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Research article

Proteomic analysis of latex from *Jatropha curcas* L. stems and comparison of two classic proteomic sample isolation methods: The phenol extraction and the TCA/acetone extraction

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ABSTRACT

Background: Jatropha curcas is a wide-spreading latex-rich biodiesel plant with high oil content in seeds that have always been under intense studies. However, studies are lacking on the latex component that is considered rich in proteins with potentially important physiological functions and secondary metabolites that are a promising source for new drugs. The proteomic analysis, which would be the first step to study these substances, was hampered by the presence of interfering components. Phenol extraction and Trichloroacetic acid (TCA)/acetone extraction, two major plant proteomic isolation methods, were used and compared in this study.
Results: We identified 459 proteins from the *J. curcas* latex proteome using the combination of the two extraction techniques. Although more number of latex proteins were identified by the phenol extraction (401 proteins vs. 123 proteins by the TCA/acetone extraction), only 65 proteins were commonly isolated by both methods. Analysis of the biochemical properties revealed that relatively more number of lower isoelectric point (pI) proteins were isolated by the TCA/acetone method (pI mode: 4.79, 6.51 for phenol). Moreover, GO, COG, and KEGG analyses showed that certain classes/categories/pathways annotated more number of proteins than others, and most of them had proportionally comparable protein counts by both the methods, however, with exemplified exceptions.
Conclusions: A large number of proteins were found and exclusively identified by either method, indicating that a better proteome coverage of plant samples in a similar context needs the combined use of multiple isolation methods. In addition, the core biological function of the latex may be uncovered by certain GO, COG, and KEGG classes/categories/pathways that annotate more proteins.

1. Introduction

Plant latex is stored in the laticifer tissue of approximately 10% of all angiosperms and is typically exuded as a white sticky sap upon physical tissue damage to the plants [1]. Despite the role of plant latex in defense against herbivores, which has so far been well evidenced in many publications, both observational and experimental, other physiological or biochemical functions are poorly understood [2]. The latex contains a variety of secondary metabolites such as isoprenoids (rubber, cardenolides, terpenoids, etc.) [3], alkaloids, and phenolics and proteins such as peptides [4,5], proteases [6], protease inhibitors, lectins, chitinases, and oxidases [2]. Studying these proteins, enzymes, peptides, and hydrocarbons (products of enzymes) is the first step to pharmaceutical developments from the plant latex as considered by reviews [7,8] and performed by researchers and in clinical trials [9,10,11] and a better understanding of the latex's biological function [12,13]. As is known to all, such studies could greatly benefit from proteomic analysis, a powerful tool to identify, characterize, and determine the properties and functions of all individual proteins from a certain biological sample, especially for nonmodel organisms with poorly reported genomic and transcriptomic data [12,13,14,15,16].

The latex-rich shrub *Jatropha curcas* L. (family: Euphorbiaceae) is presently becoming a prevalent biodiesel crop because of its easy propagation, short gestation period, rapid growth, drought endurance, marginal land adaptation, and avoidance by herbivores [17]. Until recently, published proteomic studies on *J. curcas* mostly focused on

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the high oil content of seeds. From specific tissues of seeds such as inner integument [18], endosperm, and embryo [19,20] and specific cellular organelles such as plastids [21,22] and oil body [23,24] to the endosperm development [25], the seed itself [26], the seed under cold stress [27], and oil mobilization in seed germination [28], the seeds are undoubtedly considered the major oil-producing and storing organ. However, to our knowledge, only one report of individual protein identification [6] and no proteomic analysis targeting *J. curcas* latex were reported.

Phenol extraction and TCA/acetone extraction are two of the most popular methods to extract comprehensive and representative protein populations with less contaminants from plant samples; plant samples are poor in proteins and rich in proteases, oxidative enzymes, saccharides, and mostly the secondary metabolites as contaminants [29], which may greatly hamper the isolation of high-quality proteomic samples. This problem might be even worse for plant latex because of the known large proportion of water, isoprenoids, all other kinds of contaminants, and relatively smaller proportion of proteins [1,30,31]. In this study, we for the first time determined the in-depth proteomic data of *J. curcas* latex by LC–ESI–MS/MS analysis with the combination of both the phenol and TCA/acetone extraction techniques to obtain a better coverage of the total protein population. Our data shows that each of the two isolation methods led to a group of hundreds of identified proteins, with large proportion of different proteins between the two groups. Further analysis was performed to determine the isoelectric points (pIs) and masses of these proteins, with emphasis on the pIs. All the identified proteins were subjected to GO, COG, and KEGG analyses. Bioinformatics analysis was performed on the union, intersection, and differences of the two groups of proteins identified by the two methods. Our findings will benefit the understanding and further study of the biochemical ingredients and physiological role of *J. curcas* latex.

2. Materials and methods

2.1. Latex sample collection

J. curcas seeds were collected from Liangshan, Sichuan province, China, and germinated in greenhouse conditions (24,000 Lx, 60% humidity, 25°C, 16 h; dark, 80% humidity, 18°C, 8 h) as described previously [32]. One-month-old seedlings were transferred to the test field. Six-month-old plants were subjected to the latex collection operation. To be specific, the stems of *J. curcas* were cut using lancets, and the oozing latex was collected in 10-mL Eppendorf tubes held right below the cut. At the end of the collection, the tubes were capped, sealed, and quickly froze in liquid nitrogen as soon as possible to minimize the degradation and oxidation of the latex samples. After that, the latex samples could be transferred to a freezer at −80°C for long-term storage, but it is recommended to extract the samples ASAP.

2.2. Total protein extraction, pretreatment, and LC–ESI–MS/MS analysis

For total protein extraction, the collected latex samples were first thawed on ice and then subjected to both phenol extraction and TCA/acetone extraction as detailed previously [29]. The tissue grinding step was skipped because of the liquid nature of the latex. At the end of the extraction, the dried protein pellet was resuspended in a buffer appropriate for the downstream analytical approach [42% urea (w/v), 15.2% thiourea, 1% DTT, 4% CHAPS, and 0.5% carrier ampholytes (v/v)]. The resulting protein solution was quantified with Pierce™ Coomassie Plus (Bradford) Assay Kit purchased from Thermo Fisher Scientific. Then, 50 mg/lane of total protein samples were subjected to 1D SDS-PAGE. The electrophoresed gels were then stained with Coomassie Brilliant Blue (CBB) R-250 and imaged.

The total protein samples were pretreated, and the LC–ESI–MS/MS analysis was performed as described previously [33]. Briefly, the CBB-stained protein bands were excised and washed with 0.1 M ammonium bicarbonate/acetonitrile (1:1, v/v) and incubated with occasional vortexing for 30 min, depending on the staining intensity. After the removal of the wash solution, gel pieces were covered with neat acetonitrile for 20 min at room temperature and then let dry. Subsequently, 100 μg of total protein from each latex sample was chemically reduced with 10 mM DTT (1 h, 60°C) and carboxyamidomethylated with 55 mM iodoacetamide (45 min, RT, dark). Trypsin digestion was performed at 37°C for 16 h by Trypsin Gold (Promega, Madison, WI, USA) with an enzyme-to-substrate ratio of 1/30 (w/w). After that, 10 μL of formic acid was used to acidify the digested peptide mixture for further MS analysis [34].

