Malays. Appl. Biol. (2016) 45(2): 1–10

SEXING IN RATTANS

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Accepted 14 October 2016, Published online 21 December 2016

ABSTRACT

In Malaysia, rattans belonging to *Calamus* species are regarded as the most economically important non-wood resource from forest after timber. However, the genetic resource of rattans in the wild is much depleted due to over exploitation and lose of forest habitat. Therefore, there is a need to establish large scale plantation to produce enough canes for furniture and handicraft industries. Large scale planting requires sufficient planting materials, and the establishment of seed orchards for seed production is important for supplying sufficient planting materials. However, the dioecy in rattan limits its breeding and cultivation. The dioecious *Calamus* species have distinct male and female plants, and gender is identified only after the first flowering. Early identification of male and female individuals by molecular markers can help to address the limitation of dioecy for large scale planting. In this paper, we address the dioecy and sex ratio in *Calamus* species. Subsequently, we discuss the functional genomics of *Calamus manan* and *C. palustris* in understanding the sex determination and flower development in rattans.

Key words: *Calamus*, dioecy, floral genes, genomics, rattans

INTRODUCTION

Rattan is a spiny climbing plant belonging to the family Arecaceae, and classified in the large subfamily Calamoidea for its scaled fruits. Approximately 600 different rattan species in 13 genera (*Calamus*, *Calospatha*, *Ceratolobus*, *Daemonorops*, *Eremospatha*, *Korthalsia*, *Laccosperma*, *Myrialepis*, *Oncocalamus*, *Plectocomia*, *Plectocomiopsis*, *Pogonotium* and *Retispatha*) are distributed through the tropical and subtropical regions worldwide. The rattan species are dominated by the genus *Calamus* with roughly 370 species (Dransfiled, 1992). In Peninsular Malaysia alone, there are more than 100 species (Aminuddin, 1995), indicating the high biodiversity of rattan resources in this geographical area. Of these, roughly 25 species are of commercial value, including *Calamus manan*, *C. caesius*, *C. ornatus*, *C. palustris*, *C. scipionum* and *Korthalsia* spp. (Aminuddin, 1995).

The rattan canes are used in matting, furniture, fish trap and handicraft; the leaves are traditionally used as cigarette paper, thatching; and the fruits of some species are edible and fruits from a few species of *Daemonorops* produce Dragon's blood, a deep maroon resin, used as dye, vanish and Chinese medicine (Dransfield, 1979). However, the real commercial use of rattan is of its canes for furniture making. Therefore, it is not surprising to note that rattan represents the most important non-wood forest product after timber. According to the rattan furniture statistics (data up to year 2011) by Malaysian Timber Industry Board, the total export value of rattan furniture for year 2011 was just over RM21 million, and Singapore, Australia, Belgium, China and the United Kingdom were the main export destinations (MTIB, 2016). However, there was a trend of decreasing export value from RM55 million in 2005 to RM21 million in 2011 (MTIB, 2016), and this may indicate that the rattan cane resources for furniture industry are depleting. Therefore, there is an urgent need of large scale rattan plantation for mass production of rattan canes. With the purpose of establishing rattan cultivation, our research focuses on two commercially important species – *C. palustris* and *C. manan* (Figure 1). Both the species have been listed as the seven high priority rattan species for regional and international actions (Rao & Ramanatha Rao, 1998).

Calamus palustris is locally known as rotan manau Langkawi, with its distribution in the

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Fig. 1. Rattan plants of *C. manan* (left) planted in an over-logged secondary forest and *C. palustris* (right) planted in an *Acacia* experimental plot.

northern part of Peninsular Malaysia (Nur Supardi, 1990). However, this rattan species is rather common in Myanmar, Thailand and Indochina (Dransfield, 1979; Aminuddin & Salleh, 1994). In Peninsular Malaysia, it is found growing in belukar and limestone hills. *C. palustris* is a clustering rattan species with a cane diameter of 1.5-2 cm and a length between internodes of 30 cm (Dransfield, 1979). A cluster of rattan may consist up to 10 stems (Choong *et al*., 2009). Its yellowish glossy medium-size diameter cane is of excellent general appearance for furniture making. In Peninsular Malaysia, *C. palustris* is found being planted near villages, and this might suggest that it has potential as a cultivated rattan species (Dransfield, 1979).

