Comparative proteomic analysis revealed maize (Zea mays L.) response to Setosphaeria turcica early infection

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Abstract

Background Globally, maize (Zea mays L.) is an important crop. Maize production is severely affected by northern corn leaf blight (NCLB), a destructive foliar disease caused by Setosphaeria turcica. Interaction between maize and S. turcica has not been characterised at protein level.

Results We used tandem mass tag labelling and liquid chromatography–tandem mass spectrometry to investigate the proteomes of maize leaves at 24 and 72 h post-inoculation (hpi) with S. turcica. In total, 4,740 proteins were quantified which encoded by 4,711 genes. Clustering analyses provided insight into the dynamic reprogramming of leaves proteomes by revealing the functions of different proteins during S. turcica infection. Screening and classification of differentially expressed proteins (DEPs) revealed that numerous defence-related proteins, including defence marker proteins, proteins related to the phenylpropanoid lignin biosynthesis, benzoxazinone biosynthesis and jasmonic acid signal pathway, participated in the defence responses of maize to S. turcica infection. Furthermore, the early induction of GST family proteins could contribute to the resistance of S. turcica. In addition, the protein–protein interaction network of DEPs suggested that some defence-related proteins, e.g. ZmGEB1, play key roles in defence responses against S. turcica infection.

Conclusion This study provides insight into the complex defence responses triggered by S. turcica at the protein level, as well as a new basis for studying the interaction process between maize and S. turcica at the earlier stage of infection.

Background

Northern corn leaf blight (NCLB) is a foliar disease of maize caused by Setosphaeria turcica [1–4]. The pathogen spreads with the propagation of its conidium and causes infections with its mature appressorium. The disease can reduce maize yield up to 50% in the year of serious occurrence of NCLB [5, 6], which destroys the photosynthetically active leaf during the grain-filling period [2, 5, 7].

Plants are constantly threatened by potentially pathogenic microbes, specifically, viruses, bacteria and fungi during their development. In the meantime, plants have evolved two types of defense systems—pathogen-associated molecular pattern triggered immunity (PTI) and effector-triggered immunity (ETI)—to resist attacks by microbial pathogens. PTI and ETI are the first and second tiers of plant defense systems, respectively [8, 9]. Pathogen-associated molecular patterns are present in highly conserved microbial structures, such as fungal cell wall component chitin or bacterial flagellin [8, 10], and are recognized by pattern recognition receptors (PPRs). PPRs then triggers a signaling cascade which activates PTI, which is considered as the basic defense response necessary to prevent further pathogen dissemination [11, 12]. At this stage, the host initiates a broad-spectrum resistance against all of non-adapted microbes, also described as non-host resistance. Furthermore, the pathogen evades PTI by producing specific effectors which suppress the immune responses of the host. Pathogenic effectors are translocated into the host cytoplasm, where they are recognized by cytoplasmic receptor proteins, which
touches off ETI [13]. ETI then acted as the second accelerated and amplified PTI response, reacts against adapted pathogens and often results in the death of the infected cell through hypersensitive reaction. In plant, PTI induces intracellular signal transduction pathway including mitogen-activated protein kinases and calcium signaling, the transcription of pathogen responsive genes, the production of reactive oxygen species and the deposition of callose to reinforce the cell wall at sites of infection [14]. ETI is also closely related to the accumulation of many secondary metabolites, such as flavonoids, phenolic compounds and phytoalexins [11, 15, 16].

During plants long association with pathogens, the ping-pong-type coevolution pattern have been established for plant and pathogen [8]. Pathogen continuously evolve novel virulence effectors to escape intracellular recognition by host. These virulence effectors are structurally related proteins which belong to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family [17]. The co-evolutionary arms race between plants and pathogens is the major reason for the rapid breakdown of NBS-LRR-based disease resistance in crop plants within only a few years [6, 18].

The maize inbred line B73 has been sequenced [19], which enabled us to confirm the protein expression patterns of maize during pathogen infection at the posttranscriptional level. Novel tandem mass spectrometry (MS/MS) isotopomer labels, also known as ‘tandem mass’ tags (TMTs), have been developed for the accurate quantification of proteins [20]. Newly developed tags and methods can be used to determine the relative abundances of peptides from different sample groups with high easily and accurately [21]. Up to date, very little data has been published about the interaction between S. turcica and maize except that an expression profile analysis at the level of transcription [22]. In the present study, the proteomes of maize leaves in three different stages of S. turcica infection were characterized and analyzed to obtain a comprehensive understanding of maize defense response to S. turcica infection on the protein level.