After trypsin digestion, the peptide samples were desalted with Strata X columns (Phenomenex), vacuum dried, and then resuspended in buffer A (2% ACN, 0.1% FA; 200 μL). Next, the samples were centrifuged at 20,000 × g for 10 min. The supernatant was collected, and the concentration of the peptide solution was adjusted to approximately 0.5 μg/μL. Then, 10 μL of this solution was loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by an autosampler onto a 2-cm C18 trap column. Subsequently, the sample was eluted onto a 10-cm analytical C18 column (75 μm inner diameter) that was packed in-house. The sample loading condition was set at 8 μL/min for 4 min, and then the gradient was run for 44 min at 300 nL/min starting from 2% to 35% B (98% ACN, 0.1% FA), followed by 2 min of linear gradient to 80% and 4 min of 80% B maintenance, and finally back to 5% for 1 min.

The peptide samples were ionized with a nanoelectrospray and then handled by tandem mass spectrometry (MS/MS), both inside a Q EXACTIVE (Thermo Fisher Scientific, San Jose, CA) coupled online to the HPLC. Orbitrap with a resolution of 70,000 was used to detect the intact peptides, which were selected for MS/MS by an operating mode of high-energy collision dissociation with 27.0 as the normalized collision energy setting, and a resolution 17,500 was used to detect the ion fragments. The 15 most abundant precursor ions above a threshold ion count of 20,000 in the MS survey scan following 15 s of the Dynamic Exclusion duration were dealt with an alteration between one MS scan followed by 15 MS/MS scans as the data-dependent procedure. The voltage applied for the electrospray was 1.6 kV. The spectra generated by the Orbitrap were optimized by automatic gain control, of which the target for full MS was 3e6 and 1e5 for MS2. The m/z scan ranges were 350 to 2000 Da for MS scans and 100 to 1800 for MS2.

2.3. Protein identification

Proteome Discoverer 1.2 (PD 1.2, Thermo) (5600 msconverter) was used to convert the raw data files collected from the Orbitrap into Mascot Generic Format (MGF) files. The Mascot search engine (Matrix Science, London, UK; version 2.3.02) was used to identify proteins against a 27,579-sequence-containing database (The sequence databases of Mascot include SwissProt; NCBInr; and EMBL EST divisions, contaminants, and cRAP. http://www.matrixscience.com/). The mass tolerance for intact peptide masses and fragmented ions were set to 20 (ppm) and 0.1 Da, respectively, for protein identification. One missed cleavage in the trypsin digests was allowed. We used Gln- > pyro-Glu (N-term Q), oxidation (M), and deamidated (NQ) as the potential variable modifications and carbamidomethyl (C) as a fixed modification. The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of database is generated and tested for raw spectra as well as the real database. Only peptides with significance scores (≥20) at the 99% confidence interval by a Mascot probability analysis greater than “identity” were counted as identified in this study to further reduce the probability of false peptide identification, and

at least one unique peptide should be involved for each confident protein identification.

After the comprehensive protein identification with Mascot, raw data files of MS peaks and Mascot identification results were well described and uploaded to public proteomics data repository PRIDE Archive (http://www.ebi.ac.uk/pride/archive/) with the same project name as the title of this article. A Venn diagram was graphed to show the number of proteins identified by each extraction method and both together, an electric 2-DE scatter plotting all identified proteins' pIs and masses, and a line chart emphasizing the pI modes of proteins identified by the two methods.

2.4. Functional description

The Blast2GO [35] program was used to functionally annotate the proteins against the nonredundant sequence database of proteins (NCBI). Gene ontology (GO) is a well-accepted standard of gene functional classification system. With the three ontologies of molecular function, cellular component, and biological process to be addressed, the GO provides a set of dynamic updating controlled vocabulary to describe genes and attributes of gene products in a certain organism. In addition, the COG database (http://www.ncbi.nlm.nih.gov/COG/) and the KEGG database (http://www.genome.jp/kegg/) were used to classify and categorize the identified proteins. Clusters of Orthologous Groups of proteins (COGs) is the database for protein orthologous classification, in which each COG consists of a group of proteins found to be orthologous by sequence analysis and likely derived from a common ancient conserved protein or domain. The database could be used for protein orthologous functional classification. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a popular database to reveal and understand high-level pathways and functions of biological networks of the cell, the organism, and the ecosystem based on molecular biological data generated, especially by high-throughput techniques. It is a collection of our knowledge on the molecular interaction and reaction networks of biological macromolecules.

The ID, description, and sequence of each identified protein extracted by either isolation method were collected into fasta format files, whereas more detailed information of each protein record was collected into Comma-separated Values (CSV) format and KEGG analysis results into ko and path format. These data formats could be conveniently read, screened, analyzed, and output by scripting with the programming language Python [36]. The united, intersected, and differential protein records obtained with the two extraction methods were picked out and exported to new fasta, CSV, ko, and path files by running the Python codes with Biopython [37] (handling fasta) and csv (handling CSV, ko, and path) modules. CSV, ko, and path files were all pasted to sheets of a MS Excel (.xlsx) file for convenient use as supplementary data.

3. Results

3.1. 1D SDS-PAGE of the protein samples

The total protein samples isolated by both the phenol extraction method and the TCA/acetone extraction method were separated by 1D SDS-PAGE. After the electrophoresis, the protein bands were visualized by CBB staining (Fig. 1a). As shown in Fig. 1a, the total protein samples extracted by the two methods shared many bands with identical molecular weights, however, most with different relative expression amounts (to the total loading amounts). Many bands exclusively belonging to either sample (and not to the other) were also found, implying the probable different preferences of the two methods for extracting different proteins.

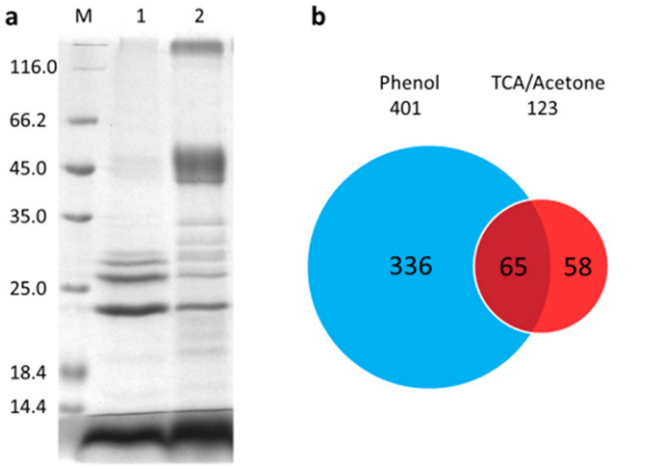

Fig. 1. Extraction and identification of proteins from *J. curcas* latex: (A) SDS-PAGE of the latex total proteins extracted by the phenol method and the TCA/acetone method (M: molecular marker, 1: phenol extracted sample, 2: TCA/acetone extracted sample); (B) Venn diagram of proteins identified by the two methods. A total of 459 proteins were identified by the two methods combined (401 by phenol method and 123 by TCA/acetone method), 65 of which could be detected by both methods.

