Proteome Analysis of Tea Pollen (*Camellia sinensis*) under Different Storage Conditions

JIANKE LI,[†] JIAN CHEN,[†,§] ZHAOHUI ZHANG,[‡] AND YINGHONG PAN*,[‡]

Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing 100093, China; Institute of Crop Science, Chinese Academy of Agricultural Sciences, The National Key Facility for Crop Gene Resources and Genetic Improvement, 12 Zhongguancun Nandajie, Beijing 100081, China; and Department of Bioengineering, Zhengzhou University, Zhengzhou 450001, China

The protein complement of tea pollen collecting from tea tree (*Camellia sinensis*) was compared under different storage conditions. Protein was partially identified using a combination of the 2D-PAGE, MALDI-TOF/MS, MASCOT, and Xproteo search engine. Two hundred and sixty-nine and 396 proteins were detected in pollen stored at room temperature (RT) and −20 °C, respectively. Forty-three of the identified proteins were assigned to defense-related functions, energy metabolism, cytoskeleton, nucleic acid metabolism, membrane transport, amino acid metabolism, stress response, protein metabolism, transcription, fat metabolism, others, and function unknown proteins. The abundance analysis showed that more proteins related to stress response, nucleic acid metabolism, fat metabolism, and membrane transport are lost at RT than at −20 °C, while proteins related to defense and energy metabolism showed a reverse relation. For the others, no differences were found between the two storage conditions. During the determination of the peptides mass fingerprinting (PMF) of each spot, 35 proteins were identified in tea pollen for the first time. Thus, our data present an initial molecular picture of bee colleted tea pollen, and our results suggest that freezing is the best way to maintain the quality of tea pollen.

INTRODUCTION

Bee pollen is collected from the male reproductive part of flowering plants. The pollen is collected by worker bees and brought to the hive where they add enzymes and nectar to the pollen. Bee pollen is promoted as a health food with a wide range of nutritional and therapeutic properties (*1, 2*). The best-documented health benefit of pollen for humans is undoubtedly the treatment of chronic prostatitis. Consumption of pollen preparations have been shown in several studies to reduce the inflammation, discomfort, and pathology of patients suffering from prostatic inflammation (*3*). Another benefit of pollen is its ability to help protect against the adverse effects of X-rays based on evidence of a radioprotective effect of ingested pollen for both animals and humans (*4*). Bee pollen could also treat chronic hepatitis B in children (*5*) and influence on lipid peroxidation, antioxidant system, and liver function in old

animals (*6*). Recent studies show that bee pollen extracts have antioxidative and radical scavenging abilities (*1, 7*). Nutritional analysis indicates that pollen storage results in a decrease in crude protein (51−28% dry weight) and lipid content (10−8% dry weight). Fatty acids comprised a higher proportion of total lipids in fresh pollen than in stored pollen (*8*). In the most active floral pollens, which tend to contain the highest levels of flavonoids/phenolic acids, pollen aged over 3 resulted in a reduction of the free radical scavenging activity by up to 50% (*9*). Ozone treated pollen causes significant changes in fatty acid composition, thiobarbituric acid value, and pigment by lipid oxidation and decoloration (*10*).

Tea pollen is one of the important bee pollens, which is collected from tea tree (*Camellia sinensis*) flowering in early November. More than 2000 tons are harvested annually in China. Because of its moderate sweet taste, light brown color, and delicate fragrance, it has been widely used in health care bee products in recent years. Tea pollen receives much attention as a health promotion food. But, knowledge about how storage condition affects its functional ingredients is still scarce. The purpose of this study was to provide solid experimental data on how to store tea pollen properly. Rapid advances in proteomic technologies, along with completion of the Arabidopsis and rice genome sequence projects and the availability of comprehensive public sequence databases, have provided tremendous impetus

* To whom correspondence should be addressed. E-mail: apislijk@126.com. Phone: +86-10-6259-1449. Fax: +86-10-6259-1449.
[†] Institute of Apicultural Research, Chinese Academy of Agricultural Science.
[‡] Institute of Crop Science/The National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Science.
[§] Zhengzhou University.

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to proteomics research into protein change of tea pollen subjected to different storage conditions. To date, the proteome analyses of mature *Arabidopsis thaliana* (*11, 12*), tomato (*13*), and *Oryza sativa* pollen (*14*) have also been conducted. Many proteins identified correspond to the known transcripts in pollen, in addition to several other proteins in each of these studies.

By combining two-dimensional gel electrophoresis (2-DE) with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) and by using the available databases for other species, as well as tea expressed sequence tags (3234 ESTs), a comprehensive analysis of the tea pollen proteome has been performed. Many of the proteins identified in this study have designated roles in defense mechanisms, energy conversion, pollen germination, pollen tube growth, and germ cell formation. To our knowledge, this is the first proteomic study on tea pollen, and several proteins reported here have not been identified in the pollen of other species.

MATERIALS AND METHODS

Chemical Agents. The immobilized pH gradient (IPG) strip (pH 3−10, linear), 2-DE marker, Biolyte (pH 3−10), and mineral oil are from Bio-Rad Laboratories (Hercules, CA). Tris-base, ammonium persulfate (AP), agarose, sodium dodecyl sulfate (SDS), *N,N,N′,N′*-tetramethylethylene diamine (TEMED), and glycin are from Sigma (St.Louis, MO). Acrylamide, *N,N′*-methylenebisacrylamide, bromophenol blue, Coomassie brilliant blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), urea, glycerol, and bovine serum albumin (BSA) are from Amresco (Solon, OH); Dithiothreitol (DTT) and iodoacetamide are from Merck (Darmstadt, Germany). Trypsin was from Roche;trifluoroacetic acid (TFA) and acetonitrile were from JT Baker (Phillipsburg, NJ).

Tea Pollen Samples. During the tea tree (*Camellia sinensis*) flowering period (November, 2006) in southern China's Zhejiang province, a major tea producing zone, a pollen trap was placed in the entrance of the bee hive to collect the tea pollen. Pollen from five bee hives were pooled as one sample. Three pooled batches were collected during the flowering period at three different times in the year. To guarantee that pure tea pollen was sampled, the pollen was picked out by its light brown color after being trapped. Then, the sampled pollen was dried in a cabinet dryer at 60 °C, a conventional way in the beekeeping industry. It was stored at −20 °C or at room temperature (RT) for 6 months.

