Fungus-mediated plant-pollinator mutualism in *Monoon laui* (Annonaceae): functional differentiation of stigmatic exudate and petal nectar

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Abstract

Background Floral nectar typically functions as a pollinator reward in mutualistic flower-pollinator interactions, with this mutualism sometimes strengthened when plants provide the pollinators with brood sites and larval food as rewards. The functional and molecular mechanisms underpinning such rewards remain unclear.

Results We present strong circumstantial evidence supporting a fungus-mediated plant-pollinator mutualism in a beetle-pollinated early-divergent angiosperm, *Monoon laui* (Annonaceae), which has flowers that produce exudates on both the stigmas and inner petals, with fungi that develop on the inner petals subsequently consumed by insect larvae. The identities of the pollinators and larvae, as well as the fungal communities borne on the pollinators and petals, indicate that the pollinators disperse fungi while ovipositing on the petals. The nutritional value of the two exudates reveals that the stigmatic exudate is sugar-rich, whereas the inner petal exudate has a greater amino acid content. Transcriptomic and proteomic comparisons between the two organs and their exudates corroborate the nutritional profiles, with a stronger immune response on stigmas.

Conclusions Both stigmatic exudate and petal nectar of *Monoon laui* function as a pollinator reward, while petals with their nectar are moreover critical in the fungus-mediated plant-pollinator mutualism, as they are likely to be closely adapted to the requirements of the pollinators by providing them with brood sites and larval food, thereby increasing their population size during the flowering season and promoting pollination success.

Keywords Stigmatic exudate, Petal nectar, Plant-pollinator mutualism, Fungi, Sugar, Amino acid, Proteome, Transcriptome, Plant immune response, Annonaceae

Background

The diversification of flowering plants is heavily influenced by their interaction with animal pollinators, often achieved via co-evolution and specialisation within ecozones [1, 2]. A large proportion of plant-pollinator interactions are mutualistic, in which the floral visitors assist in dispersing pollen and the flowers provide nutritional and energy rewards in return. Floral nectar functions as an important pollinator reward with complex composition [3], primarily consisting of water, sugars, inorganic ions, amino acids (AAs), proteins, lipids, phenols,

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reactive oxygen species (ROS), reactive nitrogen species (RNS), and other constituents in relatively low concentrations [4, 5]. Secreted by nectaries, floral nectar can occur on various organs, including the receptacle, perianth, androecium, and gynoecium [4]. Sugars, which are the main components of floral nectar, are dominated by sucrose, glucose, and fructose. As the major constituents in floral nectar, sugar concentration has a significant impact on the osmolarity of the solution and has been suggested to affect the evaporative rate and pollen germination success [4, 6]. Free AAs are also important components that are ubiquitous to floral nectar, although the concentrations are tiny compared to sugars. Free AAs in floral nectar can be of nutritional value to the pollinators, and the composition of AAs has been demonstrated to affect taste perception by insects through the stimulation or inhibition of chemoreceptor cells [7]. Nutritious floral nectar also creates a favourable medium for fungal growth on plant tissues, however [8]. Floral nectar has also been shown to contain secondary metabolites and proteins that defend against pathogens, including pathogenesis-related (PR) proteins from different families [9].

In some specialised cases, the plant-pollinator mutualism can be reinforced when the flowers provide brood sites for the pollinators, with the insect pollinators copulating and ovipositing on floral organs where the larvae subsequently develop. Sakai [10] summarised three types of brood-site pollination mutualism according to differences in oviposition sites and food sources. The first two types are associated with ovules and pollen as larval food, while the third is correlated with fungi and decaying flowers or inflorescences that abscise after anthesis. The third type mutualism generally occurs in tropical species, for which beetles and flies are the primary pollinators [10]. Studies have shown that flowers often host a diverse fungal community, with yeasts the most commonly reported [3, 8], although moulds are also occasionally observed [11]. Moist weather conditions in tropical forests can facilitate fungal development, thereby generating abundant food source for the beetles and flies. The presence of extra-stigmatic nectars and other tissues that enable the development of fungi and insect larvae during and after anthesis might be necessary to achieve the third type of mutualism, which can also be termed as fungus-mediated plant-pollinator mutualism. This type of brood-site mutualism also benefits from the rather short life cycle of the insects, which enables several generations to breed within the flowering seasons of their host plants. The increased number of pollinators, as a result, can greatly enhance the pollination and reproductive success of plants.

Monoon laui (Merr.) B.Xue & R.M.K.Saunders (Annonaceae) is a tree species that is native to lowland monsoonal forests in Hainan, growing up to 25 m in height. As with other members of the Annonaceae, *M. laui* has flowers that exhibit two whorls of petals, numerous densely packed stamens, and multiple free carpels, along with protogyny (in which the female phase precedes the male phase). The yellowish flowers are likely to exhibit the third type of brood-site pollination mutualism (sensu [10]), as they are commonly visited by beetles and flies, with insect larvae found on abscised petals on the ground, consuming the decomposing petals and fungal hyphae (Fig. 1A–J). This putative fungus-mediated mutualism is probably associated with the production of inner petal exudate (Fig. 1D) in addition to stigmatic exudate during anthesis, considering that the inner petals with exudate exposed to the air serve as suitable sites for the growth of fungi and insect larvae. In flowering plants such as Annonaceae, Winteraceae, and Orchidaceae, stigmatic exudate can provide nutritional rewards to pollinators [6, 12, 13]. This ecological role has also been proposed for petal exudate, acting as an alternative nutritional reward for stigmatic exudate, and also as a defence against microbes [14–16].

Previous studies on a potentially fungus-associated brood-site pollination mutualism have been primarily descriptive [10], such as that of *Artocarpus integer* (Thunb.) Merr. [17], lacking a clear understanding of the associated molecular mechanisms and functional regulation (e.g., nutritional composition of the extra sources of nectars, and plant immune system control of microbial multiplication). Most previous studies involving the determination of nectar composition and proteomes only focused on stigmatic exudate, and very few have comprehensively compared stigmatic exudate with other sources of floral nectars. Proteomic studies on stigmatic exudate, for example, have been conducted in *Zea mays* L. and *Nicotiana tabacum* L. [18], as well as *Lilium longiflorum* Thunb. and *Olea europaea* L. [19], with that of petal nectar conducted in *Liriodendron tulipifera* L. [14] and *Cucurbita maxima* Wall. [20], which only categorised different proteins by functions and seldom analysed the proteins in the molecular pathways. Our investigation of inner petal nectar in *M. laui* therefore aims to address these research gaps by assessing the transcriptional (RNA of nectaries) and post-transcriptional (protein of nectar) machineries underpinning the regulation of fungus-mediated plant-pollinator mutualistic interactions.

This study initially recorded the floral phenology of *M. laui* and the behaviours of floral visitors through field observations, with the identification of associated insects and fungi. We hypothesise that the larvae are conspecific with the adult floral visitors that also function as vectors for the dispersal of fungi between flowers. We
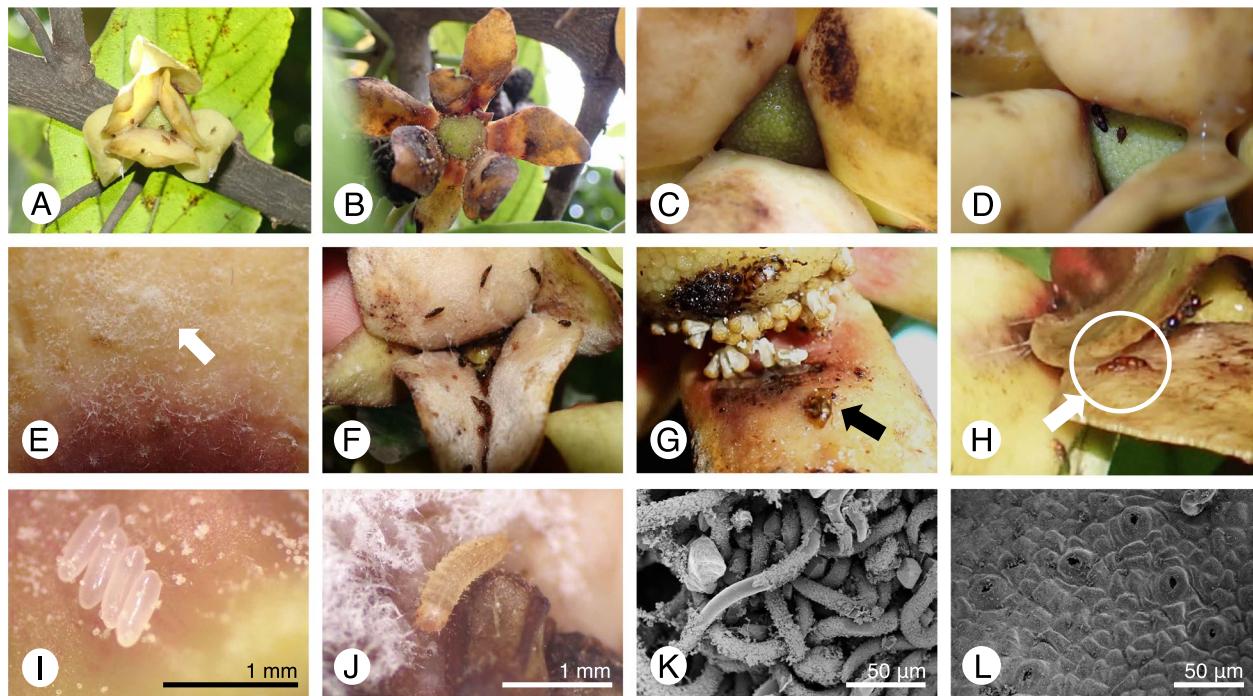
Fig. 1 Flowers, floral exudates, fungi, floral visitors, larvae, and nectaries of *Monoon laui*. **A** Pistillate-phase flower with floral visitors. **B** Staminate-phase flower. **C** Stigmas secreting exudate during pistillate phase. **D** Petals secreting exudate during pistillate phase. **E** Fungi on the inner petal. **F** Beetles resting at the base of the floral chamber, as well as between gaps among the inner petals of a pistillate-phase flower. **G** *Epuraea ocularis*, visiting a staminate-phase flower with dehisced pollen. **H** Copulation of two *E. ocularis* on the inner petals of a pistillate-phase flower. **I** Eggs on the adaxial surface of an inner petal. **J** Larvae of *E. ocularis* eating fungi on the inner petals. **K** Scanning electron micrographs of the trichome nectaries on the stigmas. **L**, Scanning electron micrographs of the mesophyll nectaries on the adaxial surface of the inner petals (Photos: A, F, H–J: B. Xue; B–E, G, K, L: Y. Chen)

additionally investigate the functions of both inner petal and stigmatic exudates by comparing their chemical compositions, including sugars, AAs, as well as their transcriptomic and proteomic profiles. Through both direct and circumstantial evidence, we infer that the inner petal exudate of *M. laui* likely functions in maintaining pollinator populations during the flowering season, thereby enhancing the reproductive success of *M. laui*.

