Planta (2018) 247:1423–1438
https://doi.org/10.1007/s00425-018-2880-3

ORIGINAL ARTICLE

Comparative multi-omics analysis reveals diverse latex-based defense strategies against pests among latex-producing organs of the fig tree (*Ficus carica*)

Sakihito Kitajima[1,2] · Wataru Aoki[3,4] · Daisuke Shibata[5] · Daisuke Nakajima[5] · Nozomu Sakurai[5] · Kazufumi Yazaki[6] · Ryosuke Munakata[6,7] · Toki Taira[8] · Masaru Kobayashi[3] · Shunsuke Aburaya[3] · Eric Hyrmeya Savadogo[1] · Susumu Hibino[1] · Haruna Yano[1]

Received: 7 November 2017 / Accepted: 3 March 2018 / Published online: 13 March 2018

Abstract

Main conclusion **Latexes in immature fruit, young petioles and lignified trunks of fig trees protect the plant using toxic proteins and metabolites in various organ-dependent ways.**

Latexes from plants contain high amounts of toxic proteins and metabolites, which attack microbes and herbivores after exudation at pest-induced wound sites. The protein and metabolite constituents of latexes are highly variable, depending on the plant species and organ. To determine the diversity of latex-based defense strategies in fig tree (*Ficus carica*) organs, we conducted comparative proteomic, transcriptomic and metabolomic analyses on latexes isolated from immature fruit, young petioles and lignified trunks of *F. carica* after constructing a unigene sequence library using RNA-seq data. Trypsin inhibitors were the most abundant proteins in petiole latex, while cysteine proteases ("ficins") were the most abundant in immature fruit and trunk latexes. Galloylglycerol, a possible defense-related metabolite, appeared to be highly accumulated in all three latexes. The expression levels of pathogenesis-related proteins were highest in the latex of trunk, suggesting that this latex had adapted a defensive role against microbe attacks. Although young petioles and immature fruit are both unlignified soft organs, and potential food for herbivorous insects, unigenes for the sesquiterpenoid pathway, which likely produces defense-associated volatiles, and the phenylpropanoid pathway, which produces toxic furanocoumarins, were expressed less in immature fruit latex. This difference may indicate that while petioles and fruit protect the plant from attack by herbivores, the fruit must also attract insect pollinators at younger stages and animals after ripening. We also suggest possible candidate transcription factors and signal transduction proteins that are involved in the differential expression of the unigenes.

Keywords Furanocoumarin · Laticifer · Multi-omics · Psoralic acid glucoside · Trans-omics

Abbreviations

DEG	Differentially expressed (uni)gene
GO	Gene ontology

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-2880-3) contains supplementary material, which is available to authorized users.

✉ Sakihito Kitajima
sakito@kit.ac.jp

[1] Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

[2] The Center for Advanced Insect Research Promotion, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

[3] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

[4] Kyoto Integrated Science and Technology Bio-Analysis Center (KIST-BIC), Shimogyo-ku, Kyoto 600-8813, Japan

[5] Kazusa DNA Research Institute, Kazusa-kamatari 2-6-7, Kisarazu, Chiba 292-0818, Japan

[6] Research Institute for Sustainable Humanosphere, Kyoto University, Uji 611-0011, Japan

[7] Université de Lorraine, INRA, UMR1121, LAE, 54 000 Nancy, France

[8] Department of Bioscience and Biotechnology, University of the Ryukyus, Senbaru, Nishihara-cho, Okinawa 903-0213, Japan

PR Pathogenesis-related
RPKM Read counts per kilobase of unigene per million mapped reads

Introduction

A laticifer is a plant cell unique in shape, differentiation and physiological function, and its cytoplasm is a sticky fluid called latex. In addition to the industrial importance of plant latex as a rubber source, such as from the para rubber tree (*Hevea brasiliensis*), latex is a component of plant defense against microbes and herbivores.

Laticifers form long tubular or branched structures running throughout the plant's body. Owing to this structure, when the plant body is cut, a large amount of latex is exuded from the cut site, and toxic proteins and metabolites contained in it attack pests. Laticifers have been found in 12,500 plant species of 22 families, including monocots and dicots, and they are estimated to exist in up to 20,000 species from 40 families (Lewinsohn 1991). Even though latexes share a common biological role in terms of pest defense, their protein and chemical constituents are highly variable among plant species (Hagel et al. 2008; Konno 2011).

In addition, protein constituents of latexes are variable even among organs in a single species (Kitajima et al. 2012, 2013). The transcriptome and proteome are different among latexes extracted from young, unlignified organs and older, lignified organs in mulberry (*Morus alba*). In the unlignified organs, such as petioles and young stems, latexes contained greater amounts of two insecticidal chitinase-like proteins, named LA-a (equivalent to MLX56 reported by Wasano et al. 2009) and its homolog LA-b. In contrast, in latexes of older lignified stems and trunks, these two proteins were weakly detected, and class I chitinase (named LA-c), which has antifungal but not insecticidal activity, was present in the greatest amount (Kitajima et al. 2012, 2013). Considering that soft, unlignified organs are food for insects such as *Lepidoptera* caterpillars, while harder lignified organs are not attacked by such insects but are subject to attack by microbes at wound sites, the differences in the latex constituents is most likely an organ-specific adaptation to different potential pests. Thus, plant defense strategies appear to be well adapted to most threatening pests through the diversity in latex structure and composition.

In contrast to mulberry, which bears small fruit and produces a limited amount of latex, the fruit of the fig tree (*Ficus carica*) (technically, it is "syconium" which has many flowers inside when immature and then becomes a ripened fruit) exudes a high amount of latex. Thus, comparative multi-omics studies on *F. carica* latexes should provide more information on the diversity of latex-associated defenses.

Ficus carica latex contains large amounts of isoforms of ficin, a cysteine protease, which is toxic to the caterpillars of *Lepidoptera* (Konno et al. 2004) and fungi (Karnchanatat et al. 2011; López-García et al. 2012), as well as isoforms of trypsin inhibitor, which is also known to be toxic to insects (Hilder et al. 1987) and fungi (Huynh et al. 1992; Terras et al. 1993). In our preliminary experiments, we found that the ficins to trypsin inhibitor ratio was different between latexes from immature fruit and young petioles, suggesting that, despite these organs both being young and unlignified, their latexes have adopted different defense strategies. We compared the proteomes, metabolomes and transcriptomes in the latex of various *F. carica* organs to investigate the diversity of defense strategies. As sources of latex, we chose three different organs: immature fruit, which are economically important as food; young and unlignified petioles, whose laticifers are expected to be connected to those in leaf veins; and > 1-year-old trunks, which are lignified and thus may have different pests from unlignified organs (Fig. 1).

Materials and methods

Plant materials

Ficus carica L. trees were maintained at the Center for Bioresource Field Science, Kyoto Institute of Technology, Kyoto, Japan.

Protein extraction

Latexes of *F. carica*, exuded separately from the cut immature fruits, young petioles and lignified trunks (> 1-year-old), were mixed immediately with equal volumes of buffer A (100 mM potassium phosphate and 10 mM EDTA, pH 6.7) supplemented with 0.1% (v/v) β-mercaptoethanol, frozen in liquid nitrogen, and stored at − 80 °C until use. Latex proteins were extracted according to the procedure described by Wang et al. (2010) for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography coupled with mass spectrometry (LC–MS) analysis.

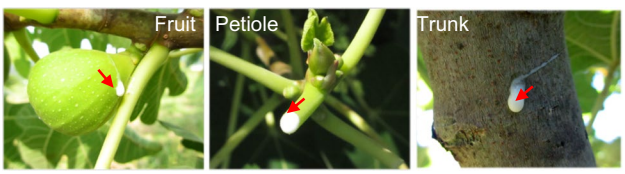

Fig. 1 The three organs of *F. carica* used in this study. Arrows indicate exuded latexes

2D-PAGE and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS)

Latex proteins (300 µg) were solubilized with Solution 2 (EzApply 2D Kit; ATTO, Tokyo, Japan) supplemented with 1% of dithiothreitol, alkylated with iodoacetamide and electrophoresed on pH range 3–10 agarose gels (agar GEL A-M310; ATTO) for the first-dimension isoelectric focusing according to the manufacturer's protocol. The gel strips were applied to an SDS–polyacrylamide gel (20% acrylamide, acrylamide:bis-acrylamide = 30:0.135, SDS-PAGE reagent set; Nacalai-tesque, Kyoto, Japan) after fixation in 10% trichloroacetic acid and equilibration in equilibration buffer [50 mM Tris–Cl, 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol, pH 6.8]. After SDS-PAGE, protein bands were stained with Coomassie brilliant blue R250 and excised. Tryptic digests were prepared according to Jimenez et al. (2003), and mass and MS/MS spectra were obtained using an Autoflex TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Leipzig, German) following the protocol recommended by the manufacturer. Protein identification was performed using the Mascot program (Matrix Science, London, UK) and the unigene database of *F. carica* constructed in this study.

Quantitative LC–MS of proteins

Latex proteins (three biological replicates for each organ's latex) were extracted as described above and digested with a Lys-C/Trypsin mix (Promega, Madison, WI, USA) for LC–MS analysis as described in Kitajima et al. (2016). Tryptic digests were labeled using a tandem mass tag 6-plex labeling kit (Thermo Fisher Scientific, Waltham, MA, USA) with reporters at *m/z* 126, 129, 130 and 131 as described in Matsui et al. (2013). An internal standard was prepared by a mixture of tryptic digests of all organs and labeled with TMT-131. To identify differentially accumulated proteins, *P* values were calculated for each protein by the empirical Bayes method using limma package ver. 3.5 (Ritchie et al. 2015) with R program (ver. 3.1.1, R Core Team 2014), and adjusted by the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

Preparation of laticifer RNA and mRNA-seq analysis

Latex samples independently exuded from the cut immature fruits, young petioles or lignified trunks (> 1-year-old) of *F. carica* were mixed immediately with nine volumes of TRIzol reagent (Thermo Fisher Scientific), frozen in liquid nitrogen, and stored at − 80 °C until use. RNA was purified using a PureLink RNA mini kit (Thermo Fisher Scientific) by a procedure described previously (Kitajima et al. 2012). Paired-end sequencing of 100-nt reads for de novo assembly and single-end sequencing of 50-nt reads for differential expression analysis were performed according to the manufacturer's standard protocol on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). Single reads sequenced by the HiSeq 2000 are available through the Sequence Read Archive under accession numbers DRR101540–DRR101542 for paired-end sequencing, and DRR101543–DRR101551 for single-end sequencing.