3.2. Protein identification by LC–ESI–MS/MS analysis

To obtain a better coverage of the *J. curcas* latex proteome and to learn better the extraction capacities and the possible preferences of the two isolation methods, we subjected the total protein samples from both methods to LC–ESI–MS/MS analysis. Owing to the series of publications and updating of the genomic data [38] and expression profiles [22,23,39,40] of *J. curcas* in recent years, we could identify the latex proteins with Mascot search engine as described above in Materials and Methods (Section 2.3. Protein identification). All identified protein records yielded from the phenol method (Supplementary Table 1) and TCA/acetone method (Supplementary Table 2) were listed in the respective fasta files with NCBI protein IDs, descriptions, and sequences. Detailed information such as protein mass, pIs, protein score, and peptide coverage has been listed (Supplementary Table 3 for phenol method and Supplementary Table 4 for TCA/acetone method). The corresponding regroupings of the identified protein records and the detailed information from the two isolation methods were generated by Python codes according to the principle that one NCBI protein ID only links to one protein sequence. These regroupings include the set of union (Supplementary Table 5 for fata and Supplementary Table 6 for detailed information), intersection (Supplementary Table 7 for fasta and Supplementary Table 8 for detailed information), and differences, which consist of the protein records exclusively yielded by the phenol method rather than the other (phenol–TCA/acetone, Supplementary Table 9 for fasta and Supplementary Table 10 for detailed information) and the antitype (TCA/acetone–phenol, Supplementary Table 11 for fasta and Supplementary Table 12 for detailed information).

The regroupings described above led to a Venn diagram (Fig. 1B) of the identified proteins by the two methods. Of the 401 proteins identified by the phenol method, 65 were also found in the 123 proteins identified by the TCA/acetone method. These common 65 proteins have only made up of 16.2% of the phenol proteins and 52.8% of the TCA/acetone proteins. Adding together, we identified 459 *J. curcas* latex proteins by these two sample extraction methods combined. With 2.26 times more proteins identified by the phenol method, it might seem that this technique worked better for *J. curcas* latex samples. However, the relative low coverage of the intersection part to total proteins identified by either method implies the probably

discrepant preferences of the two extraction methods on isolating different *J. curcas* latex proteins.

3.3. pI and protein mass analysis of the identified proteins

To further analyze the probable preferences of each isolation method on some of the crucial protein biochemical features, we scatter plotted the electric 2-DE image with all the protein spots identified by both methods, with pIs on the X-axis and protein masses on the Y-axis (Fig. 2). The ratios of the protein spot counts within that specific scale range to the total protein spot counts of the corresponding groupings (U for union, P for phenol, and T for TCA/acetone) were shown as percentages labeling the respective axis scale ranges. Generally, as shown in Fig. 2, the protein spots identified by the two extraction methods were scattered in overlapping areas. However, 43.9% TCA/acetone proteins were estimated to have pIs of 4–6, which is more than any other TCA/acetone pI scale ranges and proportionally much more than that of the phenol (P: 30.2%) method and union of both

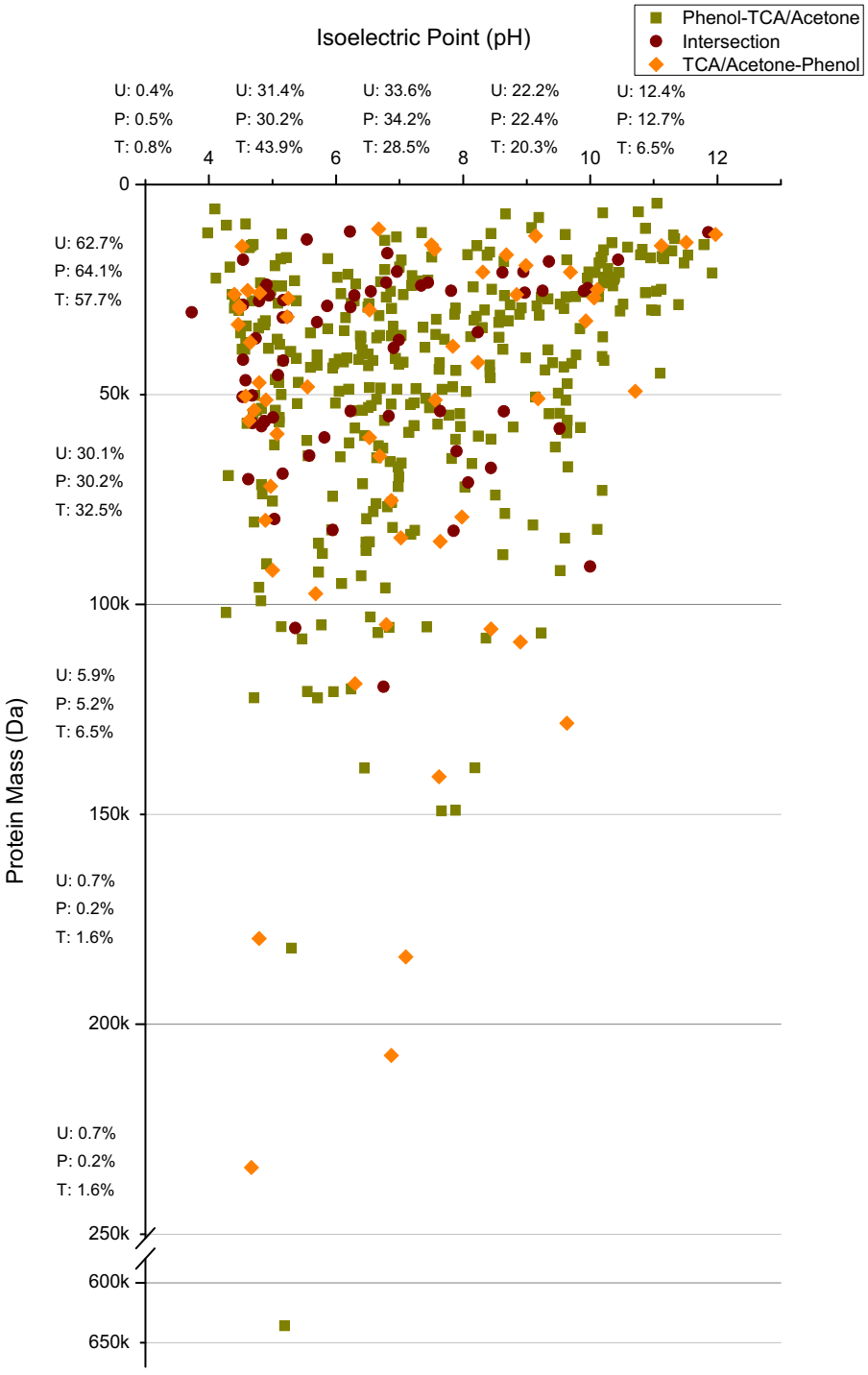

Fig. 2. Electric 2-DE scatter plot of the identified proteins. The protein spots identified only by the phenol method not by the TCA/acetone method (phenol–TCA/acetone), by both methods (intersection), and only by the TCA/acetone method not by the phenol method (TCA/acetone–phenol) were scattered over the diagram. The ratios of the protein spot counts within that specific scale range to the total protein spot counts of the corresponding groupings (U for union, the grouping of total identified proteins, P for phenol, and T for TCA/acetone) were shown as percentages labeling respective axis scale ranges.

