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Similitudes and differences between two closely related *Ficus* species in the synthesis by the ostiole of odors attracting their host-specific pollinators: A transcriptomic based investigation

Rui Hu[a], Peng Sun[b], Hui Yu[a,c,*], Yufen Cheng[a], Rong Wang[d], Xiaoyong Chen[d,**], Finn Kjellberg[e]

[a] Guangdong Provincial Key Laboratory of Applied Botany and Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou, 510650, China
[b] School of Life Sciences, Qufu Normal University, Qufu, Shandong, 273165, China
[c] Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, 511458, China
[d] School of Ecological and Environmental Sciences, Tiantong National Station for Forest Ecosystem Research, East China Normal University, Shanghai, 200241, China
[e] CEFE, CNRS, Univ Montpellier, Univ Paul Valéry Montpellier, EPHE, IRD, France

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ABSTRACT

Flower odor is often essential for attracting pollinators, and this is especially true for species-specific mutualism such as the one between *Ficus* species and their pollinating wasps. Receptive figs emit a stage and species specific odor composed of a mix of volatile organic compounds (VOC) that is attractive to their pollinators. Histological studies suggest that the ostiolar bracts are the main locus of fig floral VOC synthesis. To confirm a major role of the ostiole in VOC synthesis, detect the genes involved in VOC synthesis and analyze differences between closely related species, we compare the transcriptomes of pre-receptive and receptive figs of two closely related species, *F. hirta* and *F. triloba*. The two species presented similar numbers of expressed genes and similar annotation, classification results, and their up-regulated unigenes belonged to similar biosynthetic pathways. However, phenylpropanoid and terpene synthesis were the main enriched VOC pathways in receptive figs of *F. hirta* while terpene and jasmonate synthesis were the main enriched VOC pathways in *F. triloba*. The shift in gene expression between pre-receptive and receptive figs was much more marked in the ostiolar bracts than in the flowers of *F. hirta*, and in the fig wall, confirming an important role of the ostiole in receptive fig odor emission. Only one unigene directly involved in VOC synthesis presented signatures of positive selection, further supporting that the source of interspecific receptive fig odor differentiation is based on gene regulation rather than on gene differentiation. Regulation based receptive fig odor differentiation between species suggests a potential for rapid evolution of this interspecific barrier.

1. Introduction

Pollinators constitute a strong selective factor acting on floral traits. It may results in the evolution of suites of traits preferentially attractive to particular groups of pollinators (Kemp et al., 2019). Such suites of traits constitute pollination syndromes. They have been shown to constitute good predictors of pollinators within regional floras (Johnson and Wester, 2017), and convergent evolution of flower traits associated with pollinator shifts have been demonstrated (e.g. Rosas-Guerrero et al., 2014; Smith and Kriebel, 2018). In addition to flower colour and shape, floral odors constitute an important component of the floral display used by pollinators, as evidenced for individual species (Byers et al., 2014) and for plant communities as a whole (Kemp et al., 2019). Floral odors may vary in response to pollinator shifts (Schiestl and Johnson, 2013). Floral odor and flower colour modification are often due to the modification of regulatory genes and mutation in a single gene can control modification of a given trait (Sheehan et al., 2012; Schiestl and Johnson, 2013). Nevertheless, because of the diffuse nature of many pollination systems, the relevance of the pollination syndrome concept has been questioned: determining causality can be challenging (Ollerton et al., 2009).

Tight species-specific plant-pollinator interactions provide useful model systems to decipher the selective pressures and the adaptive responses underlying the evolution of floral odors and floral odor perception (Friberg et al., 2019). Specialized pollination is especially

* Corresponding author. Guangdong Provincial Key Laboratory of Applied Botany and Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou, 510650, China.
** Corresponding author.
E-mail addresses: yuhui@scbg.ac.cn (H. Yu), xychen@des.ecnu.edu.cn (X. Chen).

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Abbreviation

VOC	volatile organic compounds
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
KOG	Clusters of Orthologous Groups of proteins
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
TPS	terpene synthase
MVA	mevalonic acid
MEP	methylerythritol phosphate
FPP	farnesyl diphosphate
GGP	geranyl diphosphate
GGPP	geranylgeranyl diphosphate
RNAseq	High-throughput RNA sequencing
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
bp	base pairs

frequent in species rich tropical ecosystems (Johnson and Steiner, 2000). Within this context, the mutualism between *Ficus* and their pollinating fig wasps represents a unique system to investigate the evolution of olfactive signaling by plants and its perception by their pollinators (Hossaert-Mckey et al., 2010). Indeed, this mutualism forms the most extreme example of plant-insect co-diversification documented to date; coevolution between floral odor and its perception is expected (Cruaud et al., 2012).

Fig wasps breed within figs, the closed urnshaped receptacles characteristic of *Ficus*. The interior of the fig is lined with flowers. Pollinating wasps are attracted by receptive figs (ready to be pollinated), enter the receptive fig through an ostiole closed by bracts, and oviposit in some flowers, inducing galls in which their offspring develop, and also fertilise other flowers that will produce seeds (Weiblen, 2002). Fig wasps only live a few days (Weiblen, 2002). It is therefore critical for them to rapidly find receptive figs of their host species. In *Ficus*, as in nursery pollination systems in general, floral odors play a central role in pollinator attraction (Hossaert-Mckey et al., 2010). Fig wasps are attracted by volatile organic compounds (VOCs) released by receptive figs, while they are not attracted by the VOCs released by figs that are not yet receptive or past receptivity (Hossaert-Mckey et al., 1994; Chen and Song, 2008; Proffit et al., 2008). Histological investigations of figs have established that VOC emitting glands are restricted to the ostiolar bracts and, in some *Ficus* species, the fig wall epidermis (Souza et al., 2015). In combination with biological observations, these results suggest that the ostiolar bracts could be the main locus of production of the VOCs involved in wasp attraction (Souza et al., 2015).

The VOCs produced by receptive figs are mainly terpenoids, but also phenylpropanoids/benzenoids (or shikimate pathway compounds), and fatty acid derivatives (Grison-Pige et al., 2002a; Grison-Pige et al., 2002b; Chen et al., 2009; Proffit et al., 2008; Proffit and Johnson, 2009; Soler et al., 2012; Hossaert-Mckey et al., 2016). The biosynthetic pathways at the origin of these compounds are well known in plants, and the enzymes involved have been investigated for a number of plant species (Dudareva et al., 2013). While some studies have documented the regulation of floral VOC synthesis (Dudareva et al., 2013; Muhlemann et al., 2014), none of them has analyzed a specialized obligate mutualistic pollination interaction.

In this study, we focus on two closely related *Ficus* species, *F. hirta* and *F. triloba*, that are pollinated by closely related wasp species (Yu et al., 2019). The two species co-occur throughout their range (Berg, 2007) so that we may expect selection for signaling differentiation between the two species. We analyze the difference of transcription of candidate genes involved in VOC synthesis in ostiolar bracts between pre-receptive figs and receptive figs of the two *Ficus* species.

