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Differential gene expression during flower bud initiation and flower bud development of *Rhynchostylis gigantea* (Lindl.) Ridl

Bounma Phengphachanh*, Daruni Naphrom, Weenun Bundithya and Nuttha Potapohn

Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

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Generally, *Rhynchostylis gigantea* (Lindl.) Ridl usually induce flowers during winter time, January-February. There was a report which stated that growing *R. gigantea* under short day condition could induce flowering faster than natural flowering period. Thus, this study was conducted to determine the gene expression during floral induction of this plant. Plants were grown under natural condition and short day condition. Shoot tips of those plants were collected every five days, then, each sample was used for total RNA extraction and followed with cDNA synthesis. Fifty- eight arbitrary primers with oligo $dT_{12}VA$, $dT_{12}VC$, $dT_{12}VG$ and $dT_{12}VT$ were screened. It was found that combinations of 2 primers with $dT_{12}VC$, 8 primers with $dT_{12}VG$ and 12 primers with $dT_{12}VT$ were able to amplify polymorphic bands differentially expressed among treatments. Selected bands were cloned and sequenced. Thirty TDFs from these combinations, $dT_{12}VG$+OPF16; $dT_{12}VC$+OPD07; $dT_{12}VT$+OPAB17 and $dT_{12}VT$+OPF13 were retrieved for reamplification and cloning. Only 19 TDFs were subsequently sequenced. The sequence analyses revealed that TDFB1, TDFC5, TDFC6 and TDFC8 shared the homology with different species of Pseudomonase. TDFC5 and TDFC8 shared the homology with dairy cattle and marine animal, whereas 9 TDFBs (TDFB2 to TDFB10) shared the homology with 1) flower-specific gene promoters of Oncidium Gower Ramsey, 2) the transcripts encoding protein gene of *Phalaenopsis equestris* and 3) the vernalization-induced floral transition gene of *Dendrobium nobile*. The other 4 TDFs of TDFD shared the homology with mixed tissue cDNA library of *Nicotiana benthamiana*. On the other hand, TDFC1 and TDFC3 had no significant sequence homology in the database.

This study could suggest that short day was a favorable factor affecting flower bud initiation. During that period, TDFB2 to TDFB10 were detected which could be related to flower bud initiation of *R. gigantea*.

Keywords: *Rhynchostylis gigantea* (Lindl.) Ridl, day length, Gene expression, flowering.

* Corresponding author: Bounma Phengphachanh; e-mail: bm_pheng@hotmail.com

Introduction

Generally, plants can flower when they have reached a certain stage of growth or received proper conditions, e.g., temperature and photoperiod, that can induce flowering process. The initiation of flowering is the first step in flower development which can be triggered by environmental and endogenous factors (Taiz and Zeiger, 2007; Amasino and Michaels, 2010). There has been quite a number of information on flower induction of orchid species, e.g., in vitro flowering of several Dendrobium hybrids (Goh,1996); thermoperiodism on flowering of Dendrobium Second Love (Campos and Kerbauy, 2004); cool temperature on flowering of most of Phalaenopsis hybrids (Lopez *et al*., 2007) and day length and temperature as well as GA_3 application on flowering of Rhynchostylis gigantea (Theesoda, 2004; Talee, 2008; Banyai, 2009; Watthanasrisong, 2010; Phengphachanh *et al*., 2012).

The transition from vegetative to reproductive growth is controlled by genes. The flower initiation of Arabidopsis thaliana occurs in the leaves and induces systemic signals. The signalling is activated by floral promoter gene, CONSTANS (CO) and directly activates the expression of transcription factor gene, FLOWER LOCUS T (FT). FT-protein is synthesized and translocated to shoot apical meristem (SAM) to initiate flowering. FLOWER LOCUS D (FD) is formed at SAM and flowering occurs by repressing floral identity genes, APETALA 1(AP1) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC 1) (Amasino and Michaels, 2010).