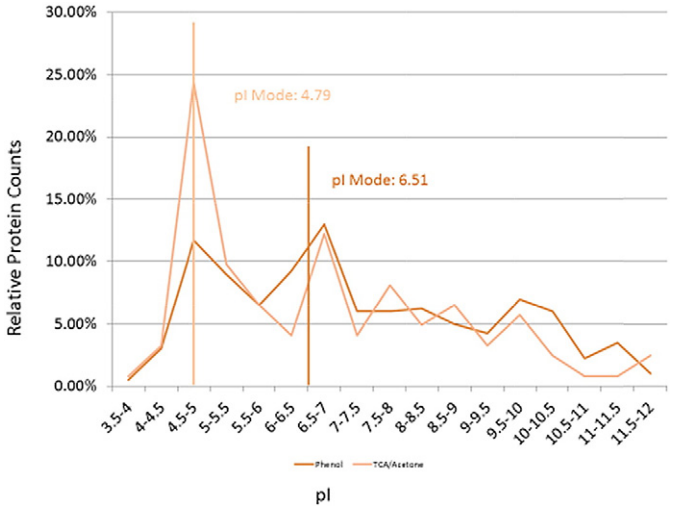

Fig. 3. Relative protein counts of each 0.5-pI scale identified by phenol extraction and TCA/acetone extraction (ratios of protein counts within that specific pI scale to the total protein counts, presented as percentages). The modes of pIs of the two extraction methods are labeled.

(U: 31.4%), whereas more phenol proteins and union proteins have neutral pIs of 6–8 (34.2% for P and 33.6% for U). Similarly, there are also proportionally more number of TCA/acetone pI 0–4 (most acidic) spots than those by the phenol method and the union (0.8% for T, 0.5% for P, and 0.4% for U) but proportionally much less at pI ≥6.

Fig. 3 illustrates the relative protein counts of each 0.5-pI scale (ratios of protein counts within that specific pI scale to the total protein counts, shown as percentages), with the modes of pIs of phenol extraction (6.51) and TCA/acetone extraction (4.79) labeled. This line chart clearly states that proportionally more number of lower pI proteins were isolated by the TCA/acetone method.

3.4. Gene ontology annotation of the identified proteins

To learn the molecular function of the identified proteins to better understand the physiological role of *J. curcas* latex, all the identified proteins were functionally annotated by the Blast2GO program against the nonredundant protein database (NCBI). The annotated GO information of different groupings of the identified proteins is given as supporting information (Supplementary Table 13–18 for phenol method, TCA/acetone method, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol).

From these data, we bar plotted the annotating GOs and classes against the corresponding numbers of annotated proteins (Fig. 4). Certain classes from all the three ontologies annotated more proteins than the rest. For example, class “cellular process,” “metabolic process,” “single-organism process,” and “response to stimulus” from the ontology “Biological process”; class “cell,” “cell part,” “organelle,” “organelle part,” and “membrane” from the ontology “Cellular component”; and class “catalytic activity” and “binding” from the ontology “Molecular function” have annotated approximately half or even more than half of the identified latex proteins and were the most used annotation terms (one protein could be annotated to multiple classes or ontologies) (Fig. 4, Table 1).

All the ratios (presented in percentages) of the annotated protein counts (APC) to the total protein counts (TPCs) of one certain grouping of the six groupings (phenol, TCA/acetone, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol) of the identified proteins for all annotating GO ontologies and classes are listed in Table 1. As shown, for most annotating GO classes, larger (or smaller) APC/TPC of one certain grouping generally occurs with larger (or smaller) APC/TPCs of other groupings. In other words, the APC/TPCs stay relatively consistent with one another among all six groupings of the identified proteins for most GO classes. However, there are exceptions such as class “membrane-enclosed lumen” from the ontology “Cellular component” and class “structural molecule activity” from the ontology “Molecular function” where proportionally more number of proteins were isolated by the phenol method compared to the others; class “transporter activity” from the ontology “Molecular function” is an opposite example.

3.5. COG orthologous classification of the identified proteins

The COG analytic results for all groupings of the identified proteins were well collected (Supplementary Table 19–24 for the phenol method, TCA/acetone method, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol).

Taken together, these data resulted in Fig. 5 and Table 2. The identified protein counts were plotted against the corresponding annotating COG functional categories in Fig. 5. All the APC/TPCs of the six groupings (phenol, TCA/acetone, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol) of the identified proteins for all annotating COG functional categories are listed in Table 2. As shown by the data of the grouping of union, just like in the GO annotation, certain COG functional categories such as “General function prediction only,” “Translation, ribosomal structure and biogenesis,” “Carbohydrate transport and metabolism,” and “Posttranslational modification, protein turnover, chaperones” orthologously classified more number of identified proteins than the others (one protein could be classified to more than one categories) (Fig. 5, Table 2). Moreover, for most annotating COG functional categories, the APC/TPCs of six groupings remain relatively consistent with each other (Table 2). However, just like the GO result, exceptional categories such as “Translation, ribosomal structure and biogenesis” and “Signal transduction mechanisms” also exist, as presented in Table 2. Our data show that proteins of the prior category were proportionally better extracted by the phenol method, whereas proteins from the next category were more compatible with the other method.

3.6. KEGG pathway analysis of the identified proteins

KEGG analysis results such as KO (KEGG orthology) ID, identity, and KO definition of each identified protein were all recorded (Supplementary Table 25–30 for phenol method, TCA/acetone method, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol), together with the classification of the identified proteins according to their KEGG pathway information (Supplementary Table 31–36 for phenol method, TCA/acetone method, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol).

Fig. 6 and Table 3 were generated from these data. The KEGG pathway analysis result, as illustrated by Fig. 6, reveals that some pathways such as “Metabolic pathways,” “Biosynthesis of secondary metabolites,” and “Ribosome” were apparently crowded with more number of identified proteins than the others (one protein could participate in more than one pathway). Interestingly, the identified proteins classified to the “Ribosome” pathway were proportionally better extracted by the phenol method as indicated by the APC/TPCs (Table 3), which is in accordance with the COG “Translation, ribosomal structure and biogenesis” functional category (Table 2). Moreover, contrary instances such as the “Phagosome” pathway could also be found. Apart from this, similar to GO and COG analysis, relative consistence was observed in the six-grouping APC/TPCs for most KEGG pathways.

