J. Crop Sci. Biotech. 2019 (December) 22 (4) : 363 ~ 369
DOI No. 10.1007/s12892-019-0171-0

RESEARCH ARTICLE

Differential Expression Pattern Of MADS Box Genes in Floral Whorls of *Garcinia indica*

Reshma V Patil, Kiran D Pawar*

School of Nanoscience and Biotechnology, Shivaji University, Kolhapur, Maharashtra, India

Received: June 18, 2019 / Revised: June 28, 2019 / Accepted: July 01, 2019

Abstract

Flower formation is one of the main developmental stages in thelife cycle of flowering plantsthat can be used as a model systemto elucidate the molecular mechanics that control the developmental process in plants. In the present study, we investigated the floral homeotic and MADS box genes in the polygamadioecious tree *Garcinia indica*. The differential gene expressions of floral homeotic and MADS box genes in male, female, bisexual flowers, and floral organs such as sepal, petal, stamen, and carpel were studied by employing quantitative real-time, PCR-based assays. Of ninedifferentially expressed floral genes of the MADS box class, AGL11, the master control gene of ovule identity was found to be expressed 2-fold higher in female carpel whereas theribosome protein involved in ovule development showed $2x10^6$-fold high expression in the female carpel. The hierarchical clustering grouped these genes into fourmajor clusters:cluster I comprised AGL11, AG, and PMADS2, cluster II comprised AP3 and AGL9, cluster III comprised SEP1 and ELFB, and cluster IV comprised AG pathway and ribosome protein. The clustering found was correlated with the quantitative and qualitative expression of genes.

Key words : *Garciniaindica*, floral genes, MADS box gene, gene expression, flower development, sex determination

Introduction

The most important event in the life cycle of higher plants, especially fruit trees, is flower development. According to the accepted ABC model, flower organs are specified by involving combinationsof floral homeotic genes with A specifying sepals, A + B + E specifying petals, B + C + E specifying stamens, C + E specifying carpels, and D specifying ovules (Angenent and Colombo 1996; Theissen and Saedler 2001). In *Arabidopsis*, genes belonging to these functional classes were APETALA1 (AP1) in class A, PISTILATA (PI) and APETALA3 (AP3) in class B, AGAMOUS (AG) in class C (Bowman et al. 1991a), SEEDSTICK/ AGAMOUS-LIKE 1 (STK/AGL11) and SHATTERPROOF (SHP) in class D (Colombo et al. 1995), and SEPALLATA (SEP1, SEP2, SEP3, and SEP4) genes in class E (Theissen 2001).In addition to floral homeotic gene functions, MADS box genes also governsthe reproductive development in eudicotyledonous flowering plants (Ng and Yanofsky 2001). Therefore, MADS-box genes are important in the studies of plant evolutionary development.

Economically important *Garciniaindica*, which is commonly known as "Kokum" belongs to the family Clusiaceae. This polygamdieocious tree species produces male (ML), female (FL), and bisexual (BS) flowers whose differentiation is possible only after the flowering stage that occurs when the plant is7 to 8 years old. It comprises pistillodeML flowers, staminode FL flowers, and different combinations of BS flowers. Geographical variation with respect to sexual polymorphism has been found in *G. indica* (Joseph and Murthy 2014). According to Barrett et al. (2010),many of the tree species have an equal proportion of sex ratios in the populations but *G. indica*populations show either a very low proportion of co-sexuals or a total absence of co-sexuals or an unequal proportion of male and female trees (Joseph and Murthy 2014). However, the sexual system of *G. indica* is contentiouswhich compels the cultivators to maintain the large population of plants for longer periods of time. However, to assess the sexual system in *G. indica,*the systematic studies have not been carried out which is likely to be accompanied by substantial diversification of floral gene families.

Kiran D Pawar (✉)
Email: kdp.snst@unishivaji.ac.in, pawarkiran1912@gmail.com
Tel: +9102312609490 Fax: +9102312692333

Studying the expression pattern bygene quantification is an important method to untangle the function of important pathways in biological samples.The real-time quantitative PCR (qRT-PCR)based investigation of the floral homeotic gene expression patterns in different flower types would help understand the molecular mechanism underlying regulation of flower formation and sex differentiation in *G. indica.*To this end, the present study reports the expression pattern of previously identified, differentially expressed MADS box genes in ML, FL, and BS flowers and their different whorls.

