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Identification of floral genes for sex determination in *Calamus palustris* Griff. by using suppression subtractive hybridization

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ABSTRACT. *Calamus palustris* Griff. is an economically important dioecious rattan species in Southeast Asia. However, dioecy and onset of flowering at 3-4 years old render uncertainties in desired female:male seedling ratios to establish a productive seed orchard for this rattan species. We constructed a subtractive library for male floral tissue to understand the genetic mechanism for gender determination in *C. palustris*. The subtractive library produced 1536 clones with 1419 clones of high quality. Reverse Northern screening showed 313 clones with differential expression, and sequence analyses clustered them into 205 unigenes, including 32 contigs and 173 singletons. The subtractive library was further validated with reverse transcription-quantitative polymerase chain reaction analysis. Homology identification classified the unigenes into 12 putative functional proteins with 83% unigenes showing significant match to proteins in databases. Functional annotations of these unigenes revealed genes involved in male flower development, including MADS-box genes, pollen-related genes, phytohormones for flower development, and male flower organ

development. Our results showed that the male floral genes may play a vital role in sex determination in *C. palustris*. The identified genes can be exploited to understand the molecular basis of sex determination in *C. palustris*.

Key words: *Calamus palustris*; Dioecious; Floral genes; Sex determination; Suppression subtractive hybridization

INTRODUCTION

Rattan is a spiny climbing plant belonging to the sub-family Calamoidea of Palmae. It represents the most important non-wood forest product after timber. *Calamus palustris* Griff., locally known as rotan Langkawi, is one of the commercially important rattan species with distribution in the northern part of Peninsular Malaysia (Nur Supardi, 1990). Its high quality, medium-sized diameter cane has a high demand in furniture and handcraft manufacturing.

The dioecious *C. palustris* has distinct male and female plants, and gender is identified after the first flowering at the age of 3-4 years (Choong et al., 2009). Therefore, species cultivation and breeding programs are confined because the sex ratio is crucial for proper seed orchard design to produce sufficient seeds as planting material for plantation. A sex ratio survey of a planted *C. palustris* population revealed an even ratio of male and female plants, indicating the need for more seed producing female plants in the seed orchard (Choong et al., 2009). Early identification of male and female individuals can help to address the limitation of dieocy.

Five percent of the flowering plants are dioecious; however, only a few contain heteromorphic sex chromosomes, which have been explicitly studied for gender determination (Charlesworth, 2002). For example, *Silene latifolia* possesses large sex chromosome, XY in male and XX in female plants, where the Y chromosome is dominant in sex determination (Mrackova et al., 2008). Nevertheless, a karyological study found no evidence of sex chromosomes in *C. palustris* or in other related species in the same genus (Indira and Anto, 2002). A recent study of *Phoenix dactylifera*, which belongs to the same family as rattan, revealed genes that were linked to gender inheritance through genome sequencing, where male determines the sex in the XY system (Al-Dous et al., 2011). *P. dactylifera* has a homomorphic sex chromosome (Siljak-Yakovlev et al., 1996) that makes the sex determination more sophisticated. If a sex chromosome exists in *C. palustris*, it is probably homomorphic, too. As of now, the reproductive biology of *C. palustris* is still uncertain, and the genetic mechanism for gender determination in *C. palustris* remains ambiguous.

Previous studies of dioecious species revealed that most of the sex-determining genes were found in male plants (Ainsworth, 2000; Al-Dous et al., 2011), which suggests that males play a vital role in the sex determination of dioecious plants. The characteristics of male plants, including prolonged and earlier flowering cycle (Pickering and Hill, 2002) and more frequent reproduction compared to females, might have suggested that male plants are somehow crucial for sex determination. In *C. palustris*, female inflorescence has functional female flowers and abortive male flowers, whereas male inflorescence has male flowers only (Choong et al., 2009). Therefore, we targeted male plants in this study to understand the gene expression in male floral tissue of *C. palustris*. Earlier, an attempt was made to study the floral genes in *C. manan* (a closely related species of *C. palustris*) through expressed sequence tag (EST) analy-

sis from male and female inflorescence cDNA libraries, and only a few floral genes were identified (Nadarajah et al., 2009). Therefore, the subtractive library used in this study provides an alternative approach to enrich sequences or transcripts related to sex. The differentially expressed genes might have potential for developing molecular markers to identify gender of immature *C. palustris* plants for large-scale cultivation and conservation.