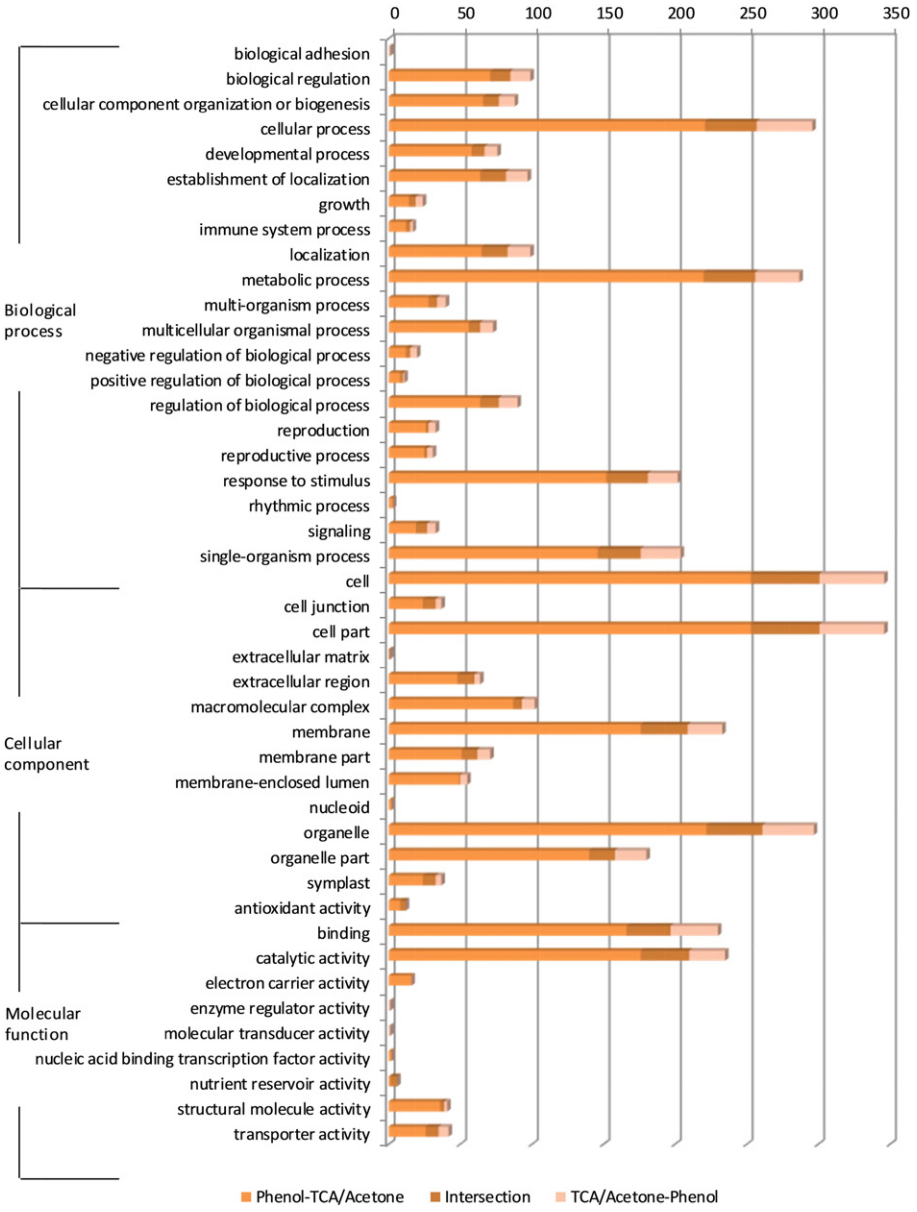

Fig. 4. Number of proteins annotated to GOs and classes. The number of proteins (phenol–TCA/acetone: identified only by the phenol method not by the TCA/acetone method; intersection: by both methods; and TCA/acetone–phenol: only by the TCA/acetone method not by the phenol method) are plotted as the length of the bars.

4. Discussion

To date, as a wide-spreading and promising biodiesel plant, most functional genomic studies on *J. curcas* were mainly focused on the seeds where the oil is synthesized and accumulated or focused on helping improve the environmental adaptation, cultivation, and yields [17]. The latex, which is known to be rich in proteins and hydrocarbons in various forms [1,2], might have important physiological roles and serve as a new source for bioactive substances. The significant value of such a source was highlighted by the pharmaceutical development efforts reviewed [8] and experimentally performed [9,10], even in clinical trials [11], by other researchers.

This manuscript describes the first reported *J. curcas* latex proteome to our knowledge. To maximize the number of identified proteins from a well-known "tough" plant tissue with high concentration of proteases, protein isolation interferents, and contaminants, we performed total protein isolation with two of the most well-accepted plant proteomic sample extraction techniques: the phenol extraction method (which alone identified 401 individual proteins) and the TCA/acetone extraction method (123 proteins) [29] (Fig. 1b). The phenomenon that more number of protein bands could be visualized on 1D SDS-PAGE using extracts obtained by the TCA/acetone method than those obtained by the phenol method (Fig. 1a), which might seem to be inconsistent with the LC–ESI–MS/MS result (Fig. 1b), may be due to three causes: (1) some uncertain contaminants that were unequally left after the extraction with the two methods might have interfered with the CBB staining, giving rise to nonprotein bands because the TCA/acetone extraction protocol does not include steps of repeated removal of water-soluble contaminants (extraction of proteins) like the phenol extraction; (2) such contaminants may also interfere with the LC–ESI–MS/MS analytical process, causing loss of protein identification; and (3) because normally the well-visualized proteins by CBB staining are relative abundant proteins of the given sample, complex samples with more proteins of low abundance and fewer proteins of high abundance may show fewer dark bands and more light bands that may be hard to distinguish. On the contrary, the LC–ESI–MS/

Table 1
Ratios (presented in percentages) of the annotated protein counts (APC) to the total protein counts (TPCs) of one certain grouping of the six groupings of the identified proteins for all annotating GOs and classes. The six groupings (with the respective TPC shown in the parenthesis) are phenol (401), TCA/acetone (123), union (459), intersection (65), phenol–TCA/acetone (P–T, 336), and TCA/acetone–phenol (T–P, 58). The classes were sorted within each ontology in a descending order of APC/TPC values of the union grouping.

Ontology	Class	Phenol	TCA/acetone	Union	Intersection	P–T	T–P
Biological process	Cellular process	64%	61%	64%	55%	66%	67%
	Metabolic process	64%	54%	63%	55%	65%	53%
	Single-organism process	44%	47%	44%	46%	43%	48%
	Response to stimulus	45%	41%	44%	45%	45%	36%
	Biological regulation	21%	23%	22%	22%	21%	24%
	Localization	21%	28%	22%	28%	19%	28%
	Establishment of localization	20%	27%	21%	28%	19%	26%
	Regulation of biological process	19%	21%	20%	20%	19%	22%
	Cellular component organization or biogenesis	19%	18%	19%	17%	20%	19%
	Developmental process	17%	15%	17%	14%	17%	16%
	Multicellular organismal process	16%	14%	16%	12%	17%	16%
	Multi-organism process	8%	10%	9%	9%	8%	10%
	Reproduction	7%	6%	7%	3%	8%	9%
	Signaling	7%	11%	7%	12%	6%	10%
	Reproductive process	7%	5%	7%	3%	7%	7%
	Growth	5%	8%	5%	8%	4%	9%
	Negative regulation of biological process	4%	7%	4%	5%	4%	9%
	Immune system process	4%	4%	4%	5%	4%	3%
	Positive regulation of biological process	2%	2%	2%	3%	2%	2%
	Rhythmic process	1%	0%	1%	0%	1%	0%
	Biological adhesion	0%	1%	0%	0%	0%	2%
Cellular component	Cell	75%	76%	75%	74%	75%	78%
	Cell part	75%	76%	75%	74%	75%	78%
	Organelle	65%	61%	65%	60%	66%	62%
	Membrane	52%	46%	51%	51%	52%	41%
	Organelle part	39%	33%	39%	28%	42%	38%
	Macromolecular complex	23%	12%	22%	9%	26%	16%
	Membrane part	15%	16%	15%	17%	15%	16%
	Extracellular region	15%	13%	14%	18%	14%	7%
	Membrane-enclosed lumen	12%	5%	12%	2%	15%	9%
	Cell junction	8%	11%	8%	14%	7%	7%
	Symplast	8%	11%	8%	14%	7%	7%
	Nucleoid	0%	0%	0%	0%	1%	0%
	Extracellular matrix	0%	1%	0%	2%	0%	0%
	Catalytic activity	52%	48%	51%	52%	52%	43%
	Binding	49%	52%	50%	48%	49%	57%
	Transporter activity	9%	13%	9%	14%	8%	12%
	Structural molecule activity	10%	4%	9%	5%	11%	3%
Molecular function	Electron carrier activity	4%	0%	3%	0%	5%	0%
	Antioxidant activity	3%	3%	3%	6%	2%	0%
	Nutrient reservoir activity	1%	4%	1%	8%	0%	0%
	Nucleic acid binding transcription factor activity	0%	0%	0%	0%	1%	0%
	Enzyme regulator activity	0%	1%	0%	0%	0%	2%
	Molecular transducer activity	0%	1%	0%	0%	0%	2%