2. Materials and method

2.1. Study species

Ficus hirta Vahl. and *Ficus triloba* Buch.-Ham. ex Voigt are closely related species, belonging to section *Eriosycea*, subgenus *Ficus*. *F. triloba* was considered as a subspecies of *F. hirta* (Berg and Corner, 2005) but has since been elevated to species rank in a revision of the species group (Berg, 2007). Most individuals of the two species are easily distinguished in the field. *F. hirta* is shrub that grows to a height of approximately 3 m while *F. triloba* is a small tree and can be 4–10 m tall. *Ficus hirta* has small figs, 10–15 mm in diameter, while those of *F. triloba* are 20–25 mm in diameter. Receptive figs of *F. triloba* emit a strong sent while the smell of receptive figs of *F. hirta* is hardly detectable to the human nose.

2.2. Sampling and sequencing

Ficus hirta was collected at the South China Botanical Garden, Guangdong Province, China (23°11′N, 113°11′E) while *F. triloba* was collected nearby, at Dinghu Mountain, Guangdong Province, China (23°09′~23°11′N, 112°30′~112°33′E). To avoid pollinator visitation, figs were bagged before fig receptivity. Ostiolar bracts were dissected from pre-receptive and receptive figs without pollinated, and were put into Sample Protector for RNA/DNA (Takara). For *F. triloba*, each sample consisted of bracts from two figs from one tree. Three replicates from three trees were collected. For *F. hirta*, because of the smaller figs and smaller ostiolar bracts, every sample consisted of bracts from 10 to 12 figs. Two samples were collected from one tree and a third sample was collected from another tree.

RNA was isolated using TRIzol™ (Tiangen). RNA degradation and contamination was monitored on 1% agarose gels. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. High-throughput RNA sequencing (RNAseq) was performed on an Illumina Hiseq platform. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

2.3. Assembly and annotation

Clean data was obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from the raw data. All the downstream analyses are based on this clean data. Error rate, Q20, GC-content were calculated. Transcriptome assembly was accomplished based on all sampled data using Trinity (Grabherr et al., 2011) with min_kmer_cov set to 2 and all other parameters set at default. Gene function was annotated using the following databases: Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database) and GO (Gene Ontology).

2.4. Differential gene expression analysis

Gene expression levels were estimated for each sample using the program RNA-Seq by Expectation-Maximization (RSEM, Li and Dewey, 2011). Differential gene expression analysis between pre-receptive and receptive figs was performed using the DESeq R package (1.10.1). To control for false discovery rate, the resulting P values were adjusted using the Benjamini and Hochberg approach. Genes with an adjusted P-value <0.05 and fold chang >2 found by DESeq were considered to be differentially expressed. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented using the GOseq R packages based on the Wallenius non-central hyper-geometric distribution (Young et al., 2010). Enrichment of differential expression genes in KEGG pathways was analyzed using the KOBAS software (Mao et al., 2005).

In *F. hirta*, the low detected numbers of differentially expressed genes between pre-receptive and receptive figs was caused by the low Pearson correlation of gene expression between FhirtaB4, which is an outlier in almost all the pairwise comparisons, and the two other samples collected at receptive stage, FhirtaB2 and FhirtaB3 (see results). Therefore, an additional analysis was done, including only the two samples FhirtaB2 and FhirtaB3. To compare variation in the level of expression of individual genes in pre-receptive and receptive fig ostiolar bracts, reads per kilo base per million mapped reads (RPKM) of the two phases were compared.

2.5. Real-time PCR

To confirm upregulation of genes at fig receptivity, 8 terpene synthases of *F. hirta* and 13 terpene synthases of F. triloba were amplified using real-time PCR. For the two species, samples were dissected and immediately put into Sample Protector for RNA/DNA (Takara, Japan). They were transferred to -20 °C as rapidly as possible. RNA was extracted using the PRC1 buffer of the HiPure Plant RNA Mini Kit (Magen, China) according to the manufacturer instructions. Then 500 ng RNA per sample were used as input material for first-strand cDNA synthesis by the Reverse Transcriptase M-MLV (RNaseH-) (Takara, Japan). Real-time PCR was carried out in the presence of the double-strand DNA-specific dye SYBR Green I (SYBR Green Premix Ex Taq, Takara, Japan) and monitored in real-time with the Roche LightCycler480 system (Roche, Switzerland). The primers were designed according to the sequences to get 70–300 bp products and the specificity of the primers was monitored by the melting curve of the real-time PCR program.

2.6. Ortholog and Ka/Ks analysis

The coding sequence of each putative unigene was extracted based on the BLASTX results. The ESTSCAN software was used to determine the coding directions of sequences for which no alignment was obtained. The resulting coding sequences extracted from putative unigenes were translated into amino acid sequences using the standard codon table. Self-to-self BLASTP was conducted for all amino acid sequences with a cut-off E-value of $1e^{-5}$. Orthologous groups were constructed from the BLASTP results with OrthoMCL v2.0.3 (Li et al., 2003) using default settings.

To assess roles of Darwinian positive selection on the evolution of the genes, we used the CodeML program from the PAML package v4.8a