Results

Floral phenology

Flowers of *Monoon laui* are bisexual and protogynous. The pistillate phase usually begins in the morning, as indicated by copious stigmatic exudate that forms a continuous layer over the stigmas (Fig. 1C). During the pistillate phase, the three inner petals loosely converge to form a floral chamber, providing a shaded and protected area for small floral visitors (Fig. 1A). There is an aperture at the apex of the floral chamber, through which floral visitors can enter and leave the flower unimpeded. Petal exudate forms at the base of the adaxial surface of the inner petals (Fig. 1D), largely coincident with stigmatic exudate

formation; the inner petal exudate is less viscous and dries more quickly than the stigmatic exudate.

The stigmas possess trichome nectaries (Fig. 1K), whereas those on the inner petals are mesophyll nectaries (Fig. 1L). Fungal hyphae were observed during late-pistillate and staminate phases on the inner petals where the nectar formed (Figs. 1E). The staminate phase normally begins around noon on the second day and ends in the evening. Stigmatic exudate usually dries prior to the onset of the staminate phase, but sometimes remains viscous until the end of the anthesis, obscuring the existence of an interim phase. Toward the end of the staminate phase, the petals gradually open further, exposing the pollen from dehisced anthers (Fig. 1B). The staminate phase ends as the stamens and petals abscise. No floral scent was detected during the pistillate phase, although a mild scent of fermenting fruit was emitted during the staminate phase. The flowers open asynchronously, with the overall flowering period of one individual tree usually extending over 1.5–2 months, with the flowering period of different individuals within the same population overlapping.

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Floral visitors and pollinators

Numerous floral visitors were observed on the stigmas and petals when the exudates formed during the pistillate phase (Fig. 1A, D, F). They repeatedly entered and left the floral chamber through the apical aperture and were observed to move between pistillate- and staminate-phase flowers (Fig. 1F, G). Fruit flies commonly rested on the upper part of the petals (Fig. 1A), while beetles typically rested at the base of the floral chambers (Fig. 1F). Visits by beetles and fruit flies continued until stamen and petal abscission, with pollen found on the bodies of beetles. Not only were the floral visitors observed to copulate on the flowers (Fig. 1H), but eggs and larvae were also found on the inner petals (Fig. 1I, J), with larvae only occurring after petal abscission. Beetle and fruit fly larvae have been recorded to consume fungi on the petals (Fig. 1J). Experimental rearing of larvae revealed that beetle larvae developed into adults in around 3 weeks, whereas fruit fly larvae matured over two to three weeks.

Nine morphologically distinct floral visitors were discovered in the flowers of *Monoon laui* (Fig. 2A): eight were beetles (six Nitidulidae and two Staphylinidae), while the remaining morphospecies was a fruit fly (Drosophilidae). Three morphologically distinct larvae were found on the abscised petals, two of which were identified as beetles and one as a fruit fly. Adult floral visitors and larvae were further identified to species level using DNA barcoding. As indicated by the UPGMA dendrogram constructed using COI and 28S sequences, each larval species—*Epuraea ocularis*, *Carpophilus* cf. *pallipennis*, and *Drosophila* cf. *bipectinata*—is clustered with one of the observed adult floral visitors (Fig. 2A). There is no evidence of sexual reproduction for the other species of floral visitors since their larvae were not discovered on the petals.

In the field surveys of the composition of floral visitors and larvae, 103 floral visitors were retrieved from 18 flowers, among which the top five commonest floral visitors were *Epuraea ocularis* (31.07%), Aleocharinae sp. (26.21%), *Epuraea* sp. (19.42%), Omaliinae sp. (7.77%), and *Drosophila* sp. (6.80%) (Fig. 2B). A total of 48 larvae were collected from 15 flowers, with the most abundant larvae belonging to *Epuraea ocularis* (68.75%), followed by *Drosophila* sp. (29.17%) and *Carpophilus* cf. *pallipennis* (2.08%) (Fig. 2B). Since more than one species was found in the genus of *Drosophila* according to molecular sequencing data, and since it was difficult to differentiate species in this genus based solely on morphology, the "*Drosophila* sp." here refers to any species in the genus. *Epuraea ocularis* was the most abundant species among the floral visitors and larvae.

Comparison of fungal communities

$78,766 \pm 869$ (mean ± SE) clean ITS reads were generated from each fungal community sample (average length 245 ± 3 bp; Additional file 1: Table S1). Rarefaction analysis indicated sufficient sequencing coverage (Additional file 1: Fig. S1A). The saturated richness of fungal OTUs varied between the two groups, with around 70–80 on petals and 20–30 on insect bodies (Additional file 1: Fig. S1A). Analysis of similarities (Anosim) based on the Binary-Jaccard (R = 1, $p = 0.012$) distances indicated statistically greater differences between than within groups (Additional file 1: Fig. S1B).

The majority of fungal OTUs from insect bodies were also discovered on petals. Around 51% of fungal OTUs on the petals were present on the bodies of floral visitors (Fig. 3A). Fifty-one and 14 fungal OTUs that were common among the replicates in each group were detected on the petals and insect bodies separately (Fig. 3B, C). The 20 most abundant fungal OTUs detected on inner petals and floral visitor bodies are listed in Additional file 1: Table S2. The diversity of the fungal community on petals was higher than that of the insect bodies at all six taxonomic ranks. The dominant phylum in both groups was Ascomycota, with a small proportion of Basidiomycota only present on petals (Fig. 3D). *Kurtzmaniella quercitrusa* was the most abundant species identified in both groups, which accounted for 22.24% of the fungal community on petals and 47.24% of that on the insect bodies (Fig. 3D, Additional file 1: Table S2). The major discrepancy between the two groups was the presence of OTUs in the genera *Cladosporium* and *Penicillium* on petals, contributing 28.83% and 13.15% of the fungal community, respectively (Fig. 3D, Additional file 1: Table S2).

