Proteomic Analysis of Sugarcane Seedling in Response to Ustilago Scitaminea Infection

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Abstract: Sugarcane smut (*U. scitaminea* Syd.), one of the most severe sugarcane diseases, could lead to considerable yield loss and reduction in cane quality. In order to provide useful information for developing rational strategies to control smut at early stage of disease development, a proteomic approach was used to analyze proteins differently expressed during the early interaction between sugarcane seedling and pathogen. Tissue cultured plantlets of sugarcane cultivar ROC22 with 6-7 leaves were inoculated with teliospores suspension at the apical portion using a syringe. Total proteins were extracted from uninoculated control and smut-inoculated plantlets 3 d post-inoculation, separated by 2-DE system, and the protein spots with differential expression in intensity ($p<0.05$) were analyzed and tentatively identified using MALDI-TOF-TOF/MS/MS. Eighteen protein spots were successfully identified, of which 15 were up-regulated and 3 were down-regulated. Based on their putative biochemical role, the proteins were classified into 7 categories, including defense response, metabolism, photosynthesis, signal transduction, protein processing, cell growth/division and unclassified. These proteins were related to oxidative burst, photorespiration and PAs synthesis and so on. The information obtained in this research provides the first proteomic analysis of the responses of sugarcane seedlings to *U. scitaminea* inoculation at the early stage of pathogen invasion.
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1. Introduction

Sugarcane is an important sugar and biofuel crop with high capability to assimilate carbon. Sugarcane smut is caused by basidiomycotina fungus *Ustilago Scitaminea* Syd., and is common, worldwide problem resulting in sugar content and cane yield diminution seriously. It is sometimes known as culmicolous, which characterizes the outgrowth of fungus from the cane stalk. Smut is transmitted mainly by teliospores in the soil infecting planted setts, and by wind-borne teliospores infecting the standing cane (Santiago et al., 2012). The infection could take place through the open stomata in the leaves, buds or wound in plant tissues. It is hard to control this disease with farm chemicals, because teliospores have strong stress resistance. Smut resistant sugarcane cultivars often begin to display increasing smut susceptibility after years of commercial production because multiple races of the pathogen can overcome host plant resistance. So the development and release of high-quality smut-resistant cultivars remain the most economical and effective strategy for sustainable management of the disease control. However, the classical sugarcane breeding and selection programme takes about 14 years from the time crosses are made to the commercial release of a new cultivar. The complexity and size of the sugarcane genome is a major limitation in genetic improvement including the development of smut-resistant cultivars (D'Hont and Glaszmann, 2001). A better knowledge of the inducible molecular defense mechanism could be useful in designing protective strategies such as over expression of defense genes in sugarcane plants. Researchers used the technology of cDNA-AFLP, DDRT-PCR and cDNA microarray to identify sugarcane genes that respond to the smut pathogen attack. Borras-Hidalgo et al. (2005) identified 62 differentially regulated genes using cDNA-AFLP. Using cDNA microarray, Que et al. (2009) successfully identified 101 differentially expressed ESTs in *E. arundinaceus* induced by *U. scitaminea*. These analysis methods are of high throughput but they are limited to the analysis of genes expression. It also has been shown the level of gene expression does not necessarily correlate with protein level. In recent years, proteomics has been successfully used for the

study of different pathosystems and symbiotic interactions (Kav et al., 2007). Yang et al. (2011) reported that the differentially expressed proteins in grape inoculated *Xylella fastidiosa* were involved in disease development and defense responses. Kim et al. (2004) identified the pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. Campo et al. (2004) used high-resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry to identify proteins that are differentially expressed in response to fungal infection in maize embryos. Geddes et al. (2008) successfully isolated and identified 43 proteins associated with resistance to fusarium head blight from six barley genotypes of varying resistance. Que et al. (2011) obtained 23 differentially expressed proteins from the smut infected sugarcanes after the smut symptoms occurred. Although there are some researches about the interaction between sugarcane and smut, they all analyzed sugarcane materials with obvious smut symptoms. If want to manage plant diseases most effectively, the control measures should be introduced at an early stage of disease development (Singh et al., 2004). But so far it has not been reported the interaction between sugarcane seedling and smut at the early stage of pathogen infection yet.

