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Composition Diversity and Expression Specificity of the *TPS* Gene Family among 24 *Ficus* Species

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Abstract: Volatile organic compounds (VOCs) released by the receptive syconia of *Ficus* species play a vital role in attracting highly species-specific pollinating fig wasps. The components of VOCs vary considerably among *Ficus* species, but are generally dominated by a few common terpenoid compounds or specific proportions of several compounds. Terpene synthase (*TPS*) is the main source of specific and diverse terpenoids, but the evolution of the *TPS* gene family in *Ficus* and the potential functions of the *TPS* genes in species-specific pollination remain largely unelucidated. In this study, using transcriptomes of ostiole bracts of receptive male figs from 24 *Ficus* species collected from South China and Southeast Asia, we comprehensively scanned and investigated the composition and evolutionary characteristics of all *TPS* genes in all 24 species. We identified 248 *TPS* genes, including 33 orthologous genes and six singletons. Sequence and phylogenetic analysis showed that a majority of the 248 *TPSs* contained the DDXXD and DTE motifs, rather than the DXDD motif, and involved all subfamilies (*TPS-a,b,c,e/f* and *g*) known in other angiosperm genomes, suggesting a very diverse and complex composition of class I *TPSs* during the receptive phase. In addition, compared to *TPS-a*, which is generally the largest subfamily in some plants, the *TPS-b* subfamily contained the highest number of genes in *Ficus* species. Expression profile comparison showed that the distribution and expression levels of different *TPSs* among different *Ficus* species differed considerably, but a few *TPS* genes were common across most species. Positive selection analysis showed that the *Ficus TPS* genes were mainly under purifying selection, with only four genes having positive selection signals and two genes having positive selection sites, and two genes having relatively fast-evolving rates. The present study demonstrates the basic evolutionary characteristics of *TPS* genes in *Ficus* and reveals the roles of *TPSs* in shaping the diversity and specificity of the fig–fig wasp symbiotic relationship.

Keywords: fig; *TPS*; DDXXD motifs; expression profile; positive selection

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1. Introduction

The interaction between plants and pollinating insects is considered to be an important driving force for the biodiversity and evolution of angiosperms in the tropics [1,2]. While the polymorphism of floral characteristics helps cater to diverse or specific flower visitors, the quantity and quality of pollen transmitted by flower visitors affect the reproductive success of plants. In nature, most of the interactions between plants and pollinators are generalized, except for a few highly obligate mutualisms, such as fig–fig wasp and yucca–yucca moth.

Ficus, which belongs to the Moraceae family, constitutes one of the largest genera of angiosperms with approximately 750 species, including trees, shrubs, epiphytic vines, and

other life forms [3,4]. The most typical feature of *Ficus* is its unique enclosed inflorescence commonly known as a syconium or fig. A syconium contains hundreds or thousands of tiny flowers and connects to the external environment via a narrow ostiole at its top. The opening of the ostiole is controlled by bracts, which are the main parts of *Ficus* that emit volatile organic compounds (VOCs) in receptive-phase syconia to attract obligate pollinators [5,6]. Only morphologically specialized fig wasps can enter the syconium and pollinate the female flowers within [7,8]. A species of *Ficus* can only attract and drive one or a few fig wasp species to pollinate [9]. The key components that attract specific pollinating wasps are different and generally include one or a few common terpenoid compounds or specific proportions of several compounds [10]. These key components strongly attract specific pollinator wasps, but not the wasps that pollinate other *Ficus* species [11]. Even for the same *Ficus* species, the specific pollinating wasps attracted by syconia in the receptive phase may be rejected by those in other developmental stages. This "push and pull" mechanism maintains the specific symbiotic relationship between pollinating wasps and their host plants [12–14].

VOCs include terpenoids, fatty acids derivatives, phenylpropanol/benzene ring compounds, and amino acid derivatives. Terpenoids represent the largest and most diverse class of chemicals, among the myriad of compounds produced by plants [15]. In *Ficus*, terpenoids provide key olfactory signals that attract pollinating wasps [10,16]. Terpenoids can be classified into monoterpenes (including 10 carbons), sesquiterpenes (including 15 carbons), diterpenes (including 20 carbons), and triterpenes (including 30 carbons). Most monoterpenes and sesquiterpenes are oily liquids that become volatile at room temperature and have special odors [17]. The synthesis of plant terpenoids generally occurs in three stages: (1) isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesized through the mevalonate (MVA) pathway in the cytoplasm and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastid, respectively; (2) under the action of allyl transferase, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and intermediates with various molecular weights are formed; (3) GPP, FPP, and GGPP, under the action of various terpene synthases (*TPSs*), generate monoterpenes, sesquiterpenes, diterpenes, and their derivatives (Figure S1). Finally, the modified enzymes catalyze hydroxylation, methylation, isomerization, and reduction to further produce terpene alcohols, acids, esters, and other derivatives [15,18].

The *TPS* gene family exists widely in plant genomes, most of which are involved in the production of secondary metabolites [19,20]. At the protein level, each TPS enzyme has two conserved domains, Pfam ID PF01397 and PF03936, distributed at the N- and C-terminals. On the basis of their related catalytic motifs, TPSs can be divided into classes I and II [21–23]. Class I TPSs, including all monoterpenes, all sesquiterpenes, and some diterpenes, contain two conserved DDXXD and NSE/DTE motifs. Class II TPSs, which only include diterpenes, contain only a conserved DXDD motif. In addition, the N-terminal domain of plant TPSs usually has a conserved R(R)X8W motif [21]. The second R of the motif R(R)X8W is not conserved, and some cases even feature the absence of the R(R)X8W motif [24].