Several genes which control flowering in orchids have been identified. In Dendrobium Madame Thong-In under in vitro condition, DOMADS 1, DOMADS 2 and DOMADS 3 were activated during floral transition (Yu and Goh, 2000). Yu *et al*. (2002) reported that DOMADS1 was a marker gene and specifically expressed in the transitional shoot apical meristem during floral transition in D. Madame Thong-In. For Oncidium Gower Ramsey, OnTI1, OnTI2 and OnTI3 were highly expressed at different stages of flower development (Hsu *et al*., 2011). In Dendrobium nobile, DnVRN1 gene, which was related to the induction of flowering, was detected in vernalized axillary buds at the middle stage of vernalization (Liang *et al*., 2012).

As a part of the study on flowering, this work was conducted in order to determine genes which are differentially expressed during floral transition process and flower development of Rhynchostylis gigantea.

Materials and methods

Plant materials

Rhynchostylis gigantea (Lindl.) Ridl., 2-3 years old plants, which flowered in the previous year, were grown under natural condition and short day condition (10 h of light) for 3 months. After that, all plants were transferred to the shaded house for further growth and development. The experiment was conducted during May 2nd, 2011 to February 2nd, 2012, at Orchid Nursery, Mae Hia Agricultural Center for Research, Demonstration and Training, Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Thailand.

According to the experiment of Talee (2008), the first visible flower bud appeared around 30 days after *R. gigantea* plants were grown under short day regardless of GA_3 application at different concentrations, 1000 and 3000 ppm. Thus, in this study, shoot samples were randomly taken on day-0 and then every 5 days until day 35th. Three plants per treatment from each of the 2 treatments, natural and short day condition were used. After that, samples were collected every 20 days until day 95th.

Identification of gene expression

RNA Extraction

Total RNA was extracted from fresh shoot tip, using TRIzol® Reagent (Invitrogen™) following the manufacturer's instructions. The RNA pellet was dissolved in 40 µl of Ultra Pure™ distilled water prior to DNase treatment. DNase-free RNA (5µl) were reverse transcribed for first strand cDNA, using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen™) with some modifications. Single strand cDNA was separated into 4 groups which could be tagged with 4 primers, i.e., oligo $dT_{12}VA$, $dT_{12}VC$, $dT_{12}VG$ and $dT_{12}VT$.

Primers screening

Fifty-eight random primers, i.e., OPD 01- OPD 20; OPF 01- OPF 20; AP-A 01- AP-A10; OPAB 11- OPAB 14 and OPAB 16- OPAB 19, were screened with each of the 4 tagged cDNA groups. Two µl (100ng/µl) of cDNA templates were mixed with reaction buffer according to Anuchai (2011) and Chaipar (2011) procedures. Polymerase Chain Reaction (PCR) was performed, started with 3 mins for activation at 94 °C, followed by 40 cycles of 94 °C for 30

sec, 42 °C for 1 min, and 72 °C for 1 min and extended at 72 °C for 8 mins. The amplified product was electrophoresed on 6% polyacryamide gel at 200 mA, 1200 V and 50 W for 5 h. The gel was silver-stained and dried under the vacuum at 60 °C for 3 h. Primers which yielded polymorphism were selected for further steps.

Re-amplification and cloning

Potential bands amplified by selected primers were excised and eluted in 20 µl of TE buffer at room temperature for 10 mins, ground using micro-tip and incubated for 15 mins at 95 °C using heat boxes and kept at -20 °C overnight. The re-amplification was done using 2 µl of eluted template under the same reaction and PCR conditions previously mentioned. The re-amplified products were electrophoresed on 1.2% agarose gel at 100 V for 40 mins. The gel was stained using 1% of ethidium bromide and photographed under ultra violet light (Gel Documentations, Lab Focus). The bands of expected size were selected and subsequently cloned into pGEM®-T Easy Vector Systems II (Promega) following the manufacturer's instructions. Plasmids containing expected inserts were submitted to Pacific Science Co., LTD (http://www.pacific-science.co.th) for sequencing. All sequences were analyzed and compared with all known database from GenBank, using BLAST and ETS programs (http://www.ncbi.nlm.nih.gov).

