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Proteomic analysis of banana xylem sap provides insight into resistant mechanisms to *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4

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Research

Keywords: Banana, xylem sap, proteome, Fusarium wilt, Tropical Race 4

Posted Date: May 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-26869/v1

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Abstract

Background: Fusarium wilt is a destructive soilborne disease of banana caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), especially Tropical Race 4 (TR4), which is a xylem-invading fungus. It was evident that xylem sap contained macromolecules, such as proteins, involved in disease-resistance processes. However, there is no research to analyze changes in banana xylem sap proteins response to TR4 to date.

Methods: To gain an integrated understanding of differential protein expression in banana xylem sap during TR4 infection, we performed a comparative proteomic analysis of xylem sap in resistant 'Pahang' and susceptible 'Brazilian' bananas inoculated with TR4.

Results: A total of 1036 proteins were detected in xylem sap of both bananas, among which some proteins are involved in 'signal transduction', 'environmental adaptation', 'biosynthesis of secondary metabolites' and 'lipid metabolism', indicating that xylem sap contained defense-related proteins. A number of 129 differential expression proteins (DEPs) were identified in 4 possible pairs between resistant and susceptible tested combinations. Of these DEPs, hypersensitive-induced response protein 1 (HIR1), E3 ubiquitin ligase (E3) might play negative roles in 'Pahang' response to TR4 attack, whereas chalcone isomerase (CHI), glycine-rich RNA-binding protein (GRP), carboxylesterase (CXE) and GDSL lipase (GLIP) might play positive roles in 'Pahang' defense against TR4 infection.

Conclusions: Banana xylem sap contained defense-related proteins, among which HIRP1, E3, CHI, GRP, CXE and GLIP involved in banana defense against TR4. To our knowledge, this is first report to analyze changes in banana xylem sap proteins response to TR4, which help us to explore molecular mechanisms of banana resistant to Fusarium wilt.

Background

Banana (*Musa* spp.) is a major staple food in many tropical and subtropical regions, and is also an important export fruit in many developing countries [1]. Banana is grown in 135 countries and with an annual output of 145 million tons worldwide [2]. Most cultivated and edible bananas are diploid or triploid derived from *Musa acuminata* (AA) selfing or from hybridization with *Musa balbisiana* (BB) [3]. The Cavendish subgroup (AAA), derived from a single triploid genotype, is responsible for approximately 45% of all productions [4].

Due to remarkably low genetic variation and monoculture of bananas, diseases and pests increasingly damage banana production [5]. Among these diseases, Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), specifically Tropical Race 4 (TR4) [5], is threatening the global banana industry [6]. TR4 affects all banana cultivars [7] and the rapid expansion of TR4 worldwide is worrisome [8–10]. Recently, Latin America was put on alert after TR4 was reported presence in Colombia [11, 12].

Fusarium wilt of banana is a typical soil-borne and vascular disease. TR4 invades roots and extends upward to rhizome along the root xylem vessels [13], further to outer leaf sheaths of the pseudostem [14].

The infected plants nearly produce no fruit bunches, and finally died from a serious shortage of water due to vascular plugging [15, 16]. The susceptible cultivars cannot be planted once the pathogen invaded, because its chlamydospores survive for decades in the soil even absence of banana [17].

Fusarium wilt of banana is disastrous to producers, unfortunately, few effective measures can be used to manage this disease, unless the commercial resistant cultivar is available [18]. Breeding resistant cultivar is the only option of effective disease management [19]. Banana conventional breeding is difficult to use because most cultivars are triploid, sterile and parthenocarpic [20]. Genetically modified banana is a perfect alternative for banana improvement [21–23]. Recently, transgenic Cavendish bananas which transformed with a resistance gene analogue 2 (RGA2), from a TR4-resistant wild diploid banana, remained healthy in 3-year infected field trial [24]. It shows promising way to confer resistance to TR4.

Proteomics, complementary to transcriptomics, provides an insight into complex biological processes at the global protein level [25–27]. In recent years, large-scale studies of proteomics have focused on dissecting interactions between bananas and *Foc* [28–30]. The proteins related to PR response, cell wall strengthening and antifungal compound synthesis were involved in banana defense to TR4 [29]. β-1,3-glucanase and chitinase were reported to function in banana against TR4 at the early defense stage [28]. The expression patterns of proteins related with cell cytoskeleton, natural killer cell mediated cytotoxicity and lipid signaling were different in banana during *Foc*1 and *Foc*4 infection, suggesting these proteins participated in mediating different resistance to *Foc*1 and *Foc*4 in banana cultivar ‘Brazilian’ [30]. These studies help us to understand the defense mechnism of banana against TR4.

Plants transport signal molecules as well as water and minerals over long distance via the xylem [31]. The signal molecules are vital for plant adaption to abiotic and biotic stress [32, 33]. The xylem sap proteomics have been applied to characterize the processes associated with plant defense to Fusarium wilt [34–38]. However, to our knowledge, there is no research to analyze changes in banana xylem sap proteins response to TR4 to date. In this study, we performed a comparative proteomics analysis of xylem sap in resistant and susceptible bananas inoculated with TR4. A total of 1,036 proteins were detected in both banana xylem sap, including proteins involved in ‘signal transduction’, ‘environmental adaptation’, ‘biosynthesis of secondary metabolites’ and ‘lipid metabolism’ pathways, suggesting that xylem sap contained defense-related proteins. To analyze differential protein expression during TR4 infection, a number of 129 DEPs were identified in 4 possible pairs between resistant and susceptible tested combinations, among which HIRP1, E3, CHI, GRP, CXE and GLIP involved in banana defense against TR4. This study provides integrated insight into the resistant mechanism of banana against Fusarium wilt.

Materials And Methods

Plant inoculation and xylem sap collection

Musa acuminata ‘Pahang’ (AA, ITC0609) obtained from the International *Musa* Germplasm Transit Centre and *Musa* Cavendish ‘Brazilian’ (AAA, commercial cultivar in China) were used in the study. Pahang

shows high resistance to *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4), while Brazilian shows high susceptible to TR4 [1, 13, 39]. The banana inoculation was performed according to our previous study [13] with minor modification. The roots of banana with 6–8 leaves were cut to 5 cm and immersed into 10^6 conidia/mL TR4 conidia suspension for 30 min, then were transplanted to plastic pots filled with sterile vermiculite. Plants were immersed into sterile distilled water as mock inoculation. The inoculated plants were placed in an artificial climate chamber at 30℃, 8 h light/16 h dark with 80% humidity (Fig. 1A). At 14 days post inoculation (DPI), the plants were rinsed off with tap water and dried with filter papers, and the pseudostems were transected at 0.5 cm above the corms with a sterile blade (Fig. 1B, C). After removed the exudate from the cut cells, the xylem sap exuded spontaneously from the remaining pseudostems was collected with a pipette in time (Fig. 1D-E). To obtain sufficient amounts of protein for analysis, the xylem sap isolated from at least 30 plants was concentrated into one independent biological replicate, and three independent biological replicates were conducted. The xylem sap was frozen with liquid nitrogen prepared for protein extraction. The Pahang and Brazilian inoculated with TR4 named P_dpi and B_dpi, respectively. The Pahang and Brazilian inoculated with sterile water named P_mock and B_mock, respectively.

(A) The inoculated banana plants were placed in an artificial climate chamber. (B) Banana with 6–8 leaves. (C) The pseudostem of banana was transected at 0.5 cm above the corms. (D) The transected pseudostem of banana was placed on the ice to collect xylem sap. (E) Banana xylem sap exuded spontaneously from the transected pseudostem. (F) Banana xylem sap was collected in a pipette.

