Kasetsart J. (Nat. Sci.) 49 : 174 - 187 (2015)

An Analysis of *cis*-Acting Regulatory Elements Related to Light Response in the 5' Flanking Region of the *Ascocenda* and *Dendrobium* Actin Genes

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ABSTRACT

In a previous study, the rice actin promoter was considered as a constitutive promoter, which could drive reporter gene expression in orchid flowers. Therefore, in this study, the 5' flanking regions of the actin genes isolated from *Ascocenda* Princess Mikasa 'Blue' and *Dendrobium* Jacquelyn Thomas 'UH44-50' orchids were validated for predicted *cis*-acting regulatory elements (CAREs) using the PlantCARE program and the PLACE database. A high frequency of predicted CAREs, which were distinct in numbers and locations, particularly the CAREs that respond to light, was found within the upstream flanking regions of the actin gene in orchids, rice and banana. Both orchid upstream sequences contained some predicted CAREs that are predominantly involved in light regulation. When orchid flowers were exposed to different light periods, the expression of vegetative actin in *Ascocenda* flower tissues (column, lip and petal) was completely up-regulated, as determined by qualitative real-time polymerase chain reaction. In addition, all of the 5' flanking regions of orchid, rice and banana actin genes contained large numbers of the transcriptional control sequences, TATA boxes, which were composed of similar sequences to the conserved TATA box of vegetative actin. The further investigation of the 5' flanking region of orchid actins for their activities will be essential in the development of these promoter regions for biotechnological use.

Keywords: promoter, actin, *Cis*-acting element, light response, orchid transformation

INTRODUCTION

The actin promoter, which was first isolated from rice, was successfully developed for plant expression vectors that are commonly used in monocotyledonous plant transformations. (McElroy *et al.*, 1990; Zhang *et al.*, 1991; Vickers *et al.,* 2006). Subsequently, more actin promoters were isolated from both monocotyledons and dicotyledons, including arabidopsis actin 1, actin 3, actin 4, actin 11 and actin 12 (An *et al*., 1996b; Huang *et al.*, 1996, 1997), banana actin 1 (Hermann *et al.*, 2001) and rice actin 2 (He *et al.*, 2009). To develop constitutive promoters to drive foreign genes, functional analyses of these promoter regions have been conducted in terms of their effects on differential expression and tissue specificities (McElroy *et al.*, 1990, 1991; Reece *et al.*, 1990; Himmelbach *et al*., 2010; Park *et al.*, 2010; Li *et al*., 2013). The tissue-specific properties of these actin genes have allowed for their classification as isovariant molecules

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Received date : 29/05/14 Accepted date : 29/08/14

(McDowell *et al*., 1996; Kandasamy *et al*., 2002) within vegetative and reproductive classes based on expression patterns and phylogenetic attributes (McElroy *et al*., 1990; Slajcherova *et al*., 2012). Consequently, general functional regulators located upstream of the transcription initiation sites of the actin promoter, which are required for maximal gene expression in transformed rice, were identified as small introns that interrupt the coding sequences, such as leader sequences (leader Exon1) or small blocks of *cis*-acting regulatory elements (CAREs) (McElroy *et al*., 1990; An *et al*., 1996a; Huang *et al*., 1996, 1997; He *et al*., 2009; Zhang *et al*., 1991; Park *et al*., 2010). Apart from those conserved elements, two other essential regulatory elements are poly (dA-dT) and CCCAA repeat regions that positively and negatively regulate actin promoter activity, respectively (McElroy *et al*., 1990, 1991; Zhang *et al*., 1991).

In transient over expression experiments, the rice actin 1 promoter was shown to drive the expression of reporter genes in the flower petals (Suwannaketchanatit *et al*., 2006) and in the protocorm like-bodies (Suwannaketchanatit *et al*., 2007) of *Dendrobium* orchids. Growing demand for novel desirable trait improvements in orchids via gene transfer technology prompted the isolation of the actin promoters from two cultivated orchid species, *Ascocenda* sp. and *Dendrobium* spp., which represent major economic crops in Thailand. The current study reports the cloning and characterization of the 5′ flanking upstream sequences of the actin gene from *Ascocenda* Princess Mikasa 'Blue' and *Dendrobium* Jacquelyn Thomas 'UH44-50'. All of the predicted CARE motifs within these regions—whether conserved or unique—have been investigated and characterized for their structures and organization compared with the CAREs observed in the rice and banana actin genes. This study contributes to an understanding of the genetic variation found in the regulatory regions of actin promoters of monocotyledonous orchid plants. The results suggest the possibility that these upstream sequences represent potential candidate promoters for biotechnological use.

