**iTRAQ-based proteomic analysis of defence responses triggered by the
necrotrophic pathogen *Rhizoctonia solani* in cotton**

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Abstract

The soil-borne necrotrophic pathogen fungus *Rhizoctonia solani* is destructive, causing disease in various important crops. To date, little is known about the host defence mechanism in response to invasion of *R. solani*. Here, an iTRAQ-based proteomic analysis was employed to investigate pathogen-responsive proteins in the disease tolerant/resistant cotton cultivar CRI35. A total of 174 differentially accumulated proteins (DAPs) were identified after inoculation of cotton plants with *R. solani*. Functional categorization analysis indicated that these DAPs can be divided into 12 subclasses. Notably, a large portion of DAPs are known to function in reactive oxygen species (ROS) metabolism and the expression of several histone-modifying and DNA methylating proteins were significantly induced upon challenge with the fungus, indicating that the redox homeostasis and epigenetic regulation are important for cotton defence against the pathogen. Additionally, the expression of proteins involved in phenylpropanoid biosynthesis was markedly changed in response to pathogen invasion, which may reflect a particular contribution of secondary metabolism in protection against the fungal attack in cotton. Together, our results indicate that the defence response of cotton plants to *R. solani* infection is active and multifaceted and involves the induction of proteins from various innate immunity-related pathways.

KEY WORDS: proteomic, cotton, *Rhizoctonia solani*, defence response

Significance

Cotton damping-off is a destructive disease caused by the necrotrophic fungus *Rhizoctonia solani*. To date, the host defence mechanism involved in the disease protection remains largely unknown. Here, we reported the first proteomic analysis on cotton immune responses against *R. solani* infection. Employing iTRAQ technique, we obtained a total of 174 differentially accumulated proteins (DAPs) that can be classified into 12 functional groups. Further analysis indicated that ROS homeostasis, epigenetic regulation and phenylpropanoid biosynthesis were tightly associated with

the innate immune responses against *R. solani* infection in cotton. The obtained data provide not only important information for understanding the molecular mechanism involved in plant-*R. solani* interaction but also application clues for genetic breeding of crops with improved *R. solani* resistance.

1. Introduction

The soil-borne necrotrophic fungal pathogen *Rhizoctonia solani* is widely dispersed and invades a broad range of plants, including staple food crops such as rice, wheat and potato, as well as the cash crops cotton and sugar beet [1]. The fungus attacks the root, stem and other aerial parts of the plant, and causes diseases such as root rot, sheath blight and seedling damping-off [2]. Based on their hyphal anastomosis reaction, the isolates of *R. solani* can be classified into fourteen anastomosis groups (AGs) [3]. Due to the lack of stable genetic resistance source against the pathogen, traditional breeding for *R. solani* resistance remains a great challenge for many crop hosts.

During recent years, much evidence has accumulated for the role of plant innate immunity in *R. solani* infection [4]. Global identification of defence-related proteins/pathways against *R. solani* infection in plants indicates that signal transduction, reactive oxygen species (ROS) production and redox regulation, as well as metabolic changes, are involved in the native defence processes [4-8]. Meanwhile, a number of genes were identified to be related to protection against *R. solani* invasion, including those encoding receptor protein kinases, glutathione peroxidase, chitinase, molecular chaperones, enzymes in the glycolytic pathway, and pathogenesis-related proteins (PRs) [4, 5, 7, 9-11]. The function of these genes in protection of the host plant against pathogen infection has been demonstrated by transgenic approaches. For example, overexpression of *OsWRKY4* in rice or the protein kinase gene *NtPK* in tobacco led to the up-regulation of defence-associated genes and enhanced resistance to *R. solani* [12, 13], while *snakin-1* gene overexpression improved disease resistance in potato [14]. Additionally, ectopic expression of cotton *GhWRKY3* or the zinc finger protein gene *GhZFP1* conferred increased tolerance to *R. solani* in transgenic tobacco [15, 16], and overexpression of *PR* genes such as *PR-2* (β -1,3-glucanase gene), *PR-3* (chitinase gene) and *PR-5* (thaumatin-like protein gene) led to enhanced resistance to *R. solani* in various plants

[17-19]. These studies have made a substantial step towards understanding the interaction between *R. solani* and plant hosts, and provide important clues for the breeding of *R. solani* resistant/tolerant host plants.