Xylem Sap Protein Extraction

At least 3 mL xylem sap was resuspended with benzyltriphenylphosphonium chloride (BPP) solution (containing 1% Poly vinyl pyrrolidone pvp, PVPP) in the ratio of 1:3, and vortexed for 10 min at 4℃. Equal volume of Tris-saturated phenol was added, and shook for 10 min at 4℃. The mixture was centrifuged with 12,000 g at 4℃, and the bottom phenol phase was transferred to a new reaction tube and re-extracted by adding an equal volume of BPP solution, and shook for 10 min at 4℃. The mixture was centrifuged with 12,000 g at 4℃, and the bottom phenol phase was collected. The proteins were precipitated from the phenol phase with ammonium carbinol acetate solution in the ratio of 1:5 at -20℃ overnight. Proteins were pelleted by centrifugation. Pellets were washed twice with acetone. Proteins were dissolved in 8 M urea and 1% sodium dodecyl sulfate (SDS). After centrifuging at 4℃ the supernatant was collected.

Itraq Labeling

Protein concentrations were determined by Bicinchoninic acid (BCA) method by BCA Protein Assay Kit (Pierce, Thermo, USA), and the total of protein should be no less than 100 μg. Protein digestion was performed according to the standard procedure and the resulting peptide mixture was labeled using the 8-plex iTRAQ reagent (Applied Biosystems, 4390812) according to the manufacturer's instructions.

High Ph Rplc Separation

Samples were fractionated using high pH reverse phase separation techniques to increase the depth of the proteome. The peptides were resuspended with a loading buffer (2% acetonitrile in ammonium hydroxide solution, pH 10), and separated by high pH reversed-phase liquid chromatography (RPLC, Acquity Ultra Performance LC, Waters, USA). The gradient elution was carried out on a high pH RPLC column (ACQUITY UPLC BEH C18 Column 1.7 μm, 2.1 mm × 150 mm, Waters, USA) at 400 μL/min with a gradient increased for 66 min (Phase B: 5 mM Ammonium hydroxide solution containing 80% acetonitrile, pH 10). Twenty fractions were collected from each sample and these fractions were pooled to form 10 total fractions per sample.

Mass Spectrometry Analysis

The experiment was conducted on a Q Extraction mass spectrometer in combination with Easy-nLC 1200. 4 μL of each fraction was injected into nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Peptide mixture (2 μg) was loaded into a C18-reversed phase column (75 μm × 25 cm, Thermo, USA) in buffer A (2% acetonitrile and 0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a 300 μL/min flow rate. An electrospray voltage of 1.8 kV was used at the inlet of the mass spectrometer. Q Exactive mass spectrometer was operated in the data-dependent mode and automatically switched between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350–1300) were measured with a mass resolution of 70,000, followed by 20 consecutive high-energy collisional dissociation (HCD) MS/MS scans with a resolution of 17,500. In all cases, a micro-scan was recorded with a dynamic exclusion of 18 sec, and the MS/MS normalized collision energy was set at 30.

Sequence Database Searching

MS/MS spectra were searched using ProteomeDiscoverer (Thermo Scientific, Version 2.2) against *Musa acuminata* database (http://www.uniprot.org/proteomes/UP000012960) and the decoy database as the following parameters. The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. The parameters for protein searching were set as follows: tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as fixed modification, and oxidation of methionine and protein N-terminal acetylation as variable modifications. False discovery rate (FDR) of peptide identification was set as FDR ≤ 0.01. A minimum of one unique peptide identification was used to support protein identification.

Quality Control Of Raw Data

The quality evaluation for each sample of the original MS data was conducted, including the matching error of peptides, the distribution statistics of peptide number, the length distribution of identified peptides and the distribution of protein molecular weight.

Itraq Quantitative Proteomics Analysis

The basic information analysis process for iTRAQ quantitative proteomics using the free online platform of Majorbio Cloud Platform (www.majorbio.com). First, the raw mass spectrums generated by the mass spectrometer were subjected to the peak identification. Secondly, the reference proteomic database of banana (http://www.uniprot.org/proteomes/UP000012960) was established to identify peptides and proteins. All identified proteins were functional annotated using Cluster of Orthologous Groups of proteins (COG, http://eggnogdb.embl.de/#/app/home), Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) with E-value ≤ 1e-5 and identity ≥ 0.98. The DEPs were identified with fold change > 1.2 (upregulation), fold change < 0.83 (downregulation) and P value < 0.05, and analyzed including DEPs Venn and expression pattern analysis.

Results

Data and quality control evaluation

Xylem saps of Pahang (resistant) and Brazilian (susceptible) inoculated with TR4 or mock at 14 dpi were collected for comparative proteomic analysis. A total of 261,038 spectra were acquired through iTRAQ quantitative proteomics analysis, among which 31,450 spectra were matched to 6,503 peptides, 3,291 proteins and 1509 protein groups (Fig. 2A). Since this experiment was divided into two labeled groups, some proteins only appeared in one labeled group, and 1036 proteins that existed in both labeled groups were used for subsequent analysis. The distribution of peptide matching error analysis indicated error distribution between the true and theoretical values of the relative molecular weights of all matched peptides was acceptable (Fig. 2B). There were 126 proteins only with one peptide, and approximately 87.84% of the proteins contained at least two peptides. Protein number decreased with increasing number of peptides (Fig. 2C). The length of the peptide ranged from 6 to 34 amino acids, among which the peptide of 10 amino acids was most abundant (Fig. 2D). The molecular weight of almost all proteins (99.5%) ranged from 1 kDa to 121 kDa. 21–41 kDa proteins account for 40.54%, followed by proteins with 41–61 kDa and 1–21 kDa (Fig. 2E). In terms of protein's sequences coverage distribution, about 70.14% of proteins constituted more than 10% protein's sequences coverage, and 45.56% of proteins constituted more than 20% protein's sequences coverage (Fig. 2F). It indicated that the identified proteins have good peptide coverage, and the data had highly confidence.

(A) Protein information. (B) The distribution of peptide matching error. (C) Peptide number distribution. (D) Peptide length distribution. (E) Protein molecular weight distribution. (F) Distribution of protein's

sequences coverage.

All Proteins Function Annotation And Expression Analysis

All identified proteins (1,036) were conducted functional analysis, of which 938, 923 and 779 proteins were annotated with COG, GO and KEGG databases, respectively.

In terms of COG (Fig. 3), these proteins were assigned into 23 functional categories, and the top 3 largest categories were ‘posttranslational modification, protein turnover, chaperones’ (113 proteins), ‘energy production and conversion’ (85 proteins) and ‘carbohydrate transport and metabolism’ (75 proteins). The categories related to plant in response to pathogen are ‘lipid transport and metabolism’ (35 proteins), ‘cell wall/membrane/envelope biogenesis’ (16 proteins), ‘secondary metabolites biosynthesis, transport and catabolism’ (13 proteins), ‘signal transduction mechanisms’ (18 proteins) and ‘defense mechanisms’ (6 proteins).

(A) Chromatin structure and dynamics. (B) Energy production and conversion. (C) Cell cycle control, cell division, chromosome partitioning. (D) Amino acid transport and metabolism. (E) Nucleotide transport and metabolism. (F) Carbohydrate transport and metabolism. (G) Coenzyme transport and metabolism. (H) Lipid transport and metabolism. (I) Translation, ribosomal structure and biogenesis. (J) Transcription. (K) Replication, recombination and repair. (L) Cell wall/membrane/envelope biogenesis. (M) Cell motility. (N) Posttranslational modification, protein turnover, chaperones. (O) Inorganic ion transport and metabolism. (P) Secondary metabolites biosynthesis, transport and catabolism. (Q) General function prediction only. (R) Function unknown. (S) Signal transduction mechanisms. (T) Intracellular trafficking, secretion, and vesicular transport. (U) Defense mechanisms. (V) Nuclear structure. (W) Cytoskeleton.

