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Investigation of Differential Genes Expression in the Genome of *Epipactis flava* Seidenf. (Orchidaceae) under Flooded Condition Using cDNA-SRAP Analysis

Waroon Suwankitti[1], Surin Peyachoknagul[1], Somjit Homchan[1], Pattamon Sang-In[1], Anupan Kongbangkerd[1] and Maliwan Nakkuntod[1*]

[1]Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand.

Authors' contributions

This work was carried out in collaboration between all authors. Author WS performed the experiment, statistical analysis and wrote the first draft of the manuscript. Authors MN and SP designed and managed the analyses of the study. Authors SH, PSI and AK prepared the plant samples and some protocol. All authors read and approved the final manuscript.

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ABSTRACT

Epipactis flava Seidenf. is classified into a rare type of orchid species, a rheophyte. The specific habitat for its life cycle requires a strong current and flooding time. In this study, differential gene expression between submerged and non-submerged conditions was studied using the cDNA-SRAP method. RNAs were extracted from rhizomes at 0, 6, 12, 24 and 48 hours after submergence. Seven candidate genes were selected to study the relative gene expression by real-time RT-PCR and found to be upregulated in submergence condition. Six of these, RNA polymerase sigma factor gene, ATPase gene, Plant homeodomain finger Alfin-like genes, DNA-3-methyladenine glycosylase gene, and Allene oxide synthase gene were induced to the highest level within 12 hrs. Subsequently, the expression of five genes decreased at 24 and 48 hrs of treatment except for

**Corresponding author: E-mail: lotharmali@yahoo.com;*

UDP-glucosyl transferase gene which showed stable expression at all time. Apart from others, dolichyl-diphosphooligosaccharide glycosyl transferase gene was induced to the highest level at 24 hrs and was stable until 48 hrs. This suggested that the induction of genes expression by flooding occurred within 12-24 hrs and most genes in this finding involved in stress responses which were easily upregulated in rheophytic plants during submergence. The more differentially expressed genes should be analysed for a further understanding of the rheophytic habit of this plant.

Keywords: Stream orchid; rheophytic; cDNA-SRAP; gene expression.

1. INTRODUCTION

Orchidaceae is one of the largest families of flowering plant with five subfamilies and about 870 genera. Some species in this family are threatened while others are endemic. Plant numbers have been reduced by human activities such as over-collection due to their ornamental and medicinal values [1] or deforestation. Orchidaceae can be divided into four basic types by habitat as an epiphyte, terrestrial, lithophyte, saprophyte [2] and a rare type, rheophyte which requires a special habitat of a fast current stream and flooding area for a time to complete life cycle [3].

Epipactis flava Seidenf. is the only one species that was classified as a stream orchid (rheophytic orchid) which was found only in Thailand, Laos and Vietnam [3]. It consequently becomes an endangered species. The rheophytic lifestyle seems to be a derived feature in *Epipactis*. The same is assumed to be the case in most other rheophytic genera [4] and recurrent evolution of a rheophytic lifestyle in *Crinum* L. was convincingly documented by Borja et al. [5]. Rheophytes are rare in Orchidaceae, however, *E. flava* is an exceptional one [6].

All known occurrences of *E. flava* in Thailand are located in limestone areas along the path of waterfall at Tak and Kanchanaburi provinces [3]. The other place in which *E. flava* is found is Wah canal in Nan province, where the bedrock is also mixed with widespread of limestone (P. Suksathan, pers. comm.). *E. flava* does not only grow terrestrially but also as a true lithophyte with its rhizome attaching directly to the surface of limestone rocks. It appears to be unique in the genus and might be seen as an adaptation to its rheophytic habit. Thus, the ability to attach firmly to rocks probably reduces the risk of being uprooted by the strong current. The habit of forming pure stands is a common feature of the rheophytes [7]. In October–January when the water level remains low, it produces a vegetative growth such as stems and leaves and reaches a reproductive growth in February–March. In the rainy season during May–September, when the water level rises, the morphology of *E. flava* starts to change. The leaves and flowers get detached, only rhizomes are left attached to the rock beneath the water [4,8]. At present, there are a few studies about genetic information on gene expression of this unique type of plant. This information can help in understanding how this species can survive underwater for four months and changing some morphological characters at the molecular level.