On the other hand, *C. manan* is more well-known in Malaysia for its large diameter cane. Known locally as rotan manau, *C. manan* is a solitary rattan species, with a cane diameter reaching up to 8 cm and a length between internodes of 40 cm (Dransfield, 1979). It is distributed through Peninsular Malaysia, and it can also be found in Sumatra, South Thailand and Borneo (Dransfield, 1979). It grows well on steep slopes in hill dipterocarp forest at an altitudinal range of 50-1000 m. Its large diameter canes are excellent quality for large size furniture. Undoubtedly, *C. manan* is one the most exploited rattan species in Malaysia until its distribution range in the wild has been very much reduced.

Planting of rattan for cane production is still in developing stage. The enormous spines and climbing behavior of rattan plants somehow pose a challenge in rattan cultivation. Rattan planting systems for small-diameter and large-diameter cane species and the planting challenges have been discussed in detailed by Tan (1992) and Nur Supardi & Aminuddin (1992). Attempts have been made to grow *C. manan* and *C. palustris* with different planting systems, including intercropping with rubber and acacia trees and planting in logged-over forest (Nur Supardi & Aminuddin, 1992; Tan, 1992).

DIOECY

Most rattan species are dioecious with the presence of male and female plants, except rattan species from genera *Korthalsia*, *Oncocalamus*, *Laccosperma* and *Eremospatha* which are monoecious (Dransfield, 1979). These four monoecious genera only consist roughly 50 species. For the dioecious rattans, the male inflorescence (from male plant) of *Plectocomia*, *Plectocomiopsis* and *Myrialepis* bears only male flowers and the female inflorescence of these rattans bears only female flowers (Dransfield, 1979). On the other hand, the dioecious rattans of genera *Calamus*, *Daemonorops*, *Calospatha* and *Cerotolobus* are different from the former three dioecious genera. The male inflorescence of these later four genera

bears only male flowers while their female inflorescence consists of fertile female flowers and sterile male flowers in pair (Dransfield, 1979; Raja Barizan, 1992). The presence of different dioecy forms may indicate the complexity of dioecy in rattans.

Raja Barizan (1992) documented in detail the dioecy of *C. manan* by illustrating well the structures of the male and female inflorescences. On the other hand, Kidyoo & McKey (2012) discussed and illustrated in detail the dieocy of *C. castaneus*. It seems that both *C. manan* and *C. castaneus* have similar structure of male and female inflorescences. The structures of the male and female inflorescences are distinguishable. In male inflorescence, the flowers (only male flowers) are borne on the third rachilla order. On the other hand, flowers (fertile female and sterile male flowers in pair) of the female inflorescence are borne on the second rachilla order (Raja Barizan, 1992). At many times, the inflorescences of *C. manan* are high in the canopy of forest, and the sex of the plants are easily distinguished in the field by looking at the inflorescence structure assisted by a pair of good binoculars. The inflorescence structure of *C. palustris* (Figure 2) is similar to *C. manan* (Choong *et al*., 2009). Therefore, we expect the mechanism of dioecy development in both *C. manan* and *C. palustris* to be similar.

Roughly 5% of the flowering plants are dioecious. However, only a few contain heteromorphic sex chromosomes, which have been explicitly studied for gender determination in plants (Charlesworth, 2002). A good example is in a few *Silene* species which possess large sex chromosomes, XY in male and XX in female plants, where the Y chromosome is dominant in sex determination (Mrackova *et al*., 2008). A study of *Phoenix dactylifera*, in 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). Furthermore, the sex chromosomes in *P. dactylifera* are homomorphic (Siljak-Yakovlev *et al*., 1996), making the sex determination more sophisticated. *Calamus* rattans are usually diploid species with chromosome number 2n = 26 or 28 (Sarkar & Datta, 1985; Renuka *et al.,* 1998; Indira & Anto, 2002; Wang *et al*., 2005). The chromosome number for *C. palustris* is 2n = 28 (Indira & Anto, 2002), but the chromosome number for *C. manan* is unknown. Nevertheless, karyological studies found no evidence of sex chromosomes in *C. palustris* and other related species in the same genus (Indira & Anto, 2002; Wang *et al*., 2005). As of now, the genetic mechanism for gender determination in *Calamus* rattans remains unclear.