Results

Quantitative analysis of proteome data and quality validation of LC-MS/MS data

To expand our understanding of the response of maize during infection with the pathogenic fungus S. turcica, the maize leaves which inoculated with S. turcica after 24 and 72 hours (hpi) were collected as treatments and 0 hpi as control (CK). Firstly, the expression level of two pathogenesis-related genes (Zmprp4, Zm00001d018738 and Zmprp5, Zm00001d031158) were analyzed to explore whether the artificial inoculation was successful. The semi-RT-PCR result shown that the expression level of Zmprp4 and Zmprp5 increased with the infection time (Table S1, Fig 1a), this indicated that the maize had initiated its defense response. Further, total protein was extracted from each sample, and an integrated method involving TMT labelling and liquid chromatography–tandem MS (LC-MS/MS) was used for the spectrophotometric quantification of the protein abundance of different samples. Pearson correlations were computed for all nine samples (three replicates × three stages) to assess the reproducibility and accuracy of the biological replicates. Averages of 0.76 for the three proteome replicates were obtained
MS data was used to detect the peptides after quality validation. The mass delta values of all identified peptides are shown in Fig. 1c. Almost 92.5% of all obtain peptides had lengths of 7–19 amino acid residues (Fig. 1d). The similarity between these results and the properties of known tryptic peptides suggests that the prepared samples met the required standards.

By using the 24,114 unique peptides identified in our study, a total of 5,646 proteins (protein groups, detected at least two replicates), encoded by 5,587 gene were identified. Amongst of the 5,646 proteins, 4,740 were quantified which encoded by 4,711 genes (Table S2). We compared the protein abundances of a number of selected genes with those of well-studied genes to further evaluate the quality of our proteome data. We found that the pathogenesis-related protein (ZmPRP5, Zm00001d031158), β-1,3-glucanases (ZmGEB1, Zm00001d042143) and lipoxygenase 4 (ZmLOX4, Zm00001d033624), which are defense marker genes with functions in maize defense response [23-25], were up-regulated at the 24 and 72 hpi (Fig. S1). The increment in the protein abundances of the selected marker genes after infection with S. turcica indicated that maize had initiated its defense response process in protein level and the method used for quantification of protein abundance processed well.

Integration of maize protein activity and cellular function during S. turcica infection

In order to review the detail cellular function of the detected proteins, the k-means clustering algorithm was used to group them into 10 clusters, and each cluster contained proteins with similar expression patterns. Further, the MapMan term was carried out to assign proteins to functional categories (Table S3).

The modules can be divided into three categories on the basis of cluster analysis results (Fig. 2): induced-expression, depressed-expression and uncertain-expression pattern modules. The induced-expression modules (represented by modules C1, C2, C3 and C7), comprised the proteins that may positively regulate the defense response. Indeed, we found there were 27 well studied proteins which related to biotic stress in these module, including ZmPRP3, ZmPRP5 and the NB-ARC domain-containing disease resistance protein (Zm00001d018734). Moreover, these modules were typified by the overrepresentation of protein synthesis elongation, protein folding, tricarboxylic acid cycle, glycolysis, cell vesicle transport, signalling calcium and signalling G-proteins (Table S3). These characteristics indicated that the defense process of maize requires high energy consumption and frequent signalling conduction. The depressed-expression modules, which were represented by C4, C8 and C10. The proteins related to these modules may negatively regulate the defense response or be suppressed by the S. turcica. Further analysis shown that these modules enrichment by the reduced expression of the PS lightreaction photosystem, serine protease, jasmonate degradation, redox thioredoxin and cell cycle peptidylprolyl isomerase. These results suggested that the photosynthetic rate and cell proliferation of maize leaves decreased during S. turcica infection. Modules C5, C6 and C9 included proteins which exhibited irregular expression patterns and were related to protein synthesis and degradation, hormone metabolism and mitochondrial electron transport. Taken together, these data show that the defense responses of maize during infection partially originated from the highly coordinated dynamics amongst the abundances of different proteins.