De novo assembly, annotation and differential expression analysis

mRNA-seq data were manipulated using Biolinux 8 software (Field et al. 2006). To create the unigene sequence library, paired-end reads of latex mRNA from the three organs were mixed together and de novo assembled using the Trinity assembler ver. 2.2.0 (Grabherr et al. 2011) with the default parameter settings. The outputted sequences were filtered with a cutoff length of 400 nt. After merging with mRNA sequences that had been deposited in NCBI database (https://www.ncbi.nlm.nih.gov/), similar sequences were clustered by CD-HIT-EST (Huang et al. 2010). The obtained unigene sequences have been deposited at DDBJ/EMBL/GenBank under the accessions IACP01000001–IACP01078277.

The obtained unigene sequences were annotated based on the results of a homology search performed using a BLASTX (Altschul et al. 1997) against the *Arabidopsis thaliana* TAIR10 database (https://www.arabidopsis.org/) and refseq protein databases of NCBI with a cutoff *E* value of 1E−15.

To evaluate the expression level of each unigene, the single-end reads (three biological replicates for each organ's latex) were mapped to the unigene sequences using the Bowtie2 program (Langmead and Salzberg 2012), and the mapped read counts per kilobase of unigene per million mapped reads (RPKM) were calculated. To identify differentially expressed unigenes (DEGs), the fold change of expression level and *P* value were calculated for each unigene by a quasi-likelihood *F* test based on the read counts using edgeR program ver. 3.16.5 (Robinson et al. 2010), and the *P* values were adjusted by the Benjamini–Hochberg method. Unigenes satisfying of the following: $\log_2$ (fold change) ≥ 2; adjusted *P* value ≤ 0.01; and at least one RPKM value in paired samples ≥ 2, were considered to be differentially expressed and subjected to further analyses.

DEGs were classified based on the gene ontology (GO, http://www.geneontology.org/) or KEGG metabolic pathway (http://www.genome.jp/kegg/) of the most similar proteins of *A. thaliana* (*E* value < 1E−15). The GO and pathway enrichment analyses were performed using Fisher's exact

test (Fisher 1922) versus the entire unigenes in the *F. carica* latexes, and the *P* values were adjusted by the Benjamini–Hochberg method.

Reverse transcription PCR cloning

To clone the cDNA of a latex protein, total RNA isolated from latex of *F. carica* fruit was reverse-transcribed using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and an oligo(dT) primer. PCR was carried out using KOD-plus-Neo DNA polymerase (Toyobo) and a pair of gene-specific primers designed based on the unigene's sequence. The accession numbers in DDBJ/GenBank/EBI databases are listed in Suppl. Table S1.

Metabolite analysis

Latexes exuded separately from the cut immature fruits, young petioles or lignified trunks (> 1-year-old) of *F. carica* were collected, immediately frozen in liquid nitrogen, and stored at − 80 °C until use. Metabolites were extracted with three volumes of methanol. After vigorous mixing, mixtures were centrifuged (12,000*g*, 10 min, 4 °C). The supernatant was filtered through a C18 Spin Column (GL Sciences, Tokyo, Japan), and the filtrate was subjected to LC–MS analysis. LC–MS was performed using an high-performance liquid chromatography system (model 1200; Agilent Technologies, Santa Clara, CA, USA) coupled to an LTQ Orbitrap XL-MS system (Thermo Fisher Scientific), equipped with an electrospray ionization (ESI) source operating in the positive ion mode and with a lockspray interface for accurate mass measurements. Five different chemicals (lidocaine, prochloraz, reserpine, bombesin and aureobasidin A) were employed as the lock-mass compounds. The injection volume was 5 µL. Analytical conditions were as follows: liquid chromatograph column, TSK-GEL ODS-100V (5 µm, 3 × 50 mm; Tosoh, Tokyo, Japan); solvent system, solvent A (0.1% (v/v) formic acid in water) and solvent B (acetonitrile including 0.1% formic acid); gradient program, 97% A/3% B at 0 min, 3% A/97% B at 15 min, 3% A/97% B at 20.0 min, 97% A/3% B at 20.1 min and 97% A/3% B at 25 min. The flow rate was set to 0.4 mL/min, and the column oven temperature was set at 40 °C. Compounds were detected in ESI-positive mode over the *m/z* range 100–1500. The duty cycle included one MS1 acquisition with the top four most intense precursor ions subjected to MS/MS analysis. MS/MS analyses were carried out using collision-induced dissociation in a linear ion trap detector with a normalized collision energy of 35.0% and an isolation width of 2.0 (*m/z*). FT-Orbitrap detectors were used at a mass resolution of 60,000 (at *m/z* 400). The ESI settings were a spray voltage of 4.0 kV and capillary temperature of 300 °C. The nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units, respectively. To monitor the high-performance liquid chromatography eluate, a photodiode array detector was used with a wavelength range of 190–950 nm. Four biological replicates of latex exudates from each tissue were conducted. The same procedures without plant samples were performed as the negative control (mock).

These data were acquired with Xcalibur software (Thermo Fisher Scientific) and processed with PowerGet software (Sakurai et al. 2014) and MassChroViewer program ver. 1.3.2 (http://www.kazusa.or.jp/komics/software/MassChroViewer) for the alignment and annotation of metabolites. Peaks reproducibly detected in more than three of four biological replicates and absent in the mock data were used as valid peaks for further analyses. Flavonoid aglycones were searched using FlavonoidSearch software (Akimoto et al. 2017). A principal component analysis (PCA) was performed using the prcomp function of R program based on the variance–covariance matrix. The peak area values transformed to log base 10 and normalized by the median value of all peaks in the sample were used for the PCA. Missing values were filled with one tenth the minimum value among all of the samples. To identify differentially accumulated metabolites, *P* values were calculated for each metabolite using the empirical Bayes method and adjusted by the Benjamini–Hochberg method, as described above.

Results

Construction of the unigene database of *F. carica* latexes

Prior to proteome and transcriptome analyses of *F. carica* latexes, we constructed a sequence database of unigenes that were expressed in laticifer cells of *F. carica* by the de novo assembly of paired-end reads of 100 nt in length obtained from the RNA-seq analyses of immature fruit (11,430,175 pairs of reads), young petioles (17,708,672) and lignified trunk (> 1-year-old) (17,280,043). The unigenes were then annotated based on their similarities to proteins of *A. thaliana* and the refseq protein databases with a cutoff *E* value < 1E−15. In summary, we obtained 78,316 unigenes with an average length of 1387 nt and an N50 (50% of the total assembled sequence was contained in sequences of this length or longer) of 1869 nt. Among these, 53,190 unigenes were similar to *Arabidopsis* proteins with *E* values < 1E−15, and 19,464 unigenes did not show a similarity to any protein in these databases with *E* values < 1E−15. To determine nucleotide sequences of some unigenes, such as ficins, trypsin inhibitors and chitinases, their cDNAs were cloned by RT-PCR using gene-specific primers. Results are shown in Suppl. Table S1. The unigene database was used

to identify proteins by MS and to map RNA-seq reads for the evaluation of mRNA abundance.

Comparative proteome analyses of latex produced from each of the three organs

To identify proteins accumulated at high levels in each latex exuded from the three organs of *F. carica*, we performed 2D-PAGE followed by the identification of the protein spots using MALDI-TOF/MS (Fig. 2; Table 1). At least six isoforms of ficin (cysteine protease) were found. Several isoforms of ficin and trypsin inhibitor were major proteins in these latexes. As described above, these two protein families are toxic to insects and fungi. Although *F. carica* and mulberry are both Moraceae plants, the proteomes of their latexes were quite different from each other. In mulberry, an antifungal chitinase isoform was most abundant in the latex of lignified parts, while two anti-insect chitinase-like proteins were the most abundant in latexes of young unlignified parts (Kitajima et al. 2010, 2012). In addition to ficin and trypsin inhibitor, *F. carica* latexes contained other defense-related proteins, including chitinases, which hydrolyze chitin (one of the component of fungal cell walls), pathogenesis-related (PR) protein 4, acid phosphatase and a PLAT/LH2 family protein. One isoform of acid phosphatase from *Arabidopsis* is toxic to insects (Liu et al. 2005). Defense-related functions of the PLAT/LH2 family proteins, which are characterized by having PLAT domains, have not been reported but may exist because the expression of a gene having this domain was inducible by a tobacco mosaic virus infection in hot pepper (*Capsicum annuum*) (Shin et al. 2003). The above proteins were detected in the latex samples of the three organs tested but their abundance levels were different among organs (Fig. 2; Table 1). In the latexes of fruit and trunk, ficin isoforms were more abundant than trypsin inhibitor, but trypsin inhibitor isoforms were more abundant in petiole latex. These two proteins have common roles in their toxicity to insects and fungi, but the differential accumulation pattern suggests that *F. carica* might use them for different purposes; for example, against organ-specific pests. The amounts of other proteins may also differ between different latexes.