Results

R. gigantea plants were grown under natural condition and short day condition for 3 months, after that all plants were transferred to natural condition, under shaded house. Under short day condition, 70% of plants could give visible flower buds at approximately 140 days after treatment whereas 79% of plants under natural condition could give visible flower buds at approximately 163 days after treatment. Flower bud development of all plants grown under natural condition and short day condition could yield flowers and all flowers senesced around the same time.

Screening of primers

Fifty-eight primers were used for primer screening. The results revealed that twenty primers could providing polymorphic bands. Eight primers, OPD10, OPD18, OPD20, OPF11, A-PA07, OPAB13, OPAB16 and OPAB17 yielded polymorphism with oligo $dT_{12}VG$-tagged samples. Two primers, OPD07 and OPF16 yielded polymorphism with oligo $dT_{12}VC$-tagged samples.

Twelve primers, OPD05, OPD16, OPF04, OPF06, OPF09, OPF12, OPF13, OPF14, OPAB12, OPAB16, OPAB17 and OPAB18 yielded polymorphism with oligo $dT_{12}VT$-tagged samples. OPAB16 and OPAB17 primers yielded polymorphism with oligo $dT_{12}VG$ and oligo $dT_{12}VT$-tagged samples. Samples which were tagged with oligo $dT_{12}VA$ showed barely or weak band. These 20 primers were used to determine the differential gene expression during flower bud initiation and flower bud development of *R. gigantea*. Result showed that the combinations of oligo $dT_{12}VG$+OPF16, oligo $dT_{12}VC$+OPD07 and oligo $dT_{12}VT$+OPAB17 could yield polymorphic bands during flower bud initiation stage whereas oligo $dT_{12}VT$+OPF13 could yield polymorphic bands during flower bud development stage.

The combination of oligo $dT_{12}VG$+OPF16 could yield bands at the range of 204 - 2,520 bp. The bands were visible for almost all samples. However, the 204 bp band was specific for short day condition on day 5th, 10th and 35th after treatment. These 3 bands were selected for furthur re-amplification and cloning (Table 1).

The combination of oligo $dT_{12}VC$+OPD07 could yield bands at the range of 1,534 - 3,439 bp. The bands were visible for almost all samples. However, the 1,534 bp band was specific for natural condition on day 5th, 10th, 20th, 25th, 30th and 35th and for short day condition on day 10th, 15th, 30th and 35th after treatment. These 10 bands were selected for furthur re-amplification and cloning (Table 1).

The combination of oligo $dT_{12}VT$+OPAB17 could yield bands at the range of 367- 658 bp The bands were visible for almost all samples. The 476 and 428 bp bands were specific for natural condition on day 25th after treatment and for short day condition on day 5th, 10th, 20th and 25th after treatment. These 9 bands were selected for furthur re-amplification and cloning (Table 1).

The combination of oligo $dT_{12}VT$+OPF13 could yield bands at the range of 4,081-5,847 bp The bands were visible for almost all samples. The 4,081 and 5,115 bp bands were specific for natural condition on day 55th , 75th and 95th after trtetment and for short day condition on day 55th after treatment. These 8 bands were selected for furthur re-amplification and cloning (Table 1).

Analysis of sequences

Thirty transcript-derived fragments (TDF) were reamplified, and 19 TDFs, 10 of TDFB, 4 of TDFC and 5 of TDFD, showed expected sizes. These 19 TDFs were sequenced (Table 2). TDFB1and TDFC6 were matched to *Pseudomonas fluorescens* with 82-91% homology. TDFB2 to TDFB10 were matched to orchids genes with 93-94 % homology.

TDFC1 and TDFC3 had no significant sequence homology in the database. TDFC5 and TDFC8 were matched to dairy cattle genes with 94% homology and marine animal Hydractinia echinata with 95% homology. TDFD2 to TDFD8 were matched with Nicotiana benthamiana sequence with 93-96% homology.