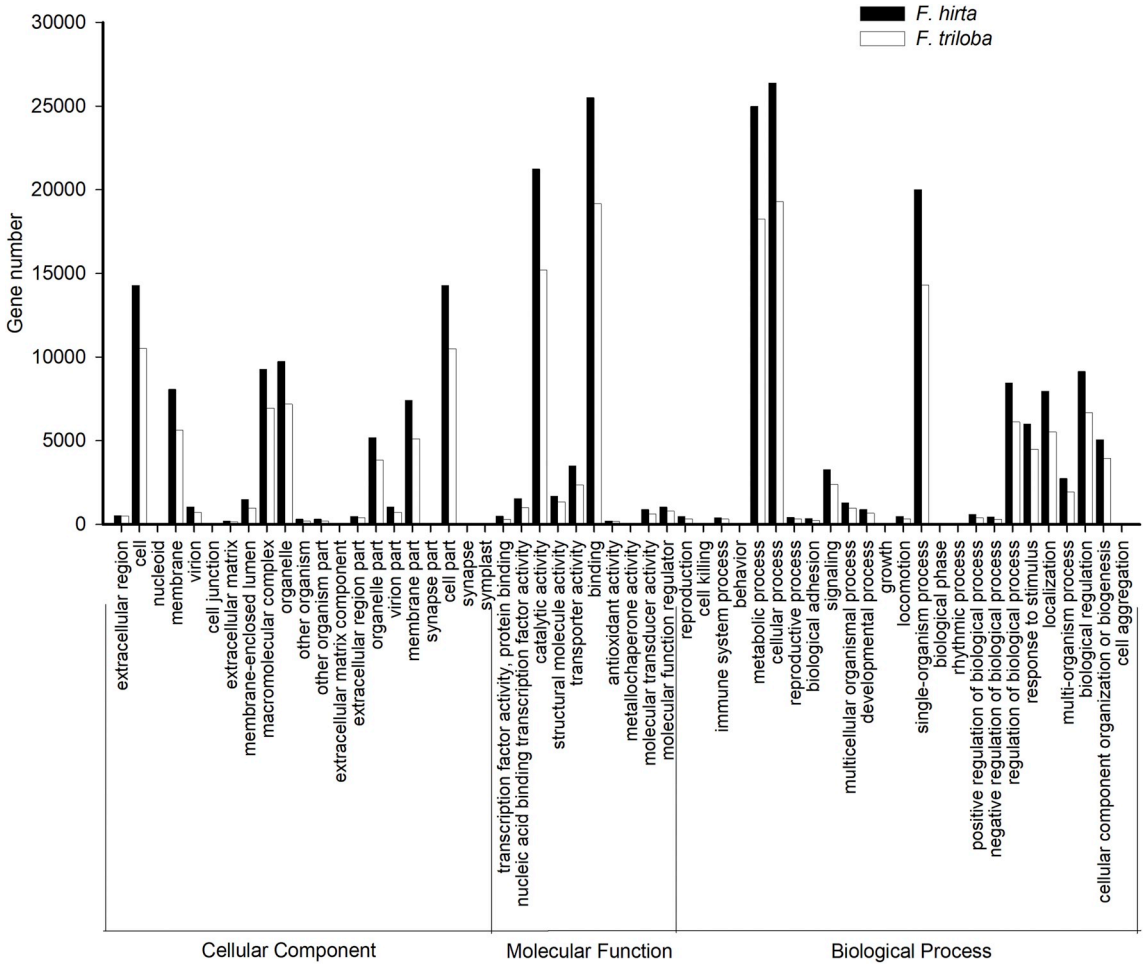

Fig. 1. The GO category distribution of unigenes for *F. hirta* and *F. triloba*.

(Yang, 2007) to perform positive selection tests. Ka is the number of nonsynonymous substitutions per non-synonymous site and Ks is the number of synonymous substitutions per synonymous site. The ratio Ka/Ks (ω) of orthologous gene pairs is an indicator of selective pressure acting on a protein-coding gene and were estimated. Signature of positive selection is indicated by ω > 1 (Yang and Nielsen, 2002; Yang et al., 2005; Zhang et al., 2005).

3. Results

3.1. Sequencing and de novo assembly

For *F. hirta* pre-receptive and receptive figs, we obtained 43, 311, 613 and 44, 910, 372 clean reads amounting to 6.50G and 6.74G, respectively. For *F. triloba* pre-receptive and receptive figs, we obtained 42, 566, 854 and 44, 320, 990 clean read amounting to 6.38G and 6.65G, respectively (Table S1). The Q20 of all the samples were above 93% and the GC content of all the samples were comprised within the interval 45–47% (Table S1). For *F. hirta*, 111,065 unigenes were obtained with an average size of 1048 bp, and for *F. triloba*, 83,407 unigenes were obtained with an average size of 1032 bp. The length distribution of the unigenes is shown in Fig. S1. The Pearson correlation coefficients show high correlation in gene expression of a plant between pre-receptive and receptive figs. This is especially true for *F. triloba* for which the three highest correlation values correspond to the three within plant-between stage comparisons (Fig. S2). Sample FhirtaB4 is an outlier presenting the lowest correlation coefficients with other samples. Further, sample FhirtaB4 presents the highest number of reads and the lowest GC content of all the samples confirming its outlier status (Table S1).

3.2. Annotation and classification of the assembled unigenes

Annotation results are summarized in Table S2 and Fig. S3. 61,456 (55.33% of the total) unigenes of *F. hirta* and 45,894 (55.02% of the total) unigenes of *F. triloba* were annotated in at least one database. The top hit species for *F. hirta* and *F. triloba* were the same, namely *Prunus mume*, *Prunus persica*, *Vitis vinifera*, *Pyrus x*, and *Theobroma cacao* (Fig. S4). These five taxa and *Ficus* are members of the rosid clade of the Angiosperms. The GO category (GO), the unigene function categories (KOG) and the KEGG database distributions of the unigenes of *F. hirta* and *F. triloba* were highly similar (Fig. 1, Fig. 2A, Fig. 2B). The identified pathways include the metabolism of terpenoids, lipids, and amino acids, which are volatile organic compounds (VOCs) and/or precursors of VOCs.

3.3. Volatile organic compound synthase genes

For *F. hirta*, we found 205 unigenes coding for 51 enzymes involved in terpenoid biosynthesis (Table S3). Among these, 99 unigenes coded for 29 terpenoid backbone synthases, 44 unigenes coded for 9 sesquiterpenoid and triterpenoid synthases, 44 unigenes coded for 10 diterpenoid synthases and 18 unigenes coded for 3 monoterpenoid synthases. For *F. triloba*, we found 175 unigenes coding for 47 enzymes involved in terpenoid biosynthesis (Table S3). Among these, 77 unigenes coded for 28 terpenoid backbone synthases, 47 unigenes coded for 9 sesquiterpenoid and triterpenoid synthases, 37 unigenes coded for 8 diterpenoid synthases and 14 unigenes coded for 2 monoterpenoid