We conducted an LC-based quantitative proteomic analysis in combination with isotope-coded affinity tag technology. In total, 54 proteins were reproducibly found in latex of at least one of the three organs, although some proteins were not detectable in the 2D-PAGE analysis. Most were proteins toxic to microbes (ficins, trypsin inhibitors, chitinases, osmotin, PR proteins 1 and 4, and lectins) and to insects (ficins, trypsin inhibitors and acid phosphatase). PLAT/LH2 family proteins, which were found in the 2D-PAGE analysis, were also found. Protease inhibitors (serine protease inhibitor and cystatin) other than trypsin protease inhibitor were also

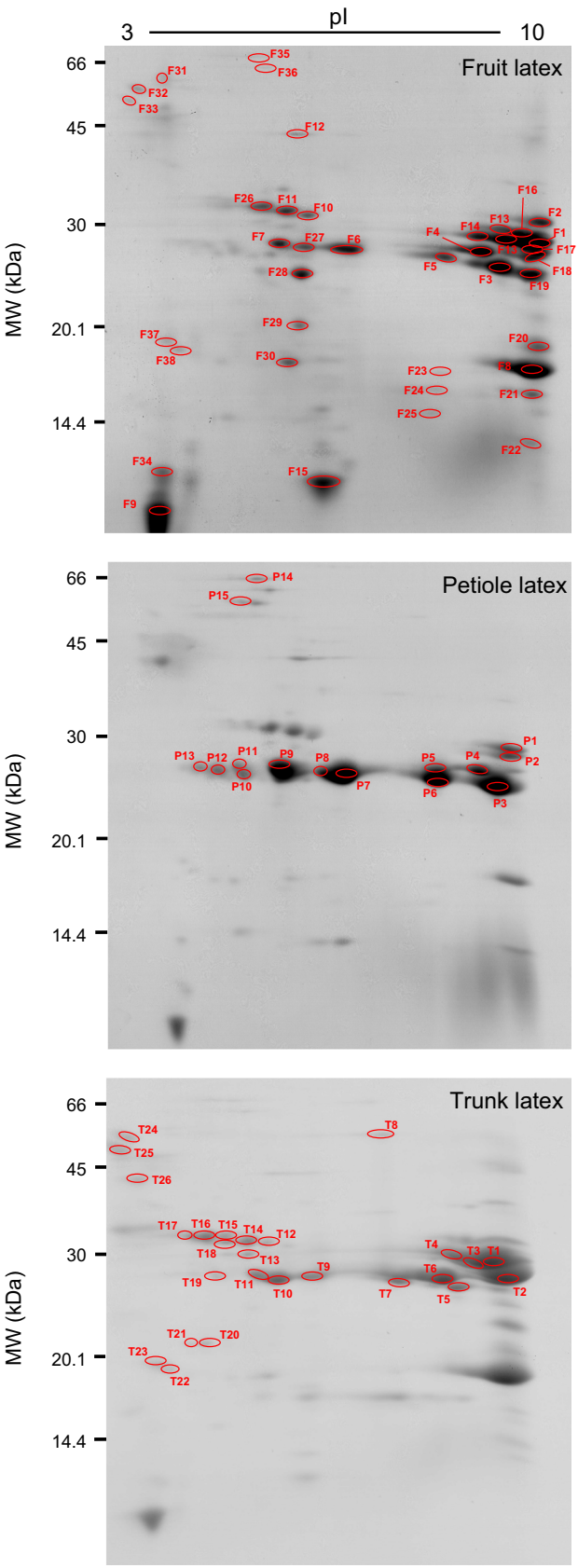

Fig. 2 Proteins detected in 2D-PAGE of latexes isolated from the three organs. Proteins identified by MALDI-TOF/MS analysis are listed in Table 1

Table 1 Proteins detected in 2D-PAGE of latexes isolated from three organs

Spot	Unigene (acc. no.)[a]	Mascot score	Most similar sequence in Blastx results[b]		
			AGI code/acc. no.	*E* value	Gene name
Fruit latex					
F1	Ficin 1c (LC222279)	212	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
F2	Ficin 5 (LC222285)	163	AT5G43060.1	4.E−119	Granulin repeat cysteine protease family protein
F3	Trypsin inhibitor 2 (LC222262)	306	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F4	Ficin 1a (LC222277)	189	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F5	Trypsin inhibitor 2 (LC222262)	268	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F6	Trypsin inhibitor 2 (LC222262)	343	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F7	Trypsin inhibitor 2 (LC222262)	330	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F8	Ficin 1a (LC222277)	184	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F9	Ficin 1a (LC222277), Ficin 1b (LC222278), Ficin 1c (LC222279), Ficin 6b (LC222286)	65	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F10	Acid phosphatase (LC222263)	289	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
F11	Acid phosphatase (LC222263)	113	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
F12	Class V chitinase (LC222269)	283	AT4G19810.1	1.E−149	Glycosyl hydrolase family protein with chitinase insertion domain
F13	Ficin 1c (LC222279)	89	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
F14	Ficin 6b (LC222286)	55	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F15	Ficin 1a (LC222277), Ficin 1b (LC222278), Ficin 1c (LC222279)	90	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F16	Ficin 1c (LC222279)	235	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
F17	Ficin 4 (LC222280)	152	AT5G43060.1	2.E−126	Granulin repeat cysteine protease family protein
F18	Ficin 1c (LC222279)	143	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
F19	Trypsin inhibitor 2 (LC222262)	400	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F20	Ficin 6b (LC222286)	81	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F21	Ficin 1a (LC222277)	152	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F22	Trypsin inhibitor 2 (LC222262)	152	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F23	Pathogenesis-related protein 4 (LC222264)	79	AT3G04720.1	1.E−82	Pathogenesis-related 4
F24	Pathogenesis-related protein 4 (LC222264)	98	AT3G04720.1	1.E−82	Pathogenesis-related 4
F25	Ficin 6b (LC222286)	37	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F26	Acid phosphatase (LC222263)	165	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
F27	Trypsin inhibitor 2 (LC222262)	289	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F28	Ficin 6b (LC222286)	134	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F29	Ficin 6b (LC222286)	216	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F30	Trypsin inhibitor 2 (LC222262)	191	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
F31	Ficin 1a (LC222277), Ficin 1b (LC222278), Ficin 1c (LC222279), Ficin 6b (LC222286)	67	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F32	Peroxidase (LC222265)	170	AT5G06730.1	3.E−140	Peroxidase superfamily protein
F33	Peroxidase (LC222265)	154	AT5G06730.1	3.E−140	Peroxidase superfamily protein
F34	Ficin 1a (LC222277), Ficin 1b (LC222278), Ficin 1c (LC222279), Ficin 6b (LC222286)	72	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
F35	Subtilase 2 (LC222268)	187	AT3G14067.1	0.E+00	Subtilase family protein
F36	Subtilase 2 (LC222268)	114	AT3G14067.1	0.E+00	Subtilase family protein

Table 1 (continued)

Spot	Unigene (acc. no.)[a]	Mascot score	Most similar sequence in Blastx results[b]		
			AGI code/acc. no.	*E* value	Gene name
F37	PLAT/LH2 family protein (LC222270)	212	AT4G39730.1	3.E−69	Lipase/lipoxygenase, PLAT/LH2 family protein
F38	PLAT/LH2 family protein (LC222270)	298	AT4G39730.1	3.E−69	Lipase/lipoxygenase, PLAT/LH2 family protein
Petiole latex					
P1	Ficin 1c (LC222279)	222	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
P2	Ficin 1c (LC222279)	135	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
P3	Trypsin inhibitor 2 (LC222262)	284	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P4	Ficin 1a (LC222277)	213	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
P5	Trypsin inhibitor 2 (LC222262)	521	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P6	Trypsin inhibitor 2 (LC222262)	510	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P7	Trypsin inhibitor 2 (LC222262)	303	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P8	Trypsin inhibitor 2 (LC222262)	309	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P9	Trypsin inhibitor 2 (LC222262)	236	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P10	Trypsin inhibitor 2 (LC222262)	227	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P11	Trypsin inhibitor 2 (LC222262)	324	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P12	Trypsin inhibitor 2 (LC222262)	195	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P13	Trypsin inhibitor 2 (LC222262)	227	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
P14	Subtilase (LC222268)	127	AT3G14067.1	0.E+00	Subtilase family protein
P15	Mandelonitrile lyase (LC222273)	161	AT1G73050.1	0.E+00	Glucose–methanol–choline (GMC) oxidoreductase family protein
Trunk latex					
T1	Ficin 1c (LC222279)	164	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
T2	Ficin 4 (LC222280)	109	AT5G43060.1	2.E−126	Granulin repeat cysteine protease family protein
T3	Ficin 6b (LC222286)	165	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
T4	Ficin 1c (LC222279)	218	AT5G43060.1	2.E−129	Granulin repeat cysteine protease family protein
T5	Trypsin inhibitor 2 (LC222262)	286	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T6	Ficin 1a (LC222277)	246	AT5G43060.1	4.E−135	Granulin repeat cysteine protease family protein
T7	Trypsin inhibitor 2 (LC222262)	321	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T8	Class III chitinase (LC222272)	433	AT5G24090.1	1.E−105	Chitinase A
T9	Trypsin inhibitor 2 (LC222262)	380	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T10	Trypsin inhibitor 2 (LC222262)	470	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T11	Trypsin inhibitor 2 (LC222262)	418	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T12	Acid phosphatase (LC222263)	401	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T13	Acid phosphatase (LC222263)	405	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T14	Acid phosphatase (LC222263)	429	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T15	Acid phosphatase (LC222263)	434	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T16	Acid phosphatase (LC222263)	317	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T17	Acid phosphatase (LC222263)	537	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T18	Acid phosphatase (LC222263)	259	AT4G25150.1	1.E−66	HAD superfamily, subfamily IIIB acid phosphatase
T19	Trypsin inhibitor 2 (LC222262)	150	XP_010088113	2.E−31	Hypothetical protein L484_000853 (*Morus notabilis*)
T20	Class I chitinase (LC222274)	203	AT3G12500.1	1.E−77	Basic chitinase
T21	Class I chitinase (LC222274)	198	AT3G12500.1	1.E−77	Basic chitinase
T22	PLAT/LH2 family protein (LC222270)	346	AT4G39730.1	3.E−69	Lipase/lipoxygenase, PLAT/LH2 family protein
T23	PLAT/LH2 family protein (LC222270)	418	AT4G39730.1	3.E−69	Lipase/lipoxygenase, PLAT/LH2 family protein
T24	Peroxidase (LC222265)	319	AT5G06730.1	3.E−140	Peroxidase superfamily protein
T25	Peroxidase (LC222265)	315	AT5G06730.1	3.E−140	Peroxidase superfamily protein

Table 1 (continued)

Spot	Unigene (acc. no.)[a]	Mascot score	Most similar sequence in Blastx results[b]		
			AGI code/acc. no.	*E* value	Gene name
T26	Chitinase (LC222275)	260	AT3G12500.1	2.E−126	Basic chitinase

[a]The sequences were determined by RT-PCR cloning in this study

[b]Search results using a BLASTX algorithm-based search against the Arabidopsis protein database (TAIR10). If there was no hit with an *E* value of < 1E−15, then results using the refseq protein database of GenBank are indicated

Table 2 Quantitative proteomic analysis of the three organs' latexes ($n = 3$)