Detection of differentially expressed proteins (DEPs) at different infection stages
Among of the 4,711 quantified proteins, 710, 1,096 and 465 were identified as DEPs (minimum fold-change of ±1.3 or greater and P < 0.05) for 24 hpi vs CK, 72 hpi vs CK and 72 vs 24 hpi, respectively (Table S4). The number of up-regulated DEPs was higher than that of down-regulated DEPs (Fig. 3a). Moreover, the distribution of the DEPs was illustrated in a Venn diagram (Fig. 3b). This diagram shows that the three compare shared 131 DEPs in common. Amongst the 131 common DEPs, 105 were up-regulated, and almost 80% (81) DEPs were continuously induced. For instance, the wound-induced protein1 (ZmWIP1, Zm00001d008548), which is associated with hypersensitivity reaction (HR) defense response in maize [26], was up-regulated by 3.51-fold at 24 hpi and 12.55-fold at 72 hpi, indicating the important role of ZmWIP1 for maize HR defense response to *S. turcica*. These results suggest maize has initiated HR defense response at 24 hpi and gradually increased as the infection progresses and eventually leads to cell death lesions. Furthermore, the functions of DEPs were annotated by using GO terms (Fig. S2). Overall, the distribution proportion of the DEPs function was similar among the three comparison (24 hpi vs CK, 72 hpi vs CK and 24 hpi vs 72 hpi). Notably, the distribution proportion of the term ‘response to stimulus’ at 72 vs 24 hpi was significantly higher (Fisher’s exact test P < 0.05) than that at CK vs 24 hpi. These results suggests that, although, the DEPs of different infection stages were different, the similar defense reaction may be involved in maize response to infection and the response strength is increasing during the infection process.

To further understanding the defense response of maize, the GO enrichment analysis was performed. For the enriched GO terms, some were exists in 24 hpi vs CK (Fig. 3c) and 72 hpi vs CK (Fig. 3d), such as ‘response to biotic stimulus’, ‘defense response’, ‘COPI vesicle coat’, ‘oxidoreductase activity’, ‘hydrogen peroxide metabolic process’ and ‘eukaryotic translation elongation factor 1 complex’, suggesting the proteins involved in the above terms may function with regulatory roles in basal responses. Some were specifically enriched in one stages. To illustrate, ‘regulation of protein metabolic process’, ‘protein insertion into membrane’ and ‘lipid transport’ were enriched in 24 hpi, while ‘hydrolase activity’, ‘disaccharide and oligosaccharide biosynthetic process’ and ‘trehalose biosynthetic process’ were enriched in 72 hpi. In addition, when compared 72 hpi to 24 hpi (Fig. 3e), the GO term ‘hydrolase activity’, ‘glucosyltransferase activity’, ‘amino acid transport’, ‘anchored component of membrane’ and ‘photosystem I reaction center’ were enriched. Taken together, these results indicated some induced proteins function in basal responses, whereas some were specifically involved in invasion.

**DEPs related to the glutathione S-transferase (GST) family**

To further understanding the defense response of maize, the DEPs function were annotated by mapman. Compared with the DEPs in 24 hpi vs CK, more DEPs of 72 hpi vs CK were took part in the defense response. It’s worth noting that, the number of DEPs belong to the GST family had grown from one to ten in 72 hpi (Fig. 4a). Among of them, the ZmGST23 (Zm00001d020780) which had proved to be associated with modest levels of resistance to NCLB in maize [27], was up-regulated at 72 hpi (Fig. 4A). To have an insight into the function of GSTs, we analyzed the expression pattern of the above ten DEPs in transcriptional level by using the published RNA-Seq, which contain two inbred lines with opposite resistance (B73: Susceptible; B73Htn1: contain the NCLB resistance gene Htn1) with four infected stage
Almost all of above GSTs were highest expression at the latest infection stage (240 hpi) of the studies in B73 (Fig. 4b), while, in the B73Htn1, they were highly expression in the earlier period of infection (9 and 72 hpi) (Fig. 4c). These results indicated the glutathione-dependent detoxification participate in defense response and the earlier time of induction of which may contribute to the resistance to *S. turcica*.