MS analysis is much better for identifying low-abundant proteins. Thus, in our opinion, the number of distinct SDS-PAGE CBB bands does not have to be strictly correlated with the number of total MS-identified proteins in a given complex sample.

In total, 459 individual latex proteins of *J. curcas* were identified with certain bioinformatics means [36,37] after combining the protein records isolated separately by the two methods with no replications (Fig. 1b). We believe that this set of information would greatly accelerate future research on *J. curcas* latex and drug discovery based on it, which is already underway in our lab.

Our bioinformatics analysis has found that, as mentioned before, the intersection part of the identified proteins (the 65 proteins shared by both methods) only made up of 16.2% of the phenol proteins and 52.8% of the TCA/acetone proteins (Fig. 1b). Additional analysis (Fig. 2) revealed that although on average no remarkable differences in protein pIs or masses between the two methods were found, proportionally much more number of lower pI proteins were identified by the TCA/acetone method than by the other method (pI mode 4.79 vs. 6.51, Fig. 3). This finding is consistent with the fact that proteins precipitate best below their pIs (so low-pI-proteins precipitate better with the acidic TCA reagent). Taken together, these findings indicate that the two "mainstream" proteomic sample isolation methods do differ in dealing with complex sample context and extracting proteins with certain differential biochemical properties.

The molecular function analysis and annotation of all the identified protein groupings in six ways (phenol, TCA/acetone, union, intersection, phenol–TCA/acetone, and TCA/acetone–phenol) was performed with GO, COG, and KEGG analyses. On the one hand, certain GO, COG, or KEGG functional classes labeled more proteins (as described before) that may better correlate with the major physiological function of *J. curcas* latex as we speculated. Thus, we have provided evidence from a proteomic study to illustrate the probable composition and biological function of the plant tissue. Judging from more protein-labeling functional classes, our findings coincide with those of previous studies that the latex is actually specialized cytoplasm for containing membrane-bound organelles and particles, and it is very important for plant metabolism for containing large parts of the catalytic activities and metabolites (Fig. 4, Fig. 5, Fig. 6 and Table 1, Table 2, Table 3) [41,42]. The secondary metabolic role of the latex has already been widely reported [2,43], although the metabolism (primary metabolism) of which and other unverified functions revealed by our data is now under further in-depth research in our lab.

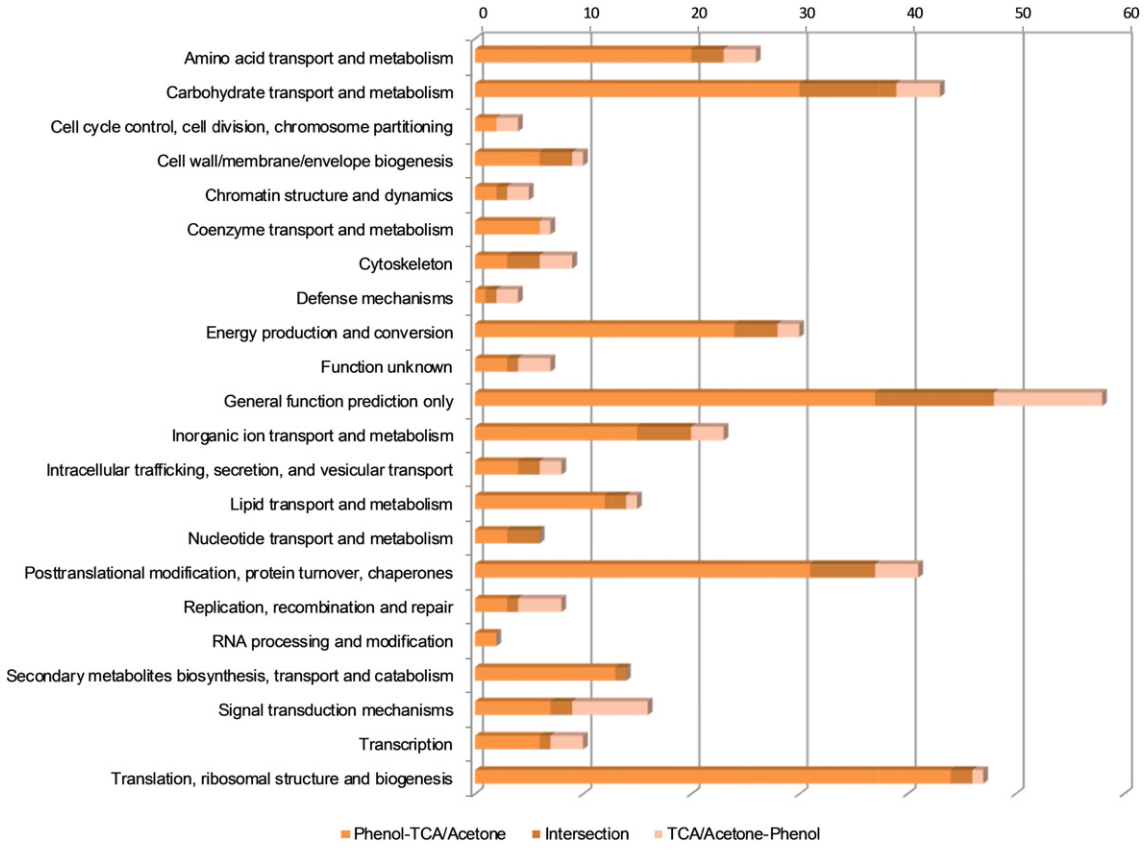

Fig. 5. Number of proteins orthologously categorized to COG functional categories. The number of proteins (phenol–TCA/acetone: identified only by the phenol method not by the TCA/acetone method; intersection: by both methods; and TCA/acetone–phenol: only by the TCA/acetone method not by the phenol method) are plotted as the length of the bars.

