

Expression analysis of fertilization/early embryogenesis-associated genes in *Phalaenopsis* orchids

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ABSTRACT

One of the distinct reproductive programs in orchid species is pollination-triggered ovule development and megasporogenesis. During sexual reproduction, fertilization occurs days to months after pollination. The molecular mechanisms evolved to carry out this strategic reproductive program remain unclear. In the August issue of *Plant Physiology*¹, we report comprehensive studies of comparative genome-wide gene expression in various reproductive tissues and the molecular events associated with developmental transitions unique to sexual reproduction of *Phalaenopsis aphrodite*. Transcriptional factors and signaling components whose expression is specifically enriched in interior ovary tissues when fertilization occurs and embryos start to develop have been identified. Here, we report verification of additional fertilization-associated genes, *DOMAINS REARRANGED METHYLTRANSFERASE 1 (PaDRM1)*, *CHROMOMETHYLTRANSFERASE 1 (PaCMT1)*, *SU(VAR)3-9 RELATED PROTEIN 1 (PaSUVR1)*, *INDOLE-3-ACETIC ACID inducible 30-like 1 (PaIAA30L1)*, and *ETHYLENE INSENSITIVE 3-like 1 (PaEIN3L1)*, and discuss their potential roles in gametophyte development, epigenetic reprogramming, and hormone regulation during fertilization and establishment of embryo development in *Phalaenopsis* orchids.

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Fertilization and embryo development starts by fusion of the sperm cell and egg from the female gametophyte. Successful fertilization and seed development requires coordinated development of male and female gametophytes and intensive communication between male and female reproductive cells. Tremendous progress has been made recently in understanding the molecular mechanism of fertilization and embryo development in plants. Particularly, studies on regulation of gametophyte development, prevention of polyspermy, and epigenetic reprogramming after fertilization have provided new insights into hormone signaling and the gene regulatory paradigm for plant sexual reproduction.²⁻⁴

In our previous study, we reported that pollen tubes enter the ovule at approximately 65 d after pollination (DAP) followed by expression of an egg-specific marker *EGG CELL 1 Like 1 (PaECIL1)* in *P. aphrodite*.⁵ These data provide cellular and molecular evidence that fertilization occurs at approximately 70 d after pollination (DAP) in *P. aphrodite*. In addition to the egg-specific marker, several transcription factors, methyltransferases, hormone signaling components, and cell cycle regulators⁶ have also been shown to display fertilization-associated expression patterns.¹ Isolation of these genes provides a novel opportunity to study the unique sexual reproduction of *Phalaenopsis* orchids.

Development of reproductive cells and establishment of embryogenesis

Among the over-represented transcription factors enriched during fertilization and early embryo development (70-80 DAP) are type I MADS domain transcription factors. Plant MADS domain transcription factors play important regulatory roles at various stages in plant development.⁷ Type I MADS domain transcription factors are involved in regulation of plant reproduction, particularly female gametophyte, embryo, and endosperm development. For example, Arabidopsis *AGAMOUS-LIKE 23 (AGL23)*, which is regulated by the epigenetic regulator PRC2-type polycomb group complex, is expressed in developing ovules, embryo, and endosperm. *AGL23* is required for successful development of female gametophyte and embryogenesis.⁸ Expression of *AGL62*, another Type I MADS domain transcription factor, is detected in antipodal cells of the female gametophyte and in developing endosperm. *AGL62* regulates cellularization of developing endosperm during seed development.⁹ In addition to Type I MADS domain transcription factors, the divergent MADS domain transcription factors (MIKC*) have been shown to regulate male reproductive cells. Members of the Arabidopsis and rice MIKC* subgroup are predominantly expressed during the late stage of pollen development.^{10,11} In line with the expression patterns, mutations in MIKC*-type transcription factors cause reduction in pollen viability and defects in pollen tube growth.^{11,12}

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Table 1. Transcript abundances of the indicated genes enriched in interior ovary tissues collected at 70/80 DAP of *P. aphrodite* by RNA-seq analysis. Expression pattern of the transcripts was confirmed by qRT-PCR (Fig. 1).

