

Isolation of an *actin* promoter for strong expression of transgenes in the orchid genus *Dendrobium*

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Abstract We isolated and functionally characterized a *Dendrobium Actin1* (*DmACT1*) promoter that drives strong gene expression in the orchid genus *Dendrobium*. A genomic fragment containing the region 3227 bp upstream of the coding region of *DmACT1* was obtained by inverse PCR. Detailed comparison of the full-length cDNA and genomic sequences revealed that *DmACT1* has a 1374 bp first intron in the 5' UTR. However, the 5' flanking sequences upstream of the coding region showed no obvious sequence similarities compared to those of known promoters, including plant actin promoters. Serial deletion constructs of the 5' flanking region from the translation initiation codon were fused to the coding sequence of a GUS/luciferase fusion reporter to identify the regulatory elements necessary for promoter activity. Transient assays in the flowers of *Dendrobium* revealed that the 5' UTR-intron greatly enhanced promoter activity. Moreover, the *DmACT1* promoter with its 5' UTR-intron yielded approximately 10-fold higher reporter activity than the rice *Act1* promoter-intron. Our data suggest that the *DmACT1* promoter with its 5' UTR-intron is a useful tool for strong expression of transgenes in *Dendrobium* orchids.

Keywords 5' UTR-intron, transient expression

Abbreviations CaMV, Cauliflower mosaic virus; GUS, β -Glucuronidase; X-GlcA, 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid cyclohexylammonium; *DmACT1*, *Dendrobium moniliforme Actin1*; RACE, rapid amplification of cDNA ends; UTR, untranslated region

Introduction

Orchids in the genus *Dendrobium* are some of the most important commercial orchid plants in the floricultural industry because many species in this genus produce a wide variety of beautifully colored flowers with unique morphologies and diverse fragrances. Furthermore, chemical compounds from orchids in this genus are used in traditional Chinese medicine (Wood 2006). Extensive conventional breeding programs based on intra- and inter-specific hybridization have been conducted over the last decade in *Dendrobium* species to improve floricultural traits such as color, plant shape, and growth. However, these approaches have resulted in limited success due to the lack of genetic resources, the sterility of inter-specific hybrids, and difficulty obtaining a genotype with all favorable genetic alleles present in the hybrids (Shibata 2008). Recently, technical advances in genetic transformation technology in *Dendrobium* have made it possible to modify target traits by direct incorporation of related genes with few undesirable effects on the existing plant phenotype (Chia et al. 1994; Kuehnle and Sugii 1992; Yang et al. 2003; Yu et al. 2001).

Strong, constitutive promoters are generally required for ectopic expression of target genes in a transgenic plant. Several promoters have been investigated as useful tools for strong expression of target genes in plants. Promoters obtained from *CaMV* 35S RNA (Guilley et al. 1982), the maize polyubiquitin 1 (*Ubi 1*) gene (Elliott et al. 1999), and the chimeric HBT promoter consisting of the *CaMV* 35S enhancer fused to the basal promoter and the 5' leader sequence of the maize C4PPDK gene (Sheen et al. 1995) have been evaluated in *Dendrobium* by genetic transformation or transient expression analysis (Chia et al. 1994; Tee et al. 2003; Yu et al. 2001). The HBT and *Ubi1* promoters showed relatively low GUS expression compared to that of the *CaMV* 35S promoter in *Dendrobium sonia* (Tee et al. 2003). Similarly, the rice *Actin1* (*Act1*) (McElroy

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et al. 1990) and maize *Ubi1* promoters showed lower activity than the CaMV 35S promoter in some non-cereal monocot plants, namely *Lolium*, *Liliaceae*, and *Gladiolus* (Kamo et al. 1995; Wilpink et al. 1995). These results suggest that typical monocot promoters, such as *Act1* and *Ubi1*, although they perform well in some monocot species, do not perform well in all monocot species. In contrast, the CaMV 35S promoter drove developmental- and tissue-specific expression of transgenes in transgenic rice and cotton plants, but was not constitutive (Sunilkumar et al. 2002; Tereda and Shimamoto 1990; Zhang and Wu 1988). Thus, for successful transformation of a target plant species, the promoter should be chosen with care.

Actin is a ubiquitous protein that plays essential roles in plant developmental processes such as cytoskeletal function, cell division, cell elongation, cellular trafficking, transportation of organelles, and pollen tube growth (Wasteneys and Galway 2003). High levels of *Actin* transcript were observed in all tissues and development stages in rice plants (McElroy et al. 1990). Likewise, the *Actin1* (*Act1*) promoter directed high *GUS* expression in all tissues of transgenic rice plants (Zhang et al. 1991). Recently, the rice *Actin2* promoter, which drives high-level constitutive expression of *GUS* in transgenic rice, was also isolated for monocot transformations (He et al. 2009). However, the feasibility of using rice *actin* promoters in *Dendrobium* remains to be evaluated.

