

Quantification of the Expression of the MADS-Box Gene *DthyAG* in the Orchid *Dendrobium thyrsiflorum*

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Abstract

MADS-box genes encode transcription-factor proteins with key roles in many aspects of plant development. MADS-box genes are named after the highly conserved DNA-binding motif, initially described in the *Saccharomyces* gene MCM1, the *Arabidopsis* gene AG, the *snapdragon* gene DEF, and in the human protein Serum response factor. Phylogenetic analysis of MADS-box genes isolated from multiple plant species show that they comprise a large family of genes consisting of several different subgroups, related to different developmental functions in plants. Transcriptional quantification of MADS-box genes in plants is important in elucidating possible roles of different organs and tissues in plants. Hybridization techniques are unsuitable for this purpose due to possible cross hybridization between similar genes. An alternative method that offers high sensitivity and precision, such as real-time RT-qPCR, is ideal. Here we analyse the expression of the MADS-box gene *DthyAG* (the ortholog of AG in *Arabidopsis*) in different tissues in the orchid *Dendrobium thyrsiflorum*, using RT-qPCR.



Figure 1. Inflorescence of *Dendrobium thyrsiflorum* flowers.

Objective

To use RT-qPCR to quantify *DthyAG* transcription in flowers and during ovule development in *D. thyrsiflorum*, to elucidate possible roles of *DthyAG* in these structures.

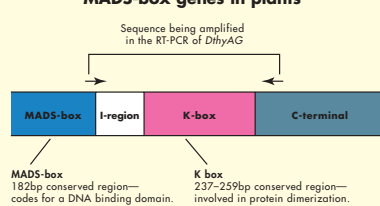
Introduction

The MADS-box gene family

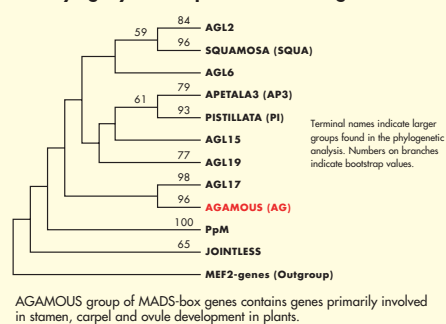
MADS-box genes are found in animals, fungi, and plants¹. In plants, the MADS-box genes have diversified enormously, and due to the complete sequencing of the *Arabidopsis* genome more than 100 different MADS-box genes are known².

Plant MADS-box genes containing a K box are often referred to as MIK or MIKC genes.

Structure of MIKC-type MADS-box genes in plants



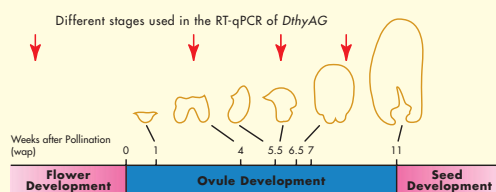
Phylogeny of 198 plant MADS-box genes³



AGAMOUS group of MADS-box genes contains genes primarily involved in stamen, carpel and ovule development in plants.

Gene of interest: *DthyAG*, a member of the AGAMOUS group of MADS-box genes

Flower, ovule and seed development in orchids



Unlike most other plants, ovule formation in *Dendrobium* orchids is entirely dependent on the pollination of the flowers. The developmental process leading to the fertilization is long and can last up to 11 weeks. This makes *Dendrobium* orchids excellent organisms for the study of molecular development of female reproductive structures.

Protocol

Reverse Transcription

Total RNA was extracted from different tissues using FastRNA[®] Kit-Green from Qbiogene (Cat # 6040-600). A poly (t)16 primer was used in a first strand cDNA synthesis on 0.5µg total RNA using M-MLV Reverse Transcriptase according to the manufacturer's recommendations (Promega, Cat # M1705).

Template

Standard curves are based on 10-fold dilution series of *Actin* and *DthyAG* amplicons cloned into a pCR2.1-TOPO vector (Invitrogen Cat # K4500-1).

Quantification of *Actin* and *DthyAG* were done on the following tissues: Stage0 = Leaf, stage1 = 4-5mm inflorescence, stage2 = ovules (2-3 weeks after pollination, wap), stage3 = ovules 5-6 wap, and stage4 = ovules 8-9 wap.

Primers and amplicons

Ordered from TAG Copenhagen (Copenhagen, Denmark).

Actin

Actin forward: 5'-GCTGGTCTGACCTGACTGA-3'

Actin Reverse: 5'-ACGGAACCTCTCAGCTCCAA-3'

Amplicon = 228bp

DthyAG

DthyAG forward: 5'-TTCTCAAGTCGGTTCGCCTCTA-3'

DthyAG Reverse: 5'-GGTGAGCATAATGATCATTGGGATC-3'

Amplicon = 513 bp

Experimental layout

This experiment was a one-color assay using SYBR[®] Green I (DyNamo[™] Hot Start) with all standards done in duplicate and samples in triplicate.

Results

Standard curves were generated for both the housekeeping gene (*Actin*) and the gene of interest (*DthyAG*), using Opticon Monitor[™] software (ver. 2.02).

Reaction Composition

Component	Volume	Final Concentration
DNA	5.0µl	1ng cDNA/20µl
Forward Primer	2.0µl	1mM
Reverse Primer	2.0µl	1mM
DyNamo HS 2X	10µl	1X
ddH ₂ O	1µl	
Total Volume	20µl	

DyNamo HS was from Finnzymes (Cat# F-410L)

The reaction was set up in Hard-Shell[™] microplates (MJ Research HSP-9655 and sealed with ultra clear strip caps (MJ Research TCS-0803).