Sequence Related Amplified Polymorphism (SRAP) technique, was developed [9] to construct a linkage map in *Brassica oleracea* L. This marker has been adapted for various purposes such as germplasm identification, map construction, gene tagging, genomic and cDNA fingerprinting and map-based cloning [10]. SRAP markers have been used in many plants including two species of *Dendrobium* SW. [11,12], saffron (*Crocus sativus* L.) [13], *Pinus* species [14] and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) [15]. Furthermore, the cDNA-SRAP technique was applied for differential gene expression study in *Spartina angelica* under salt stress [16], studying comparative gene expression in the restore and maintainer lines of cabbage [17], analysis of genes related to seed-coat color in *Brassica napus* L. [18] and exploration of molecular mechanism of drought resistance in *Erianthus arundinaceus* (Retz.) Jesw. [19]. These studies demonstrate that the SRAP technique is a simple, efficient and reliable tool which could be applied to cDNA-SRAP to study the differential genes expression at specific conditions, which correlate with the changing phenotype. Compared with the other differential expression study techniques, cDNA-SRAP is simple and has better repeatability which makes it more reliable and economical.

Drought and waterlogging stresses are serious problems affecting growth and development of most terrestrial plants. These conditions induced

the expression of various stress-responsive genes [20], hormone signalling pathway [21], energy production and conversion [22]. However, in rheophyte *E. flava,* surviving in a strong current and remaining as rhizome in submerged condition is a common life cycle habit. Thus, its genes expression in this circumstance should be investigated. In this study, the differences in gene expression levels between submerged and non-submerged conditions of *E. flava* were compared using the cDNA-SRAP analysis and qRT-PCR.

2. MATERIALS AND METHODS

2.1 Plant Materials

Epipactis flava from the Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand were grown in a normal dry condition for 2-3 months to produce the uniform rhizomes of the same size (approximately 1 cm) before placing under the artificial waterfall for 48 hours. Their rhizomes were randomly collected from three different plants at 0, 6, 12, 24 and 48 hours after submergence.

2.2 RNA Extraction and cDNA Synthesis

Total RNA was isolated by TRIzol reagent kit (Invitrogen, USA) using a manufacturer's protocol with some modifications. The quality and quantity of total RNA were checked by 1.5% agarose gel electrophoresis and NanoDrop spectrophotometer. First strand cDNA was synthesised using OneScript cDNA Synthesis Kit (Applied Biological Materials, Canada) for further amplification studies.

2.3 Screening and Selection of cDNA-SRAP Primers

A set of five forward and ten reverse SRAP primers (Table 1) was used. The equal amount of first strand cDNA from 6, 12, 24 and 48 hrs treatments were pooled together and used to compare with control (0 hr) cDNA. The reaction was performed by adding the following components to PCR tubes for each PCR amplification reaction: 13.5 µl of RNase-free water, 2 µl of 10X PCR Buffer, 2.0 µl of dNTP mix (2 mM each dNTP), 0.5 µl of each forward and reverse primer (10 µM), 0.5 µl of first strand cDNA, 0.2 µl of *Taq* polymerase (5 U/µl). The PCR reaction conditions were, initial denaturation at 94ºC for 5 min, 5 cycles of denaturation at 94ºC for 1 min, annealing at 35ºC for 1 min and extension at 72ºC for 1 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min and extension at 72ºC for 1 min and final extension at 72ºC for 5 min. All the PCR products were stored at 4ºC until visualised on 1.7% agarose gel prepared in 1X TAE buffer containing 0.5 µg/ml ethidium bromide (EtBr). The selected primers were utilised again and the products were separated by 4.5% non-denaturing polyacrylamide gel electrophoresis, and visualised by silver nitrate staining.