MATERIAL AND METHODS

Plant material

Male and female inflorescences of *C. palustris* were obtained from the experimental population planted since 2001 in Bangi Campus, Universiti Kebangsaan Malaysia. The inflorescences in early developmental stages with enclosed flower buds were collected. Flower buds were separated from rachillae, and only fertile female flower buds from female plants (inflorescence) and fertile male flower buds from male plants (inflorescence) were used. The fertile female flower buds were designated as female floral tissues, and the fertile male flower buds were designated as male floral tissues.

RNA isolation and subtracted cDNA library construction

Total RNA was extracted from the male and female floral tissues using the RNeasy Midi Kit (Qiagen, Germany) and treated with on-column DNase I digestion. The extraction was done according to the manufacturer protocol with a minor modification: 1% polyethylene glycol was added to the ground floral tissue prior to adding the lysis buffer. The cDNA was obtained using the SMARTer Pico polymerase chain reaction (PCR) cDNA Synthesis Kit (Clontech Laboratories, USA). A total of 1 µg total RNA was used to synthesize double-stranded cDNA.

The suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) was performed using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories) following manufacturer instructions. cDNA from the male floral tissue was used as a tester, and cDNA from the female floral tissues was used as a driver. Both tester and driver cDNAs were digested with the restriction enzyme *Rsa*I. The tester cDNA was divided into two portions and separately ligated to adaptors 1 and 2R. Two hybridizations with excess driver were performed followed by PCR amplification of differentially expressed cDNAs. The subtraction efficiency was evaluated by PCR amplification of the *GAPDH* gene.

The secondary PCR products were cloned into the pGEM-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* JM109 (Promega, USA). *E. coli* cells were plated on Luria broth (LB) agar containing 100 mg/mL ampicillin, 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), and 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plates were then incubated at 37°C overnight, and clones were selected through blue-white screening.

Plasmid extraction and amplification of cDNA inserts

White colonies were selected and cultured in LB medium containing 100 mg/mL am-

picillin for 20-24 h. Plasmids were extracted using Montage 96-well Plasmid Miniprep Kit (Millipore, USA), according the manufacturer protocol. The cDNA inserts were amplified with M13 forward and reverse primers with the following PCR protocol: 96°C for 2 min; 35 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR amplicons were analyzed by electrophoresis on 1.2% agarose gels for quality and insert size analysis.

Differential screening by reverse Northern analysis

In order to identify positive clones, a reverse Northern dot blot was performed using Digoxigenin (DIG) High Prime DNA Labeling and Detection Starter I Kit (Roche, Germany). Probes for tester and driver cDNAs were randomly labeled with DIG-11-dUTP. Two micrograms of high-quality clones was denatured and dot blotted on nylon membranes. The membranes were pre-hybridized at 42°C for 30 min and then hybridized overnight with DIG-labeled probe at 42°C with gentle agitation. Membrane washing and clone color detection were carried out following manufacturer instructions. The color intensity signals that were formed on membranes were documented, and the differentially expressed positive clones were selected for sequencing.

DNA sequencing and analysis

The selected clones were sequenced from the forward direction using a universal M13 forward primer. Adaptor and vector sequences were removed using the FASTX-Toolkits program (http://hannonlab.cshl.edu/fastx_toolkit/). A homology search was performed using the translated DNA Basic Local Alignment Search Tool (BLASTX) algorithm against the National Center for Biotechnology Information (NCBI) GenBank protein database. Sequences with an E-value $\leq 10^{-5}$ were considered to be significantly homologous. The cDNA sequences were then manually analyzed and grouped into protein functional groups according to the Munich Information Center for Protein Sequences (MIPS).

