Maize and Common Bean Seed Exudates Mediate Part of Nonhost Resistance to *Phytophthora Sojae* before Infection

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Abstract

Purpose: *Phytophthora sojae* does not infect the nonhost maize (*Zea mays* L.) but could infect the nonhost common bean (*Phaseolus vulgaris* L.). Soybean seed exudates participate in mediating host resistance to *P. sojae* before infection. This study aimed to elucidate the role of nonhost seed exudates in mediating nonhost resistance to *P. sojae* before infection.

Methods: The response behavior of *P. sojae* zoospores to the seed exudates was determined using an assay chamber and a concave slide, and the proteomes of *P. sojae* zoospores treated with the seed exudates were analysed with the tandem mass tag (TMT) method. The key proteins were quantified by parallel reaction monitoring (PRM).

Results: Maize seed exudates exerted a repellent effect on zoospores, whereas common bean seed exudates did not exhibit any attraction to zoospores but could dissolve the cysts. The key proteins related to zoospores chemotaxis showed no significant changes in response to maize seed exudates, but the key proteins in arachidonic acid pathway were downregulated and controlled the repellent behavior of zoospores. Proteins protecting the cell membrane structure were significantly downregulated in zoospores responding to common bean seed exudates, which confirmed the bacteriolytic effect of common bean seed exudates on cysts.

Conclusion: Maize and common bean seed exudates mediate part of the nonhost resistance via different mechanisms prior to *P. sojae* infection. The immune of maize to *P. sojae* is due to the repellent effect of maize seed exudates on zoospores. Common bean seed exudates participate in mediating nonhost resistance by dissolving cysts.

1 Introduction

Phytophthora sojae Kaufmann & Gerdemann only infects cultivated soybean [*Glycine max* L. (Merr)] in nature (Davison 1998) and causes Phytophthora root and stem rot which results in great losses per year in soybean production (Sui et al., 2010). It does not infect nonhost maize (*Zea mays* L.) but infects nonhost common bean (*Phaseolus vulgaris* L.) under inoculation (Schmitterenner 1985).

P. sojae overwinters in soil as sexual oospores (Schmitthenner 1985). These oospores germinate to produce sporangia that release zoospores (motile spores with two flagella and no cell walls) to infect host seeds and roots; hence, zoospores are the primary infectious propagules (Barz and Mackenbrock 1994). The zoospore develops into an adhesive cyst with a cell wall once it comes into contact with the seed or root surface and germinates to produce a germ tube that penetrates the plant to complete colonization (Zuo et al. 2005).

The chemotaxis of zoospores plays an important role in the success of *P. sojae* infection (Hua et al. 2015). Soybean is the only natural host of *P. sojae*, which depends on the chemotactice effects of isoflavones in soybean seed and root exudates on zoospores (Morris and Ward 1992). Zoospores swim towards soybean seed and roots when sensing the chemotactic substances (Tyler et al. 1996) and a series of programmed developmental changes are initiated (Penington et al. 1989), including cyst formation and germination. Some substances in seed and root exudates act as signal molecules that regulate the zoospores chemotaxis and germination (Deason and Saxena 1998).

Researchers have only focused on the attraction of soybean seed or root exudates to zoospores, which determines part of host resistance to *P. sojae* prior to infection (Zhang et al. 2020). As a matter of fact, nonhost seed or root exudates also exert inhibitory effects on the pathogen. Several defence-related proteins in cowpea (*Vigna unguiculata* L.) (Rose et al. 2006) and wishbone bush (*Mirabilis jalapa* L.) seed exudates protect seeds from nonhost pathogens (Cammue et al. 1992). Common bean root exudates also inhibit the mycelial growth of *Botrytis cinerea* (El-Gali 2015), while maize root exudates exhibit inhibitory effects on *Fusarium oxysporum* and *Phytophthora parasitica* var. *nicotianae* (Zhang et al. 2015; Ma et al. 2016). But the stable and durable nonhost resistance mechanisms of common bean and maize to *P. sojae* is still unclear.

It has been found that the G protein signaling pathway, as a molecular switch of *P. sojae* zoospores, participates in the specific recognition of zoospores to their hosts (Hua et al. 2015). G protein also participates in the response of zoospores to extracellular isoflavones in seed or root exudates (Hua et al. 2015) and regulates the chemotaxis of zoospores (Zhang et al. 2020). Many other proteins related to G protein also participate in the regulation of *P. sojae* physiological activities. GTP (guanosine triphosphate)-activated protein, which binds to and inactivates the Gα subunit (Khafizov et al. 2009), participates in the response of zoospores to soybean with different resistances (Zhang et al. 2020). Palmitoylation can regulate the function of G protein-coupled receptors, which can affect downstream signal transduction (Liu et al. 2012). G protein-dependent signal transduction pathways are also involved in chemotaxis regulation: These include the cAMP (cyclic adenosine monophosphate) and MAPK (mitogen-activated protein kinase) pathways, which have been extensively studied (Ye et al. 2016). The MAPK-like protein PsMPK1 and the C_2H_2 zinc finger protein PsCZF1 of *P. sojae* not

only affect zoospore production (Wang et al. 2009), but also participate in the response of *P. sojae* to soybean seed exudates (Zhang et al. 2020). The Gβ subunit, Ca^{2+}-transport ATPase and Ca^{2+} channel proteins positively regulate the developmental behavior (encystment) of zoospores (Zhang et al. 2020). The G protein-related proteins are involved in the pathogenic process and affect mycelial growth, sporangia formation and germination of *P. sojae* (Zhang et al. 2016b).

In addition to G protein, some other proteins in *P. sojae* also participate in regulating its developmental behavior. PsTATD4, a key protein regulating DNA degradation during cell apoptosis, plays an important role in sporangia production (Tani et al. 2005; Chen et al. 2014). At the stage of zoospore formation, the protein kinase PIPKZ1 is upregulated. The interaction of the BZIP transcription factor with PIPKZ1 also affects the behavior and chemotaxis of zoospores (Ye et al. 2013).

Currently, proteomics is widely used to analyse the key proteins in pathogen infection. By iTRAQ (isobaric tags for relative and absolute quantitation), Taylor et al. (2008) found that 72 proteins participate in the *Fusarium graminearum* infection process to maize and wheat. Grenville et al. (2005) also found that some proteins in *Phytophthora infestans* involved in amino acid synthesis and metabolism regulate the appressorium formation and sporangium germination.

The G protein regulated the chemotaxis of zoospores to signal substances in host seed exudates and determined host selection (Zhang et al. 2020). However, the responding behavior of zoospores to nonhost seed exudates have been ignored. This study thus aimed at understanding the dialogue mechanisms between *P. sojae* and nonhosts in the rhizosphere prior to infection. We accordingly analysed the behavior and proteome of zoospores in response to nonhost seed exudates. This work not only lays a foundation for understanding the pathogenic mechanism of soilborne *P. sojae* and nonhost resistance mechanism but provides a theoretical basis for the prevention of *P. sojae* infection.