In terms of GO (Fig. 4), these proteins were assigned into 43 GO terms and classified into three groups, included biological process, cellular component and molecular function. For the biological process, ‘metabolic process’ (561 proteins) was the most abundant terms, followed by ‘cellular process’ (526 proteins) and ‘single-organism process’ (442 proteins). For the cellular component, the top 3 most enriched terms were ‘cell’ (528 proteins), ‘cell part’ (521 proteins) and ‘organelle’ (343 proteins). For the molecular function, the top 3 enriched terms were ‘catalytic activity’ (521 proteins), ‘binding’ (442 proteins) and ‘transporter activity’ (66 proteins). In addition, among of all pathways, ‘response to stimulus’ (137 proteins), ‘signaling’ (35 proteins), ‘detoxification’ (30 proteins), ‘immune system process’ (6 proteins) and ‘antioxidant activity’ (26 proteins) were normally regarded as disease resistance related categories.

(A) Biological process. (B) Cellular component. (C) Molecular function.

In terms of KEGG (Fig. 5), these proteins were assigned into 18 pathways, and divided into 5 categories. ‘Carbohydrate metabolism’ (163 proteins), ‘signal transduction’ (14 proteins), ‘transport and catabolism’ (85 proteins), ‘folding, sorting and degradation’ (93 proteins) and ‘environmental adaptation’ (10 proteins)

were the most abundant pathways in metabolism, environmental information processing, cellular processes, genetic information processing and organismal systems categories, respectively. The proteins involved in ‘signal transduction’ and ‘environmental adaptation’ as well as ‘biosynthesis of secondary metabolites’ (45 proteins) and ‘lipid metabolism’ (45 proteins) might play important roles in plant against pathogen (Supplementary Table 1), such as nucleoside diphosphate kinase (XP_009384691.1, XP_009413273.1), pathogenesis-related protein 1 (XP_009388942.1), coronatine-insensitive protein homolog (XP_009416210.1), calcium-dependent protein kinase (XP_009379843.1), calcium-binding protein CML7 (XP_009418740.1), phenylalanine ammonia-lyase (XP_009399473.1), 4-coumarate-CoA ligase (XP_009384735.1), cinnamoyl-CoA reductase (XP_009395948.1, XP_009413954.1), cinnamyl alcohol dehydrogenase (XP_009397914.1), peroxidase (XP_009384773.1, XP_009396783.1, XP_009390142.1), caffeoyl-CoA O-methyltransferase (XP_009407208.1), chalcone synthase (XP_009404102.1), flavonoid 3',5'-hydroxylase (XP_009411862.1, XP_009386727.1), dihydroflavonol-4-reductase (XP_009396003.1), long chain acyl-CoA synthetases (XP_009394139.1, XP_009413949.1), phospholipase D α1 (XP_009407292.1, XP_009381115.1, XP_009408984.1), etc.

(A) Organismal systems. (B) Genetic information processing. (C) Cellular processes. (D) Environmental information processing. (E) Metabolism.

Differential Expression Proteins Analysis

A total of 129 DEPs were identified in 4 possible pairwise comparisons among resistant and susceptible tested combinations (Fig. 6A and 6B), further performed expression and function annotation (Supplementary Table 2) analysis. To analyze differential protein expression during TR4 infection, the DEPs were identified through comparing TR4 inoculated samples with mock samples (P_dpi vs P_mock, B_dpi vs B_mock). A number of 29 DEPs were obtained. 19 DEPs (Fig. 6C) were changed significantly in Pahang against TR4 attack (P_dpi vs P_mock), among which 14 DEPs decreased and 5 DEPs increased. 11 DEPs (Fig. 6D) were changed significantly in Brazilian responding to TR4 (B_dpi vs B_mock), among which 4 DEPs decreased and 7 DEPs increased. In these DEPs, only one protein (fumarylacetoacetase, XP_009398768.1) increased in both infected bananas. These results indicated that the susceptible and resistant banana genotypes might adopted different strategies to combat TR4. Hypersensitive-induced response protein 1 (XP_018684918.1) and E3 ubiquitin ligases (XP_009408396.1) decreased in abundance in Pahang under TR4 infection. Chalcone-flavonone isomerase (XP_009384766.1) and glycine-rich RNA-binding proteins (XP_009394303.1) increased in abundance in Pahang response against TR4 infection. These DEPs might play center roles in banana response to TR4.

To further explore the associated resistance mechanisms of Pahang, the DEPs were identified by comparing TR4 inoculated Pahang with that of Brazilian (P_dpi vs B_dpi). A number of 52 DEPs were obtained, of these were 31 up-regulated DEPs and 21 down-regulated DEPs. 84 DEPs were identified by comparing mock inoculated Pahang with that of Brazilian (P_mock vs B_mock). Among which 61 DEPs were up-regulation, and 23 DEPs were down-regulation. A number of 26 DEPs (Fig. 6E) were identified by

comparing TR4 inoculated Pahang with that of Brazilian, and the DEPs in mock inoculated Pahang versus that of Brazilian were excluded. Carboxylesterases (XP_009406873.1) and GDSL esterase/lipases (XP_009382515.1) increased abundance in TR4 inoculated Pahang compared with that of Brazilian, but no changes in Pahang mocks compared with that of Brazilian. These DEPs might play crucial roles in making Pahang resistant to TR4.

(A) Number of identified in 4 pairwise comparisons among resistant and susceptible tested combinations. (B) Venn diagram analysis of identified in 4 pairwise comparisons. (C) DEPs identified by comparing TR4 inoculated Pahang with mock samples (P_dpi vs P_mock). (D) DEPs identified by comparing TR4 inoculated Brazilian with mock samples (B_dpi vs B_mock). (E) DEPs identified by comparing TR4 inoculated Pahang with that of Brazilian, and the DEPs, in Pahang mock samples versus that of Brazilian, were excluded ('P_dpi vs B_dpi'–'P_mock vs B_mock'). The protein abundances are normalized to the same total peptide amount per channel and scaled.

Discussion

Fusarium wilt is a destructive soilborne disease of banana caused by *Foc*, especially by TR4 which is a xylem-invading fungus. TR4 colonizes in the xylem of banana and completes its life cycle [40]. It was evident that xylem sap contained macromolecules, such as proteins, involved in disease-resistance processes [34, 35, 38]. To gain an integrated understanding of the changes of banana xylem sap proteins during TR4 infection, we performed a comparative proteomic analysis of xylem sap in resistant diploid 'Pahang' and susceptible triploid 'Brazilian' inoculated with TR4 at 14 DPI. A total of 1,036 proteins were detected in xylem sap of both bananas, and were further researched into functions. Some proteins are involved in 'signal transduction', 'environmental adaptation', 'biosynthesis of secondary metabolites' and 'lipid metabolism' which are normally regarded as disease-resistance pathway, indicated that xylem sap contained defense-related proteins.

Signal Transduction

There is no doubt that signal transduction pathways are responsible for induction of plant defense response [41, 42]. Of these pathways, the mitogen-activated protein kinase (MAPK) cascades and plant hormone signals play pivotal roles in plant disease resistance [43]. Once plant perceives the invading pathogen, the activation of MAPKs is one of the earliest signaling events [44]. In the present studies, 10 proteins were related with MAPK signaling pathway-plant (Supplementary Table 1), such as nucleoside diphosphate kinase (NDPK, XP_009384691.1 and XP_009413273.1) inducing MPK3/6 expression through phosphorylation leading to hypersensitive response (HR) cell death in plant response to pathogen attack [45].