**DEPs related to the Secondary metabolites pathway**

Many secondary metabolites found in plants have a role in defence against pathogens. It was well known that the phenylpropanoids and benzoxazinoids (BXs) are involved in plant defense in plant [29, 30]. So, the DEPs related to phenylpropanoid and BXs biosynthesis were analyzed further (Fig. 5). In total, 19 DEPs were annotated as phenylpropanoid lignin biosynthesis pathway enzyme, and they belong to the other 8 enzymes, except F5H (ferulate 5-hydroxylase) and C3H (*p*-coumarate-3-hydroxylase) (Fig. 5a). We found two DEPs (ZmPAL1, Zm00001d017274; ZmPAL2, Zm00001d003016) which belong to the phenylalanine ammonia-lyase the rate-limiting enzyme of phenylpropanoid biosynthesis pathway were down-regulated in 24 and 72 hpi. Moreover, the DEPs of the key enzymes in lignin biosynthesis which produced through the activity of the phenylpropanoid pathway were analyzed. The Cinnamate 4-hydroxylase (ZmC4H, Zm00001d009858) and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (ZmHCT, Zm00001d050455 and Zm00001d020530) were significantly increased in infected plants at 24 and 72 hpi. While, Cinnamyl alcohol dehydrogenase (ZmCAD, Zm00001d015618), Cinnamyl alcohol dehydrogenase (ZmCCR, Zm00001d032152) and Caffeic acid 3-O-methyltransferase (ZmCOMT, Zm00001d049541) were down-regulated significantly in 24 and 72 hpi. These results shown that there were a complex response pattern of phenylpropanoid lignin biosynthesis pathway enzymes to *S. turcica* infection. In addition, four DEPs which belong to BXs biosynthesis pathway were identified (Fig. 5b). Except the benzoxazinone synthesis 10 (BX10) which catalyze the conversion of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-Glc (DIMBOA-Glc) to 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-Glc (HDMBOA-Glc), the BX4, BX6 and BX7 which are responsible for the synthesis of DIMBOA-Glc were down-regulated. Furthermore, we found the β-glucosidase (ZmGLU1, Zm00001d023994) which was responsible for hydrolyzing DIMBOA-Glc to DIMBOA and glucose [31], was up-regulated 2.2 and 7.46 fold in 24 hpi and 72 hpi, respectively. These results suggested the enzymes responsible for synthesis of DIMBOA-Glc were reduced and the decomposition were increased in maize leaves when response to *S. turcica*.

**DEPs related to the jasmonic acid biosynthesis pathway**

The responses mediated by jasmonic acid (JA) and salicylic acid (SA) play a key role in plant defenses against pathogens [32]. Form the mapman results, we found the DEPs related to JA were almost up-regualted, while, the DEPs related to SA was not detected in our study, indicating the JA may play the most importance roles in the defense response to *S. turcica* infected. Furthermore, the JA biosynthesis pathway were analyzed next. In this study, 18 proteins which are related to JA biosynthesis were quantified and 11 of which were DEPs (Fig. 6). Among of the 11 DEPs, 5 DEPs were different expressed
both in 24 and 72 hpi when compared with CK, including three lipoxygenases (ZmLOX1; ZmLOX4 and ZmLOX5) which catalyzes the oxygenation of polyunsaturated fatty acids and two 12-Oxo-PDA-reductase (ZmOPR1 and ZmOPR4) which catalyzes the reduction of cyclopentenone rings. In addition to those of ZmLOX1 and ZmLOX5, the expression levels of ZmLOX4, ZmOPR1 and ZmOPR4 were up-regulated at 24 hpi relative to those at 72 hpi. In addition, ZmAOS2 was up-regulated only at 24 hpi, whereas ZmLOX2, ZmLOX6, ZmLOX11, ZmAOS1 and ZmOPR8 were only differentially expressed at 72 hpi. Combined with the above results, the DEPs that related to jasmonic acid biosynthesis, excepted for ZmLOX6 and ZmLOX11, were up-regulated at 24 or 72 hpi. In addition, we noticed that the Zmlox6 gene expression was down-regulated in maize during infection with Cochliobolus heterostrophus [23]. This result suggested that Zmlox6 may play a negative role in responses to fungal infection. Moreover, the proteins which were the downstream of JA signal transduction pathway were also up-regulated, for instance, two 60 kDa jasmonate-induced protein, Zm00001d004573 was up-regulated in 24 hpi and 72 hpi, Zm00001d004591 was specifically up-regulated in 72 hpi; The MYC2 (Zm00001d030028), which is a major regulator in the JA signaling pathway [33], was also up-regulated in 24 hpi (Table S4). The above results suggested that jasmonic acid biosynthesis was induced during S. turcica infection, and the downstream proteins of JA had been activated.

**Protein-protein interaction (PPI) networks of the DEPs**

In order to further interpret the DEPs in the biological context, the PPI networks of DEPs were constructed PPI which could be used to predict the relationship between DEPs. In total, 499 DEPs of 0 vs 24 hpi and 841 DEPs of 0 vs 72 hpi were identified as network nodes by using the medium confidence cut-off (confidence score > 0.4). The PPI networks showed significantly more interactions than a random set of proteins of similar sizes \( P < 1.0e-16 \) (Table S5), indicating the DEPs were at least partially biologically connected. PPI nodes involved in biological processes were strongly associated with response to biotic stimulus (GO:0009607, \( P = 9.7e-12 \) and 3.4e-20 at 24 and 72 hpi, respectively) and defense response to ‘fungus, incompatible interaction’ (GO:0009817, \( P = 0.0017 \) and 7.1e-08 at 24 and 72 hpi, respectively). Further, the PPI nodes which were related to the term ‘defense response to fungus, incompatible interaction’ were analyzed further. We found that 5 and 12 nodes at 24 hpi and 72 hpi were belong to this GO term and interacted with 26 and 80 nodes, respectively (Fig. 7). Furthermore, the nodes of interaction network at 72 hpi almost overlapped with those identified at 24 hpi (Fig. S3), suggesting the maize had initiated additional reactions to response the infection at 72 hpi. In addition, some of the interacted nodes were also related to defense response. These proteins included the well-studied proteins ZmWIP1, ZmPRP5 and chitinase (ZmCHN1, Zm00001d043988) [25, 26, 34].