2.4 Extraction and Purification of Differentially Expressed Bands

The differently amplified bands were cut from polyacrylamide gel and soaked in 20 µl of TE buffer, crushed piece of gel with pipette tip and heated at 95ºC for 20 min. 1 µl was used for reamplification following initial denaturation at 94ºC for 5 min, 35 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min and extension at 72ºC for 1 min and final extension at 72ºC for 5 min. PCR products were visualised on 1.5% agarose gel electrophoresis and the ones which were larger than 300 bp were selected and purified for sequencing.

2.5 Sequencing, Bioinformatics Analysis and Primers Design

DNA fragments were sequenced by Macrogen Inc., South Korea (Sanger sequencing). Sequences were blasted in the GenBank database (http://www.ncbi.nlm.nih.gov) and gene-specific primers were designed according to the gene sequences (Table 2).

2.6 Validation of Differentially Expressed Genes

The RNA samples were reverse transcribed and primarily screened by RT-PCR using all the designed primers. PCR products were visualised on 1.5% agarose gel electrophoresis. The genes which expressed differentially were further analysed by qRT-PCR.

Quantitative real-time RT-PCR (qRT-PCR) analysis was used to verify the candidate genes. The RNA samples were reverse transcribed, then first strand cDNA from 3 plants at each time point were pooled as a representative of 3

Table 1. Names and sequences of SRAP primers used

Name	Forward primer sequence (5´-3´)	Name	Reverse primer sequence (5´-3´)
M1	TGA GTC CAA ACC GGA AA	E1	GAC TGC GTA CGA ATT AAC
M2	TGA GTC CAA ACC GGA AG	E2	GAC TGC GTA CGA ATT AAT
M3	TGA GTC CAA ACC GGA AC	E3	GAC TGC GTA CGA ATT GAC
M4	TGA GTC CAA ACC GGA AT	E4	GAC TGC GTA CGA ATT GCA
M5	TGA GTC CAA ACC GGA GC	E5	GAC TGC GTA CGA ATT CAA
		E6	GAC TGC GTA CGA ATT CAG
		E7	GAC TGC GTA CGA ATT CAC
		E8	GAC TGC GTA CGA ATT CGT
		E9	GAC TGC GTA CGA ATT TGA
		E10	GAC TGC GTA CGA ATT TGC

Table 2. Names and sequences of gene specific primers used

Genes name	Primers sequence (5´-3´)
RNA polymerase sigma factor	Forward:AGA AAA GCC ATT TTC CAG CA Reverse:ATT GAA ATG GGG TTT TTG GA
Dolichyl-Diphosphooligosaccharide glycosyl transferase	Forward:CCG ATG GAA AGG TGA GTT TT Reverse:CGA ATT CAA TAG GGG AAC GA
ATPase	Forward:GCA GCA GAC ACA GAA CCT GA Reverse:ATC TGA AGT CCC TCG GTG TG
Alfin like	Forward:GAT CAA GTG ACG GGC AGA TT Reverse:GCA ACT GGG GCA TTT GTA CT
DNA-3-methyladenine glycosylase	Forward:GAT ACC CCC TAA GCC TGC TC Reverse:ACG CAC GTG CAT TTT CAG TA
Allene oxide synthase	Forward:ATG CTG TGG CTG AGA ACC G Reverse:ATT GGC CAA AAA CAA TCG AC
UDP-glucosyl transferase	Forward:TTG GAG ACC AAC ACT TGA ACA C Reverse:TCC CTC TTT CGA GAT TCT GC