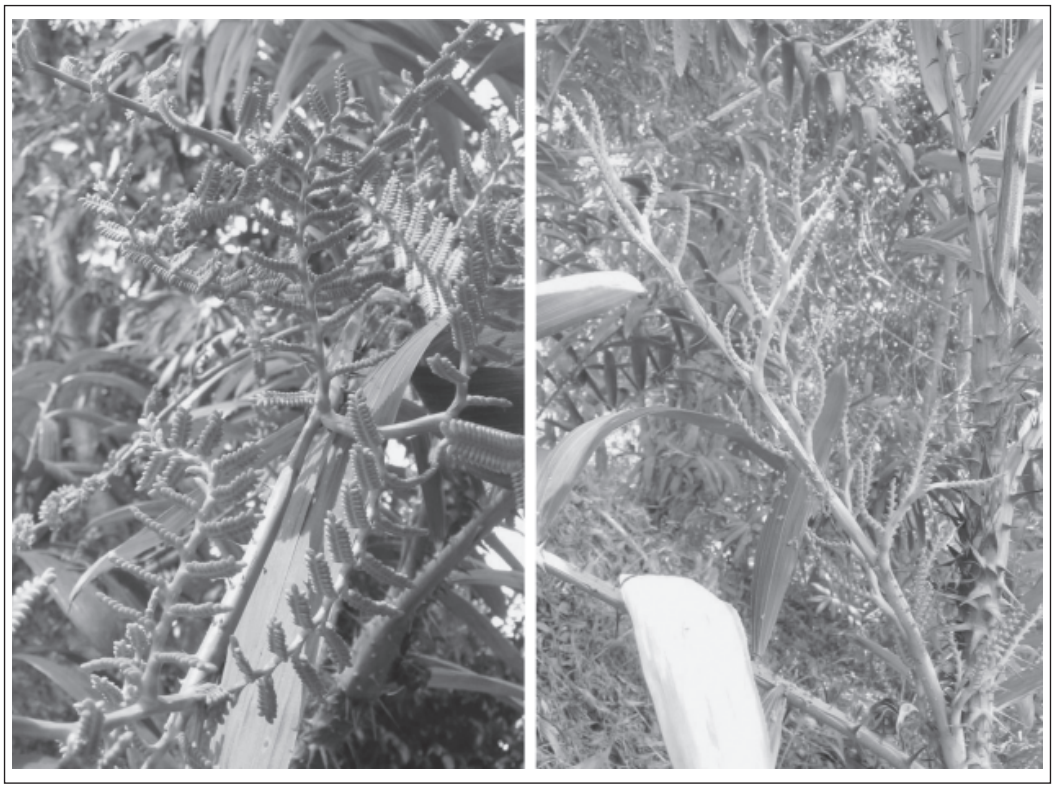

Fig. 2. Inflorescences of *C. palustris*. Left: male inflorescence, and right: female inflorescence.

The gender of the dioecious *Calamus* rattan is basically distinguishable only by its reproductive part – the inflorescence. Therefore, the gender of the plants is only identified after the first flowering. There is no study on the first flowering age of *Calamus* rattan in the wild. However, planted populations showed that *C. manan* comes into first flowering at an age of 5.5 years (Manokaran, 1985) while *C. palustris* starts its first flowering at an age of 3-4 years after planting (Choong *et al*., 2009). The unknown gender at early growth stage always poses a challenge for rattan cultivation and breeding as a seed orchard design with proper sex ratio of male and female plants is imperative to produce sufficient seeds for plantation establishment.

SEX RATIO

Sex ratio study is always an important aspect for dioecious plant species. Primary sex ratio is expected to be kept in an evolutionary balance near one to one (Meagher, 1981). An equal primary sex ratio is to be expected on the basis that each offspring produced will contain equal gene complements from male and female parents in a dioecious sexually reproducing population. However, skewed sex ratios have been reported in natural populations for a number of plant species (Meagher, 1981; Armstrong & Irvine, 1989; Sakai, 1990). The skewed sex ratios could be the result of prezygotic factors of the differential success of male and female determining gametes, which bring about unequal numbers of male and female progeny, or they could be the result of postzygotic life history differences between males and females (Lloyd & Webb, 1977; Meagher, 1981; Sakai, 1990). The implication of skewed sex ratios in dioecious plant species is particularly vital in the planning of seed production areas, studies on genetic diversity and conservation of near-extinct species (Opler & Bawa, 1978).