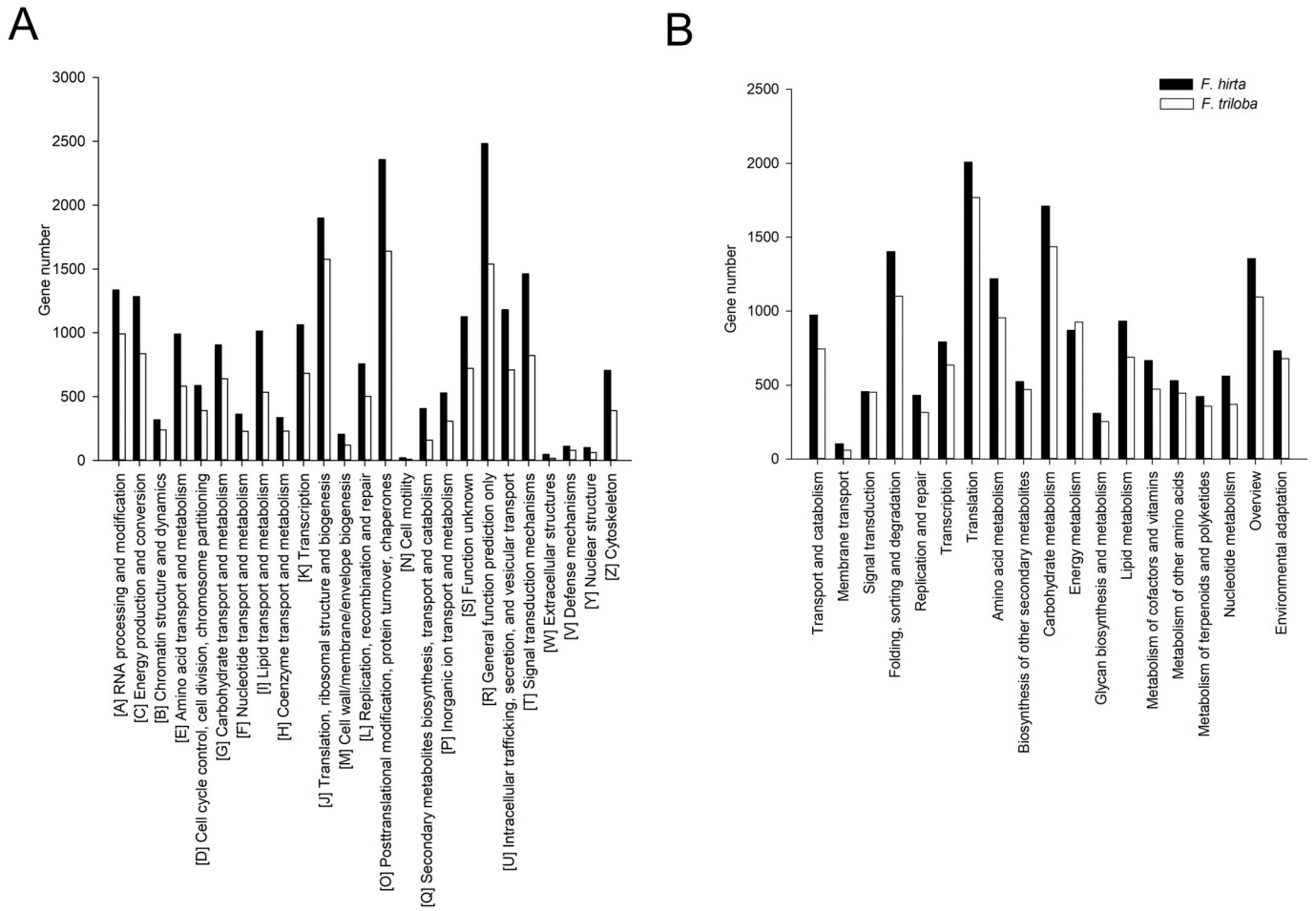

Fig. 2. The distributions of unigene function categories predicted by KOG (A) and KEGG (B) for *F. hirta* and *F. triloba*.

synthases (Table S3).

All the terpene backbone enzymes expressed in *F. hirta* were also expressed in *F. triloba*, except for isopentenyl-diphosphate delta-isomerase (IDI), which catalyzes the reversible conversion of IPP and DMAPP. The two species expressed the same sesquiterpene and triterpene synthases, but *F. hirta* expressed one more monoterpene synthase ((−)-alpha-terpineol synthase) and two more diterpene synthases (ent-cassa-12, 15-diene 11-hydroxylase and ent-copalyl diphosphate synthase) than *F. triloba*.

Phenylpropanoid/benzenoid pathway genes and fatty acid derivatives synthase genes were also annotated (Table S4). For *F. hirta*, we found 251 unigenes encoding 17 synthases involved in phenylpropanoid/benzenoid synthesis, and 91 unigenes encoding 14 synthase involved in alpha-linolenic acid metabolism pathways. For *F. triloba*, we found 238 unigenes encoding 16 synthases involved in phenylpropanoid/benzenoid synthesis, and 75 unigenes encoding 14 synthase involved in alpha-linolenic acid metabolism pathways. Overall, *F. hirta* and *F. triloba* expressed the same phenylpropanoid/benzenoid pathway synthases, except for one additional synthase (caffeoylshikimate esterase) expressed by *F. hirta.* The two species expressed the same fatty acid derivatives synthase.

3.4. Differentially expressed unigene analysis

For *F. hirta*, among the 60,299 unigenes detected in both pre-receptive and receptive figs, 187 (0.31%) unigenes were found to be differently expressed. Out of these, 79 unigenes were up-regulated in the receptive phase while 108 unigenes were down-regulated. For *F. triloba*, among the total 53,267 unigenes detected in both pre-receptive and receptive figs, 1413 (2.65%) unigenes were found to be differently expressed. Out of these, 773 unigenes were up-regulated in receptive phase and 640 unigenes were down-regulated. In *F. hirta*, we did an additional analysis excluding the outlier, sample FhirtaB4. All the following differential gene expression analyses in *F. hirta* are based on this reduced dataset. Excluding sample FhirtaB4, many more unigenes were differentially expressed (3007; 5.0%), out of which 1487 were up-regulated and 1520 were down-regulated in receptive figs. To detect metabolic pathways that are particularly active at fig receptivity, the KEGG enrichment of the up-regulated genes was analyzed (Fig. 3 and Table S5). In *F. hirta*, the up-regulated pathways included ribosome, phenylpropanoid biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, terpenoid backbone biosynthesis, carotenoid biosynthesis, monoterpenoid biosynthesis, isoflavonoid biosynthesis, brassinosteroid biosynthesis, and so on (Fig. 3A). In *F. triloba*, the up-regulated pathways included plant-pathogen interaction, fatty acid elongation, galactose metabolism, terpenoid backbone biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, monoterpenoid biosynthesis, alpha-linolenic acid metabolism, ether lipid metabolism, and phenylpropanoid biosynthesis (Fig. 3B). Up-regulated genes of *F. triloba*were enriched in fatty acid elongation, alpha-linolenic acid metabolism, ether lipid metabolism, which were different from that of *F. hirta*.

3.5. Differentially expressed volatile organic compound synthase genes

For *F. hirta*, several genes involved in VOC synthesis were up-regulated at fig receptivity (Table 1 and Table S6). Twenty-four differentially expressed unigenes belonged to the phenylpropanoid pathways. The following enzyme coding genes were up-regulated. Five genes that encoded 4-coumaroyl-CoA ligase (4CL) which could catalyze the formation of cinnamoyl-CoA, coumaroyl-CoA and caffeyoyl-CoA. These were precursors of phenylpropanoid compounds, such as cinnamaldehyde, p-coumaraldehyde, caffeylaldehyde. Among the differentially expressed genes, there were also two caffeic acid O-methyltransferase (COMT), that catalyzing the methaltransfer in precursors of the dehydes, and five dehydrogenase, that catalyzing the transformation of dehydes into alcohols. These dehydes and alcohols could constitute the VOCs produced by the phenylpropanoid pathways. Seven unigenes encoded peroxidases (Mei et al., 2009), which catalyzing the degrading of volatile phenylpropanoid compounds. In terpene synthesis, eight backbone genes, five sesquiterpene (four TPS1 and one alpha-farnesene synthase), two triterpene and three monoterpene genes (TPS14) were differentially expressed (Table 1). These results suggest that phenylpropanoid and terpene synthesis may constitute an important component of receptive fig odor in *F. hirta*.