Transcript ID	Annotation	FPKM values									
		30/40 DAP	50/60 DAP	70/80 DAP	90/100/120 DAP	140/160 DAP	180/200 DAP	PLB	Protocorm	Young leaves	Stalk buds
orchid.id141182.tr411193	PaDRM1	0.0	0.0	7.5	0.4	0.0	0.0	0.0	0.0	0.0	0.0
orchid.id175307.tr208591	PaCMT1	13.9	30.7	103.7	15.3	7.8	4.6	7.1	9.0	11.4	8.5
orchid.id159191.tr200827	PaSUVR1	0.7	4.6	13.5	3.9	1.9	1.0	0.2	0.7	1.3	0.5
orchid.id153148.tr220461	PaAA30L1	0.0	0.0	8.9	0.2	0.0	0.0	0.0	0.0	0.1	0.2
orchid.id123237.tr401072	PaEIN3L1	2.8	0.8	8.4	1.9	0.0	0.0	0.1	0.1	0.4	0.0

Our previous study reported that expression of *PaMADS39*, *PaMADS51*, and *PaMADS63* are tightly associated with the stage (70–80 d after pollination) when the female and male gametophytes are established and fertilization occurs (Table 1).¹ *PaMADS39* and *PaMADS51* belong to the $M\alpha$ -subclass of type I MADS-box genes and are closely related to Arabidopsis *AGL23* and *AGL62*.¹ Similar to temporal expression patterns of *AGL23* and *AGL62*, *PaMADS39* and *PaMADS51* are expressed in reproductive tissues when fertilization occurs and embryo development initiates. It will be of future interest to investigate the tissue specific localization and molecular functions of *PaMADS39* and *PaMADS51* during sexual reproduction of *Phalaenopsis* orchids.

Phylogenetic analysis puts *PaMADS63* in the MIKC* subclass of type II MADS domain transcription factors.¹ The MIKC* type MADS domain proteins belong to a divergent class of type II MADS domain proteins and are expressed

specifically in mature pollen of Arabidopsis¹³ and rice,¹¹ and in gametophytic generation of moss *Funaria hygrometrica*.¹⁴ Functional characterization of MIKC* heterodimers has shown that they function as master regulators of pollen maturation programs.^{11,12} Similarly, *PaMADS63* mRNA was preferentially expressed in pollinia, and pollinia in hydrated (one day after pollination) and germinated (3 d after pollination) conditions (Fig. 1A and Fig. 1B). *PaMADS63* mRNA was hardly detectable in root tips, floral stalks, PLBs, and young leaves (Fig. 1B). This suggests that *PaMADS63* may play an important role during pollen development in *Phalaenopsis* orchids.

DNA methylation and fertilization

Dynamic regulation of DNA methylation is important for plant sexual reproduction.¹⁵ In plants, *de novo* and maintenance of