biological replications and used for the qRT-PCR. The qRT-PCR was carried out using an Eppendorf Mastercycler S real-time PCR system (Eppendorf, Germany). SYBR green reaction mix (SYBR® Green qRT-PCR Master Mix, Kapa biosystem, Cape Town, South Africa) was used in qRT-PCR reactions, following the manufacturer's instructions. For each reaction, 0.5 µl of cDNA was mixed with 5 µl 2X SYBR green reaction mix, 0.5 µl of 10 µM each of forward and reverse primers and RNase free water was added to make a final volume of 15 µl. The conditions for qRT-PCR amplification were as follows: polymerase activation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. The fluorescence signal was measured once in every cycle. *Actin I* gene was used as an endogenous control gene in the qRT-PCR detection and the relative expression levels of genes were calculated by $2^{-\Delta CT}$ method. All qRT-PCR were performed in 3 technical replications. Data were analysed with one-way ANOVA in a randomized complete block design using Duncan's multiple range test (P = .05) (SPSS ver.17).

3. RESULTS AND DISCUSSION

3.1 cDNA-SRAP Analysis of Differential Gene Expression in *E. flava* under Submerged Condition

E. flava's rhizome is a difficult material for RNA extraction. However, after some modifications from the standard TRIzol protocol, RNA extraction was successful. Out of the 50 primer combinations of SRAP, only 36 primer combinations showed good amplification (Fig. 1). The results from polyacrylamide gel electrophoresis showed 143 upregulated and 83 downregulated bands from all combinations studied. The fragment size of amplicons produced by an individual primer combination ranged from 100 to 1500 base pair. The fragments sized above 300 bp were selected for sequencing step. Sequences blast indicated that 16 differentially expressed genes showed homologies to certain genes from 50% to 96% in GenBank, while the other showed no significant homology with any reported genes.