For *F. triloba*, differentially expressed genes belonged to several types of VOC biosynthesis pathways especially the terpene pathway. In the terpenoid synthesis pathway, 13 unigenes were up-regulated at fig receptivity (Fig. 4 and Table 1). These synthases included 5 backbone synthases, and 8 TPS family unigenes, including 4 sesquiterpene, 1 triterpene and 2 monoterpene genes (TPS14). Different from *F. hirta*, 6 fatty acid derivatives synthesis pathway genes were up-regulated, including lipoxygenase (LOX2S), hydroperoxide dehydratase (AOS),

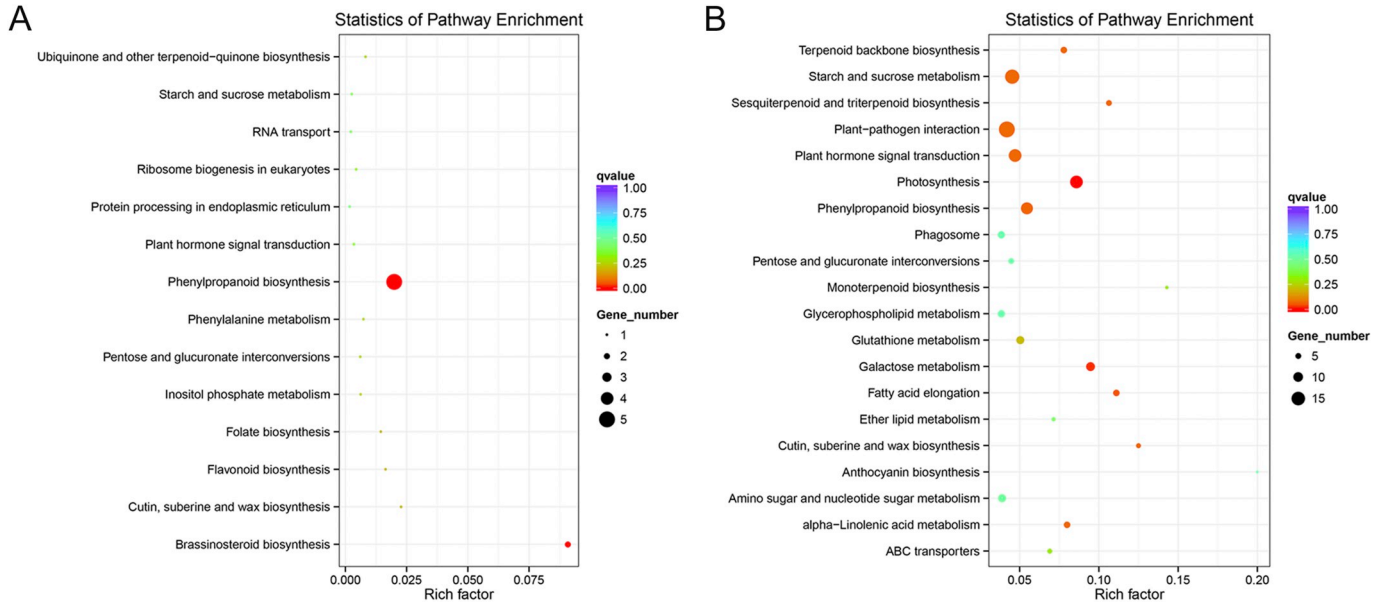

Fig. 3. KEGG enrichment of the differentially expressed genes of *F. hirta* (A) and *F. triloba* (B).

Table 1
Unigenes encoding terpenoid pathway synthase up-regulated in receptive compared with pre-receptive phase of *F.hirta* and *F. triloba*

		Gene_id	Name	Abbrevation in this paper	Homologous genes of *Arabidopsis*
F. hirta	Backbone synthase	Cluster-12957.42642	1-deoxy-D-xylulose 5-phosphate synthase	DXS	DXS
		Cluster-12957.32504	hydroxymethylglutaryl-CoA synthase	HMGS	HMGS
		Cluster-12957.17615	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	HDS	HDS
		Cluster-12957.36388	geranylgeranyl pyrophosphate synthase	GGPPS	GGPPS
		Cluster-12957.31617	hydroxymethylglutaryl-CoA reductase	HMGR	HMGR
	Sesquiterpene synthase	Cluster-12957.13509	farnesol dehydrogenase	FLDH	FLDH
		Cluster-12957.3618	(-)-germacrene D synthase	TPS1	TPS1
		Cluster-12957.2277	(-)-germacrene D synthase	TPS3	TPS1
		Cluster-12957.3619	valencene/7-epi-alpha-selinene synthase	TPS2	TPS1
	Triterpene synthesase	Cluster-12957.59461	squalene monooxygenase	SQLE	SQLE
	Monoterpene synthase	Cluster-14540.0	(3S)-linalool synthase	TPS14	TPS14
		Cluster-12957.2989	(3S)-linalool synthase	TPS15	TPS14
	Diterpene synthase	Cluster-12957.25452	gibberellin 2-oxidase	GO	
F. triloba	Backbone synthase	Cluster-21669.40714	prenylcysteine alpha-carboxyl methylesterase	PCME	PCME
		Cluster-21669.41527	geranylgeranyl reductase	chlP	chlP
		Cluster-21669.67549	mevalonate kinase	MVK	MVK
		Cluster-21669.60291	1-deoxy-D-xylulose-5-phosphate synthase	DXS	DXS
		Cluster-21669.47566	geranylgeranyl diphosphate synthase, type II	GGPS	GGPS
		Cluster-21669.29167	geranylgeranyl diphosphate synthase, type II	GGPS	GGPS
		Cluster-21669.45994	geranylgeranyl diphosphate synthase, type II	GGPS	GGPS
		Cluster-21669.54684	1-deoxy-D-xylulose-5-phosphate reductoisomerase	DXR	DXR
	Sesquiterpene synthase	Cluster-21669.67617	alpha-farnesene synthase	AFS1	
		Cluster-21669.38878	(-)-germacrene D synthase	TPS1	TPS1
		Cluster-21669.67705	(-)-germacrene D synthase		TPS1
		Cluster-21669.41693	(-)-germacrene D synthase	TPS2	TPS1
		Cluster-21669.62265	valencene/7-epi-alpha-selinene synthase		TPS1
	Triterpene synthesase	Cluster-21669.5622	squalene monooxygenase	SQLE	
		Cluster-21669.53323	premnaspirodiene oxygenase		
	Monoterpene synthase	Cluster-21669.5677	(3S)-linalool synthase	TPS15	TPS14
		Cluster-21669.6522	(3S)-linalool synthase	TPS14	TPS14
		Cluster-21669.5677	(3S)-linalool synthase		TPS14

allene oxide cyclase (AOC), and acetyl-CoA acyltransferase (ACAA1) (Table S6). These were all jasmonate synthesis pathway enzymes. Jasmonate could generate methyl-jasmonate by jasmonate O-methyltransferase.

Hence, *F. hirta* and *F. triloba* presented similitudes and differences in the up-regulation at receptivity of genes involved in metabolic pathways that can be associated with the production of VOCs. In addition, three unigenes encoding ATP-binding cassette transporters (ABC transporters) also show a differential expression pattern between pre-receptive and receptive figs of *F. triloba* (Table S5), while in *F. hirta* no genes in this pathway show significantly different expression. These three unigenes include two ATP-binding cassettes, subfamily B (MDR/TAP), and one ATP-binding cassette, subfamily A (ABC1).