**Parallel Reaction Monitoring (PRM) validation**

In order to confirm the LC-MS/MS quantification results, PRM method were used to validate the abundance of 14 functional DEPs (Table S7). For the validation DEPs, they included the pathogenesis-related proteins, GST family protein, proteins related to phenylpropanoid and jasmonic acid biosynthesis and other proteins with important functions, which were mention in the above results. High consistency
(correlation coefficients were all greater than 0.90, \( P < 0.001 \)) between PRM and LC-MS/MS quantification were found (Fig. 8). For instance, in the defense response process, the ZmPRP6, ZmWIP1 and ZmGEB1 were significantly induced, while the ZmPAL and ZmCCR was significant down-regulation. These results further lent confidence to our LC-MS/MS data.

**Discussions**

In the past few years, microarray and RNA-Seq data have shown that hundreds of genes are up- and down-regulated on the transcript level in compatible and incompatible plant–pathogen interactions [22, 35–38]. Nevertheless, although proteomics approaches have been used to investigate plant–pathogen interactions, high-throughput proteomic data remain insufficient [39–43]. In this study, we used the LC–MS/MS technique to characterize the proteomics of the interactions between maize and *S. turcica*.

The response of maize to fungal infection is highly complex, and requires cooperation and interaction amongst a vast number of proteins. Thus, confirming the responses of different proteins is necessary to expound to expound the maize–*S. turcica* interaction. In our study, we used two infection stages (24 and 72 hpi) which corresponded to penetration of the leaf epidermis and biotrophic growth stages, respectively, to explore the interaction progress. In fact, we found three categories clusters with dynamic change in proteins abundance of maize during *S. turcica* infection (Fig. 2). It’s worth noting that some proteins in the cluster showing dynamic changes have important functions. For example, ZmACCO31 (Zm00001d024843), which catalyzes the final step in ethylene biosynthesis, was allocated to C1 and was up-regulated at 24 hpi (2.97-fold change, 0 vs 24 hpi), but was down-regulated in 72 hpi (2.10-fold change, 24 vs 72 hpi). As we known, ethylene play important roles in regulating plant defense responses against pathogens [44]; The DEPs of GST family were all clustered in to the induced-expression module C2 and C7, indicating the important roles of GST family proteins in the defense response. Furthermore, we found the GSTs were induced earlier in the resistance line than the susceptible line (Fig. 4). Previous research have proved that the differences between susceptibility and resistance host are related to the differences in changes of the timing and magnitude of resistance correlation genes rather than expression of different sets of genes [35, 45]. Hence, these further confirmed the above speculate that GST family proteins could participate in the defense to *S. turcica* and the induced time of that may contributed to the resistance to *S. turcica*. In the case of the important function of these proteins, the purpose of the abundance dynamic change in the abundances of proteins during *S. turcica* infection require further clarification, and the regulators responsible for coordinating the defense response of maize to *S. turcica* infection remain to be identified.

Plants have evolved diverse strategies to fend off pathogen attack during long term battle between plants and pathogenic fungi [8, 45–47]. Indeed, in our study, we found that numbers of well-studied genes associated with infection resistance, for instance pathogenesis-related proteins and genes related to jasmonic acid biosynthesis were up-regulated. Furthermore, some of these, such as ZmPRP5, ZmPRP6 (Zm00001d028816) and ZmGEB1, were continuously induced during plant–pathogen interaction. Moreover, our functional annotation results showed that numbers GO terms were enriched amongst DEP.
These terms included ‘response to biotic stimulus’, ‘defense response’. These results indicate that the maize have acted to resist fungal infection. In addition, our PPI network showed that defense response protein tend to interact and work together to respond to infection. Among of these proteins, ZmGEB1 (Zm00001d042143), ZmGBP2 (Zm00001d033422), ZmSIP1 (Zm00001d021901) and ZmPCK1 (Zm00001d028471) are presented in the two PPI network and may have basal roles in the defense process (Fig. 6). ZmGEB1 interacted with seven and nine DEPs at 24 and 72 hpi, respectively. These DEPs include ZmCHN1, ZmPRP5 and ZmSIP1. ZmGEB1, a stress-induced marker, could contribute to biotic and abiotic stress responses [45, 48]. ZmGBP2 is a GTP-binding protein in maize. AtAGB1, the homologue of ZmGBP2 in Arabidopsis, was involving in jasmonic acid mediated signalling pathway and participates in defense response to fungal infection [49, 50]. The PPI network showed that ZmGBP2 interacted with 8 and 13 nodes at 24 hpi and 72 hpi, respectively. Among of the interacted nodes, ZmRACK1, ZmMPK1, ZmCDPK1 and ZmCDPK23 are all related to signal transduction and are involve in defense response [51–53]. ZmSIP1, a member of the thaumatin-like protein family inhibits fungal infection in maize [54]. Its interacted nodes were also related to defense response, for instance ZmGEB1, ZmWIP1, ZmSCI1, ZmCHN1, and ZmPRP5. All in all, our PPI network analysis provided insight into the complex defense responses of maize, and some of these may acts hub proteins in the network. Nevertheless, where and how the nodes functional interactions require further confirmation.