Table 1. Primer combination, treatment and ID of selected clones

Combination	Treatment	Clone ID	Combination	Treatment	Clone ID
oligo dT_{12}VG	SD at D5	TDFA1		SD at D10	TDFC3
+	SD at D10	TDFA2		SD at D10	TDFC4
OPF16	SD at D35	TDFA3	Oligo dT_{12}VT	SD at D20	TDFC5
	NC at D5	TDFB1	+	NC at D25	TDFC6
	NC at D10	TDFB2	OPAB17	NC at D25	TDFC7
	SD at D10	TDFB3		SD at D25	TDFC8
	SD at D15	TDFB4		SD at D25	TDFC9
Oligo dT_{12}VC	NC at D20	TDFB5		NC at D55	TDFD1
+	NC at D25	TDFB6		NC at D55	TDFD2
OPD07	NC at D30	TDFB7		SD at D55	TDFD3
	SD at D30	TDFB8	Oligo dT_{12}VT	SD at D55	TDFD4
	NC at D35	TDFB9	+	NC at D75	TDFD5
	SD at D35	TDFB10	OPF13	NC at D75	TDFD6
Oligo dT_{12}VT	SD at D5	TDFC1		NC at D95	TDFD7
+	SD at D5	TDFC2		NC at D95	TDFD8
OPAB17					

Discussion

Rhynchostylis gigantea plants were grown under different conditions, short day and natural conditions. It was found that plants grown under short day could give flower 23 days earlier than those grown under natural condition. That means flower initiation should have occurred at different time, resulting from earlier induction by short day condition.

Sequence analysis of differentially-expressed TDFs revealed positive matches to the database. Nine TDFs, i.e., TDFB2, TDFB3, TDFB4, TDFB5, TDFB6, TDFB7, TDFB8, TDFB9 and TDFB10, were matched with orchid genes, i.e., 1) flower-specific gene promoters of Oncidium Gower Ramsey, from flower organ (Hsu *et al*., 2011), 2) the transcripts encoding protein gene of Phalaenopsis equestris, gene from flower bud (Tsai *et al*., 2006) and 3) the vernalization-induced floral transition gene of Dendrobium nobile (20+30) d-vernalized cDNA library, detecting from axillary buds of D. nobile at middle stage of vernalization (Liang *et al*., 2012). These TDFs were found in plants grown under short day and natural conditions with some differences. Under short day, TDFs could be detected

on days 10th, 15th, 30th and 35th whereas TDFs of natural condition could be found on days 10th, 20th, 25th, 30th and 35th. In addition, TDFA and TDFC could be noticed only in plants grown under short day condition. Unfortunately, ones from TDFA could not be reamplified and cloned due to technical difficulties. These TDFAs are very interesting in that the expression profiles could be involved with flowering since they were found in plants grown under short day at days 5th, 10th and 15th, but none was present in plant grown under natural condition. Further investigation regarding this type of TDF should be conducted to elucidate gene expression controlling flower induction and initiation.

Other TDF results showed general information on living things. The results matched to dairy cattle and marine animal. However, the result from TDFB1 and TDFC6 showed matching with the complete genome of *Pseudomonas fluorescens* Pf-5 and SBW25, respectively. *P. fluorescens* are common soil bacteria that can improve plant health through nutrient cycling, pathogen antagonism and induction of plant defenses by producing secondary metabolites that suppress plant pathogens (Paulsen *et al*., 2005; Silby *et al*., 2009). Interestingly, *R. gigantea* is an ephiphytic orchid and gene expression from this species is similar to those found in plants grown in soil. This might help explain why this type of orchid can survive without any growing medium.