On the other hand, the two isolation methods exhibited identical preferences for extracting *J. curcas* latex proteins categorized to most GO, COG, or KEGG functional classes because the APC/TPCs of the six groupings remained relatively unchanged for most classes (Table 1, Table 2, Table 3). Moreover, proteins of exceptional categories were proportionally better extracted by one method rather than the other as exemplified in the Results section (Table 1, Table 2, Table 3). Such diversity in the extracting capacity of the two isolation methods for proteins of certain functional classes may to some extent explain the interesting finding that only 65 proteins, a relatively small fraction, were commonly isolated by both methods, which might also partly be due to discrepant isolating preferences of the two methods for

Table 2
APC/TPCs of the six groupings of the identified proteins for all annotating COG functional categories. The six groupings (with the respective TPC shown in the parenthesis) are phenol (401), TCA/acetone (123), union (459), intersection (65), phenol–TCA/acetone (P–T, 336), and TCA/acetone–phenol (T–P, 58). The functional categories were sorted in a descending order of APC/TPC values of the union grouping.

Functional categories	Phenol	TCA/acetone	Union	Intersection	P–T	T–P
General function prediction only	12%	17%	13%	17%	11%	17%
Translation, ribosomal structure and biogenesis	11%	2%	10%	3%	13%	2%
Carbohydrate transport and metabolism	10%	11%	9%	14%	9%	7%
Posttranslational modification, protein turnover, chaperones	9%	8%	9%	9%	9%	7%
Energy production and conversion	7%	5%	7%	6%	7%	3%
Amino acid transport and metabolism	6%	5%	6%	5%	6%	5%
Inorganic ion transport and metabolism	5%	7%	5%	8%	4%	5%
Signal transduction mechanisms	2%	7%	3%	3%	2%	12%
Lipid transport and metabolism	3%	2%	3%	3%	4%	2%
Secondary metabolites biosynthesis, transport and catabolism	3%	1%	3%	2%	4%	0%
Transcription	2%	3%	2%	2%	2%	5%
Cell wall/membrane/envelope biogenesis	2%	3%	2%	5%	2%	2%
Cytoskeleton	1%	5%	2%	5%	1%	5%
Replication, recombination and repair	1%	4%	2%	2%	1%	7%
Intracellular trafficking, secretion, and vesicular transport	1%	3%	2%	3%	1%	3%
Coenzyme transport and metabolism	1%	1%	2%	0%	2%	2%
Function unknown	1%	3%	2%	2%	1%	5%
Nucleotide transport and metabolism	1%	2%	1%	5%	1%	0%
Chromatin structure and dynamics	1%	2%	1%	2%	1%	3%
Cell cycle control, cell division, chromosome partitioning	0%	2%	1%	0%	1%	3%
Defense mechanisms	0%	2%	1%	2%	0%	3%
RNA processing and modification	0%	0%	0%	0%	1%	0%

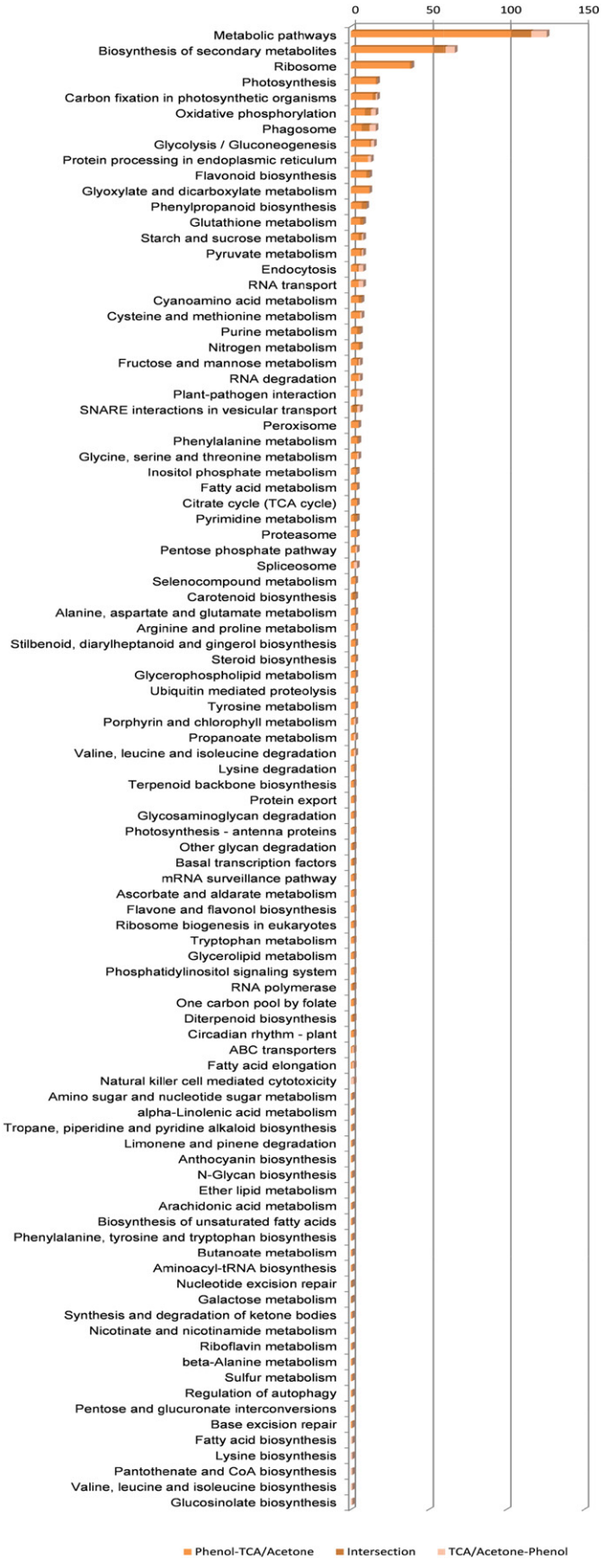

Fig. 6. Number of proteins classified to KEGG pathways. The number of proteins (phenol–TCA/acetone: identified only by the phenol method not by the TCA/acetone method; intersection: by both methods; and TCA/acetone–phenol: only by the TCA/acetone method not by the phenol method) were plotted as the length of the bars.

Table 3
APC/TPCs of the six groupings of the identified proteins for all annotating KEGG pathways. The six groupings (with the respective TPC shown in the parenthesis) are phenol (401), TCA/acetone (123), union (459), intersection (65), phenol–TCA/acetone (P–T, 336), and TCA/acetone–phenol (T–P, 58). The pathways were sorted in a descending order of APC/TPC values of the union grouping.