Preparation of Protein Samples. Protein extraction was according to Li et al. (*15*). To protect from protein hydrolysis and breakdown during the extraction procedures, protein was always extracted on ice. Totals of 5 g of bee collected tea pollen (1 mg pollen/10 μL buffer) were added to phosphate buffer (PB) pH 7.6, containing 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 400 mM NaCl. The pollen were homogenized for 20 min on ice and sonicated for 2 min, centrifuged at 12 000*g* and 4 °C for 10 min, and then further centrifuged at 15 000*g* and 4 °C for 10 min. The supernatant was transferred to another tube for further analysis. The pellets (1 mg pollen/2 μL buffer) were resuspended in PB and then centrifuged at 15 000*g* and 4 °C for 10 min. The supernatant was removed and added to the tube containing supernatant as a PB-soluble proteins extract while the pellets (1 mg pollen/10 μL buffer) of PB-insoluble proteins were mixed in lysis buffer (LB) (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Biolyte pH 3−10). The mixture was homogenized for 10 min on ice and sonicated for 2 min, and centrifuged at 15 000*g* and 4 °C for 10 min. The supernatant was removed and added to the tube containing PB-soluble protein extract, and the debris was discarded. Trichloroacetic (TCA) was added to the collected supernatants at a final concentration of 10%, and then, the mixture was kept on ice for 10 min for protein precipitation and desalting. Subsequently, the mixture was centrifuged twice at 15 000*g* and 4 °C for 10 min. The supernatant was discarded, and the pellets (1 mg pollen/4 μL buffer) were dissolved in LB. Then, the mixture was homogenized for 5 min on ice, sonicated for 2 min, and subsequently adjusted to pH 7.0 with 2 M NaOH. The pollen

protein extract was stored at −70 °C for further use. Protein concentration was determined according to the method developed by Bradford (*16*) using BSA as the standard. The absorption was measured at 595 nm (Beckman, spectrophotometer DU800).

Two-Dimensional Gel Electrophoresis (2-DE). A 400 μg protein sample extracted from 5014 μg of pollen was suspended in LB and then mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, 0.2% Biolyte pH 3−10). The mixture was loaded on a 17 cm IPG strip (pH 3−10, linear, Bio-Rad Hercules, CA). Isoelectric focusing electrophoresis (IEF) was performed at 18 °C (Protean IEF Cell. Bio-Rad Hercules, CA) according to the following program: 14 h at 50 V; 250 V for 30 min × 4; 1000 V for 60 min; 9000 V for 5 h; 9000 V to 60 000 V·h. Before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the IPG strips were first equilibrated for 15 min in equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2% DTT) and then placed in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetoamide) for 15 min. After equilibration, the strip was overlaid onto the SDS-PAGE gel, 12% T separating gel (1.00 mm). The second dimension electrophoresis, SDS-PAGE, was performed in a Protean II Xi Cell (Bio-Rad Hercules, CA) at 25 mA/gel for 6.5 h.

Image Acquisition and Analysis. Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) orthophosphoric acid, washed with water (3 × 20 min), and stained with colloidal Coomassie blue G-250 (CCB). After washing with water, gels were scanned, annotated, and analyzed for spot number using PDQuest V 7.3.0 (Bio-Rad Hercules, CA). Five replicate gels were run for each of three pooled pollen samples collected from different batches of the bee colony.

To accurately compare samples with variations in spot size and intensity not due to protein abundance, normalization was done by defining a quantity of Actin-3 as the protein standard. Significance of the normalized volume in total density of identified proteins in all gels was tested by ANOVA (Version 6.12, SAS Institute, Cary, NC) using a Duncan's parametric test. An error probability of $P < 0.05$ was considered to be statistically significant.

Tryptic Digestion. CBB stained spots were excised and destained for 30 min using 100 mL of acetonitrile (50%) and 25 mM $(NH_4)HCO_3$ pH 8 (50%) until the gels were transparent. They were dried for 10 min with acetonitrile (100%) and then for 30 min using a Speed-Vac system. At this point, 2.5 mL of 25 mM $(NH_4)HCO_3$ was added to 25 μg of trypsin (final concentration 10 ng/μL); 10 μL of this solution was pipetted on each dried protein spot and incubated for 60 min at 4 °C. The supernatant was discarded to minimize auto digestion of trypsin. The Eppendorf tube was then turned upside down, and the sample was incubated for 14 h at 37 °C. To extract the peptide fragments from the tryptic digests, 20 μL of 5% (v/v) TFA was added and incubated for 60 min at 37 °C. Then, 20 μL of 50% (v/v) acetonitrile (containing 2.5% (v/v) TFA) was added to the gel and incubated for 60 min at 30 °C. After each step, the supernatants were pooled and dried using a Speed-Vac system.