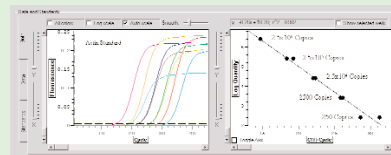
Cycling Conditions

Amplification was performed on the DNA Engine Opticon[™] 2 using the following cycling protocol:

- 95°C 10min
- 94°C 20sec
- 55°C 45sec
- 72°C 45sec
- 75°C 1sec
- Plate read
- Go to line 2 for 44 times
- 72°C 5min
- Melting curve analysis: 75°C to 90°C, 0.1°C/read, 2sec hold

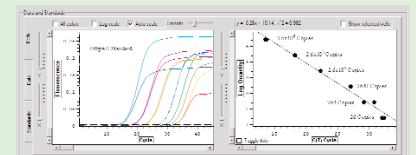
END

Standard curves for *Actin*



Standards based on a 10-fold dilution of *Actin*, which has been cloned into a pCR2.1-TOPO vector.

Standard curves for *DthyAG*



Standards based on a 10-fold dilution of *DthyAG*, which has been cloned into a pCR2.1-TOPO vector.

Table 1: *DthyAG* expression at different stages of development

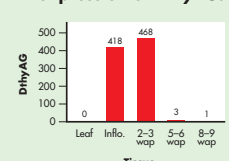
Tissue	Stage	<i>Actin</i>		<i>DthyAG</i>		<i>DthyAG/Actin</i>
		Ct	copies/ng total RNA	Ct	copies/ng total RNA	
Leaf	0	17.27	152863±24702	None	0	
Inflorescence	1	17.09	173124±35958	20.29	18663±1512	0.1078
2-3 wap	2	20.69	16602±2504	23.99	2005±14	0.1208
5-6 wap	3	19.47	36802±6938	31.21	28±14	7.82E-04
8-9 wap	4	18.26	79848±8046	31.65	21±2	2.58E-04

RNA concentrations for each sample were calculated by interpolating Ct values against the standard curve. *DthyAG* expression for each of the samples was normalized (to control for the efficiency of the reverse transcription) by dividing the RNA concentration of *DthyAG* by that of *Actin*.

All samples were done in triplicate. Based on these average Ct values, copy numbers, and standard deviations were calculated. Stage0 = Leaf, stage1 = 4-5mm inflorescence, stage2 = ovules (2-3 weeks after pollination, wap), stage3 = ovules 5-6 wap, and stage4 = ovules 8-9 wap.

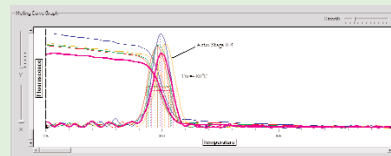
Expression of *DthyAG* was normalized using *Actin*.

Table 2: Relative changes in expression of *DthyAG*.



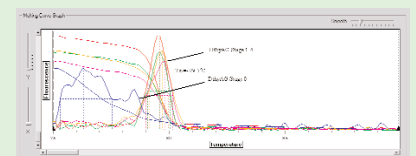
The changes in *DthyAG* expression during ovule development were calculated relative to the stage with the lowest expression level (stage 4), which was calibrated to an expression level of 1. No expression of *DthyAG* was detected in leaves. The relative expression of *DthyAG* is more than 400 fold higher in stage 1 (inflorescence) and 2 (ovules 2-3 wap) than in stage 4 (ovules 8-9 wap).

Melting curves for the *Actin* amplicon at stages 0-4.



Specific amplification was obtained from all 5 stages. The calculated melting temperature (<http://www.pitt.edu/~rsup/OligoCalc.html>) is 81°C.

Melting curves for the *DthyAG* amplicons at stages 0-4.



No specific amplification was obtained from stage 0. The calculated melting temperature (<http://www.pitt.edu/~rsup/OligoCalc.html>) is 80°C.

Summary

- We have successfully amplified the *DthyAG* MADS-box gene from the Orchid *Dendrobium thyrsiflorum*. As expected, no expression of *DthyAG* was detected in leaves, but only in flowers and developing early-stage ovules. This is in accordance with the findings in other plant species⁴. The dramatic decrease in expression of *DthyAG* at 5-9 wap indicates that *DthyAG* does not play a significant role in late ovule development in *D. thyrsiflorum*.
- Reverse-transcriptase quantitative PCR has shown to be a valuable tool in the study of expression patterns of genes within large gene families like the MADS-box family.
- Future work will continue the study of MADS-box genes in *D. thyrsiflorum* using RT-qPCR.

References

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- Martinez-Castilla LP, Alvarez-Buylla ER: Adaptive evolution in the *Arabidopsis* MADS-box gene family inferred from its complete resolved phylogeny. *Proc Natl Acad Sci U S A* 2003, 100(23):13407-12.
- Johansen B, Pedersen LB, Skipper M, Frederiksen S: MADS-box gene evolution-structure and transcription patterns. *Mol Phylogenet Evol* 2002, 23:458-480.
- Pinyopich A, Ditta GS, Savidge B, Liljgren SJ, Baumann E, Wisman E, Yanofsky MF: Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* 2003, 424(6944):85-8.

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