The understanding of sex ratio in rattan species would contribute towards better management of natural stands and the development of seed orchards to ensure efficient seed production for rattan plantation. No conclusive study has been made to determine the sex ratio of rattan species in wild populations. This is mainly due to the difficulty of finding undisturbed population in the wild. Nevertheless, the estimation of sex ratio of some rattan species has been made in established plantations and experimental plots (Aminuddin & Nur Supardi, 1993; Chia, 2000). Aminuddin & Nur Supardi (1993) surveyed a planted *C. manan* in a secondary forest located in FRIM Kepong, and they found an male-biased sex ratio population (male:female = 1.55:1). On the other hand, Chia (2000) surveyed two planted populations of *C. subinermis*, one in a secondary logged-over forest (male:female = 1:1.04) and the other in an *Acacia* plantation (male:female = 1:1.75), and it was noted that sex ratios were biased towards female.

We conducted sex ratio surveys on a planted population of *C. manan* (Jaya Kumar *et al*., 2005) and a planted population of *C. palustris* (Choong *et al*., 2009). The *C. manan* population is in a secondary logged-over forest located in FRIM Kepong, and the plants were planted in 1978. This *C. manan* population is the same as that surveyed by Aminuddin & Nur Supardi (1993), but they only managed to identify gender on 69 plants in that population. Our survey was conducted from December 2003 to July 2004. A total of 80 male and 55 female plants were identified, giving a male-biased sex ratio (male:female = 1.45:1) for the planted population of *C. manan* (Jaya Kumar *et al*., 2005). Our result is not much different from that of Aminuddin & Nur Supardi (1993) – male bias. The deviant sex ratio could be due to the differential mortality rate between male and female plants, with the assumption that the seed selection from the seed lot used for planting was not biased. Differential mortality rates have been put forth as a factor resulting in male-biased sex ratios in several dioecious plant species (Meagher 1981; Sakai, 1990). Female plants are more prone to an early demise than male plants because of greater energy investment in fruits/seeds and associated structures.

On the other hand, the planted population of *C. palustris* is in an *Acacia* experimental plot, located in Bangi Campus of Universiti Kebangsaan Malaysia. The plants were planted in 2001. The field survey on the *C. palustris* population was conducted from September 2003 to July 2006. A total of 46 male and 43 female plants were identified in the experimental plot, giving a near equal sex ratio (male:female = 1.1:1) (Choong *et al*., 2009). The near even sex ratio of the planted *C. palustris* population might indicate absence of postzygotic sex selection and unbiased sex selection during seed germination.

Natural populations of many dioecious plant species tend to have equal sex ration (Rottenberg, 1998, 2000) and some may bias in favour of male (Dupont & Kato, 1999). The female-biased sex ratio for dioecious plants is infrequent (Stehlik & Barrett, 2005). However, it is worth to note that the planted population of *C. subinermis* in an *Acacia* plantation (Chia, 2000) is female-biased sex ratio. It is not clear if the male-biased sex ratio observed in the planted *C. manan* population and the near equal sex ratio observed in the planted *C. palustris* would also be applied to natural populations. Nevertheless, the sex ratio in planted populations is of much interest for the purpose of seed production for rattan plantations.

FUNCTIONAL GENOMICS

Information on the molecular biology of flowering in rattan species is important for understanding the development of its flowers and to identify the genes that are expressed in various stages of flower development as well to identify those that are being expressed specifically in either male or female plants. This knowledge is essential for the development of molecular markers for sex identification in dioecious rattan plants. We attempted to study the floral genes in *C. manan* through expressed sequence tags (ESTs) generated from male and female inflorescence cDNA libraries (Thi, 2004; Nadarajah *et al*., 2009b). In another more recent work, we investigated the differentially expressed floral genes in the male inflorescence of *C. palustris* using suppression subtractive hybridization (SSH) approach (Ng *et al*., 2014).