Among the plant hormone signals, salicylic acid (SA) and jasmonic acid (JA) are essential components of plant defense against pathogen [46]. SA and JA antagonize each other [47]. It is generally considered that SA enhances resistance to biotrophs, while JA is effectively against necrotrophs and insects [48, 49].

However, there is exception that SA metabolism activation and signal transduction can improve banana resistance to TR4 [50]. In this study, 2 proteins was associated with SA and JA signals (Supplementary table 1), including pathogenesis-related protein 1 (PR1, XP_009388942.1), a marker for systemic acquired resistance (SAR) from SA signaling pathway [51]; and coronatine-insensitive protein homolog (COI1, XP_009416210.1), a key regulator for JA-dependent induced systemic resistance (ISR) [48, 52]. Further research is needed to determine whether SA-dependent SAR and JA-dependent ISR were simultaneously activated in banana.

Environmental Adaptation

In the natural environment, plants were threatened by various abiotic and biotic stress. Over the evolutionary course during plant-pathogen interaction, plants have developed multi-layered innate immune system to defend against pathogen. The preliminary layer of immune is pathogen-associated molecular pattern (PAMP) perceived by pathogen recognition receptors (PRRs), and induces a series of physiological changes leading to PAMP-triggered immunity (PTI) [53]. These physiological changes include bursts of reactive oxygen species (ROS) and changes in calcium (Ca^{2+}) concentrations [54, 55]. Ca^{2+} acts as an important second messenger whose concentration is sensed by Ca^{2+}-binding proteins, such as calcium-dependent protein kinase (CDPK, XP_009379843.1) and calcium-binding protein CML7 (CaMCML, XP_009418740.1) detected in the banana xylem sap (Supplementary Table 1), further initiate downstream signaling processes [56], such as HR and cell wall reinforcement.

Biosynthesis Of Secondary Metabolites

Plant secondary metabolites contribute to all aspects in plant and pathogen interactions [57]. In the biosynthesis of secondary metabolites, phenylpropanoid and flavonoid biosynthesis have been proved to encompass a wide range of constitute and inducible immunity through lignin and phytoalexin synthesis [58]. In the present study, 21 proteins were divided into phenylpropanoid biosynthesis (Supplementary Table 1). The synthetic enzymes of lignin leading to the reinforcement of the cell wall [59], including phenylalanine ammonia-lyase (PAL, XP_009399473.1), 4-coumarate-CoA ligase (C4L, XP_009384735.1), cinnamoyl-CoA reductase (CCR, XP_009395948.1 and XP_009413954.1), cinnamyl alcohol dehydrogenase (CAD, XP_009397914.1) and peroxidase (POD, XP_009384773.1, XP_009396783.1 and XP_009390142.1) were detected in xylem sap (Supplementary Table 1). In addition, caffeoyl-CoA O-methyltransferase (CCoAOMT, XP_009407208.1) associated with lignin production resulting in quantitative resistance to multiple pathogens [60], existed also in xylem sap. 13 proteins were assigned to flavonoid biosynthesis, such as chalcone synthase (CHS, XP_009404102.1) as the gatekeeper of flavonoid biosynthesis which can help plant to produce more flavonoids, isoflavonoid-type phytoalexins [61], the P450 enzyme flavonoid 3',5'-hydroxylase (F3'5'H, XP_009411862.1 and XP_009386727.1) and dihydroflavonol-4-reductase (DFR, XP_009396003.1) as precursors for the production of catechins and pro-anthocyanidins involved in plant resistance [62].

Lipid Metabolism

Lipids and fatty acids involved in lipid metabolism were considered as signal transduction mediators of plant disease resistance [63, 64]. In this study, long chain acyl-CoA synthetases (LACS, XP_009394139.1 and XP_009413949.1) involved in fatty acids metabolism that acting the synthesis of cutin conferred plant resistance to fungal pathogen [65, 66], and phospholipase D α1 (PLDα1, XP_009407292.1, XP_009381115.1 and XP_009408984.1) involved in lipid metabolism which promote phosphatidic acid and ROS affecting plant immunity [67] were detected in xylem sap (Supplementary Table 1).

Differential Protein Expression Response To Tr4 Infection

To analyze differential protein expression response to TR4 infection, a number of 129 DEPs were identified in 4 possible pairs between resistant and susceptible tested combinations, but only 19 and 11 DEPs were identified in P_dpi vs P_mock and B_dpi vs B_mock, respectively (Supplementary Table 2). It suggested that TR4 did not induce highly dramatic changes in the overall xylem sap proteome. This result was similar to the proteomic analysis of melon phloem sap in response to viral infection [68]. However, these limited DEPs present in phloem sap might also play important roles in banana combatting with TR4.

Hypersensitive-induced response protein 1 (HIR1) may act as regulators of plant immunity by triggering hypersensitive cell death [69, 70]. In the study, HIR1 (XP_018684918.1) decreased in abundance in Pahang but no significant changes occurred in Brazilian under TR4 infection, suggesting Pahang might decrease HIR1 expression to suppress the cell death, and enhanced resistant to TR4 due to *Foc* usual as hemibiotroph or necrotroph [71, 72].

Ubiquitin involved in the ubiquitination system are key for plant immunity [73]. Ubiquitination is mediated by a three-step enzymatic cascades including activating (E1), conjugating (E2) and ligating (E3) enzymes [74]. E3 has received more attention in research. *CaRING1*, E3 ubiquitin ligase RING1 gene, played a positive role in pepper (*Capsicum annuum*) response to microbial pathogens [75]; whereas a homologous triplet of U-box type E3 ubiquitin ligases acted as negative regulators of PTI in *Arabidopsis* [76]. In the study, E3 (XP_009408396.1) with a RING zinc-finger domain decreased in abundance only in Pahang response to TR4, however, further studies are needed to prove whether this protein played a negative role in banana response to TR4.

Chalcone isomerase (CHI) is a key enzyme of flavonoid pathway involved in the production of phytoalexin [77], which plays an important role in plant defense against pathogen. Overexpression of CHI enhanced resistance of soybean (*Glycine max*) against *Phytophthora sojae* [78]. In this study, chalcone-flavonone isomerase (also regard as CHI, XP_009384766.1) was increased in abundance in Pahang under TR4 infection, as well as in Pahang mocks compared with Brazilian mocks. It implied that this protein increased resistance against TR4 in banana.

Glycine-rich RNA-binding proteins (GRPs) function as regulators in diverse cellular processes, including response to stress in plants [79, 80]. Over expressing TaRZ1, a wheat (*Triticum aestivum*) zinc finger-containing GRP, in *Arabidopsis thaliana* increased resistance against necrotrophic bacteria *Pseudomonas syringae* [81]. In the present study, GRP (XP_009394303.1) containing an RNA recognition motif (RRM) domain was increased in abundance in Pahang response to TR4, but no significant changes in other pairwise comparisons. It indicated that this protein might play a positive role in banana response against TR4.

Deps Associated Resistance In Pahang

To further explore the associated resistance mechanisms of Pahang, a number of 26 DEPs were identified by comparing TR4 inoculated Pahang with that of Brazilian, and the DEPs in mock inoculated Pahang versus that of Brazilian were excluded. Among which, 7 proteins whose function is unknown, but some proteins are highly associated with resistance to pathogen in plant, such as carboxylesterase and GDSL esterase/lipase (Supplementary Table 2).