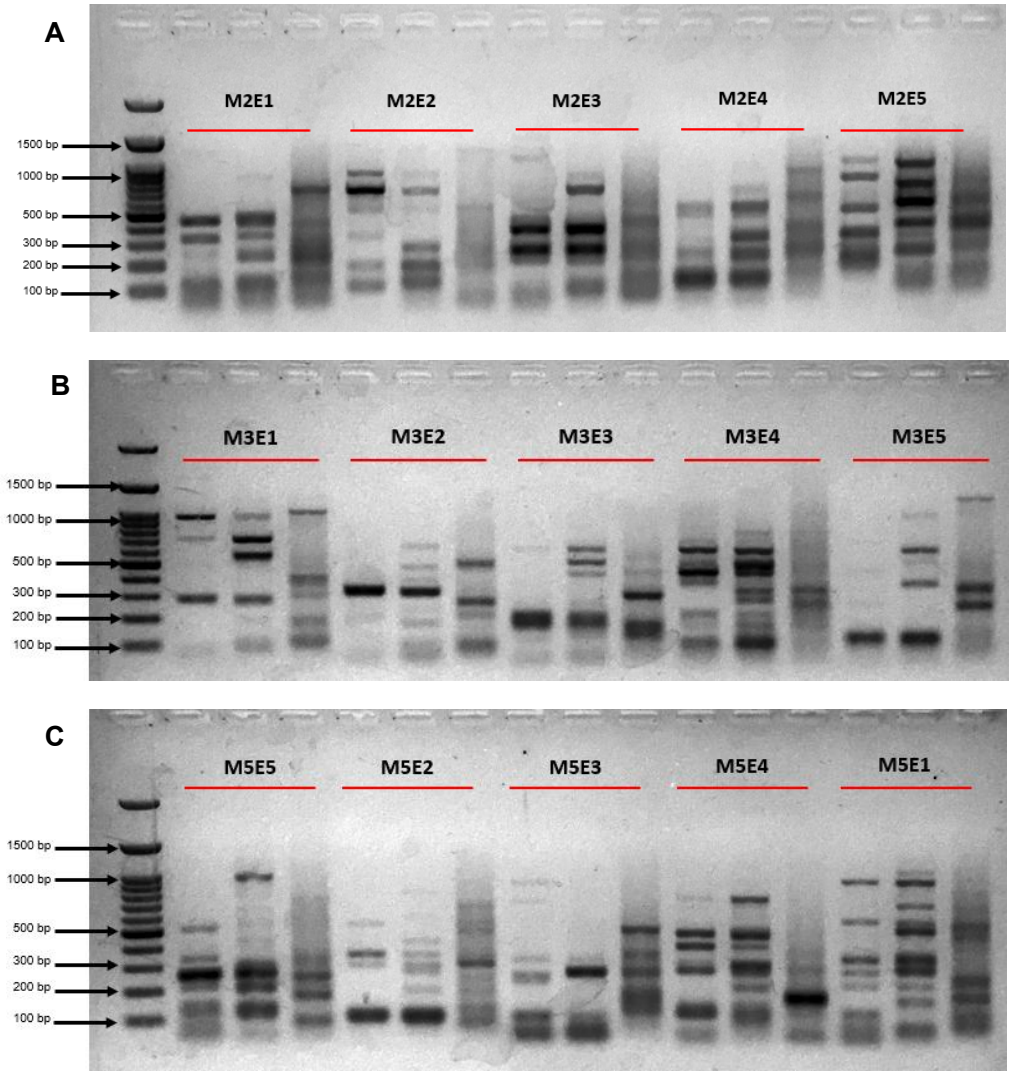


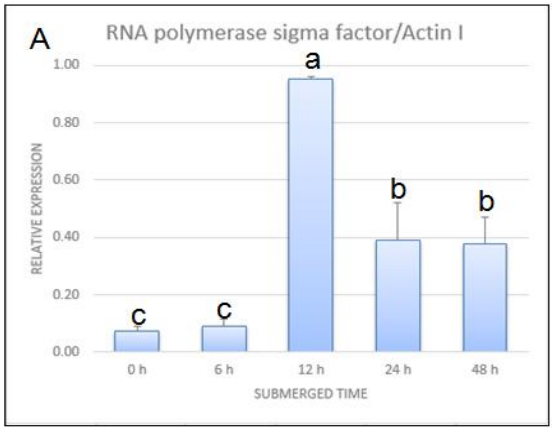
Fig. 1. Agarose gel electrophoresis of products amplified by cDNA-SRAP method of *Epipactis flava* using specified primers; M2E1-5(A), M3E1-5(B), M5E1-5(C). Each group of three samples are control (non-submerged), treatment (submerged) and genomic DNA (for comparison of amplification from the cDNA), respectively and the first lane is 100 bp ladder

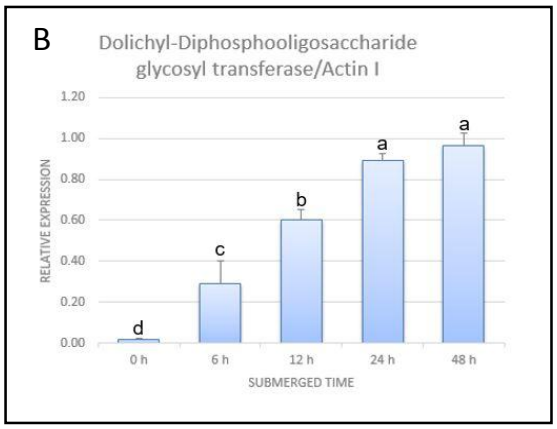
3.2 Validation of Differential Expression of Genes by RT-PCR and qRT-PCR