At present, genome-wide comparative analysis of the evolutionary characteristics of the *TPS* gene family has been performed in more than 50 species [22,25]. Meanwhile, the number of plant species for which *TPS* gene family information has been obtained, on the basis of individual genome screening, is increasing [9,23,26–28]. Previous studies showed that the plant *TPS* gene family can be divided into seven subfamilies, namely, *TPS-a*, *TPS-b*, *TPS-c*, *TPS-d*, *TPS-e/f*, *TPS-g*, and *TPS-h* [21,22]. These subfamilies are specific for angiosperms, gymnosperms, land plants, vascular plants, and *Selaginella moellendorffii* Hieron, respectively [21]. *TPS-a*, *TPS-b*, and *TPS-g* encode proteins that produce sesquiterpenes and monoterpenes, respectively.

Owing to the importance of terpenoids in plant growth, chemical interactions, and protection in the environment [15], the cloning, expression, and functional characterization of *TPS* genes have also been completed in many plant species [16,19,26,28–30]. For

example, He et al. [28] demonstrated the dynamic regulation of *TPS* genes in the emission of herbivore-induced plant volatiles after studying the expression and promoter activity of cucumber *TPS* genes in response to different herbivores. Recently, eight *TPS* genes from *F. carica* L. were isolated and confirmed to play a key role in their defense against pests during syconium development by triggering diverse and dynamic changes in volatile emissions [16].

Although numerous studies have provided an important basis for understanding the functions and contributions of *TPS* genes, existing studies only focused on a few species and paid more attention to plant protection. In this study, to reveal the *TPS* diversity of *Ficus* and the evolutionary characteristics of the special gene family, we investigated the *TPS* genes in 24 *Ficus* species using RNA-seq. We aimed to (1) identify the potential *TPS* genes that function in *Ficus* pollination attraction, (2) determine the gene structures, phylogenetic relationships, and expression divergence of the *TPS* genes among different *Ficus* species, and (3) evaluate the probable effects of positive selection on the *Ficus TPS* genes. The findings of this study could lead to an improved understanding of the production mechanism of VOCs in *Ficus* and, most importantly, may shed light on the evolution of the *TPS* gene family across the genus.

2. Results

2.1. Characteristics of the TPS Genes in the Receptive Phase of Male Figs of 24 Ficus Species

After RNA-sequencing, we obtained 41.52–56.03 million raw reads from the ostiole bract transcriptomes of 24 *Ficus* species. For different species, we achieved 40.17–55.09 million clean reads after adapter clipping and quality control. Using the Trinity program, the clean reads of each species were assembled into 34,454–90,120 transcripts. The high-quality transcripts of the 24 species were subjected to cluster and assembly analyses, resulting in coding of 18,344–68,322 unigenes, N50 lengths of 877–1970 bp, and GC contents of 43.77–52.24%. All assembly statistics are summarized in Table S1.

Using blast scanning, a total of 248 *TPS* genes with two conserved N-terminal and C-terminal domains (PF01397 and PF03936) were identified from the unigenes of the 24 ostiole bract transcriptomes (Table S2). Among the genes, 180 *TPSs* were found to have complete coding sequences, and 68 were found to have incomplete coding regions (Table S2). All *TPS* genes containing a typical Terpene_Synth_C (or Terpene_cyclase_plant_C1) conserved domain were confirmed after revalidation by the NCBI Online Batch CD-Search. At the protein level, those complete *TPS* genes showed an obvious sequence length polymorphism ranging from 405 aa in *F. variolosa* to 850 aa in *F. microcarpa* (Table S2). Different numbers of *TPS* genes were found to be expressed during the receptive phase of male figs. Among the 24 *Ficus* species. *F. altissima* showed the most *TPS* genes (19), while *F. fistulosa* and *F. montana* showed the fewest (3) (Table 1).

Table 1. Twenty-four *Ficus* species used in this study.

Subgenus	Section	Subsection	Species	Name Abbreviations	*TPS* No.	Site	Latitude (N)	Longitude (E)
Ficus	*Eriosycea*	*Eriosycea*	*F. chartacea*	ficha	8	China—Guangdong	8.776	99.724
			F. fulva	fiful	14	China—Guangdong	8.776	99.724
			F. grossularoides	figro	3	Thailand—Narathiwat	5.799	101.762
			F. hirta	fihir	16	China—SCBG	23.171	113.349
			F. langkokensis	filan	11	China—Guangdong	24.227	112.006
			F. ruficaulis var. *antaoensis*	firuf	4	Taiwan	21.962	120.811
			F. triloba	fitri	13	China—SCBG	23.180	112.537
	Ficus	*Frutescentiae*	*F. abeli*	fiabe	12	China—Guangdong	23.636	113.780
			F. erecta var. *beecheyana*	fiere	11	China—Guangdong	23.765	113.915
			F. formosa	fifor	6	China—Guangdong	23.623	113.811
			F. heteromorpha	fihet	15	China—Guangdong	24.918	113.033
			F. ischnopoda	fiisc	11	Thailand—Chiang Mai	18.504	98.665
			F. pandurata	fipan	13	China—Guangdong	24.252	112.036
			F. pyrifomis	fipyr	12	Thailand—Chiang Mai	18.504	98.665
			F. variolosa	fivar	13	China—Guangdong	23.624	113.797

Table 1. *Cont.*

Subgenus	Section	Subsection	Species	Name Abbreviations	*TPS* No.	Site	Latitude (N)	Longitude (E)
Sycidium	*Sycidium*	*Sycidium*	*F. montana*	fimon	3	Thailand	7.557	99.776
Sycomorus	*Sycomorus*	*Sycocarpus*	*F. fistulosa*	fifis	3	China—Guangdong	23.156	112.511
			F. hispida	fihis	8	China—SCBG	23.180	113.350
		Neomorphe	*F. variegata*	fivae	9	China—Guangdong	23.176	112.538
		Hemicardia	*F. semicordata*	fisem	11	Thailand—Chiang Mai	19.362	98.922
Urostigma	*Urostigma*	*Conosycea*	*F. altissima*	fialt	19	China—SCBG	23.188	113.363
			F. benjamina	fiben	7	China—SCBG	23.186	113.358
			F. microcarpa	fimic	13	China—SCBG	23.178	113.352
		Urostigma	*F. rumphii*	firum	13	Myanmar	21.966	96.069

A total of 10 motifs were extracted from the 248 *TPS* genes, but three (DDXXD, DTE, and RXR) showed the highest degree of conservation. Generally, these *TPS* genes contained DDXXD and DTE motifs at the C-terminus (Figure 1A).