Carboxylesterases (CXEs) have been implicated in plant defense. A conserved NbCXE inhibited accumulation of *Tobacco mosaic virus* (TMV) in *Nicotiana benthamiana*, which enhanced plant resistance [82]. Constitutive expression of *PepEST*, a fungus-inducible carboxylesterase in peper (*Capsicum annuum*) increased resistance against the hemibiotrophic anthracnose fungus (*Colletotrichum gloeosporioides*) [83]. In the present study, a CXE (XP_009406873.1) was increased abundance in TR4 inoculated Pahang compared with that of Brazilian, but no changes in Pahang mocks compared with that of Brazilian.

GDSL esterase/lipases (GLIP) have been identified in many vascular plants and have been demonstrated that involved in plant defense against pathogens [84]. Overexpressing *GLIP1* in Arabidopsis improved resistance against hemibiotrophic and necrotrophic pathogens [85, 86]. In the study, a GLIP (XP_009382515.1) was increased abundance in TR4 inoculated Pahang compared with that of Brazilian, but no changes in other pairs. It further validated our previous transcriptomic study that one GLIP gene was activated by TR4 attack [59].

Conclusions

To gain an integrated understanding of the changes of banana xylem sap proteins during TR4 infection, we performed a comparative proteomic analysis of xylem sap in resistant diploid ‘Pahang’ and susceptible triploid ‘Brazilian’ inoculated with TR4 at 14 DPI. A total of 1036 proteins were detected in xylem sap of both bananas, among which some proteins are involved in ‘signal transduction’, ‘environmental adaptation’, ‘biosynthesis of secondary metabolites’ and ‘lipid metabolism’ which are normally regarded as disease-resistance pathway, indicated that xylem sap contained defense-related proteins. A number of 129 differential expression proteins (DEPs) were identified in 4 possible pairs. 19 DEPs identified by comparing TR4 inoculated Pahang with mock samples (P_dpi vs P_mock), and 26

DEPs identified by comparing TR4 inoculated Pahang with that of Brazilian, and the DEPs, in Pahang mock samples versus that of Brazilian, were excluded ('P_dpi vs B_dpi'–'P_mock vs B_mock'). Of these DEPs, hypersensitive-induced response protein 1 (HIRP1), E3 ubiquitin ligase (E3) might play negative roles in 'Pahang' response to TR4 attack, whereas chalcone isomerase (CHI), glycine-rich RNA-binding protein (GRP), carboxylesterase (CXE) and GDSL lipase (GLIP) might play positive roles in 'Pahang' defense against TR4 infection. To our knowledge, this is first report to analyze changes in banana xylem sap proteins response to TR4 to date, which provided insight into resistant mechanisms of banana defense against Fusarium wilt.

Abbreviations

Foc TR4: *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4; DEPs: Differential expression proteins; HIR1: Hypersensitive-induced response protein 1; E3: E3 ubiquitin ligase; CHI, Chalcone isomerase; GRP: glycine-rich RNA-binding protein; CXE: Carboxylesterase; GLIP: GDSL lipase; RGA2: Resistance gene analogue 2; DPI: Days post inoculation; BPP: Benzyltriphenylphosphonium chloride; PVPP: Poly vinyl pyrrolidone pvp; SDS: Sodium dodecyl sulfate; BCA: Bicinchoninic; iTRAQ: Isobaric tags for relative and absolute quantitation; RPLC: Reversed-phase liquid chromatography; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; HCD: High energy collisional dissociation; FDR: False discovery rate; COG: Cluster of Orthologous Groups; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: Mitogen-activated protein kinase; NDPK: Nucleoside diphosphate kinase; HR: Hypersensitive response; SA: Salicylic acid; JA: Jasmonic acid; PR1: Pathogenesis-related protein 1; SAR: Systemic acquired resistance; COI1: Coronatine-insensitive protein homolog; ISR: Induced systemic resistance; PAMP: Pathogen-associated molecular pattern; PRRs: Pathogen recognition receptors; PTI: PAMP-triggered immunity; ROS: Reactive oxygen species; CDPK: Calcium-dependent protein kinase; CaMCML: Calcium-binding protein CML; PAL: Phenylalanine ammonia-lyase; C4L: 4-coumarate-CoA ligase; CCR: Cinnamoyl-CoA reductase; CAD: Cinnamyl alcohol dehydrogenase; POD: Peroxidase; CCoAOMT: Caffeoyl-CoA O-methyltransferase; CHS: Chalcone synthase; F3'5'H: Flavonoid 3',5'-hydroxylase; DFR: Dihydroflavonol-4-reductase; LACS: Long chain acyl-CoA synthetases; PLDα1: Phospholipase D α1; RRM: RNA recognition motif; TMV: *Tobacco mosaic virus*.

Declarations

Competing interests

The authors declare that no competing interests exist.

Authors' contributions

L.Z. performed the experiments, analyzed the data and wrote the paper; L.L., S.L., T.B., S.X., H.F., K.Y. and P.H. analyzed the data; Y.W. and W.T. proof writing; S.-J.Z. conceived, designed the experiments and proof writing. All authors read and approved the final manuscript.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository [87] with the dataset identifier PXD018261.

Acknowledgements

This work was supported by Yunling Scholar Programme of Yunnan Provincial Government, the National Natural Science Foundation of China (NSFC31560505), the Science and Technology Department of Yunnan Provincial Government (2018BB016), Yunnan University of Chinese Medicine (30270104879),Key Laboratory of Green Prevention and Control of Agricultural Transboundary Pests of Yunnan Province, the CGIAR Research Program on Roots, Tubers and Bananas (RTB) and the National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences.

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Supplementary Information

Supplementary table 1 List of proteins involved in ‘signal transduction’, ‘environmental adaptation’, ‘biosynthesis of secondary metabolites’ and ‘lipid metabolism’.

Supplementary table 2 List of DEPs identified in P_dpi vs P_mock, B_dpi vs B_mock, and ‘P_dpi vs B_dpi’–‘P_mock vs B_mock’.