Transcriptomes of stigmas and inner petal nectaries

A total of 132,639,943 paired-end clean reads and 39.67 Gigabyte (Gb) of clean data (Q30 > 93.38%) were generated in transcriptome sequencing of the six samples (Additional file 1: Table S3). Stigmas showed a greater number of unique transcripts as well as more highly expressed shared transcripts compared to inner petal nectaries (Additional file 1: Fig. S2A, D). Greater variations in the transcript expression patterns were found between than within the two organs, as revealed by the principal component analysis (PCA) (Additional file 1: Fig. S2B) and pairwise Pearson's correlation coefficient (PCC) heatmap (Additional file 1: Fig. S2C). Fifty-three thousand two hundred eighty-eight unigenes were obtained from Trinity assembly, the N50 length of which was 1792. Totally 35,113 unigenes were functionally annotated. The number of differentially expressed genes (DEGs) discovered in the comparison between stigmatic and inner petal nectaries was 7020, consisting of 3184
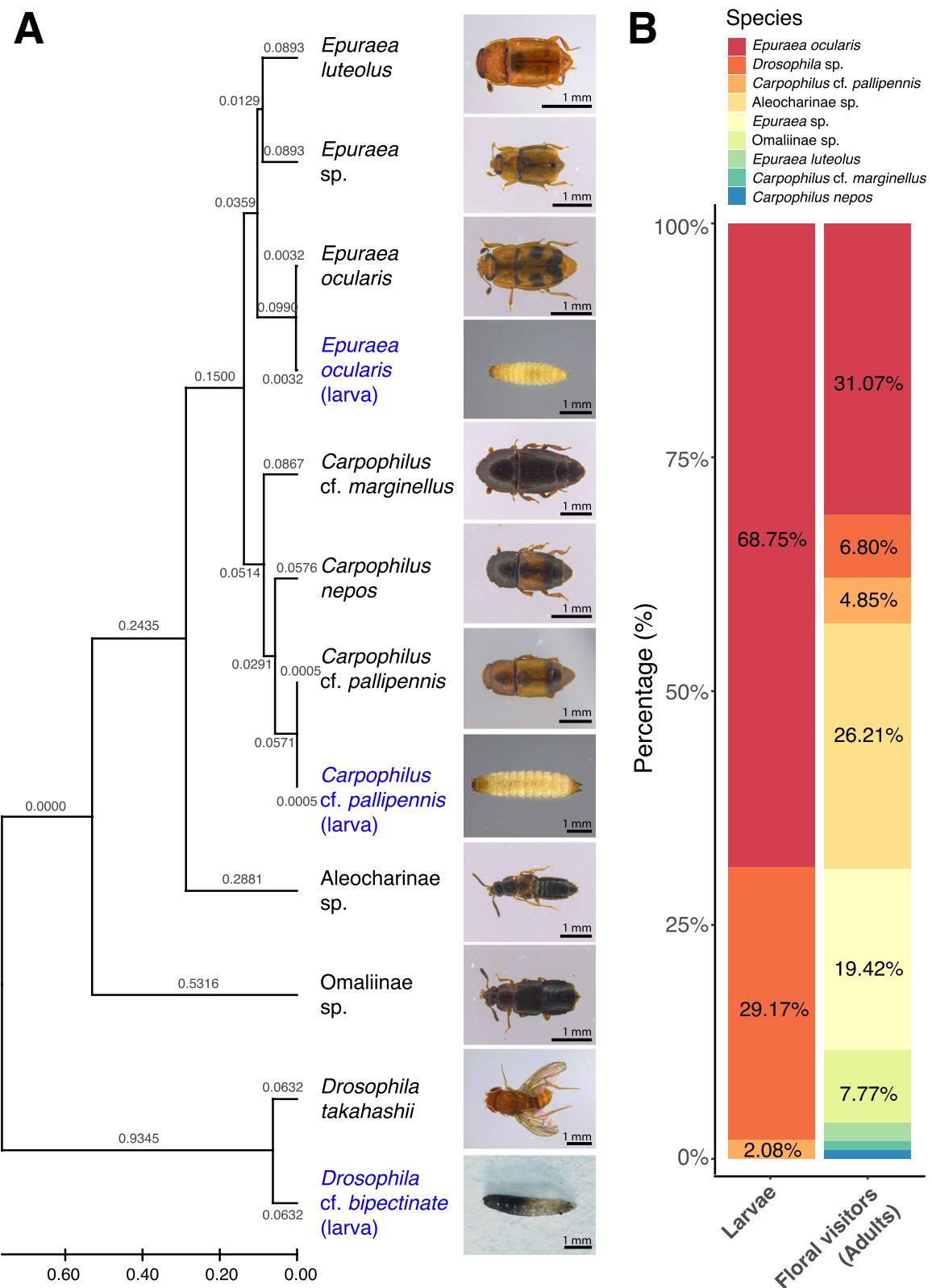
Fig. 2 Community composition of the floral visitors and larvae retrieved from flowers of *Monoon laui*. **A** UPGMA dendrogram of COI and 28S sequences of the adult floral visitors and larvae, showing the values of branch lengths. Adult floral visitors are shown in black font and larvae in blue, together with their morphologies. **B** Relative proportion of adult floral visitors and larvae retrieved from the flowers. Abbreviation: UPGMA = Unweighted Pair Group Method with Arithmetic mean
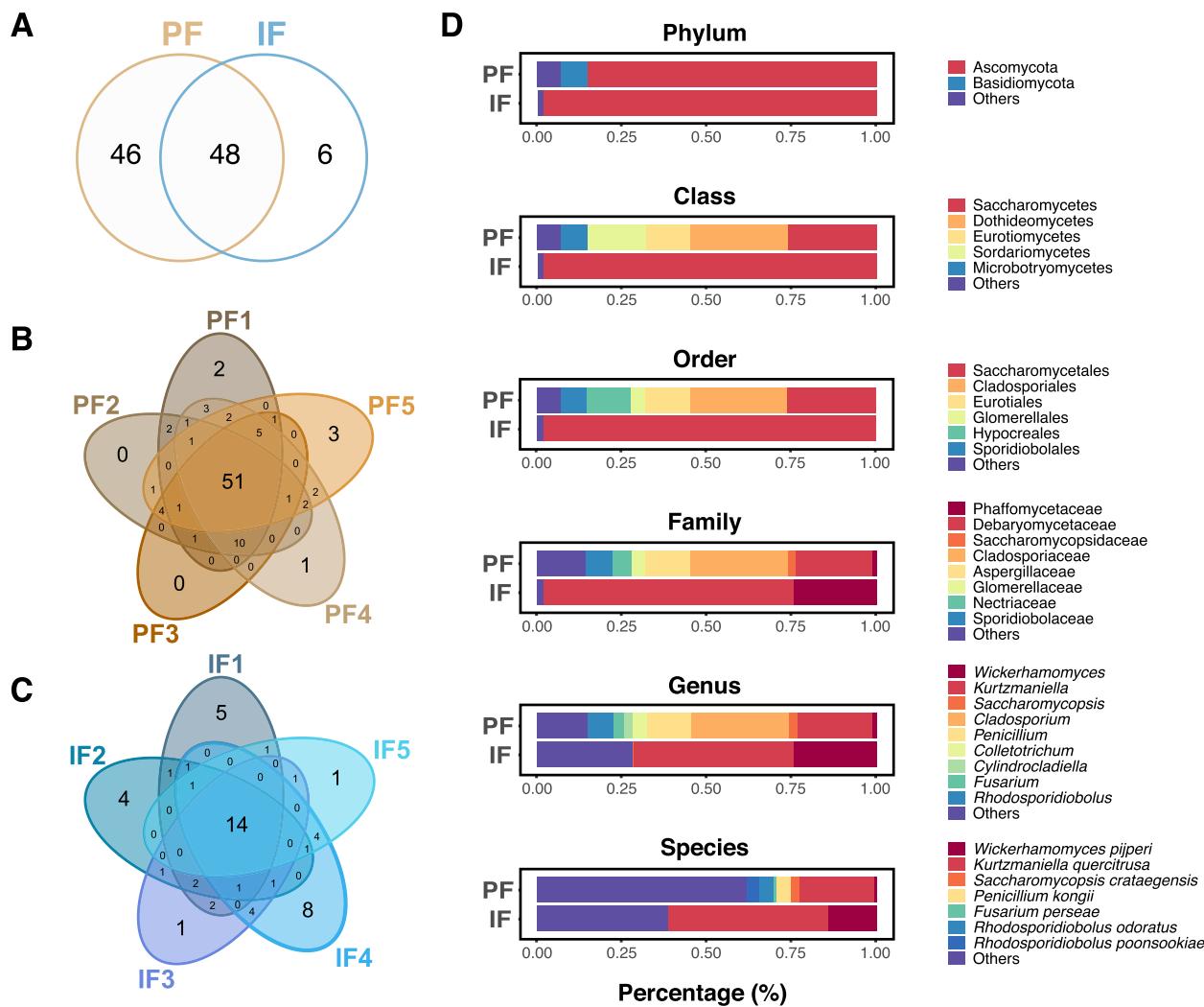
Fig. 3 Fungal community composition on the floral visitor bodies and the inner petals of *Monoon laui*. **A** Venn graph showing the number of common and exclusive OTUs on petals and insect bodies. **B** Venn graph showing the comparison among the five biological replicates of the fungal community on petals. **C** Venn graph showing the comparison among the five biological replicates of the fungal community on insect bodies. **D** Stacked bar charts revealing the composition of fungal communities at different taxonomic levels: phylum, class, order, family, genus, and species. Abbreviations: PF = samples collected from inner petals; IF = samples collected from insect bodies

upregulated and 3836 downregulated DEGs in inner petal nectaries compared to stigmas (Additional file 1: Fig. S3A, B). Several pathways of interest in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database contained more upregulated DEGs than downregulated ones in inner petal nectaries compared to stigmas, viz. carbon metabolism (ID: ko01200) and biosynthesis of AAs (ID: ko01230) (Additional file 1: Fig. S2E). In contrast, a few pathways of interest consisted of more downregulated DEGs in inner petal nectaries, including plant hormone signal transduction (ID: ko04075) and plant-pathogen interaction (ID: ko04626) (Additional file 1: Fig. S2E). The result of the KEGG enrichment analysis of the DEGs between stigmas and inner petal nectaries based on hypergeometric tests showed that the plant hormone signal transduction (ID: ko04075, 53 DEGs, $q < 0.005$) and the starch and sucrose metabolism (ID: ko00500, 74 DEGs, $q < 0.005$) were included in the five most enriched pathways (Additional file 1: Fig. S2F).

Proteomes of stigmatic and inner petal exudates

A total of 3121 proteins were found in the concatenated floral exudates. The two exudates shared 1669 proteins in common, with 1008 exclusively existing in the stigmatic exudate and 444 solely in inner petal exudate (Additional file 1: Fig. S4A), demonstrating that proteins were

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more abundant in stigmatic exudate than in inner petal exudate. PCA and PCC heatmap showed that the three replicates in each organ were very similar, with greater difference between the two organs (Additional file 1: Fig. S4B, C). A total of 849 differentially expressed proteins (DEPs) were identified, among which 215 were upregulated and 634 downregulated in inner petal exudate compared to stigmatic exudate (Additional file 1: Fig. S3C, D). The number of downregulated proteins was higher than those that were upregulated in inner petal exudate when compared to stigmatic exudate (Additional file 1: Fig. S4D). The number of DEPs annotated in the KEGG database was 439, among which ribosome (ID: ko03010), biosynthesis of AAs (ID: ko01230) and carbon metabolism (ID: ko01200) were the three most frequently annotated pathways (Additional file 1: Fig. S4E). DEPs that were categorised into broad classifications of cellular processes, genetic information processing and organismal systems were generally downregulated in inner petal exudate compared to stigmatic exudate. In particular, DEPs that were classified under plant-pathogen interaction (ID: ko04626) were all downregulated in inner petal exudate, whereas DEPs were upregulated in many pathways under the category of metabolism. Many pathways that are associated with AA metabolism contained more upregulated than downregulated proteins in inner petal exudate, such as the biosynthesis of AAs (ID: ko01230), alanine, aspartate and glutamate metabolism (ID: ko00250), arginine and proline metabolism (ID: ko00330), and arginine biosynthesis (ID: ko00220). The KEGG enrichment analysis on the DEPs showed that the proteasome (ID: ko03050, 17 DEPs, $q < 0.05$), phagosome (ID: ko04145, 21 DEPs), and protein processing in endoplasmic reticulum (ID: ko04141, 39 DEPs) were the three most enriched pathways (Additional file 1: Fig. S4F), with the proteins in which mostly downregulated in inner petal exudate (Additional file 1: Fig. S4E).

Sugar composition and associated pathways

The concentrations and the corresponding proportions of fructose, glucose, and sucrose in stigmatic and inner petal exudates are shown in Fig. 4A, B and Additional file 1: Table S4. The glucose concentration and the overall sugar concentration were statistically significantly higher ($p < 0.05$) in stigmatic exudate relative to inner petal exudate (Fig. 4A). The variances of the concentrations of total sugar ($F_{2,3} = 165.72$, $p < 0.005$), fructose ($F_{2,3} = 937.40$, $p < 0.0005$), glucose ($F_{2,3} = 181.00$, $p < 0.005$), and sucrose ($F_{2,3} = 29.50$, $p < 0.05$) were significantly higher in inner petal exudate than that in stigmatic exudate (Additional file 1: Table S4). Fructose and glucose are both common hexose sugars in floral nectar. The sucrose/hexose ratio

in stigmatic exudate was 0.4823, while that in inner petal exudate was 0.6803 (Fig. 4B).

The regulation and expression levels of transcripts and proteins that were closely associated with glucose concentration in the KEGG pathway of starch and sucrose metabolism are visualised in Fig. 4C. Transcripts and proteins that can produce and increase the concentration of glucose, including trehalase (EC: 3.2.1.28), glucan endo-1,3-beta-glucosidase (EC: 3.2.1.39), and beta-glucosidase (EC: 3.2.1.21), were generally downregulated in inner petal nectaries or exudate, whereas beta-fructofuranosidases (EC: 3.2.1.26) were upregulated in both transcriptomic and proteomic levels on petals. 4-alpha-glucanotransferase DPE2 (EC: 2.4.1.25) was upregulated at the transcriptomic level, but downregulated at the proteomic level. Hexokinases (EC: 2.7.1.1) that can decompose D-glucose were moreover upregulated in inner petal nectaries and exudate.

Amino acid composition and associated pathways

Stigmatic and inner petal exudates contained nine essential and 26 non-essential AAs. The AA profiles were very diverse in both stigmatic and inner petal exudates, but more abundant in the latter (Additional file 1: Table S5). Figure 5A shows the concentration of the 10 most abundant AAs in the secretions in descending order: the most abundant was arginine, followed by glutamic acid and hydroxyproline. Arginine accounted for 22.44 and 49.97% of the total AAs in stigmatic exudate and inner petal nectar, respectively. The concentrations of arginine ($p < 0.05$) and cystine ($p < 0.05$) were significantly higher in inner petal exudate than in stigmatic exudate, while the concentrations of aspartic acid ($p < 0.001$) and proline ($p < 0.05$) were significantly lower in inner petal exudate (Fig. 5A). The higher concentration of arginine largely accounts for the higher total AA content in inner petal exudate. The proportion of arginine was also much higher in inner petal exudate than in stigmatic exudate (Fig. 5B).