The EST analysis is much of use to analyze expressed genes in a specific tissue within any given genome. Functional analysis on ESTs has been conducted to understand gene expression, developmental regulation, physiological functions, disease resistance as well as sex determination (Tan *et al*., 2005; Ho *et al.*, 2007; Guo *et al*., 2010). We constructed eight *C. manan* cDNA libraries covering four flowering developmental stages of male and female inflorescences (Thi, 2004). From there, we generated 1529 good quality ESTs with an average size of 400 bp (Table 1). Of these, 915 ESTs were from the female inflorescence cDNA libraries and 614 ESTs were from the male inflorescence cDNA libraries. The clustering process on these ESTs successfully assembled 229 contigs containing 805 ESTS, and the remaining 724 ESTs were singletons. This gives an EST redundancy of 24%. This redundancy rate was lower compared to those of other EST projects such as in *Arabidopsis thaliana* (Hofte *et al*., 1993) and *Citrus sinensis* (Bausher *et al*., 2003). The number of ESTs being generated for each cDNA library in our study in comparatively smaller, and this would reduce the probability of repeated reads. The ESTs were classified into four categories (significant known function; significant unknown function and hypothetical; not significant; and no match) based on BLASTX analysis (Figure 3). The ESTs of the significant known function category were further analyzed by grouping into functional groups based on a modified MIPS classification system (Figure 4). Eight floral-related genes were identified from the EST database (Thi, 2004; Nadarajah *et al*., 2009b), i.e. MADS 8 ($1e^{-47}$), stamen specific fil1 ($2e^{-12}$), CONSTANS ($3e^{-34}$), stigma/stylar cysteine-rich adhesion precursor ($1e^{-28}$), Men-7 ($1e^{-7}$), flower-specific gamma-thionine precursor ($9e^{-19}$), FRIGIDA ($1e^{-6}$), anther-specific proline-rich protein (0.022) and

Table 1. Summary of sequencing and clustering of *C. manan* floral ESTs

Output	Amount
cDNA clones sequenced	2688
ESTs generated	2063
Good quality ESTs	1529
Average length of ESTs (bp)	400
Contigs	229
Singletons	724
Sequence clusters	953

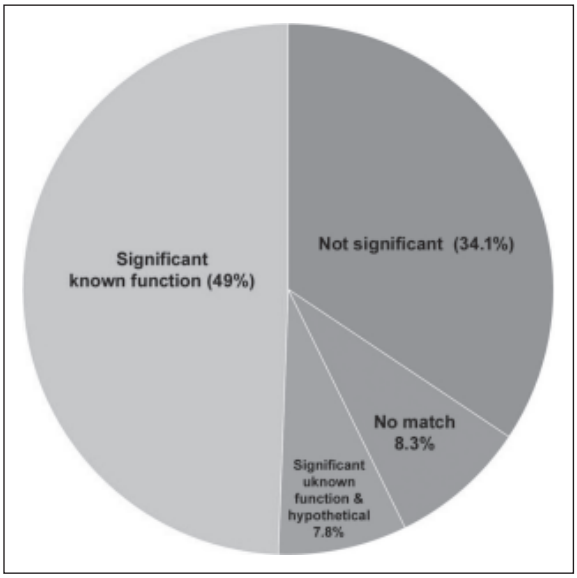

Fig. 3. 1529 ESTs from *C. manan* inflorescence libraries categorized into four groups based on BLASTX analysis.

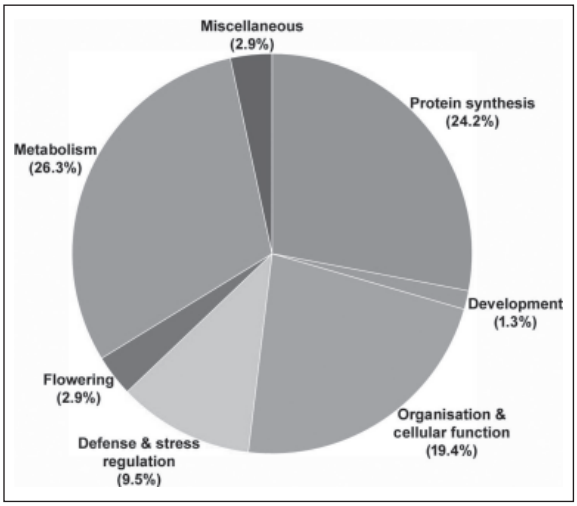

Fig. 4. Distribution of significant known function ESTs of *C. manan* according to functional groups based on a modified MIPS classification system.