2 Materials And Methods

2.1 Strain and varieties

Phytophthora sojae strain Eps597-3, a transgenic strain that was previously used in our laboratory (Li et al. 2012), was utilized for the experiments. The strain has an enhanced green fluorescent protein (EGFP) label and exhibits no changes in either developmental behaviour or pathotype compared with the wild-type strain (Li et al. 2012). It was selected for this study because it grows rapidly on carrot agar medium and produces abundant zoospores to meet the protein extraction requirement of zoospores sample. The nonhost maize variety Suiyu23, which is widely planted in Heilongjiang Province, China and rotated routinely with soybean, is immune to *P. sojae* both in nature and under inoculation conditions. The nonhost common bean variety Zihuayoudou cannot be infected by *P. sojae* in nature but can be infected under inoculation conditions and exhibits a hypersensitive reaction (HR).

2.2 Collection of seed exudates

Seed exudates were collected as described by Zhang et al. (2020). The disinfected seeds (20 g) of maize and common bean were placed in 20 mL of sterile distilled water and maintained at 25°C in the dark overnight. A certain amount of sterile distilled water was added to obtain a total volume of 20 mL. The seeds were immersed in the water at 25°C for 48 h, and the water containing seed exudates was then collected and filtered through a 0.22-μm Millipore filter (Xiya Reagent, Shanghai, China).

2.3 Preparation of zoospore suspension and chemotaxis assay

The preparation of zoospore suspension and chemotaxis assay were performed as described by Zhang et al. (2020).

2.4 Protein extraction, trypsin digestion and tandem mass tag (TMT) labelling

The fresh zoospore suspension (2×10^4 zoospores/mL) was treated with nonhost seed exudates (diluted with four volumes of sterile water) at 25°C for 30 min and then centrifuged at 6,000 r/min for 5 min, and the zoospore precipitate was frozen in liquid nitrogen before being stored in a freezer at -80°C for protein extraction. Sterile distilled water instead of seed exudates was used as the blank control.

The protein extraction, trypsin digestion and TMT labeling of zoospore samples were performed as described by Zhang et al. (2020).

2.5 HPLC fractionation and LC-MS/MS analysis

The tryptic peptides were fractionated by high-performance liquid chromatography (HPLC) using an Agilent 300 Extend C18 column (5-μm particles, 4.6-mm ID, 250-mm length). The chromatographic process was designed according to the method described by Zhang et

al. (2020). MaxQuant (v1.5.2.8) was used to retrieve the secondary mass spectrometry data. The retrieved parameter settings are detailed in the *Phytophthora sojae* proteome database (25,721 sequences).

2.6 Protein annotation and analysis

A Gene Ontology (GO) annotated proteome was derived from the UniProt-GOA database (http://www. ebi.ac.uk/GOA/). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for the annotation of protein pathways. Two-tailed Fisher's exact test was employed to test the enrichment of differentially expressed proteins against all identified proteins. GO terms and KEGG pathways with a corrected *P*-value < 0.05 were considered significant.

2.7 Parallel reaction monitoring (PRM) verification

The zoospore sample was ground in liquid nitrogen into cell powder and then transferred to a 5 mL centrifuge tube. The protocols used for protein extraction of zoospore samples were described by **2.4**.

The tryptic peptides were dissolved in 0.1% formic acid (solvent A) and directly loaded onto a homemade reversed-phase analytical column. The gradient comprised increases from 6–23% solvent B (0.1% formic acid in 98% acetonitrile) over 38 min, 23–35% over 14 min and from 35–80% over 4 min, followed by maintenance at 80% for the last 4 min. The flow rate was maintained constant at 700 nL/min, and an EASY-nLC 1000 ultra performance liquid chromatography (UPLC) system was used for the separation.

The peptides were subjected to an nanoelectrospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) with a Q Exactive™ Plus instrument (ThermoFisher, Shanghai, China) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range for the full scan was 350 to 1,000, and intact peptides were detected with the Orbitrap at a resolution of 35,000. Peptides were then selected for MS/MS using the normalized collision energy (NCE) setting of 27, and the fragments were detected with the Orbitrap at a resolution of 17,500. A data-independent procedure that alternated between one MS scan followed by 20 MS/MS scans was used. Automatic gain control (AGC) was set to 3E6 for the full MS scan and 1E5 for the MS/MS scan. The maximum IT was set to 20 ms for the full MS scan and to auto for the MS/MS scan. The isolation window for MS/MS was set to 2.0 m/z.

2.8 Data analysis

The statistical analyses of the results from the behavior tests were performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). The resulting MS data were processed using Skyline (v.3.6). The peptide settings were the following: The enzyme was set to trypsin [KR/P], and the maximum number of missed cleavages was set to 2. The peptide length was set to 8 to 25, the variable modification was set to carbamidomethyl on Cys and oxidation on Met, and the maximum number of variable modifications was set to 3. The transition settings were set to the following: the precursor charges were set to 2 and 3, the ion charges were set to 1 and 2, and the ion types were set to b, y, and p. The product ions were set from ion 3 to the last ion, and the ion match tolerance was set to 0.02 Da.

3 Results

3.1 Behavior of zoospores in response to nonhost seed exudates

The number of zoospores attracted by maize seed exudates was significantly lower ($P < 0.05$) than that of control, which indicated that maize seed exudates repelled the zoospores. However, common bean seed exudates exerted no significant effect on the chemotaxis of zoospores ($P < 0.05$, Fig. 1a).

Both maize and common bean seed exudates significantly promoted encystment ($P < 0.05$), and no significant difference was found between the two exudates (Fig. 1b). Two hours after treating with the seed exudates, the cysts subjected to the control (Fig. 2a) and maize treatment (Fig. 2b) were intact, but those treated with the common bean exudates had dissolved (Fig. 2c).

3.2 Quantitative proteomic analysis of zoospore responses to seed exudates

TMT technology was used to analyse differentially expressed proteins in zoospores responding to the seed exudates. A total of 2,714 proteins were quantitatively detected, and these included 245 (146 up- and 99 downregulated) and 428 (264 up- and 164 downregulated) differentially expressed (a fold change greater than 1.3 was considered to indicate significant up- or downregulation) proteins found in zoospores treated with maize and common bean seed exudates, respectively. Some key proteins identified in the zoospores were divided into two clusters based on their expression patterns (Fig. 3). The proteins in the red cluster were upregulated, whereas the proteins in the

blue cluster were downregulated (Fig. 3). The roles of these proteins in the responses of zoospores to seed exudates are analysed in the following sections.