Unigene	Petiole/fruit[a]		Petiole/trunk[a]		Trunk/fruit[a]		Most similar sequence in Blastx results[c]		
	$\log_2$ (fold change)	Adjusted *P*[b]	$\log_2$ (fold change)	Adjusted *P*[b]	$\log_2$ (fold change)	Adjusted *P*[b]	AGI code/acc. no.	*E* value	Gene name
Class I chitinase (LC222274) [*4]	NA	NA	−3.23	0.0000	NA	NA	AT3G12500.1	1.0E−77	Basic chitinase
34286_c1_g1_i1	NA	NA	NA	NA	−0.70	0.2936	AT4G11650.1	2.0E−118	Osmotin 34
3193_c3_g1_i1	NA	NA	NA	NA	−1.69	0.0123	AT3G12490.1	3.0E−22	Cystatin B
Class III chitinase (LC222272)[d]	5.10	0.0000	−2.91	0.0000	8.13	0.0000	AT5G24090.1	1.0E−105	Chitinase A
Chitinase (LC222275)[d]	3.46	0.0000	0.50	0.0769	3.08	0.0000	AT3G12500.1	2.0E− 126	Basic chitinase
40947_c2_g4_i2	2.65	0.0000	7.00	0.0000	-4.23	0.0000	AAL85343.1	4.0E-69	Trypsin-like protease inhibitor (*Ficus carica*)
47626_c0_g1_i1	2.54	0.0001	2.53	0.0004	0.13	0.7323	AT1G29670.1	1.0E−124	GDSL-like lipase/acylhydrolase superfamily protein
Mandelonitrile lyase (LC222273)[d]	2.47	0.0000	3.97	0.0000	−1.38	0.0004	AT1G73050.1	0.0E+00	Glucose-methanol-choline (GMC) oxidoreductase family protein
30637_c0_g1_i1	2.39	0.0000	0.29	0.3956	2.22	0.0001	AT3G14067.1	1.0E−32	Subtilase family protein
39401_c0_g2_i1	2.06	0.0001	0.40	0.2229	1.78	0.0004	AT3G14067.1	8.0E−118	Subtilase family protein
40773_c4_g1_i1	1.80	0.0001	2.56	0.0000	−0.64	0.0323	NA	NA	NA
39401_c0_g1_i3	1.68	0.0000	0.27	0.2899	1.53	0.0001	AT3G14067.1	1.0E−92	Subtilase family protein
28163_c2_g1_i1	1.58	0.0000	0.11	0.5529	1.58	0.0000	AT4G36190.1	0.0E+00	Serine carboxypeptidase S28 family protein
Trypsin inhibitor (LC222262)[d]	1.42	0.0002	3.57	0.0000	−2.03	0.0000	AAL85343.1	8.0E−92	Trypsin-like protease inhibitor (*Ficus carica*)
37694_c4_g2_i2	1.22	0.0003	2.31	0.0000	−0.97	0.0032	AT1G55980.1	2.0E−22	FAD/NAD(P)-binding oxidoreductase family protein
38673_c1_g1_i1	1.22	0.0844	0.34	0.6329	1.00	0.0410	AT1G78850.1	6.0E−124	D-mannose binding lectin protein with Apple-like carbohydrate-binding domain
40947_c3_g8_i4	1.18	0.0002	3.01	0.0000	−1.71	0.0001	AAL85343.1	8.0E−92	Trypsin-like protease inhibitor (*Ficus carica*)
Pathogenesis-related protein 4 (LC222264)[d]	1.13	0.0057	−3.74	0.0000	4.99	0.0000	AT3G04720.1	1.0E−82	Pathogenesis-related 4
29681_c0_g2_i1	0.99	0.0005	0.55	0.0221	0.56	0.0240	AT5G11540.1	0.0E+00	D-Arabinono-1,4-lactone oxidase family protein
AF479622.1	0.97	0.0007	3.56	0.0000	−2.47	0.0000	AF479622.1	0.0E+00	Trypsin-like protease inhibitor (*Ficus carica*)
40689_c7_g1_i2	0.84	0.0312	1.58	0.0029	−0.62	0.0657	AT5G06860.1	7.0E−72	Polygalacturonase inhibiting protein 1
40689_c7_g1_i10	0.70	0.1373	−0.15	0.6992	0.97	0.0277	AT5G06860.1	9.0E−76	Polygalacturonase inhibiting protein 1
21590_c0_g2_i1	0.44	0.0242	0.51	0.0320	0.04	0.7710	NA	NA	NA
40780_c4_g1_i2	0.40	0.3029	0.69	0.0961	−0.18	0.5650	AT5G06860.1	4.0E−38	Polygalacturonase inhibiting protein 1
14641_c1_g1_i2	0.34	0.0771	1.46	0.0003	−1.00	0.0002	AT1G55265.1	6.0E−29	Protein of unknown function, DUF538
22795_c0_g1_i1	0.22	0.3862	−2.09	0.0001	2.44	0.0000	AT4G33720.1	1.0E−64	CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 protein) superfamily protein
Acid phosphatase (LC222263)[d]	0.10	0.4931	−1.22	0.0003	1.44	0.0001	AT4G25150.1	1.0E−66	HAD superfamily, subfamily IIIB acid phosphatase
29341_c0_g1_i1	−0.02	0.8947	1.56	0.0001	−1.46	0.0001	AT1G47710.1	3.0E−76	Serine protease inhibitor (SERPIN) family protein
Subtilase 1 (LC222268)[d]	−0.09	0.8491	2.95	0.0005	−2.92	0.0007	AT5G67090.1	3.0E−151	Subtilisin-like serine endopeptidase family protein
40079_c0_g1_i2	−0.23	0.2507	0.91	0.0047	−1.02	0.0007	AT4G34260.1	0.0E+00	1,2-Alpha-L-fucosidases
Class V chitinase (LC222269)[d]	−0.26	0.1373	−0.23	0.2125	0.09	0.6281	AT4G19810.1	1.0E−149	Glycosyl hydrolase family protein with chitinase insertion domain
PLAT/LH2 family protein (LC222270)[d]	−0.76	0.0179	−2.02	0.0003	1.38	0.0004	AT4G39730.1	3.0E−69	Lipase/lipooxygenase, PLAT/LH2 family protein
27981_c0_g1_i1	−0.76	0.0277	−0.95	0.0147	0.31	0.3106	AT4G15210.1	0.0E+00	Beta-amylase 5
62746_c0_g1_i1	−0.92	0.0003	0.82	0.0035	-1.62	0.0000	AT1G76020.1	7.0E-80	thioredoxin superfamily protein
Ficin 1b (LC222278)[d]	−0.99	0.0003	−0.82	0.0047	−0.04	0.7723	AT5G43060.1	2.0E−137	Granulin repeat cysteine protease family protein
41239_c38_g6_i2	−1.13	0.0000	0.55	0.0152	−1.57	0.0000	AT5G43060.1	9.0E−108	Granulin repeat cysteine protease family protein
Ficin 1c (LC222279)[d]	−1.25	0.0001	−1.01	0.0009	−0.12	0.5053	AT5G43060.1	2.0E−129	Granulin repeat cysteine protease family protein
Ficin 1a (LC222277)[d]	−1.40	0.0000	−1.08	0.0004	−0.20	0.2528	AT5G43060.1	4.0E−135	Granulin repeat cysteine protease family protein
40908_c3_g2_i1	−1.41	0.0103	−3.17	0.0004	1.88	0.0001	AT2G09990.1	1.0E−89	Ribosomal protein S5 domain 2-like superfamily protein
12480_c0_g2_i1	−1.42	0.0002	−0.98	0.0050	-0.32	0.1858	AT1G13440.1	9.0E−112	Glyceraldehyde-3-phosphate dehydrogenase C2
17253_c0_g1_i1	−1.43	0.0001	−0.12	0.6014	−1.18	0.0005	AT3G52590.1	3.0E−90	Ubiquitin extension protein 1
34239_c4_g2_i2	−1.51	0.0002	−3.58	0.0000	2.19	0.0001	AT3G12500.1	3.0E−141	Basic chitinase
33297_c0_g2_i1	−1.52	0.0051	−2.52	0.0005	1.12	0.0064	AT4G39730.1	1.0E−67	Lipase/lipooxygenase, PLAT/LH2 family protein
Ficin 3 (LC222284)[d]	−1.69	0.0000	0.08	0.6496	−1.64	0.0000	AT5G43060.1	2.0E−126	Granulin repeat cysteine protease family protein
41128_c2_g1_i3	−1.82	0.0003	−0.28	0.2931	−1.42	0.0038	AT1G24310.1	4.0E−23	Unknown protein
31918_c1_g1_i1	−1.82	0.0000	−3.39	0.0000	1.68	0.0001	AT4G16260.1	4.0E−146	Glycosyl hydrolase superfamily protein
36339_c0_g1_i1	−1.91	0.0005	−0.35	0.4236	−1.45	0.0010	AT3G56340.1	4.0E−33	Ribosomal protein S26e family protein
38225_c0_g1_i1	−2.01	0.0003	−1.68	0.0037	−0.22	0.5597	AT1G47128.1	0.0E+00	Granulin repeat cysteine protease family protein
41239_c38_g6_i7	−2.09	0.0000	0.26	0.2556	−2.23	0.0000	AT3G19390.1	4.0E−120	Granulin repeat cysteine protease family protein
Ficin 6a (LC225767)[d]	−3.16	0.0000	0.30	0.2556	−3.34	0.0000	AT5G43060.1	2.0E−122	Granulin repeat cysteine protease family protein
Ficin 5 (LC222285)[d]	−8.56	0.0000	−0.65	0.0961	−7.79	0.0000	AT5G43060.1	4.0E−119	Granulin repeat cysteine protease family protein

[a]Proteins detected in both samples in all three biological replicates. NA indicates the protein was not detected in one or both samples. Red, upregulated; blue, downregulated

[b]*P* value was calculated using the empirical Bayes method and corrected by the Benjamini–Hochberg method. Adjusted *P* values < 0.05 are indicated in gray

[c]Search results using a BLASTX algorithm-based search against the Arabidopsis protein database (TAIR10). If there was no hit with an *E* value of < 1E−15, then results using the non-redundant protein database are indicated. NA indicates no hit with an *E* value of < 1E−15

[d]The sequences were determined by RT-PCR cloning in this study

found. Polygalacturonase-inhibiting protein inhibits fungal infections of host plants by interacting with fungal polygalacturonase, which can degrade plant cell walls (Federici et al. 2006). Of the detected proteins, peroxidase 1 (accession no. LC222265) was found solely in trunk latex. Other proteins were found in latexes of two or three organs, and some of them were differentially accumulated among latexes of organs (Table 2). The levels of trypsin inhibitors (40947_c2_g4_i2, LC222262, 40947_c3_g8_i4, AF479622.1) were all higher in petiole latex than in trunk and fruit latexes. Levels of ficin isoforms were higher in fruit and trunk latexes than in petiole latex, with some exceptions. These patterns were consistent with the results of the 2D-PAGE analysis. Chitinases were most abundant in trunk latex. Class I chitinase (LC222274) was not detected in fruit latex and its level was $2^{3.23}$ times higher in trunk latex than in petiole latex. The Class III chitinase (LC222272) level was $2^{5.10}$ and $2^{8.13}$ times higher in the latexes of petiole and trunk, respectively, than in fruit latex. Another chitinase (LC222275) also had higher levels in petiole and trunk latexes than in fruit latex. The acid phosphatase (LC222263) level was also highest in trunk latex. In contrast, the levels of two PLAT/LH2 family proteins (LC222270 and 33297_c0_g2_i1) were higher in the order of trunk latex > fruit latex > petiole latex. Thus, the proteins used in the defense against pests were present in high levels in all the latexes, but their levels were differentially regulated based on the organ.