Proteomic analysis is a powerful tool that can be used to investigate cellular changes during plant-pathogen interactions. Recently, using the proteomics approach, a number of proteins from different AGs of *R. solani* were determined to be related to host infection, including those involved in plant cell wall degradation, redox regulation, and the pathogenicity or virulence-associated proteins [20, 21]. On the host side, a proteomic analysis of the defence response against *R. solani* infection was also reported, and 17 proteins were identified as defence-responsive proteins, including β -1, 3-glucanase, chitinase, ascorbate peroxidase, and glyceraldehyde phosphate dehydrogenase [9]. It has been recognized that isolates from different AGs of *R. solani* tend to have a distinct host range, and recent evidence indicates that *R. solani* isolates attack plants through complex ways, including degradation of host cell walls, inactivation of host defence factors, and the release of toxic metabolites [22]. Hence, it is speculated that multiple defence pathways are required to achieve effective resistance in different hosts [22]. In this regard, continuous efforts are required to identify proteins that function in different hosts relative to a certain pathogen isolate to gain a comprehensive understanding of the *R. solani*-host interaction mechanisms.

Cotton is the most important natural fibre crop. During the early stage of growth, particularly under cold and wet weather conditions, cotton seedlings are susceptible to attack by *R. solani*, which can cause damping-off disease and may result in great yield losses in the most cotton-producing areas [23]. Despite the threat of the pathogen in cotton production, very little is known about the defence responses against *R. solani*. In this study, we conducted a proteomic comparison using a high-throughput iTRAQ-based technique to explore the pathogen-responsive proteins and biological processes in the cotton cultivar CRI35, which shows moderate resistance to *R. solani* infection. Our study identified a number of defence-related cotton proteins and revealed several important pathways associated with the defence response. These data provide not only important information for our understanding of the molecular mechanism involved in the plant-*R. solani* interaction but also application clues for the genetic breeding of crops with improved *R. solani* resistance.

2. Material and Methods

2.1 Plant growth

Seeds of upland cotton varieties CRI35 and TM-1 were treated with H₂SO₄ (98%), then rinsed with water several times. The germinated seeds were sowed into the soil and grown in a growth chamber at 28°C under a 16 h light/8 h dark cycle with a relative humidity of 65%.

2.2 Pathogen cultivation and inoculation

The *R. solani* strain used in this study was originally isolated from an infected cotton plant and was kindly provided by Prof. Gui-Liang Jian at the Institute of Plant Protection in Chinese Academy of Agricultural Sciences. The fungal mycelia were incubated on 10% V-8 agar medium overlaid with cellophane at 28°C for 48 h according to procedures described previously [24]. The inoculation method was based on the reported protocol [15] with some modifications. Briefly, sterilized soil (soil:vermiculite = 3:1) was thoroughly mixed with mycelia powder with a concentration of 50 or 100 mg/L. Seedlings treated with sterile water were used as a mock control. The germinated seeds were then transplanted into the mixed soil, and roots were harvested at 36, 60, 84, or 108 hours post inoculation (hpi), and then frozen at -80°C for RNA or protein extraction.

Disease index was calculated based on the following formula: disease index = [(A × 0) + (B × 1) + (C × 2) + (D × 3) + (E × 4) + (F × 5) × 100]/(total number of seedlings used × 5) [25]. The arabic numerals represent: 0, no symptoms; 1, lesion width less than 1/4 stem diameter; 2, lesion width between 1/4 and 1/2 of stem diameter; 3, lesion width between 1/2 and 3/4 of stem diameter; 4, lesion width more than 3/4 of stem diameter; 5, seedling died during infection. The capital letters in the formula refer to the number of plants.