Fragments from the pathways of arginine biosynthesis, arginine and proline metabolism, and alanine, aspartate, and glutamate metabolism are connected and collectively illustrated in Fig. 6 to disclose the regulation and expression levels of the associated transcripts and proteins. The expression of the transcripts and proteins related to aspartic acid consumption was more activated on petals than on stigmas. Pathways that provide substrates for the urea cycle, including the reactions catalysed by argininosuccinate synthase (EC: 6.3.4.5), acetylornithine deacetylase (EC: 3.5.1.16), acetylornithine aminotransferase (EC: 2.6.1.11), glutamate N-acetyltransferase (EC: 2.3.1.35), and aminoacylase (EC: 3.5.1.14), were upregulated in inner petal nectaries and exudate, providing greater
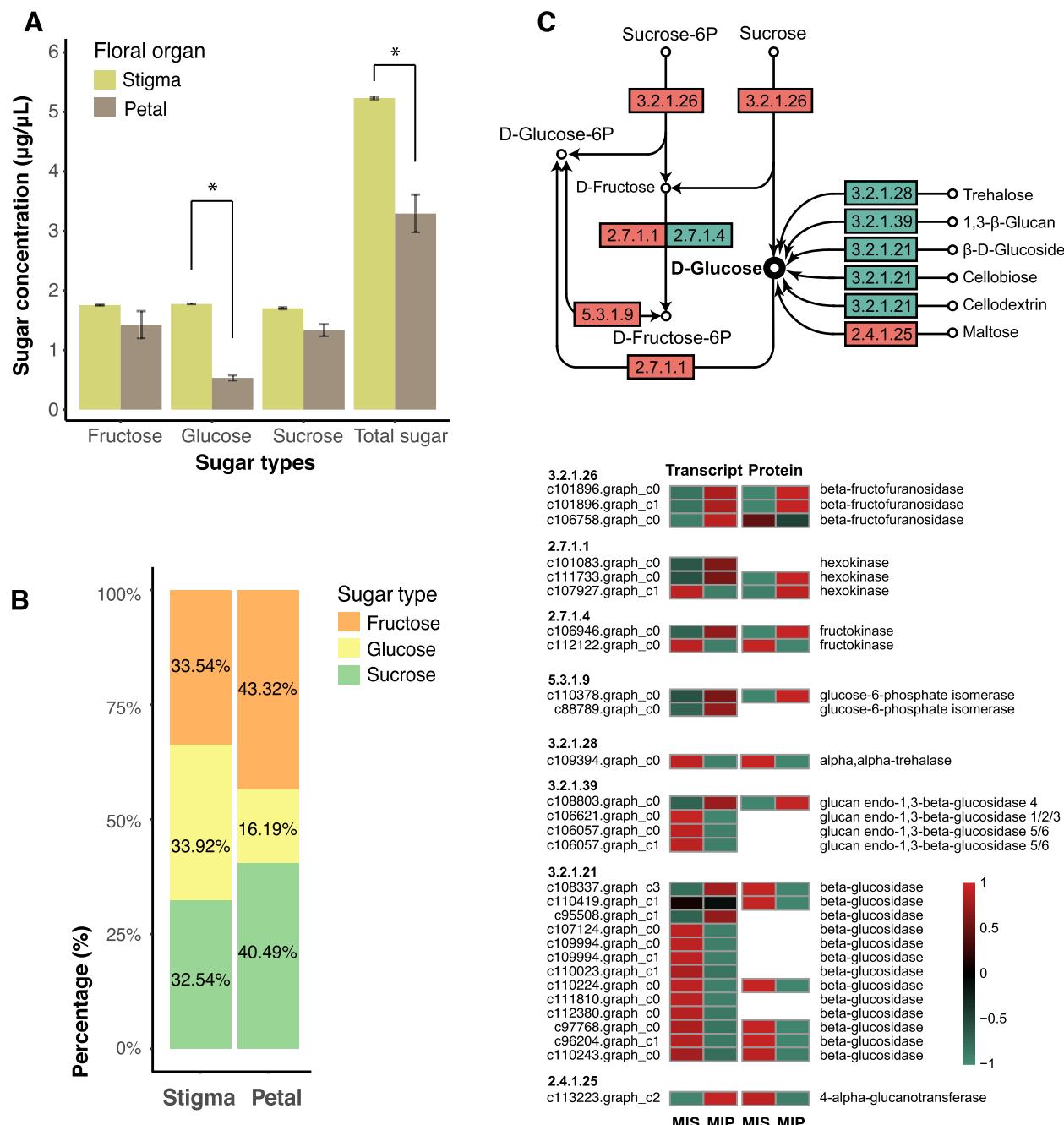
Fig. 4 Concentrations and composition of sugars, as well as the possible regulating pathways on the stigmas and inner petals of *Monoon laui*. **A** Concentrations of fructose, glucose, sucrose, and total sugar in stigmatic and inner petal exudates. **B** Relative proportions of different types of sugars in the stigmatic and inner petal exudates. **C** Schematic illustration of the expression levels of transcripts and proteins in part of the KEGG pathway of starch and sucrose metabolism. Red represents upregulation in inner petal nectaries or exudate, while green represents downregulation. Single asterisk indicates significant differences at $p < 0.05$ based on Student's t test. Abbreviations: MIS = stigmas or stigmatic exudate of *M. laui*; MIP = inner petal nectaries or exudate of *M. laui*

feedstock for arginine synthesis. Expression of argininosuccinate lyase (EC: 4.3.2.1) was suppressed on petals compared to stigmas, while the expression of arginase (EC: 3.5.3.1) was also downregulated, showing less direct synthesis and consumption of arginine. Pyrroline-5-carboxylate reductase (EC: 1.5.1.2), which catalyses the last step of proline biosynthesis, was downregulated at both transcriptomic and proteomic levels on petals.

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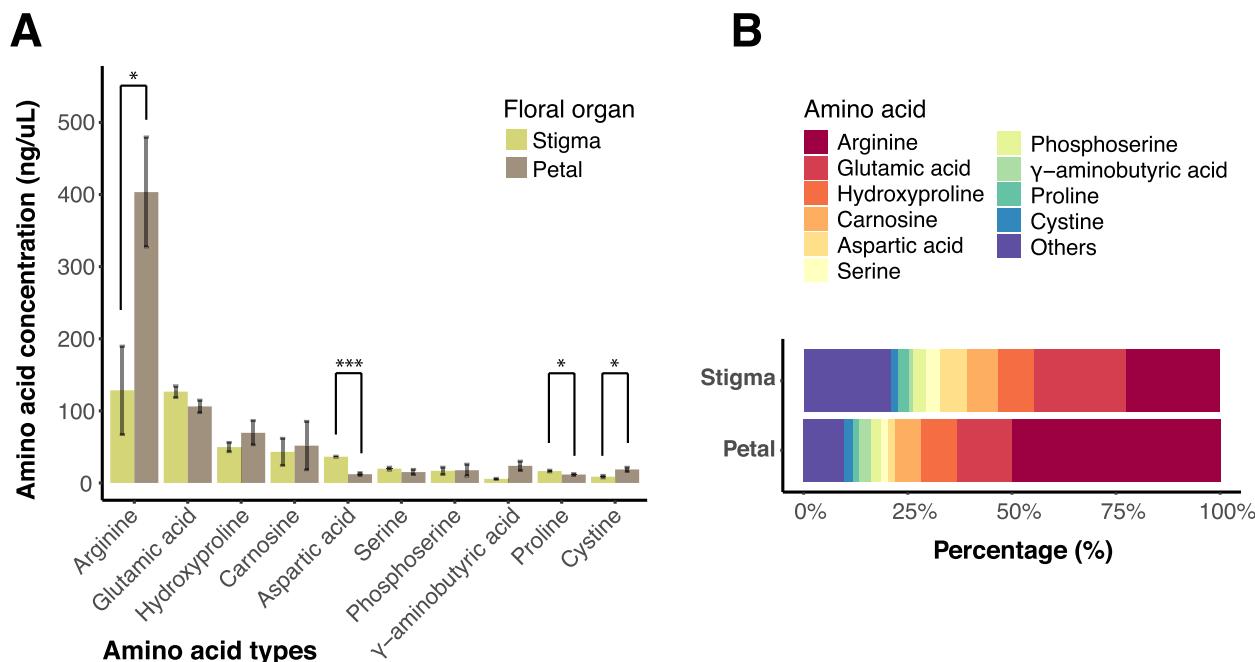
Fig. 5 Concentrations and composition of amino acids (AAs) in stigmatic and inner petal exudates. **A** Concentrations of the 10 most abundant AA in stigmatic and inner petal exudates. **B** Relative proportions of the 10 most abundant AAs in stigmatic and inner petal exudates. Single asterisk indicates significant differences at $p < 0.05$ based on Student's t test; three asterisks represent significant differences at $p < 0.001$

Defence responses and plant-pathogen interactions

Transcripts and proteins related to plant-pathogen interaction, plant hormone signal transduction, and proteasome were selected, with their regulation and expression levels shown in Fig. 7. Several transcripts associated with the recognition of pathogen invasion were downregulated on petals, including CERK1 and CNGCs. Transcripts involved in the PAMP-triggered immunity (PTI) response were generally upregulated on petals at the transcriptomic level, including CDPK, Rboh, CaM/CML, and FLS2, which were downregulated at the proteomic level. Conversely, the expression of transcripts and proteins required for the effector-triggered immunity (ETI) response was largely suppressed on petals, including Pti6, PR1, RPM1, RPS2, SGT1, and HSP90. Transcripts and proteins in the plant hormone signal transduction pathways, such as jasmonic acid (JA) and salicylic acid (SA), were shown to be generally downregulated on petals compared to stigmas. Proteasome was the most significantly enriched pathway at the proteomic level (Additional File 1: Fig. S4F). DEPs in the proteasome were all downregulated on petals compared to stigmas, and the corresponding transcripts at the transcriptomic level mainly showed lower expression on petals as well.