Comparative metabolomic analysis of latexes produced in three organs

In addition to proteins, some secondary metabolites are also involved in the defense against pests. Thus, we compared latex metabolomes among the three organs. Methanol-soluble metabolites were extracted from latex samples and subjected to LC–MS analysis. In positive ion mode, 1015 metabolite peaks (817 in fruit latex, 790 in petiole latex and 808 in trunk latex) were detected reproducibly, including unidentified peaks (Suppl. Table S2). Several metabolites, such as candidates of 1-*O*-galloylglycerol (peaks 1022, 1031, 1037, 1042, 1046, 1054 and 1061) produced high peak intensities in all three organs' latexes, suggesting that they might have accumulated at high amounts. In the case of 1-*O*-galloylglycerol candidates, the peak intensities were, in particular, several 10s of times higher in fruit and petiole latexes than in trunk latex. This metabolite is reported in *Ficus lyrata* (Farag et al. 2014), and its possible hydrolysis product, gallic acid, has been reported in *F. carica* (Veberic et al. 2008). Gallic acid is known to have antifungal activity (Friedman et al. 2003; Nohynek et al. 2006; Chanwitheesuk et al. 2007; Gañan et al. 2009). The differences among the latex samples were studied using the PCA of their peak intensities (Fig. 3; Suppl. Table S2). A score scatterplot from the PCA showed that the latex metabolomes were different among the three organs (Fig. 3a). A loading scatterplot showed that some of the metabolites strongly contributed to PC1 (red dots in Fig. 3b) or PC2 (black dots). These metabolites were marked in Suppl. Table S2, although most of them, unfortunately, were not identified.

Comparative transcriptome analyses of latexes produced in the three organs

To further investigate the diversity in defense systems of the latexes and their regulatory mechanisms, we conducted an RNA-seq analysis and compared the expression levels of the unigenes in the three organs' latexes. The averages of RPKM

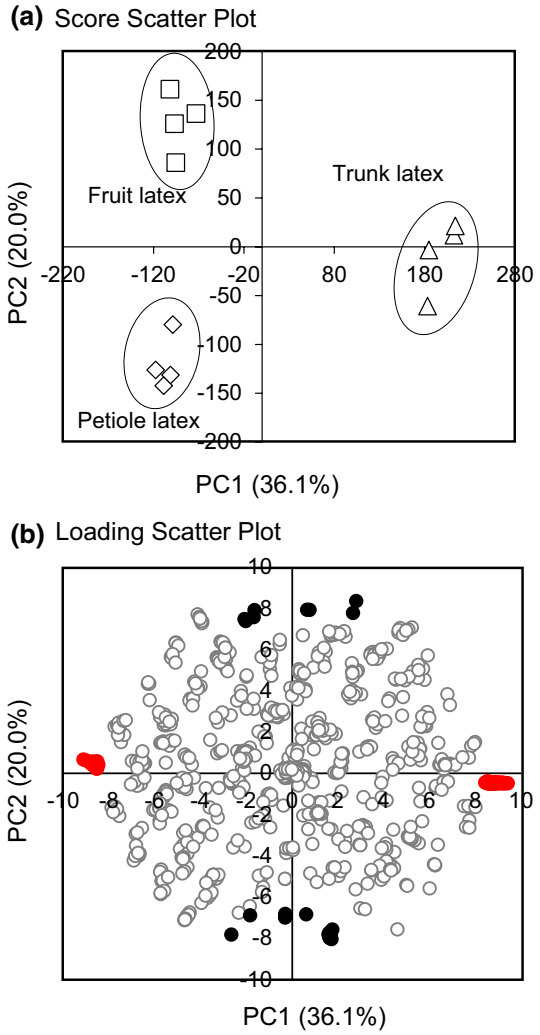

Fig. 3 Principal component analysis of metabolites in the three organs' latexes. Scores (**a**) and loadings (**b**) of PC1 and PC2 are plotted. Squares, diamonds and triangles indicate latex of fruit, petioles and trunk, respectively. Peak intensities and annotations are indicated in Suppl. Table S2. Metabolites shown in black and red are marked in the same colors in Suppl. Table S2

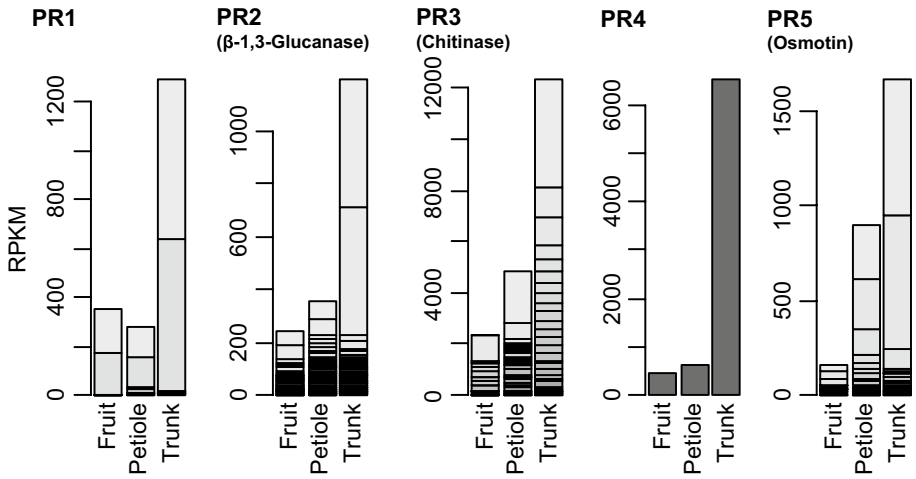

Fig. 4 The average RPKM values ($n = 3$) of unigenes encoding PR proteins in the three organs' latexes. Unigene in each part of the stacked bar graphs is indicated in Suppl. Table S3

values ($n = 3$) and the fold changes between latex pairs are indicated in Suppl. Table S1.

The expression levels of PR proteins, groups 1–5, which are related to defense against pathogens (Van Loon 1999), were different among the organs (Fig. 4; Suppl. Table S3). All of the PR protein groups showed their highest expression levels in trunk latex and lowest levels in fruit latex, except the PR1 group.

Many unigenes were differentially expressed with $\log_2$ (fold change) > 2 and adjusted P values < 0.01. After removing low-expressed unigenes with RPKM values < 2 in both of the paired samples, the DEG numbers were 2871, 604, 172, 369, 2877 and 1103 for petiole > fruit, fruit > petiole, petiole > trunk, trunk > petiole, trunk > fruit and fruit > trunk, respectively. In total, 6163 unigenes were differentially expressed. Of them, the DEGs showing similarity to *Arabidopsis* genes in BLASTX algorithm-based comparisons with E values < 1E−15, were 2162, 348, 72, 306, 1750 and 773, respectively. These six DEG groups were classified based on the GO of *Arabidopsis* homologs (Fig. 5a). Compared with the whole transcriptome as the background, GO terms associated with response to biotic stresses (GO:0009620, GO:0009871 and GO:0080027) were significantly enriched, in particular, in DEG groups of petiole > fruit and trunk > fruit. The DEGs in these GO terms included chitinases, transcription factors, metabolic enzymes and blue-copper-binding proteins. Thus, the defense system was more highly diverse in petiole and trunk latexes than in fruit latex.

Unigenes for secondary metabolic pathways

Some metabolites of phenylpropanoid and terpenoid pathways are involved in the defense against pests. When the DEGs were classified based on KEGG metabolic pathways of *Arabidopsis* homologs (Fig. 5b), the DEG group of petiole > fruit was significantly enriched in phenylpropanoid biosynthesis (KEGG ath00940), and sesquiterpenoid and triterpenoid biosynthesis (ath00909). Expression levels of unigenes in sesquiterpenoid and triterpenoid biosynthesis and terpenoid backbone biosynthesis (ath00900), which supply farnesyl pyrophosphate, a precursor of sesquiterpenoid and triterpenoid, are indicated in Fig. 6 and Suppl. Table S4. The RPKM values suggested that farnesyl pyrophosphate may be synthesized in larger amounts in petiole latex and supplied for the biosynthesis of sesquiterpene. A similarity search of the unigenes against the protein database suggested that the products of sesquiterpenoid were germacrene D, germacrene A, 7-epi-α-selinene, δ-cadinene and/or humulene. Of these, germacrene D and δ-cadinene have been reported in *F. carica* (Gibernau et al. 1997; Oliveira et al. 2010; Lazreg-Aref et al. 2012; Mawa et al. 2013). Germacrene D, a volatile sesquiterpenoid, may have insecticidal activity against mosquitos (Kiran and Devi 2007) and act as a repellent against aphids (Bruce et al. 2005) and ticks (Birkett et al. 2008).

In the phenylpropanoid pathway, the synthesis of *p*-coumaroyl-CoA could be interesting. This product is a precursor of toxic furanocoumarins (Karamat et al. 2014; Munakata et al. 2016), such as psoralen and bergapten, which were both reported in *F. carica* (Mawa et al. 2013 for review). Candidates of glycosylated furanocoumarin were also found in our metabolome analysis (peaks 1890, 1892, 1893, 1894, 1924, 1944, 2165 and 2167 in Suppl. Table S2). These metabolites may be psoralic acid glucoside, which accumulates at high levels in leaves of *F. carica* (Takahashi et al. 2014, 2017). The pathway from phenylalanine to *p*-coumaroyl-CoA appeared to be more active in the petiole latex (Fig. 7; Suppl. Table S5). The prenyl group used in furanocoumarin biosynthesis comes from the terpenoid backbone biosynthesis pathway, which was also more active in the petiole latex (Fig. 6; Suppl. Table S4).