3.3 GO and KEGG analysis of differentially expressed proteins

A Gene Ontology (GO) functional analysis of the proteins in zoospores of *P. sojae* was performed (Fig. 4). Differentially expressed proteins were analysed in three categories of GO secondary annotation (biological process, cellular component and molecular function). These proteins (fold change greater than 1.3) were analysed and derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). The results showed that the proteins in zoospores that responded to both seed exudates participate in the same processes (Fig. 4). The significant enrichment of biological processes mainly affected metabolic processes (89 and 162 proteins in zoospores responding to maize and common bean seed exudates, respectively) and cellular processes (66 proteins to maize and 139 to common bean). Significant enrichment of the cellular components was mainly found for membrane (48 proteins to maize and 103 to common bean) and cell (44 proteins to maize and 76 to common bean). In addition, the effects of seed exudates on molecular function mainly included binding activity (103 proteins to maize and 184 to common bean) and catalytic activity (97 proteins to maize and 151 to common bean) (Fig. 4). The abundances of the differentially expressed proteins found in zoospores responding to common bean seed exudates were two-fold higher than those observed in response to maize. Proteins participating in metabolic processes are closely related to the chemotaxis and encystment of zoospores. Some upregulated proteins in zoospores responding to nonhost seed exudates participate in fatty acid metabolism and biosynthesis pathways to regulate behavioral activity. Proteins on the cell membrane participate in intercellular communication, transport and maintenance of the stability of the cell structure, which indicates that nonhost seed exudates communicate with zoospores through these membrane proteins. Most signal transduction proteins with binding and catalytic activity participate in the zoospores response process.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) classification results indicated that 14 and seven proteins in zoospores were significantly upregulated or downregulated in response to maize seed exudates, respectively (Fig. 5a). Among the upregulated proteins, four participate in the unsaturated fatty acid biosynthesis, five are involved in the fatty acid metabolism, three participate in the fatty acid biosynthesis, and two take part in the biotin metabolism. Among the downregulated proteins, three, two and two proteins participate in leucine, valine and isoleucine degradation, arachidonic acid (AA) metabolism and starch and sucrose metabolism, respectively.

The results also showed that 75 and 10 proteins of zoospores in response to common bean seed exudates were significantly upregulated or downregulated, respectively (Fig. 5b). Among the upregulated proteins, 19 participate in the antibiotic biosynthesis, while 13, 11 and five proteins participate in carbon metabolism, the amino acid biosynthesis and cysteine and methionine metabolism, respectively. Six, five and four proteins take part in glycine, serine and threonine metabolism, unsaturated fatty acid biosynthesis and fatty acid biosynthesis, respectively. Three differentially expressed proteins are involved in the biotin metabolism pathway and sulphur metabolism pathway, and six proteins participate in fatty acid metabolism. Among the downregulated proteins, four participate in glutathione metabolism, three participate in the mismatch repair, and three participate in AA metabolism.

3.4 Correlation between the phenotype and proteome of zoospores responding to maize seed exudates

The chemotaxis assay of *P. sojae* zoospores showed that maize seed exudates repel the zoospores (Fig. 1a). The significant repellent effect of maize seed exudates on zoospores indicates that the nonhost resistance of maize is mediated by seed exudates prior to *P. sojae* infection. The repelling mechanism of zoospores can be explained as follows: When zoospores sense the signal molecules in maize seed exudates, the Gα subunit on the cell membrane, which regulates the chemotaxis of zoospores, shows no significant change in expression. This result is consistent with the behavior that zoospores show no chemotaxis in response to maize seed exudates.

Zoospores transform signal molecules from extracellular signals into intracellular signals through G proteins on the cell membrane after sensing signal molecules in maize seed exudates. The expression of G protein showed no significant change, but the key downstream protein, intracellular phosphatidylinositol-4-kinase (PI4K, G4ZCQ6 in the phosphatidylinositol signalling pathway) was upregulated, which is involved in maintaining the dynamic balance of the phosphatidylinositol system. (Fig. 6, online resource 1). A calcium channel protein downstream of PI4K (G4ZC26) was also upregulated, which resulted in continuous promotion of the production of phospholipase A_2 (PLA_2) (Fig. 6, online resource 1). PLA_2 is a key enzyme in the regulation of cellular metabolism and is responsible for information transmission. This enzyme is also a marker of the occurrence of tissue damage and inflammation, which indicates that maize seed exudates exert adverse effects on the normal life activities of zoospores.

PLA_2 is also a rate-limiting enzyme in the production of AA and prostaglandin bioactive substances. The upregulation of PLA_2 and the downregulation of prostaglandin-D-synthetase G4YV08 reduced the synthesis of prostaglandin prostaglandin D_2 (PGD_2) (Fig. 6, online resource 1), which blocked the physiological functions of PGD_2.

AA is a second messenger that conducts intracellular signals. AA can also promote or amplify other second messenger systems, such as cAMP and cyclic guanosine monophosphate (cGMP), by regulating their concentration in response to extracellular signals. Therefore, the downregulation of proteins involved in AA metabolism weakens the chemotaxis of zoospores.

Maize seed exudates also affected the normal activities of zoospores by decreasing the degradation of valine and isoleucine, protein synthesis and glutathione metabolism in zoospores (G4Z179, G4ZY60 and G4YKE2 in online resource 1). The Ca^{2+} channel protein G4ZC26 was upregulated, which resulted in the opening of the Ca^{2+} channel and thereby the activation of protein kinase C (PKC) (Fig. 6, online resource 1) on the plasma membrane. When zoospores sensing maize seed exudates, the Ca^{2+} concentration increased to promote the encystment, which is also consistent with the encystment behavior of zoospores.

3.5 Correlation between the phenotype and proteome of zoospores responding to common bean seed exudates

Common bean seed exudates exerted no effect on the chemotaxis of zoospores because no significant changes were detected in both the expression of G protein and some other proteins that participate in the phosphatidylinositol pathway to transmit the chemotactic signal. Common bean seed exudates promoted the encystment of zoospores. During the response of zoospores to common bean seed exudates, the expression of the key proteins β-glucosidase G4ZCH5 and 1,4-β-glucanase G5A663 involved in cellulose degradation decreases, which lead to the increasing of cellulose synthesis (Fig. 7, online resource 2). However, after these effects, some cysts (resting zoospores) are dissolved by common bean seed exudates. The proteome results also showed that glutathione peroxidase G4ZXP6 (Fig. 7, online resource 2), which is responsible for protecting the cell structure, was significantly downregulated in zoospores responding to common bean seed exudates. The increasing glutathione is also an early signal of apoptosis. This finding explains the dissolution of some zoospores after 2 h of treatment with common bean seed exudates; common bean seed exudates also participate in the nonhost resistance process by dissolving zoospores.

When zoospores stop swimming to form a resting spore (cyst), they do not need energy for movement. This process is accompanied by flagella shedding and cell wall formation because the proteins (G4Z179, G4YKE2 and G4ZXP6) that power zoospores were significantly downregulated and the glucose for energy supply was reduced due to the downregulation of G4ZCH5 (Fig. 7, online resource 2). The changes in these proteins explain the significant promotion of the encystment behavior of zoospores by common bean seed exudates.

Pyruvate and phosphodikinase G4YKE2 (online resource 2), a terminal enzyme of oxidative phosphorylation responsible for adenosine triphosphate (ATP) synthesis, were downregulated, which resulted in significant inhibition of ATP synthesis. Approximately 95% of the ATP molecules that maintain normal activities originate from the oxidative phosphorylation process. In cells, a decline in oxidative phosphorylation leads to an abnormal physiological state. The effect of common bean seed exudates on the oxidative phosphorylation level affects the life activity and preference of zoospores. As proteins upstream of oxidative phosphorylation, acetyl-CoA carboxylase G5A3T5 (online resource 2) and very-long-chain 3-oxocarbonyl CoA reductase G4ZKM7 (online resource 2), which are involved in fatty acid metabolism in zoospores, led to an abnormal cellular state. In addition, the content of glutathione, which participates in detoxification and free radical scavenging in zoospores, was increased (due to the downregulation of G4YKE2) (online resource 2), which indicates that common bean seed exudates are involved in nonhost resistance to *P. sojae*.