MALDI-TOF/MS and Protein Identification. Before obtaining the mass spectra of the peptide mixture, the digested proteins were desalted and cleaned with ZipTip C18 pipet tips (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. All analyses were performed using a Bruker Daltonics Autoflex system (Bruker Daltonics Billerica, MA) operated in the delayed extraction (190 ns) and reflector mode with an accelerating voltage of 20 kV. Peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics Billerica, MA) in 50% acetonitrile/0.1% TFA. External calibration was performed using a peptide calibration standard (Bruker Daltonics Billerica, MA, part no. 206195) and internal calibration with trypsin autoproteolytic fragments. Masses of proteolytic peptide fragments were determined by peptide mass fingerprinting (PMF), which is a MALDI-TOF/MS based protein identification technique. To interpret the mass spectra of protein digests, the generated peak lists of the tryptic peptide masses were searched against MASCOT (http://www.matrixscience.com/search_form_select.html) and Xproteo (http://xproteo.com:2698). Search parameters for MASCOT were as follows: trypsin cleavage; allow up to one missed cleavage; no restriction on protein mass; peptide mass tolerance of 100 ppm;
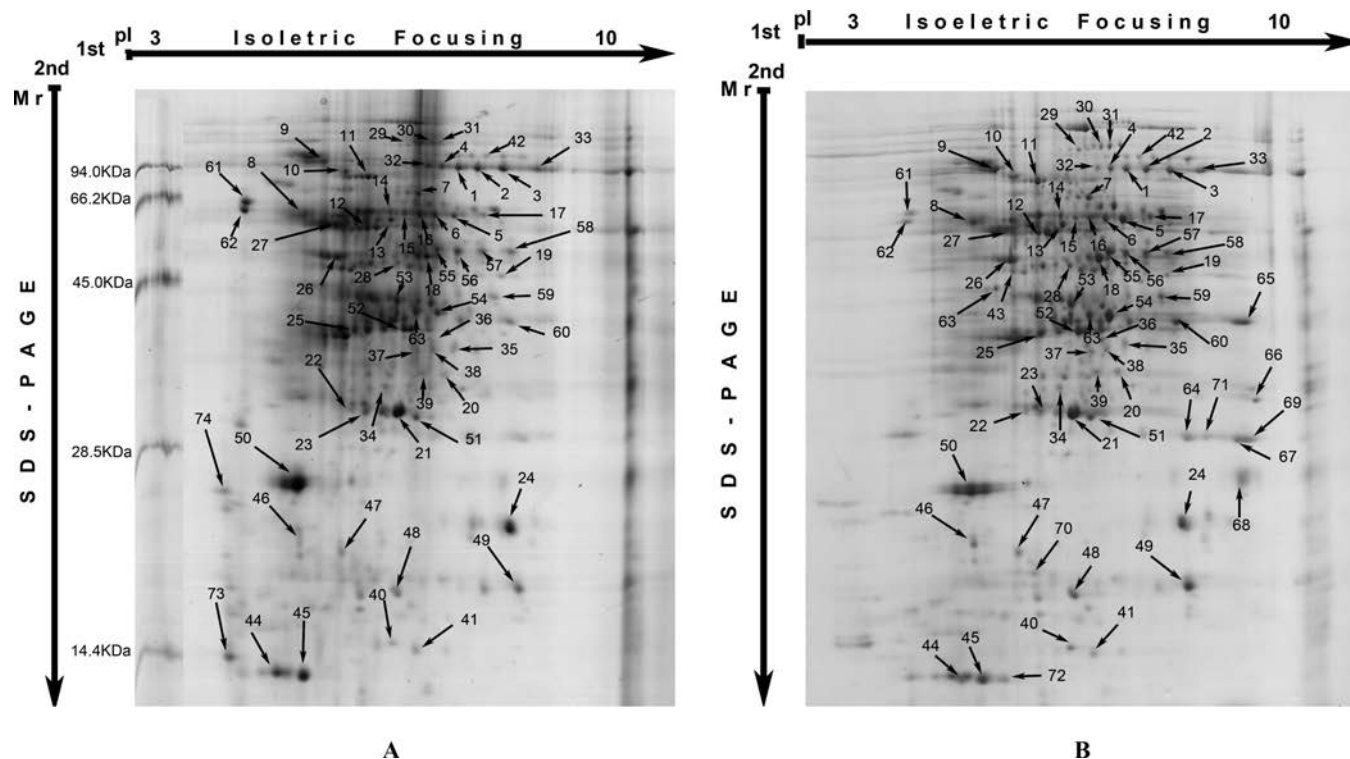
Figure 1. Protein spots subject to tryptic digestion, MALDI-TOF/MS, and identification. Shown is a representative profile of tea pollen collecting from tea tree (*Camellia sinensis*). A total of 400 μg of tea pollen sample was subjected to 2-DE and stained by CCB G-250. Images of tea pollen stored under (**A**) room temperature and (**B**) −20 °C. Number-labeled spots were cut out and subjected to tryptic digestion for mass spectrometry analysis.

quantitative modification, carbamidomethyl (C); and nonquantitative modification, oxidation (M). Search parameters for Xproteo were as follows: trypsin cleavage; allow up to one missed cleavage; protein mass of 0−300 kDa; protein pI 1−14; and peptide mass tolerance of 100 ppm. Proteins identified with a Mowse score greater than 79 and $D' > 4$ (significant at 95% confidence interval) are reported.

RESULTS

2-DE Images of Tea Pollen at Frozen and RT. **Figure 1** is a representative gel image of the best five runs from three different pooled pollen samples collected from different batches of the bee colony, showing the soluble proteins extracted from tea pollen, separated by 2-DE on pH 3−10 IPG strips, and stained with CCB. A total of 261 and 396 reproducible protein spots were detected on pollen stored at RT and −20 °C, respectively. The number of protein spots observed at RT was significant lower than those at −20 °C ($P < 0.05$).

Seventy-four proteins with higher abundance were selected throughout the molecular mass and isoelectric point (pI) ranges of 3−10 gels and analyzed by MALDI TOF/MS. Of these, 62 spots (**Figure 1**, spots 1−62) well resolved to the images both of RT and −20 °C, while 10 (63−72) and 2 spots (73, 74) specific to −20 °C and RT were selected to identify (**Figure 1**).

Forty-three proteins, representing 38 distinct proteins, were successfully analyzed. Despite the limited availability of tea tree protein sequence data, it was possible to identify 58% of the selected spots. The identified proteins, along with the gene index number and score, are listed in **Table 1**. Thirty-one proteins could not be identified because the Mowse score could not meet the significant threshold, and this is the limitation of MALDI TOF/MS.

Functional Grouping and Quantitative Analysis of Identified Proteins. The identified proteins were categorized into 12 functional groups (**Figure 2**) based on predicted protein function.

Nearly half of the identified proteins were in four major groups (i.e., energy (13.2%), nucleic acid metabolism (10.3%), and cytoskeleton (10.5%) and amino acid metabolism (7.9%)). The largest group is the identified proteins with unknown functions (21.1%) when checked by sequence or domain homology with other organisms. The other groups included proteins involved in membrane transport, amino acid metabolism, stress response, protein metabolism, transcription, fat metabolism, and so forth (**Figure 2**).