Table 2. Expression pattern, reamplification and sequence search of selected clones

Day after treatment	**TDFA** oligodT$_{12}$VG+OPF16		**TDFB** oligodT$_{12}$VC+OPD07		**TDFC** oligodT$_{12}$VT+OPAB17		**TDFD** oligodT$_{12}$VT+OPF13	
	Natural condition	Short day	Natural condition	Short day	Natural condition	Short day	Natural condition	Short day
D0								
D5		Δ	√			O ⊗		
D10		Δ	√	√		O ⊗		
D15				√				
D20			√			√		
D25			√		√ ⊗	√ ⊗		
D30			√	√				
D35		Δ	√	√				
D55							⊗ √	⊗ √
D75							⊗ √	
D95							⊗ √	

Note: Δ: could not re-amplify; √: matched to database; O: not match to any information in data base; ⊗: Not expected size

Table 3. Characterization of genes differentially expressed in flower bud initiation and flower buds development of *R. gigantea* (Lindl.) Ridl

Clone	**Sequence size (bp)**	**Data from gene bank**			
		Accession Number	Homology to	Comparisons score	Homology (%)
TDFB1	270	CP000076.1	Pseudomonas fluorescens Pf-5, complete genome	254	84
		CP002585.1	Pseudomonas brassicacearum subsp. brassicacearum NFM421, complete genome	210	82
		CP000075	Pseudomonas syringae pv. syringae B728a, complete genome	130	88
TDFB2	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	381	94
TDFB3	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	381	94
TDFB4	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	381	94
TDFB5	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	381	94
TDFB6	272	HS522463	Oncidium Gower Ramsey, flower cDNA library	374	93
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94

		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	387	94
TDFB7	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	363	93
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	243	93
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	375	94
TDFB8	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	363	93
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA library	375	93
TDFB9	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA	381	94
TDFB10	271	HS522463	Oncidium Gower Ramsey, flower cDNA library	368	94
		CK857414	Flower bud of Phalaenopsis equestris, cDNA 5', mRNA sequence	248	94
		HO199381.1	Dendrobium nobile (20+30)d-vernalized cDNA	381	94
TDFC1	476		No significant sequence homology in the database		
TDFC3	476				
TDFC5	476	AV725203	Bos taurus cDNA clone	472	94
		DN602668	Hypothetical protein	95.3	95
TDFC6	476	AM181176	Pseudomonas fluorescens SBW25 complete genome	366	91
		CP003041	Pseudomonas fluorescens A506, complete genome	322	88
TDFC8	555	AV725203	Bos taurus cDNA clone	472	94
		DN602668	Hypothetical protein of Hydractina echinata	93.5	95
TDFD2	475	CK285246	Nicotiana benthamiana mixed tissue cDNA library	263	93
		CK285245		84.2	96
TDFD4	475	CK285246	Nicotiana benthamiana mixed tissue cDNA library	263	93
		CK285245		84.2	96
TDFD6	475	CK285246	Nicotiana benthamiana mixed tissue cDNA library	263	93
		CK285245		84.2	96
TDFD8	476	CK285246	Nicotiana benthamiana mixed tissue cDNA library	263	93
		CK285245		84.2	96

Conclusion

The identification of gene expression during flower bud initiation and flower bud development was studied. Fifty-eight arbitratry primers were screened on cDNA of R. gigantea. Twenty primers showed polymorphisms. Combinations of oligo dT_{12}VG+OPF16, oligo dT_{12}VC+OPD07, oligo dT_{12}VT+OPAB17 yielded polymorphic bands at flower bud initiation while oligo dT_{12}VT+OPF13 at flower bud development. Thirty TDFs from these 4 combinations were reamplified, cloned and sequenced.

The sequence analysis revealed TDFB1, TDFC5, TDFC6 and TDFC8 having homology with different species of *Pseudomonas*. TDFC5 and TDFC8 had homology with dairy cattle and marine animal. Nine TDFBs (TDFB2 to TDFB10) shared the homology with flower-specific gene promoters of Oncidium Gower Ramsey; the transcripts encoding protein gene of P. equestris and the vernalization- induced floral transition gene of D. nobile. Four TDFDs shared sequences with mixed tissue cDNA library of N. benthamiana. TDFC1 and TDFC3 had no significant sequence homology in the database.

This primary information on molecular practice would guide the study on the flowering process of *R. gigantea* in the future. It could facilitate further characterization of the key players in flowering process pathways. Further study is needed in order to understand and determine genes controlling flower induction, initiation and development of *R. gigantea*.

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