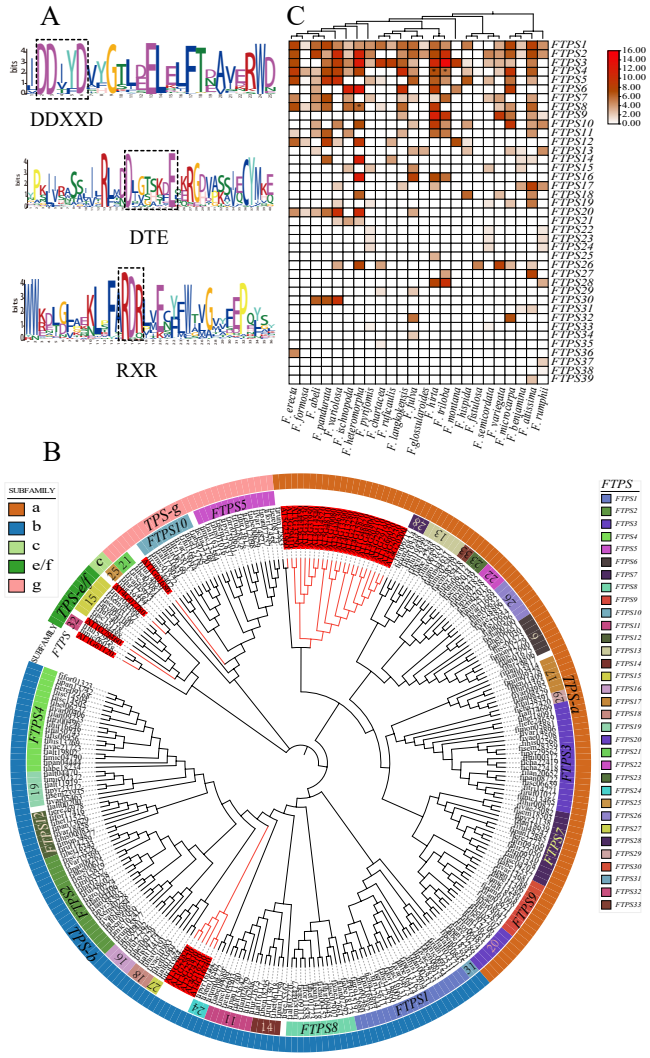

Figure 1. Sequence characteristic, phylogeny, subfamily classification, and expression profile of 248 *FTPS* genes. (**A**) Three conserved motif logos of the 248 *Ficus* genes, showing DDXXD, DTE, and RXR motif, respectively. (**B**) A maximum likelihood tree based on amino acid sequences of 248 *FTPS* genes and 33 *A. thaliana* genes, showing that these *FTPSs* are classified into five subfamilies and 33 orthologous genes (*A. thaliana* genes are colored in red). (**C**) Expression heatmap of 248 *FTPS* genes across 24 species. * Genes with positive sites.

We classified 248 *TPS*s into 33 orthologous genes (*FTPS1–FTPS*33) and six singletons (*FTPS34–FTPS39*) using OrthoMCL clustering analysis and by manually checking the sequence similarity (Figure 1B, Table S2). Subsequently, we annotated these genes using the nonredundant protein database (Table S3). Consequently, we found that these *FTPSs* were probably matched to 18 known terpene synthases and their analogs, including frequently occurring myrcene synthase, (−)-germacrene D synthase, linalool synthase, and *E*-beta-caryophyllene synthase. Generally, these expressed *FTPSs* tended to encode acyclic monoterpenes (12 acyclic and five cyclic) and cyclic sesquiterpenes (five acyclic and 15 cyclic), indicating the complex configuration of the compounds. In addition, 10 pair paralogous *TPSs* were found in eight *FTPS* genes of 10 species (Table S4).

2.2. Subfamily Analysis of the FTPS Genes

A phylogenetic tree for 248 *FTPS* genes and 33 *Arabidopsis TPS* genes was constructed in RAxML using the ML method (Figure 1B). In the ML tree, the *FTPS* genes were classified into four separate clades. According to the classification of the *Arabidopsis TPSs* in each clade, the four clades corresponded to four *TPS* subfamilies, namely, *a*, *b*, *g*, and *c* and *e/f*. Most *FTPSs* were clustered into the *TPS-b* (114), *TPS-a* (94), and *TPS-g* subfamilies (29). In contrast, the number of genes in *TPS-e/f* and *TPS-c* subfamilies was greatly reduced. Two *TPS-c* genes were mixed into the nine *TPS-e/f* genes.

2.3. Expression Analysis of the FTPS Genes

To check the expression differences of the *FTPSs*, we compared the TPM values of the 39 *FTPSs* (Figure 1C, Table S5). For those paralogous pairs (Table S4), only the higher expression values were used. Consequently, we found that the expression of *FTPS* genes varied among the 24 species. Although most *FTPS*s were expressed in a limited number of species, a few genes were common across multiple species. For example, six genes (*FTPS1–FTPS5, FTPS7,* and *FTPS8*) were expressed in more than half of the 24 *Ficus* species. Remarkably, *FTPS1* was expressed in 21 species. In contrast, six singletons were highly species-specific, and only one species (*F. rumphii*) had two singletons (Figure 1C, Table S4). Nevertheless, each species usually has a specific *FTPS* expression profile. However, two distantly related species, *F. pyrifomis* and *F. semicordata*, shared very similar *FTPS* expression profiles (Figure 1C).