On the basis of the normalized quantity, proteins of nucleic acid metabolism, membrane transport, stress response, and fat metabolism were significant higher at −20 °C than those at RT ($P < 0.05$) respectively (**Figure 3D**, **E**, **G**, **J**). While proteins of defense and energy metabolism were significant higher at RT than those at −20 °C, respectively (**Figure 3A**, **B**). Other proteins involving cytoskeleton, amino acid and protein metabolism, and transcription (**Figure 3C**, **F**, **H**, **I**) observed no significant differences between RT and −20 °C, respectively ($P > 0.05$). One major royal jelly protein (MRJP) 3 was identified at −20 °C; MRJP 2 was found not to differ under the two conditions (**Figure 3K**). As for eight identified proteins with functions unknown (spots 5, 6, 12, 16, 20, 24, 29, 39), the abundance of three spots (12, 24, 39) was significantly lower at RT than at −20 °C ($P < 0.05$), while others observed no differences between the two conditions ($P > 0.05$, **Figure 3L**). For the 19 selected but not identified spots, 8 were significantly lower at RT than at −20 °C, 9 were not significantly different under the two conditions, and only 2 spots were significantly higher at RT than at −20 °C (**Figure 3M**).

DISCUSSION

On the basis of 2-DE patterns of pollen stored at RT and −20 °C, the detected number of proteins is significantly lower at RT than at −20 °C, indicating that pollen kept at RT lose

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Table 1. Proteins Identified in Tea Pollen Collecting from Tea Tree (Camellia sinensis)[a]

spot no.	pI/MW	sequence coverage	matched peptides	score	protein identity	accession no.	database
					defense-related proteins		
1	8.62/63.41	16.0%	10(34)	87	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
2	8.6/63.45	20.1%	10(33)	D' = 4.6	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
3	8.6/63.45	23.5%	11(33)	D' = 9.4	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
4	8.62/63.41	24.0%	11(19)	120	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
32	8.62/63.41	18.0%	8(21)	81	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
33	8.62/63.41	25.0%	12(37)	109	pollen-specific protein (Camellia sinensis)	gi|114152675	NCBInr
36	5.83/20.24	58.0%	7(52)	78	antibiotic resistance protein (Methanosarcina acetivorans)	Q8TRH3_METAC	MSDB
					energy metabolism		
10	5.11/68.77	22.0%	9(37)	73	vacuolar ATP synthase catalytic subunit A (Arabidopsis thaliana) (mouse-ear cress)	VATA_ARATH	SwissProt
13	5.23/55.56	29.0%	11(35)	84	ATP synthase subunit alpha, mitochondrial (Oenothera biennis) (German evening primrose)	ATPAM_OENBI	SwissProt
19	5.77/36.54	20.0%	5(8)	80	OSJNBa0044K18.22 (Oryza sativa) (Japonica cultivar-group)	gi|38605779	NCBInr
23	5.76/47.25	25.0%	8(30)	84	thiamine biosynthesis protein (Victivallis vadensis ATCC BAA-548)	gi|150385419	NCBInr
25	6.03/33.08	36.0%	6(21)	78	porphobilinogen deaminase precursor (EC 2.5.1.61) (Mesorhizobium sp. BNC1)	Q3WXP8_9RHIZ	MSDB
					cytoskeleton		
8	4.77/49.82	25.0%	11(41)	97	β-tubulin (Oryza sativa) (Japonica cultivar-group)	gi|303842	NCBInr
15	6.07/158.50	12.0%	15(34)	87	ankyrin repeat domain 30A (Pan troglodytes)	gi|114630218	NCBInr
26	5.31/41.61	22.0%	7(10)	82	actin-3 (Pisum sativum) (garden pea)	ACT3_PEA	SwissProt
35	9.90/25.18	38.0%	6(24)	81	ankyrin repeat domain 54 (Ornithorhynchus anatinus)	gi|149622678	NCBInr
					nucleic acid metabolism		
14	9.13/45.99	30.0%	10(39)	80	exodeoxyribonuclease VII large chain Escherichia coli (K12) (EC 3.1.11.6) (Candidatus Kuenenia stuttgartiensis)	Q1PV41_9BACT	MSDB
17	7.78/24.38	41.0%	7(30)	78	adenylate kinase (EC 2.7.4.3) (Francisella tularensis subsp. holarctica) (strain LVS)	Q2A425_FRATH	MSDB
28	9.56/24.40	46.0%	7(23)	89	endonuclease V (Methylococcus capsulatus)	NFI_METCA	SwissProt
38	9.96/47.44	26.0%	9(45)	78	transposase, IS605 OrfB (Crocosphaera watsonii)	Q4C6F4_CROWT	MSDB
					membrane transport		
11	5.04/24.25	31.0%	6(17)	79	Ypt-like GTP-binding protein (yeast) (Pichia pastoris)	JC7590	MSDB
37	8.64/162.49	16.0%	18(66)	85	sec7 domain protein (Aspergillus clavatus NRRL 1)	gi|121704660	NCBInr
40	8.65/59.41	21.0%	8(24)	81	peptide ABC transporter, ATP-binding protein (Pseudomonas stutzeri A1501)	gi|146282075	NCBInr
					amino acid metabolism		
21	5.76/35.64	24.0%	7(18)	80	putative aspartate carbamoyltransferase catalytic subunit (uncultured crenarchaeote)	gi|42557731	NCBInr
30	6.09/84.54	20.0%	9(44)	72	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (Solenostemon scutellarioides) (Coleus blumei)	METE_SOLSC	SwissProt
31	6.09/84.54	21.0%	10(49)	74	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (Solenostemon scutellarioides) (Coleus blumei)	METE_SOLSC	SwissProt
					stress response		
9	5.03/71.34	16.0%	12(28)	78	heat shock cognate 70 kDa protein 2 (Arabidopsis thaliana) (mouse-ear cress)	HSP72_ARATH	SwissProt
22	8.98/33.01	25.0%	7(38)	80	urease accessory protein UreD (Mesorhizobium sp. BNC1)	gi|110635014	NCBInr
					protein metabolism		
7	5.38/13.06	13.0%	14(34)	84	ubiquitin-specific protease, putative (Arabidopsis thaliana)	gi|30681938	NCBInr
41	9.80/11.41	56.0%	5(28)	68	60S ribosomal protein L30 (Saccharomyces cerevisiae) (Baker's yeast)	RL30_YEAST	SwissProt
					transcription		
18	6.43/20.35	50.0%	6(25)	78	CP000270 NID (Burkholderia xenovorans LB400)	ABE31588	MSDB
42	6.62/127.88	13.0%	10(35)	68	transcription-repair-coupling factor (Rickettsia typhi)	MFD_RICTY	SwissProt
					fat metabolism		
27	6.35/59.57	26.0%	7(25)	80	long-chain fatty-acid-CoA ligase (Blastopirellula marina DSM 3645)	gi|87310773	NCBInr
					other function		
34	6.83/51.04	32.0%	15/51	103	major royal jelly protein 2 precursor (MRJP-2) (Apis mellifera)	MRJP2_APIME	SwissProt
43	6.5/61.66	20.6%	11(36)	D' = 4.8	major royal jelly protein 3 (Apis mellifera)	gi|5585142	NCBInr
					unknown function		
5	6.05/19.99	48.0%	6(27)	86	hypothetical protein BP2144 (Bordetella pertussis Tohama I)	gi|33593154	NCBInr
6	7.43/15.65	14.0%	14(37)	79	Hypothetical protein (Ustilago maydis) (smut fungus)	Q4P5M2_USTMA	MSDB
12	7.11/32.84	23.0%	7(20)	81	probable phage protein (Pseudomonas fluorescens Pf-5)	gi|70731107	NCBInr