2.3 Protein extraction and SDS-PAGE

Fresh samples were ground into powder in liquid nitrogen in the presence of polyvinylpyrrolidone (PVPP) and quartz sand. Protein extraction and purification were carried out according to previous report [26] with some modifications. Briefly, ground powders were suspended in extraction buffer containing 50 mM

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate, 25 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 5 mM tris-(2-carboxyethyl) phosphine (TCEP), 50 mM vitamin C and 2% SDS, pH 7.4. Proteins were then enriched and purified by chloroform/methanol precipitation. Protein pellets were dissolved in a buffer containing 8 M urea, 200 mM TEAB, 0.1% SDS, 1 mM TCEP, 1 mM PMSF, and 2% 3-[(3-Cholamidopropyl)dimethylammonio]-propanesulfonate (CHAPS). Total soluble protein concentration was determined using the 2D-Quant Kit (GE healthcare). Extracted proteins were separated by 12% SDS-PAGE to evaluate the protein quality.

2.4 iTRAQ labelling

For each sample, 100 μg of proteins were reduced, alkylated and trypsin-digested using the iTRAQ Reagents 8-plex Kit (AB Sciex, Inc., Foster City, CA, USA) according to the manufacturer's instructions. Control peptide samples collected at 36, 60, 84, and 108 hpi were labelled with iTRAQ tags 113, 115, 117, and 119, respectively, and their counterparts labelled with 114, 116, 118, and 121, respectively. After labelling, the peptide samples were mixed and vacuum-dried.

2.5 Fractionation of iTRAQ-labelled peptides

The mixed peptide samples were resuspended in buffer A (20 mM ammonium formate, pH 10.0) and separated on an LC-20AT HPLC system (Shimadzu, Japan) with a Gemini 5 μm C18 column (4.6 $\times$ 250 mm; Phenomenex) at a rate of 0.5 ml/min. Peptides were eluted from the column by a 2%-95% linear gradient of buffer B (20 mM ammonium formate, 80% acetonitrile, pH 10.0) for over 100 min. The eluted peptide samples were pooled into 20 fractions and vacuum-dried.

2.6 LC-MS/MS

The collected peptide fractions were resuspended in TEAB (tetraethylammonium bromide) buffer for further reversed-phase HPLC separation on a Thermo Scientific Easy-nLC 1000 system. The separation was conducted on an Easy-spray column (C18, 5 μm , 75 μm $\times$ 15 cm) at a flow rate of 400 nl/ μl with a 3%-90% liner gradient of buffer B for over 60 min. The MS/MS analysis was performed on an Orbitrap Fusion

system. The parameters for MS1 were set as follows. Orbitrap resolution: 120000; scan range: 400-1600 m/z; data dependent mode: top speed; cycle time: 3s; dynamic exclusion duration: 40s. The parameters for MS2 were set as below. Orbitrap resolution: 15000; isolation mode: quadrupole; isolation window: 1.6; activation type: HCD; collision energy: 38%; data type: profile.

2.7 Database search and quantitative proteomic analysis

Mascot was used to analyse all MS/MS samples (Matrix Science, London, UK; version 2.5.1), and was set up to search the *Gossypium hirsutum* L. acc. TM-1 sequence database. The identification parameters included follows. Type of search: MS/MS ion search; enzyme: trypsin; mass values: monoisotopic mass; max missed cleavages: 1; fragment mass tolerance: 0.05 Da; peptide mass tolerance: 15 ppm; variable modifications: oxidation (M), acetyl (protein N-term), Gln- > pyro-Glu (N-termQ), iTRAQ8plex (Y); fixed modifications: methylthio (C), iTRAQ8plex (N-term), and iTRAQ8plex (K). Verification of peptide and protein identifications based on MS/MS was carried out by scaffold (version Scaffold_4.4.5, Proteome Software Inc., Portland, OR). Peptide and protein identifications were both filtered by a FDR less than 1% and proteins were required to have at least two unique peptides. The charge states of peptides were +2, +3 or +4. All normalization calculations were performed based on Oberg's approach [27]. *P*-values were calculated according to Student's *t*-test. Proteins with a Log2 fold change ≥ 0.43 or ≤ -0.43 (inoculated vs mock at 36, 60, 84 and 108 hpi) and a *P*-value ≤ 0.05 were selected as differentially accumulated proteins (DAPs).