Some 5' UTR-introns, but not all, greatly enhance gene expression in humans (Jonsson et al. 1992), mice (Palmiter et al. 1991), *Arabidopsis* (Rose and Last 1997), rice (Jeon et al. 2001; Zhang et al. 1991), and *Caenorhabditis elegans* (Ho et al. 2001). Although 5' UTR-intron mediated gene expression (IME) exists in a diverse range of organisms, the precise mechanism underlying IME is likely not general. The length of 5' UTR-introns is highly variable across species, and 5' UTR-introns tend to be similar in size or larger than intragenic introns (Bradnam and Korf 2008). Moreover, it has been shown that the most highly expressed genes in humans and *C. elegans* tend to have shorter introns (Castillo-Davis et al. 2002), whereas in rice and *Arabidopsis*, highly expressed genes tend to have longer introns (Ren et al. 2006). Furthermore, analysis of the positional effect of IME has shown that IME effects decrease with distance from the transcription start site within a single gene in transgenic *Arabidopsis* (Rose 2004). Hence, the mechanisms underlying IME still remain to be elucidated.

One of the major barriers in genetic transformation of *Dendrobium* is the lack of promoters able to drive strong

ectopic expression of target genes. To overcome this problem, we isolated the *Actin1* (*DmACT1*) promoter from a species of *Dendrobium*, because *DmACT1* transcripts are abundantly expressed in the roots, leaves, and flowers of species in this genus. To identify the promoter region necessary for efficient gene expression, we examined the promoter activity of *DmACT1* 5' flanking sequences using a GUS/luciferase fusion reporter system. Transient assays in *Dendrobium* flowers revealed that the *DmACT1* 5' UTR-intron is essential for transgene expression. Moreover, the *DmACT1* promoter showed approximately 8- to 10-fold higher activity than the rice *Act1* promoter. Thus, the *DmACT1* promoter can be used to drive strong ectopic gene expression in *Dendrobium* orchids.

Materials and methods

Plant Materials

Dendrobium moniliforme "Jindo" (Whang et al. 2011) was cultivated under a 16-h light/8-h dark cycle at 24°C in a greenhouse facility at Chonbuk National University. Leaf and root samples were harvested from young seedlings to isolate total RNA. Flower buds were harvested just after flowering in late May and throughout June for total RNA isolation or transient expression assays.

Northern blot analysis

Flowers, roots, and leaves were ground in liquid nitrogen using a mortar and pestle, and total RNA was isolated using Trizol reagent (Gibco BRL, NY, USA) according to the manufacturer's instructions. To prepare a *DmACT1*-specific probe, 120 bp of the 5' UTR was amplified by radioactive PCR with the primers ActProbe-F (5'-GCCCTGCTAGTCGGCTGC-3') and ActProbe-R (5'-CTTTTACAA GCCTAAACAGAAG-3'). Five micrograms of total RNA was denatured and separated on a 2% agarose gel containing formaldehyde. After transferring the RNA onto a Hybond-N plus membrane (Amersham Biosciences, USA), the RNA was hybridized with ³²P-labeled probe for 8 h at 65°C in Church buffer and washed as described previously (Koo et al. 2004). The blot was exposed to a Phosphor Screen (Molecular Dynamics, Amersham Bioscience, Inc.) to visualize the signal. The pixel intensity of each band was quantified and analyzed using TotalLab100 software (Nonlinear Dynamics, USA). The relative expression levels are shown as the ratio of the expression in the leaves to that in the other tissues.

Cloning of partial *Actin1* cDNA by degenerate RT-PCR

Partial *DmACT1* cDNA was isolated using RT-PCR with degenerate primers. The primers dACT-F (5'-GARAARA TGACNCARATHATG-3') as the upstream forward primer and dACT-R (5'-TCNACRTCRCAYTTCATDAT-3') as the downstream reverse primer were designed based on a multiple alignment of orchid actin genes including those isolated from *Dendrobium* hybrid cultivars (EF612438.1 and GU301090.1), *Phalaenopsis* (AF246715.1), and *Cymbidium* (JQ360575.1 and JN177721.1). Five micrograms of total RNA was used to synthesize first strand cDNA using the SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., CA, USA) according to the manufacturer's instructions. PCR was performed with one-tenth of the synthesized cDNA, 20 μM dACT-F, and 20 μM dACT-R in 20 μl of PCR premix (Clontech Laboratories Inc., CA, USA). The PCR conditions were as follows: initial denaturation at 95°C for 2 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by a final extension of 72°C for 7 min. PCR products were separated on a 0.8% agarose gel (w/v). Specifically amplified DNA was recovered from the agarose gel using a PCR DNA purification Kit (GeneAll, Korea) and cloned into the pTOP TA V2 vector (Enzymomics, Inc., Korea). The nucleotide sequence of the insert was determined using vector-

derived primers and analyzed using the BlastN program of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Isolation of the full-length *DmACT1* cDNA by 5'- and 3'-RACE

Full-length *DmACT1*cDNA was isolated by 5'- and 3'-RACE using the SMARTer RACE cDNA Amplification Kit according to the manufacturer's instructions with minor modifications. Briefly, 5 μg of total RNA was used to synthesize first strand cDNA using the SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., CA, USA). One-tenth of the first strand cDNA was subjected to PCR with the *DmACT1*-specific reverse primer, Act-RACE-R (5'-GTCGTGCGGCCACTAGCATATAG-3') for 5'-RACE or the forward primer, Act-RACE-F (5'-CTATT GGAGCTGGGAGATTTAGGTGC-3'), for 3'-RACE and vector derived universal primers to synthesize *DmACT1*, respectively. The PCR profile was as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. PCR products were purified using a PCR DNA purification Kit (GeneAll, Korea) and cloned into the pTOP TA V2 vector (Enzymomics, Inc., Korea) and sequenced with vector-derived primers. The complete *DmACT1* cDNA sequence is available at Genbank (accession number KC171011, Fig. 1).