MATERIALS AND MATHODS

Plant materials

Ascocenda Princess Mikasa 'Blue' (*Vanda* Alliance Ascda Royal Sapphire × *Vanda* coerulea) and *Dendrobium* Jacquelyn Thomas 'UH44-50' (*Dendrobium gouldii* × *Dendrobium phalaenopsis* 'Lyon's Light No.1 were purchased from an orchid nursery in Bangkok, Thailand. To isolate the actin promoter, genomic DNA was extracted from the first fully expanding young leaves of 1 yr-old *Ascocenda* Princess Mikasa 'Blue' and *Dendrobium* Jacquelyn Thomas 'UH44-50' using the CTAB method (Murray and Thomson, 1980).

Isolation of the 5′ flanking regions of the actin gene

Thermal Asymmetric Interlaced-Polymerase Chain Reaction technique

The 5′ flanking regions of the actin genes were amplified from *Dendrobium* genomic DNA using the Thermal Asymmetric Interlaced-Polymerase Chain Reaction (TAIL-PCR) method as described by Liu *et al.* (1995). Fifty nanograms of orchid gDNA was amplified in a 25 µL PCR reaction containing 1X buffer, 0.3 mM of each dNTP, 0.5 µM of the forward primer at the -3,000 base pairs (bp) position of rice actin 1 (PAct-F-3000), 0.5 µM of the reverse primer complementary to position 429 of the coding sequence of rice actin 1 (Act-R-429) and 0.5 units of KAPA HiFi Fidelity DNA Polymerase (Kapa Biosystems; Wilmington, MA, USA). Next, the primary PCR products were diluted 40-fold prior to being used in a secondary PCR reaction using nested inner forward (PAct-F-2500) and reverse (Act-R-429) primers (Figure 1a and Table 1). The thermal cycling conditions of the primary and secondary PCR are shown in Table 2.

Hi-efficiency Thermal Asymmetric Interlaced-Polymerase Chain Reaction technique

The primers used in the hi-efficiency TAIL-PCR (hiTAIL-PCR) reactions (Liu and Chen, 2007) were developed by replacing random degenerated nucleotides in the long arbitrary degenerated (LAD) primer with repetitive and frequently found interspersed sequences from rice actin 1. In this study, two nested, reverse-specific primers, with high melting temperatures (Tm) of approximately 70 °C, were designed from the conserved sequences within the coding of *Ascocenda* actin gene (accession no. HQ596370). hiTAIL-PCR reactions were performed using the initially amplified 5' flanking regions of the actin gene from both orchids using forward primers (PAct-F-LAD1) and a gene-specific reverse primer

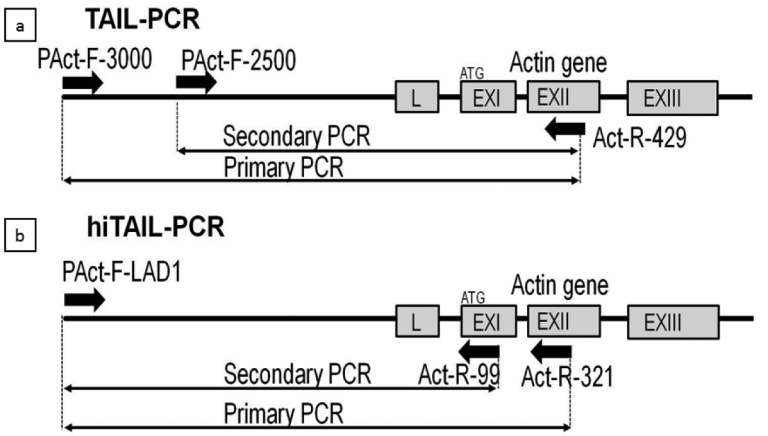

Figure 1 Schematic diagrams of the structure of the 5' flanking regions of the actin gene and the two sets of primers for: (a) Thermal Asymmetric Interlaced-Polymerase Chain Reaction (TAIL-PCR); and (b) hi-efficiency TAIL-PCR (hiTAIL-PCR). The boxes indicate exons (EX) and the 5' leader sequence (L). Lines indicate the untranslated regions. The relative positions of the PAct-F (forward primer for actin promoter) and Act-R (reverse primer specific to actin gene) primers are indicated according to the translational start site (ATG) of the actin gene. The primers in each group are divided between two types for primary and secondary polymerase chain reaction (PCR) reactions as indicated by the double-headed arrows.

Table 1 List of primer sequences used for Thermal Asymmetric Interlaced-Polymerase Chain Reaction (TAIL-PCR) and hi-efficiency TAIL-PCR (hiTAIL-PCR).