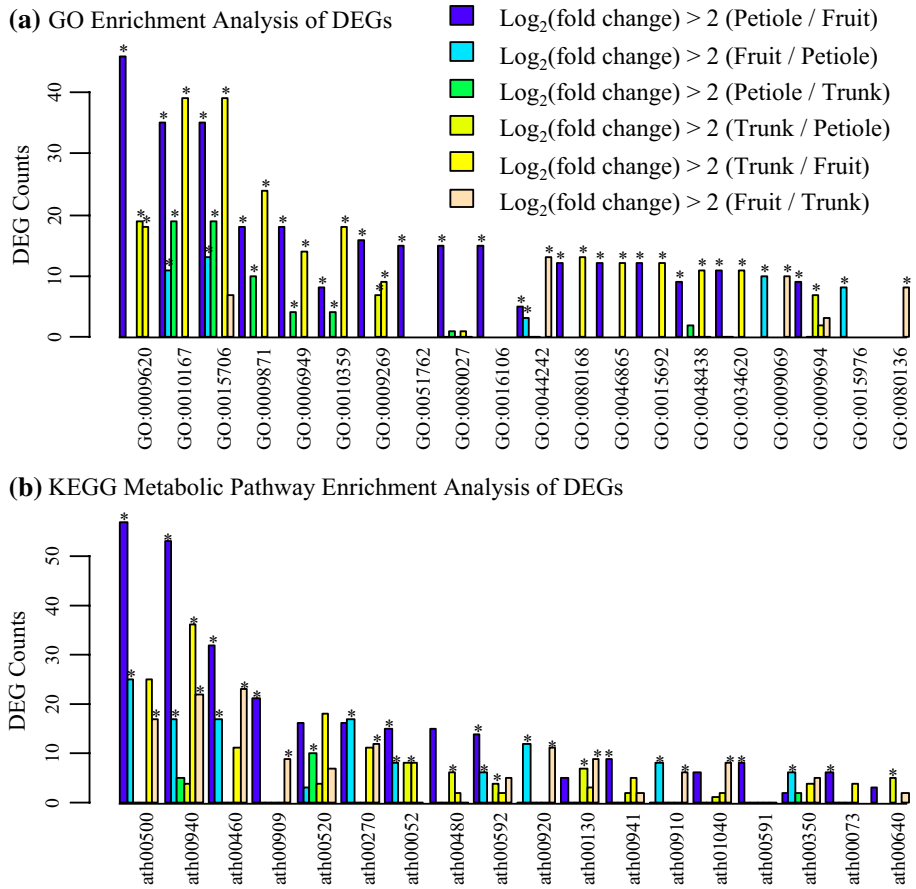

Fig. 5 Enrichment analysis of DEGs found in the RNA-seq analysis of latexes of three organs. DEGs, satisfying $\log_2$ (fold change) between paired latex samples > 2, adjusted *P* value < 0.01, and RPKM value ≥ 2 in at least one of paired latex samples, were subjected to GO enrichment analysis (**a**) and KEGG metabolic pathway enrichment analysis (**b**) based on sequence homologies to *Arabidopsis* proteins. Asterisk indicates adjusted *P* values in Fisher's exact test < 0.01 compared with the whole transcriptome of the latexes as the background. GO:0009620, response to fungus; GO:0010167, response to nitrate; GO:0015706, nitrate transport; GO:0009871, jasmonic acid and ethylene-dependent systemic resistance, ethylene mediated signaling pathway; GO:0006949, syncytium formation; GO:0010359, regulation of anion channel activity; GO:0009269, response to desiccation; GO:0051762, sesquiterpene biosynthetic process; GO:0080027, response to herbivore; GO:0016106, sesquiterpenoid biosynthetic process; GO:0044242, cellular lipid catabolic process; GO:0080168, abscisic acid transport; GO:0046865, terpenoid transport; GO:0015692, lead ion transport; GO:0048438, floral whorl development; GO:0034620, cellular response to unfolded protein; GO:0009069, serine family amino acid metabolic process; GO:0009694, jasmonic acid metabolic process; GO:0015976, carbon utilization; GO:0080136, priming of cellular response to stress; ath00500, starch and sucrose metabolism; ath00940, phenylpropanoid biosynthesis; ath00460, cyanoamino acid metabolism; ath00909, sesquiterpenoid and triterpenoid biosynthesis; ath00520, amino sugar and nucleotide sugar metabolism; ath00270, cysteine and methionine metabolism; ath00052, galactose metabolism; ath00480, glutathione metabolism; ath00592, α-linolenic acid metabolism; ath00920, sulfur metabolism; ath00130, ubiquinone and other terpenoid-quinone biosynthesis; ath00941, flavonoid biosynthesis; ath00910, nitrogen metabolism; ath01040, biosynthesis of unsaturated fatty acids; ath00591, linoleic acid metabolism; ath00350, tyrosine metabolism; ath00073, cutin, suberine and wax biosynthesis; ath00640, propanoate metabolism

DEGs for transcription factors and signal transduction proteins

The differential expression of these unigenes should be regulated by transcription factors and possibly signal transduction proteins. Of the 6163 DEGs, the RPKM values of 323 DEGs related to transcription factor (GO:0003700), and 120 DEGs associated with signal transduction (GO:0007165) but not with GO:0003700, were compared among the three organs' latexes (Fig. 8; Suppl. Table S6). Many of these unigenes were more highly expressed in the latexes of petiole or trunk than in fruit latex. These included homeobox domain-like transcription factors (InterPro ID: IPR009057), K-box domain transcription factors (IPR002487), AP2/ERF domain transcription factors (IPR001471), heat shock factor-type transcription factors (IPR000232), and zinc finger C2H2-type transcription factors (IPR013087), as well as leucine-rich repeat-containing proteins (IPR001611) and serine/threonine-protein kinase (IPR008271). The DEGs related to defense against biotic stresses, such as PR proteins

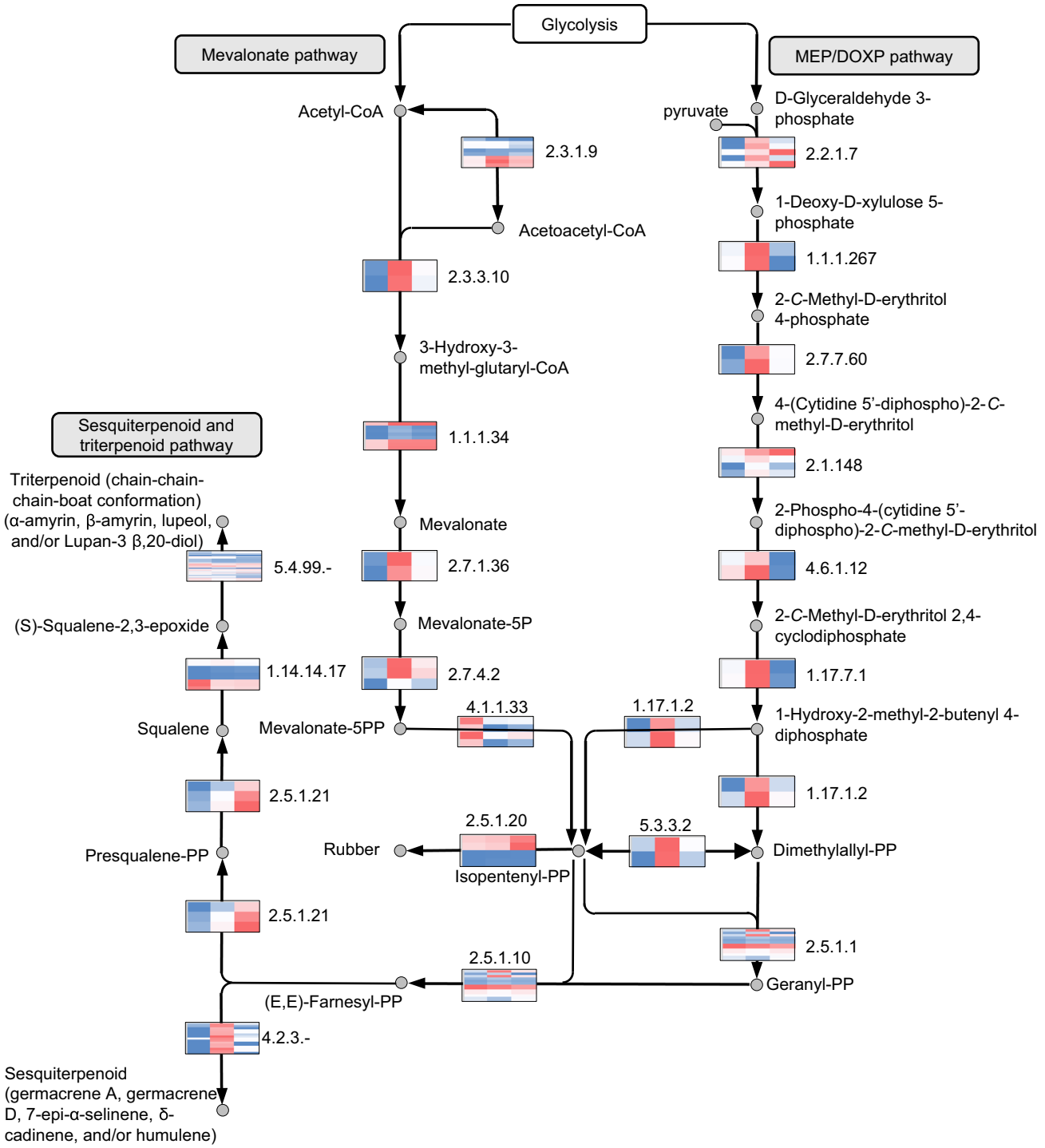

Fig. 6 Heatmap of average RPKM values ($n = 3$) for unigenes encoding enzymes for terpenoid backbone, sesquiterpenoid and triterpenoid biosynthesis. Unigenes with RPKM values < 2 in all three latex samples are not indicated. The biosynthetic pathway is drawn according to the KEGG pathways ath00900 and ath00909 with some modifications. RPKM values are shown from the left in the order of fruit, petiole and trunk latexes in each heatmap. Red and blue indicate high and low RPKM values, respectively. A list of unigenes and their RPKM values appears in Suppl. Table S4