Figures

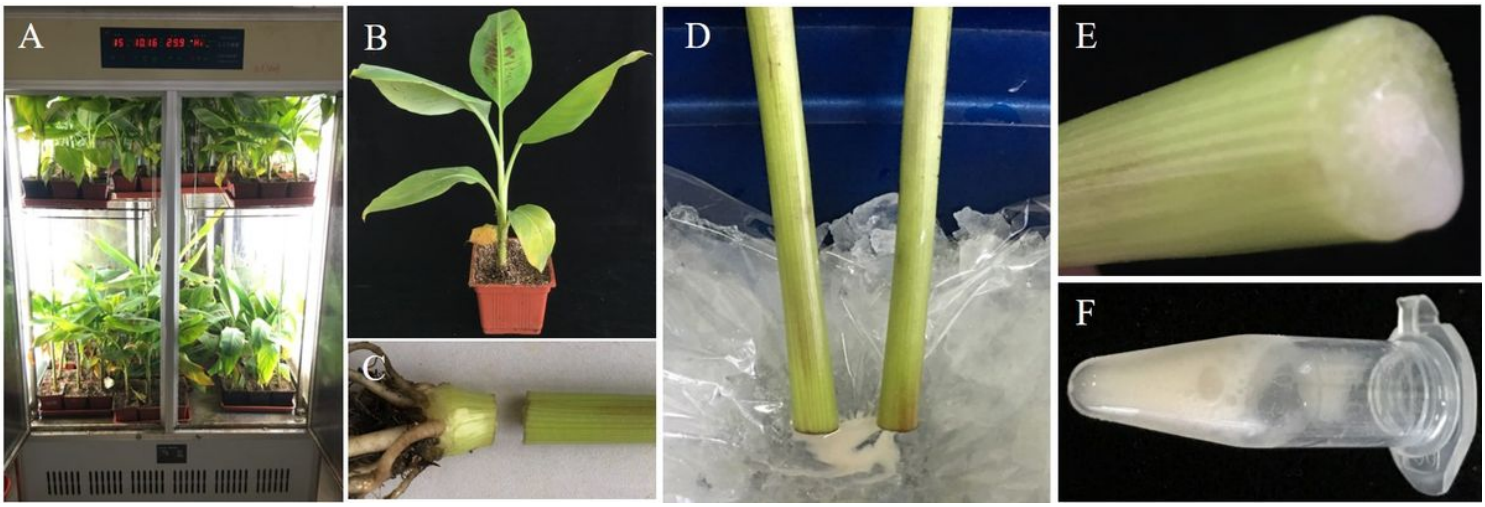

Figure 1

Banana xylem sap collection. (A) The inoculated banana plants were placed in an artificial climate chamber. (B) Banana with 6-8 leaves. (C) The pseudostem of banana was transected at 0.5 cm above the corms. (D) The transected pseudostem of banana was placed on the ice to collect xylem sap. (E) Banana xylem sap exuded spontaneously from the transected pseudostem. (F) Banana xylem sap was collected in a pipette.

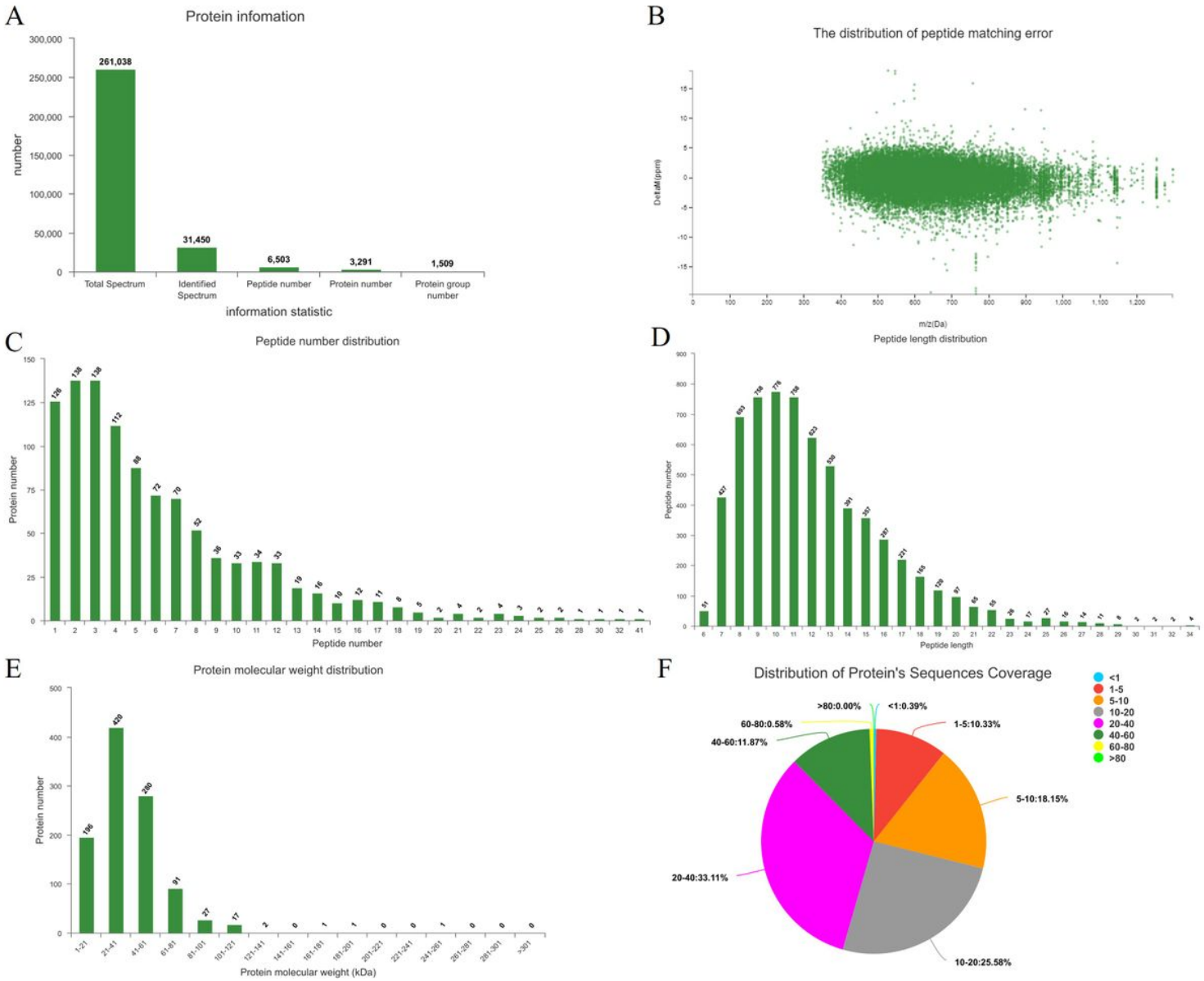

Figure 2

The general information of identified proteins from all samples. (A) Protein information. (B) The distribution of peptide matching error. (C) Peptide number distribution. (D) Peptide length distribution. (E) Protein molecular weight distribution. (F) Distribution of protein's sequences coverage.