3.6 Protein-protein interaction (PPI) analysis

In the PPI network map (used for establishing protein networks), the most significantly enriched proteins in zoospores in response to nonhost seed exudates, which exhibited high connectivity, are shown in Fig. 8.

Little interaction was found among the differentially expressed proteins during the response process of zoospores to maize seed exudates. The interacting proteins mainly participate in protein synthesis, ATP synthesis, amino acid metabolism, phosphatidylinositol signal transduction and glutathione metabolism. When zoospores sense the signal molecules in maize seed exudates, the extracellular signals are transformed into intracellular signals, and the energy supply and nutrient metabolism in the zoospores are significantly affected (Fig. 8a).

However, the interaction network of the differentially expressed proteins during the response process of zoospores to common bean seed exudates is complex. The proteins in the interaction network are mainly responsible for regulating the citric acid cycle, amino acid metabolism, phosphatidylinositol signalling pathway, DNA replication, transcription and translation, and oxidative phosphorylation. When zoospores sense the signal molecules in common bean seed exudates, the extracellular signals are transformed into intracellular signals. The metabolism of various nutrients (sugar, lipid and protein), the process of gene replication and the supply of cellular energy in zoospores are significantly affected (Fig. 8b).

3.7 PRM verification of the candidate differentially expressed proteins

The analyses of proteomics revealed that the proteins significantly changed during the responses of zoospores to nonhost seed exudates. Maize seed exudates mediate stronger nonhost resistance than common bean seed exudates that were selected to verify the changes of some candidate proteins.

PRM (a type of multiple reaction monitoring for peptides) mass spectrometry was used to verify the target peptides found in the TMT analysis of the zoospores treated with maize seed exudates, and the 11 candidate peptides of the target protein were subjected to LC-PRM/MS analysis. The peptides used to quantify the candidate proteins are shown in Table 1.

Table 1
Quantitative results for eight candidate proteins determined by the PRM and tandem mass tag (TMT) methods

Protein ID	Peptide sequence	PRM	TMT	Consistency between PRM and TMT	Participating pathways	Protein type
G4ZC26	GFSALTDLAEK//SAQAAQQLLQVENPR	1.65	1.33	Yes	Phosphatidylinositol pathway	Ca2+ osmotic pressure gate cation channel protein
G5A3T5	SVLQGYFAPEDLTQK	1.70	1.51	Yes	Fatty acid metabolism and biosynthesis pathway	Acetyl-CoA carboxylase
G4ZKM7	SFGQWGVVTGATDGIGK	1.88	1.38	Yes	Fatty acid metabolism pathway	Very-long-chain 3-oxocarbonyl CoA reductase
G4YV08	NALANFDGFSLK	0.87	0.59	Yes	Arachidonic acid metabolism	Haematopoietic prostaglandin D synthase
G4ZZG9	NVPGWVAGQSPFHSDR	1.73	2.79	Yes	Glutathione metabolisms	NADH dehydrogenase
G4Z179	FFDFLPLNNK	0.86	0.71	Yes	Degradation of valine and isoleucine	Acetyl-CoA carboxylase mitochondria
G4ZY60	VVVLCNLK//STGAILLVANDK	0.80	0.52	Yes	Protein synthesis	tRNA amidation cofactor
G4YKE2	ALGVTFADAANPLLVSVR//VHTSSGAEVVLR	0.90	0.44	Yes	Glutathione metabolism	Pyruvate and phosphodikinase

A quantitative analysis with LC-PRM/MS of the expression levels of eight candidate proteins associated with response-induced resistance was performed to validate the TMT results. The trends obtained for the candidate proteins by quantitative PRM results were similar to those found with TMT, which indicated that the data obtained using the experimental TMT technique combined with LC/MS are reliable (Table 1).

4 Discussion

P. sojae, a typical soil-borne pathogen, only infects host soybean seed and root through zoospores under suitable conditions. The specific recognition and movement of zoospores towards soybean is the first and crucial step which determines the host selection of *P. sojae* (Tyler 2002). *P. sojae* cannot infect other nonhost plants (Schmittenner 1985). Interestingly, *P. sojae* exhibits pathogenicity to

legume common bean under inoculation conditions but cannot infect gramineous plants maize (Sui et al. 2010). We previously found that soybean seed exudates participate in mediating part of host resistance prior to infection (Zhang et al. 2020). In this study, the repellent effect of nonhost maize seed exudates on zoospores of *P. sojae* also indicated that seed exudates are involved in mediating part of nonhost resistance before infection.

The signaling molecules in maize exudates which repelled the zoospores determined the immunity of maize to *P. sojae*. Maize root exudates contain some inhibitory substances, such as hydroxamic acid, which widely exists in Gramineae crops with broad-spectrum resistance (Niemeyer 1985). Hydroxamic acid can inhibit the spore germination and mycelial formation of *Septoria nodorum* (Baker and Smith 2010), *Exserohilum turcicum* and *Fusarium moniliforme* (Couture et al. 1971). 6-Methoxy-2-benzoxazolinone (MBOA) and benzothiazole (BZO) in maize root exudates also significantly inhibit the zoospore activity, cyst germination and mycelial growth of *P. sojae* (Zhang et al. 2019). Maize seed exudates exert a repellent effect on zoospores, which confirms that maize is immune to *P. sojae*.

Common bean seed exudates can dissolve some cysts, and the remaining cysts look for an opportunity to infect, which confirms that the HR response of common bean to *P. sojae*. Previous studies have shown that cumin acid in *Cuminum cyminum* seed exudates can destroy the cell wall and inhibit the cyst germination of *Phytophthora capsici* (Feng et al. 2012). Further research is needed to identify the substances responsible for dissolving cysts in common bean seed exudates.

The numbers of differentially expressed proteins are quite different in zoospores responding to the two nonhost seed exudates. Specifically, 428 differentially expressed proteins were found in response to common bean seed exudates. This number was 1.7-fold higher than that found in response to maize seed exudates. The finding indicated that the response mechanisms of zoospores to common bean seed exudates were more complex. This could because only repellent signal molecules in maize seed exudates act on *P. sojae*, whereas both chemotactic signal molecules and substances that can dissolve cysts in common bean seed exudates act on *P. sojae*.

As a membrane protein with an important signal transduction function, G protein of *P. sojae* has been widely studied. G protein is involved in the regulation of zoospore chemotaxis to isoflavones in soybean seed exudates (Hua et al. 2015; Zhang et al. 2020). In this experiment, the expression of the G protein α subunit almost did not change during the response of zoospores to nonhost seed exudates. The G protein α subunit showed 1.24- and 1.16-fold upregulation in zoospores in response to susceptible and resistant soybean seed exudates, respectively (Zhang et al. 2020). But host and nonhost seed exudates had no significant difference in the degree of promotion. However, host and nonhost seed exudates exerted quite different effects on zoospore behaviors. It has been speculated that the G protein α subunit regulates the behavior of zoospores through protein modification rather than the expression level. We also found that PI4K, a key protein of the phosphatidylinositol signalling pathway that is also involved in signal transduction, showed 2.71-fold upregulation in response to maize seed exudates. But 13.63-fold and 5.05-fold upregulation of PI4K was found in response to susceptible and resistant soybean seed exudates, respectively (Zhang et al. 2020). This finding suggests that PI4K might transmit different signals from host and nonhost seed exudates through different expression levels. Maize, susceptible soybean and resistant soybean seed exudates significantly upregulated Ca^{2+} channel proteins in zoospores by 1.33-fold, 1.36-fold and 1.29-fold, respectively. This finding indicated that Ca^{2+} channel proteins are involved in the identification of zoospores to host and nonhost by expression multiples. The responses of zoospores to host and nonhost seed exudates significantly downregulated cellulose-degrading enzymes and promoted cell wall formation, which was consistent with the significant promotion of encystment obtained after treating with host and nonhost seed exudates (Zhang et al. 2020).