Reverse transcription-quantitative PCR (RT-qPCR) analysis

The selected differentially expressed genes (glutamate decarboxylase, pollen ole e 1 allergen, hydroxymethylglutaryl-CoA synthase, β-galactosidase 13, and β-glucosidase 1) were further validated by RT-qPCR. Three biological cDNA replicates each for male and female floral tissues were synthesized using SuperScript III First-Strand cDNA Synthesis System (Invitrogen, USA) according to manufacturer instructions. A total of 2 µg total RNA was added to synthesize the first-strand cDNA. Specific primers were designed for the selected genes (Table 1).

RT-qPCRs were carried out using the i-Cycler iQ5 PCR System (BioRad, USA). Each PCR contained 7.5 µL Power SYBER Green PCR Master Mix (Applied Biosystems, USA), 1.2 µL 2.5 µM forward and reverse primers, 1.0 µL cDNA template, and 4.1 µL ddH_2O. The PCR thermal profile was as follows: pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s. The specificity of PCR amplification was assessed by generating melt-curve data within 55-95°C, and data were collected for 10 s with each 0.5°C temperature increase.

The PCR for each target gene was carried out in triplicate. The relative expression

value was calculated from the three biological replicates and normalized to two reference genes (actin and α-tubulin) using the method of Vandesompele et al. (2002).

Table 1. Specific primers for reverse transcription-quantitative PCR analysis.

Gene	Sequence (5'-3')	Amplicon size (bp)
Actin	F: TGCTGATCGTATGAGGCAAGG R: AATCCACATCTGCTGGAAGG	145
α-*tubulin*	F: CATGATCTCCAACTCCACCAG R: CCTCAGCAGACTCAGCACCA	197
β-galactosidase *13*	F: ACTCGTTGCGGAGAGCCAAC R: CGCCACCAGCTTCCTCAAAC	106
β-*glucosidase 1*	F: CCCCATCGTCAATTGCCTTC R: CAGCCCGGAAACGTCACTCT	103
Pollen ole e 1 allergen	F: GAGGCTTGCATTGTTAGGGTC R: GCTCAACCTTACAACTCCTGC	141
Hydroxymethyglutaryl-CoA synthase	F: GCCAAATGCTCCCATTGCTT R: CACTTGCAAGGTTGGGCTTG	89
Glutamate decarboxylase	F: AGCCGTCCTTCGTGTCGTG R: TTGCGGTGCTCTGACTTGG	119

RESULTS

Isolation of differentially expressed cDNAs

In order to determine the differentially expressed genes from male floral tissue in dioecious *C. palustris*, an SSH library was constructed using the male flower bud as a tester and the female flower bud as a driver. The cDNAs synthesized from the floral tissues underwent subtraction to remove complementary sequences, and then they were exponentially amplified to enrich differentially expressed sequences. The library subtraction efficiency was evaluated using the housekeeping gene *GAPDH* (Figure 1). PCR amplicons for subtracted samples were observed after 33 cycles, whereas PCR amplicons for unsubtracted samples were observed after 18 cycles. The abundance of the *GAPDH* gene was reduced in the subtracted sample, indicating the efficiency of the SSH. Therefore, the differentially expressed genes were enriched in the library.

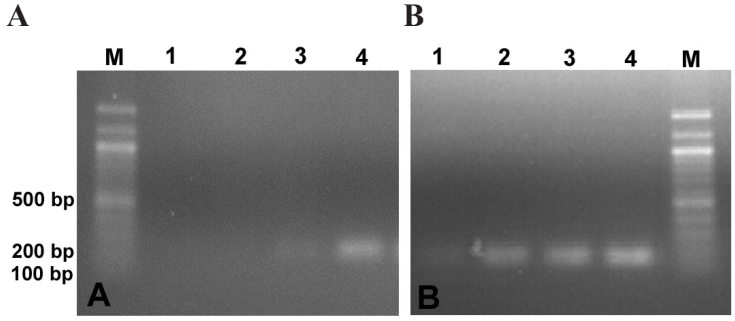

Figure 1. Subtraction efficiency with the *GAPDH* primer. **A.** Subtracted samples; **B.** unsubtracted samples. *Lane 1* = 18 cycles; *lane 2* = 23 cycles; *lane 3* = 28 cycles; *lane 4* = 33 cycles; *lane M* = 100-bp marker (New England BioLabs, USA).