Primer name	Primer sequences	Technique
PAct-F-3000	5′-CCA TAT AAG TTC CCT TGT CG-3′	TAIL-PCR
PAct-F-2500	5′-ACC TTG GCA TGT ATA TTC GG-3′	TAIL-PCR
PAct-F-LAD1*	5′-GAG AAG AAC TGG ATC GCG GCC GCH NHN NNC TAT-3′	hiTAIL-PCR
Act-R-429	5′-AGA GAG CAC AGC CTG AAT GG-3′	TAIL-PCR
Act-R-99	5′-GAA GAC AGC CCT GGG AGC ATC ATC-3′	hiTAIL-PCR
Act-R-321	5′-GAG AAG AAC TGG ATG TTC TTC TGG GGC GAC ACG AAG CTC ATT GTA-3′	hiTAIL-PCR

*PAct-F-LAD1 contains a consensus nucleotide with primer Act-R-32 1 (bolded) and repetitive sequence from rice Act1 (underlined). The recognition site for *Not*I (GC^GGCCGC) is boxed.

(Act-R-321). Next, a secondary PCR reaction was performed using the primary PCR products with PAct-F-LAD1 and the gene-specific reverse primer (Act-R-99) (Table 1 and Figure 1b). The thermal cycling conditions of the primary and secondary PCR reactions are shown in Table 2. Large PCR products amplified from both orchids, over 1,000 base pairs (bp), were gel extracted using a HiYield Gel/PCR DNA Fragment Extraction kit (RBC Bioscience, Taiwan), cloned into the pGEM®-T easy vector (Promega; Madison, WI, USA) and transformed into XL1-blue *Escherichia coli*. The positive clones were sequenced using DNA Automated Sequencing (Marcrogen, Korea) with vector-specific SP6 and T7 primers.

Sequence analysis of the actin promoter

The DNA sequences of the 5' flanking region of the actin genes were analyzed using the Basic Local Alignment Search Tool with the nucleotide collection (nr/nt) database (http://blast.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997) and alignment by T-coffee (http://tcoffee.crg.cat/apps/tcoffee/index.html; Tommaso *et al.*, 2011). First, the non-coding exon (Leader exon) was predicted using NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/; Hebsgaard *el al*., 1996). The *cis*-acting elements within the actin regulatory sequences were found to contain putative *cis*-acting elements using online software PlantCARE (plant *cis*-acting regulatory element; http://bioinformatics.psb.ugent.be/webtools/plantcare/html; Lescot *et al.,* 2002) and PLACE (a database of plant *Cis*-acting regulatory DNA elements; http://www.dna.affrc.go.jp/PLACE/; Higo *et al.*, 1999). The sequences of the 5′ flanking regions of the orchid actin genes were compared to the regulatory element sequences of the reported rice actin 1 gene.

Gene expression analysis by qualitative real-time polymerase chain reaction

Total RNAs were immediately extracted from three tissues (column, lip and petal) of fully opened orchid flowers, which were collected from the nursery at 0800 hours (2,430 lux intensity), 1400 hours (5,280 lux) and 2000 hours (0 lux)

Table 2 Thermal conditions for polymerase chain reaction (PCR) used in this study.

Reaction	Step	# Cycle	Denaturation temperature (°C) /time (min:s)	Annealing temperature (°C) /time (min:s)		Extension temperature (°C) /time (min:s)
				TAIL-PCR	hiTAIL-PCR	
Primary PCR	1	1	95/5:00	-	-	-
	2	10	98/0:30	57/0:30	72/0:30	72/2:00
	3	1	98/0:30	27/0:30*	25/0:30*	72/2:00
	4	2	98/0:30	57/0:30	72/0:30	72/2:00
	5	1	98/0:30	48/0:30	68/0:30	72/2:00
	6	9	Go to step 4			
	7	1	-	-		72/5:00
Secondary PCR	1	1	95/5:00	-	-	-
	2	2	98/0:30	57/0:30	72/0:30	72/2:00
	3	1	98/0:30	52/0:30	53/0:30	72/2:00
	4	9	Go to step 1			
	5	1	-	-		72/5:00

TAIL-PCR =Thermal Asymmetric Interlaced-Polymerase Chain Reaction; hiTAIL-PCR = hi-Efficiency TAIL-PCR; # = Number of; * = Ramping temperature rate set to 0.3 °C/s

using the lithium chloride (LiCl) extraction method (Lievens *et al.*, 1997) and were treated with RNase-free DNase-I (Promega, USA). Five micrograms of the total RNAs was reverse transcribed into cDNA using Act-R (5′-GCT CCT GTT CAT AGT CCA A-3′) and 5.8S rRNA-R (5′-GCT TGA AGC CCA GGC AGA CG-3′) primers and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare; Little Chalfont UK). Next, the 218 bp corresponding to the actin genes were amplified using the following primers: Act-F (5′-ATT CTG GTG ATG GTG TTA GC -3′) and Act-R. In addition, the 198 bp of *5.8S rRNA,* the reference gene, was amplified by 5.8S rRNA-F (5′-ATG ACT CTC GAC AAT GGA TTT-3′) and 5.8S rRNA-R using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems; Wilmington, MA, USA). Each reaction, which contained 0.2 µL of cDNA template and 0.625 µM of each primer in a final reaction volume of 10 µL, was performed in triplicate to ensure the reproducibility of the results. The qualitative real-time polymerase chain reactions **(**qPCRs) were performed using the following conditions: denaturing for 2 min at 95 °C followed by 35 cycles of amplification with 1 s of denaturing at 95 °C, 20 s of annealing and extension at 58 °C using the Eppendorf Mastercycler® ep realplex^4 S real-time PCR instrument (Eppendorf; Hauppauge, NY, USA).