Materials and Methods

To conduct this study, we confirmed, identified, and collected ML, FL, and BS flowers of Kokum from different locations in the Konkan regions of Western Maharashtra, India during flowering season in the months of January-February. After collection, the flowers were immediately stored in RNALater (Sigma Aldrich, Waltham, Massachusetts, US) at 4°C until further use for RNA extraction. The extraction of total RNAs, quality check, and their quantifications were done as described previously (Patil and Pawar 2019). The high quality working stocks of 10 ng/µl were prepared and used to synthesize first strand cDNA with the Ready Script® cDNA synthesis mix (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's protocol. The cDNA samples were diluted 1:10 with nuclease-free water prior to the qRT-PCR analysis. The differentially expressed 25 MADS box genes were selected from the previous comparative floral transcriptomic study of *G. indica* (Patil and Pawar 2019). The sequences of cDNAs of endogenous candidate reference genes such as Actin, Elongation factor, and Ubiquitin (ACT7, ELF4, and UBQ4) were retrieved from the NCBI GeneBank database. Primers were designed using the Primer3 software according as described by Thornton and Basu (2011). The qRT-PCR reactions were performed on Biorad CFX96 Real-time system (Bio-Rad, Richmond, CA, USA) using the SYBR Green-based PCR assay. The qRT-PCR reaction mixture (20 µl) contained 10 µl SYBR Green PCR mix (Genei, Bangalore, India), 6.0 µl of 1:10 diluted cDNA and 0.4 mM of each primer. The qRT-PCRs were run as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 61°C-67°C for 30 s, and 65°C-95°C dissociation for 10 s. Using melt curve analysis function of qRT-PCR, 25 primers pairs were screened initially for real-time expression and their specificities were verified by the presence of a single peak in the melt curve analysis. In qRT-PCR based quantification, the threshold cycle (Ct) was measured and used to define the expression level of each reference gene. The best reference genes were determined using three Excel-based tools namely geNorm, NormFinder, BestKeeper and RefFinder (Andersen 2004; Pfaffl et al. 2004; Vandesompele et al. 2002; Xie et al. 2012). Using $2^{-\Delta\Delta CT}$ method, average Ct values were calculated to estimate relative expression level of each gene under study.

Results and Discussion

In the case of the present study, the stability ranking of candidate reference genes in 13individual samples were estimatedby using four software programsand presented in Fig. 1. Based on these estimations,a comprehensive ranking of candidate reference genes in the decreasing order of their ranking was found as follows: ELF4, UBQ4, and ACT7 with their lower stability (1.189, 1.414, and 3.0, respectively) (Table 1). Therefore, ELF4was used as control gene to normalize the expression of floral whorls of *G. indica* (Fig. 2). The previous MADS Box gene discovery from flower transcriptomes and its statistics showed 63 counts in BS, 54 counts in FL, 67 counts in ML transcriptome libraries whereas, 45 common counts were found among three libraries analyzed (Patil and Pawar 2019). From these, we selected and shortlisted 25 differentially expressed MADS box gene for qRT-PCR-based analysis (Table 2). Based on our qRT-PCR analyses, 9

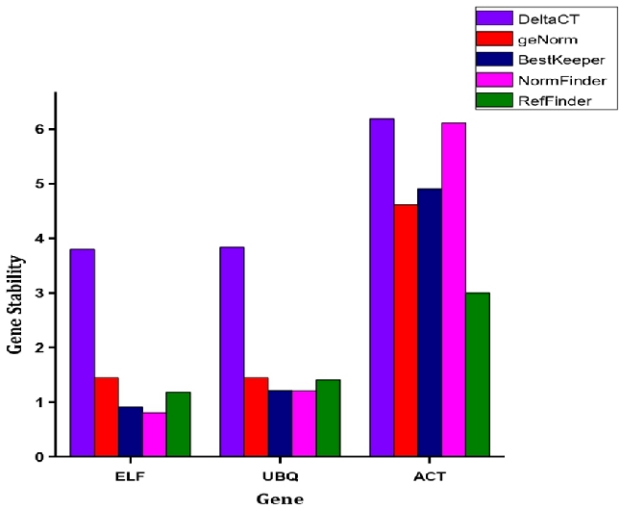

Fig. 1. Overall ranking of candidate reference gene by using different software in floral organs of *G. indica.*

Table 1. Gene Stability of candidate reference by using different software in floral organs.