Screening of SSH clones

The constructed cDNA library was screened to detect high-quality, differentially expressed clones. Of all 1536 clones in the library, 1419 clones (92.4%) showed a single amplicon after amplification with M13 primers. Reverse Northern screening with color detection was applied to reduce false-positive clones, and only 313 clones were selected after the screening. These clones were expressed at a higher level in the male flower than in the female flower. Sequencing the selected differentially expressed clones produced 292 high-quality sequences. Assembly analysis of the high-quality sequences produced 205 unique genes consisting of 32 contigs and 173 singletons. The EST redundancy rate of this library was 42.4%. The size of contigs ranged from 263 to 723 bp, and the size of singletons ranged from 151 to 771 bp.

Functional classification of unigenes

The 205 unigene sequences were subjected to homology search with the nucleotide BLAST algorithm against the NCBI database. A total of 171 unigenes were significantly matched with known sequences, and the remaining 34 unigenes had no significant match or no match in the database. The significant matched unigenes were assigned to the functional category of protein according to the FunCat of MIPS. The unigenes were classified into 12 functional categories: metabolism, transport facilitation, protein fate and regulation, signal transduction, defense, energy, transcription, flower development, biogenesis of cellular component, protein synthesis, cell cycle and DNA processing, and hypothetical genes (Figure 2). Genes of particular interest that were found in the SSH were MADS-box genes, pollen-specific and pollen-related genes (pollen ole e 1 allergen, pollen-specific C2 domain containing protein, SWEET, and hydroxymethylglutaryl-CoA synthase), phytohormones (gibberellin 20 oxidase 1, cytokinin riboside 5'-monophosphate phosphoribohydrolase, auxin-induced 5NG4 protein, and gibberellin-regulated protein 10), and flower development genes (β-galactosidase 13, β-glucosidase 1, glucose-6-phosphate dehydrogenase, glutathione synthase, and glutamate decarboxylase).

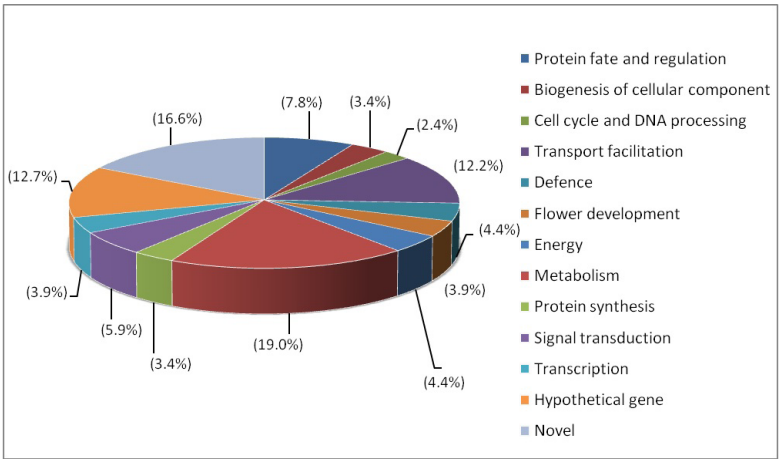

Figure 2. Functional classification of genes differentially expressed in male SSH library.

RT-qPCR analysis

RT-qPCR analysis was used to confirm the expression level of five differentially expressed genes (glutamate decarboxylase, pollen ole e 1 allergen, hydroxymethylglutaryl-CoA synthase, β-galactosidase 13, and β-glucosidase 1) in male and female flowers of *C. palustris*. Optimization was carried out with a standard curve, and all of the genes showed a PCR efficiency of 90-110% and $r^2 > 0.980$. All the five genes were expressed at a higher level in male samples than in female samples. After normalization with the two reference genes (actin and α-tubulin), the graphs were plotted to show the different expression levels of the genes (Figure 3). The results showed that the differentially expressed genes from male flower tissue were successfully isolated in the SSH library. The β-galactosidase 13 gene showed the highest differential expression level (1530-fold), pollen ole e 1 allergen expression was 256-fold higher, β-glucosidase 1 expression was 52-fold higher, glutamate decarboxylase expression was 1.6-fold higher, and hydroxymethylglutaryl-CoA synthase expression was 4-fold higher in male samples than in female samples. The melt curve analysis had a single peak, indicating that the amplicon was specific.