RESULTS

Primary structure of the 5′ flanking regions of orchid actin genes

The modified TAIL-PCR and hiTAIL-PCR reactions, which primed the 5'-end of the actin promoter with specific forward primers designed from the rice actin1 promoter sequences (PAct-F-3000, PAct-F-2500 for TAIL-PCR), modified forward primers (PAct-F-LAD1 for hiTAIL-PCR) and specific reverse primers for the *Ascocenda* actin sequences, successfully amplified the 5′ flanking regions of the actin genes from *Ascocenda* and *Dendrobium* gDNA, respectively. The primary PCR products were smeared with various sizes of randomly amplified products. The subsequent secondary PCR reactions amplified more selective, non-smeared products, with amplicons ranging in size from 260 to 2,800 bp. Large PCR fragments of 1,260 bp and 1,120 bp were obtained from the secondary PCR reactions using *Ascocenda* and *Dendrobium* gDNA by TAIL- and hiTAIL-PCR, respectively. Their sequences were submitted to GenBank under the accession number HQ596371 for the *Ascocenda* DNA fragment containing 744 bp upstream of the translational start site and KJ690614 for the *Dendrobium* DNA fragment containing 928 bp upstream of the translational start site.

Sequence analysis of the 5′ flanking region

By pairwise comparison, the upstream sequences of the *Ascocenda* and *Dendrobium* actin genes contained no significant identity, with less than 5% sequence similarity to any previously described genes or promoters and the other related DNA sequences deposited in GenBank. Therefore, to understand the organization of the regulatory region of the orchid actin genes, the 5′ flanking upstream sequences of the actin genes obtained from *Ascocenda* actin 1 (HQ596371) and *Dendrobium* actin 1 (KJ690614) were aligned with those of the rice (S44221) and banana (AF285176) actin 1 genes. The results revealed that most of the sequences exhibited a significantly distinct genetic relationship, except for short conserved sequences located within two exon regions, as shown in Figure 2.

Prediction of *cis*-acting regulatory elements within the 5′ flanking regions of the orchid actin genes

The predicted CAREs within the 5′ flanking regions of the *Ascocenda*, *Dendrobium*, rice and banana actin 1 promoters were identified by PlantCARE analysis. A maximum number of 21 total CARE types were found within the rice actin 1 promoter, whereas significantly less

CARE types were found within the *Ascocenda* and *Dendrobium* actin 1 promoters (Table 3). By searching for enriched CARE sequences, the most frequently predicted CAREs were light response CARE types associated with stress responsiveness. The *Ascocenda* and banana actin 1 promoters contained 3 Box4 (ATTAAT) elements, whereas the rice actin 1 promoter contained 17 Sp1 (CC(G/A)CCC) elements. Neither orchid actin 1 promoter contained Sp1 motifs. Both the Box4 elements within the orchid actin 1 promoter and the Sp1 element within the rice actin 1 promoter acted as light-responsive elements during stress response.

Distribution of CAREs within the actin promoter

The distribution of all of the predicted CAREs was mapped within the 5′ flanking region in 100-bp increments and was observed within the -1 to -600 regions, which might represent the proximal gene regulatory region of the actin promoters. The rice actin 1 sequences within the -400 to -600 bp region appeared to contain an increased density of CAREs associated with stress-induced light response. Unlike the rice actin 1 promoter, both the orchid and banana actin 1 promoters contained significantly less CAREs within these upstream regions (Figure 3). Among the numerous potential CAREs identified within these actin 1 promoters, they appeared particularly relevant for regulation of the actin gene under daily, regular and periodic light exposure periods, including a regular 24 hr-light condition. Therefore, qPCR was used to approximate the expression of the actin gene during specific light exposure periods, which is rapid and easy for functional analysis of the actin promoter.