The response of *P. sojae* to nonhost seed exudates destroys the immune function. Pyruvate, phosphodikinase and acetyl-CoA carboxylase were downregulated and inhibited the metabolic process, which are the key proteins in leucine, isoleucine and valine metabolism. Thus, the biosynthesis of macrolides and type II polyketide skeletons, which serve important roles in immunity and pathogenicity, was also inhibited (Adusumilli et al. 2010). In response to nonhost seed exudates, key proteins in zoospores that participate in fatty acid metabolism, unsaturated fatty acid biosynthesis and biotin metabolism were upregulated. *Beauveria bassiana* can reduce the external oxidative pressure from plants by increasing lipid catabolism and sugar metabolism function to protect DNA and enzymes from being damaged by external pressure (Zhang et al. 2016a). This finding indicates that some substances in nonhost seed exudates play a role in nonhost resistance through putting oxidative pressure on zoospores. The secondary metabolites salicylic acid and ferulic acid in seed exudates also participate in nonhost resistance (Nelson 2004). It is necessary to identify the substances in maize and common bean seed exudates that act in nonhost resistance against *P. sojae*.

The upregulation proteins in zoospores responding to common bean seed exudates participate in carbon metabolism, sulphur metabolism, amino acid synthesis and glycine-serine-threonine-methionine-cysteine metabolism. Many fungicides affect the carbon

metabolism process of pathogens through the above processes (Wang et al. 2016). During the response of zoospores to nonhost seed exudates, glutathione-Px and glutathione-S-transferase are downregulated, resulting in the inhibition of GSH oxidation process, the enhancement of glutathione reduction process and the increasing glutathione to mediate nonhost resistance. Previous studies have found that the content of glutathione in *Fusarium oxysporum* (Cohen et al. 1986) and *Phytophthora infestans* (Ellner 1990) increases after treating with fungicides. Glutathione and its metabolites act as detoxification and free radical-scavenging substances in cells to protect important proteins from oxidation and ensure energy metabolism.

In this experiment, the key protein of AA metabolism was downregulated in zoospores responding to maize seed exudates, which led to a decrease in the cAMP concentration. This change is involved in regulating the repellence of zoospores to maize seed exudates. The concentration of cAMP was closely related to the process of protein phosphorylation (Randall et al. 2005). Therefore, The pathogenicity might was affectted by the decreasing in the concentration of the second messenger cAMP in zoospores of *P. sojae* after treating with maize seed exudates. In response to maize seed exudates, key proteins of zoospores involved in fatty acid metabolism and synthesis were upregulated: These effects included phosphorylation, acetylation and deacetylation of a large number of proteins (Sun et al. 2017). Protein modification might also be related to the pathogenicity, recognition and selectivity of *P. sojae* to maize seed exudates. Further research will investigate the function of these proteins in the response of zoospores to nonhost seed exudates.

All the above-described results indicated that nonhost seed exudates are involved in mediating part of nonhost resistance via different mechanisms prior to *P. sojae* infection. The immune response of maize to *P. sojae* is due to the repellent effect of maize seed exudates on zoospores, and this response involves the downregulation of the key proteins in the AA pathway. Common bean seed exudates participate in mediating nonhost resistance by dissolving cysts, and this effect is manifested by the downregulation of proteins that protect the cell membrane structure in zoospores.

Abbreviations

TMT Tandem mass tag

PRM Parallel reaction monitoring

EGFP Enhanced green fluorescent protein

HR Hypersensitive reaction

GTP Guanosine triphosphate

cAMP Cyclic adenosine monophosphate

MAPK Mitogen-activated protein kinase

iTRAQ Isobaric tags for relative and absolute quantitation

EGFP Enhanced green fluorescent protein

HPLC High-performance liquid chromatography

GO Gene ontology

KEGG Kyoto encyclopedia of genes and genomes

UPLC Ultra performance liquid chromatography

NSI Nanoelectrospray ionization

MS mass spectrometry

NCE Normalized collision energy

AGC Automatic gain control

AA Arachidonic acid

PI4K Phosphatidylinositol 4-kinase

PLA2 Phospholipase A2

PGD2 Prostaglandin prostaglandin D2

cGMP Cyclic guanosine monophosphate

PKC Protein kinase C

(ATP) Adenosine triphosphate

PPI Protein-protein interaction

MBOA 6-Methoxy-2-benzoxazolinone

BZO benzothiazole

Declarations

Conflict of Interest:

The authors declared that they have no conflict of interest.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were mainly performed by Zhuoqun Zhang, Yifan Zhao, Tai An, Haixu Liu, Xiangqi Bi, Han Yu, Ying Xu, Yufei Chen, and Zhiyue Yang. The first draft of the manuscript was written by Zhuoqun Zhang with help from Jingzhi Wen, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

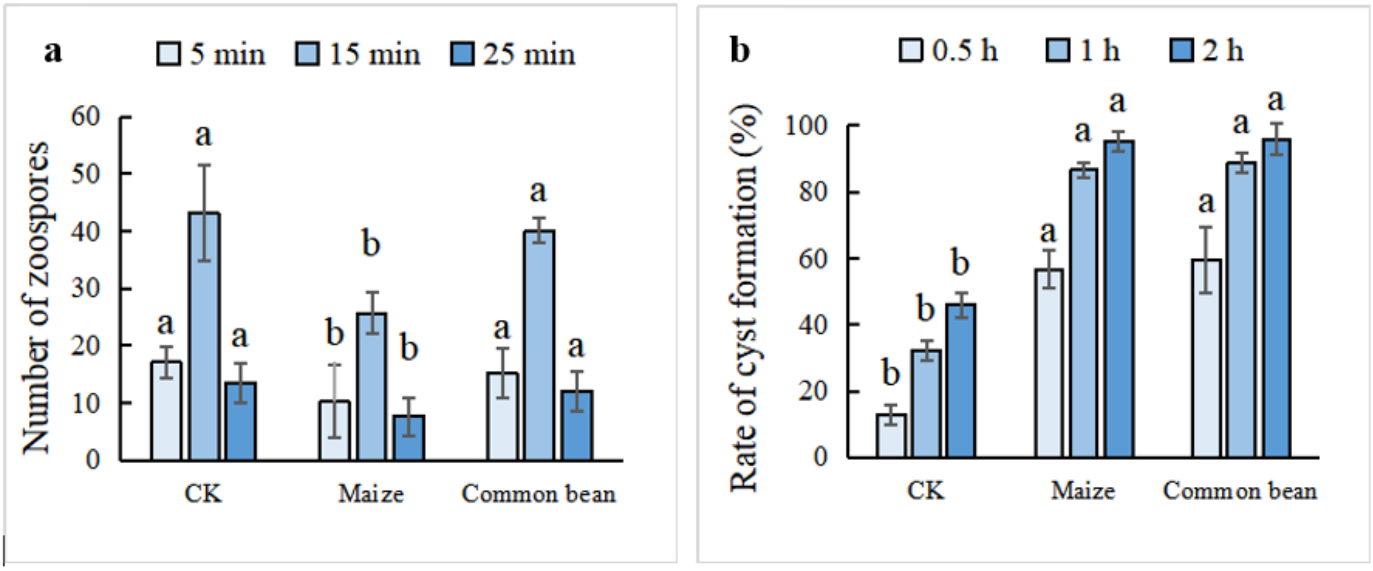

Figure 1

(a) Number of Phytophthora sojae zoospores attracted by seed exudates of nonhost maize and common bean. (b) Effects of the two seed exudates on the cysts formation of Phytophthora sojae. Maize and common bean refer to the maize and common bean seed exudate treatments, respectively. Different letters indicate significant differences in the number of zoospores and in the rate of cyst formation among the different treatments at the same time at a significance level of 0.05.