2.8 Bioinformatics

Blast2go (<http://www.blast2go.com/b2g>), Tair (<http://www.arabidopsis.org/>) and AgriGO (<http://bioinfo.cau.edu.cn/agriGO/manual.php>) were used for the annotation and functional classification of identified protein species. KOBAS 2.0 (<http://kobas.cbi.pku.edu.cn/>) was utilized for the protein pathway analysis. The classical secreted proteins were predicted using SignalP and TargetP 1.1 (<http://www.cbs.dtu.dk/services/SignalP/>; <http://www.cbs.dtu.dk/services/TargetP/>). The non-classical secreted proteins were predicted by SecretomeP

(<http://www.cbs.dtu.dk/services/SecretomeP/>). Heatmaps were constructed and visualized using the Perseus 1.4.1.3 package.

2.9 Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from infected and control cotton roots using an RNA Extraction Kit (QIAGEN). cDNA was synthesized using an RNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's instructions. The conditions used for qRT-PCR were as follows: 5 min at 95°C; followed by 40 cycles at 95°C for 30 s, 58°C for 20 s and 72°C for 30 s. The cotton *UBQ7* gene was used as the internal control. The relative expression levels of all genes were calculated by the $2^{-\Delta\Delta CT}$ method [28], and Student's *t*-test statistical analysis was performed to evaluate significant differences between the three experimental repeats. The primers for RT-PCR were shown in Table S1.

3. Results and Discussion

3.1 Phenotype and symptom examination of the cotton cultivar CRI35 for resistance against *R. solani* infection

CRI35 is a cotton cultivar showing moderate resistance to *R. solani* infection [29]. To examine its disease resistance under our experimental condition, we inoculated CRI35 with a mixture of soil and hypha powder (50 mg/L), and the standard upland cotton variety TM-1 was used to compare their disease-related phenotypes. While the mock-inoculated control plants of both varieties looked healthy (Fig. 1A), a clear difference in the development of disease symptoms could be observed between the two kinds of plants. Compared to CRI35, TM-1 showed more severe disease symptoms: brown sunken lesions covered the hypocotyl-root junction and all seedlings were damping-off at 7 dpi. The difference in disease index between the two plants showed that TM-1 had a higher mean disease index compared to CRI35 (Fig. 1B). To confirm the disease resistance of CRI35, plants were inoculated with 2x higher concentration of inoculum (100 mg/L). No obvious symptom was observed at 36 hpi on the seedlings; only some small, brown-colored spots were detected around the hypocotyl-root junction at 60 hpi; at 84 hpi, browning necrotic lesions appeared

and damage at the junction region could be seen; at 108 hpi, typical disease symptoms were seen as brownish sunken lesions around the junction region (Fig. 1C). This behaviour of CRI35 after *R. solani* challenge indicates that it has moderate resistance to the pathogen; thus, it was suitable for the subsequent proteomic analysis.

3.2 Identification of DAPs in response to *R. solani* infection

To gain a global view of root proteome changes and to identify defence-related proteins in the cotton cultivar CRI35 challenged with *R. solani*, a multiplex iTRAQ-based quantitative proteomic approach was used. Roots were selected for analysis because they represent the first barrier against *R. solani* invasion. The procedure for iTRAQ analysis is shown in Fig. 2. Because the cotton root contains a high content of secondary metabolites including polysaccharides and polyphenols, we first made an effort to optimize the protein extraction method by manipulating the buffers and the procedure of protein extraction to reduce these substances. We also avoided using the thiols and primary amine reagents to obtain a high peptide labelling efficiency. By the above means, we obtained a protein extraction of high purity and quality, which contributed to complete protein digestion and efficient labelling of iTRAQ isobaric tags.

After searching the cotton sequence database, we obtained a total of 7,560 quantitative proteins with at least 2 unique peptides ($\text{FDR} \leq 0.01$) and 5,726 of which (Table S2) were identified in all three biological replicates. The CV (coefficient of variance) values of more than 95% of the quantified protein species were less than 20% based on calculating the variation of quantitative ratios between inoculated and mock at each time point. A total of 174 DAPs with Log2 ratios of either ≥ 0.43 or ≤ -0.43 were quantified as dramatic changes according to the accumulative change relative to their controls at 36, 60, 84, and 108 hpi (Table 1, Table S3). Approximately, the number of up-accumulated proteins was 1.5-fold more than that of the down-accumulated proteins (Fig. 3). A heatmap was generated to display the expression profile of these *R. solani*-responsive protein species. As can be seen in Fig. 4, some DAPs were continually activated in response to the pathogen attack as they were identified from more than one infection stages, whereas majority of DAPs were

identified at a particular infection stage (either at 60 or 84 hpi), indicating that significant proteome changes occurred at these stages when the symptoms appeared and developed quickly. The number of identified proteins at 108 hpi decreased, especially for the up-accumulated proteins, suggesting that *R. solani*-response in cotton was attenuated at this time point.