Early Flowering 5 (0.17). These floral-related ESTs may be valuable for use to understand the sex differentiation in *Calamus* rattan. On the other hand, the ESTs (191 ESTs containing 24 contigs and 167 singletons) from categories significant unknown function and hypothetical proteins and no match (Figure 3) were further analyzed by motif search based on InterPro motif database in order to predict the putative functions of these ESTs (Nadarajah *et al*., 2009a). A total of 66 types of motifs were detected. The motif search on these categories of ESTs yielded six functional groups (Figure 5). However, no floral-related gene was detected on these two categories of ESTs by motif search.

SSH can be used to efficiently isolate differentially expressed genes between different samples (Diatchenko *et al*., 1996; Sahebi *et al*., 2015). In order to determine the differentially expressed genes from male floral tissue in dioecious *C. palustris*, we constructed an SSH cDNA library using the male flower buds as a tester and the female flower bud as a driver (Ng *et al*., 2014). The SSH library contained 1536 clones. False positive clones were removed by screening with amplification and reverse Northern analysis. After the screening, only 313 clones were selected for further analysis. These clones were expressed at a higher level in the male flower than the female flower. Sequencing on the selected differentially expressed clones produced 292 high quality sequences. Assembly analysis of the high quality sequences produced 205 unique genes (unigenes) consisting 32 contigs and 173 singletons. Therefore, the redundancy rate of this SSH 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. The 205 unigene sequences were subjected to homology search with the nucleotide BLAST algorithm against 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 classified into 12 functional categories of protein according to the FunCat of MIPS (Figure 6). Diversity of the functional categories might suggest that male flower tissue development in *Calamus* rattan is a complex biological process.

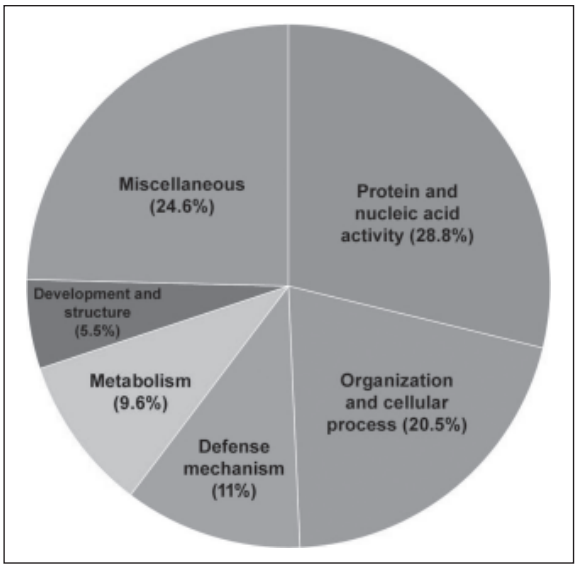

Fig. 5. Distribution of significant unknown function and hypothetical protein and no match ESTs of *C. manan* according to functional groups based on motif search at InterPro database.