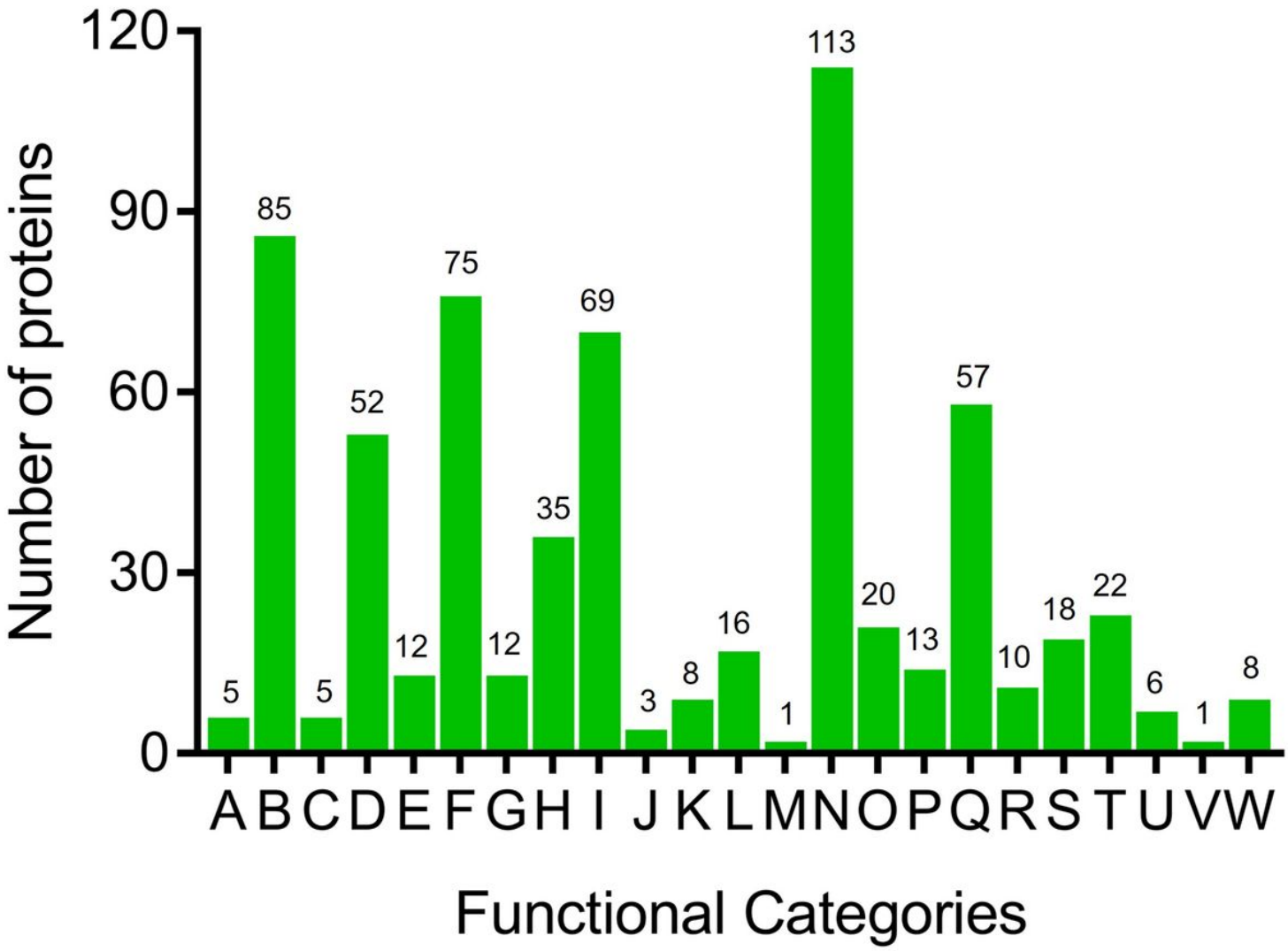

Figure 3

COG functional classification of all identified proteins. (A) Chromatin structure and dynamics. (B) Energy production and conversion. (C) Cell cycle control, cell division, chromosome partitioning. (D) Amino acid transport and metabolism. (E) Nucleotide transport and metabolism. (F) Carbohydrate transport and metabolism. (G) Coenzyme transport and metabolism. (H) Lipid transport and metabolism. (I) Translation, ribosomal structure and biogenesis. (J) Transcription. (K) Replication, recombination and repair. (L) Cell wall/membrane/envelope biogenesis. (M) Cell motility. (N) Posttranslational modification, protein turnover, chaperones. (O) Inorganic ion transport and metabolism. (P) Secondary metabolites biosynthesis, transport and catabolism. (Q) General function prediction only. (R) Function unknown. (S) Signal transduction mechanisms. (T) Intracellular trafficking, secretion, and vesicular transport. (U) Defense mechanisms. (V) Nuclear structure. (W) Cytoskeleton.

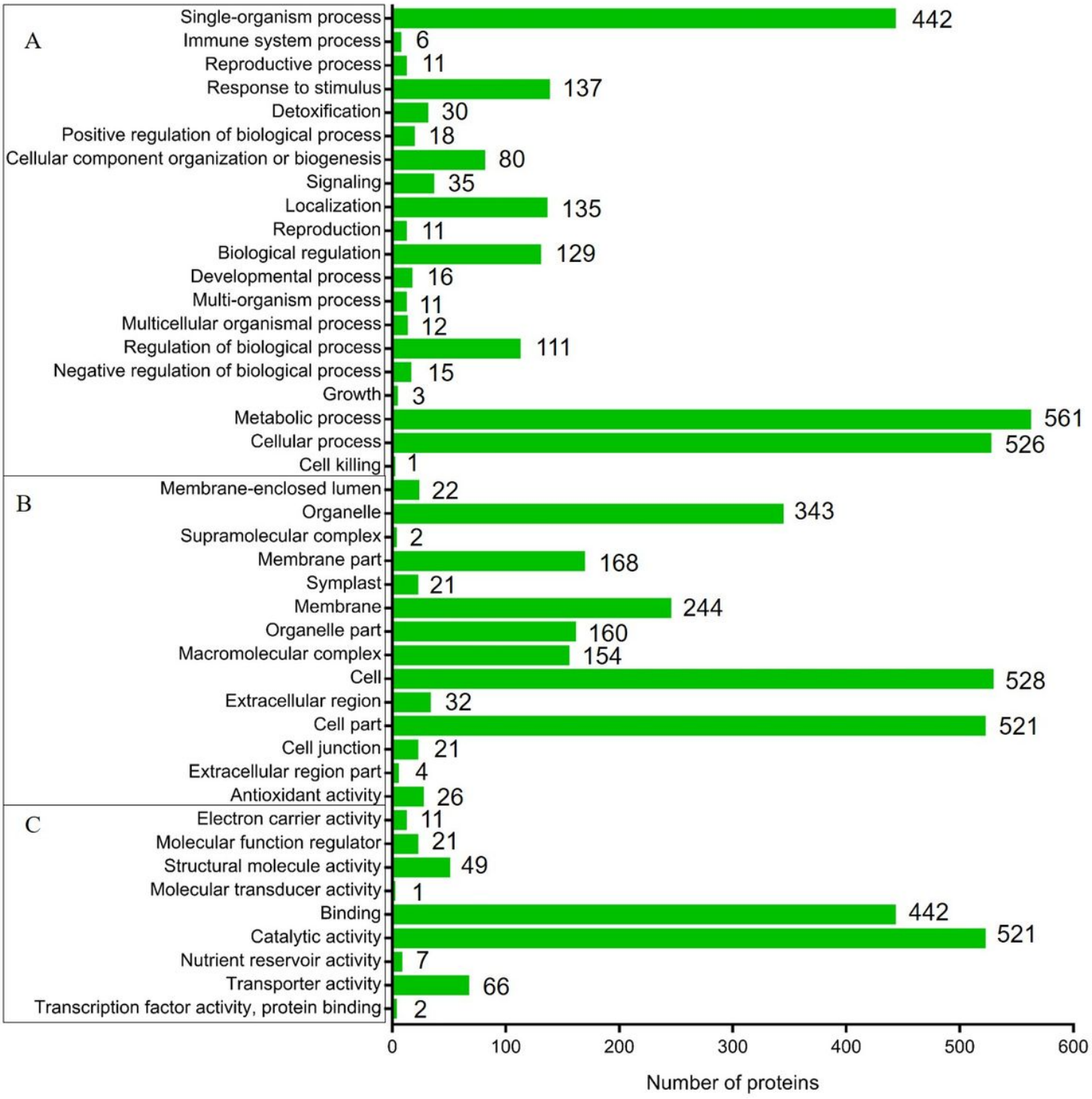

Figure 4

GO functional classification of all identified proteins. (A) Biological process. (B) Cellular component. (C) Molecular function.