The secondary metabolic pathway of plants is closely related to plant response to external stress and pathogen invasion [55, 56]. Many studies have shown that BXs play a role in the defense of herbivorous insects and pathogens in plants [30, 57]. In maize, when inoculated S. turcica on the maize leaves, the genes related to BXs biosynthesis were down-regulated and the contents of BXs in the maize leaves were reduced, indicating the important roles of BXs in the defense response to S. turcica [28]. Moreover, the mutations in BXs biosynthesis genes (Bx1, Bx2 and Bx6) increase NCLB resistance [28]. In our research, we found ZmBX4, ZmBX6 and ZmBX7 were down-regulated, while the ZmBX10 were up-regulated, which leads to the synthesis of DIMBOA-Glc was reduced and the decomposition was increased. In addition, the up-regulated of ZmGLU1 would further accelerated the decrease of DIMBOA-Glc. Previous had reported that Zmglu1 induction earlier could contributed resist to Bipolaris maydis [58]. Finally, these results in reduced the content of downstream product of DIMBOA-Glc, such as 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM2BOA-Glc). Importantly, in the ZmBX13 knockout mutant, which block DIMBOA-Glc to form DIM2BOA-Glc, the decrease of the DIM2BOA-Glc cloud increase in NCLB resistance during the early infection phase [28]. However, it may be that the protein abundance of ZmBX13 was too low, so that our experiment did not detect its expression. In summary, combine our proteome data with the previous reports of the relate mutants, we speculate that the DIMBOA-Glc maybe a key intermediate that can determine whether resistant or susceptible to S. turcica.

Phenylpropanoids can function as antimicrobial compounds and signal molecules in plant–microbe interactions [59]. In our study, we found the abundance of proteins that related to the phenylpropanoids biosynthesis were dynamic regulated. The ZmPAL1 and ZmPAL2, which are the key enzyme of phenylpropanoids biosynthesis [60], were down-regulated at 24 and 72 hpi, and the ZmPAL1 relative abundance wad further confirm by the PRM (Fig. 7). In previous studies on transcription level, the
expression level of ZmPAL was up-regulated when inoculated pathogen in maize leaves [61]. In the published RNA-Seq data of maize leaves inoculation with *S. turcica* [28], we also found the ZmPAL was also up-regulated (Fig. S4). These results suggest that, on the one hand the ZmPAL is able to participate in the response process to pathogens, one the other hand the transcription level of *ZmPAL* is not consistent with the protein level. However, the induced pattern of other enzymes, such as ZmC4H, ZmCAD, ZmCCR and ZmCOMT were accordant with the transcription level. Hence, some of regulatory factors can be regulated ZmPAL after transcription, such as the translation efficiency of *ZmPAL* mRNA is slower or the rate of ZmPAL degradation is lower. Therefore, other experiments are needed to verify the regulation mechanism. In addition,

**Conclusions**

In this study, we analyzed the maize proteome during the early stage of *S. turcica* infection (24 and 72 hpi). The results of our study suggest that maize response to *S. turcica* infection may be associated with dynamic changes in the abundances of pathogen-inducible defense proteins, JA related proteins, BXs biosynthesis and phenylpropanoid lignin biosynthesis related proteins. Specifically, the GST family proteins earlier induction may contribute to the resist to *S. turcica*. The results provide a new perspective for the earlier interaction between maize and *S. turcica* at the protein level. Previous research have proved that the difference between different resistant hosts is not due to the difference of gene set, but to the gene response time and intensity. Therefore, our results also provide a meaningful candidate list for maize to resist *S. turcica* at protein level.