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1  gccctgctagtcggctgctccgacgacggactcgcttctaggctcttgaggcttaaggtctacatagaagaggaggaagggcgctttgaa
    M A D V D D I Q P L V C D N G T G
91  tcgatcctcgctcagattcttctgtttaggcttgtaaaagATGGCGGATGTTGATGATATTCAGCCTCTTGTGGATAATGGAAGTGA
    M V K A G F A G D D A P R A V F P S I V G R P R H T G V M V
181 ATGGTTAAGGCTGGATTGCTGGTGACGATGCGCCAGGCGCGTGTCCCAAGCATTGTGGGCCGACCTCGTACACTGGTGCATGGTT
    G M G Q K D A Y V G D E A Q S K R G I L T L K Y P I E H G I
271 GGCATGGGCCAAAAGGATGCTTATGTGGGAGATGAAGCCAGTCCAAAAGAGGTATCCTCACCTTGAAATATCCATTGAACATGGAAT
    V S N W D D M E K I W H H T F Y N E L R V A P E E H P V L L
361 GTAAGCAACTGGGATGATATGGAAAAGATTGGCCATCACACCTTCTACAATGAACCTCCGTGTGGCCCTGAGGAGCACCCGTACTGCTA
    T E A P L N P K A N R E K M T Q I M F E T F N V P A M Y V A
451 ACTGAAGCTCCTCTCAATCCCAAGGCAAAACAGAGAAAAGATGACACAGATAATGTTCCGAGACTTTCAATGTGCCATGATGTTGCC
    I Q A V L S L Y A S G R T T G I V L D S G D G V S H T V P I
541 ATTTCAAGCTGTTCTTTCCCTATATGCTAGTGGCCGACGACAGGTATTGTGCTTGATTCTGGTGTGGTGTAGCCATACAGTCCCTATT
    Y E G Y A L P H A I L R L D L A S R D L T D S L M K I L T E
631 TATGAGGGTTATGCACTCCCCCATGCCATCCTTCGATTGGATCTTGGAGCCGAGATCTCACAGACTCCTTGATGAAGATCCTCACTGAG
    R G Y S F T T T A E R E I V R D I K E K L A Y V A L D Y E Q
721 AGAGGATATTCTTCCACTACTGCAGAGCGTGAATTTGTCAGGACATCAAGGAGAAGCTAGCCTATGTTGCCCTTGATTATGAGCAG
    E L E T S K S S S S I E K S Y E L P D G Q V I T I G A G R F
811 GAGTTGGAACCTCCAAGAGCAGCTTCCCATAGAAAAGAGCTATGAACCTCCTGATGGTCAGGTTATTACTATTGGAGCTGGGAGATTT
    R C P E V L F Q P S L I G M E A A G I H E T T Y I N S I M K C
901 AGGTGCCAGAAAGTTCTCTCCAGCCATCTCTGATGGAATGGAAGCTGCTGGAATTCATGAGACAACCTACAACCTCATTATGAAATGC
    D V D I R K D L Y G N I V L S G G S T M F P G I A D R M S K
991 GACGTGATATCAGAAAGGATTTGTATGGAACATGTTCTTAGTGGAGGCTCCACCATGTTCCCGCCATTGCTGATCGCATGAGCAAA
    E I T A L A P S S M K I K V V A P P E R K Y S S V W I G G S I
1081 GAGATAACTGCACCTTGCTCCTAGCAGCATGAAGATCAAGTGGTGCCTCCACCAGAGAGGAATACAGTGTCTGGATTGGAGGATCTATC
    L A S L S T F Q Q M W I S K A E Y D E S G P A I V H R K C F
1171 CTCGCTCCCTCAGTATTTCCAGCAGATGTGGATTTCCAAGGCCGAGTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
1261 tgagctccatatttgctttggtctcgtaactgctgattagctgtttttctggctttgctttccagagattgttgaaatthaagtaaggggg
1351 gtttggtgatttacttctcagatggttttataattttcagatggtcttctgattgtttggtgctgctcacaatttacaaggcagcagaa
1441 cttctgttccatattgtctatcagacatgcccagtttgatccagatggctggttcttctggttctatttacgaacttttaaacagagata
1531 aatgagtcggtaccttctatttaaaaaaaaaaaaaaaaaaaaaa
    
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Fig. 1 Nucleotide and deduced amino acid sequence of *DmACT1* (Genbank accession No. KC171011). The lower-case letters represent the 5' and 3' UTR sequences. Underlined sequences indicate the sequences isolated by degenerate RT-PCR