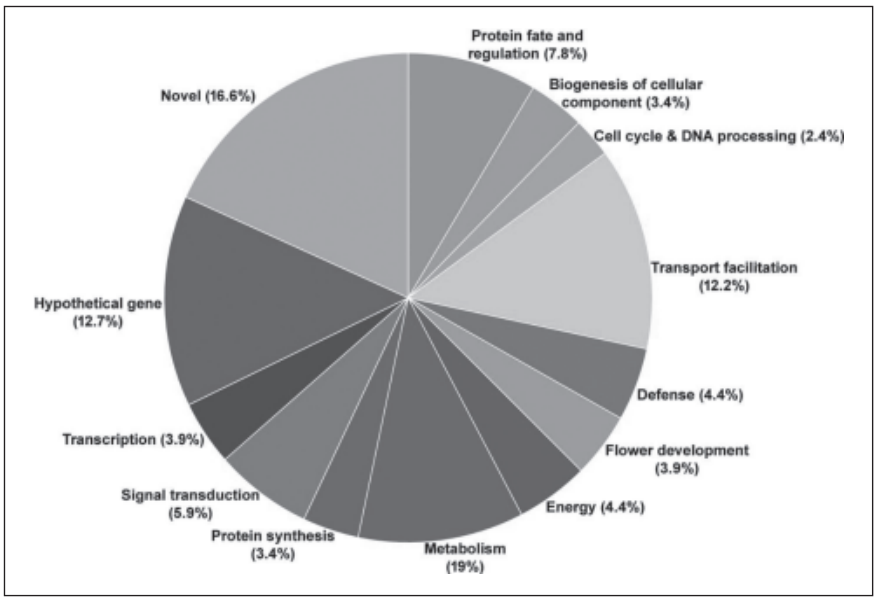

Fig. 6. Functional classification of genes differentially expressed in male SSH library of *C. palustris.*