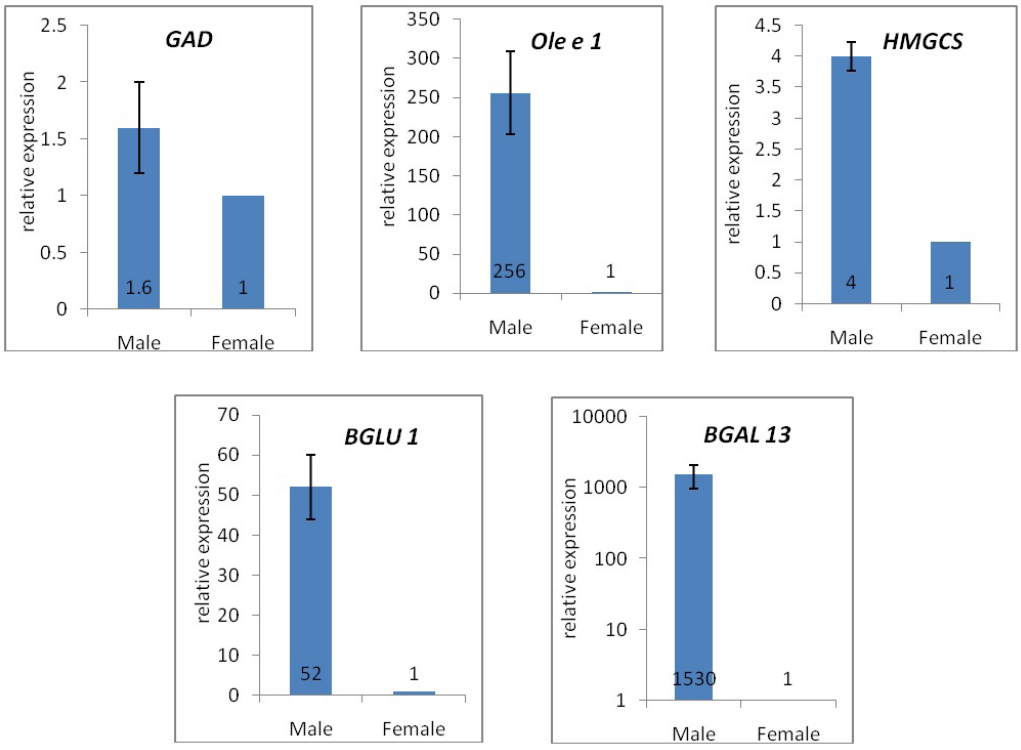

Figure 3. Expression analyses of differentially expressed genes using RT-qPCR. *GAD* = glutamate decarboxylase; *Ole e 1* = pollen ole e 1 allergen; *HMGCS* = hydroxymethyglutaryl-CoA synthase; *BGAL 13* = β-galactosidase 13; *BGLU 1* = β-glucosidase 1.

DISCUSSION

SSH can be used to efficiently isolate differentially expressed genes between different samples (Diatchenko et al., 1996) and to construct a subtractive cDNA library. Although SSH has been widely used in various plant species, this SSH male flower tissue library was the first use of SSH in *C. palustris*. Most studies revealed that sex-linked markers are abundantly linked to maleness and the male is the heterogametic sex for species with sex chromosomes (Ainsworth, 2000). The male flower consists of stamens for the development of pollen grains. The stamen consists of anthers and stalk-like filaments, and male gametogenesis occurs within the anther to develop pollen grains. The mechanism involved in the development of the unique and sex-determining organs of male plants, the stamen and pollen, consists of various special events that are different from the processes taking place in the meristem. The uniqueness of this event makes the study of male flower genes crucial to understanding the molecular biology and the regulation of dioecy in flowering plants. Thus, finding differentially expressed genes will be less complex in male samples than in female samples.