**Methods**

**Plant materials and the inoculation method**

Experiments were performed with maize inbred lines B73 which is highly susceptible to *S. turcica*. The maize seeds of B73 were acquired form Jingjuan Yu lab (State Key Laboratory for Agrobiotechnology, College of Biological Sciences, China Agricultural University). Maize plants were grown under greenhouse conditions at Hebei Agriculture University during April 2018 to May 2018. Conidia for inoculation were obtained by culturing the hyphae of *S. turcica* strain 01-23 on PDA medium at 25°C for 10 days in the dark. Sixth-leaf-stage maize plants were inoculated with *S. turcica* conidia (1×10⁵) by spray-inoculating the entire plant. The top two leaves of maize (B73 inoculated with 01-23) were harvested at 0, 24 and 72 hours post-inoculation (hpi). All samples were collected rapidly, immediately frozen in liquid nitrogen, and then stored at −80°C prior to processing and use in subsequent experiments. Each sample was obtained from at least four maize plants.

**Protein Extraction**

The leaves of samples were collected and ground by liquid nitrogen and then the cell powder was transferred into 5 mL centrifuge tube. After adding four volumes of lysis buffer [8 M urea, 1% Triton-100,
10 mM dithiothreitol (DTT), and 1% protease inhibitor cocktail] in centrifuge tube, the samples were sonicated three times on ice using a high intensity ultrasonic processor (Scientz, Ninbo, China). The remaining debris was removed by centrifugation at 20,000 g at 4 °C for 10 min. Finally, the protein was precipitated at −20°C for 2 h with cold 20% trichloroacetic acid. After centrifugation (12,000 g, 4°C, 10 min), the supernatant was discarded. The remaining precipitate was washed three times by using cold acetone. Finally, the protein was redissolved in 8 M urea and the BCA kit was used to determine the protein concentration.

**Trypsin digestion and TMT labeling**

For trypsin digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at 25°C in darkness. Finally, trypsin (Promega, Madison, WI, USA) was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second digestion 4 h. After trypsin digestion, protein samples was desalted by Strata X C18 SPE column (Phenomenex, Torrance, CA) and then vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer’s protocol for TMT kit (ThermoFisher Scientific, Waltham, USA). Briefly, one unit of TMT reagent were thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 h at 25°C and pooled, desalted and dried by vacuum centrifugation.

**High Performance Liquid Chromatography (HPLC) fractionation**

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length, Agilent, Santa Clara, CA, USA). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 18 fractions and dried by vacuum centrifuging.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis**

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15 cm length, 75 μm i.d.). The gradient was comprised of an increase from 9% to 25% solvent B (0.1% formic acid in 98% acetonitrile) over 40 min, 25% to 36% in 12 min and climbing to 80% in 4 min then holding at 80% for the last 4 min, all at a constant flow rate of 400 nL/min on an EASYnLC 1000 UPLC system (Thermo Fisher Scientific, Waltham, USA). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo Fisher Scientific, Waltham, USA) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Isolation window was set as 2.0. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap (Thermo Fisher Scientific, Waltham, USA) at a resolution of 17,500. A data dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 30.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.
Database search

The MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against MaizeGDB *Zea mays* protein database concatenated with reverse decoy database, respectively. Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl (Cys) was set as a fixed modification, whereas oxidation (Met) and acetylation (protein N-terminal) were set as variable modifications. False discovery rate (FDR) thresholds for proteins, and peptides were set at 1%. For the quantification method, TMT-10-plex was selected. All the other parameters in MaxQuant were set to default values. The quantitative value of the unique peptide was calculated according to the ratio of the ion signal intensity in secondary spectrum. Then, the mean value of all unique peptides that related to each protein was used to quantify to protein expression. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013370.

PRM validation

To determine the reliability of TMT results, a Parallel Reaction Monitoring (PRM) assay was performed using the original nine protein samples (CK-1, CK-2, CK-3, 24_1 hpi, 24_2 hpi, 24_3 hpi, 72_1 hpi, 72_2 hpi, 72_3 hpi) [62]. The DEPs selected for PRM validation were based on the LC-MS/MS results. Full details are given in Supplementary Methods 1.

Hierarchical clustering

Hierarchical clustering was performed by using the TCseq package with 10 clusters through the soft clustering (cmeans) method. Transformed and normalized gene expression values with log2 (protein abundance + 1) were used for hierarchical clustering. The functional category enrichment of each cluster was evaluated through MapMan annotation [63]. Prior to MapMan annotation, the longest protein of each gene of AGP B73v4 was chosen as the representative protein. The selected proteins were annotated by using Mercator with default settings. The overrepresentation of functional categories in one cluster was determined through Fisher's exact test. *P*-values were adjusted to Q values by using the Benjamini-Hochberg correction equation. A false discovery rate of 5% was applied.