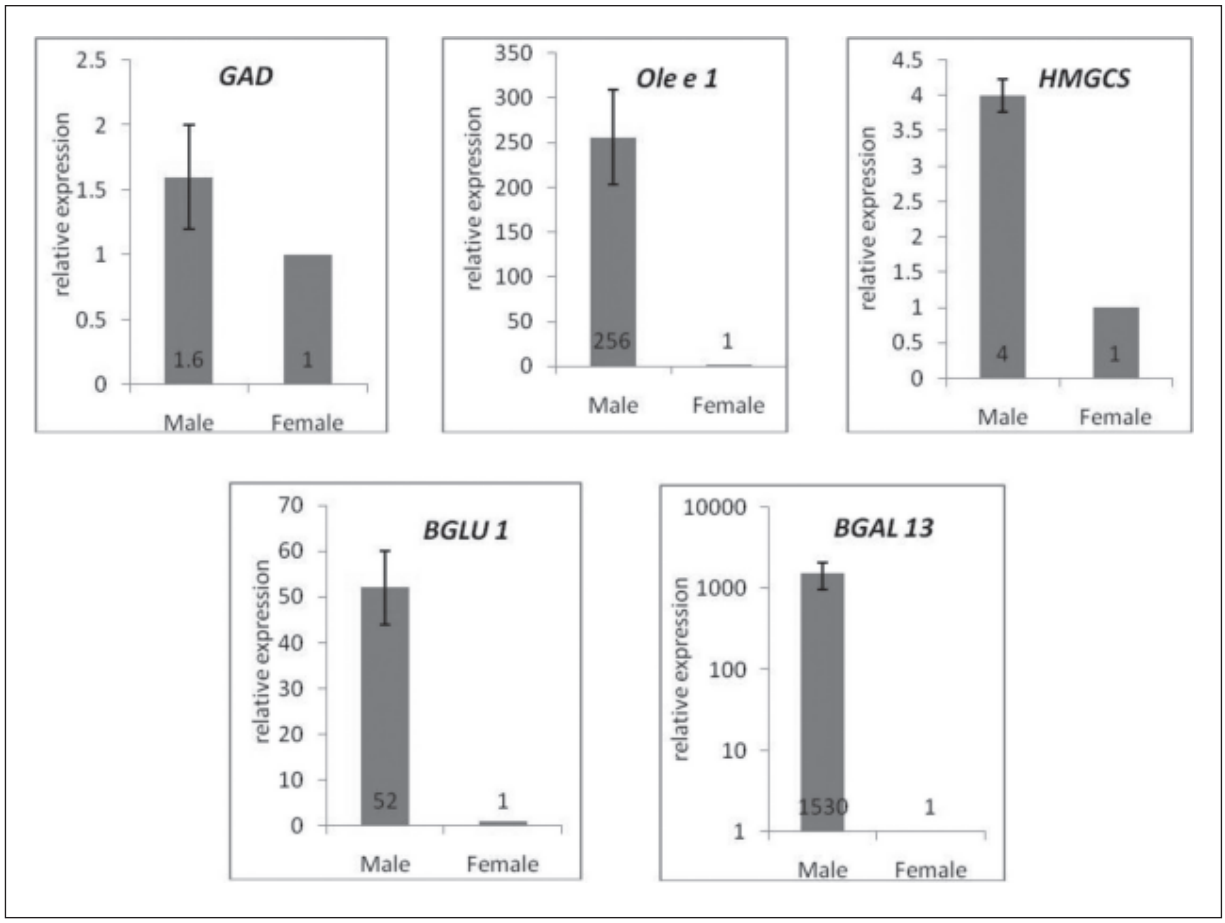

Fig. 7. Expression analyses of differentially expressed genes in male and female flower tissues of *C. palustris* using RT-qPCR. GAD: glutamate decarboxylase, Ole e 1: pollen ole e 1 allergen, HMGCS: hydroxymethyglutaryl-CoA synthase, BGAL 13: β-galactosidase 13 and BGLU 1: β-glucosidase 1.

Genes of particular interest that were found in the SSH were MADS-box genes, pollen specific and related genes (pollen ole e 1 allergen, pollen-specific C2 domain containing protein, SWEET and hydroxymethyglutaryl-CoA synthase), phyto-hormones (gibberellin 20 oxidase 1, cytokinin riboside 5'-monophospahte phosphoribohydrolase, auxin-induced 5NG4 protein and gibberellin-regulated protein 10) and flower development genes (beta galactosidase 13, beta-glucosidase 1, glucose-6-phosphate dehydrogenase, glutathione synthase and glutamate decarboxylase) (Ng *et al*., 2014). These genes are directly and indirectly involved in flora and pollen development (de Dios *et al*., 1999; Palanivelu *et al*., 2003; Hruba *et al*., 2005; Krizek & Fletcher, 2005; Guan *et al*., 2008; Krizek, 2011; Olimpieri *et al*., 2011; Zechmann & Russell, 2011). However, it is noted that two floral-related genes (Men-7 and proline-rich anther-specific protein) detected in the EST analysis of *C. manan* (from female cDNA library) (Thi, 2004; Nadarajah *et al*., 2009b) were absent in the SSH male flower cDNA library of *C. palustris* (Ng *et al*., 2014). It is most likely that these two genes were expressed in the female floral tissue of *Calamus* rattan.

The expression level of five differentially expressed gene (glutamate decarboxylase, pollen ole e 1 allergen, hydroxymethyglutaryl-CoA synthase, β-galactosidase 13 and β-glucosidase 1) was further analyzed by RT-qPCR in male and female flowers of *C. palustris*. All the genes were expressed at a higher level in male samples than in female samples (Figure 7). Gen β-galactosidase 13 showed the highest differential expression level (1530 folds), pollen ole e 1 allergen was 256 folds higher, β-glucosidase 1 was 52 folds more expressed and glutamate decarboxylase and hydroxymethyglutaryl-CoA synthase were 1.6 and 4 folds higher respectively. Those genes with highly differential expression between male and female floral tissues such as ole e 1 allergen, β-galactosidase 13 and β-glucosidase 1 are of particular interest for the development of gene-based molecular markers for sex identification in *C. palustris* or C. *manan*.

CONCLUSIONS

Cultivation and breeding for dioecious plant species are always problematic due to the inability to differentiate the gender of the plants at an early growth stage. This seems to be true for rattan species as it takes 5.5 years to identify the gender of *C. manan* by inflorescences and 3-4 years for *C. palustris*. The absence of sex chromosome and the various types of dioecy in rattan species may infer the complexity of dioecy mechanism in rattan species. The sex ratio studies on the planted populations of *C. manan* and *C. palustris* could provide us some valuable information to establish seed producing orchards for large scale cultivation. Sex identification of rattan species, particularly for the economically important and high priority rattan species such *C. manan* and *C. palustris*, is crucial for their survival in the wild. The limited functional genomic studies of *C. manan* and *C. palustris* provide us some understanding of the flower development and sex determination in *Calamus* rattans, and this undoubtedly has brought us closer to the development of molecular markers for sex identification in *C. manan* and *C. palustris*.

ACKNOWLEDGEMENTS

The work reported here was supported by Intensified Research Priority Grant 09-02-02-0036-EA132 and Science Fund 02-01-02-SF0404 under the Ministry of Science, Technology and Innovation, Malaysia; and Fundamental Research Grant UKM-ST-FRGS0048-2006 under the Ministry of Higher Education, Malaysia.

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