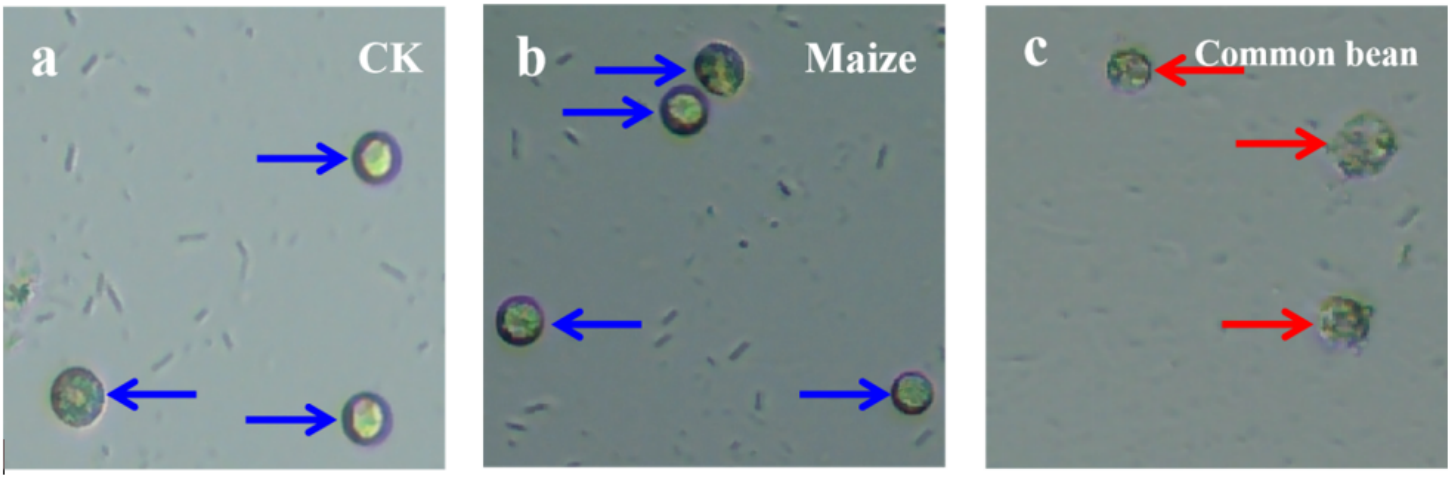

Figure 2

Intact cysts were observed after 2 h of the CK (a) and maize seed exudate (b) treatments, whereas dissolved cysts were found after 2 h of treatment with common bean seed exudates (c). The blue arrows point to intact cysts, and the red arrows indicate dissolved cysts. Maize and common bean indicate the common bean and maize seed exudate treatments, respectively.

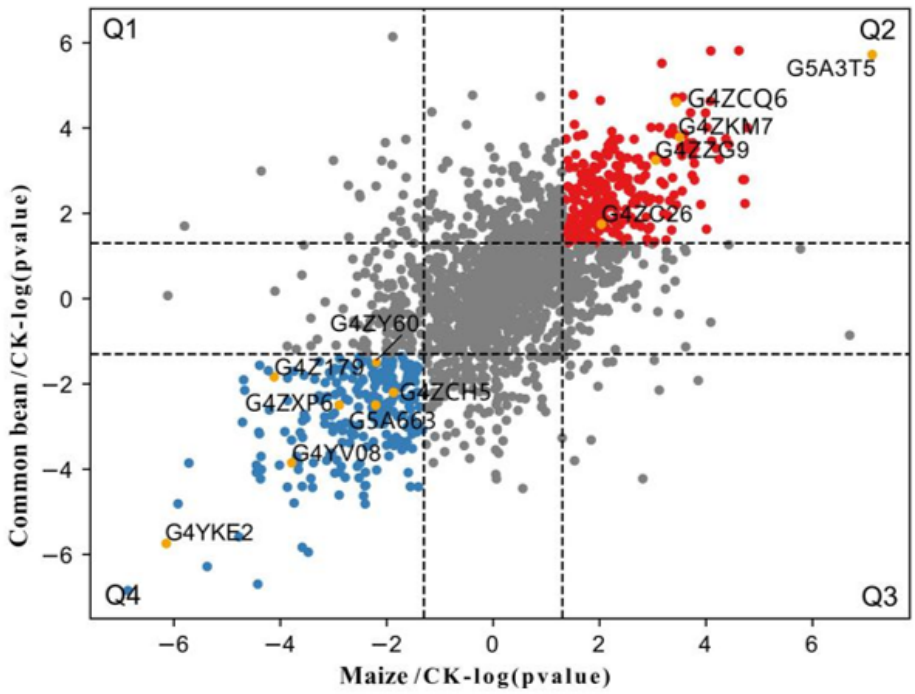

Figure 3

Volcano plot of differentially expressed proteins in Phytophthora sojae zoospores in response to maize and common bean seed exudates. The red and blue dots indicate upregulated and downregulated proteins (fold change greater than 1.3), respectively, after maize or common bean seed exudate treatments. The yellow dots indicate some key proteins that are mentioned in the text to explain the mechanisms involved in the response of zoospores to seed exudates. Maize and common bean indicate the common bean and maize seed exudate treatments, respectively.