2.4. Positive Selection Analysis

The ML tree of the 24 species of *Ficus* constructed from the concatenated *TPS* genes (Figure S2) conformed to the taxonomy of the species [31]. Using the ML tree as a guide, we performed a positive selection analysis for 20 of the 33 *FTPS* genes, distributed across >4 species.

Using one ratio model calculation, the ω range of 20 *FTPS* genes was found to be 0.250 (*FTPS15*) to 0.758 (*FTPS16*) (Table 2). In order to better detect the probable selective constraint on each *FTPS* gene in the evolutionary process, the free ratio model, which allows varied ω ratios in each branch, was implemented by the likelihood ratio test (LRT). Two genes, *FTPS10* and *FTPS12* showed significant selection signals (Table 2), suggesting divergent evolutionary rates of the genes among different *Ficus* species.

Table 2. Positive selection analysis of *FTPS* genes occurred in more than four species under branch model test.

Gene	Model	np	Mates of Parameter	lnL	df	2ΔlnL	*p*-Value [a]
FTPS1	Free-ratio	79	Variable ω	−4424.469	38	35.058	0.606
	One-ratio	41	0.522	−4441.998			
FTPS2	Free-ratio	67	Variable ω	−5413.296	32	33.835	0.379
	One-ratio	35	0.449	−5430.214			
FTPS3	Free-ratio	67	Variable ω	−5872.822	32	37.559	0.229
	One-ratio	35	0.679	−5891.602			
FTPS4	Free-ratio	59	Variable ω	−5024.747	28	21.298	0.813
	One-ratio	31	0.496	−5035.396			
FTPS5	Free-ratio	51	Variable ω	−3504.033	24	19.138	0.745
	One-ratio	27	0.345	−3513.602			
FTPS6	Free-ratio	23	Variable ω	−3252.649	20	3.613	0.999
	One-ratio	13	0.465	−3254.456			
FTPS7	Free-ratio	43	Variable ω	−3883.714	20	19.883	0.465
	One-ratio	23	0.503	−3893.656			
FTPS8	Free-ratio	43	Variable ω	−6311.560	20	14.685	0.794
	One-ratio	23	0.504	−6318.902			
FTPS9	Free-ratio	23	Variable ω	−3433.342	10	8.137	0.615
	One-ratio	13	0.479	−3437.411			
FTPS10	Free-ratio	35	Variable ω	−4081.220	16	26.337	0.049 *
	One-ratio	19	0.426	−4094.389			
FTPS11	Free-ratio	27	Variable ω	−3358.780	12	18.882	0.091
	One-ratio	15	0.499	−3368.221			
FTPS12	Free-ratio	27	Variable ω	−3715.070	12	23.205	0.026 *
	One-ratio	15	0.517	−3726.673			
FTPS13	Free-ratio	23	Variable ω	−4886.043	10	6.841	0.740
	One-ratio	13	0.382	−4889.464			
FTPS14	Free-ratio	15	Variable ω	−3614.771	6	10.329	0.111
	One-ratio	9	0.637	−3619.935			
FTPS15	Free-ratio	15	Variable ω	−3086.012	6	4.530	0.605
	One-ratio	9	0.250	−3088.278			
FTPS16	Free-ratio	15	Variable ω	−3125.859	6	6.115	0.410
	One-ratio	9	0.758	−3128.916			
FTPS17	Free-ratio	19	Variable ω	−3127.501	8	3.009	0.934
	One-ratio	11	0.447	−3129.006			
FTPS19	Free-ratio	15	Variable ω	−2834.753	6	4.801	0.570
	One-ratio	9	0.407	−2837.153			
FTPS20	Free-ratio	23	Variable ω	−3453.672	7	3.639	0.820
	one-ratio	13	0.548	−3455.491			
FTPS26	Free-ratio	23	Variable ω	−5711.069	8	14.155	0.078
	one-ratio	13	0.489	−5718.147			

[a] Significantly different values according to the χ^2 value with specific k degrees of freedom at a significance level of 0.05 (*).

In the branch-site model, we identified three genes (*FTPS*3, *FTPS*16, and *FTPS20*) with significant statistics values ($p < 0.01$ or $p < 0.05$) in four species-specific (*F. montana* or *F. pyrifomis*, *F. fistulosa*, and *F. formosa*) clades (Table 3), suggesting a divergent evolutionary rate of the genes in these species. In addition, we also found that three and one positive selection sites in *FTPS4* and *FTPS8* in *F. hirta–F. triloba* and *F. heteromorpha* clades, respectively (Table 3).

Table 3. Positive selection analysis of *FTPS* genes occurred in more than four species under branch site model test.

Gene	Clade	Models Compared	np	*p*-Value [a]	lnL	ω Values	Positive Sites (BEB) [b]
FTPS3	fimon	Model A	38	1.490×10^{-2} *	−5595.802	p_0 = 0.023 ω1 = 1.000 ω2 = 44.817	
		Model A null	37		−5598.162	p_0 = 0.023 ω1 = 1.000 ω2 = 1.000	
	fipyr	Model A	38	6.105×10^{-4} **	−5490.893	p_0 = 0.015 ω1 = 1.000 ω2 = 315.194	
		Model A null	37		−5496.038	p_0 = 0.018 ω1 = 1.000 ω2 = 1.000	
FTPS4	fihir-fitri	Model A	34	0.051	−4860.385	p_0 = 0.000 ω1 = 1.000 ω2 = 92.201	258S * 345Y * 424M *
		Model A null	33		−4861.730	p_0 = 0.000 ω1 = 1.000 ω2 = 1.000	
FTPS8	fihet	Model A	26	0.059	−5773.442	p_0 = 0.047 ω1 = 1.000 ω2 = 28.837	217H **
		Model A null	25		−5776.607	p_0 = 0.047 ω1 = 1.000 ω2 = 1.000	
FTPS16	fifis	Model A	13	1.129×10^{-3} **	−3118.162	p_0 = 0.417 ω1 = 1.000 ω2 = 314.584	
		Model A null	12		−3122.826	p_0 = 0.408 ω1 = 1.000 ω2 = 1.000	
FTPS20	fifor	Model A	16	6.005×10^{-7} **	−3399.251	p_0 = 0.588 ω1 = 1.000 ω2 = 246.640	
		Model A null	15		−3427.039	p_0 = 0.626 ω1 = 1.000 ω2 = 1.000	

[a] Significantly different values according to the χ^2 value with specific k degrees of freedom at a significance level of 0.01 (**) or 0.05 (*). [b] Significantly different values with p > 95% (*) and p > 99% (**).

