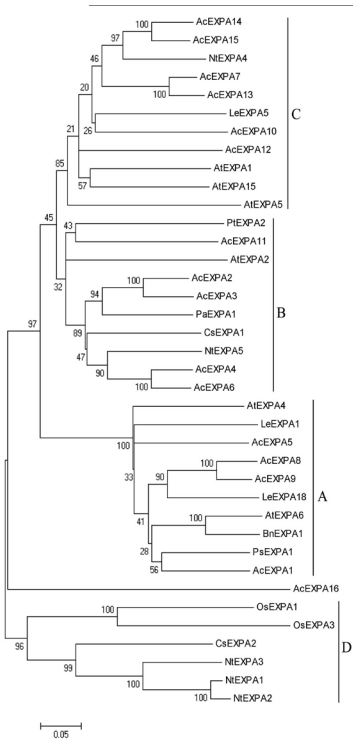

Figure 6. Phylogenetic analysis of α-expansin genes. Subgroups A to D as defined by Link and Cosgrove (1998). GenBank accession numbers of expansin genes from *Arabidopsis thaliana*: *AtEXPA1* (U30476), *AtEXPA2* (U30481), *AtEXPA4* (129526), *AtEXPA5* (U30478), *AtEXPA6* (U30480), *AtEXPA15* (126361); *Brassica napus*: *BnEXPA1* (AJ000885); *Cucumis sativus*: *CsEXPA1* (U30382), *CsEXPA2* (U30460); *Lycopersicon esculentum*: *LeEXPA1* (U82123), *LeEXPA5* (AF059489), *LeEXPA18* (AJ004997); *Nicotiana tabacum*: *NtEXPA1* (AF049350), *NtEXPA2* (AF049351), *NtEXPA3* (AF049352), *NtEXPA4* (AF049353), *NtEXPA5* (AF049354); *Oryza sativa*: *OsEXPA1* (Y07782), *OsEXPA3* (U30479); *Prunus armeniaca*: *PaEXPA1* (U93167); *Pisum sativum*: *PsEXPA1* (X85187); *Pinus taeda*: *PtEXPA2* (U64890). The tree was unrooted.

DISCUSSION

To date, model plants such as *Arabidopsis*, rice, and poplar have been fully sequenced. Therefore, it is very easy to clone target genes in these species. However, due to limited genomic information, it is still very difficult to clone the genes of interest in most plants, such as *A. chinensis*, to carry out further research. In this report, we present an efficient method carried out by cloning the conserved motif first, then amplifying full-length genes by RACE and genomic walking to clone genes in this unknown species.

The genomes of model plants contain many α-expansin genes (https://homes.bio.psu.edu/expansins/genes.htm). Despite the deduction that the mechanisms of all these α-expansins are similar, whether their functions are different is still not entirely clear (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2000; Cosgrove et al., 2002), as various genes are expressed at different levels in different organs or tissues, and also at different development stages (Cho and Cosgrove, 2002; Hiwasa et al., 2003; Gray-Mitsumune et al., 2004; Ishimarua et al., 2007; Sharova, 2007; Figueroa et al., 2009; Jieun et al., 2010). In the present study, we cloned 16 α-expansin genes in *A. chinensis*. This suggests that they might have different functions in cell wall extension. It is also possible that their expression and function are redundant and synergistic, as all *A. chinensis* α-expansins exhibited a high level of conservation not only in protein length, but also as specific amino acids, including eight C residues at the N terminus, the HFD motif, and four W residues at the C terminus (Figure 5) (Shcherban et al., 1995). *A. chinensis* α-expansins might also be regarded as a gene resource for plant molecular engineering.

α-expansins possess highly conserved motifs, not only in *Arabidopsis* and rice, but also in *A. chinensis* and other species. Thus, the degenerate primers described in this manuscript can also be used to amplify α-expansins in other species. Additionally, conservative α-expansins might serve as molecular markers to study plant genomic duplication and evolution.