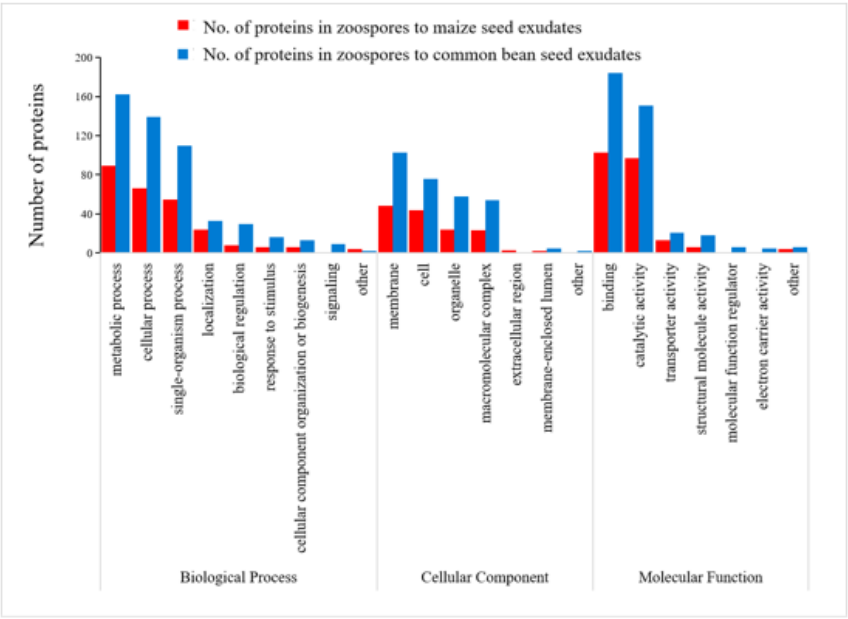

Figure 4

Distribution of biological processes, cellular components and molecular functions associated with the differentially expressed proteins in Phytophthora sojae zoospores in response to nonhost maize and common bean seed exudates. A differentially expressed protein was defined as a protein showing a fold change greater than 1.3 and a significance P-value < 0.05.

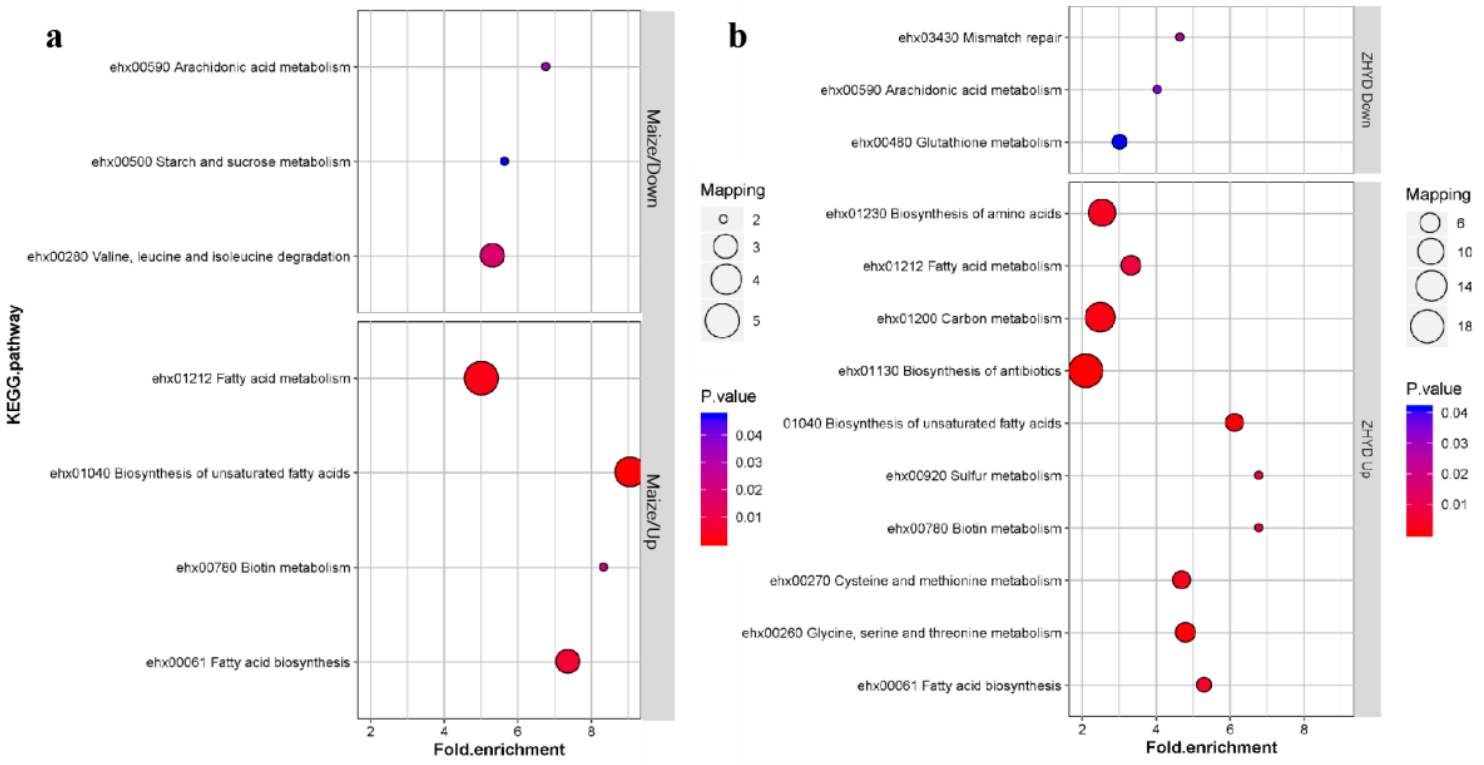

Figure 5

Enrichment of metabolic pathways involving the differentially expressed proteins in Phytophthora sojae zoospores in response to maize (a) and common bean (b) seed exudates. Maize and common bean indicate the common bean and maize seed exudate treatments, respectively.