3. Discussion

TPS is an important enzyme that is primarily responsible for catalyzing the synthesis of various terpenoids [23]. In the present study, using ostiole bract transcriptomes, we compared the genic characteristics of expressed *TPS*s among 24 *Ficus* species. The number, distribution, and expression level of the *TPS* genes among different *Ficus* species were obviously inconsistent, suggesting a diverse composition of terpenes in *Ficus* species.

3.1. Numbers of TPS Genes in 24 Ostiole Bract Transcriptomes

The *TPS* gene family is a mid-size family of angiosperms [21]. The number of *TPSs* in angiosperms varies widely, ranging from two *TPS* in *Zostera marina* [22] to 152 in *Vitis vinifera* [21,22,24]. In particular, *TPS* genes typically follow a lineage-specific evolutionary pattern, resulting in diverse terpenoid products [32,33]. In addition, given that the *TPSs* are expressed in a spatiotemporal differential and organ-specific manner, the number of *TPS* genes identified from the transcriptomes is probably smaller than the number localized in plant genomes. In this study, within *Ficus*, using tissues at the same development stage, we also found that the number of *TPS* genes was quite different among the 24 species. For example, there were only two *TPS* in *F. fistulosa*, but 19 in *F. altissima* (Table 1). In addition, for species within the same subgenus, even within the same section of *Ficus*, we did not find any similarities in the distribution pattern of the *TPS* genes. To further estimate the occurrence frequency of the *TPS* genes during the receptive stage of male syconia, we scanned two known genomes of *F. microcarpa* and *F. hispida* and compared their *TPS* number at a genome-wide level to the number identified in our study. We identified 33 and 59 *TPS* genes in the two genomes and found that only 39% (13) and 13% (eight) *TPS* genes of two species occurred during the receptive stage (Table 1). This result suggests that the number and occurrence frequency of *TPS* genes in the receptive stage of male syconia largely depend on the species in *Ficus*. This situation is consistent with the hypothesis that different species of *Ficus* release specific volatiles to attract specific pollinating wasps [34].

3.2. Sequence Characteristics and Subfamily Categories of the FTPS Genes

According to the amino acid motifs and catalytic mechanism, TPS enzymes can be divided into two classes: class I and II [21–23]. Class I TPSs contain two conserved motifs, DDXXD and (N,D)DXX(S,T,G)XXX(E,D) (also named DTE), which facilitate ionization of an isoprenoid diphosphate moiety and generate a reactive carbocation intermediate. In contrast, Class II TPSs typically contain only a DXDD motif, which protonates substrates, catalyzes scaffold rearrangements without cleavage of the diphosphate ester bond, and produces initial carbocation intermediates [35]. In this study, at the protein level, the DXDD motif was only found in one sequence of FTPS25 (*fihir39312*). Phylogenetic analysis showed there was another paralog of the sequences in *F. hirta* (*fihir17484*). However, owing to the low expression levels of the two sequences (Table S3), we could not detect sufficient reads to assemble complete coding sequences, including the DXDD domain in the second sequence. Because the DXDD motif is the characteristic of Class II *TPS* gene, the *FTPS*s identified in our transcriptomes should be dominated by Class I TPS enzymes [36]. This suggestion was also supported by the subsequent phylogenetic analysis.

On the basis of genome-wide comparisons of multiple plants, the *TPS* family has been split into seven subfamilies, i.e., *TPS-a*, *TPS-b*, *TPS-c*, *TPS-d*, *TPS-e/f*, *TPS-g*, and *TPS-h* [21,22,37]. Among the subfamilies, *TPS-d* and *TPS-h* are distributed only in Gymnosperms and *Selaginella moellena*, respectively [21]. Notably, in this study, we identified all of the other five *TPS* subfamilies that can be distributed in angiosperms in the 24 *Ficus* species, suggesting a diverse and complicated composition of terpenes in the receptive stage of male syconia. In addition, compared with other plants such as *A. thaliana* [38], grapevine [24], and tomato [30], in which *TPS-a* genes are the most abundant genes, here, we found that a majority of *FTPSs* were categorized into *TPS-b* (46%) rather than the *TPS-a* (38%) subfamily (Figure 1B). Previous studies have shown that the *TPS-b* subfamily is mainly composed of angiosperm monoterpene synthases genes [39,40], except a sesquiterpene, (*E*,*E*)-α-farnesene synthase gene, found in apple [41], poplar [42], and soybean [43]. Here, we also identified an orthologous gene encoding (*E*,*E*)-α-farnesene synthase (*FTPS8*) in 12 *Ficus* species (Table S3). More interestingly, our study revealed that most *FTPSs* protein sequences in the *TPS-b* subfamily (63%) lack the second R of the R(R)X8W motif, i.e., lacking the RR domain (Figure 2). Previous authors confirmed that the RR domain was functionally important to isomerize GPP to a cyclizable intermediate [44]. However, the RR domain may be absent in the monoterpene synthases producing only acyclic compounds, which do not require isomerization [45]. Therefore, our study suggests that a majority of the *Ficus TPS-b* genes likely encode acyclic terpene synthases. This suggestion is supported by our current annotations that 70% of monoterpenes were acyclic (Table S3). However, the result is inconsistent with previous findings that many of the enzymes in the plant *TPS-b* group produce cyclic monoterpenes [21]. Therefore, the production of rich highly volatile acyclic monoterpenes may be a distinguishing characteristic for the *Ficus* TPS-b enzymes.