Discussion

Evidence of a putative fungus-mediated plant-pollinator mutualism

Our study provides evidence of an intimate association among the flowers of *Monoon laui*, pollinators, and fungi. The protogynous flowers of *M. laui* are visited by numerous small beetles and fruit flies that consume stigmatic and inner petal exudates, copulate, and oviposit. After anthesis, the petals abscise with eggs that subsequently develop into larvae and consume the fungi generated on the dehydrated inner petal exudate. In approximately two to three weeks, the larvae pupate and emerge as adults, possibly helping promote further pollination of *M. laui* by boosting the pollinator population size. This process is mutualistic, with insects pollinating *M. laui* flowers, and with the petals simultaneously providing a brood site for the insects that rely on fungi and decomposed organic matter as a larval food source. This cycle can therefore be interpreted as a fungus-mediated plant-pollinator mutualism (Fig. 8).

Flowers of *Monoon laui* not only produce copious stigmatic exudate, but also abundant inner petal exudate. Similar phenomena of petal exudate production have been observed in other Annonaceae flowers, including *Pseuduvaria froggattii* (F.Muell.) Jessup [21], *Asimina obovata* (Willd.) Nash, *A. pygmaea* (W.Bartram) Dunal [22], *Xylopia aromatica* (Lam.) Mart. [23], and *Alphonsea*
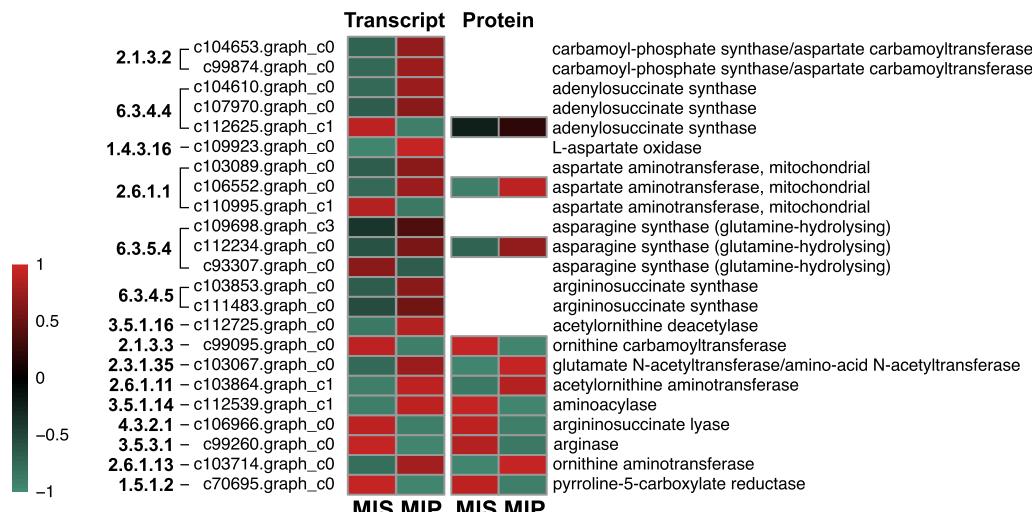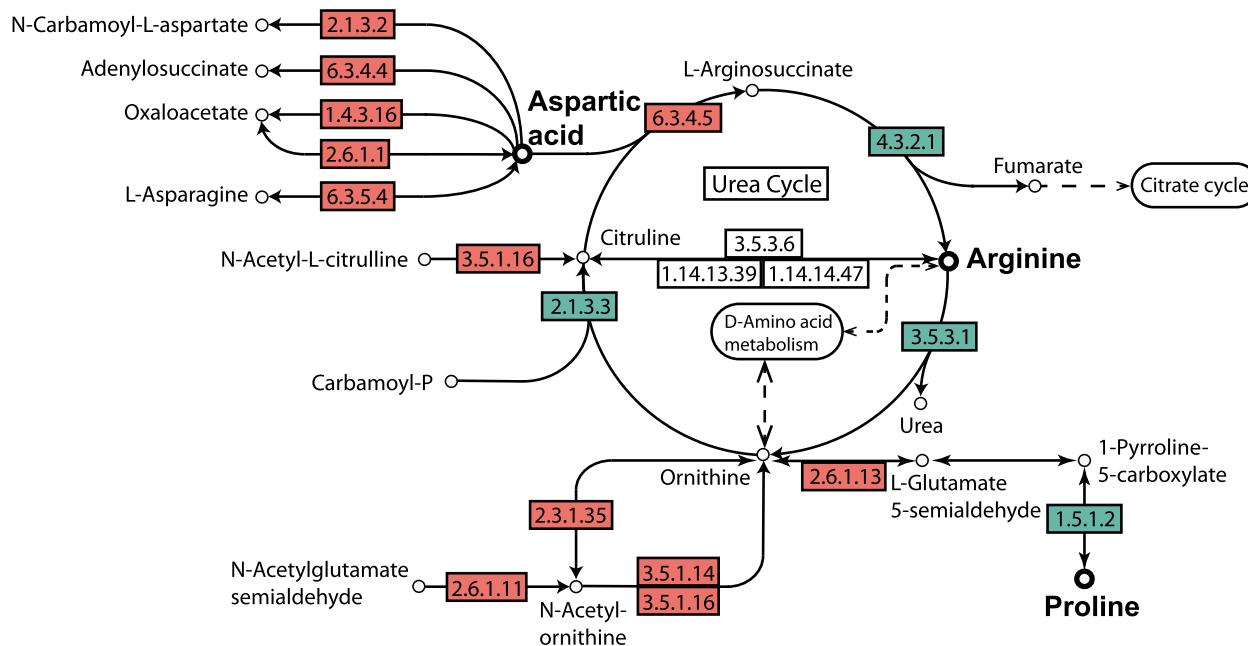
Fig. 6 Schematic illustration of the expression levels of transcripts and proteins in part of the KEGG pathways of arginine biosynthesis, arginine and proline metabolism, and alanine, aspartate, and glutamate metabolism. Red represents upregulation in inner petal nectaries or exudate, while green represents downregulation. Abbreviations: MIS = stigmas or stigmatic exudate of *M. laui*; MIP = inner petal nectaries or exudate of *M. laui*

glandulosa Y.H.Tan & B.Xue [15]. Flowers of *Pseudu-varia froggattii*, for example, possess large dark globose nectary glands on the adaxial surface of the inner petals, with the nectar shown to be consumed by fruit flies, sap beetles, and click beetles [21]. Petal nectar has been demonstrated in many cases to be consumed by floral visitors, and one of its functions is therefore clearly as a food reward for pollinators. The longevity of the inner petal nectar in *M. laui* is shorter than that of stigmatic exudate, however, suggesting that the inner petal exudate might not function as an alternative food reward after drying of the stigmas. The production of inner petal nectar is nevertheless likely to provide enhanced food rewards to pollinators due to an increase in the total volume of the exudates generated in a single flower.

Insect visitors to *M. laui* undertake diverse activities on the inner petals, including foraging, resting, copulation, and oviposition. The most abundant larva found on the petals was *Epuraea ocularis* (Nitidulidae), which was congruent with the most common floral visitors. *Epuraea*
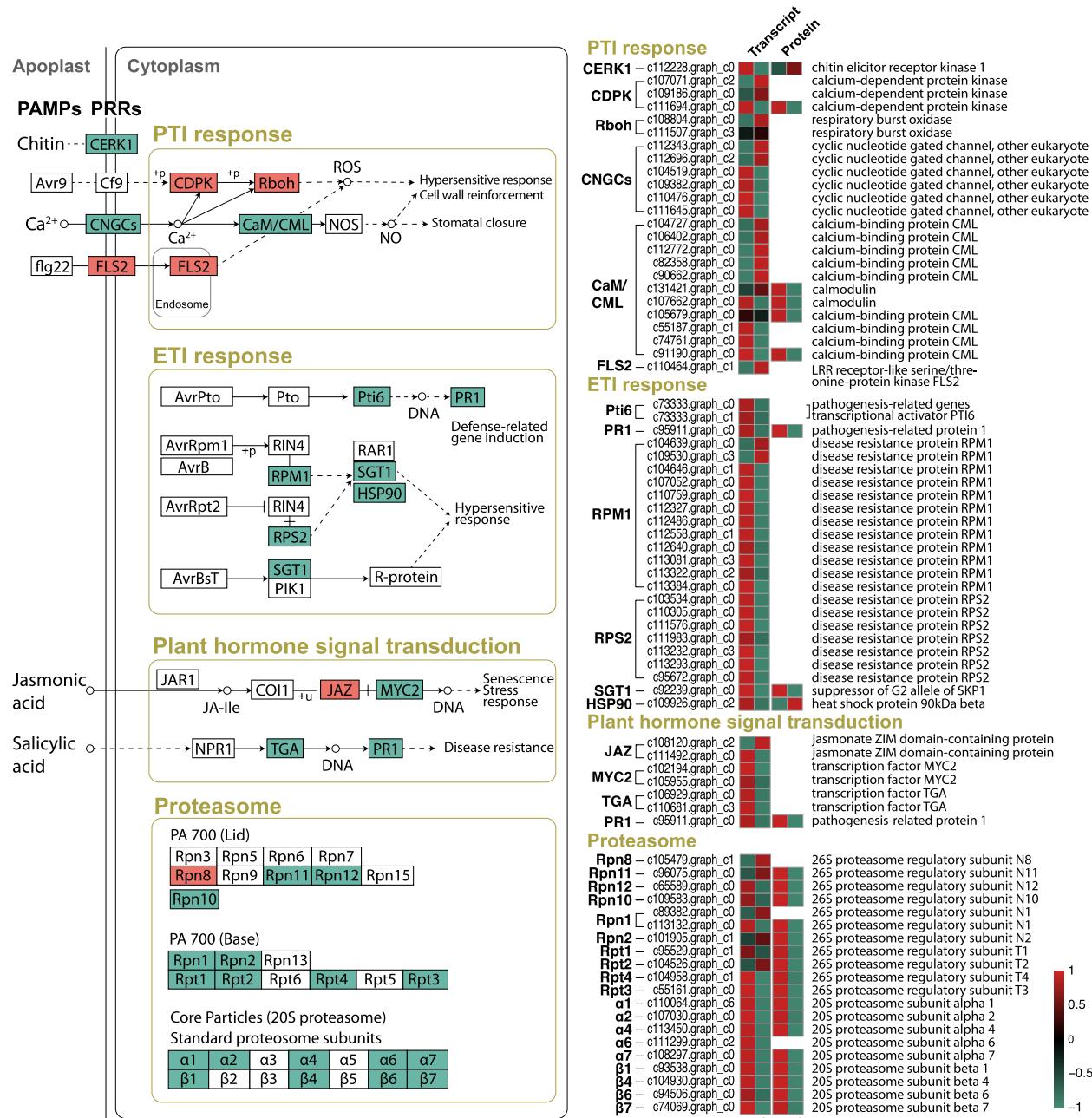
Fig. 7 Schematic illustration of the defence responses on the stigmas and inner petals of *Monoon laui*, showing the expression levels of transcripts and proteins in part of the KEGG pathways of plant-pathogen interaction, plant hormone signal transduction, and proteasome. Red represents upregulation in inner petal nectaries or exudate, while green represents downregulation. Abbreviations: MlS = stigmas or stigmatic exudate of *M. laui*; MlP = inner petal nectaries or exudate of *M. laui*