Actin gene expression over specific photoperiods at 8:00 am, 2:00 pm and 8:00 pm

The existing predicted light responsive elements within upstream regulatory sequences might be associated with actin expression. To confirm this possibility, qPCR was employed to quantify the level of actin expression in various orchid flower tissues (petal, lip and column)

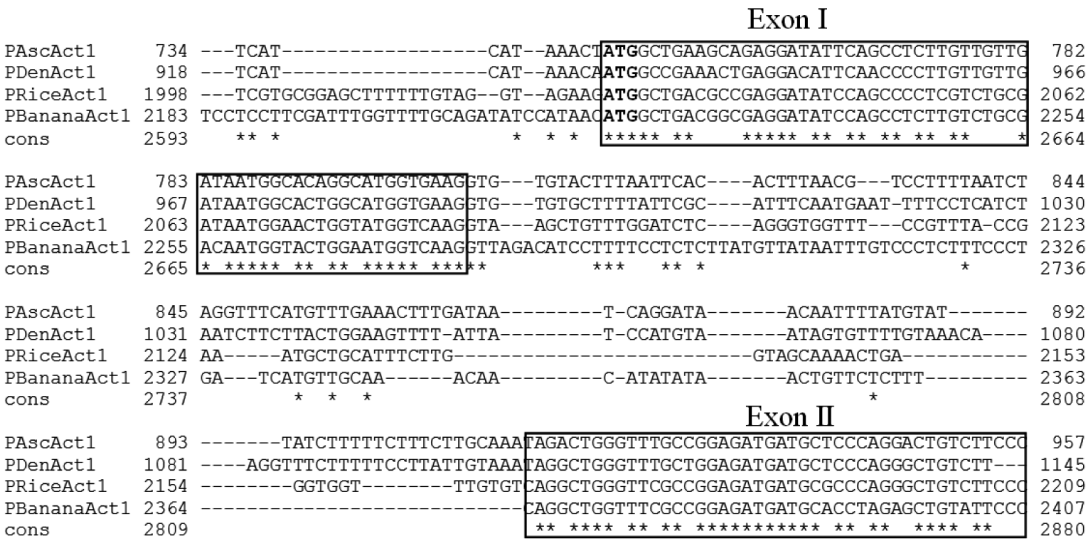

Figure 2 Conserved sequence alignments of the four 5' flanking upstream sequences of actin genes isolated from *Ascocenda* (PAscAct1), *Dendrobium* (PDenAct1), rice (PRiceAct1) and banana (PBananaAct1). The actin exons are boxed. The identical residues are indicated by *, strongly homologous residues by :, weakly homologous residues by . and no consensus residues by -.

Table 3 Predicted *cis*-acting regulatory elements present in upstream sequence of actin promoters isolated from *Ascocenda* (Asc), *Dendrobrium* (Den), *Oryza sativa* L. (rice), *Musa* spp. (banana).

Name	Sequence	Asc	Den	Rice	Banana	Function	Group*
5′-UTR Py-rich stretch	TTTCTTCTCT	-	1	1	-	Conferring high transcription levels	D
CAT-box	GCCACT	-	-	1	-	Meristem expression	D
O2-site	GATGACATGA	1	1	1	-	Zein metabolism regulation	D
Skn-1_motif	GTCAT	-	-	-	1	Endosperm expression	D
ABRE	TACGTG	-	-	1	-	Abscisic acid (ABA) responsiveness	H
CGTCA-motif	CGTCA	-	-	1	1	MeJA-responsiveness	H
GARE-motif	AAACAGA	-	1	-	-	Gibberellin-responsive element	H
Motif-IIb	CCGCCGCGCT	-	-	1	-	Abscisic acid (ABA) responsive element	H
TCA-element	GAGAAGAATA	-	-	1	1	Salicylic acid responsiveness	H
TGACG	TGACG	1	-	-	-	MeJA-responsiveness	H
ARE	TGGTTT	1	-	2	3	Anaerobic induction	S
GC-motif	CCCCCG	-	-	1	-	Anoxic specific inducibility	S
HSE	AAAAAATTTC	-	1	-	-	Heat stress responsiveness	S
LTR	CCGAAA	1	-	-	-	Low temperature responsive element	S
MBS	CAACTG	-	-	1	2	MYB binding site involved in drought-inducibility	S
W -Box	TTGACC	-	-	-	2	Fungal elicitor responsive element	S
ACE	**AAAACGTTTA**	**-**	**-**	**1**	**-**	**Light responsiveness**	**L****
ATC-motif	**TGCTATCCA**	**-**	**-**	**1**	**-**	**Part of light responsiveness**	**L**
BOX 4	**ATTAAT**	**3**	**-**	**-**	**3**	**Part of light responsiveness**	**L**
GAG-motif	**GGAGATG**	**-**	**1**	**1**	**-**	**Part of a light responsive element**	**L**
GATT-motif	**CTGCAGATTTCT**	**-**	**-**	**-**	**1**	**Part of a light responsive element**	**L**
G-box	**TACGTG**	**-**	**-**	**1**	**2**	**Light responsiveness**	**L**
I-box	**GATAAGATA**	**-**	**-**	**-**	**1**	**Part of a light responsive element**	**L**
SP1	**CC(G/A)CCC**	**-**	**-**	**17**	**1**	**Light responsive element**	**L**
TGG-motif	**TGGTGGCTA**	**-**	**1**	**-**	**-**	**Part of a light responsive element**	**L**