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Table 1. Continued

| spot no.	pl/MW	sequence coverage	score	matched peptides	protein identity	accession no.	database
16	6.94/81.73	18.0%	82	10(35)	hypothetical protein Rpic12DDRAFT_4652 (*Ralstonia picketti* 12D)	gi|153885525	NCBInr
20	8.99/77.46	21.0%	91	11(40)	putative polyprotein (*Oryza sativa*) (Japonica cultivar group)	gi|18057120	NCBInr
24	9.97/41.46	23.0%	80	5(9)	hypothetical protein BC1G_01640 (*Botryotinia fuckeliana* B05.10)	gi|154321531	NCBInr
29	5.69/169.00	11.0%	78	11(27)	GA10274-PA (fragment) (*Drosophila pseudoobscura*) (fruit fly)	Q29PC6_DROPS	MSDB
39	8.83/57.63	19.0%	84	9(23)	hypothetical protein (*Paramecium tetraurelia*)	gi|145515824	NCBInr

[a] Protein scores greater than 67 and $D' \geq 4$ are significant ($P < 0.05$) in Mascot and Xproteo database, respectively. Spot number corresponds to the number of a protein spot in **Figure 1**. MW (molecular weight) and pI (isoelectric point) represent the theoretical results identified in Genbank, MSDB, or SwissProt. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of pairing an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e. GenBank).

some functional proteins during the storage period. The results are proteomes of bee collected pollen dried at 60 °C: some protein may be hydrolyzed in this process. So, the fresh sample should be included in the future study to obtain a complete proteome sample of bee collected tea pollen. Since there is no sequence database available for the tea tree (*Camellia sinensis*), most identified proteins in this study are homologous to those found in other plants and organisms. However, the identified proteins are only part of high abundance proteins, and there is no direct relation with the health effects found, which might be located in the other fractions of bee collected tea pollen.

Defense-Related Proteins. Proteins in this group are identified as pollen-specific proteins (*Camellia sinensis*), viz. multi-copper oxidase or ascorbate oxidase (**Table 1**, spots 1, 2, 3, 4, 32, 33), performing the first line of defense against potentially damaging external oxidants such as ozone, SO_2, and NO_2 and are highly expressed in pollen (*17*). Spots 1−4, 32, and 33 migrate as multiple isoforms on 2-DE gels for several reasons. Naturally occurring protein isoforms can result from in vivo PTMs such as phosphorylation, glycosylation, or acetylation. These modifications do not significantly change the molecular weight of a protein, but they will likely cause a shift in pI in either the acidic or basic direction. These proteins also have effects on pollen vigor and filament fertility (*17, 18*). The antibiotic resistance protein (**Table 1**, spot 36) in tea pollen could be part of the survival strategy to fight against disease. This is equivalent to *Arabidopsis thaliana* expressing this resistant gene in its pollen (*19*). The abundance of pollen-specific proteins and antibiotic resistance protein were significantly higher at RT than at −20 °C, indicating the biotic stresses are suppressed at lower temperature (**Figure 3A**).

Energy Metabolism. The high percentage (13.2%) of proteins related to energy metabolism correlates well with the large number of these proteins identified in the pollen of *Arabidopsis thaliana* (*20*), tomato (*13*), and *Oryza sativa* (*14*). These include proteins associated with the electron transport chain, vacuolar ATP synthase catalytic subunit A (**Table 1**, spot 10), and ATP synthase subunit α (in mitochondria) (**Table 1**, spot 13), with the TCA cycle, mitochonrial NAD^+-dependent isocitrate dehydrogenase (**Table 1**, spot 19), and thiamine as a coenzyme of thiamine pyrophosphate (**Table 1**, spot 23) and with the synthesis of porphyrin, porphobilinogen deaminase (**Table 1**, spot 25). Although the transcriptome of tea pollen is not yet available, in *Arabidopsis thaliana* pollen, the transcripts of energy-related proteins are under-represented (*21*). However, Holmes-Davis et al. showed an inverse relationship of high abundance energy related proteins between the corresponding mRNA in *Arabidopsis thaliana* pollen (*12*). For the abundance in this group, three of them (isocitrate dehydrogenase, porpho-bilinogen deaminase, and thiamine biosynthesis protein) are significantly higher at RT than at −20 °C (**Figure 3B**), suggesting the energy metabolic rate of tea pollen could be decreased at lower temperature.