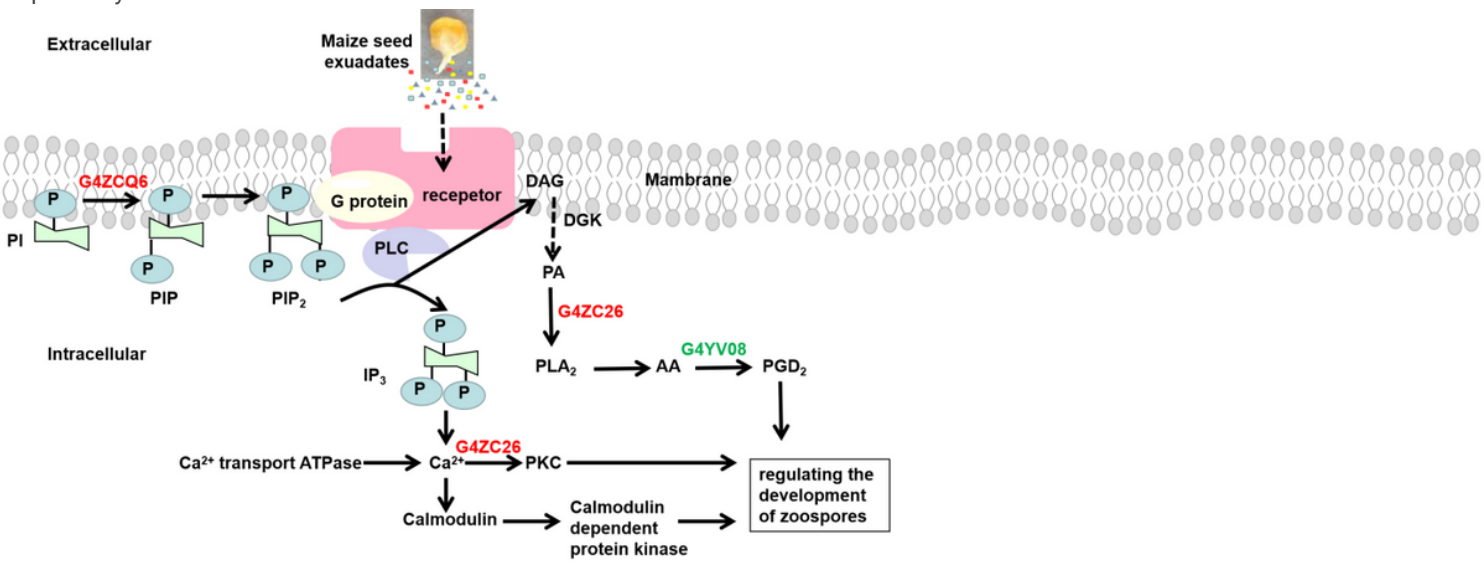

Figure 6

Schematic diagram of the G protein signal and arachidonic acid metabolism pathways in Phytophthora sojae zoospores. The red and green text indicates upregulated and downregulated proteins in zoospores in response to maize seed exudates, respectively. PI: phosphatidylinositol; PIP: phosphatidylinositol phosphate; PIP2: phosphatidylinositol diphosphate; PLC: phospholipase C; IP3: inositol triphosphate; DAG: diacylglycerol; DGK: diacylglycerol kinase; PA: phosphatidic acid; PLA2: phospholipase A2; AA: arachidonic acid; PGD2: prostaglandin D2; PKC: protein kinase C.

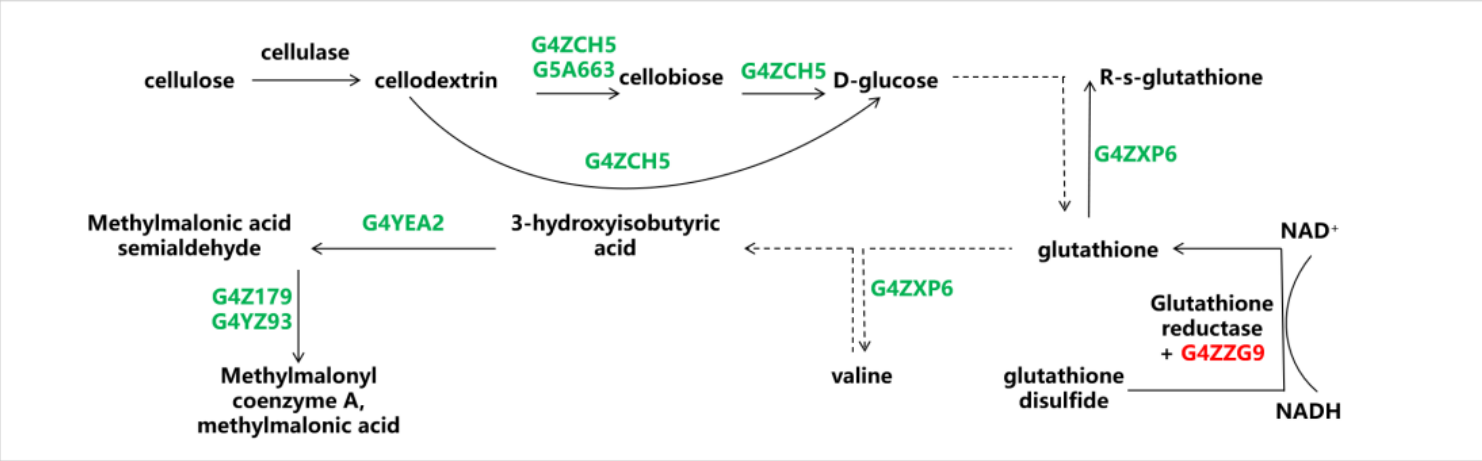

Figure 7

Schematic diagram of starch and sucrose metabolism, degradation of valine and isoleucine and glutathione metabolism in Phytophthora sojae zoospores. The red and green text indicates upregulated and downregulated proteins in zoospores in response to common bean seed exudates, respectively.

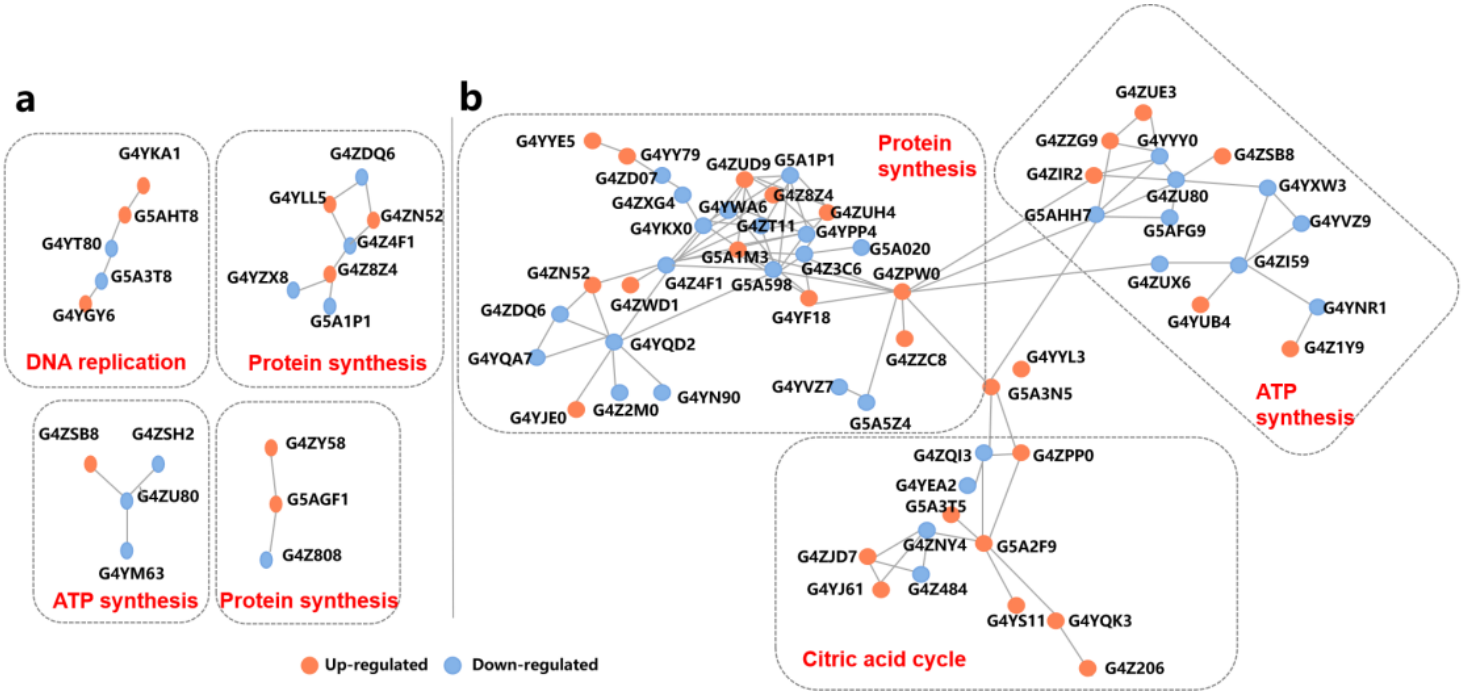

Figure 8

Protein-protein interaction (PPI) network of the differentially expressed proteins in zoospores in response to seed exudates of maize (a) and common bean (b). The orange points indicate the upregulated proteins, and the blue points indicate the downregulated proteins. The processes in which the proteins in each circle participate are shown in red text.

Supplementary Files

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