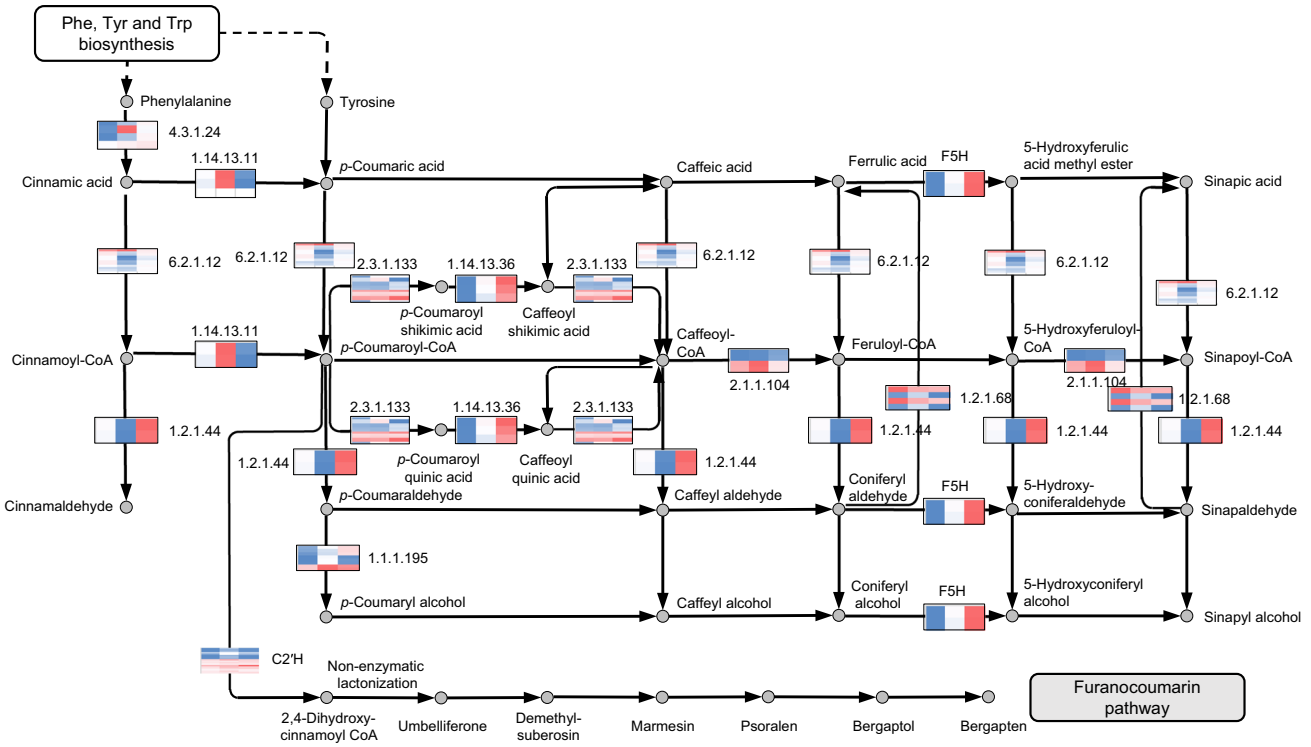

Fig. 7 Heatmap of average RPKM values ($n=3$) for unigenes encoding enzymes for the phenylpropanoid and furanocoumarin pathway. Unigenes with RPKM values < 2 in all three latex samples are not indicated. The biosynthetic pathway is drawn according to the KEGG pathway ath00940 with some modifications. RPKM values are shown from the left in the order of fruit, petiole and trunk latexes in each heatmap. Red and blue indicate high and low RPKM values, respectively. A list of unigenes and their RPKM values appears in Suppl. Table S5

and trypsin inhibitors, as well as those related to the metabolic pathways, might be regulated by these transcription factors and signal transduction proteins.

Discussion

In this study, we compared the proteomes, metabolomes and transcriptomes of latexes of immature fruit, young and unlignified petioles, and older and lignified trunks of *F. carica* to understand the diversity of latex-mediated defense strategies against pests. In any of the three organs' latexes, the proteins present in the highest amounts were isoforms of ficin and trypsin inhibitor. In addition, candidates of galloylglycerol, which produces a possible hydrolysis product that is an antimicrobial gallic acid, may be highly accumulated in all of the latexes. These findings support latex being a potent defensive element against pests in all three organs of *F. carica*. However, a quantitative analysis indicated that the latex contents were highly divergent among the three organs.

The expression levels of unigenes for PR proteins were highest in trunk latex. The higher expression level of the antifungal chitinase in trunk latex was consistent with our previous study on latexes in mulberry (Kitajima et al. 2012, 2013), and it may be a response to the severity of the fungal infection. For example, herbivorous insects may be the most threatening pests in unlignified organs, whereas resilient fungi may be more threatening to lignified organs.

The constituents in latexes of young petioles and immature fruit were highly different from each other, although they are similarly unlignified soft organs. Although fruit latex contained high amounts of ficins and trypsin inhibitors, the expression levels of other defense-related unigenes were likely less active than in petiole latex. This might be because, in contrast to petiole (and leaf) which must be always protected from attack by pests, the immature fruit of *F. carica* needs to attract fig wasps for pollination, and after ripening, the fruit needs to be eaten by animals to disperse the seeds.

In addition to the proteins that had previously been reported to be toxic to pests, such as proteases and chitinase, we found unigenes that were highly expressed or differentially expressed at transcript or protein levels that had no previously reported anti-pest functions. Moreover, some of the metabolites accumulated differentially among the three organs' latexes or may have accumulated at high amounts in all of them, although we could not identify many

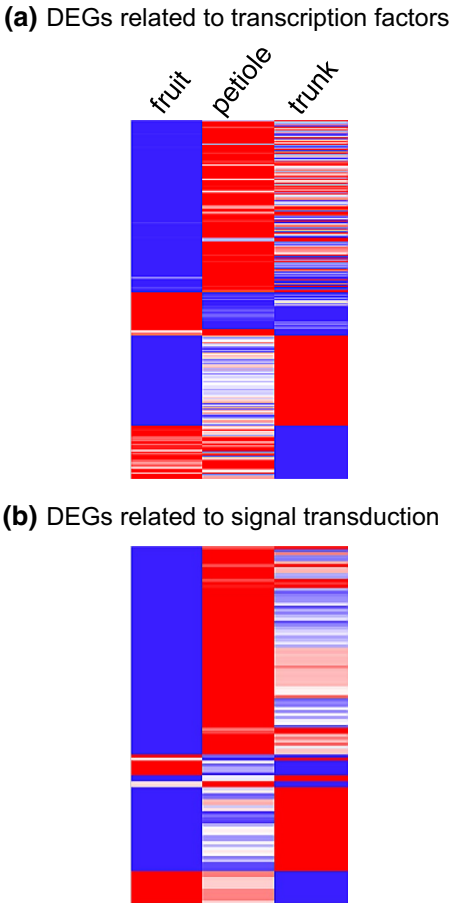

Fig. 8 The average RPKM values ($n=3$) of DEGs related to transcription factors and signal transduction. Of 6163 DEGs satisfying $\log_2$ (fold change) between paired latex samples > 2, adjusted P value < 0.01, and RPKM value > 2 in at least one of the paired latex samples, 323 DEGs related to transcription factor (GO:0003700) are shown in **a**, and 120 DEGs associated with signal transduction (GO:0007165) but not with GO:0003700 are shown in **b**. A list of DEGs and their RPKM values appear in Suppl. Table S6. Red and blue indicate high and low RPKM values, respectively

of these compounds. These unigene products or metabolites are possible candidates for novel defense-related proteins or chemicals.

Regulatory mechanisms of gene expression in laticifer cells have not been studied well. We found 443 unigenes, related to transcription factor or signal transduction, were differentially expressed among the three organs' latexes. They are possible candidates for regulators of the latex-mediated defense against pests.

In conclusion, through a multi-omic study, we revealed the diversity of latex-related defense strategies in organs of *F. carica*. The diversity might relate to different pests. The latex of the hardened trunk protects the plant mainly from attack by microbes; that of the young and soft petiole (and leaf) protects the plant mainly from attack by herbivores, and fruit need to not only protect the fruit but must also attract insect pollinators at younger stages and animals after ripening.

Author contribution statement SK conceived and designed research, conducted RNA-seq analysis, analyzed data and wrote the manuscript. TT and MK assisted in conceiving the research. EHS, SH and HY conducted computational analysis of RNA-seq data, 2D-PAGE and RT-PCR cloning. WA and SA conducted LC–MS-based proteome analysis. DS, DN and NS conducted metabolome analysis. KY and RM were involved in pathway analysis. All authors read and approved the manuscript.

Acknowledgements This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (to SK, No. 16K07641). The LC-Orbitrap analysis of proteome was technically supported by the Kyoto Integrated Science and Technology Bio-Analysis Center. We wish to thank Dr. Jun Wada for his generous aid in the proteome analysis.