Cytoskeleton. β-Tubulin (**Table 1**, spot 8) is a globular protein as a part of cytoskeleton. It has a different role at different intracellular locations. In higher plants, β-tubulin is the main component of the cytoskeletal apparatus in the pollen fertilization process (*22*). Relevantly, this protein has already been detected in the pollen of *Oryza sativa* (*14*) and *Arabidopsis thaliana* (*12, 20*). During the process of pollen grain germination and pollen tube growth, Actin-3 (**Table 1**, spot 26) serves as the molecular tracks for cytoplasmic streaming and provides the way of transporting new components of cell wall and plasma membrane to the apical meristem (*23*). This protein has been
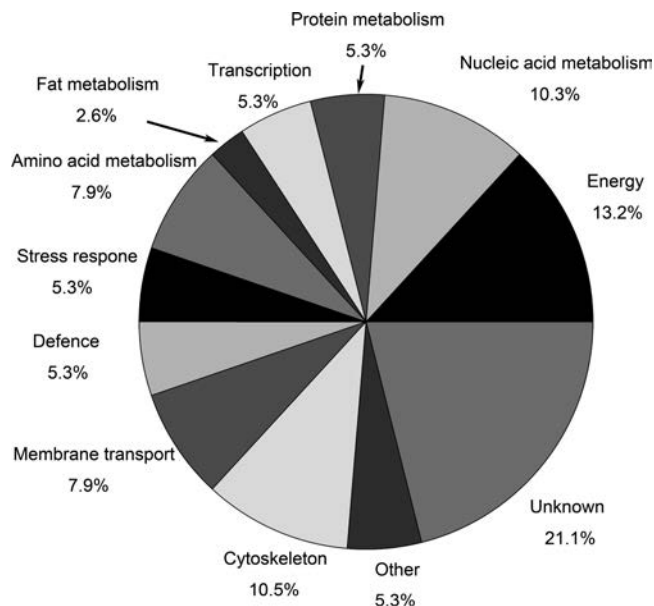
Figure 2. Functional categorization of proteins identified from tea pollen collecting from tea tree (*Camellia sinensis*).

identified in the pollen of other plant species (*12–14*). Ankyrin repeat domain (**Table 1**, spots 15 and 35) primarily mediates protein−protein interactions. This domain should be considered an important regulatory employed by numerous proteins and had been reported in the pollen of *Arabidopsis thaliana* (*24*). The transcription patterns of pollen cDNAs have been profiled from lily and found that a cDNA coding ankyrin repeat protein exists (*25*). This group of protein showed no significant difference in abundance, suggesting they are not sensitive to temperature (**Figure 3C**).

Nucleic Acid Metabolism. Adenylate kinase (**Table 1**, spot 17) has a distinct signaling role in setting the cellular response to stress through activation of AMP-dependent processes (*26*). This kinase was already detected in pollen of lily (*25*) and *Populus* (*27*). Endonuclease V (**Table 1**, spot 28), used for recognizing the cyclobutane pyrimidine dimmer, is very common in bacteria. This protein detected in tea pollen is likely because the bacteria had parasitized one of two hosts (pollen or honeybee), as similar to the pollen of *Oryza sativa* (*28*). Three proteins in this group showed significantly higher abundance at −20 °C than at RT, suggesting these enzymes are degraded at higher temperature (**Figure 3D**).

Membrane Transport. GTP-binding protein (**Table 1**, spot 11) has the activity of endogenous GTP enzyme and has been reported in tomato (*14*) and *Arabidopsis thaliana* pollen (*20*), showing to be important regulators of pollen germination and polar pollen tube growth in signal transduction (*29*). Sec7 domains (**Table 1**, spot 37) catalyze the exchange of guanine nucleotides on ADP-ribosylation factor (ARF) and display a characteristic hydrophobic groove that forms the binding site for ARF. It plays an important role for vesicle transportation and signal transduction and was found at higher levels in mutant pollen (*30*). The ATP-binding cassette transporter (**Table 1**, spot 40, ABC transporter) belongs to a member of the ABC superfamily that is one of the largest known, with over 120 members in both the pollen of *Arabidopsis thaliana* (*12*) and *Oryza sativa* (*14*). To be relevant to this protein, a tobacco WBC subfamily ABC transporter gene, *NtWBC1*, whose transcript levels are the highest in stigmata and styles before and during anthesis, has been identified. NtWBC1 contains a lipid-binding motif primarily involved in sterol-dependent signal transduction pathways. This domain is also recognizable in the *Arabidopsis*

thaliana At3g55090 (AtWBC16) protein, the nearest equivalent to NtWBC1 from this source, which transports lipids (*31*). The abundance of Sec7 domains and ABC transporter were significant higher at −20 °C than at RT, implying lower temperature could slow down the transportation of membrane protein in this regard (**Figure 3E**).

Amino Acid Metabolism. The function of this group is the synthesization of essential amino acids. Aspartate transcarbamoylase (EC 2.1.3.2, **Table 1**, spot 21) is an allosteric enzyme exhibiting motropic cooperativity with respect to both substrates, heterotropic inhibition by CTP, which is enhanced by UTP, and heterotropic activation by ATP, the product of the parallel purine biosynthetic pathway. However, it was reported that there was a deficiency in adenylates in *Arabidopsis thalian* pollen development (*11, 13*). 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (**Table 1**, spots 30, 31) catalyzes the formation of methionine by transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine. It requires a cobamide coenzyme. The enzyme can act on mono- or tri-glutamate derivatives. This group of proteins had no significant difference between being stored at −20 °C and at RT in abundance, suggesting the higher and lower temperature have no affect on their metabolism (**Figure 3F**).