Name	Sequence	Asc	Den	Rice	Banana	Function	Group*
AAGAA-motif	GAAAGAA	-	1	1	1	Unknown	U
AC-I	(T/C)C(T/C)(C/T) ACC(T/C)ACC	-	-	1	-	Unknown	U
AC-II	(C/T)T(T/C)(C/T) (A/C)(A/C)C(A/C)A (A/C)C(C/A)(C/A)C	-	-	1	-	Unknown	U
ATGCAAAT-Motif	ATACAAAT	-	1	-	-	Associated with the TGAGTCA motif	U
MPE	AAAAAAAAAAA AAGAAAGAAAA AAAAGAAAAAG AAAAA	-	-	1	-	Unknown	U
TATCCAT/C-motif	TATCCAT	-	-	-	1	Unknown	U
CAAT	CAAT***	6	4	4	6	Common CARE in promoter and enhancer regions	C
TATA	TATA****	10	12	18	20	Core promoter element around -30 of transcription start	C
Total types of CARE		7	10	21	15		
Total CARE		23	24	58	46		
Promoter length		744	928	1250	1500		
Promoter length/CAREs		32.35	38.67	21.55	32.61		

D = Plant development; H = Hormonal regulation; S = Stress response; L = Light response; U = Unknown function; C = Core promoter.

The light response CAREs are bolded.

*** = CAAT, CAAAT, CAATT, gGCAAT.

**** = ATATAT, TATA, TTTTA, TATAAA, TATAAAT, TATAAATT, ccTATAAAaa, TATACA.

collected from day to night at 0800 hours (low light intensity with 2,430 lux and a short period of light exposure for 2 hr), at 1400 hours (high light intensity with 5,280 lux and a long period of light exposure for 7 hr) and at 2000 hours (no light with 0 lux or dark for 2 hr) under shading conditions. As expected, the qPCR analysis revealed that the actin gene was actively expressed in all three types of orchid flower tissues and was maximally up-regulated when the orchid flower tissues were collected after midday at 1400 hours. Subsequently, the expression of actin in the orchid tissues declined during the dark conditions at 2000 hours (Figure 4).The abundance of actin transcripts detected under high light intensity condition at 1400 hours was significantly ($P < 0.05$) increased compared to during the dark conditions by 150-, 28.7- and 7.5-fold in the column, lip and petal tissues, respectively.

DISCUSSION

Currently, the hiTAIL-PCR procedure has been modified to produce more validated PCR products from non-specific targets (Zhou *et al.*, 2012) compared with conventional TAIL-PCR (Liu *et al.*, 1995). In the current study, both modified TAIL-PCR and hiTAIL-PCR methods were used to amplify larger and more PCR products from *Ascocenda* and *Dendrobium* gDNA, respectively, because the synthesis of small fragments was inhibited by the formation

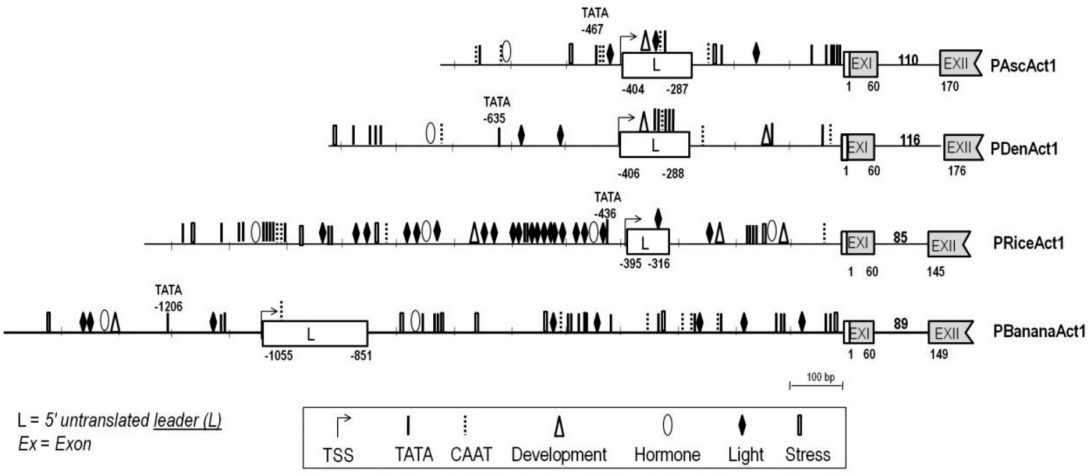

Figure 3 Map of the four actin promoters—*Ascocenda* (PAscAct1), *Dendrobium* (PDenAct1), rice (PRiceAct1) and banana (PBananaAct1) actin promoters. The consensus sequences corresponding to various predicted *cis*-acting regulatory elements described in the text are indicated. The positions are numbered with respect to the first base of the translation start site ATG of the exon (EX). The lines and open boxes indicate untranslated and Leader (L) sequences, respectively.