3.6. Confirmation of differential gene expression by real-time PCR

To confirm transcriptome data, ostiolar bracts of pre-receptive and receptive figs were subjected to real-time PCR. Eight supposedly up-regulated terpene synthases unigenes of *F. hirta* and 13 terpene synthases unigenes of *F. triloba* were selected (Table S7). For *F. hirta*, 6 unigenes showed consistency between the real-time PCR and the transcriptomic data (75% consistency, Fig. 5 and Table S7) while this number was 8 for *F. triloba* (61.5% consistency, Fig. 6 and Table S7). The expression of 4 *F. hirta* synthases that were shown to be up-regulated in the ostiolar bracts of receptive figs was compared between tissues by amplifying the genes in extracts from the epidermis of fig walls from receptive fig. The expression of these genes was only up-regulated in the ostiolar scales (Fig. S5).

3.7. Sequence divergence of orthologs between the two species

Among 3322 identified pairs of orthologous genes (Table S8), 583 orthologous pairs of genes had Ka/Ks values larger than 1, and 1174 had Ka/Ks values below 0.1 and the remaining 1565 genes had intermediate Ka/Ks values (Table S9). Hence, 583 orthologous pairs presented signatures of positive selection. These positively selected genes were clustered to photosynthesis, RNA transport, ubiquinone and other terpenoid-quinone biosynthesis, folate biosynthesis, nucleotide excision repair, flavonoid biosynthesis, plant hormone signal transduction, and so on (Table S10). A single gene directly involved in VOC synthesis, HMGR (OG06988 in Table S10), a terpene backbone synthase in the MVA pathway, presented a signature of positive selection.

4. Discussion

The results support the hypothesis that the ostiole is an important locus of receptive fig odor production. Indeed our transcriptomic results, supported by real-time PCR, show that a series of genes involved in VOC synthesis are up-regulated in the ostioles of receptive figs. Yu et al. (2015) described the transcriptome of female flowers of *F. hirta*, as female flowers were previously thought to be the locus of VOC synthesis. They analyzed terpenoid pathway genes and discovered several differentially expressed genes. However, only genes down-regulated at receptivity were evidenced, suggesting no role of female flowers in the VOCs responsible for wasp

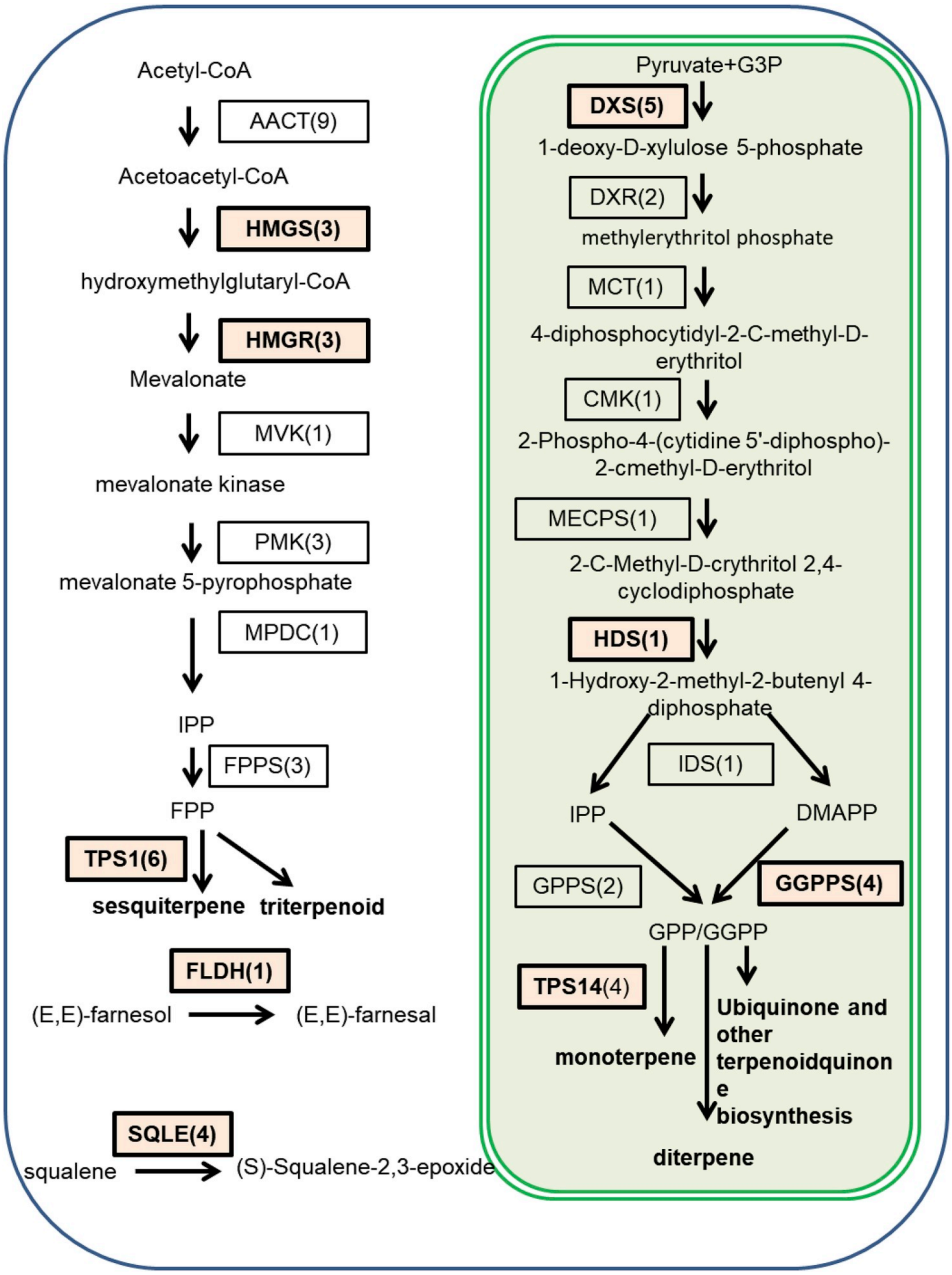

Fig. 4. An overview of terpenoid VOC biosynthesis pathway unigenes and differentially expressed genes in *F. triloba*. Enzyme are shown in box, number between bracket is the number of unigenes encoding this enzyme. Bold box includes the upregulated unigene encoded enzymes. DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MECPS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase: HDS, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase. IDS, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; AACT, acetyl-CoA C-acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, hydroxymethylglutaryl-CoA reductase (NADPH); MVK, mevalonate kinase; PMK, phosphomevalonate kinase; MPDC, diphosphomevalonate decarboxylase; FPPS, farnesyl diphosphate synthase; GGPS, geranyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase. FLDH, farnesol dehydrogenase; SQLE, squalene monooxygenase.

attraction (Yu et al., 2015). This agrees with histological data on a series of *Ficus* species (Souza et al., 2015). Further, the low expression in the fig wall epidermis of *F. hirta* of terpene synthase genes that were strongly expressed in the ostiole of receptive figs suggests a central role of the ostiole in the production of receptive fig odor.