Stress Response. Stress response is universal, and it has been observed in every organism. The literature related to the expression of heat shock protein (hsp) genes in developing pollen and in the mature male gametophyte shows mature pollen lacks a normal heat shock response. In mature pollen of several species, either no hsps are synthesized in response to heat stress, or if synthesized, only a subset is made, and the response is weak at both the transcriptional and translational levels, compared to the response in vegetative tissues. However, in developing pollen, a subset of hsps is induced in response to heat shock. In addition, certain hsp genes or heat shock cognate (hsc) genes are activated during normal pollen development in the absence of heat stress, indicating that these genes are likely to have important developmental functions (*32*). The hsps were also identified in the pollen of *Oryza sativa* (*14*), *Arabidopsis thaliana* (*12, 20*), and tomato (*13*). Ure D (**Table 1**, spot 22), most of that are very prevalent in animal and microorganism, acts as a chaperone protein that stabilizes a urease apoprotein conformation that is competent for nickel incorporation (*33*).
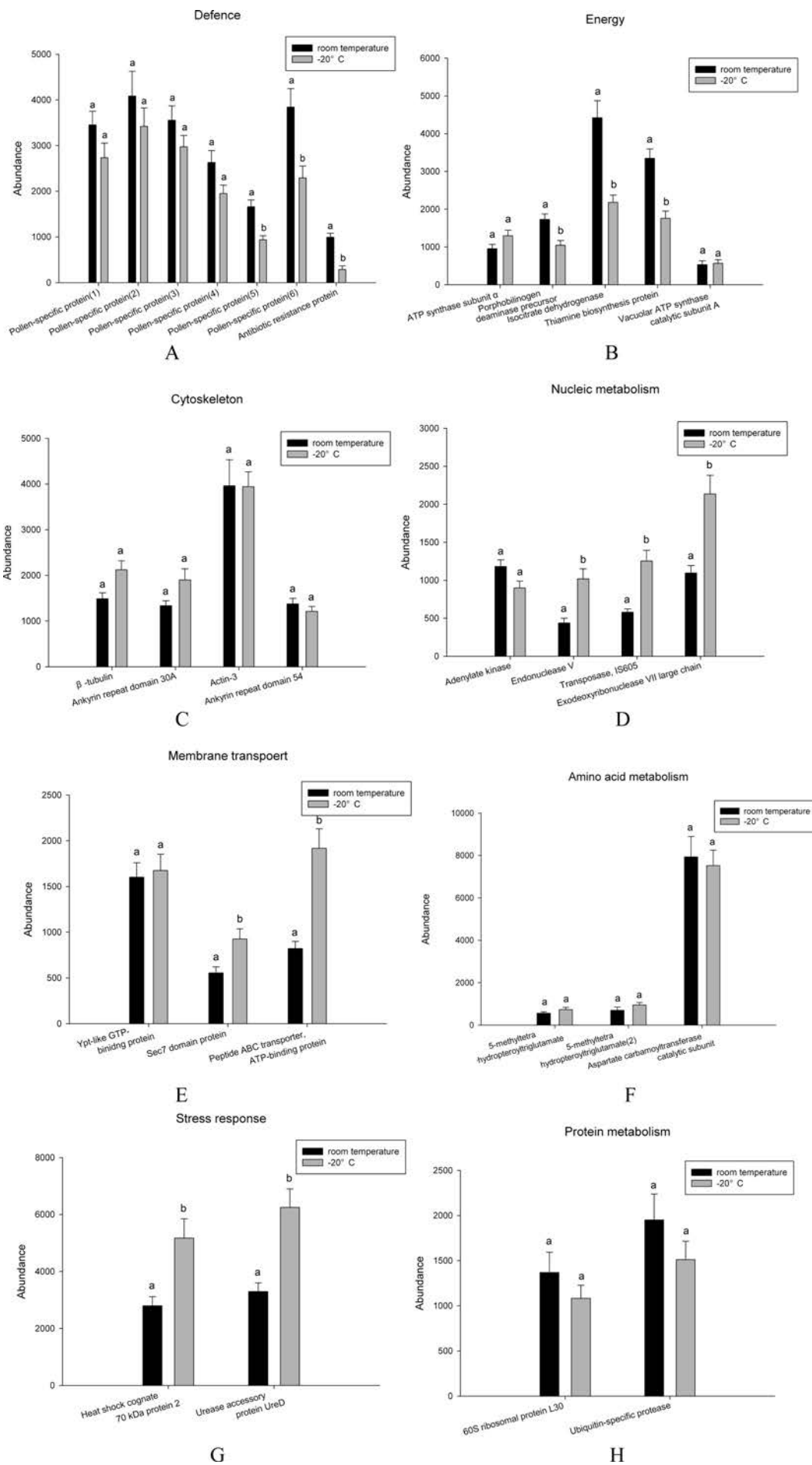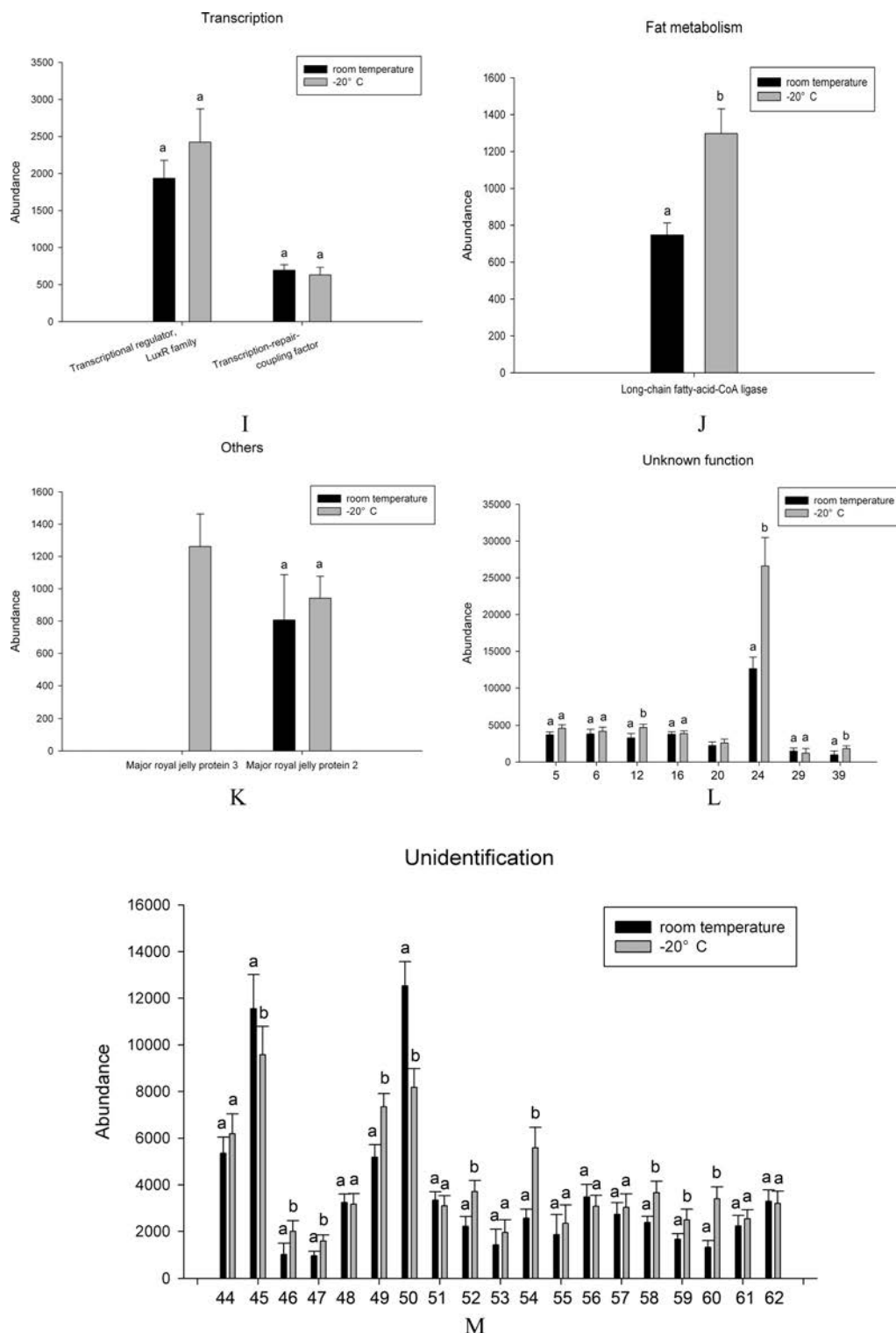
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Figure 3. (1 of 2)