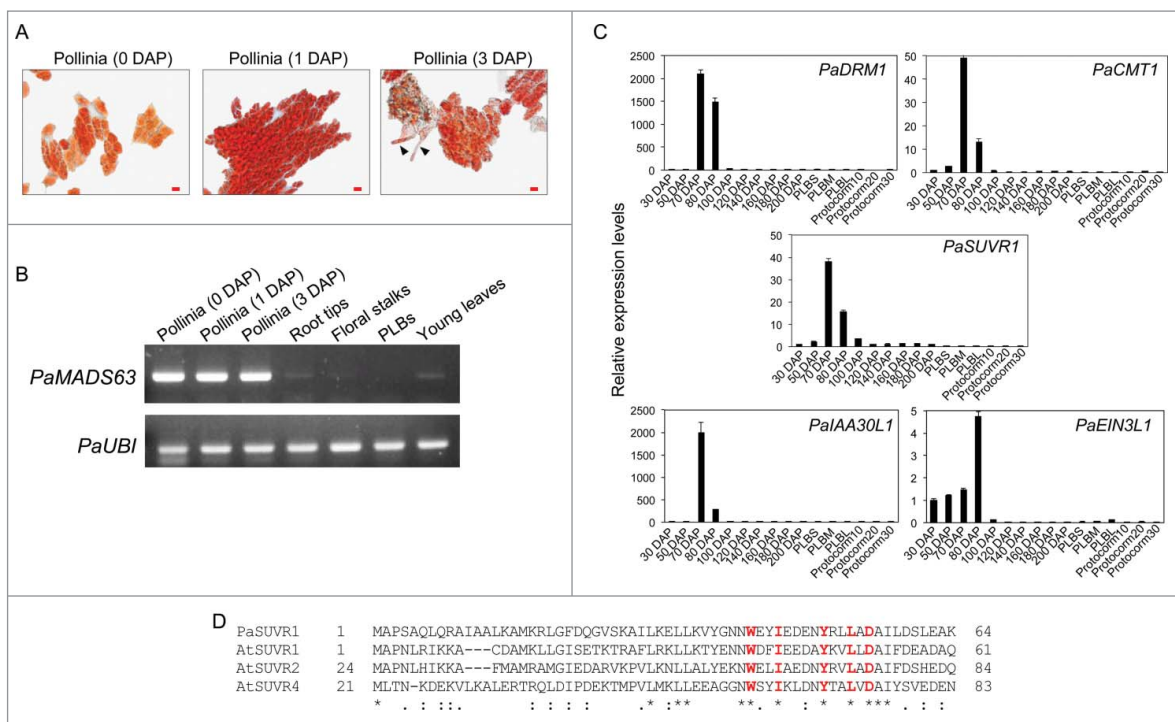


Figure 1. (A) Acetocarmine staining images showing pollinia at 0, 1, and 3 d after pollination (DAP). Arrowheads point to the pollen tubes. Scale bar = 20 μ m. (B) Semi-quantitative RT-PCR showing expression of *PaMADS63* mRNA in the indicated tissues. *PaUBI* was used as an internal control. PLB, Protocorm-like-body. (C) Quantitative RT-PCR showing expression patterns of *PaDRM1*, *PaCMT1*, *PaSUVR1*, *PaAA30L1*, and *PaEIN3L1* in interior tissues of developing ovaries from 30 to 200 d after pollination (DAP). Small-sized PLB (PLBS), medium-sized PLB (PLBM), large-sized PLB (PLBL), 10-day-old protocorms (protocorm10), 20-day-old protocorms (protocorm20), and 30-day-old protocorms (protocorm30). (D) Alignment of the WYLD domain of Arabidopsis SUVR proteins and *PaSUVR1*. AtSUVR1, AT1G04050; AtSUVR2, AT5G43990; AtSUVR4, AT3G04380.