The *TPS-a* subfamily, which included 14 *FTPS* genes (Figure 1B), is the second largest *TPS* subfamily in the *Ficus* species. Similar to other plants, most *TPSs* in the *TPS-a* clade were sesquiterpene synthase genes [21,38]. A total of 13 out the 14 *FTPS* genes identified are responsible for encoding sesquiterpenes. Only *FTPS13*, which encodes a cadinene synthase, is an acyclic monoterpene.

The third largest *TPS* subfamily in the 24 *Ficus* species is *TPS-g*. This subfamily includes 29 sequences (Figure 1B). *TPS-g* genes are often believed to show prevalence in catalyzing acyclic products [21,46]. Here, these *TPS-g* genes were categorized into three genes (*FTPS5*, *FTPS10*, and *FTPS21*) and two singletons (*ficha23769* and *fiful14859*). According to annotation by the nonredundant protein database, all five genes were matched to two acyclic monoterpenes, linalool synthase and (3*S*, 6*E*)-nerolidol synthase 1.

TPS-e/f and *TPS-c* are small subfamilies in the *Ficus* species. The *FTPS-e/f* genes are present in six species and involved in only two orthologous genes (*FTPS15* and *FTPS32*). The genes encode a diterpene (ent-kaur-16-ene synthase) and a monoterpene *S*-linalool synthase, respectively. The ent-kaurene synthases often serve as precursors to plant growth

regulators, and they exhibit a variety of useful pharmacological properties [47]. In other words, the role of the compound is more focused on pest defense. However, in sunflowers, Morris et al. [48] showed that kaurane diterpenes can be used as oviposition stimulants of insects. By contrast, *S*-linalool is often used as an attractive volatile that some flowers emit to please pollinators [49]. Despite their variability in function, in the fig–fig wasp symbiosis, terpenes are always considered to be chemical attractants to fig wasps. Therefore, the exact functions of the two *TPSs* in *Ficus* deserve further exploration. In the *TPS-c* subfamily, we only found two paralogous sequences that encoded for *FTPS25*. The two sequences were annotated as ent-copalyl diphosphate synthases and were highly specifically expressed in *F. hirta*. This obvious gene expansion event implies that the *FTPS25* may function during the receptive phase of male syconia of *F. hirta*. In addition, the presence of the classic DXDD motif in the gene suggests they function as Class II *TPS*.

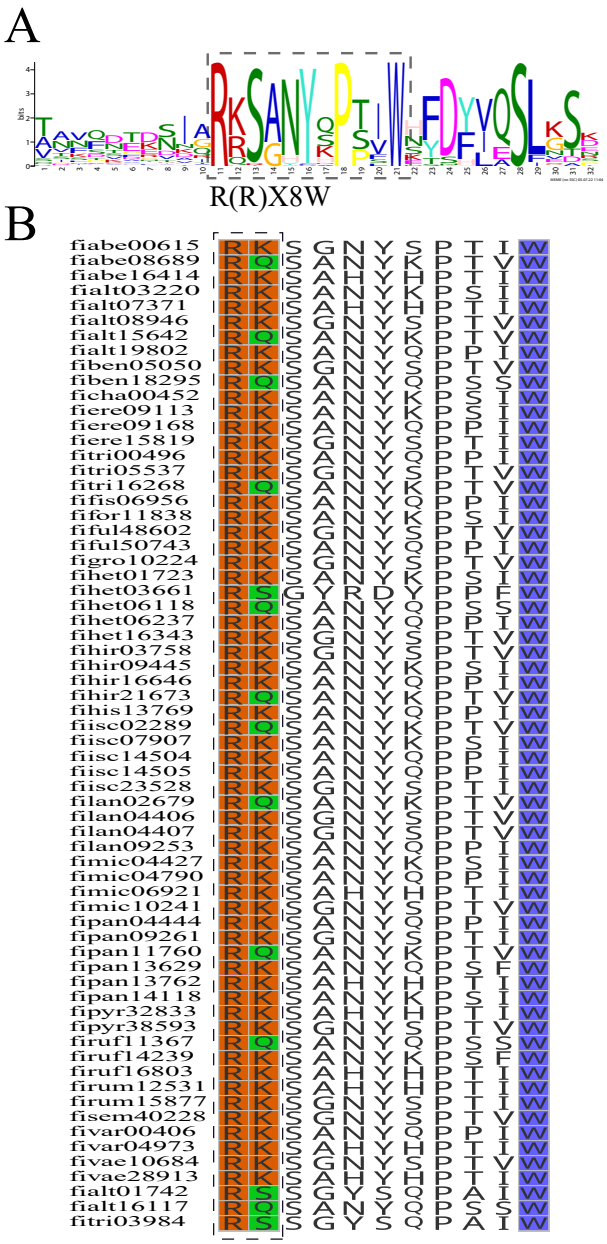

Figure 2. The R(R)X8W motif of the *FTPS* genes in the *TPS-b* subfamily. (**A**) The R(R)X8W motif logo of all *FTPS-b* subfamily genes. (**B**) Some representative sequences of *FTPS-b* subfamily genes, showing the R(R)X8W motif without the tandem RR domain.