Isolation of *DmACT1* 5'-flanking sequences by inverse PCR

Genomic DNA was extracted from fresh leaves using the CTAB method (Doyle and Doyle 1988). For inverse PCR, 1 µg of genomic DNA was fully digested with *Eco* RI at 37°C for 4 h in a reaction volume of 100 µl. The digested DNA was extracted with a PCR DNA purification kit (GeneAll, Korea). The linearized DNA was then self-ligated to form circular DNA with T4 DNA ligase (Enzyomics Inc., Korea) in a 10 µl reaction volume at room temperature overnight. Self-ligated DNA was used as a template for inverse PCR. The PCR reaction was carried out in 40 µl PCR mix containing 1 µl of self-ligated DNA and 20 µM each of the inverse PCR primers, iACT-E-3-1 (5'-GCCATCCTTCGATTGGATCTTGCGAGCCG-3') and iACT-E-5-1 (5'-CTCTAAAACAAGCCAAAACCCTCGCAG-3'). The PCR conditions were as follows: one cycle of initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 65°C for 30 s, 72°C for 3 min, followed by 72°C for 5 min. PCR products were purified using a PCR DNA purification kit and used as template for a second round of nested inverse PCR. The reaction mix and PCR condition were the same as those described above except the primers, iACT-E-3-2 (5'-CTCCTTGATGAAGATCCTCACTGAGAGAGG-3') and iACT-E-5-2 (5'-AAAGTAAGTCGCCTTATACCAAGGGCG-3') were used. Specifically amplified DNA was recovered from the agarose gel and cloned in the pTOP TA V2 vector for DNA sequencing. To obtain additional 5' flanking sequences of *DmACT1*, inverse PCR was conducted with *Nde* I-circularized genomic DNA as described above. The first round of PCR was performed using the primers iACT-

Nd-3-1 (5'-TCAAGGGTGGATCTGGAGCTACCA-3') and iACT-Nd-5-1 (5'-CAAAAGAACACAAACACCCAGATCTACC-3'). The second nested round of PCR was performed using the primers iACT-Nd-3-2 (5'-CCTGTTTGAATCGCGTTTGTAGCA-3') and iACT-Nd-5-2 (5'-CAGATCTACCAAACGGCCCACCG-3'). Specifically amplified DNA was recovered from the agarose gel and cloned into the pTOP TA V2 vector for DNA sequencing. DNA sequencing was carried out using vector-derived primers and primers designed based on internal sequences, and both strands were sequenced. Nucleotide sequences were analyzed using the BlastN program of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A database search for promoter motifs and regulatory elements was performed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>).

Vector construction

The *DmACT1* 5' flanking sequence was deposited in Genbank (accession number KC171012, Fig. 3). A 3227 bp fragment of the *DmACT1* 5' flanking sequence, containing the promoter and the first intron sequence (from -1724 to +1503 relative to the transcription start site) was amplified by LA-PCR with the primers Act1-Nd (5'-ttggatcCATATGCAAAGGACTAAACAAATTAAGTAG-3') and Act1-int-R (5'-ttaagcttCTTTTACAAGCCTGAAGAATAACTGACATG-3'). The underlined sequences indicate *Bam* HI and *Hind* III sites, respectively. The PCR product was digested with *Bam* HI and *Hind* III and cloned into the same sites in the GUS/Luciferase3300 vector, which encodes the GUS/Luciferase fusion reporter gene (Koo et al. 2007), to create *pDmACT1Nd*. In the same manner, the 1833 bp PCR product (from -1724 to +109) lacking the first intron sequence was amplified by PCR with the primers Act1-Nd and Act1-R (5'-ttaagcttAGAATCTGACGAGGATCGATTCAAAGC-3'), and cloned in the GUS/Luciferase3300 vector to create *pDmACT1NdΔint*. A 2555 bp fragment of the *DmACT1* 5' flanking sequence (from -1052 to +1503) was amplified by PCR with the primers Act1-Xb (5'-ttggatcctTCTAGAAAACTCGGTACAACCTGTATTACC-3') and Act1-int-R, and cloned into the GUS/Luciferase3300 vector to create *pDmACT1Xb*. A 1602 bp fragment of the *DmACT1* 5' flanking sequence (from -99 to +1503) was amplified by PCR with the primers, Act1-St (5'-ttggatccAGGCCTAAGCCCGTCTTGCTCAA-3') and Act1-int-R, and cloned into the GUS/Luciferase 3300 vector to create *pDmACT1St*. To compare with other monocot actin promoter, the *Pst* I and *Hind* III fragment of pCAMYS02 containing rice *Actin1* promoter (a kind

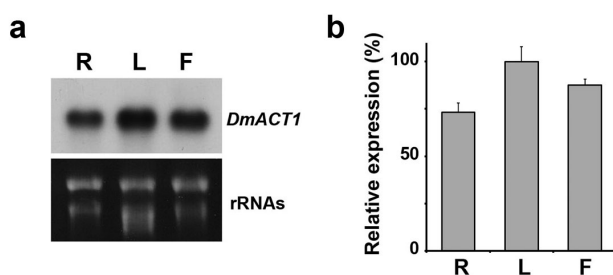


Fig. 2 Expression pattern of *DmACT1* in *Dendrobium*. (a) RNA blot analysis of *DmACT1*. Total RNA was isolated from root (R), leaf (L), and flower (F) tissues. RNA was transferred to a membrane and hybridized with a ³²P-labeled *DmACT1* 5' UTR probe. As a loading control, rRNAs was visualized after staining with ethidium bromide. (b) Quantification of *DmACT1* expression. The *DmACT1* band intensity from the RNA gel blot was measured and normalized to those of the 28S rRNA bands. Relative expression indicates the ratio of the value for the leaf to that of the other tissues. Bars indicate the SD of mean values