ocularis can be regarded as an effective pollinator of *M. laui*, as pollen was deposited on their bodies and they were observed to visit both pistillate- and staminate-phase flowers. Widespread over tropical and subtropical areas, *E. ocularis* feeds on fermenting sap and oviposits on flowers and fruits, the larvae of which subsequently

develop on the decaying plant tissues [24]. In our study, *E. ocularis* larvae were observed feeding on fungal hyphae that had multiplied on the inner petals of *M. laui*, as well as decomposed organic matter. *Epuraea ocularis* larvae typically leave the plant tissues and pupate on ventilated soil [24]; abscission of petals of *M. laui* onto the
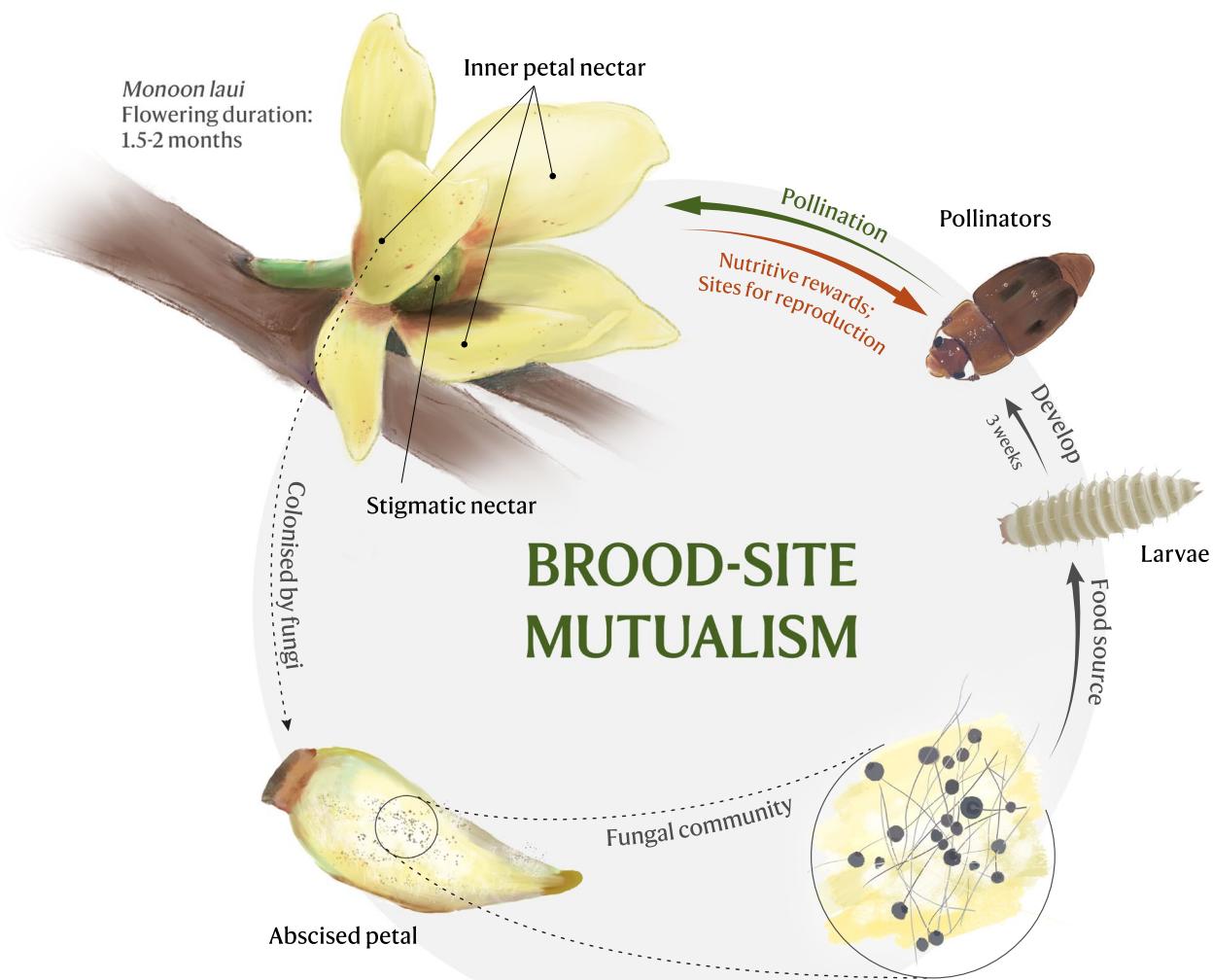
Fig. 8 Diagram illustrating the fungus-mediated plant-pollinator mutualism in *Monoon laui*, with the petals providing brood sites and the fungal hyphae on which providing larval food for the pollinators

soil therefore favours pupation. Development of *E. ocularis* from eggs to adults is reported to be 17–24 days [24], which is consistent with the results of our rearing experiments. Since larval development is shorter than the flowering season of *M. laui*, the newly pupated generations are able to pollinate other flowers within the same plant population.

Insect copulation and oviposition on petals have been reported from many beetle- and fly-pollinated plants, indicating that the petals are potential brood sites. Beetle-associated examples can be found in the Annonaceae, such as *Dasymaschalon trichophorum* Merr., *Friesodielsia borneensis* (Miq.) Steenis, *Goniothalamus tapisoides* Mat-Salleh (X. Guo, J. Y. Y. Lau & R. M. K. Saunders, unpublished data; [16]), and *Meiogyne heteropetala*

(F.Muell.) D.C.Thomas, Chaowasku & R.M.K.Saunders [25]. This phenomenon has also been recorded in various fly-pollinated species, such as *Artocarpus integer* (Moraceae; [17]), *Aristolochia maxima* Jacq., and *A. inflata* Kunth (Aristolochiaceae; [26]). In our study, *Drosophila* fruit flies (Drosophilidae) also use the petals of *M. laui* as brood sites. More than one species of fruit fly is likely to visit *M. laui* flowers, with at least two morphospecies identified among floral visitors and larvae. Although there is no evidence for pollen deposition on fruit flies, they might nevertheless serve as effective pollinators of *M. laui* as they visit both pistillate- and staminate-phase flowers and are very common.

Beetle and fruit fly larvae were significantly observed to eat fungal hyphae growing on the adaxial surface of

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the *M. laui* petals after dehydration of the exudate. Our comparisons of fungal communities indicate that floral visitors are likely to have acted as vectors for dispersing fungi between flowers, since approximately 89% of the fungi OTUs they carried are components of the fungal community on petals, accounting for 51% of fungi on petals. Among the fungal OTUs shared between the two communities, the most common species was *Kurtzmaniella quercitrusa*, a budding yeast that has been isolated from flowers, fruits, sap beetles, and insect frass in previous studies (as "*Candida quercitrusa*") [27, 28], suggesting that *K. quercitrusa* might be dispersed among flowers by beetles and the larvae can feed on this fungus. In addition to *K. quercitrus*, many saccharomycetes were also common to the two communities. The dispersal of budding yeasts among flowers is largely reliant on insect vectors, since saccharomycete spores are not suited for wind dispersal [29]. Our findings reveal that *M. laui* emits a fungal fermentation-like odour during its staminate stage. It has been shown that the volatile compounds generated by yeasts during the fermentation of plant sugars are highly likely to act as attractants for *Carpophilus* species [30, 31], indicating that the fermenting scent of *M. laui* might also attract floral visitors.

Nearly 49% of the fungal OTUs occurred exclusively on petals, which might therefore have been dispersed by abiotic means. *Cladosporium* was the most abundant genus among these, followed by *Penicillium*, collectively accounting for nearly 42% of the petal fungal community (Additional file 1: Table S2). *Cladosporium* is primarily dispersed by wind or rain-splash, with conidia that can erupt and release numerous spores into the air [32]. Filamentous *Penicillium* fungi also produce airborne spores and are likely to share a similar dispersal strategy. Since the inner petal nectar of *M. laui* is exposed to open air, spores are easily captured by the petal surfaces and develop on them. Fungi in these two genera have also been reported from other floral nectars [33]. Given the aerial dispersal of mould spores, *Cladosporium* and *Penicillium* are rarely found on floral visitors, as these fungi primarily rely on abiotic dispersal mechanisms rather than biotic vectors for propagation.

Sugar composition and associated pathways

Our evaluation of sugar content reveals that the concentration of glucose and total sugars were significantly higher in stigmatic exudate than in inner petal nectar. The evaporation of hexose (glucose and/or fructose) solutions is slower than that of sucrose solutions of equivalent mass concentration due to higher osmolality in the former [4]. A larger proportion of hexose and a higher concentration of total sugar in stigmatic exudate could therefore potentially lead to a higher osmolality and a

longer retention time than inner petal nectar, which is consistent with our field observations. It was noted that the fluctuation of the concentration of all sugar types was significantly higher in inner petal nectar than in stigmatic exudate, which might also be associated with sugar concentrations. Since the osmolality of inner petal nectar was generally lower, its evaporation rate might be more easily affected by ambient humidity. The greater longevity of stigmatic exudate is beneficial since extending stigmatic receptivity can potentially increase opportunities for fertilisation.

A higher total sugar concentration also implies an enhanced energy and nutritive value of stigmatic exudate compared to inner petal nectar. Glucose is a monosaccharide that serves as a direct substrate for a series of energy-producing metabolic processes in insect bodies, including glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation. Energy is stored as ATP, which can possibly support diverse insect activities such as flight, copulation, and oviposition. Energy production from fructose and sucrose is less direct as both sugars need to be converted to glucose before glycolysis. Moreover, glucose is easily digested by insects since its structure is relatively simple. Exudates with higher concentration of glucose might therefore possess greater nutritional value. Assuming that foraging floral visitors are initially retained by the nectar on the inner petals, they are then possibly guided deeper into the flower to consume the more sugar-rich stigmatic exudate, promoting pollen deposition on the stigmas.