The two *Ficus* species largely expressed genes encoding the same synthase. In the VOC synthesis pathways, comparatively to *F. triloba*, *F. hirta* expressed 4 additional enzymes involved in terpenoid biosynthesis, one additional synthase in the phenyl/benzenoid pathway and the two species expressed the same fatty acid derivative synthases. Further, only one terpene backbone synthase presented signatures of positive selection. Limited signals of selection on gene sequences were expected as these are classical plant biosynthetic pathways (Dudareva et al., 2013). Therefore, interspecific differentiation in receptive fig odor is most probably mainly based on gene regulation. Indeed, the two species presented some striking differences in which genes were up-regulated at receptivity: the detected number of up-regulated unigenes for *F. hirta*, and *F. triloba* were, respectively, 18 versus 13 in the terpenoid pathway, 24 versus 0 in the phenyl/benzenoid pathways and 0 versus 6 in the fatty acid pathway. Hence, variation in gene expression seems to be the main mechanism allowing rapid divergence of receptive flower odors, as in other systems (Sheehan et al., 2012; Schiestl and Johnson, 2013). Nevertheless, the large numbers of up-regulated terpene synthesis genes suggest that they are important components of receptive fig odors of both the two species. Methyl-jasmonate or its derivate might be important components in VOCs of receptive figs of *F. triloba*.

Expression profiles, especially in *F. triloba*, presented strong within plant correlation between pre-receptive and receptive figs (Fig. S2). This result suggests that different plants were in different physiological conditions. This difference in gene expression among plants should be compared with the among plants homogeneous up-regulation of VOC synthesis and other metabolic pathways in receptive figs. In *F. hirta* and in *F. triloba*, a large numbers of metabolic pathways were up-regulated at receptivity, indicating an important change in metabolism between pre-receptive and receptive figs. Unigenes encoding primary metabolism, photosynthesis, brassinosteroid biosynthesis and plant hormone

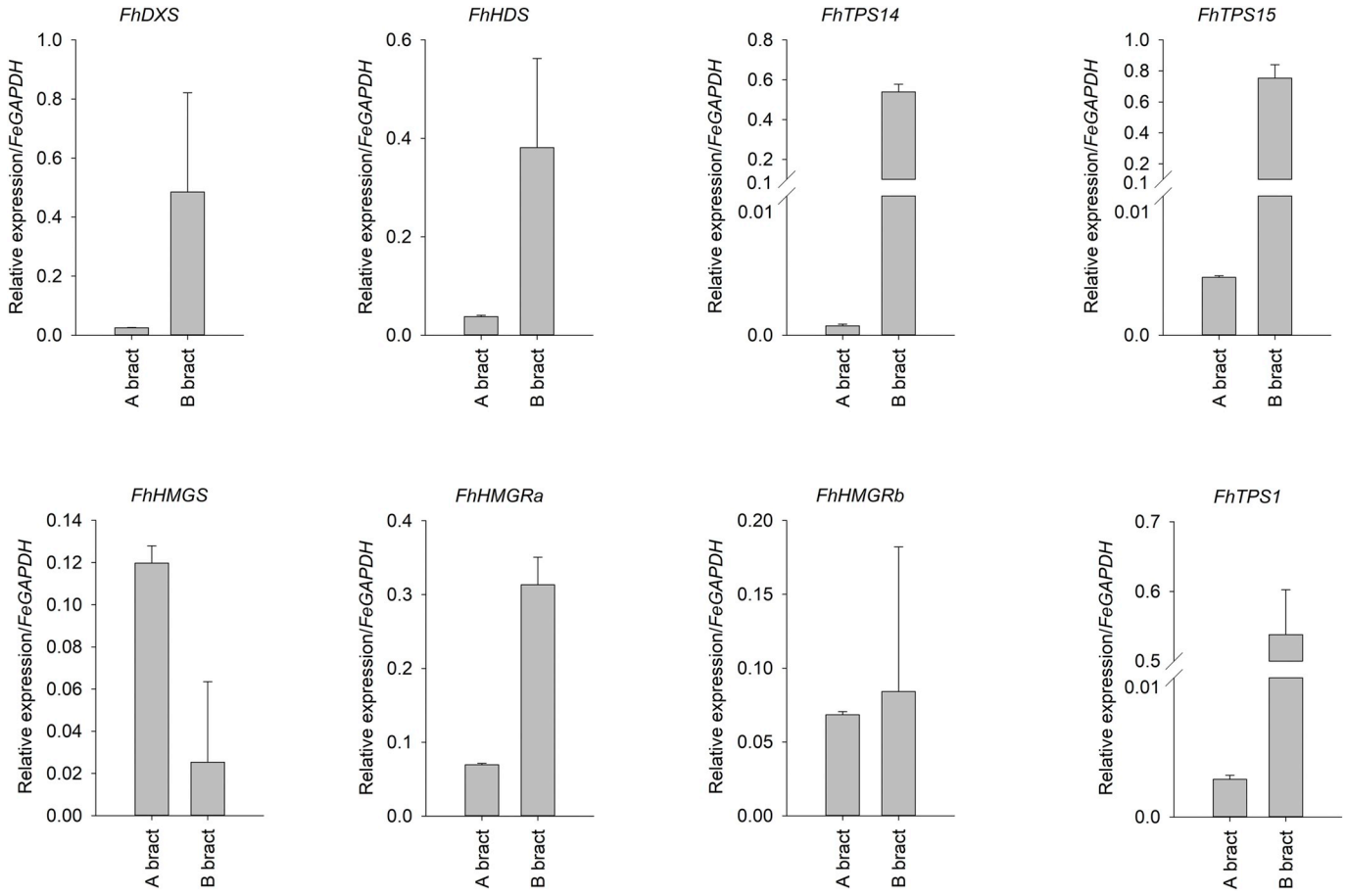

Fig. 5. Real-time PCR of some of terpenoid synthesis pathway genes of *F. hirta*. *FhGAPDH* was used as reference gene. A corresponds to pre-receptive figs, and B to receptive figs. *Fh* represents *F. hirta*; *TPS14* and *TPS15*, homologue of terpene synthase 14 gene of *Arabidopsis*; *TPS1*, homologue of terpene synthase 1 gene of *Arabidopsis*.

signal transduction were also differentially expressed. Photosynthesis provides the energy to the primary metabolism. Primary metabolism, such as starch and sucrose metabolism, glycerophospholipid metabolism, and fatty acid elongation, provide the material and energy basis required for secondary metabolism (Aharoni and Galili, 2011). Hence, this increase in gene expression may provide the metabolic background for a burst in VOC synthesis. Phytohormones play important roles in development and metabolism of plants. Brassinosteroid biosynthesis genes were up-regulated in both *F. hirta* and *F. triloba* (Table S5). Jasmonate synthase genes, including LOX2S, AOS, AOC, and ACAA1, were up-regulated in *F. triloba*. Further, transcription factor genes involved in plant hormone signal transduction such as TGA, DELLA, SAUR, ERF1, SnRK2, MYC2, were also up-regulated in *F. triloba* (Table S11). Some unigenes in these pathways might work together with unigenes of VOC pathways to regulate the synthesis of critical VOC involved in the olfactory signals used by fig wasps to locate host figs. If the ostiole is the locus of receptive fig odor emission, then signaling between ostiole and receptive female flowers is required, and the up-regulated phytohormone coding genes may be involved. Signaling between ostiole and flowers could be mediated by VOCs accumulating in the fig cavity as such a process is documented in other flowers (Boachon et al., 2019), and as receptive fig odors are known to accumulate in the fig cavity (Souza et al., 2015).