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Figure 3. Abundance analysis of identified proteins and the unidentified resolve to images of tea pollen collecting from tea tree (*Camellia sinensis*) stored at room temperature and −20 °C, respectively. (**A**−**M**) Proteins of defense-related, energy metablism, cytoskeleton, nucleic acid metabolism, membrane transport, amino acid metabolism, stress response, protein metabolism, transcription, fat metabolism, others, unknown function, and the unidentified proteins.

Nevertheless, so far there have been a number of homologues identified in other diverse plant species (such as *Arabidopsis thaliana*, potato) (*20, 34*). On account of stress response at low temperature in the organism, significant higher abundance at −20 °C than at RT in tea pollen is reasonable (**Figure 3G**).

Protein Metabolism. Spot 41 (**Table 1**), 60S ribosomal protein, is ubiquitous in eukaryota. It could potentially be important for translation of stored mRNA during pollen

germination. Labeling experiments of lily and *Tradescantia* sp. pollen tubes indicate transcriptional inactivation of rRNA genes in immature pollen grains and during pollen tube growth. However, the majority of rRNAs and ribosomal proteins are already stored in the mature pollen grain to ensure rapid germination and initial tube growth on the stigma (*35*). Ubiquitin-specific protease (**Table 1**, spot 7) is a unique hydrolase that specifically removes polypeptides covalently

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linked via peptide or isopeptide bonds to the C-terminal glycine of ubiquitin (*36*). There are relatively few proteins involved in protein synthesis compared with those in protein processing and degradation in *Camellia sinensi* pollen, as ones in other species (*12–14*). Relevant to this protein, ubiquitin-conjugating enzyme was detected in tomato pollen (*13*). No significant difference in abundance between −20 °C and RT suggests that these protein metabolisms are not temperature sensitive (**Figure 3H**).

Transcription. Transcription-repair-coupling factor (TRCF, **Table 1**, spot 42) is necessary for strand-specific repair. This protein is almost universal in bacteria but was detected because there are several bacteria parasitizing pollen or honeybee saliva. LuxR family of transcriptional regulator (**Table 1**, spot 18) is the cell density-dependent transcriptional activator. A series of LuxR proteins containing single-amino-acid alterations or deletions in the amino or carboxyl terminus have allowed mapping of specific functions to discrete regions of the protein (*37*). The abundance of this group showed no significant difference between RT and −20 °C, indicating these proteins exist in a stable state in pollen (**Figure 3I**).

Fat Metabolism. Long-chain fatty-acid-CoA ligase (**Table 1**, spot 27) plays an essential role in lipid biosynthesis and fatty acid degradation and is a central enzyme controlling the unesterified arachidonic acid (AA) level in cells. A role of fatty acid could be to promote pollen germination (*38*). The reason for this enzyme showing the higher abundance at −20 °C than at RT could be explained by the fact that fat metabolic rate is more active at lower temperature (**Figure 3J**).

Exogenous Proteins. Honeybees always mix a little of their saliva with the pollen to facilitate pollen collection during their forage activity (*1, 4*). The detected major royal jelly protein (MRJP) 2 precursor (**Table 1**, spot 34) and MRJP 3 (**Table 1**, spot 43) may be the saliva ingredient leaking from the hypopharyngeal gland. So, it is reasonable to detect MRJPs in tea pollen as Scarselli et al. reported (*39*). MRJP2 and MRJP3 have many isoforms and are sensitive to temperature, so their abundance varying between −20 °C and RT is plausible, while the complete absence of MRP3 can be best explained that it is degraded at RT as our previous results (**Figure 3K**) (*15*).

Unknown Function. Spots 5, 6, 12, 16, 20, 24, 29, and 39 (**Table 1**) are identified as hypothetical/unknown or expressed proteins from database, with no well-defined function. Similar to other plant species (*Arabidopsis thaliana*, *Oryza sativa*), function unknown proteins are also identified (*12, 14*). But, the percentage of this group is higher than *Arabidopsis thaliana* and *Oryza sativa* is likely because there is no sequence database available for this plant.

In summary, the storage condition has a significant effect on pollen proteome complement. To maintain the quality, it is better to keep tea pollen in frozen condition. Among the 43 identified proteins, 35 are homologues to other organisms, meaning they were first discovered in tea pollen. Only six pollen-specific proteins (*Camellia sinensis*) were identified as representing annotated genes of tea pollen due to the lack of a sequence database for this plant. Proteins of stress response, nucleic acid metabolism, fat metabolism, and membrane transport are less abundant at RT than those at −20 °C; while defense related and energy proteins show a reverse relationship, the others had no difference between two storage conditions. Proteins identified in this study are only a part of the high abundance proteins that serve functions in pollen development. Therefore, an alternative strategy could consist in the use of LC-ESI-MS/MS or some-thing equivalent capable of providing the sequence of peptides. This should be taken into account for future works.

ABBREVIATIONS USED

2-DE, two-dimensional electrophoresis; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; ESTs, expressed sequence tags; RT, room temperature; MRJP, major royal jelly protein; TCA cycle, tricaboxylic acid cycle; ARF, ADP-ribosylation factor; hsp, heat shock protein; hsc, heat shock cognate; TRCF, transcription-repair-coupling factor.

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