3.3. Expression Profiles of the FTPS Genes

By examining the expression profiles of the *FTPS*s, we determined the diversity and complexity of *FTPS*s expression status in 24 *Ficus* species. Here, each species had a unique expression profile indicating a highly species-specific expression array of the *FTPS* genes. However, although we identified six singletons for five species, we showed that the other 33 orthologous *FTPSs* were shared by at least two species (Figure 1C). This result suggests that the odors emitted by each *Ficus* species blend compounds of multiple common terpenoids, rather than entirely species-specific volatiles. By comparing the blends of VOCs released from receptive figs of 20 *Ficus* species, Grison-Pige et al. [50] found that a few major compounds such as β-ocimene, germacrene D, and linalool are common among floral fragrances, while the rare compounds are usually present in a very low proportion in the blends. In addition, Proffit and Johnson [11] showed that (*E*)-β-ocimene was responsible for 41.75% of VOCs in receptive syconia of *F. sycomorus*. In this study, we found that at least 10 *FTPSs* were annotated as myrcene synthases (Table S3) and were highly expressed in multiple species (Figure 1C). For example, *FTPS1* and *FTPS2* that encode different myrcene synthases were found in 21 and 19 species, respectively. Given that myrcene is an isomer of ocimenes, the myrcene/ocimene synthases should also produce common chemical compounds during the repetitive phase of male syconia for the *Ficus* species. In addition, we also found six *FTPSs* encoding germacrene D synthases were simultaneously expressed in more than 16 species (Figure 1C). In plants, germacrene D, comprising some 300 isomers ($C_{15}H_{24}$), has evolved ecological roles in the interaction of the plant with insects [51,52]. This plant sesquiterpene can specifically activate a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens* [53]. However, whether germacrene D is involved in the interaction of figs and fig wasps remains unclear.

In addition, it is worth noting that the distribution and expression intensity of the *FTPSs* across species do not always correlate with the phylogenetic relationship of the species. Recently, Yu et al. [54] investigated the volatiles emitted by receptive male figs of *F. hirta* and *F. triloba*, two phylogenetic related species, and found that the two species share 48 VOCs, but the most abundant volatiles differ. Here, we identified 11 homologous *FTPS* pairs between the two species, but the expression level of each *FTPS* pair was very different (Figure 1C). These results suggest that the proportion of compounds is more important than the composition itself.

However, interestingly, we also found that two distantly related species (*F. pyrifomis* and *F. semicordata*) had highly similar expression profiles of *FTPSs* (Figure 1C). Within the genus of *Ficus*, species *F. pyrifomis* and *F. semicordata* belong to different subgenera, *Ficus* and *Sycomorus*, respectively. In this study, the single relevance between the two species is that they were all collected from Thailand. Given that the two species have distinguished morphologies from each other and from the other fig species, sampling and identification errors can be ruled out. We also checked the other genes in the transcriptomes of the two species and found different gene lists and expression profiles, suggesting that the two RNA libraries were not misused or mixed. Previous studies confirm that similar volatile blends of fig species can trigger their evolutionary convergence to attract particular wasp species [55–57]. In turn, regionally pollinator sharing can produce gene flow between fig species [54,58]. In *Ficus*, hybridization and introgression can occur not only among fig taxa from the same section but also among taxa from different sections [58]. Therefore, the current deviant may be a special case of hybridization and introgression of distantly related species. Further confirmation research is very necessary. In addition, small doses of compounds produced by lowly expressed genes may also play some key roles in attracting special fig wasp in different symbiosis systems.

3.4. Positive Selection of FTPSs

In this study, the branch model and branch-site model methods were used to check for the positive selection of homologous *TPS* genes. Except for five genes (*FTPS10* and *FTPS12* in the branch model test and *FTPS3*, *FTPS16*, and *FTPS20* in the branch-site model test)

that showed significantly positive selection signals in some specific species (Tables 2 and 3), a majority of the *FTPSs* were still highly conserved among species and under purification selective pressure, suggesting functional stability of the *TPS* genes. The five *FTPS* genes encode four chemical compounds, linalool synthase, myrcene synthase, elemene synthase, and *E*-beta-caryophyllene synthase (Table S3). Notably, these compounds are all common in *Ficus* species rather than species-specific ones [50,54]. Grison-Pige et al. [50] confirmed that beta-caryophyllene, linalool, myrcene, and beta-elemene can be identified from fig odors of 18, 10, six, and five *Ficus* species, respectively. Yu et al. [54] revealed that (*E*)-caryophyllene and beta-element were all highly abundant compounds released by both *F. hirta* and *F. triloba*. These results imply that the *FTPS* evolution may prefer to select those common genes shared by several *Ficus* species rather than recruit some specific or rare new genes. In addition, in this study, two genes (*FTPS4* and *FTPS8*) with three and one positive selection sites in species of *F. hirta* and *F. triloba*, and *F. erecta var. beecheyana* were also identified under branch-site model, respectively. Although these sites were not located in the conserved protein domains of plants *TPS* genes, these hidden interspecies divergences may prompt the diversification and complexity on the structure and function of the *FTPS* proteins, and then enforce VOC specificity. Further in-depth functional verification should be performed.

4. Materials and Methods

4.1. Sample Collection and RNA Sequencing

Twenty-four *Ficus* species belonging to four subgenera, with five sections and eight subsections [3], were collected from South China and Southeast Asia (Table 1). To simplify species names during data analysis, abbreviated names of the 24 *Ficus* species were used (Table 1). The ostiole bracts of three receptive male syconia from three plants of each of the 24 species were dissected and placed in RNAlater™ Stabilization Solution (00936134, Takara).

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of RNA was evaluated using 1% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA). A total of 1.5 µg of RNA per species was used as the input material for an RNA library constructed using an Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Paired-end high-throughput sequencing was performed by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China) on an Illumina HiSeq 4000 platform.

4.2. RNA-Seq Assembly and Identification of TPS Genes

Data obtained by the RNA-seq of 24 *Ficus* species were assembled de novo using Trinity v2.8.5 [59] under the De Bruijn algorithm to construct unique consensus sequences. TransDecoder v5.5.0 (Haas et al., 2013) was used to predict the coding sequence (CDS) for each isoform of a gene. The raw sequence data were deposited in the Genome Sequence Archive (GSA) in the National Genomics Data Center, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/gsa/; accessed on 31 July 2022), under the accession number PRJCA009602.

To facilitate subsequent analysis, we processed all sequences with an in-house Perl script and converted the nucleotide sequence of each unigene to protein sequence. Finally, we obtained a protein database for the 24 *Ficus* species.