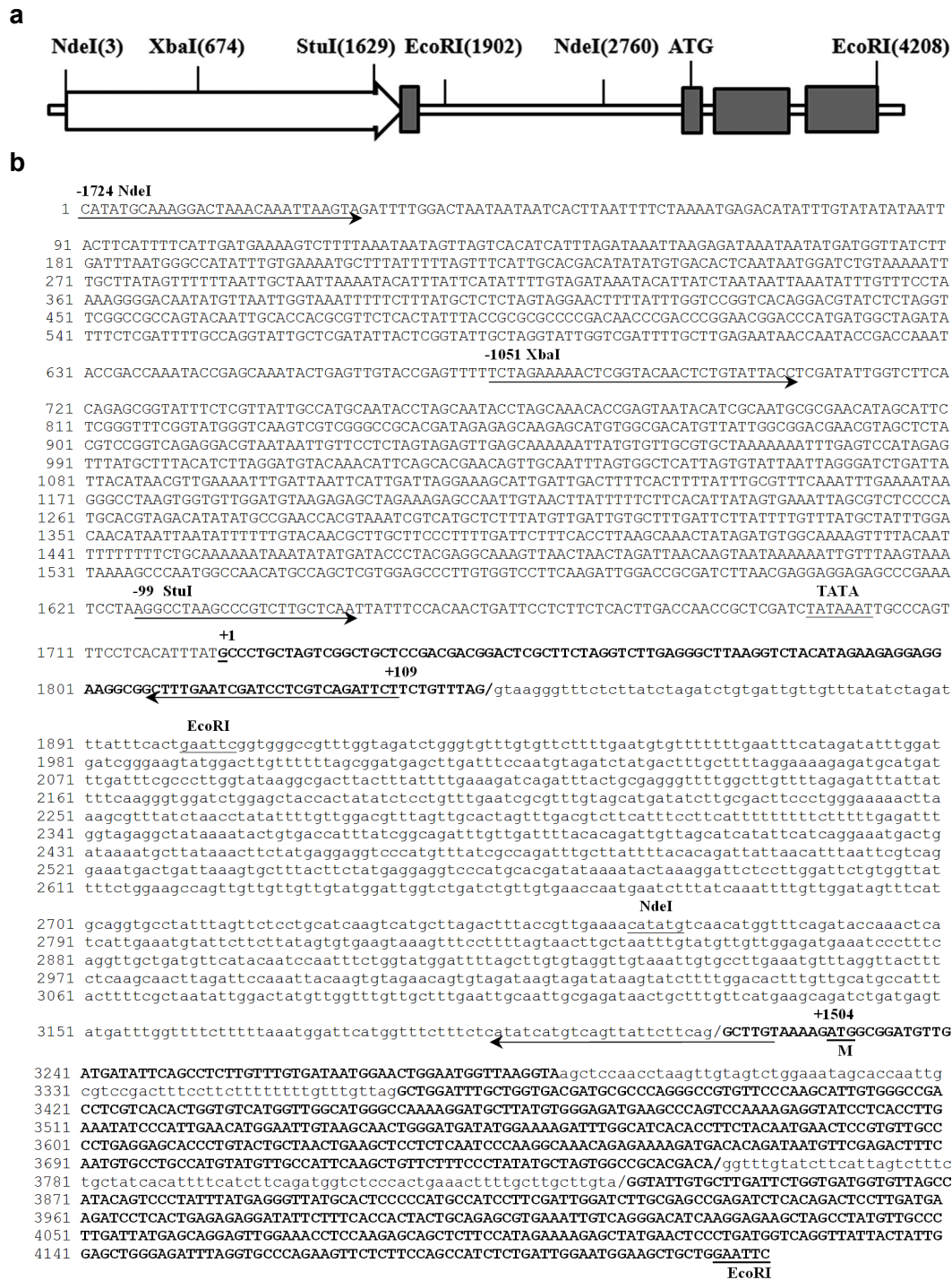


Fig. 3 Physical map and nucleotide sequence of the 5' flanking region of *DmACT1*. (a) Physical map of the 5' flanking region of *DmACT1*. Open arrow and gray boxes indicate the promoter region and exons of *DmACT1*, respectively. ATG indicates the translation initiation site. (b) Nucleotide sequence of the 5' flanking region of *DmACT1* (Genbank accession No. KC171012). Primers used for subsequent construction of promoter truncation vectors are indicated by arrows. The restriction sites used in inverse PCR are underlined. The transcription initiation site is designated as +1 and is underlined. The translation initiation site is indicated by an underlined M. The TATA box is underlined. Bold upper-case and lower-case letters represent exon and intron sequences, respectively. Slashed lines indicate exon-intron boundaries. Note that the first intron resides in the 5' UTR

gift from In-Sun Yoon, RDA, Korea) with its first intron was cloned in the same sites of GUS/Luciferase3300 vector to create *pAct1*. The DNA sequences of all constructs

were confirmed by DNA sequencing analysis. These constructs were used for transient luciferase reporter assays in *Dendrobium* flower cells.

Microprojectile bombardment

Promoter activity of constructs containing various lengths of *DmACT1* 5' flanking sequence were examined by a microprojectile bombardment-mediated transient assay (Whang et al. 2011). Approximately 3.5 µg of gold particles (0.6 µm, Bio-Rad Laboratory, USA) were coated with 5 µg of DNA construct. As an internal control, 1 µg of CaMV35S promoter-*Renilla* luciferase construct (a kind gift from H. G. Nam, DGIST, Korea) was co-bombarded. Flowers were bombarded with the DNA construct-coated gold particles at a helium gas pressure of 1,000 Psi and a target distance of 6 cm.