Three unigenes encoding ATP-binding cassette transporters (ABC transporters) also show a differential expression pattern between pre-receptive and receptive figs of *F. triloba*. ABC transporters are a transport system superfamily involved in translocation of substrates across membranes (Rice et al., 2014). Because VOCs are lipophilic liquids with low molecular weight and high vapour pressure at ambient temperatures (Pichersky et al., 2006), it has long been assumed that volatiles passively diffuse out of the cells through cell membranes (Eberl and Gershenzon, 2017). However, recent calculations have shown that for many plant volatiles, the internal concentrations could reach high values (up to 100 mM), which could lead to the buildup of toxic amounts of volatiles within the membranes (Widhalm et al., 2015). Further, in *Petunia hybrida*, PhABCG1 was demonstrated to facilitated active transport of volatiles across the plasma membrane (Adebesin et al., 2017). Hence, the up-regulated ABC transporters may be involved in VOC release by receptive figs. We did not detect variation in ABC transporters expression differentiation in *F. hirta*. This may be due to differences in ostiole structure between the two species, leading to different constraints on odor emission. Alternatively, other transporters may be involved in *F. hirta*.

5. Conclusions

This study provides the first analysis of transcriptome variation in ostiolar bracts between pre-receptive and receptive figs in two *Ficus* species. The results agree with the hypothesis that the ostiole is the locus of production of the receptive fig odors that are responsible for pollinating wasp attraction. The results suggest that differences in receptive fig odors between related species are primarily determined by variation in gene expression regulation. Regulation based receptive fig odor differentiation between species suggests a potential for rapid evolution of this interspecific barrier.

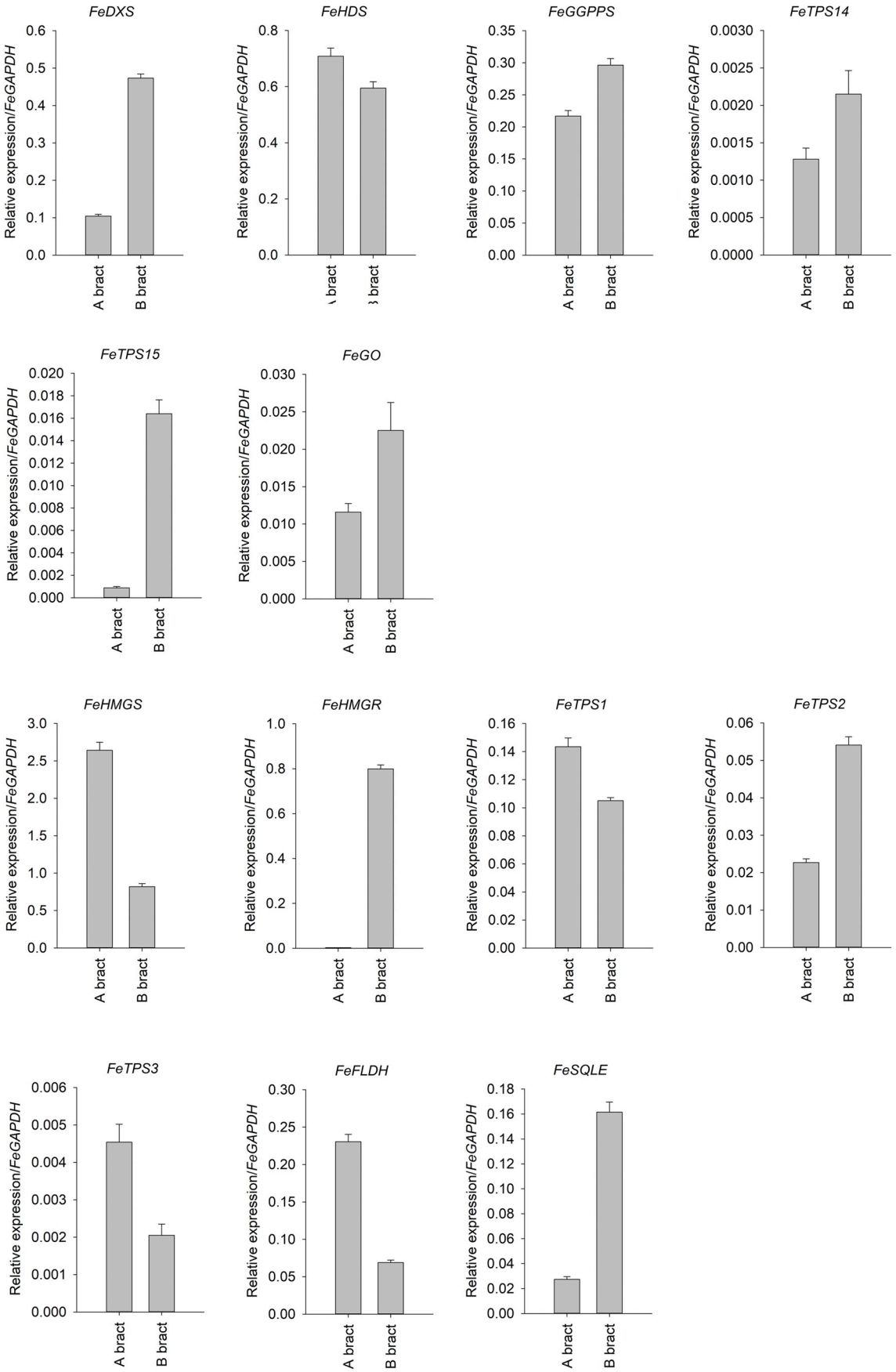

Fig. 6. Real-time PCR of some of terpenoid synthesis pathway genes of *F. triloba*. *FeGAPDH* was used as reference gene. A corresponds to pre-receptive figs, and B to receptive figs. *Fe* represents *F. triloba*; *TPS14* and *TPS15*, homologue of terpene synthase 14 gene of *Arabidopsis*; GO, gibberellin 2-oxidase; *TPS1*, *TPS2*, and *TPS3*, homologue of terpene synthase 1 gene of *Arabidopsis*.

Author contributions

Y. H. and C. X. Y. designed research. Y. H. and H. R. analyzed data and cowrote the manuscript. S. P. and K. F. cowrote the manuscript. C. Y. F. and W. R. performed laboratory work and analyzed data. All authors contributed substantially to revisions.

Data archiving statement

Raw sequence data has been submitted to NCBI's Short Read Archive (SRA). The accession number is PRJNA492455 for *F. triloba* and PRJNA491590 *F. hirta*.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actao.2020.103554.

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