DNA methylation are important to regulate genome imprinting and gene expression. Data indicate an overall reduction of DNA methylation in the germ line during gametogenesis.¹⁶ The partially demethylated genome of the zygote undergoes methylation reprogramming through de novo DNA methylation after fertilization.¹⁷ In Arabidopsis, resetting the methylation of the early-stage of embryos requires the DOMAINS REARRANGED METHYLTRANSFERASES 2 (DRM2) and CHROMOMETHYLASE 3 (CMT3), which methylate cytosine residues in the CHH, CHG, and CG context,¹⁸ and in CHG context¹⁹ respectively. It is hypothesized that DNA methylation reprogramming in the zygotes and early stage of embryos after fertilization contributes to the maintenance of genome integrity in the embryo.² Interestingly, our RNA-seq dataset identified *PaDRM1* and *PaCMT1* as overrepresented genes expressing at 70-80 DAP when fertilization occurs (Table 1). These temporal expression patterns were verified by qRT-PCR (Fig. 1C). This data suggests that *PaDRM1* and *PaCMT1* may be important in resetting methylation status after fertilization in *Phalaenopsis* orchids. In addition to *DRM* and *CMT* genes, the SET-domain histone methyltransferase has been shown to facilitate DNA methylation by enabling the propagation of CHG methylation through methylation of the lysine residue 9 of histone 3 (H3K9). Specifically, the methylated CHG DNA recruits the histone H3K9 methyltransferase SU(VAR)3-9 homolog 4 (SUVH4) that deposits H3K9me2 marks, which promote binding to CMT3.²⁰ We have identified a *Phalaenopsis* histone H3K9 methyltransferase SU(VAR)3-9 related gene, *PaSUVR1*, whose expression was specifically enriched during fertilization and early establishment of embryogenesis (Table 1 and Fig. 1C). The SUVR proteins are plant-specific SET-domain proteins that belong to a subgroup of SU(VAR)3-9-like proteins and are related to SUVH proteins.²¹ The SUVR proteins contain a conserved WIYLD domain (Fig. 1D) that has been shown to bind ubiquitin and regulate methylation of H3K9.²² It is possible that *PaDRM1*, *PaCMT1*, and *PaSUVR1* are coordinated to regulate epigenetic reprogramming after fertilization of *Phalaenopsis* orchids.

Hormone regulation and fertilization

Ethylene and auxin play essential roles in regulating fertilization in plants.²³⁻²⁵ Successful fertilization requires termination of subsequent pollen tube attraction to prevent polyspermy. The pollen tube block is mediated by *ETHYLENE INSENSITIVE 3* (*EIN3*)-dependent ethylene response signaling, which leads to programmed cell death of synergid cells that guide pollen tubes to the ovules.²⁴ Degeneration of the synergid cells ensures one-on-one pairing of male and female gametes and prevents fertilization of an egg by more than one sperm. Fertilization triggers auxin response that facilitates GA biosynthesis specifically in the ovules.²³ GA signaling is important to coordinate growth and development of fruits. The increasing auxin levels after fertilization is also important to drive central cell division and endosperm development via the AGL62-dependent pathway.²⁵

Ethylene and auxin have been shown to regulate ovary and ovule development after pollination in orchids.^{26,27} However, their roles during fertilization have yet to be determined.

Based on our RNA-seq data set, expression of *Phalaenopsis* *INDOLE-3-ACETIC ACID inducible 30 like* (*PaIAA30L1*) and *EIN3-like 1* (*PaEIN3L1*) genes is specifically enriched at 70–80 DAP when fertilization occurs and embryogenesis initiates (Table 1).¹ *PaIAA30L1* encodes an auxin-induced AUX/IAA transcriptional repressor. *PaEIN3L1* encodes an EIN3 domain transcriptional activator.²⁸ The fertilization-associated expression pattern of *PaIAA30L1* and *PaEIN3L1* was validated by qRT-PCR analysis (Fig. 1C). Given the temporal expression pattern of *PaIAA30L1* and *PaEIN3L1* and their annotated functions, we hypothesize that auxin and ethylene may play regulatory roles during fertilization and/or early embryo development in *Phalaenopsis* orchids. Similar to the expression pattern of *PaIAA30L1*, Arabidopsis *IAA30* is expressed in seeds containing zygotes. In addition, Arabidopsis *IAA30* is directly regulated by 2 embryogenesis regulators, LEAFY COTYLEDON 2 (LEC2, a B3 domain transcription factor) and AGL15. These experiments suggest that *IAA30* plays roles at an earlier stage of embryo development.^{29,30} Consistently, *IAA30* induced auxin signaling has been reported to be required for the AGL15-induced somatic embryogenesis program.³⁰ Despite the absence of *AGL15* in the *Phalaenopsis* genome, it is speculated that the 2 orchid B3 transcription factors (*PaABI3L1* and *PaABI3L2*) may coordinate with *PaIAA30L1*-induced auxin signaling to regulate orchid embryogenesis.