We selected amino acid sequences of the known *TPS* genes of *Arabidopsis thaliana* (L.) Heynh. (https://www.uniprot.org/; accessed on 31 October 2021) as query sequences, and then identified the *TPS* genes from the above constructed protein databases for the 24 *Ficus* species using a BlastP search (*E*-value < 10^{-5}). PF01397 (N-terminal) and PF03936 (C-terminal) are the query models derived from the hidden Markov model of the *TPS* genes in the PFAM database (https://pfam.xfam.org/; accessed on 31 July 2022). Using these two HMM models as the query, we identified the *TPS* genes from the protein database of 24 *Ficus* species using HMMER 3.2.1 [60]. To ensure data accuracy, the conserved domains of the *TPS*

proteins obtained using the above two methods were reconfirmed in the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; accessed on 31 July 2022). The validated *TPS* genes were annotated using the online tool eggNOG-mapper v2 (http://eggnog-mapper.embl.de/; accessed on 31 March 2022) with default parameter [61] and the nonredundant protein database (https://www.ncbi.nlm.nih.gov/; accessed on 31 March 2022) using BLASTP with the E-value cutoffs $\leq 10^{-5}$.

4.3. Phylogenetic Relationship, Subfamily Classification, and Motif Analysis of the Ficus TPS Genes

All identified *Ficus TPS* genes and 33 *TPS* genes from the *A. thaliana* genome (https://phytozome-next.jgi.doe.gov/; accessed on 1 July 2022) were combined to build a data matrix. MAFFT v.7407 software [62] was used to perform multiple sequence alignments. RAxML v8.2.12 software [63] was used to construct a maximum likelihood (ML) phylogenetic tree based on full-length amino acid sequences with the executed command "raxmlHPC-HYBRID-SSE3 -f a -x 12345 -p 12345 -# 1000 -m GTRGAMMA". The multiple Em-for-Motif-Elicitation web server (https://meme-suite.org/meme/; accessed on 21 May 2022) [64] was used for the motif analysis of the *Ficus TPS* genes using the parameters -nostatus -mod anr -minw 10 -maxw 50 -nmotifs 10.

4.4. Clustering, Annotation, and Expression Analysis of the Ficus TPS Genes

The OrthoMCL v2.0.9 [65] with a highly strict threshold of the following parameters: —evalue 1×10^{-12} —identity 0.5 —CIP 0.6 —CALP 0.6 [66] was used to cluster the identified *TPS* genes, and in-house Perl scripts were used to screen the clustering results to obtain different *TPS* orthologs.

The value of transcripts per kilobase per million mapped reads (TPM) of each *TPS* gene calculated by RSEM [67] was used to evaluate transcription abundance. To facilitate data comparison, for the *TPS* with duplicated paralogs within the same species, we retained only the *TPS* with the highest expression level for further analysis. After standardization using the trimmed mean of M values (TMM), we obtained the original data matrix of gene expression. Heat maps of the orthologous *TPS* genes were illustrated using TBtools v1.09876 (https://github.com/CJ-Chen/TBtools/; accessed on 29 June 2022) [68], and a color gradient was displayed as the $\log_2$(TPM + 1) transformed expression levels of each gene.

4.5. Positive Selection Analysis

We performed gene positive selection analyses using the branch-site model and branch model in the PAML package version 4 [69]. A concatenated *TPS* gene topology phylogenetic tree was constructed as the guide tree using RAxML v8.2.12 software [63] under the parameters "raxml HPC-HYBRID-SSE3 -f a -x 12345 -p 12345 -# 1000 -m GTRGAMMA". Species *Morus notabilis* C. K. Schneider was used as the outgroup. Because the PAML documentation recommends that the absolute minimum be four or five if the sequence divergence is optimal, the *TPS* genes that occurred in fewer than four species were ignored from further processing.

In the branch model, the one-ratio model was the null model, which means that all branches had the same ω value, while the free-ratio model considered that each branch had a different ω value. We compared the likelihood ratio of the two models and calculated the *p*-value using the LRT test.

In order to find genes that potentially experienced positive selection, the branch-site model (model = 2 and NSsites = 2) of the PAML package was used, with each branch specified as the foreground. To avoid false positive results in the branch-site model, the following rigorous criteria were used: a dN/dS ratio (ω) greater than 1 on the foreground branch, and positively selected sites with a posterior probability calculated by the Bayes empirical Bayes (BEB) method greater than 0.95 and a *p*-value ≤ 0.05 in the likelihood ratio test [70].

5. Conclusions

This study identified 248 *TPS* genes in 24 *Ficus* species, and then comprehensively analyzed their sequence characteristics, phylogenetic relationships, expression profiles, and selection divergences. Studies revealed that the *FTPSs* are dominated by class I synthase genes, and the terpenoids encoded by the *FTPSs* may involve sesquiterpenes, monoterpenes, and diterpenes. The 248 *FTPS*s can be classified into 33 homologous genes with different expression strengths and expression profiles in 24 *Ficus* species. Complex combination and divergent expression of the multiple terpenoid synthase genes may be an important factor responsible for the diversity and specificity of *Ficus* terpenoids. In short, this study deepens our understanding of the diversity of the fig *TPS* gene family.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d14090721/s1: Figure S1: The biosynthesis pathway of plant terpenoids and classification of terpene synthase (*TPS*); Figure S2: A concatenated *FTPS* phylogenetic tree for positive selection analysis; Table S1: Summary of assembly results of transcriptome data for 24 *Ficus* species; Table S2: Amino acid sequences of 248 *TPS* genes in the 24 *Ficus* species; Table S3: Annotation of *Ficus TPS* genes by the nonredundant protein database; Table S4: Expression of different *FTPS* paralogous pairs in six species; Table S5: TPM values of *FTPSs* in the 24 *Ficus* species.

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Data Availability Statement: The raw sequence data are available in the Genome Sequence Archive (GSA) at the National Genomics Data Center, Chinese Academy of Science (https://bigd.big.ac.cn/gsa; accessed on 31 July 2022), under accession number PRJCA009602.

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