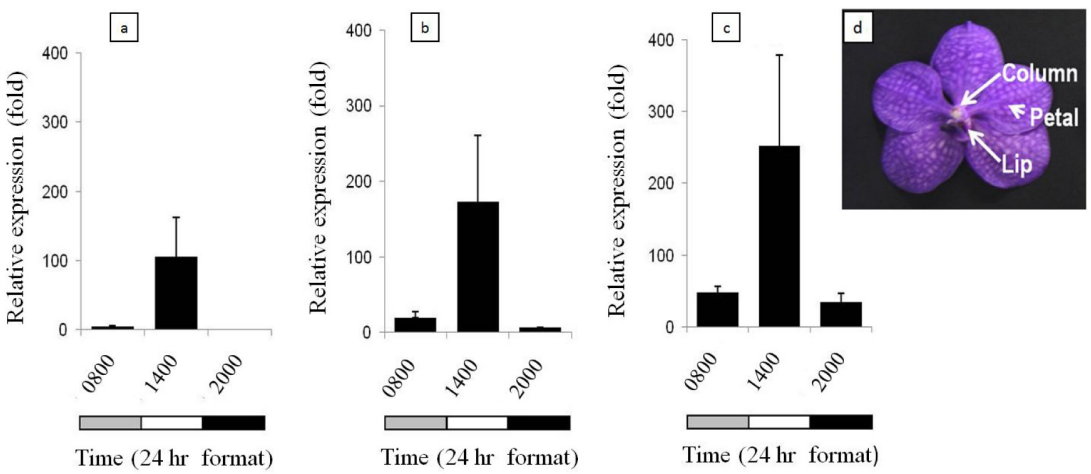

Figure 4 Qualitative real-time polymerase chain reaction validation of differentially expressed actin genes in the *Ascocenda*: (a) Column; (b) Lip; and (c) Petal at 0800 hours, 1400 hours and 2000 hours after emergence during light/dark exposures; (d) Relative position on the orchid flower of the column, lip and petal. The shaded key represents the light and dark periods. The x-axis indicates the time of examination. The y-axis is the relative expression level of the actin gene. Each value represents the mean of three replicates. The error bars indicate the SD of the independent biological replicates. Significant differences are observed in all of the mean expression levels between times in each tissue ($P < 0.05$). *P*-values were calculated using one-way analysis of variance. The calculated $\Delta\Delta^{Ct}$ value of actin expression in orchid columns collected at 0800 hours was used as a reference value of "1".

of hairpin structures and primer annealing during amplification, as previously described by Liu and Chen (2007). Thus, hiTAIL-PCR has been used more successfully to amplify several genes, such as the flowering locus T in *Cymbidium* orchids (Huang *et al.*, 2012).

Comparing nucleotide sequences within the 5′ flanking upstream region of *Ascocenda* and *Dendrobium* actin genes revealed sequence differences from the rice and banana actin 1 promoters. Consequently, the different structures of the predicted CAREs mapping along these promoter regions were evident, although there were significantly matched-motif sequences associated with plant development, hormonal regulation and stress response that were previously identified in the analysis of the arabidopsis actin (Hua *et al.*, 2001) and rice sugar transporter (Ibraheem *et al*., 2010) promoters. Moreover, in the current study, the predominant CARE in the rice actin 1 promoter comprised 17 copies of Sp1 transcription factor binding sites (CC(G/A)CCC) that are involved in the regulation of various house-keeping genes (Raiber *et al.*, 2012) and many other regulator genes (Chen *et al.*, 2013), such as that of the light-regulated RbcS promoter in green microalgae (*Ankistrodesmus convolutus*) (Thanh *et al.*, 2012). However, these regulatory sequences and the conserved Sp1 binding sites were absent within the 5′ flanking region upstream of both orchids' actin 1 genes.

Among the conserved CARE sequences, the predicted Box4 (ATTAAT) sites present within the *Ascocenda* and banana actin 1 promoters are similar to the Box4 motif sequences found in *Arabidopsis thaliana* cytosolic acyl-CoA-binding protein genes (ACBP4 and ACBP5, which are modulated by light/dark cycling (Xiao *et al.*, 2009)). Two additional light-responsive CARE types—the GAG-motif (GGAGATG) and TGG-box (TGGTGGCTA)—were found within the flanking regions of the *Ascocenda* and *Dendrobium* actin 1 promoters, as well as within the phytoene synthase gene promoter of *Oncidium* Gower Ramsey orchids (Lee *et al.*, 2013). There was only one type of O2 CARE site (GATGACATGA) associated with zein metabolism in plant development (Schmidt *et al*., 1992; Wu *et al*., 2010), which was shared among rice, *Ascocenda* and *Dendrobium* actin 1 promoters.