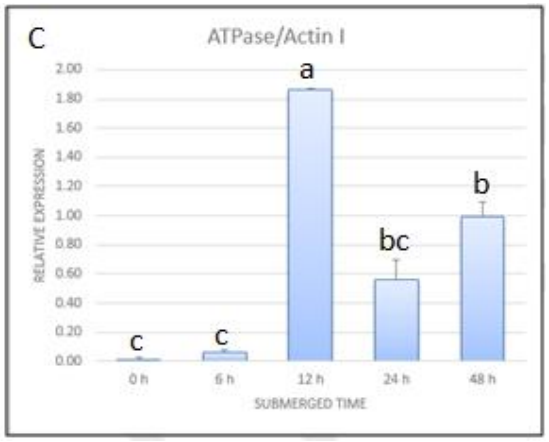
The RT-PCR assay was performed to confirm the differential expression of genes from the cDNA-SRAP analysis. The primers were designed based on the sequences of 16 differentially expressed genes identified in the cDNA-SRAP analysis, and the cDNA from *E. flava* before and after submergence was used as the template in the RT-PCR analysis. The results revealed that nine genes failed to be amplified and did not show any differential expression, which may be due to false positive from cDNA-SRAP. The other seven genes showed effective amplification, except for one fragment, the expression was opposite to that in the cDNA-SRAP analysis (false positive). The seven genes were validated to be truly differentially expressed in *E. flava* under submergence by qRT-PCR.

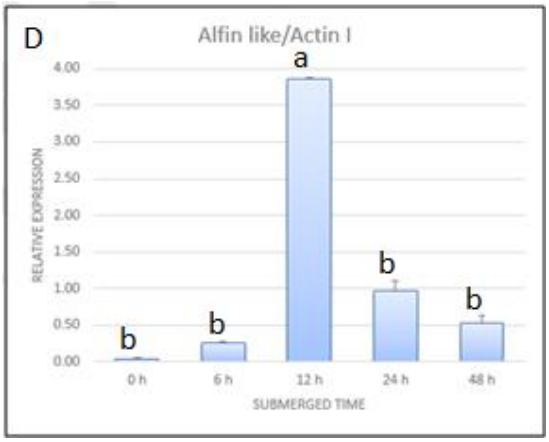
In this study, the $2^{-\Delta CT}$ method was used to measure the relative expression between treatment and control conditions instead of the $2^{-\Delta\Delta CT}$ method because the expression of some genes could not be detected in control (0 hr) (Fig. 2E, 2F, 2G). Among the seven genes found to be upregulated in submerged condition, six of them,

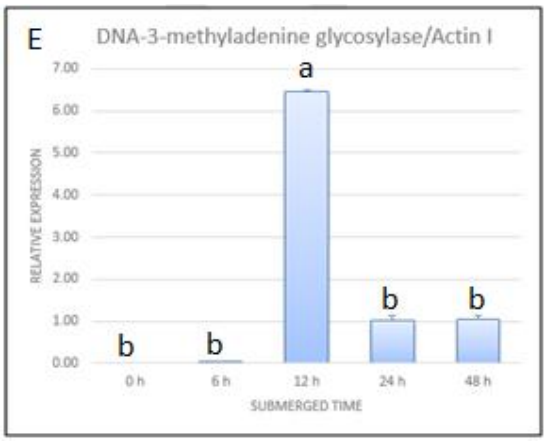
namely, RNA polymerase sigma factor gene, ATPase gene, plant homeodomain (PHD) finger Alfin-like genes, DNA-3-methyladenine glycosylase gene, Allene oxide synthase gene (Fig. 2A, 2C, 2D, 2E, 2F) were induced to the highest level within 12 hrs. Subsequently, the expression of five genes decreased at 24 and 48 hrs of treatment except for UDP-glucosyl transferase gene which showed stable expression at all the treatment time (Fig. 2G). Apart from others, dolichyl-diphosphooligosaccharide glycosyl transferase gene was induced to the highest level in 24 hrs, later than the others, and was stable until 48 hrs (Fig. 2B). This result suggested that the induction of genes expression by waterlogging or flooding occurred within 12-24 hrs.