Pathway	Phenol	TCA/acetone	Union	Intersection	P–T	T–P
Metabolic pathways	29%	19%	27%	20%	31%	17%
Biosynthesis of secondary metabolites	15%	11%	15%	12%	16%	10%
Ribosome	10%	1%	8%	2%	11%	0%
Photosynthesis	4%	1%	4%	2%	5%	0%
Carbon fixation in photosynthetic organisms	4%	2%	4%	3%	4%	2%
Oxidative phosphorylation	3%	6%	3%	6%	3%	5%
Phagosome	3%	7%	3%	8%	2%	7%
Glycolysis/gluconeogenesis	3%	2%	3%	2%	4%	3%
Protein processing in endoplasmic reticulum	3%	2%	3%	0%	3%	3%
Flavonoid biosynthesis	3%	2%	3%	3%	3%	0%
Glyoxylate and dicarboxylate metabolism	3%	0%	3%	0%	4%	0%
Phenylpropanoid biosynthesis	2%	2%	2%	5%	2%	0%
Glutathione metabolism	2%	2%	2%	3%	2%	0%
Starch and sucrose metabolism	2%	2%	2%	3%	1%	2%
Pyruvate metabolism	2%	2%	2%	2%	2%	2%
Endocytosis	1%	3%	2%	2%	1%	5%
RNA transport	1%	2%	2%	0%	1%	5%
Cyanoamino acid metabolism	2%	2%	2%	3%	1%	0%
Cysteine and methionine metabolism	1%	1%	2%	0%	2%	2%
Purine metabolism	1%	2%	1%	3%	1%	0%
Nitrogen metabolism	1%	1%	1%	2%	1%	0%
Fructose and mannose metabolism	1%	2%	1%	2%	1%	2%
RNA degradation	1%	1%	1%	0%	1%	2%
Plant-pathogen interaction	1%	2%	1%	0%	1%	3%
SNARE interactions in vesicular transport	1%	4%	1%	5%	0%	3%
Peroxisome	1%	0%	1%	0%	1%	0%
Phenylalanine metabolism	1%	1%	1%	2%	1%	0%
Glycine, serine and threonine metabolism	1%	1%	1%	0%	1%	2%
Inositol phosphate metabolism	1%	1%	1%	2%	1%	0%
Fatty acid metabolism	1%	0%	1%	0%	1%	0%
Citrate cycle (TCA cycle)	1%	0%	1%	0%	1%	0%
Pyrimidine metabolism	1%	2%	1%	3%	1%	0%
Proteasome	1%	0%	1%	0%	1%	0%
Pentose phosphate pathway	1%	1%	1%	0%	1%	2%
Spliceosome	0%	2%	1%	0%	1%	3%
Selenocompound metabolism	1%	0%	1%	0%	1%	0%
Carotenoid biosynthesis	1%	2%	1%	3%	0%	0%
Alanine, aspartate and glutamate metabolism	1%	0%	1%	0%	1%	0%
Arginine and proline metabolism	1%	0%	1%	0%	1%	0%
Stilbenoid, diarylheptanoid and gingerol biosynthesis	1%	0%	1%	0%	1%	0%
Steroid biosynthesis	1%	0%	1%	0%	1%	0%
Glycerophospholipid metabolism	1%	0%	1%	0%	1%	0%
Ubiquitin mediated proteolysis	1%	0%	1%	0%	1%	0%
Tyrosine metabolism	1%	0%	1%	0%	1%	0%
Porphyrin and chlorophyll metabolism	0%	1%	1%	0%	1%	2%
Propanoate metabolism	0%	1%	1%	0%	1%	2%
Valine, leucine and isoleucine degradation	0%	1%	1%	0%	1%	2%
Lysine degradation	0%	0%	0%	0%	1%	0%
Terpenoid backbone biosynthesis	0%	0%	0%	0%	1%	0%
Protein export	0%	0%	0%	0%	1%	0%
Glycosaminoglycan	0%	0%	0%	0%	1%	0%

Table 3 (*continued*)

Pathway	Phenol	TCA/ acetone	Union	Intersection	P–T	T–P
degradation						
Photosynthesis - antenna proteins	0%	0%	0%	0%	1%	0%
Other glycan degradation	0%	1%	0%	2%	0%	0%
Basal transcription factors	0%	1%	0%	2%	0%	0%
mRNA surveillance pathway	0%	0%	0%	0%	1%	0%
Ascorbate and aldarate metabolism	0%	0%	0%	0%	1%	0%
Flavone and flavonol biosynthesis	0%	0%	0%	0%	1%	0%
Ribosome biogenesis in eukaryotes	0%	0%	0%	0%	1%	0%
Tryptophan metabolism	0%	0%	0%	0%	1%	0%
Glycerolipid metabolism	0%	0%	0%	0%	1%	0%
Phosphatidylinositol signaling system	0%	0%	0%	0%	1%	0%
RNA polymerase	0%	1%	0%	2%	0%	0%
One carbon pool by folate	0%	0%	0%	0%	1%	0%
Diterpenoid biosynthesis	0%	1%	0%	2%	0%	0%
Circadian rhythm - plant	0%	0%	0%	0%	1%	0%
ABC transporters	0%	1%	0%	0%	0%	2%
Fatty acid elongation	0%	1%	0%	0%	0%	2%
Natural killer cell mediated cytotoxicity	0%	2%	0%	0%	0%	3%
Amino sugar and nucleotide sugar metabolism	0%	0%	0%	0%	0%	0%
Alpha-linolenic acid metabolism	0%	0%	0%	0%	0%	0%
Tropane, piperidine and pyridine alkaloid biosynthesis	0%	0%	0%	0%	0%	0%
Limonene and pinene degradation	0%	0%	0%	0%	0%	0%
Anthocyanin biosynthesis	0%	0%	0%	0%	0%	0%
N-glycan biosynthesis	0%	0%	0%	0%	0%	0%
Ether lipid metabolism	0%	0%	0%	0%	0%	0%
Arachidonic acid metabolism	0%	0%	0%	0%	0%	0%
Biosynthesis of unsaturated fatty acids	0%	0%	0%	0%	0%	0%
Phenylalanine, tyrosine and tryptophan biosynthesis	0%	0%	0%	0%	0%	0%
Butanoate metabolism	0%	0%	0%	0%	0%	0%
Aminoacyl-tRNA biosynthesis	0%	0%	0%	0%	0%	0%
Nucleotide excision repair	0%	1%	0%	2%	0%	0%
Galactose metabolism	0%	1%	0%	2%	0%	0%
Synthesis and degradation of ketone bodies	0%	0%	0%	0%	0%	0%
Nicotinate and nicotinamide metabolism	0%	0%	0%	0%	0%	0%
Riboflavin metabolism	0%	0%	0%	0%	0%	0%
Beta-alanine metabolism	0%	0%	0%	0%	0%	0%
Sulfur metabolism	0%	0%	0%	0%	0%	0%
Regulation of autophagy	0%	0%	0%	0%	0%	0%
Pentose and glucuronate interconversions	0%	0%	0%	0%	0%	0%
Base excision repair	0%	0%	0%	0%	0%	0%
Fatty acid biosynthesis	0%	1%	0%	0%	0%	2%
Lysine biosynthesis	0%	1%	0%	0%	0%	2%
Pantothenate and CoA biosynthesis	0%	1%	0%	0%	0%	2%
Valine, leucine and isoleucine biosynthesis	0%	1%	0%	0%	0%	2%
Glucosinolate biosynthesis	0%	1%	0%	0%	0%	2%

proteins with differential biochemical features such as pIs. In addition, various contaminants might differentially interfere with the two isolation processes and thus discordantly influence LC–ESI–MS/MS pretreatment and detection, as discussed above. More work is currently underway to clarify such observations. In general, our work clearly demonstrated that a better coverage of a plant proteome from such complex sample context may need combination of data from multiple isolation methods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2017.01.006.

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Conflict of interest

None declared.

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