Analysis of Gus/luciferase activity

The reporter activity of the GUS/luciferase fusion protein was examined as described previously (Koo et al. 2007). Briefly, the bombarded samples were transferred to a 24-well plate containing 0.5 mM D-luciferin or coelenterazine in MS medium, and bioluminescence was measured using a high-performance CCD camera (VersArray system, Roper Scientific Ltd., UK). Images were analyzed by MetaVue version 5.2 software (Universal Imaging Corporation, USA). To normalize the actin promoter activity, the ratio of firefly luciferase activity was calculated and represented by comparison with that of the control *Renilla* luciferase. Data are shown as mean values obtained from three independent microprojectile bombardment experiments. Histochemical staining of GUS activity was performed by overnight incubation in X-GlcA (Duchefa, The Netherlands) substrate solution as described previously (Koo et al. 2007). When necessary, samples were bleached with 70% ethanol.

Results

Molecular cloning of full-length *DmACT1* cDNA

To isolate an *actin* gene from *Dendrobium*, we used a degenerate PCR approach using primers designed to DNA sequences conserved among orchid *actin* genes (See Materials and Methods). We cloned a 513 bp partial cDNA fragment, which we named *DmACT1* (Fig. 1). Then, we isolated and characterized the 1573 bp full-length *DmACT1* cDNA by 5' and 3' RACE PCR. RACE PCR was conducted using the SMARTer RACE cDNA amplification kit that allows the cloning of the full-length sequence of a target transcript. Sequence analysis indicated that *DmACT1* contains a 129 bp 5' UTR, a 1131 bp coding sequence, and a 288

bp 3' UTR (Fig. 1). A database search revealed that the nucleotide and deduced amino acid sequences of *DmACT1* were the most similar to the nucleotide and amino acid sequences of *Phalaenopsis ACT2* (Accession No. AF246715.1) (87% and 99%, respectively).

Expression of *DmACT1* in *Dendrobium*

To determine whether *DmACT1* expression was ubiquitous in *Dendrobium*, we performed RNA gel blot analysis using total RNA isolated from roots, leaves, and flowers. Actins are ubiquitous proteins in plant cells and are encoded by a multigene family (Šlajcherová et al. 2012). Because coding sequences are highly conserved among actin genes, the 5' UTR of *DmACT1* was amplified for use as a probe in RNA gel blot analysis. As shown in Fig. 2, *DmACT1* transcripts were abundantly expressed in all organs that we examined. Although transcript levels were slightly higher in leaf than the other tissues, our results nevertheless indicate that *DmACT1* is ubiquitously expressed in *Dendrobium*.

Isolation of genomic fragments containing the 5' flanking region of *DmACT1*

The 5' flanking sequences of *DmACT1* were isolated by two rounds of inverse PCR. Circularized genomic DNA digested with *Eco* RI was used as a template in the first round of inverse PCR. However, a sequence comparison with the full-length *DmACT1* cDNA revealed that the isolated genomic clone contained only the exon-intron boundaries and the truncated form of the intron without the sequence corresponding to the 5' UTR (Fig. 3). Thus, we isolated upstream sequences by a second round of inverse PCR using circularized genomic DNA digested with *Nde* I. Figure 3 shows the full-length sequence of the isolated genomic clone. Sequence analysis revealed that the genomic clone contained a 3216 bp region upstream of the coding sequence of *DmACT1*. Because the nucleotide sequence of the full-length *DmACT1* cDNA matched the base at position 1725 exactly, this was designated +1 as the putative transcription start site. In a motif search, a TATA box sequence was found between bases -29 and -23. The translation initiation codon was found at position +1504 in the second exon. Interestingly, the 5' UTR of *DmACT1* contains a 1374 bp long intron from +119 to +1492. However, the 5' UTR-intron of *DmACT1* is somewhat longer than that reported in other plant species (Bradnam and Korf 2008), and shared no sequence similarity with known sequences in a database search.

DmACT1 promoter analysis by GUS/luciferase reporter transient assays

5' UTR-introns can enhance promoter activity in animals and plants (Bradnam and Korf 2008). Thus, we generated four promoter constructs with or without the 5' UTR-intron to examine the promoter activity of the 5' flanking sequence of *DmACT1*.

For analysis of promoter activity, choice of reporter system is very important to avoid artifacts arising from the stability or multimerization of reporter protein. We previously demonstrated that the GUS/luciferase fusion protein has the advantages of both GUS and luciferase proteins, namely high resolution histochemical GUS staining and real-time bioluminescence imaging with a luciferin-dependent rapid turn-over rate (Koo et al. 2007). Hence, we used the GUS/luciferase fusion reporter system for analysis of 5' flanking sequence of *DmACT1*. As shown in Figure 4, a 2778 bp fragment of the flanking sequence of *DmACT1* 5' (from -1724 to +1054) was fused to the coding sequence of the GUS/luciferase reporter gene to create *pDmACT1Nd*. To examine the effect of the 5' UTR-intron on gene expression, we fused the 1833 bp fragment (-1724 to +105) lacking the 5' UTR-intron to the coding sequence of the reporter gene to create *pDmACT1Nd Δ int*. Similarly, *pDmACT1Xb* and *pDmACT1St* constructs containing 2105 bp (-1051 to +1054) and 1153 bp (-99 to +1054) fragments of the 5' flanking sequence of *DmACT1*, respectively, were generated by fusion with the coding sequence of Gus/luciferase.