Candidate gene	Delta CT	geNorm	BestKeeper	Normfinder	RefFinder
ELF	3.8	1.449	0.91	0.806	1.18
UBQ	3.84	1.449	1.21	1.204	1.41
ACT	6.19	4.611	4.9	6.107	3

Table 2. The primer sequences of 25 genes validated by qPCR.

No	Gene	Gene ID	Upstream	Downstream
1	AP3	NM_001281031.1	GGAGATTAGGCAGAGGATGGG	GGGCAAATACCGGAGAGCTT
2	AGL9	XM_002514847.2	ATTGTTGGAGGCCAGCTTGA	GGATGCTTCTTGTTCCTGCTTG
3	AGL11	XM_015530186.1	GCCGTGAAGGCACCATTTTT	CACCCATCAAATGCCTGTTCG
4	AG	XM_006467514.1	GCTGCCAAGCTGAGGATACA	CGGCTATCTTTGCTCGGAGA
5	SEP1	XM_012232674.2	GTCCCTGCAACGAACTCAGA	CTGACGCTCTAGCTGCTCAA
6	AG pathway (HUA2)	AT5G23150.1	GTGGATCCTTGCTTCCCCAA	TGCAAGCACTACCGTCAGTT
7	PMADS2	XM_012222932.2	GCTGCACAGTCGCTGAAAAA	ATGGCTCCTCCCCTGTGTAT
8	Selenoprotein	AT1G05720.1	CTCGACGAAGACGGTGAACA	GGCTTGACCTTCTCTCGCAT
9	Pollen allergen	AT1G29140.1	TCTCGAGGGGGCAAAGGTAA	TGGTAGTTACCGGCGTTGTC
10	RNA poly	AT5G41010.1	TCTTGTCCGGTGGCTTGAAA	CCACAATCTCCGCAGACGTA
11	ENTH/GAT family	AT1G25240.1	CTTGTCCACGGTGTGCTTTG	AACCCCAAGTTTTGCTGGGA
12	VRN5, fibronectin	AT3G24440.1	TCAGCCAATGCAGGTAGAGATTT	TACTCCGTGCAAGGCTCAAG
13	NAC domain containing protein	AT5G41090.1	GGGCCCAACATACAGAGCTT	TTCCATAGCCAGGCAAGTCG
14	PLC-like phosphodiesterases superfamily protein (GDPD2)	AT5G41080.1	AAAGGCACATTGCTGACGTG	TGCTCAACTCTAAACCTTCGGA
15	Ribosomal protein L14p/L23e family protein (HLL)	AT1G17560.1	AGCATCCTCTCTCAGCAACA	CCGTACACGACCATCCCTTT
16	oxidase 1D (AOX1D)	AT1G32350.1	CCACTATGCTTCGGATATACAATTC	GCTTCGTGTCCAGGCAGTAT
17	RNAligase (RNL),	AT1G07910.1	GAAACGACGTTGTGTTCAAGT	GCGTGATCTCTCTTTTTCTTGC
18	Peroxidase superfamily protein (PER4),	AT1G14540.1	GCTCTCTCAGGAGCTCACAC	AATCCCGCGTCAATGTCACT
19	Ricinus SVP	XM_002513991.2	GGGAGAGGATCTGCAAGGAC	ATTGGTGACGGACTCCGATG
20	Vitis MADS box TF15	XM_019226355.1	GGGGCCGGGATATGAGAAAG	TAGGGGTCACCTCCATCCTG
21	Cellulase	AT3G26140.1	CTATCCCCGCTTCAAGGACC	TGTAGCATGCAAAGTCCGGT
22	ELFB, ViP6 binding protein	AT2G06210.1	CTATCCCCGCTTCAAGGACC	TGTAGCATGCAAAGTCCGGT
23	AGL11 Jatropha	XM_020677533.1	AGGACACGGGCATTTTCCAT	ACCAGTTCAATGGCCAGAGG
24	TCP family transcriptional factor	AT5G41030.1	TGTGCAAACATCCAGCAGGT	GAGCAGCCATCACAGCAGTA
25	ENS, ABH1, CPP80	AT2G13540.1	GGACGCGTCTTATCCTCTGG	GCCCACTTGGGAAGATCCAA
Ref1	ELF4	>NC_003076.8:24288596-24291020	ACAGTCATTGATGCCCCAGG	AGGCGAAAATAGAAAAAGACGCA
Ref2	UBQ5	>NC_003074.8:23037018-23038009	CTGGGACTTTCATGGCGAGT	ACCGCTACAACAGATCAAGC
Ref3	ACT	>NC_003076.8:3052062-3054691	CCATCGCTCATCGGAATGGA	AGAGGCGGAATTGGGATTTT