References

Akimoto N, Ara T, Nakajima D, Suda K, Ikeda C, Takahashi S, Muneto R, Yamada M, Suzuki H, Shibata D, Sakurai N (2017) FlavonoidSearch: a system for comprehensive flavonoid annotation by mass spectrometry. Sci Rep UK 7:1243. https://doi.org/10.1038/s41598-017-01390-3

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389–3402. https://doi.org/10.1093/nar/25.17.3389

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B Methodol 57:289–300

Birkett MA, Al Abassi S, Kröber T, Chamberlain K, Hooper AM, Guerin PM, Pettersson J, Pickett JA, Slade R, Wadhams LJ (2008) Antiectoparasitic activity of the gum resin, gum haggar, from the East African plant, *Commiphora holtziana*. Phytochemistry 69(8):1710–1715. https://doi.org/10.1016/j.phytochem.2008.02.017

Bruce TJ, Birkett MA, Blande J, Hooper AM, Martin JL, Khambay B, Prosser I, Smart LE, Wadhams LJ (2005) Response of economically important aphids to components of *Hemizygia petiolata* essential oil. Pest Manag Sci 61(11):1115–1121. https://doi.org/10.1002/ps.1102

Chanwitheesuk A, Teerawutgulrag A, Kilburn JD, Rakariyatham N (2007) Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk. Food Chem 100(3):1044–1048. https://doi.org/10.1016/j.foodchem.2005.11.008

Farag MA, Abdelfattah MS, Badr SE, Wessjohann LA (2014) Profiling the chemical content of *Ficus lyrata* extracts via UPLC–PDA–qTOF–MS and chemometrics. Nat Prod Res 28(19):1549–1556. https://doi.org/10.1080/14786419.2014.926353

Federici L, Di Matteo A, Fernandez-Recio J, Tsernoglou D, Cervone F (2006) Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends Plant Sci 11(2):65–70. https://doi.org/10.1016/j.tplants.2005.12.005

Field D, Tiwari B, Booth T, Houten S, Swan D, Bertrand N, Thurston M (2006) Open software for biologists: from famine to feast. Nat Biotechnol 24(7):801–803. https://doi.org/10.1038/nbt0706-801

Fisher RA (1922) On the interpretation of χ^2 from contingency tables, and the calculation of P. J R Stat Soc 85(1):87–94. https://doi.org/10.2307/2340521

Friedman M, Henika PR, Mandrell RE (2003) Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J Food Prot 66(10):1811–1821. https://doi.org/10.4315/0362-028X-66.10.1811

Gañan M, Martínez-Rodríguez AJ, Carrascosa AV (2009) Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. Food Control 20(8):739–742. https://doi.org/10.1016/j.foodcont.2008.09.012

Gibernau M, Buser HR, Frey JE, Hossaert-McKey M (1997) Volatile compounds from extracts of figs of *Ficus carica*. Phytochemistry 46(2):241–244. https://doi.org/10.1016/S0031-9422(97)00292-6

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29(7):644–652. https://doi.org/10.1038/nbt.1883

Hagel JM, Yeung EC, Facchini PJ (2008) Got milk? The secret life of laticifers. Trends Plant Sci 13(12):631–639. https://doi.org/10.1016/j.tplants.2008.09.005

Hilder VA, Gatehouse AM, Sheerman SE, Barker RF, Boulter D (1987) A novel mechanism of insect resistance engineered into tobacco. Nature 330(6144):160–163. https://doi.org/10.1038/330160a0

Huang Y, Niu B, Gao Y, Fu L, Li W (2010) CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26(5):680–682. https://doi.org/10.1093/bioinformatics/btq003

Huynh QK, Borgmeyer JR, Zobel JF (1992) Isolation and characterization of a 22 kDa protein with antifungal properties from maize seeds. Biochem Biophys Res Commun 182(1):1–5. https://doi.org/10.1016/S0006-291X(05)80103-2

Jimenez CR, Huang L, Qiu Y, Burlingame AL (2003) In-gel digestion of proteins for MALDI-MS fingerprint mapping. In: Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT (eds) Current protocols in protein science. Wiley, Hoboken, pp 16.4.1–16.4.5

Karamat F, Olry A, Munakata R, Koeduka T, Sugiyama A, Paris C, Hehn A, Bourgaud F, Yazaki K (2014) A coumarin-specific prenyltransferase catalyzes the crucial biosynthetic reaction for furanocoumarin formation in parsley. Plant J 77(4):627–638. https://doi.org/10.1111/tpj.12409

Karnchanatat A, Tiengburanatam N, Boonmee A, Puthong S, Sangvanich P (2011) Zingipain, a cysteine protease from *Zingiber ottensii* Valeton rhizomes with antiproliferative activities against fungi and human malignant cell lines. Prep Biochem Biotechnol 41(2):138–153. https://doi.org/10.1080/10826068.2011.547347

Kiran SR, Devi PS (2007) Evaluation of mosquitocidal activity of essential oil and sesquiterpenes from leaves of *Chloroxylon swietenia* DC. Parasitol Res 101(2):413–418. https://doi.org/10.1007/s00436-007-0485-z

Kitajima S, Kamei K, Taketani S, Yamaguchi M, Kawai F, Komatsu A, Inukai Y (2010) Two chitinase-like proteins abundantly accumulated in latex of mulberry show insecticidal activity. BMC Biochem 11(1):6. https://doi.org/10.1186/1471-2091-11-6

Kitajima S, Taira T, Oda K, Yamato KT, Inukai Y, Hori Y (2012) Comparative study of gene expression and major proteins' function of laticifers in lignified and unlignified organs of mulberry. Planta 235(3):589–601. https://doi.org/10.1007/s00425-011-1533-6

Kitajima S, Yamamoto Y, Hirooka K, Taki C, Hibino S (2013) Laticifers in mulberry exclusively accumulate defense proteins related to biotic stresses. Plant Biotechnol 30(4):399–402. https://doi.org/10.5511/plantbiotechnology.13.0326a

Kitajima S, Miura K, Aoki W, Yamato KT, Taira T, Murakami R, Aburaya S (2016) Transcriptome and proteome analyses provide insight into laticifer's defense of *Euphorbia tirucalli* against pests. Plant Physiol Biochem 108:434–446. https://doi.org/10.1016/j.plaphy.2016.08.008

Konno K (2011) Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. Phytochemistry 72(13):1510–1530. https://doi.org/10.1016/j.phytochem.2011.02.016

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. Plant J 37(3):370–378. https://doi.org/10.1046/j.1365-313X.2003.01968.x

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9(4):357–359. https://doi.org/10.1038/nmeth.1923

Lazreg-Aref H, Mars M, Fekih A, Aouni M, Said K (2012) Chemical composition and antibacterial activity of a hexane extract of Tunisian caprifig latex from the unripe fruit of *Ficus carica*. Pharm Biol 50(4):407–412. https://doi.org/10.3109/13880209.2011.608192

Lewinsohn TM (1991) The geographical distribution of plant latex. Chemoecology 2(1):64–68. https://doi.org/10.1007/BF01240668

Liu Y, Ahn JE, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K (2005) Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. Plant Physiol 139(3):1545–1556. https://doi.org/10.1104/pp.105.066837

López-García B, Hernández M, Segundo BS (2012) Bromelain, a cysteine protease from pineapple (*Ananas comosus*) stem, is an inhibitor of fungal plant pathogens. Lett Appl Microbiol 55(1):62–67. https://doi.org/10.1111/j.1472-765X.2012.03258.x

Matsui K, Bae J, Esaka K, Morisaka H, Kuroda K, Ueda M (2013) Exoproteome profiles of *Clostridium cellulovorans* grown on various carbon sources. Appl Environ Microbiol 79(21):6576–6584. https://doi.org/10.1128/AEM.02137-13

Mawa S, Husain K, Jantan I (2013) *Ficus carica* L. (Moraceae): phytochemistry, traditional uses and biological activities. Evid Based Complement Altern Med 2013:974256. https://doi.org/10.1155/2013/974256

Munakata R, Olry A, Karamat F, Courdavault V, Sugiyama A, Krieger C, Silie P, Foureau E, Papon N, Grosjean J, Yazaki K (2016) Molecular evolution of parsnip (*Pastinaca sativa*) membrane-bound prenyltransferases for linear and/or angular furanocoumarin biosynthesis. New Phytol 211(1):332–344. https://doi.org/10.1111/nph.13899

Nohynek LJ, Alakomi HL, Kähkönen MP, Heinonen M, Helander IM, Oksman-Caldentey KM, Puupponen-Pimiä RH (2006) Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. Nutr Cancer 54(1):18–32. https://doi.org/10.1016/j.foodchem.2010.04.064

Oliveira AP, Silva LR, de Pinho PG, Gil-Izquierdo A, Valentão P, Silva BM, Pereira JA, Andrade PB (2010) Volatile profiling of *Ficus carica* varieties by HS-SPME and GC–IT–MS. Food Chem 123(2):548–557. https://doi.org/10.1016/j.foodchem.2010.04.064

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) *limma* powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acid Res 43(7):e47–e47. https://doi.org/10.1093/nar/gkv007

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital

gene expression data. Bioinformatics 26(1):139–140. https://doi.org/10.1093/bioinformatics/btp616

Sakurai N, Ara T, Enomoto M, Motegi T, Morishita Y, Kurabayashi A, Iijima Y, Ogata Y, Nakajima D, Suzuki H, Shibata D (2014) Tools and databases of the KOMICS web portal for preprocessing, mining, and dissemination of metabolomics data. Biomed Res Int 2014:194812. https://doi.org/10.1155/2014/194812

Shin R, Kim MJ, Paek KH (2003) The *CaTin1* (*Capsicum annuum* TMV-induced clone 1) and *CaTin1-2* genes are linked head-to-head and share a bidirectional promoter. Plant Cell Physiol 44(5):549–554. https://doi.org/10.1093/pcp/pcg069

Takahashi T, Okiura A, Saito K, Kohno M (2014) Identification of phenylpropanoids in fig (*Ficus carica* L.) leaves. J Agric Food Chem 62(41):10076–10083. https://doi.org/10.1021/jf5025938

Takahashi T, Okiura A, Kohno M (2017) Phenylpropanoid composition in fig (*Ficus carica* L.) leaves. J Nat Med 71(4):770–775. https://doi.org/10.1007/s11418-017-1093-6

Terras FR, Schoofs HM, Thevissen K, Osborn RW, Vanderleyden J, Cammue BP, Broekaert WF (1993) Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. Plant Physiol 103(4):1311–1319. https://doi.org/10.1104/pp.103.4.1311

Van Loon LC (1999) Occurrence and properties of plant pathogenesis-related proteins. In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press LLC, Boca Raton, pp 1–20

Veberic R, Colaric M, Stampar F (2008) Phenolic acids and flavonoids of fig fruit (*Ficus carica* L.) in the northern Mediterranean region. Food Chem 106(1):153–157. https://doi.org/10.1016/j.foodchem.2007.05.061

Wang X, Shi M, Lu X, Ma R, Wu C, Guo A, Peng M, Tian W (2010) A method for protein extraction from different subcellular fractions of laticifer latex in *Hevea brasiliensis* compatible with 2-DE and MS. Proteome Sci 8(1):35. https://doi.org/10.1186/1477-5956-8-35

Wasano N, Konno K, Nakamura M, Hirayama C, Hattori M, Tateishi K (2009) A unique latex protein, MLX56, defends mulberry trees from insects. Phytochemistry 70(7):880–888. https://doi.org/10.1016/j.phytochem.2009.04.014