Differentially expressed genes in male flowers

Reverse Northern screening was performed in order to eliminate false-positive clones and select the differentially expressed genes in the SSH library. This result was substantiated by the RT-qPCR of five genes (glutamate decarboxylase, pollen ole e 1 allergen, hydroxymethylglutaryl-CoA synthase, β-galactosidase 13, and β-glucosidase 1) that were selected from the differentially expressed gene data. The RT-qPCR result verified the validity of the male SSH library, where all five genes showed higher expression levels in the male flower than in the female flower, indicating the consistency of the SSH library in determining the differentially expressed genes in *C. palustris*.

Of the 205 differentially expressed unigenes that were identified in the SSH of *C. palustris*, the presence of gene contents agrees with the activity in the development of male gametophytes. The formation of male flowers occurs by the growth and differentiation of meristem tissue to form vegetative tissue and organ (stamen). The formation of male gametophytes occurs in the stamen, the male reproductive organ. The stamen and pollen development are consistent with genes involved with features such as rapid growth, storage compound accumulation, and desiccation and dehiscence of water movement and stress compound (Scott et al., 2004). The functional annotation placed the differentially expressed genes in the SSH library into 12 groups of putative protein categories (Figure 2).

The diversity of the functional categories might suggest that male flower tissue development in *C. palustris* is a complex biological process. We noted a few genes of particular interest in the SSH library: MADS-box genes, pollen-specific and pollen-related genes (pollen ole e 1 allergen, pollen-specific C2 domain containing protein, SWEET, and hydroxymethylglutaryl-CoA synthase), phytohormones (gibberellin 20 oxidase 1, cytokinin riboside 5'-monophosphate phosphoribohydrolase, auxin-induced 5NG4 protein, and gibberellin-regulated protein 10), and flower development genes (β-galactosidase 13, β-glucosidase 1, glucose-6-phosphate dehydrogenase, glutathione synthase, and glutamate decarboxylase). An EST analysis of *C. manan* (a closely related species of *C. palustris*) inflorescences led to the discovery of male-related genes (*Men-7* and proline-rich anther-specific protein) in the female

floral tissue (Nadarajah et al., 2009). Nevertheless, these two floral genes were absent in our male SSH library of *C. palustris*. It is most likely that they were expressed in the female floral tissue of *C. palustris*, and they might have been subtracted from the male SSH library.

MADS-box genes

MADS-box genes encode transcription factors that control plant developmental processes (Krizek and Fletcher, 2005). The expression of MADS-box genes is integrated in a complex biological event. Based on the ABCDE model, floral MADS-box genes can be divided into five classes, and interactions within the homeotic genes refer to different floral organ development (Becker and Theissen, 2003). The various functions of MADS-box genes can be exemplified where class A genes control sepal development in whorl 1, and classes A and B in whorl 2 are involved in petal formation. In whorl 3, expression of classes B and C will regulate the stamen development. Class C genes in whorl 4 alone will control the carpel development, while class D genes specify the ovule, and class E genes are expressed for formation of all floral organs (Becker and Theissen, 2003).

Three MADS-box genes were detected in the male SSH library: MADS-box transcription factor 7 (*MADS 7*) and Agamous-like MADS-box protein (*AGL 9*) belonging to class E, and MADS-box transcription factor 16 (*MADS* 16) is in class B. All of these MADS-box genes are crucial in male floral development to induce the formation of the stamen. A study conducted on paddy *MADS 16* genes showed that the transgenic mutant line produced sterile males by causing changes in the stamen and lodicule structures, and some stamens were even converted into carpels (Xiao et al., 2003). Both *MADS 7* and *AGL 9* are essential in floral organ development, and the absence of these class E genes will affect the floral organ forming into a sepal or leaf (Ditta et al., 2004). The *MADS 7* gene is also involved in flowering time (Kang et al., 1997).