To assess the reliability of the expression change of the identified proteins, we used qRT-PCR to analyse the gene expression for eight identified proteins at 60 hpi (Fig. 5). The transcript and protein expression data of all the tested genes/proteins was consistent, showing the reliability of the identification results in our study.

3.3 GO annotation and functional classification of the identified *R. solani*-responsive proteins

GO annotation, KEGG analysis and manual searching were performed to classify the obtained DAPs (Table S4, Table 2). The *R. solani*-responsive proteins covered a broad range of functions, including epigenetic regulation (9.20%), Phenylpropanoid and lignin synthesis (7.47%), oxidative-reduction balance (16.09%), jasmonic acid (JA)/ethylene (ET) signalling (3.45%), PRs (4.02%), and protein metabolism (19.54%) (Fig. 6, Table 1, Table S3). To obtain a deeper understanding of the roles of the identified proteins, we focused our functional analysis on the DAPs with evident changes during cotton's responses against *R. solani* infection.

3.3.1 Redox homeostasis

ROS (reactive oxygen species) burst is a key event in plant defence responses upon pathogen attack [30]. Many proteins function in redox regulation, and their expression levels are tightly coordinated to maintain ROS homeostasis [31]. In our study, 28 ROS-related proteins were identified and most of them were up-accumulated (Table1) upon the *R. solani* attack. Two oxoglutarate-dependent oxygenases (CotAD_20620 and CotAD_40118) were up-accumulated in response to *R. solani* infection. The oxoglutarate-dependent oxygenases catalyze a variety of oxidative transformations and produce ROS as a result of incomplete catalytic cycles [32, 33]. In addition to these oxygenases, several ROS-removing proteins (CotAD_08788, CotAD_39520 and CotAD_64340) were down-accumulated, which could also favour the

pathogen-responsive ROS generation. Two glutaredoxins (CotAD_13268 and CotAD_37648) and two thioredoxins (CotAD_76239 and CotAD_20888) were up-accumulated. These proteins have been reported to play important roles in regulation of redox signalling [34, 35]. Recently, the thioredoxin GbNRX1 was found to be involved in ROS homeostasis in defence against *Verticillium dahliae* in cotton [36]. Five glutathione S-transferases (GSTs) (CotAD_11533, CotAD_11535, CotAD_11530, CotAD_18322, and CotAD_11531) and a glutathione peroxidase (CotAD_39520) were significantly up-accumulated upon pathogen infection. GSTs and glutathione peroxidase are known as antioxidant enzymes with the ability to catalyse the reduced form of glutathione (GSH) and xenobiotic substrates for detoxification. These enzymes are important for removing active oxygen species and in protecting plants from oxidative damage. Identification of these ROS-related proteins indicates that ROS homeostasis is well-controlled during cotton defence against *R. solani*.

3.3.2 Phenylpropanoid and lignin biosynthesis

Another notable category of DAPs was involved in phenylpropanoid and lignin biosynthesis as indicated by the GO annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-based Annotation System (KOBAS) analysis (Table 1 and 2, Table S4, Fig. S1). In this category, seven proteins were up-accumulated and 6 were down-accumulated. The up-accumulated proteins included 3 UDP-glycosyltransferases and a coumarate COA ligase involved in flavonoid biosynthesis (CotAD_26516, CotAD_23978, CotAD_70424, and CotAD_24031), and 3 class III peroxidases in the downstream phenylpropanoid biosynthesis pathway (CotAD_04115, CotAD_61121, CotAD_20099, and EC 1.11.17). Several studies have shown that plant phenolic compounds are toxic to soil-borne pathogens, nematodes or phytophagous insects [37, 38], and particular phenolic compounds such as isoflavonoids, flavonoids, cajanin, and glyceolin can act as phytoalexins [39, 40]. Here, we found that proteins involved in flavonoid biosynthesis were activated, suggesting that phytoalexins derived from flavonoid analogues play an important role in the cotton defence against *R. solani* infection. The down-accumulated proteins