The overall map of the 5′ flanking regions revealed that more TATA boxes than CAAT boxes are located within the upstream translational start site region of all of the promoters examined in this study. In addition, the CAREs associated with plant development were more frequently observed than those associated with hormone response. Actin promoters can be regulated at the proximal regions, which might be associated with tissue-specific activities and activation in most sporophytic cell types and gametophytic pollen tissues (Zhang *et al.*, 1991). In fact, a 38-bp poly (dA-dT) region and a CCCAA pentameric repeat region positioned at -548 to -699 bp of the translational start site represent both positive and negative regulator sites, respectively, of the expression of the rice actin gene (Wang *et al*., 1992). Examination of the length of an efficient regulatory element revealed that the flanking region located at -1 to -376 bp was sufficient for driving the constitutive expression of foreign genes in transgenic rice (He *et al*., 2009). In contrast, the CAREs in the *Ascocenda* and *Dendrobium* actin 1 promoters were distributed across the +1 to -600 bp upstream regions. Thus, these promoters might be regulated by a distal region due to a limitation of sequence length; longer upstream sequences of these promoters might reveal more information regarding CARE distribution.

Sawant *et al.* (1999) previously reported sequence variations within the core promoter, which contributes to determining light-dependent gene expression and leads to the formation of alternative transcription complexes in different physiological settings (Kiran *et al.,* 2006). Based on tissue-specific activity, actin genes have been classified as reproductive and vegetative actins

(McElroy *et al*., 1990; Slajcherova *et al*., 2012). The sequences of each putative TATA box of reproductive actins, such as TATA(T/C)TT in Arabidopsis actin 1/actin 3 (An *et al.*, 1996b), GATAA in Arabidopsis actin 4 and TATTA in arabidopsis actin 12 (Huang *et al.*, 1996), were found to be variable. In contrast, the putative TATA boxes in several vegetative actin promoters exhibited significant sequence similarities, such as TATATA in Arabidopsis actin 2/actin 7/actin 8 (An *et al.*, 1996a; McDowell *et al.,* 1996) and TTAATA in banana actin 1 (Hermann *et al.*, 2001), which are closely related to light-dependent TATA box sequences. All of the putative TATA boxes (ATATAT, TATA, TTTTA, TATAAA, TATAAAT, TATAAATT, ccTATAAAaa, TATACA) of orchid, rice and banana actin 1 promoters reported here were conserved and similar to the vegetative TATA box of vegetative actin promoters (Table 3).

The qPCR results in this study clearly demonstrated that the expression of actin within the column, lip and petal tissues of orchid flowers is regulated by light at the transcription level. The light-activated expression of vegetative actin has been shown to be involved in various cellular functions, such as vegetative growth (Kakinuma *et al.*,2004), plant response to external stimuli, including hormones (Kandasamy *et al.*, 2002), proper cell morphogenesis (Hussey *et al.*, 2006) and organelle adjustment, such as movement of chloroplasts to optimize light absorption (DeBlasio *et al.*, 2005, Krzeszowiec *et al.*, 2007) or stomal movement in arabidopsis (Gao *et al.*, 2008). This phenomenon has also been described in previous reports of blue-light-dependent nuclear positioning that is regulated by the reorganization of the actin cytoskeleton in arabidopsis plants (Iwabuchi *et al.*, 2010).

CONCLUSION

The study cloned and characterized the 5′ flanking upstream sequences of the actin gene from *Ascocenda* Princess Mikasa 'Blue' and *Dendrobium* Jacquelyn Thomas 'UH44-50 for predicted CARE motifs. Taken together, light-regulated CAREs are present along the 5′ flanking sequences of the orchid actin 1 genes and likely account for the observed increase in expression levels during exposure to light. However, the sensitivity of light CAREs identified within the actin promoters in terms of gene expression control and, more generally, in terms of their physiological significance should be further investigated in reporter gene experiments. Therefore, isolated actin sequences of the flanking regions of the actin gene that represent potential promoters for use in orchid transformation are being constructed in expression vectors by fusion with *gus* genes for subsequent transformation experiments.

ACKNOWLEDGEMENTS

This project was supported by the Science Achievement Scholarship of Thailand (SAST) contract no.02/2551, Chulabhorn Research Institute (CRI) and the Kasetsart University Research and Development Institute (KURDI), Thailand.

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