of 25 analyzed MADS box genesshowed differential expression levels in all the testedsamples. As shown in Fig. 2, expression level of floral homeotic gene AG "C class" was high in ML petals and BS carpel while in AG pathway (HUA2); it was highly expressed in FL flowers. As a class C gene, AGAMOUS, is a key player in floral morphogenesis whereas HUA2 is known to specifically promote the processing of AGAMOUS pre-mRNA (Cheng et al. 2003). HUA2 is also identified as genes that act in the specification of stamen and carpel identity and control of floral determinacy (Chen and Meyerowitz1999). Similarly, it is reported that the expression pattern of AGAMOUS is not limited to early flower development but continues to fully differentiate floral whorls (Bowman et al. 1991b). In the present study, it was found that the expression level of AG pathway (HUA2) was the same in the stamens and carpels of ML, FL, and BS flowers suggesting its role in floral morphogenesis in early flower development. On the other hand, the AGAMOUS gene was found significantly expressed in the FL carpel specifying FL reproductive organ identity.

Further AGL11, the master control gene of ovule identity was specifically and highly expressed in FL carpel with 2-fold higher expression as compared to low expression in FLsepals (0.1 fold) and petals (0.06 fold). AGL11 was suspected to be involved in specifying ovule identity (Becker et al. 2003) since AGAMOUS (AG) like, orthologous to class D floral homeotic genes AGL11 in *Petunia* was found to be involved in control of ovule identity (Angenent and Colombo 1996). In agreement with this possibility, AGL11 was found specifically expressed in only FL carpel of *G.*

*indica*suggesting its specificity and role in ovule identity. However, AGL9 "AGL2-like genes" was found highly expressed in all the samples except ML and FL sepals. It is known that the second sub-family AGL2-like genes include AGL2, AGL3, AGL4, and AGL9 among which AGL9 is responsible for second, third, and fourth whorl floral organ primordial expression (Becker et al. 2003). In the case of *G. indica,* AGL9 was found to express in all these three whorls suggesting the significant role of AGL9 in flower development.

In comparison, "SEP1", the new "Class E" gene was observed least expressed in all the analyzed samples except ML and BS flowers whereas an ancestral "B class" gene AP3 was highly expressed in ML stamen distinguishing it from FL (Fig. 2). In our study,higher expression of SEP1 in