included 5 proteins belonging to class III peroxidases and 1 caffeate O-methyltransferase that functions in lignin biosynthesis (CotAD_50518, CotAD_08956, CotAD_68830, CotAD_10348, CotAD_54288, EC 1.11.17, and CotAD_28867). Previous studies have shown that some pathogens such as *Fusarium solani* f. sp. *Glycines* and white-rot fungi have ability to inhibit lignin biosynthesis to facilitate their infection and colonization [41, 42]. The repression of enzymes functioning in lignin synthesis observed in our study suggested that such a strategy was also employed by *R. solani* during the process of cotton infection.

3.3.3 Epigenetic regulation

It has been well-recognized that epigenetic modulations such as DNA methylation and histone modifications play important roles in plant immunity by regulating the expression of defence-related genes [43, 44]. In our study, there were a total of sixteen proteins identified in this category (Table 1). Three proteins were up-accumulated at different time points, including 2 proteins involved in DNA methylation (CotAD_05977 and CotAD_33077), and 1 protein involved in chromatin remodelling (CotAD_37810) [45]. The up-accumulation of these proteins indicates that chromatin remodelling and DNA methylation are activated after *R. solani* challenge. As for the other proteins, seven were down-accumulated at 60 hpi, including 2 histone proteins (CotAD_03471 and CotAD_38818), 2 rRNA methyltransferases (CotAD_31765 and CotAD_46929) and 3 histone deacetylases (CotAD_37785, CotAD_18554 and CotAD_30279). Interestingly, three of these proteins [1 histone H2B (CotAD_38818), 1 ribosomal RNA methyltransferase NOP2 (CotAD_46929) and 1 histone deacetylase (CotAD_30279)] appeared up-accumulated later at 84 hpi. Since a previous study indicated that some toxins produced by necrotrophic pathogens can inhibit histone deacetylases and thereby suppress the immune response to facilitate infection [46, 47], we speculated that the down-accumulation of some proteins was caused by *R. solani* toxicity. The up-accumulation of these proteins at the later stage reflects that the plants broke the inhibition, thus possessing ability against *R. solani* infection. Together, the identification of these proteins suggests that epigenetic regulation plays an important role in cotton's defence against the necrotrophic fungus *R. solani*.

3.3.4 Proteins in other categories

We also found other categories of proteins, including JA/ET signalling, PRs, protein metabolism, carbohydrate and energy metabolism, transcriptional regulation, transport, development regulation and others (Table S3). The pathogen response of proteins in these categories reflects the wide range of changes in the plant proteome. Based on expression profiles of the proteins (CotAD_55755, CotAD_32615, CotAD_35839, CotAD_08742, CotAD_08743 and CotAD_18126), we found that the JA/ET mediated plant defence was activated during *R. solani* infection, which is consistent with the important role of this hormone signalling to necrotrophic pathogens in plants [48]. A number of PRs and some other DAPs involved in downstream defence-related processes were also identified. Some of them were key proteins with significant expression changes in response to pathogen infection, including three member of the major pollen allergen Bet v 1 protein family (CotAD_63911, CotAD_20887 and CotAD_54169) that were shown to participate in defence against *Verticillium* wilt [49, 50], two chitinases (CotAD_40314 and CotAD_26499) that were well-documented for induced expression after pathogen attack and are known to contribute to the defence against fungi by the hydrolysis of polysaccharides of fungal cell walls [51-53]. The identification of these proteins indicates that the cotton-*R. solani* interaction is active and complex, and involves multiple defence-related pathways in plant native immunity.