Our comparative genome-wide transcriptome analysis in reproductive tissues has allowed us to identify fertilization/embryogenesis-associated genes in *P. aphrodite*. Further characterization of these genes will help understanding the molecular mechanisms governing fertilization and establishment of embryogenesis in *Phalaenopsis* orchids.

Methods

Plant materials and growth conditions

Phalaenopsis aphrodite Subsp. *formosana* (m1663) plants in 2.5- or 3-inch pots were purchased from Chain Port Orchid Nursery (Ping Tung, Taiwan). Plants were grown in a growth chamber with alternating 12 h light (23°C)/12 h dark (18°C) cycles.

Sample collection and acetocarmine staining

Orchid flowers were hand pollinated and developing ovaries were harvested as described previously.¹ To visualize pollen and pollen tubes, samples were stained with acetocarmine as described previously.⁵ Protocorms and PLBs were categorized and harvested for RNA extraction as described previously.¹ Pollen, root tips with sizes smaller than 2 cm in length, 5-10 cm long floral stalks, and young leaves were collected for RNA extraction. The tissues samples were flash frozen in liquid nitrogen and stored in a freezer at -80°C.

RNA isolation and RACE-PCR

RNA was isolated as described previously.¹ DNA-free RNA was reverse transcribed in the presence of a mixture of oligo dT and

Table 2. List of primer pairs and annealing temperature used for semi-quantitative RT-PCR and qRT-PCR.

Gene name	Forward primer	Reverse primer	Amplicon size (bp)	TM(°C)
PaDRM1	5'-ACATCAAGCCGGAGTTTGTC-3'	5'-ATGCCTCTGGATGGTTTTG-3'	132	58
PaCMT1	5'-TATGACCATCGGCCTCTACC-3'	5'-GCTTCATGGATGGGTCAAGT-3'	145	58
PaSUVR1	5'-TGGGAGGTCTTTCAATGGTT-3'	5'-TTGAGGACTCTGAAGCACCA-3'	134	62
PaIAA30L1	5'-GGAAAAGAGCAGGCCTTT-3'	5'-TCCAAATGAAGTCCATCCA-3'	159	58
PaEIN3L1	5'-CAAGACCTCTCCGATACCA-3'	5'-GCGGAGGTACATTCTTCGTC-3'	170	60
PaUBI1	5-AACTCCATCGCCTTCTT-3'	5'-TGAAGCATGGCATCAATTC-3'	101	55 to 62
PaMADS63	5'-AGGGAGGAAAACAGCAAGT-3'	5'-ATGCCCTGAGAGTGTGTCC-3'	248	59

random primers (9:1 ratio) using the GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions.

RACE-PCR was carried out using a SMARTer RACE cDNA amplification kit according to the manufacturer's instructions (Clontech). The amplified *PaCMT1*, *PaDRM1*, *PaSUVR1*, and *PaEIN3L1* cDNAs were verified by sequencing. The Genbank accession numbers of *PaCMT1*, *PaDRM1*, *PaEIN3L1*, *PaIAA30L1*, and *PaSUVR1* are KX759037, KX759038, KX759039, KX759040, and KX759041 respectively.

Quantitative RT-PCR and semi-quantitative RT-PCR

The RNA samples used for quantitative RT-PCR were independent from samples used for RNA-seq. For quantitative and semi-quantitative RT-PCR, 10 μ l of PCR reaction containing 2.5 μ l of 1/20 diluted cDNA, 0.2 μ M of primers, and 5 μ l of 2 \times KAPA SYBR FAST master mix (KAPA Biosystems). PCR was programmed as follows: 95°C for 1 min, 40 cycles at 95°C for 5 s and 55 to 62°C for 20 s. Each reaction was performed in triplicate. Primer pairs and the annealing temperature used for quantitative PCR are listed in Table 2. *Ubiquitin (PaUBI1)*⁶ was used as an internal control.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author Contributions

SCF conceived and designed the experiments. JCC, and MJW performed the experiments and analyzed the data. SCF wrote the paper.

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