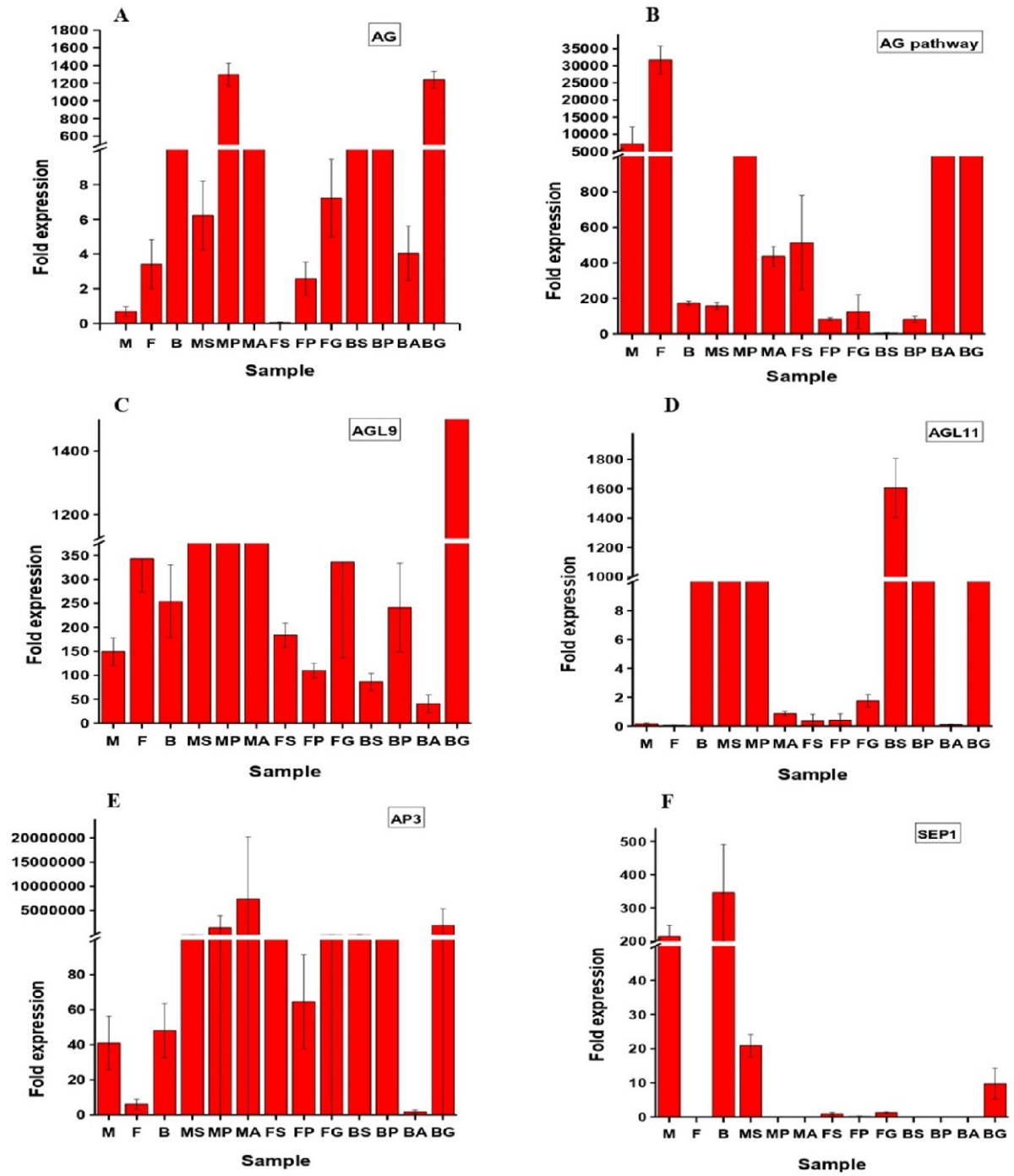

Fig. 2. Expression analysis of ninedifferentially expressed MADS Box gene in different floral whorls using qPCR. The data represents the mean ±standard errors obtained from three independent biological and technical repetitions. A-I indicates differential expressed MADS box genes for each floral whorl.