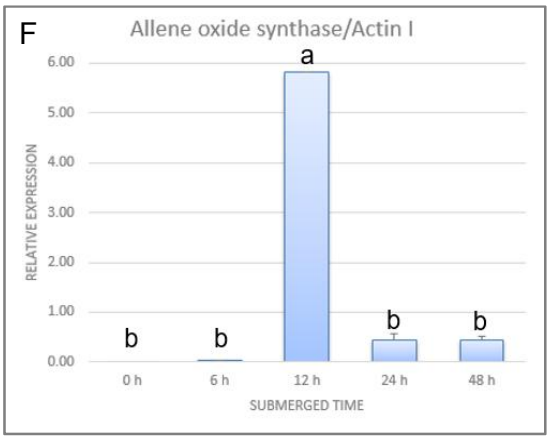












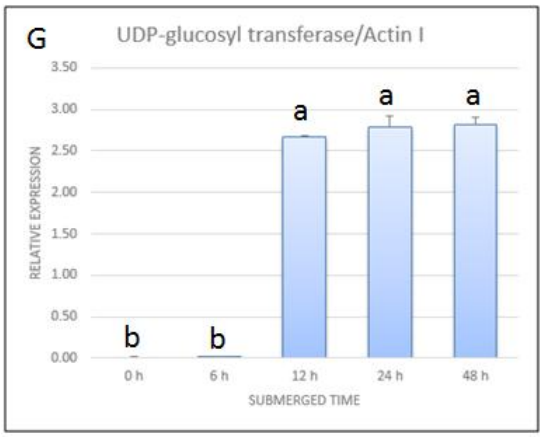


Fig. 2. Relative expression of genes in *Epipactis flava* determined by qRT-PCR and statistical analysis by one-way ANOVA with Duncan's multiple range test. The values are mean of three replications±standard error. The bars with the same letters are not significantly different at *P* =.05

3.3 Expression Profiling of Each Gene

The expression of genes in rhizome in submerged condition was more than ten folds higher than control in all genes and the highest was found in DNA-3-methyladenine glycosylase gene (Fig. 2E). The two genes, dolichyl-diphosphooligosaccharide glycosyl transferase gene and UDP-glucosyl transferase gene showed stable expression until 48 hrs treatment. This suggested that the products of these two genes might be required for longer times in submerged condition.

From the cDNA-SRAP analysis, both upregulated and downregulated genes were found but after purification, sequencing and testing by RT-PCR, only seven genes left, and all showed upregulation by qRT-PCR. Among these, the RNA polymerase sigma factor gene encoded RNA polymerase enzyme which was involved in mediated chloroplast transcription. In *Arabidopsis*, six sigma factors (SIG1-6) were encoded in the nuclear genome and was functional in flowering plant [23,24]. In a recent study, the role of sigma factors has been related to the biosynthesis of plastid. However, *SIG5* is a stress-responsive gene [25]. The result showed the possibility of a candidate gene in *E. flava* to be *SIG5* which was upregulated in this condition (Fig. 2A). However, the role of sigma factor-dependent signaling pathways in plants is still unclear. The activities of plastid sigma factors are regulated in response to distinct stress. It is important to identify more regulation and function of sigma factors to obtain a more informative of SIG-dependent signalling pathways within the context of plant growth and development.

Dolichyl-diphosphooligosaccharide glycosyl transferase genes in *Arabidopsis* (*DGL1)*, human (*OST48*), yeast (*WBP1*), and rice (*OsDGL1*) encode the dolichyl-diphosphooligosaccharide-protein glycosyl transferase 48 kDa subunit precursor [26]. The *OsDGL* displays a change of matrix polysaccharides in its root cell wall, shorter cell length, smaller root meristem and cell death in the root and plays an important role in formation and growth of adventitious root in rice [27]. In this study, this gene was upregulated until 24 and 48 hours (Fig. 2B) which might play a role in producing more adventitious root to stick to the rock under water.