Due to the 3'-oligo(dT) primer limit, an erroneous PCR product might sometimes be produced in the 3'-UTR region. For example, the length of the 3'-UTR containing PolyA of *AcEXPA1* and *AcEXPA5* in our first PCR products was much shorter than that of the other *AcEXPA* genes, being 121- and 42-bp long, respectively. These 3'-UTR lengths were much shorter than those of *Arabidopsis* (http://www.ncbi.nlm.nih.gov/nucleotide). This illustrates the fact that they were not complete sequences containing PolyA. Therefore, downstream primers were designed with genomic DNA sequences following the false PolyA for amplification of their cDNA, cooperating with upstream primers. The sequences of the PCR products were aligned with prior 3'-UTR sequences. The result is shown in Figure 7. The false PolyA is basically consistent with the sequence of the corresponding site in the second PCR product (Figure 7). This indicates that the oligo(dT) with adaptor primer was annealed at the site possessing a tandem A base in front of the PolyA in the mRNA in RT-PCR of 3'-RACE, thereby producing the short 3'-UTR.

Phylogenetic analysis showed that α-expansin genes fall into four clades (A-D; Figure 5) similar to those previously described by Link and Cosgrove (1998). It has previously been postulated that the α-expansins within each clade may act in a manner similar to each other (Link and Cosgrove, 1998). In the phylogenetic tree, *AcEXPA1*, *AcEXPA5*, *AcEXPA8*, and *AcEXPA9* aligned within subgroup A, which contains several expansin genes expressed in primary and secondary vascular tissues, such as the *AtEXPA4*, *AtEXPA6* (S. Turner, data published online at http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl), and *LeEXPA18* genes (Reinhardt et al., 1998). All of the subgroup proteins have common conserved domain as RIPGV after the predicted signal peptide cleavage site and KNFRV at the C terminal. *AcEXPA2*,

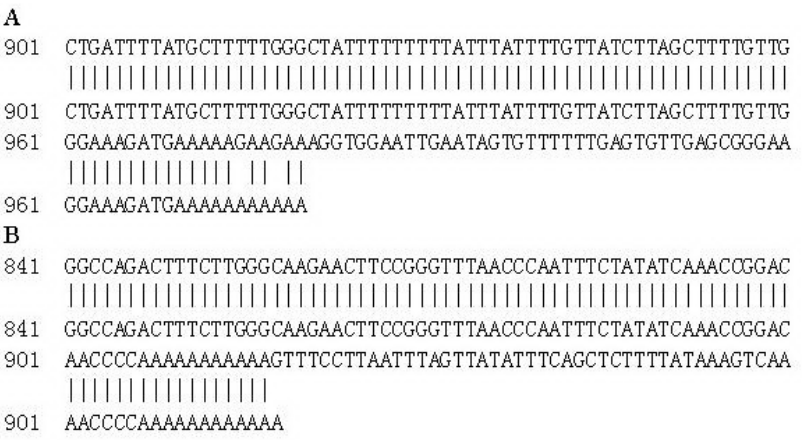

Figure 7. Sequences of PCR production with the downstream primers after the false PolyA were aligned with prior cDNAs. Upper line is sequence of second-time PCR production and lower line is cDNA sequence with false PolyA. **A.** *AcEXPA1*; **B.** *AcEXPA5*.

AcEXPA3, *AcEXPA4*, *AcEXPA6*, and *AcEXPA11* belong to subgroup B, which contains genes expressed in growing roots and hypocotyls. Members of this subgroup, such as *PtEXPA2*, are expressed during adventitious root formation and in the base of the hypocotyl in response to indole-3-butyric acid treatment in loblolly pine (*Pinus taeda*) (Hutchison et al., 1999), and *CsEXPA1*, expressed in cucumber hypocotyl (Shcherban et al., 1995). Other *A. chinensis* genes, *AcEXPA7*, *AcEXPA10*, *AcEXPA12*, *AcEXPA13*, *AcEXPA14*, and *AcEXPA15*, belong to subgroup C, which includes *Arabidopsis AtEXPA1* and *AtEXPA5*, and tomato *LeEXPA5*. *AtEXPA1* is known to express and accelerate stomatal opening in *Arabidopsis* (Zhang et al., 2011), while *AtEXPA5* is mainly expressed in the aerial parts of *Arabidopsis*, especially in the inflorescence stems and flowers (Park et al., 2010). Another member of subgroup C, *LeEXPA5*, has been shown to be expressed in expanding fruit, with expression levels being highest in full-size maturing green fruit and which decline during the early stages of ripening in tomato (Brummell et al., 1999).

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