The primary source of sugars in floral nectar comes from the phloem sap and the parenchyma that can photosynthesise or store starch [34]. The main sugar in phloem sap is sucrose, the hydrolysis of which produces equivalent proportions of glucose and fructose molecules, and therefore an unequal proportion of the two hexose sugars might be influenced by other metabolisms related to carbohydrates [3]. The relative proportion of sucrose, glucose, and fructose can be modified by enzymes in the nectaries and nectar [4]. The synthesis of glucose on petals was suppressed while the utilisation of glucose was facilitated at the transcriptomic and proteomic levels: the proportion and regulation of glucose at the molecular level were therefore consistent. Beta-fructofuranosidases (EC: 3.2.1.26), which are invertases that can hydrolyse the non-reducing terminal beta-fructofuranoside residues [35], were specifically upregulated in inner petal nectaries and nectar. Trehalase (EC: 3.2.1.28), glucan endo-1,3-beta-glucosidase (EC: 3.2.1.39), and beta-glucosidase (EC: 3.2.1.21) can hydrolyse trehalose, 1,3-beta-D-glucans, and the non-reducing terminal beta-D-glucosyl residues, respectively, to produce D-glucose [36–38], the regulation of which were mostly suppressed

in inner petal nectaries and nectar. Free glucose can also be released from the glucosyl-transferring reactions catalysed by 4-alpha-glucanotransferase (EC: 2.4.1.25) [39], the transcript and protein of which were regulated in opposite directions, with the protein being downregulated in inner petal nectar. In addition to the suppressed production of D-glucose on petals, its consumption rose on petals, which can be indicated from the upregulation of the transcripts and proteins of hexokinase (EC: 2.7.1.1), an enzyme that can phosphorylate D-glucose for the down-stream steps of glycolysis or the pentose phosphate pathway [40].

Amino acid composition and associated pathways

Arginine was the most copious AA in both stigmatic and inner petal exudates, with concentrations significantly higher in the latter ($p < 0.05$). Different AAs can stimulate different chemoreceptors on the insect labella and cause variant responses, with arginine classified as a class II AA with high concentrations inhibiting chemoreceptor cells [7]. As an essential AA [41], arginine provides crucial nutritional and energetic values to both adult and larval insects. For example, L-arginine can be reversibly catalysed by arginine kinase and engaged in the phosphagen system, supporting rapid and intense energy-demanding tissues, such as the flight muscles [42]. The arginine kinase phosphagen system can be critical to the development of insect larvae, as the activity of arginine kinase increases notably during the prepupal and eclosion periods in *Drosophila melanogaster* [43]. Arginine is moreover a precursor to the synthesis of proline, a non-essential AA that can be synthesised by insects themselves. Arginine can be catalysed by arginase, and further by ornithine aminotransferase and pyrroline-5-carboxylate reductase, to yield proline in insect bodies [44]. Proline can be utilised by insects as fuel for the initiation of flight, because its rapid catabolism releases a large amount of ATP, supporting the burst of muscular activity during take-off and providing ATP to the primary steps of glucose metabolism for long-distance flight [45].

The concentration of arginine in plants is directly affected by the activities of argininosuccinate lyase (EC: 4.3.2.1) and arginase (EC: 3.5.3.1), which have opposing influence on the concentration: argininosuccinate lyase catabolises argininosuccinate to generate arginine and fumarate, whereas arginase hydrolyses arginine to form ornithine and urea [46]. The regulation of both enzymes was downregulated on petals, suggesting that the formation and consumption of arginine was lower in inner petal nectaries and nectar. The biosynthesis and metabolism of arginine are closely correlated with other AAs, such as aspartic acid, glutamate, citrulline, ornithine, and proline, and hence regulation of the metabolism of

the associated AAs was also compared between the two floral organs. The concentration of aspartic acid was significantly higher in stigmatic exudate than in inner petal nectar ($p < 0.001$). At the transcriptomic and proteomic levels, the enzymes associated with the reversible and irreversible consumption of aspartic acid were upregulated on petals relative to stigmas, possibly accounting for the higher concentration of aspartic acid in stigmatic exudate. Among those upregulated enzymes, argininosuccinate synthase (EC: 6.3.4.5) can participate in a step prior to the synthesis of arginine from aspartic acid and citrulline in the urea cycle [46]. In addition to aspartic acid and citrulline, ornithine is also a precursor of the biosynthesis of arginine in the urea cycle. The enzymes involved in the irreversible formation of ornithine were upregulated in inner petal nectaries and nectar. The overall upregulated biosynthesis of the reactants in the urea cycle (Fig. 6) might accord with an increase in the arginine concentration on petals. The downregulation of pyrroline-5-carboxylate reductase on petals, which catalyses the synthesis of proline in plants, was furthermore consistent with the lower concentration of proline on petals.

Defence responses and plant-pathogen interactions

Due to different longevities and functions of stigmas and petals of *M. laui*, their responses to pathogens might differ at the molecular level. Plant immune systems can react promptly to infections and restrict microbial colonisation in two ways, viz. PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI) and the effector-triggered immunity (ETI) [47]. Our results indicate a higher regulation of respiratory burst in PTI response on petals than on stigmas, although with a generally suppressed regulation of ETI response on petals at the transcriptomic level. PTI response functions when the pattern recognition receptors (PRRs) on the surfaces of plant cells detect the PAMPs, which are conserved structures shared by many microbes such as the chitin on fungi or the flagellin on bacteria [48]. Compared to PTI, ETI response is stimulated by pathogen effectors, which are more specific molecules, causing accelerated and intensified plant responses [47]. Even though PTI and ETI are triggered by variant molecules and react in different intensities, they can lead to many common downstream reactions, such as the stimulation of mitogen-activated protein kinase (MAPK) signalling pathways and hypersensitive response (HR) [48]. In the pathogenic defence of *M. laui*, the expression of the disease resistance proteins RPM1 and RPS2 was suppressed on petals, negatively influencing the downstream HR. The expression of pathogenesis-related protein 1 (PR1) furthermore declined on petals at both transcriptomic

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and proteomic levels. PR1 can exhibit antifungal activities, which might also be associated with the systemic acquired resistance (SAR) response that can expand the immune reaction to a larger scale [49, 50].

Plant defence against pathogens can be regulated by plant hormones, especially salicylic acid (SA) and jasmonic acid (JA), which have been jointly regarded as the backbone of the plant immune signalling network [51]. SA has been considered as a signalling molecule positively associated with resistance against biotrophic pathogens, which can serve as a key regulator in the immune responses of SAR, PTI, and ETI [48, 52, 53]. The SA signalling pathway in the petals of *M. laui* was downregulated compared to stigmas, which was implied by the downregulation of a transcription factor TGA and an SAR marker protein PR1 [54]. Transcripts of the helix-loop-helix transcription factor MYC2 were less activated on petals, which indicated suppressed JA-regulated defence response on petals.

A high plasticity of the proteome is vital for the plant PTI and ETI responses, possibly involving the degradation of proteins by the ubiquitin–proteasome system [55]. Proteasome is the most significantly enriched KEGG pathway at the proteomic level, with all proteins engaged being downregulated on petals compared to stigmas. It has been demonstrated previously that the subunits of the proteasome play important roles in the plant immune response: for instance, the Rpn1 subunit on the 19S regulatory particles has been demonstrated to be positively correlated with resistance against fungal pathogens by affecting the regulation of several regulators, such as PR1 and the resistance (R) proteins, with the latter recognising the pathogen effectors in ETI response [56]. The expression of the subunits $\alpha3$, $\alpha6$, and $\beta1$ can be activated by the fungal elicitor cryptogein, with $\beta1$ playing important roles in the oxidative burst [57]. The ubiquitin–proteasome system has moreover been shown to participate in the degradation of FLS2, NPR1, and many other proteins in PTI response and plant defensive signal transduction [55, 58, 59]. Among the subunits mentioned above, the regulation of Rpn1, $\alpha6$, and $\beta1$ were all downregulated on petals compared to stigmas, indicating stronger protein degradation activities on stigmas, which might be advantageous to the defence against pathogens.

Collectively, the immune responses of stigmas tended to be stronger and more effective compared to petals as shown by the transcriptomic and proteomic pathways. Less active defence of petals might not harm the plants, since the petals will abscise after anthesis. The accumulation of fungi on petals can furthermore provide food source for the development of insect larvae, which is an important premise for a "type three" brood-site pollination mutualism.

Implications, limitations, and future research

Our findings on *Monoon laui* may extend to other systems where plants offer decaying floral tissues as rewards to sustain mutualistic relationships. Examples include *Artocarpus integer* (Thunb.) Merr. [17], *Balanophora tobiracola* Makino [60], *Gastrodia foetida* Koidz. [61], *Arisaema thunbergii* Blume [62], and *Arisaema urashima* H.Hara [63], all of which involve fungal participation in pollination process. While this study highlights the potential ecological significance of fungi in mediating plant-pollinator dynamics, several limitations should be noted. First, it remains unclear whether fungi are essential for maintaining the symbiotic relationship between plants and pollinators. Second, even in the present study, the dietary preferences of insects—whether they primarily consume fungal or plant tissues—have yet to be fully elucidated.

Future studies are needed to address these limitations by employing stable isotope analysis to directly determine insect dietary preferences. Because fungi are heterotrophic, their isotopic signatures exhibit significant enrichments in ^{2}H, ^{13}C, and/or ^{15}N compared to their substrates and coexisting autotrophic plants [64–66]. This makes stable isotope analysis a powerful tool for differentiating between nutritional sources derived from fungi and plants. Furthermore, the transcriptomic and proteomic approaches utilised in this study exhibit broad applicability for investigating plant-pollinator interactions. These methods can be further deployed to uncover changes in plant product chemistry and function, offering deeper insights into the mechanisms underpinning mutualistic relationships across diverse systems.

Conclusions

The present study provides compelling circumstantial evidence for a fungus-mediated plant-pollinator mutualism (Fig. 8) in *Monoon laui*, a common woody species in the evergreen broad-leaf forests in China, supported by associated transcriptomic and proteomic regulation. The protogynous flowers of *M. laui* are used as brood sites by their pollinators, especially the inner petals, the nutritional value of the exudate on which has the potential to support the growth of insect larvae, with exudate nutrition notably affected by the expression of a series of transcripts and proteins involved in sugar and AA metabolism. Apart from air-borne fungi, adult floral visitors also carry many fungi between flowers, which act as a food source for their larvae. The presence of nutrient-rich inner petal nectar provides a favourable environment for fungal growth. The defence response on the plant tissues impacts the survival of fungi, although this is more evident on stigmas than inner petals. It was assumed that the comparatively weak defence on petals might benefit

the mutualism through the preservation and growth of some fungi on the petals. Given that the pollinators and fungi have distinct life histories and habitat preferences, *M. laui* might accommodate their characteristics and achieve this fungus-mediated brood-site mutualism through molecular regulation of the nutritional composition and defence response. In this context, petal nectar not only functions as an alternative nutritional resource to stigmatic exudate for pollinators, but also strengthens the connection between the plant and successive generations of pollinators. This interaction likely enhances the reproductive success and the overall fitness of the plant.