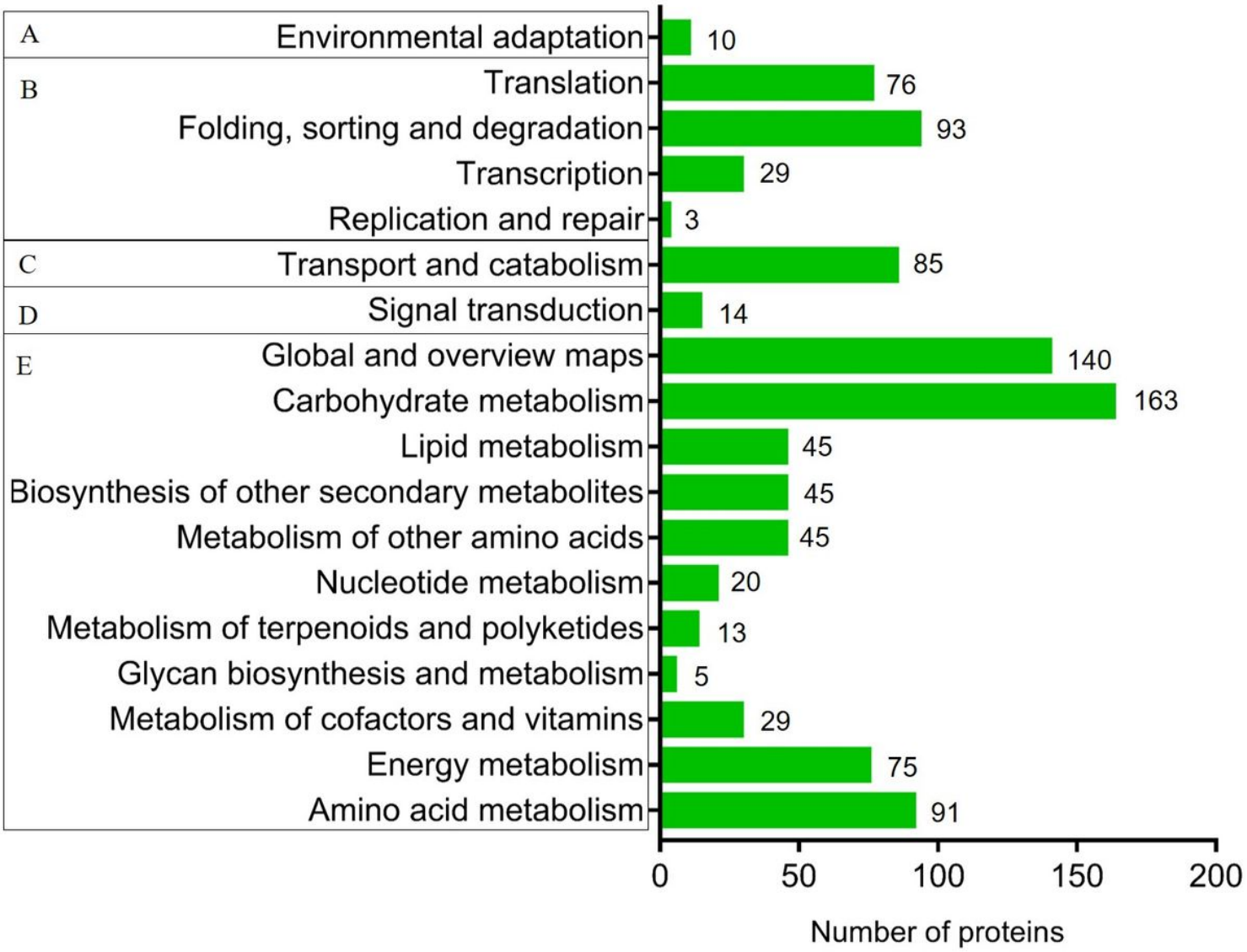

Figure 5

KEGG functional classification of all identified proteins. (A) Organismal systems. (B) Genetic information processing. (C) Cellular processes. (D) Environmental information processing. (E) Metabolism.

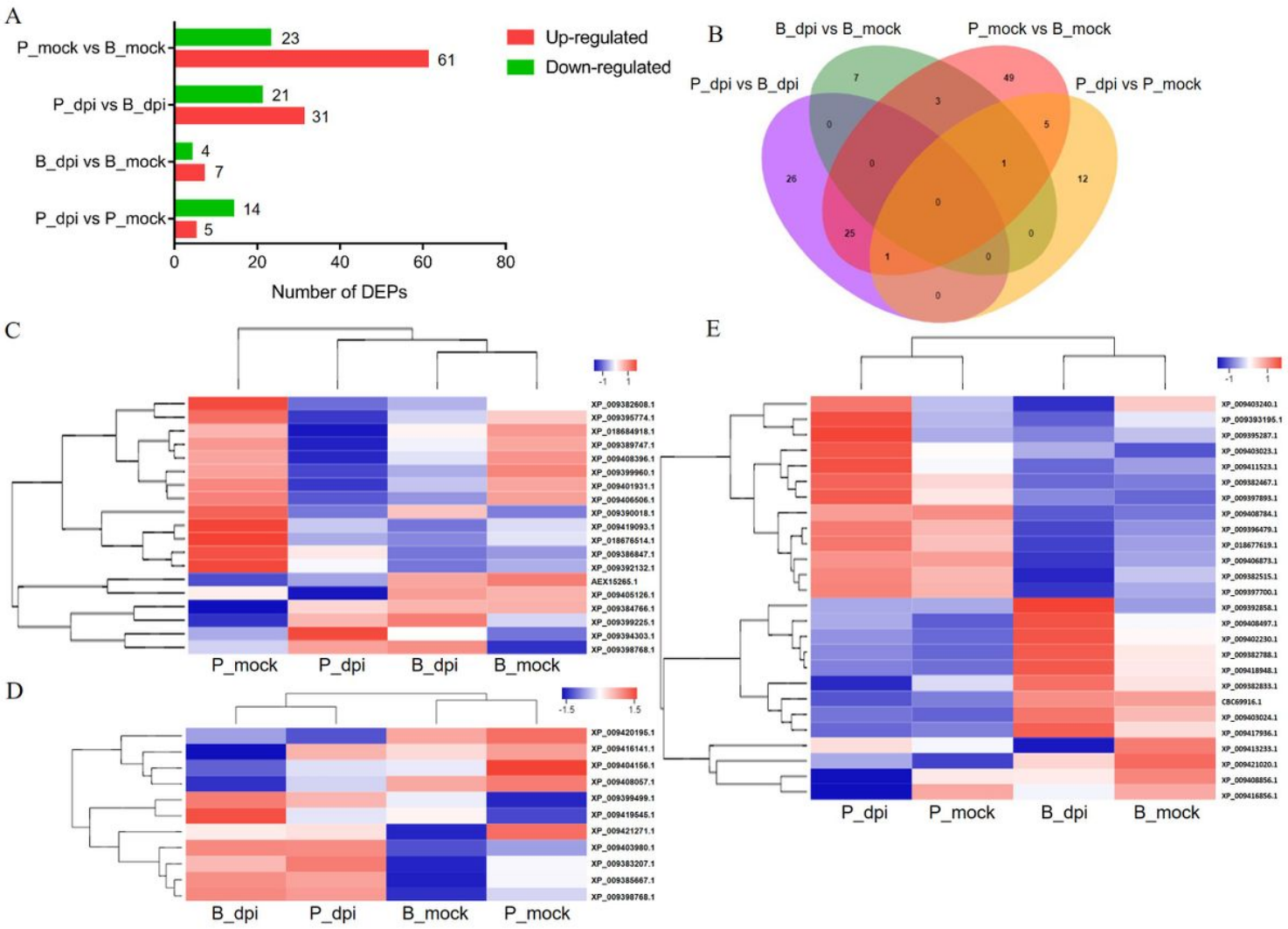

Figure 6

Number of DEPs, Venn diagram and hierarchical cluster analysis of DEPs. (A) Number of identified in 4 pairwise comparisons among resistant and susceptible tested combinations. (B) Venn diagram analysis of identified in 4 pairwise comparisons. (C) DEPs identified by comparing TR4 inoculated Pahang with mock samples (P_dpi vs P_mock). (D) DEPs identified by comparing TR4 inoculated Brazilian with mock samples (B_dpi vs B_mock). (E) DEPs identified by comparing TR4 inoculated Pahang with that of Brazilian, and the DEPs, in Pahang mock samples versus that of Brazilian, were excluded ('P_dpi vs B_dpi'–'P_mock vs B_mock'). The protein abundances are normalized to the same total peptide amount per channel and scaled.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable2.xls
- SupplementaryTable1.xls