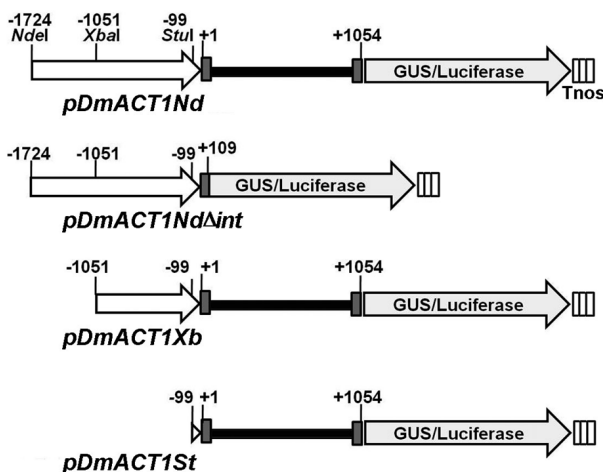


Fig. 4 Physical map of the *DmACT1* 5' flanking sequence-GUS/luciferase fusion constructs. Various portions of the 5'-flanking and/or 5'-noncoding UTRs of the *DmACT1* gene were fused to the coding sequence of a GUS/luciferase fusion reporter gene. White arrows show the promoter region. Gray and black boxes represent the exon and 5'UTR intron, respectively

The flower is a very important plant characteristic in the floricultural industry and genetic manipulation of orchids. Therefore, many researches primarily target the improvement of flower traits. RNA blot analysis indicated that *DmACT1* is also highly expressed in flowers (Fig. 2). Thus, we analyzed the promoter activity of the 5' flanking sequence of *DmACT1* in flowers of *Dendrobium* by transient expression assays.

Since transient expression system was highly affected by DNA concentration, efficiency of bombardment and conditions of plant samples, *CaMV35S* promoter-*Renilla* luciferase construct was used in the experiment as an internal control to normalize the promoter activity. An unbombarded sample was used as a negative control. The rice actin promoter (*pAct1*), which is widely used in monocot plants, was also included in the experiment to compare promoter activities. The promoter activity of each bombarded sample was monitored by the time-lapse bioluminescence imaging using a high-performance CCD camera. Then, the value of firefly luciferase activity driven by actin promoter constructs was normalized with that of *Renilla* luciferase driven by *CaMV35S* promoter. As shown in Figure 5, flowers bombarded with *pDmACT1Nd* and *pDmACT1Xb* containing the 5' UTR-intron showed high levels of luciferase activity, although *pDmACT1Nd* had 1.5-fold higher activity than *pDmACT1Xb*. Luciferase activity driven by *pDmACT1Nd* and *pDmACT1Xb* increased gradually after bombardment, and steady-state levels were reached after 28 to 30 h of incubation. Furthermore, *pDmACT1Nd* showed 8- to 10-fold higher luciferase activity than the rice actin promoter (*pAct1*). However, flowers bombarded with *pDmACT1Nd Δ int* displayed undetectable levels of luciferase activity. These results suggest that the 5' UTR-intron is necessary for efficient promoter activity of *DmACT1*. Flowers bombarded with *pDmACT1St*, which lacks the large portion of promoter region, showed near basal-levels of luciferase activity.

The promoter activity of the 5' flanking sequence of *DmACT1* was further confirmed by histochemical GUS staining. Similar with luciferase activity, the strong histochemical GUS stains were observed in flowers bombarded with *pDmACT1Nd* and *pDmACT1Xb*, whereas no detectable activity was observed in flowers bombarded with *pDmACT1Nd Δ int* and *pDmACT1St*. These results confirm that the 5' UTR-intron is necessary for efficient promoter activity of *DmACT1*. As expected, the rice Act1 promoter (*pAct1*) displayed weaker GUS stains than the *DmACT1* 5' flanking sequence. Taken together, our results demonstrate that the 5'-flanking sequence of *DmACT1* can promote strong gene expression in *Dendrobium*.