Gene Ontology analysis

Quantified proteins were annotated based on the basis of the annotation data of maizeGDB and classified through Gene Ontology GO annotation into three categories: Biological Process, Cellular Compartment and Molecular Function. Two-tailed Fisher's exact test was employed to test the enrichment of differentially expressed proteins (DEPs) in each category against that of all identified proteins. The GO annotation with a corrected P-value < 0.05 was considered significant.

Protein–protein interaction network analysis
For the protein–protein interactions (PPIs) predicted analysis, all of the DEPs were first searched against the STRING database version 10.5 (http://string-db.org/). The interactions between proteins which belonged to the searched dataset (Zea mays) were selected. The “confidence score” of STRING was used and the confidence score > 0.4 (medium confidence) between proteins was used for further analyze. Interaction network generated by STRING were visualized in Cytoscape [64].

List Of Abbreviations

NCLB: Northern corn leaf blight; PTI: pattern triggered immunity; ETI: effector-triggered immunity; PPR: pattern recognition receptor; NBS-LRR: nucleotide-binding site-leucine-rich repeat; MS/MS: tandem mass spectrometry; TMT: tandem mass tags; HR: hypersensitivity reaction; DEP: differentially expressed protein; GST: glutathione S-transferase; JA: jasmonic acid; SA: salicylic acid; PRM: parallel reaction monitoring; BX: benzoxazinoid; DIMBOA-Glc: 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-oneGlc; HDMBOA-Glc: 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-Glc; DIM2BOA-Glc: 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013370.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions
GSQ and DJG conceived and designed the experiments. LYW and GXD participated in the analysis and wrote the paper. ZQH performed the experiments. LZP and HJM collect the experimental materials. All authors read and approved the final manuscript.

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Figures
Figure 1

Quality control validation of MS data. (a) Expression level of two pathogenesis-related genes analyzed by semi-RT-PCR; (b) Pearson's correlation of protein quantitation; (c) Mass delta of all identified peptides; (d) Length distribution of all identified peptides.
Figure 2

Co-expression modules of proteins quantitation. C represent Cluster; PA represent proteins abundance.
Figure 3

Analysis of the differentially expressed proteins (DEPs). (a) The number of DEPs in the compare of each two samples; (b) Venn diagram of the DEPs in the compare of each two samples; GO enrichment analysis of DEPs in 0 hpi vs 24 hpi (c), 0 hpi vs 72 hpi (d) and 24 hpi vs 72 hpi (e);
Figure 4

DEPs related to glutathione S-transferase (GST) family. (a) Proteins relative abundance of B73 (Susceptible); (b) mRNA relative abundance of B73 (Susceptible); (c) mRNA relative abundance of B73Htn1 (Resistance, contain the NCLB resistance gene Htn1).
Figure 5

DEPs related to secondary metabolites pathway. (a) DEPs related to phenylpropanoid lignin biosynthesis. Solid line and dotted box mean the enzyme detected or not in this experiment, respectively. Red boxes mean up-regulated protein and green mean down-regulated. Left block indicate the DEPs of 24 hpi vs CK and right indicate the DEPs of 72 hpi vs CK; (b) DEPs related to benzoxazinoids (BX) biosynthesis.
pathway. Schematic representation of the enzyme involved in BX biosynthesis pathway in maize, the bar chart represent the relative abundance of protein.

Figure 6

DEPs related to jasmonic acid biosynthesis pathway. Schematic representation of the enzyme involved in jasmonic acid biosynthesis pathway in maize (Left) and Heat map showing proteins abundance of different jasmonic acid biosynthesis enzyme family members in the three interaction stages (Right). * indicated significant differences in 0 hpi vs 24 hpi and 0 hpi vs 72 hpi; # indicated significant differences in 0 hpi vs 24 hpi; ## indicated significant differences in 0 hpi vs 72 hpi. (The abbreviation in the picture please see table S6).
Figure 7

Interaction network of the DEPs related to defense response. The DEPs of protein-protein interaction network in 0 hpi vs 24 hpi (a) and 0 hpi vs 72 hpi (b). Red node indicated protein up-regulated, green node indicated protein down-regulated. (The abbreviation in the picture please see table S6).
Figure 8

Comparison between the LC-MS/MS and parallel reaction monitoring (PRM) results. Blue bar indicating the protein relative abundance detected by PRM; red line indicating the protein relative abundance detected by LC-MS/MS.

Supplementary Files

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