Waterlogging or flooding resulted in oxygen deficiency that affected mitochondrial respiration and leads to insufficient ATP for energy consumption processes [28-31]. The glycolysis pathway is the major responsibility of waterlogging stress [32,33]. Typically, the domain in the ATPase has the conserved function of binding ATP and converting the energy from ATP hydrolysis into a force that promotes cell division, expansion, growth and development [34,35]. In this study, the ATPase gene was induced to the highest level at 12 hours after submergence (Fig. 2C) indicating the need for more energy during submergence.

Plant homeodomain (PHD) finger Alfin-like genes function in several important biological processes

in plants. For example, MALE MEIOCYTE DEATH 1 (*MMD1*) regulates gene expression during meiosis [36], MALE STERILITY 1 (*MS1*) functions in anther development [37], OBERON1 (*OBE1*) and OBERON2 (*OBE2*) function in apical meristem maintenance [38]. Alfin1 from *Medicago sativa* (alfalfa) belongs to a plant-specific sub-family of PHD finger proteins; transcription of Alfin1 is salt-induced, may potentially improve the salt tolerance of transgenic plants [39]. In this study, Alfin-like genes were induced to the highest level at 12 hours after submergence (Fig. 2D) as the response to water stress.

The origin and evolution of land plants is an important event in the history of life. From water to terrestrial environments, plants need to overcome the enhanced ultraviolet (UV) radiation and many other DNA-damaging agents. Evolving new genes with the function of DNA repair is critical for land plants. In bacteria, the DNA-3-methyladenine glycosylase (MAG) recognises a variety of base lesions and initiates the process of the base excision repair for damaged DNA. The homologs of the *MAG* gene are present in all major lineages of streptophytes. Both the phylogenic and sequence similarity analyses have revealed that green plant *MAG* gene has originated through an ancient horizontal gene transfer event from bacteria [40]. This suggested that *MAG* gene might be important in DNA repair mechanism in *E. flava* too, therefore it was induced in high level in submerged condition for cellular maintenance if the DNA damage occurred.

Allene oxide synthase gene is one of the enzymes included in the biosynthesis pathway of jasmonic acid which has been shown to function in stress responses in plants [41]. In this study, this gene showed the highest expression at 12 hours after submergence (Fig. 2F) suggesting that it was triggered by ethylene production from waterlogging stress [42].

UDP-glucosyl transferase gene is a part of abscisic acid (ABA) pathway which is a sesquiterpene hormone that plays an important role in regulating plant growth and development, including seed maturation and dormancy, seed germination, and growth of roots as well as mediating adaptations to environmental stresses, such as cold, drought, and salinity [43-45]. In plants, the effect of ABA is determined by its concentration. When plants encounter abnormal environments, the level of ABA raises to trigger ABA signalling networks to initiate stress responses [46].

The upregulated genes found in this study involved in waterlogging stress, energy production, possible induction of adventitious root and DNA repair function.

4. CONCLUSION

Recently, determination of the differentially expressed genes in plants is becoming popular especially between normal and stressed environments. Flood is a major abiotic stress for plants. However, *E. flava* is a rheophytic plant which can survive under fast running water. During the transitional environment from common dry condition to a sudden flooded condition, caused by heavy rain at the beginning of monsoon period of the year, the whole plant gets submerged under water and remain in that situation for 3-4 months. Although this is the common habit of this orchid, it has been found that at the early stage of the transition the plant remains in stressed condition. Many kinds of stress-responsive genes were induced to the highest level within 12-24 hrs having more than ten folds of expression over the control condition. Two genes, dolichyl-diphosphooligosaccharide glycosyl transferase gene and UDP-glucosyl transferase gene, were also maintained at the highest level until 48 hrs and might be prolonged for a longer time. This indicated that stress-responsive genes are the first groups of genes that are easily upregulated in rheophytic plants during submergence. Therefore, more differentially expressed genes should be analysed for a further understanding of the rheophytic habit of this plant.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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