The objective of this study is to identify differentially expressed proteins in sugarcane seedling during the early interaction with *U. scitaminea* Syd. The information obtained will give an insight on the possible mechanisms involved in sugarcane seedling response to smut infection and aim in the development of rational control strategies for managing smut at an early stage of disease development.

2. Material and Methods

2.1 Materials and Inoculation

Tissue cultured plantlets of sugarcane (*Saccharum* spp.) cultivar ROC22 were grown in pots (35 cm in diameter, 40 cm in depth) containing soil mix (soil/sand/organic fertilizer, 6:2:2(w/w/w)), which got heavy sun exposure all day and sprayed 50% carbendazim, in greenhouse at Guangxi University, Nanning (China). The robust and uniform shoots were selected for inoculation with the teliospores when sugarcane seedlings had 6-7 leaves. The teliospores were collected from the infected plants of sugarcane cultivar ROC22 in the field and diluted into the concentration of 5×10^6 spores/mL with ddH_2O for inoculation. Plantlets were inoculated at the apical portion using a syringe with 100 μL of teliospores suspension (Santiago et al. 2009). The control was carried out by applying only an equivalent volume of ddH_2O.

All the plantlets were gorwn under controlled conditions (28°C, 80% relative humidity), and plants were sampled after 3 days inoculation by cutting the portion around the point of inoculation, and directly put into liquid nitrogen. There was no difference between smut-inoculated and control plantlets in morphology when sampled.

2.2 Protein extraction and quantification

Total proteins were extracted following the modified procedure of phenol extraction method (Yang et al. 2011). Two grams of plant tissue were ground to fine powder in a mortar precooled with liquid nitrogen. The tissue powder was suspended and further ground in 8 mL of cold extraction buffer (30% sucrose, 0.25 M Tris-HCl pH 7.5, 50 mM EDTA-Na_2, 2% SDS, 0.1 M KCl, 2% β-mercaptoethanol, 2% polyvinylpyrrolidone (PVP)) for 10 min. Ten mL of phenol saturated with 0.25 M Tris-HCl pH 8.0 was added and continuously ground until homogenized, then adding 8 mL of cold extraction buffer. The extracts were transferred to a centrifuge tube and stirred for 2 min, then centrifuged at room temperature (12,000×g, 30 min). The supernatant was gently collected and the precipitant was re-extracted twice with equal volume of extraction buffer without PVP, followed by centrifugation (12,000×g, 10 min). The supernatants were combined and proteins were precipitated from the phenolic phase with five volumes of cold methanol contained 100 mM ammonium acetate and 10 mM β-mercaptoethanol for 4 h at −80°C followed by centrifugation at 12,000×g for 10 min at 4°C. The protein pellet was rinsed triple with ice-cold methanol followed by centrifugation at 4°C (12,000×g, 10 min). The protein pellet was air dried under ice and dissolved in rehydration solution (7 M urea, 2% CHAPS, 2 M thiourea, 40 mM dithiothreithol (DTT), 5 mM EDTA-Na_2, 1% IPG buffer pH 4–7) for 5 h at 28°C. The protein solution was centrifuged at 12,000×g for 30 min at 4°C to remove all insoluble particulates. Protein concentration of each sample was determined according to the method described by Bradford using bovine serum albumin (BSA) as the standard.