Pollen-specific and pollen-related genes

Homologs of several pollen-specific and pollen-related genes (pollen ole e 1 allergen, pollen-specific C2 domain containing protein, SWEET, and hydroxymethylglutaryl-CoA synthase) were identified in the male SSH library. The genes expressed in pollen give good insight into the molecular basis of pollen and the interaction between pollen and floral organ development. There are two pollen-specific genes that were detected in this study (pollen ole e 1 allergen and pollen-specific C2 domain containing protein). The pollen ole e 1 allergen gene was expressed during the development of pollen in *Olea europaea* (de Dios et al., 1999). However, the function of this gene is still unknown, although it was expressed during pollination (de Dios et al., 1999). Another pollen-specific gene in the male SSH library is pollen-specific C2 domain containing protein (*NaPCCP*), which is one of the proteins found in *Nicotiana bonariensis* pollen (Lee et al., 2009). This gene controls the interaction between protein and lipid, and it also functions as a transporter within the cell for pistil arabinogalactan protein and phosphatidylinositol 3-phosphate during pollination. Lee et al. (2009) also noted that *NaPCCP* was attached to the plasma membrane and vesicles of the pollen tube during pollination.

The bidirectional sugar transporter *SWEET 8* (SWEET 8/ruptured pollen grain 1) and bidirectional sugar transporter *SWEET 6a* genes are categorized in the family of SWEET

genes. These bidirectional SWEET genes are important in producing nectar, seeds, and pollen. *SWEET 8* encodes a protein in the plasma membrane for developing exine microspores in *Arabidopsis thaliana* (Guan et al., 2008). The *SWEET 8* gene is highly expressed in microspores and the tapetum during meiosis in male flowers, and its mutant showed decreased male fertility (Guan et al., 2008).

Hydroxymethylglutaryl-CoA synthase (*HMGCS*) is involved in the mevalonate pathway in the biosynthesis of isoprenoids such as sterol (Wang et al., 2012). In *A. thaliana*, *HMGCS* is encoded by the flaky pollen 1 (*FKP 1*) gene. A study of the *FKP 1* mutant revealed that the *HMGCS* enzyme is important in the mevalonate pathway for developing the tapetum-specific organelle and the fertility of pollen (Ishiguro et al., 2010). A null allele of *FKP 1* produced male gametophytes that were lethal before the elongation of the pollen tube, and its absence caused sterile male gametophytes (Suzuki et al., 2009).

Phytohormones

Phytohormone signaling plays an important role in regulating plant growth and determining the formation of floral organs. The main classes of phytohormones consist of gibberellins, ethylene, abscisic acid, auxins, cytokinins, brassinosteroids, and jasmonates. Several genes encoding for the biosynthesis, perception, and signaling of phytohormones have been identified from the complex pathway of phytohormones (Stamm and Kumar, 2010).

Gibberellin plays an important role in plant development, including vegetative, flower, and fruit development, and it is the regulatory hormone for plants. Gibberellin 20 oxidase 1 (*GA20ox1*) was differentially expressed in the SSH library of *C. palustris*. It is involved in catalyzing gibberellins. The suppression of *GA20ox1* in tomato revealed deteriorating effects to the pollen development (Olimpieri et al., 2011). Another gibberellin-related gene that was found in the SSH library was giberellin-regulated protein 10, which belonged to the hormone-regulated gene family. The expression of this gene in flower buds may engage various biological processes such as germination, seed development, and flower induction (Roxrud et al., 2007).

Gene activation by phytohormones is important in flower development. Auxin-induced 5NG4 protein was found in the SSH library and is activated by the presence of auxin. Auxin is the regulator of flower development. It determines the site for flower growth and regulates organ growth and reproduction activity (Krizek, 2011). Auxin can reverse the male flower organ sterilization that is caused by high temperatures in *Hordeum vulgare* and *Arabidopsis* (Sakata et al., 2010), indicating the importance of auxin in male organ development.

Cytokinin is an important phytohormone in the development of post-embryo aerial organs such as leaf, stem, and flower in meristem. Lonely guy acts as an activation enzyme for cytokinin at the meristem of paddy and *Arabidopsis* (Chickarmane et al., 2012). The probable cytokinin riboside 5'-monophosphate phosphoribohydrolase (lonely guy like 8/*LOGL 8*) from paddy was differentially expressed in the SSH library. The *LOG* gene is required to maintain meristem activity, and the absence of the *LOG* gene causes abnormal termination of growth (Kurakawa et al., 2007).