Methods

Floral phenology and nectary structure of *Monoon laui*

A general floral phenological study of *Monoon laui* was conducted in the South China National Botanical Garden in Guangzhou, with 71 flowers monitored during April–June in 2017, 2020, and 2021. Each flower was observed and recorded three times per day to obtain an approximate floral phenology, with photos taken to record their morphology and interactions with pollinators.

Fresh floral materials were collected, fixed in formalin-acetic acid-alcohol (FAA) solution for 24 h and preserved in 70% ethanol. Samples were dehydrated through a graded ethanol series and then critical-point dried. After coated with Au/Pd, the external morphology of the stigmatic and inner petal nectaries was visualised using a Hitachi S-4800 FEG Scanning Electron Microscope (Hitachi, Tokyo, Japan).

Floral visitors and pollinators

Field surveys of the community composition of floral visitors and larvae were conducted from April to May in 2017 and 2021. Petals with insect eggs were collected during the late staminate phase before abscission and the larvae were then reared in the laboratory. The foraging behaviour of larvae was filmed with a Leica EZ4W Stereomicroscope (Wetzlar, Germany). A combination of morphological and molecular data was used to confirm the identity of floral visitors and larvae. Collected insects were sorted into different morphospecies, with the number of individuals of each recorded and their proportion among adults or larvae calculated. The specimens of the floral visitors have been deposited in The University of Hong Kong Herbarium (HKU).

DNA barcoding was employed to confirm the taxonomic identity of floral visitors and larvae. Detailed experimental procedures are provided in Additional File 2 [67, 68]. Partial sequences of mitochondrial cytochrome c oxidase subunit I (COI) and large subunit (28S) ribosomal DNA regions were amplified, sequenced, and BLAST (Basic Local Alignment Search Tool) searched against the National Center for Biotechnology and Information (NCBI) nucleotide and the Barcode of Life Data (BOLD) Systems databases. To reveal the genetic relationship between the floral visitors and larvae, COI and 28S sequences of the same species were assembled and aligned, which were then used to construct a dendrogram in MEGA11 ver. 11.0.10 (www.megasoftware.net) applying the UPGMA (unweighted pair group method with arithmetic mean) algorithm.

Comparison of fungal communities

Fungal communities on the adaxial surface of inner petals and the bodies of floral visitors were sampled at random. Inner petals were collected at the staminate phase prior to abscission and preserved in sterile Petri dishes for 5 days to enable fungal growth without contamination. Floral visitors were collected directly from the flowers and placed into sterile Sabouraud dextrose agar plates for 3 days. The inoculated agar plates were then incubated for ten more days to generate a sufficient volume of fungi for DNA metabarcoding. A total of five fungal community replicates were collected respectively from the inner petals and the agar plates inoculated by floral visitors using sterile swabs. Total genomic DNA was extracted from each sample using a Magnetic Soil and Stool DNA Kit (TIANGEN, Beijing, China) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of the nuclear ribosome was amplified using the primers ITS1F and ITS4. DNA libraries were prepared using purified and quantified PCR products with a SMRTbell Template Prep Kit (PacBio, Menlo Park, USA), which were subsequently sequenced on a PacBio Sequel II system (PacBio, Menlo Park, USA) by Biomarker Technologies (Beijing, China). Further details on the experimental procedures are provided in Additional File 2 [69, 70]. The shared and exclusive fungal operational taxonomic units (OTUs) among samples were assessed, with the reads of different OTUs used to represent their abundance in different fungal communities. Analysis of similarities (Anosim) between and within the two fungal communities based on the Binary-Jaccard distances were performed using the package vegan ver. 2.6–4 in R ver. 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). The difference in the abundance of each genus between the two fungal communities was compared using the Student's *t* test based on the OTU abundance.

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Sample collection for the examination of exudate chemistry, transcriptomes, and proteomes

Stigmatic and inner petal nectaries and exudates were collected from pistillate-phase flowers using sterile razors and micropipettes, respectively. A constant humidity was maintained to avoid climatic variation and the flowers were bagged prior to anthesis to minimise the contamination of free AAs derived from stray pollen [6]. Nectary tissues from five flowers and exudates from 20 flowers of the same organ were combined as a single sample, snap frozen with liquid nitrogen in the field, and then transferred to a −80 °C freezer for longer-term preservation. For exudate chemistry analysis, at least three replicates of exudate in each organ were examined for sugar and AA composition using a HPLC system and an AA analyser, respectively. For the transcriptomic and proteomic studies, a total of six samples were collected, with three replicates each of stigmatic and inner petal nectaries/exudates.

Transcriptomes of stigmas and inner petal nectaries

Total RNA in the stigmas and inner petal nectaries was extracted using the RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The concentration and integrity of RNA were checked for each sample, with samples meeting the following standards selected for library preparation and sequencing: $1.7 < OD_{260}/OD_{280} < 2.5$; $0.5 < OD_{260}/OD_{230} < 2.5$; $RIN \geq 6.5$; $28S/18S \geq 1$. More than 1 µg RNA from each sample with a minimum concentration of 20 ng/µL was used to set up the cDNA libraries with a VAHTS mRNA-seq V3 Library Prep Kit (Vazyme Biotech, Nanjing, China). cDNA libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, CA, USA) by Biomarker Technologies (Beijing, China) to generate raw data, subject to quality control. Clean reads were obtained from raw data by removing the adaptors and filtering the low-quality reads and those with poly-N.

Clean reads were pooled from all samples and assembled de novo using Trinity ver. 2.5.1 [71] with default settings (k-mer = 25). Contigs smaller than 200 bp were discarded. The longest isoform was selected for each gene. Clean reads in each sample were mapped onto the assembled unigenes, with the expression level of each gene quantified as fragments per kilobase of transcript per million mapped reads (FPKM). PCC values were calculated, and a PCA was conducted with the expression data. Differential expression analysis of the transcriptome was performed in R using the package DESeq2 ver. 1.6.0 [72]. Transcripts were identified as differentially expressed genes when the corrected p value < 0.01 and the fold change (FC) value ≥ 2 or ≤ 0.5.

Transcriptomic annotation was conducted using BLAST ver. 2.2.31 [73] to search against the following databases: NR (NCBI non-redundant protein sequence), UniProt, GO (Gene Ontology), COG (Cluster of Orthologous Groups of proteins), KOG (Eukaryotic Orthologous Groups of proteins), and KEGG (Kyoto Encyclopedia of Genes and Genomes). The threshold e-value for BLAST search results was set at < 1e−5. KOBAS ver. 2.0 [74] was used to discover significantly enriched KEGG pathways.

Proteomes of stigmatic and inner petal exudates

Protein extraction and mass-spectrometry were performed by Biomarker Technologies (Beijing, China). Total proteins in each sample were extracted using a Plant Total Protein Extraction Reagent Kit (Bangfei Biosciences, Beijing, China), with a sample volume of 100 µL for protein extraction. The Bradford method [75] was adopted for measuring protein concentration. Standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to examine the molecular weight of proteins in each sample. Proteins were cleaved into peptides after trypsin digestion. Peptides in each sample were separated using a nanoElute liquid chromatography system (Bruker Daltonics Inc., Bremen, Germany), which was coupled to a CaptiveSpray nano ion source installed in a trapped ion mobility spectrometry quadrupole time-of-flight (timsTOF) mass spectrometer (Bruker Daltonics Inc., Bremen, Germany).

Proteomic data analysis was performed using MaxQuant ver. 1.6.17 (http://maxquant.org). Raw data were searched against the transcriptome of *M. laui*, with the proteomic annotation results directly extracted from those of the transcriptomic annotation. Protein quantification was implemented using the MaxLFQ algorithm [76] embedded in the MaxQuant software, which allows label-free quantification. Proteins that were differentially expressed between stigmatic and inner petal exudates were identified if the fold change (FC) value ≥ 2 or ≤ 0.5, as well as the false discovery rate (FDR)/corrected p value of the statistical t-test < 0.05.

Abbreviations

AA	Amino acid
Anosim	Analysis of similarities
BLAST	Basic local alignment search tool
BOLD	Barcode of Life Data
COG	Cluster of Orthologous Groups of proteins
COI	Mitochondrial cytochrome c oxidase subunit I
DEG	Differentially expressed gene
DEP	Differentially expressed protein
ETI	Effector-triggered immunity
FAA	Formalin-acetic acid-alcohol
FC	Fold change
FDR	False discovery rate
FPKM	Fragments per kilobase of transcript per million mapped reads
GO	Gene Ontology
HR	Hypersensitive response

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ITS	Internal transcribed spacer
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	Eukaryotic Orthologous Groups of proteins
MAPK	Mitogen-activated protein kinase
NCBI	National Center for Biotechnology and Information
NR	NCBI non-redundant protein sequence
OTU	Operational taxonomic units
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PCC	Pearson's correlation coefficient
PR	Pathogenesis-related protein
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS-PAGE	Standard sodium dodecyl sulphate polyacrylamide gel electrophoresis
UPGMA	Unweighted pair group method with arithmetic mean

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-025-02308-6.

Additional file 1: Tables S1-S5 and Figures S1-S4. Table S1. Descriptive statistics of the ten sequenced samples of the fungal communities on inner petals and floral visitor bodies. Table S2. Proportions of 20 most abundant OTUs under different taxonomic levels. Table S3. Descriptive statistics of six Illumina sequencing samples of *Monoon laui*, including three replicates of stigmas and three replicates of inner petal nectaries. Table S4. Sugar composition of stigmatic and inner petal exudates of *Monoon laui*. Table S5. Amino acid composition of stigmatic and inner petal exudates of *Monoon laui*. Fig. S1. Comparison between the fungal communities on inner petals (group 1: PF) and the bodies of floral visitors (group 2: IF) of *Monoon laui*. Fig. S2. Comparison of the transcriptomes between the stigmas and inner petal nectaries of *Monoon laui*. Fig. S3. Identification of differentially expressed genes (DEGs) and proteins (DEPs). Fig. S4. Comparison of the proteomes between the stigmatic and inner petal exudates of *Monoon laui*.

Additional file 2: Supplementary methods for DNA barcoding of pollinators, meta-barcoding of fungal communities and the examination of sugar and amino acid composition.

Data availability
All data generated or analysed during this study are included in this published article and publicly available repositories. The RNA sequencing raw data of *Monoon laui* were deposited in NCBI under the accession number PRJNA1049325. The raw reads of the fungal metabarcoding are available in NCBI at PRJNA1052628. Supplementary videos for the insect larvae consuming fungi can be found at https://doi.org/10.6084/m9.figshare.24750450.v1.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors consent to the publication of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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