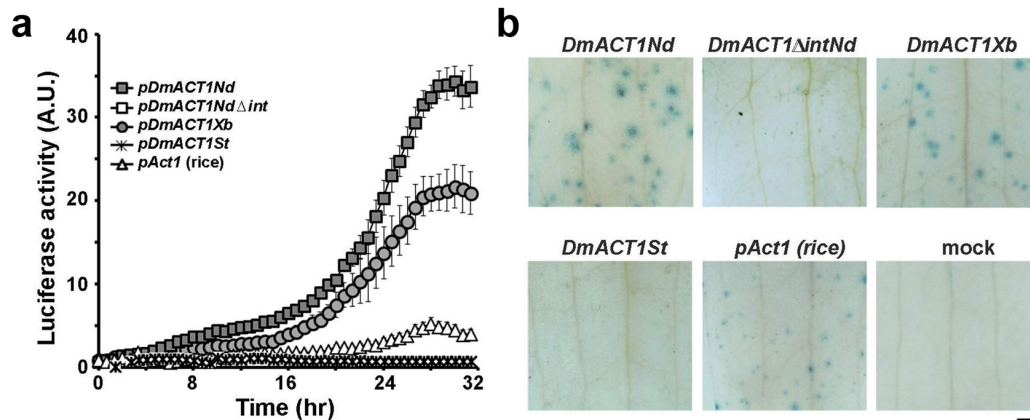


Fig. 5 Analysis of transient GUS/luciferase activity driven by various portions of the *DmACT1* 5'-flanking sequence in *Dendrobium* flowers. Each construct was co-bombarded with pCaMV35S-*Renilla* luciferase. Expression of the promoter from the rice actin promoter (*pAct1*) in *Dendrobium* was also evaluated for comparison purposes. (a) Relative luciferase activity in bombarded flowers. Bombarded sample was divided into two parts and transferred to medium containing luciferin and coelenterazine in a 24-well plate. Bioluminescence was measured using a high-performance cool CCD every 40 min for 32 hr. The values were subtracted from those of unbombarded samples (mock) and normalized relative to the *Renilla* luciferase activity. Note that *pDmACT1NdΔInt* and *pDmACT1St* showed background levels of bioluminescence. Bars indicate SD of the mean value ($n=3$). (b) GUS histochemical staining obtained after 32 hr of incubation. Representative images from three independent experiments are shown. The scale bar indicates 1 mm

Discussion

Promoters that drive strong and constitutive gene expression are important for plant biotechnology applications. Several promoters have been developed for use in monocot transformation including maize *Ubi1* (Elliott et al. 1999) and rice *Act1* (McElroy et al. 1990). Although these promoters drive strong expression of genes in rice, they show lower activity than the CaMV 35S promoter in some monocot plants (Kamo et al. 1995; Wilmink et al. 1995). This is also the case in *Dendrobium*. GUS expression driven by the *Ubi1* promoter was lower than that of GUS expression driven by the *CaMV 35S* promoter in *Dendrobium sonia* (Tee et al. 2003). Furthermore, the efficiency of the *Act1* promoter in orchids, including *Dendrobium*, has not been evaluated. Actin genes belong to a multigene family in plants, with some actin isoforms constitutively and strongly expressed in plants (An et al. 1996; McElroy et al. 1990; Šlajcheroová et al. 2012). In this study, we demonstrated that *DmACT1* expression is ubiquitous and abundant in the flowers, leaves, and roots of *Dendrobium* by RNA blot analysis (Fig. 1). Thus, we isolated the *DmACT1* promoter to determine if it could drive strong expression of a reporter gene in *Dendrobium*.

We isolated the 5'-flanking sequence of *DmACT1* by two round of inverse PCR. Sequence analysis indicated that it contained a 1374 bp intron in the 5' UTR region. To examine if this region had promoter activity, we fused various lengths of *DmACT1* 5' flanking sequence to a GUS/luciferase fusion reporter (Koo et al. 2007) to monitor

promoter activity by real-time luciferase imaging and to assess GUS activity by histochemical staining. We also utilized a transient expression system for promoter analysis. Although cellular expression patterns can be determined in stable transgenic plants in contrast to transiently transfected plants, undesirable effects may occur in measurement of promoter activity due to the presence of flanking sequences in the T-DNA insertion site, multiple gene copies, and silencing or co-suppression of genes. Transient GUS/luciferase expression analysis revealed that the *DmACT1* 5' flanking sequence had 8- to 10-fold higher promoter activity in the flower organs of *Dendrobium* orchids than the rice actin promoter (*pAct1*). Hence, the *DmACT1* promoter can be used to drive strong ectopic expression of transgenes in *Dendrobium*.

It has been shown that some 5' UTR-introns contribute to high levels of transgene expression in both dicot and monocot transgenic plans including *Arabidopsis*, maize, and rice (Bradnam and Korf 2008; Jeon et al. 2001; Lu et al. 2008; Zhang et al. 1991; Rose and Last 1997). Although the underlying mechanism remains unclear, it has been shown that the effect of the 5' UTR-intron decreases gradually as its distance from the translation initiation site increases (Rose 2004). Furthermore, the 5' UTR-intron does not drive gene expression when located in the 3' UTR. Second, the IME effect of the 5' UTR-intron is not general to all organisms. The rice *Act1* intron and the maize *Ubi1* intron greatly enhances gene expression in rice, but have negligible effects in non-cereal monocot plants (Kamo et al. 1995; Tee et al. 2003; Wilmink et al.

1995). Moreover, the combination of the CaMV35S promoter and maize *Adh1* 5' UTR-intron resulted in decreased GUS expression in the protoplasts of sugarcane (Wilmink et al. 1995), whereas addition of the maize *shrunk-1* locus 5' UTR-intron to the same promoter resulted in greatly increased CAT gene expression in the protoplasts of various grass species (Vasil et al. 1989). Third, IME differs between plant and animal cells. Highly expressed genes have a shorter 5' UTR-intron in animal cells than plant cells, and vice versa (Castillo-Davis et al. 2002; Ren et al. 2006). Fourth, the effect of IME may be due to transcriptional or post-transcriptional events. In some cases, IME has been shown to be related to increased enzyme activity or protein accumulation rather than increased mRNA levels (Bourdon et al. 2001; Kamo et al. 2012; Mascarenhas et al. 1990; Samadder et al. 2008; Rose 2004). Moreover, the effect of the 5' UTR-intron of rice ubiquitin (*rub3*) on promoter activity has been shown to be tissue- and cell-type specific (Lu et al. 2008). Thus, it would be interesting to explore the IME mechanism of the 5' UTR-intron in *DmACT1* in future studies. Taken together, our data suggest that the *DmACT1* promoter will be of great use in orchid biotechnology as it drives high levels of transgene expression, and represents a good genetic resource for the development of even more efficient orchid promoters.

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