Flower development genes

Flower development is an essential stage of plant reproduction. The genetic regulation

during flower development is a complex biological process, which involves various genes that are likely to have critical roles encoding proteins in different pathways. β-galactosidase 13 was identified in the SSH library of *C. palustris*. The β-galactosidase gene in most plants has a role in degrading pectins, xyloglucans, or arabinogalactan proteins at the cell wall (Tanthanuch et al., 2008). This gene has been observed to have a certain role in plant reproduction. In tobacco, β-galactosidase is expressed in microspores during pollen development (Hrubá et al., 2005). This gene is expressed in *Beta vulgaris* at the final stage of anther differentiation as well as during the elongation of the pollen tube (Majewska-Sawka et al., 2004). The function of this gene is still in doubt, but it has been suggested that it may play a role in degrading the cell wall during pollen dispersal after mitosis of microspores during pollination (Hrubá et al., 2005).

The β-glucosidase enzyme in plants, which has been well studied, belongs to the glycosyl hydrolase family. This enzyme is crucial for plant defense, regulation of phytohormones, catabolism of oligosaccharides, and lignin formation. β-glucosidase 1 (*BGLU 1*) is one of the differentially expressed genes in the SSH library of *C. palustris*. *BGLU 1* is highly expressed in flowers of *Oryza sativa*, and it is suggested that *BGLU 1* functions in hydrolysis and recycling of oligosaccharides during cell wall development, seed germination, and flower blossoming (Opassiri et al., 2003). The coexistence of *BGLU 1* and gibberellin glucosidase showed the consistency and function of this gene in the activation of phytohormones where gibberellin is mandatory in floral organ and anther development (Hasegawa et al., 1994).

Glutathione is an important antioxidant in plant growth and defense mechanisms during pollination. Two clones homologous to glucose-6-phosphate dehydrogenase and glutathione synthase, which are involved in glutathione metabolism, were found in the SSH library. The study on *A. thaliana* revealed that glutathione synthesis is important during *in vitro* pollination and that inhibitors of glutathione synthesis affect auxin metabolism and threaten the pollination process (Zechmann and Russell, 2011).

Glutamate decarboxylase is differentially expressed in the SSH library. It is a crucial enzyme involved in the biosynthesis pathway of gamma-aminobutyric acid (GABA). It acts as a catalase in the decarboxylation of glutamate into GABA and CO_2. GABA is the molecule that signals for pollen tube elongation. In *Arabidopsis*, pollen tube elongation is controlled by pollen-pistil-interaction 2 (*Pop 2*), which regulates the concentration of GABA (Palanivelu et al., 2003). The concentration of GABA increases during the elongation of the pollen tube toward female flower tissue, creating a concentration gradient in the pistil. The *Pop 2* gene encodes a transaminase enzyme, which degrades GABA during pollen tube elongation. The interaction between the transaminase enzyme and GABA creates a guidance path for the pollen tube toward the ovule micropyle (Bouché and Fromm, 2004).

Development of molecular markers for sex identification

Cultivation and breeding programs for dioecious species are problematic because of the inability to differentiate the gender of the plant at an early growth stage. This seems to be true for *C. palustris*, where it takes 3-4 years to identify the plant gender through inflorescences (Choong et al., 2009). Thus, the development of molecular markers is much needed for sex identification at an early growth stage.

The identification of a handful of interesting transcripts in the SSH library that are linked to floral development is of particular interest to develop molecular markers for sex iden-

tification in *C. palustris*. Furthermore, the RT-qPCR results showing highly differential expression of pollen ole e 1 allergen, β-galactosidase 13 and β-glucosidase 1 between male and female floral tissues of *C. palustris* (Figure 3) are of particular interest for the development of gene-based molecular markers for sex identification. We strongly feel that the other differentially expressed transcripts from the SSH library need to be validated using RT-qPCR to give a clearer picture of the genes that are involved in male floral tissue development in *C. palustris*.

CONCLUSIONS

The mechanism of sex determination and flower development in the dioecious rattan *C. palustris* is a complicated biological process. Nevertheless, through SSH, we have identified some important genes that are involved in the development of the male floral organ. The differentially expressed transcripts from the SSH library provide a vital resource for understanding the molecular basis of sex determination and the development of molecular markers for sex identification in *C. palustris*.

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