2.3 2-DE and image analysis

Immobilized pH gradient (IPG) strip (17 cm, pH 4-7, Bio-Rad) was used for isoelectric focusing (IEF). Protein sample was mixed with trip rehydration solution (8 M urea, 4% CHAPS, 65 mM DTT, 0.2% IPG buffer pH 4–7, 0.001% bromophenol blue). The total volume was 350 μL, containing 2000 μg protein. First dimension IEF was carried out in a Protean IEF Cell apparatus (Bio-Rad). The following protocol was used at 20°C: 50 V for 12 h; 250 V for 1 h; 1000 V for 1 h; 3000 V for 1 h; 10000 V for 2 h; 10000 V for 50000 V hours (Vh), and electrical current was maintained as 50 μA/tripe. Prior to the second dimension analysis, strips were equilibrated twice for reduction and alkylation in buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 20 % glycerol, 2 % SDS and 2 %

DTT) and buffer II (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS and 2.5% iodoacetamide) respectively, following Bio-Rad protocol. For the second dimension separation, the strips were placed onto SDS polyacrylamide gels (12.5%) and sealed with 0.5% low melt agarose. The gels were run on a Bio-Rad PROTEAN II system at 50 V for 30 min and then 200 V until the bromophenol blue dye reached the end of the gel. The running temperature was maintained at 20 ^{o}C by water flow from a thermostatic circulator. Proteins were stained with the Commassie blue staining. Three technical replicates per biological sample for both inoculated and mock inoculated period 3 days were obtained and analyzed. Stained gels were scanned with the GS-800 Calibrated Densitometer (Bio-Rad). Spots detection, matching, background subtraction, normalization and statistical analysis were facilitated using PDQuest advanced 2-D analysis software (version 8.0 Bio-Rad) and re-evaluated by visual inspection, focusing on the protein spots which altered expression. The spots that showed at least a 2-fold-change, with statistical significance at a level of $p < 0.05$ based on Student's t-tests, were selected for the further research.

2.4 Protein identification

Differentially expressed protein spots were manually excised and washed three times with Milli-Q water. The relevant spots were destained with 100 μL of bleaching solution (50% acetonitrile (ACN), 40 mM NH_4HCO_3) for 2 min, then added with 400 μL Milli-Q water to stop the reaction. After two washing with 200 μL of 40 mM NH_4HCO_3 for 2 min twice and with 100 μL of ACN for 10 min once, the gel pieces were dried under vacuum concentrator for 10 min. The protein digestion in gel was done with coverage solution (40 mM NH_4HCO_3, 10% ACN) containing 10 ng/μL of trypsin for 45 min in ice-bath, followed by adding 40 μL coverage solution and incubating for 14 h at 37^{o}C. The supernatant of protein digestion was transferred to a new EP tube and the gel pellet was washed twice for 15 min with extraction buffer (50% ACN, 0.1% Trifluoroacetic Acid (TFA)). The peptide solutions were pooled together and dried for 3 h under concentrated drain of vacuum concentrator.

The eluted peptides were re-suspended in 0.1% TFA. Samples were spotted on a 384 apertures MALDI target plate with air drying. Then both single MS and MS/MS analysis of peptides were performed by 4800 MALDI TOF/TOF plus analyzer (Applied Biosystems Sciex, USA). The information of MS was first obtained using Reflector Mode with the 4000 laser intensity. MS/MS spectra were collected in 2 kV Positive Mode with fragments generated by collision-induced dissociation (CID). The scope of Peptide Mass Fingerprinting (PMF) was from 800 to 4000 Da.

Raw data were searched by GPS Explorer (Applied Biosystems, USA) using Mascot as a search engine and NCBInr protein databases. The search parameters were trypsin, product ions tolerances ±0.3 Da, modifications allowed for alkylation modification and phosphorylation modification, and homology identification was retained with a probability set at 95 %.

The function characterization of the proteins that were successfully identified was determined by the Gene Ontology.

3. Results

3.1 Smut pathogen detection by PCR

High quality genomic DNAs of both smut-inoculated and control plantlets were obtained using the SDS method (Figure 1a). The plants inoculated with teliospores showed a positive PCR reaction for the *b*E mating-type gene of *Ustilago scitaminea* although they did not have visible smut symptoms, while the control plantlets yielded a negative reaction (Figure 1b). The result revealed smut pathogen had begun to colonize in the smut-inoculated sugarcane seedlings.