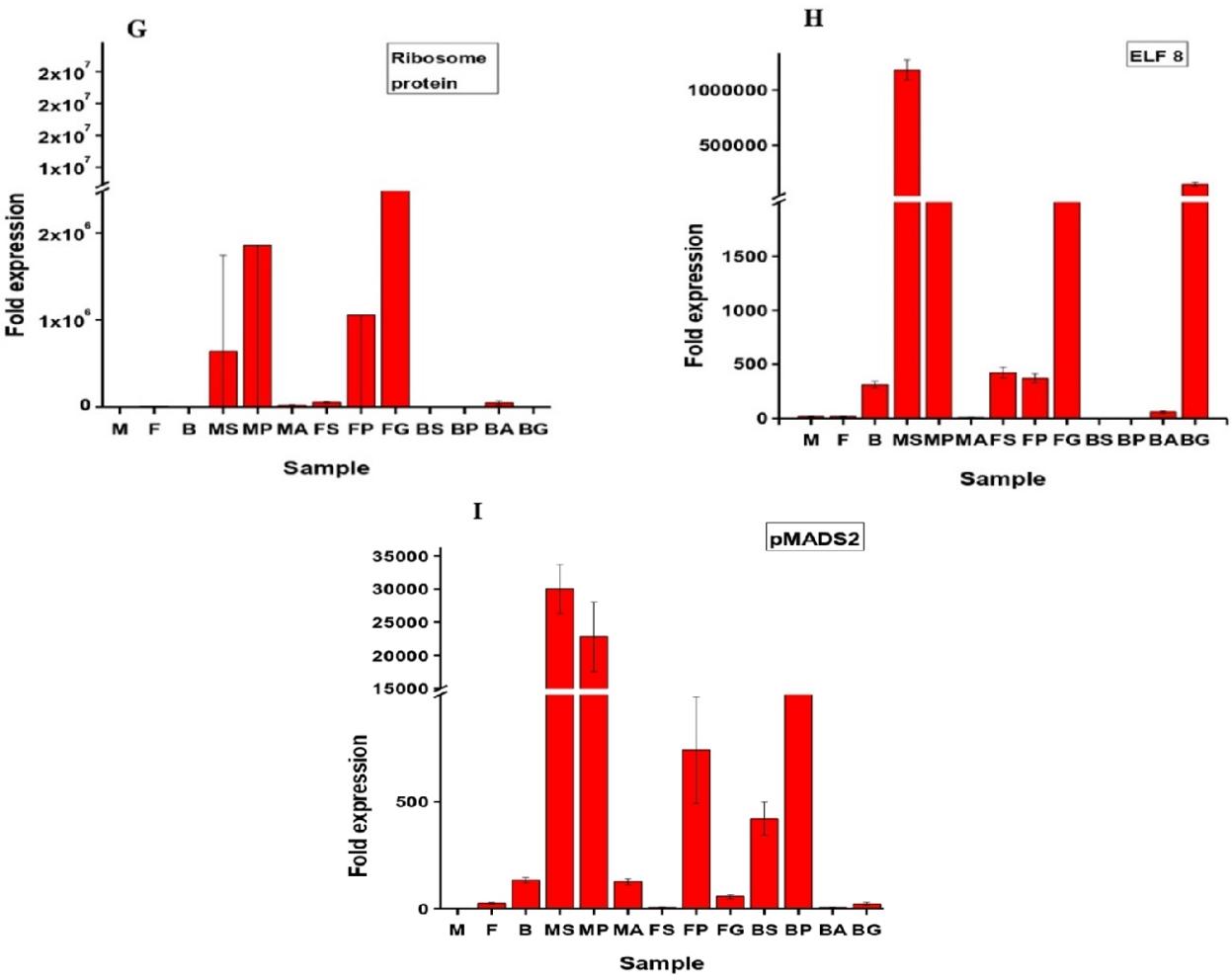

Fig. 2. Expression analysis of ninedifferentially expressed MADS Box gene in different floral whorls using qPCR. The data represents the mean ±standard errors obtained from three independent biological and technical repetitions. A-I indicates differential expressed MADS box genes for each floral whorl. (Continued.)

ML and BS flowerswas observed. There is one more class of ancestral genes DEF + GLO like genes found in gymnosperms of which only DEF like gene AP3 is present in *Arabidopsis* genome (Goto and Meyerowitz 1994). These B genes may have an ancestral function (realized in extant gymnosperms) in distinguishing ML reproductive organs (where B gene expression is on) from FL reproductive organs (B gene expression off) (Winter et al. 2002). The data obtained in *G. indica* showeda higher expression of AP3 genes in both ML and FL reproductive organs suggesting their significant and ancestral function of distinguishing between reproductive organs.

In addition, it was observed that the ribosomal protein responsible for ovule development was significantly expressed in FL carpel with more than $2x10^6$-fold expression as compared to FL sepals and petals (< $1x10^6$ fold). It is known that in *Arabidopsis,* ovule growth and patterning is essentially regulated by a ribosomal protein HUELLENLOS (HLL) gene similar to L14 ribosomal protein of eubacteria. Our expression pattern clearly indicated the highest expression of HLL gene in FL carpel which may be responsible for ovule growth and development. This would suggest a higher demand of energetic and metabolic burden in areas undergoing growth responsible for more number of mitochondrial mRNA in developing flowering (Huang et al. 1994; MacKenzie and McIntosh 1999).