3.4 Computational prediction of the secreted proteins

GO annotation (cell component) indicated that a batch of DAPs was localized at the apoplast or cell wall (Fig. 7, Table S4). These results point to the significant role of secreted proteins in cotton defence against *R. solani* infection. It is known that plant cells defend themselves from pathogen infection by secreting antimicrobial metabolites and proteins into the cell wall space [54]. Secretion is very important for the delivery of antimicrobial molecule components to combat microbes [55]. Based on this background, we analysed the identified DAPs using TargetP and SignalP software to predict secreted proteins containing a signal peptide, and SecretomeP to predict secreted proteins without a signal peptide. It turns out that 36% of 174 DAPs

were putative secreted proteins, and 21 and 41 DAPs were assigned to classic and non-classic secreted proteins, respectively (Fig. 8A). AgriGO for cell components confirmed the localization of these proteins to the cell wall and external encapsulating structures (Fig. 8B), and AgriGO for biological processes and molecular functions indicated that most of them are directly related to plant defence responses (Table S5). We also calculated the proportion of secreted proteins based on the functional classification. The results showed that the secreted proteins occupied a large proportion (61.54%) in the subclasses of phenylpropanoid and lignin biosynthesis, and were also plentiful in the subclasses of PR proteins, oxidative-reduction balance and JA/ET signalling (Fig. 8C, Table 1, Table S3). These data indicated further that secreted proteins play a critical role in cotton defence against *R. solani*. Interestingly, it was recently reported that *R. solani* isolates release a large number of secreted proteins during the invasion of host plants [56] and that the secreted proteins we identified from the cotton plant could function to counteract the secreted proteins from the pathogen in the apoplast, which is recognized as a battlefield for the plant-pathogen interaction.

4. Conclusions

Through an iTRAQ-based proteomic approach, we identified 174 defence-related protein species from cotton roots challenged with the necrotrophic fungus pathogen *R. solani*. These proteins are categorized into a number of defence-related pathways. The accumulation of proteins belonging to categories of redox homeostasis, phenylpropanoid and lignin biosynthesis, epigenetic regulation, and JA/ET signalling were significantly changed upon pathogen infection, which is consistent with the data from transcriptomics and metabolomics studies on plant defence against *R. solani* [7, 57]. In addition, the identification of predicted secreted proteins from the DAPs suggests that these proteins are important for plant immunity against *R. solani* infection. Collectively, our results provide convincing evidence demonstrating the involvement of native immunity in response to *R. solani* invasion, thus supplementing the current knowledge about plant defence against necrotrophic pathogens.

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Figure captions:

Fig. 1 Examination of disease symptoms in the cotton varieties CRI35 and TM-1 upon *R. solani* infection. (A) Symptoms of CRI35 and TM-1 seven days after pathogen inoculation. (B) Comparison of the disease index between CRI35 and TM-1. (C) Disease development in CRI35 at 36, 60, 84, and 108 hpi.

Fig. 2 Diagram outlining major steps of workflow for the iTRAQ 8-plex-based quantitative proteomic analysis. (A) Sampling of one run of iTRAQ 8-plex from mock inoculated or inoculated roots at 36, 60, 72 and 108 hpi, n=number of sampled plants. (B) Protein quality evaluation by SDS-PAGE. (C) Fractioning of the pooled peptides using high pH reversed-phase fractionation. (D) A representative MS/MS spectrum showing a peptide with quantitative information.

Fig. 3 Numbers of differentially accumulated proteins (DAPs) at 36, 60, 84, and 108 hpi in response to *R. solani* infection.

Fig. 4 Expression patterns of the 174 identified DAPs. Expression ratios were plotted on a heatmap on a Log₂ scale. Red and green colours indicate up- and down-accumulated proteins, respectively. Black represents no significant change in expression.

Fig. 5 RT-PCR analysis on the expression of genes encoding some DAPs. Changes in transcript and protein expression levels at 60 hpi are compared ($P \leq 0.05$). Selected DAPs: allene oxide synthase (CotAD_35839), probable glutathione S-transferase (CotAD_11533), beta-1,3-glucanase (CotAD_57620), V-type proton atpase subunit (CotAD_73136), major pollen allergen Bet v 1-E (CotAD_20887), endochitinase (CotAD_26499), mitochondrial-processing peptidase (CotAD_22970) and basic salivary proline-rich protein 3 (CotAD_44955).

631

632 Fig. 6 Functional classification of the identified DAPs.

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634 Fig. 7 Cellular localization of the DAPs predicted by AgriGo.

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636 Fig. 8 Analysis of the secreted proteins among DAPs. (A) Prediction of secreted
637 proteins by SignalP, TargetIP and SecretomeP. (B) AgriGO confirmation of the
638 secreted proteins ($P \leq 0.001$). (C) Percentage of predicted secreted proteins among
639 different functional groups.

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