The other gene flowering time protein ELF8, VIP6 binding showed similar expression in ML sepals, petals, and carpels of FL and BS. FLC acts as a central role in controlling flowering time where ELF8/VIP6 is required for activation of FLC. ELF8 may regulate select clade of MADS box gene for floral transition (He et al. 2004). In accordance with this, it was observed that the expression of theFLC gene was strongly activated by FRI and repressed by the autonomous pathway genes. However, the regulation of FLC gene was noted insensitive to photoperiodic flowering genes (Sheldon et al. 1999).In*G. indica*, the higher expression of ELF8/VIP6 in ML sepals and petals as well as FL and BS ovules were observed indicating their role in FLC regulation in flowering. Likewise, the transcription factor pMADS2 responsible for

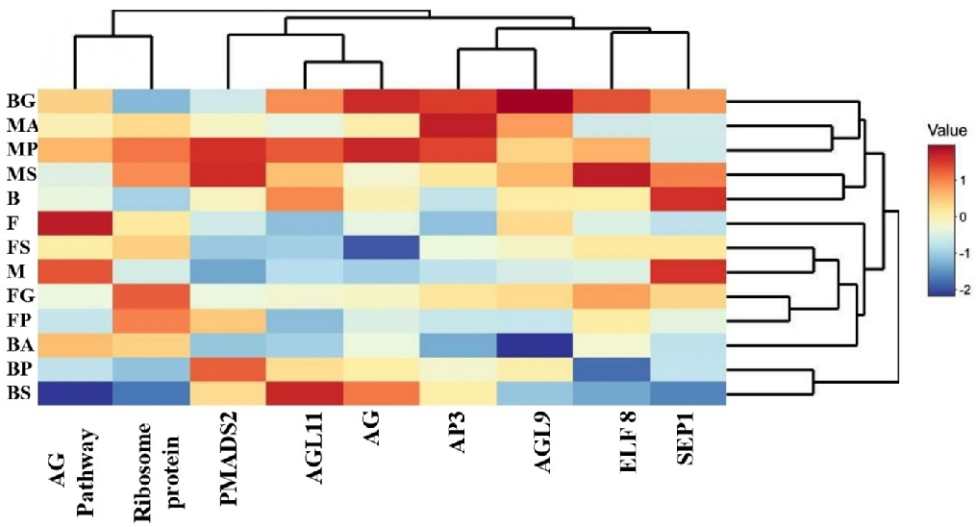

Fig. 3. Heatmap showing differential expression of MADS box gene for different floral whorls of *G. indica*.

flowering development showed higher expression in sepals and petals of all three flower types. Similar to the expression pattern in *P. hybrida,* the expression of pMADS2 in *G. indica* also showed similar pattern where pMADS2 was found highly expressed in second whorl while it was least expressed in the third whorl indicating pMADS2 as a co-suppressor for pMADS1 gene (van der Krol et al. 1993).

Further, we performed the hierarchical clustering analysis to study how various analyzed samples were related in terms of qPCR-based expressions of the tested MADS box genes and vice versa.Clustering of gene expressions data was performed using UPGMA method (Fig. 3). Hierarchical clustering arranged different floral whorls and MADS box genes in four major clusters. Cluster I comprised AGL11, AG, and PMADS2, Cluster II comprised AP3 and AGL9, Cluster III comprised SEP1 and ELFB, and Cluster IV comprised AG pathway and ribosome protein. The results reveal that the quantitative and qualitative expressions of genes in each cluster are correlated. Likewise, three clusters of tested floral whorls were qualitatively related to the floral developmental functions.Cluster I comprised MA, MP, MS, and B whereas BGwas not found to cluster withmale whorls indicating difference between male whorls and bisexual carpel. Cluster II comprised FS, M, FG, and FP while F (flower) was not observed to cluster with female whorls. Cluster III comprisedBS and BP indicating they were significantly different from all other floral whorls. These results indicated that expression patterns of the tested genes in ML and FL floral whorls were closely related but different from the expression pattern in BS floral whorls.

Conclusion

In conclusion, the current study evaluated the expression stability of three candidate genes across different floral whorls of *G. indica*. On the basis of this data, ELF4 was found suitable reference gene for normalization which enabled us to quantify the relative expression levels of different classes of MADS box gene such as AP3 and pMADS2 of class B, AG, and AG pathway of class C, AGL9, and AGL11 of class D and SEP1 of class E whereas ribosome protein (HLL) and ELF8 (FLC) genes for flower development. We also identified two most significant genes AGL11 (XM_015530186.1) and ribosome protein (AT1G 17560.1) involved in the differentiation of the different floral whorls for sex determination. These results represented a first step towards illuminating the molecular mechanisms of sex determination in *G. indica*.

Acknowledgements

This study was funded by DST-SERB (YSS/2015/ 000319).

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