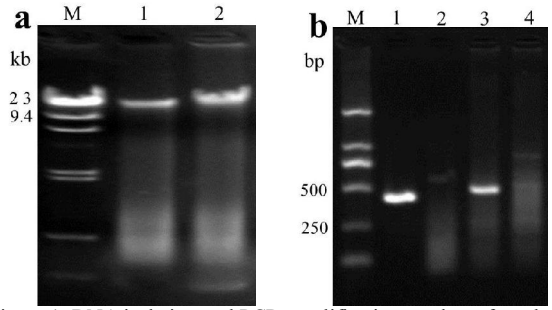

Figure 1. DNA isolation and PCR amplification products from both smut-inoculated and control plantlets. a) DNA isolation: M 23kb ladder; 1 genomic DNA of control plantlets; 2 genomic DNA of smut-inoculated plantlets; b) PCR amplification products: M 2000 DL ladder; 1 positive control, *U. scitaminea* DNA; 2 negtive control, without plantet DNA; 3 smut-inoculated plantlets; 4 control plantlets

3.2 Differentially Expressed Proteins

Differentially expressed proteins were observed in smut-inoculated and control plantlets (Figure 2). It has been found that over 90% of the protein spots appeared at *pI* 4~7 from IPG strip (pH 3-10) and SDS-PADE gels. Therefore, to obtain the optimal resolution of the differentially expressed protein profiles, IPG strips with narrow pH range (pH 4-7) were applied in the present study. More than 600 protein spots were detected on each 2-DE gel (Figure 2). The spots that changed consistently in all gels were analyzed by MS. A total of thirty-four differentially expressed protein spots were found significantly ($p < 0.05$) change in intensity after 3 d inoculation, as compared to the control. Twenty-six of the differential proteins were up-regulated and six were down-regulated.

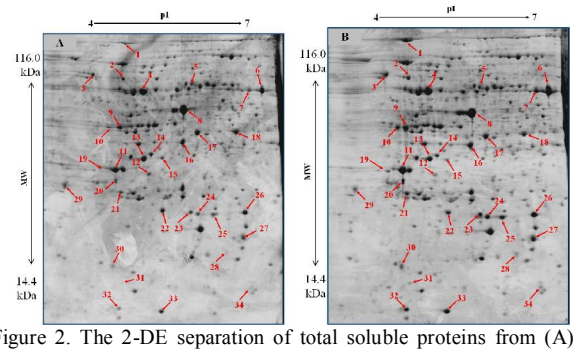

Figure 2. The 2-DE separation of total soluble proteins from (A) sugarcane inoculated with ddH_2O (as control) and (B) sugarcane inoculated with smut teliospores (after 3 d inoculation). The profiles were obtained using pH 4-7 in the first dimension and 12.5% SDS-PAGE in the second. Proteins labeled with arrows were excised.

3.3 Protein Identification

Protein identification was performed with mass spectrometry MALDI-TOF/TOF and eighteen protein spots were successfully identified (Table 1). The 18 protein spots represent 17 different proteins, as spots 6 and 7 represent the same protein but displayed different *pI* values because of isoforms or undiscovered post-translational modifications (Coumans et al., 2009). Seventeen proteins (94.4%) had known functions or sequences similar to those of known proteins, whereas 1 protein (5.6%) was novel and had not been assigned any functions. Fifteen protein spots exhibited a higher abundance in the smut-inoculated plantlets, while 3 protein spots showed higher abundance in control as compared with smut-inoculated treatment (Figure 3). Based on their putative biochemical role, the proteins were classified into 7 categories as follows: defense response (spot 3, 12, 21, 27 and 30), metabolism (spot 8, 9, 10, and 18), photosynthesis (spot 6, 11, and 32), signal transduction (spot 24), protein processing (spot 2 and 29), cell growth/division (spot 19), and unclassified (spot 33) (Figure 4). It should be noted that any given protein may be responsible for multiple biological processes and, therefore, could be classified into more than one biological process.

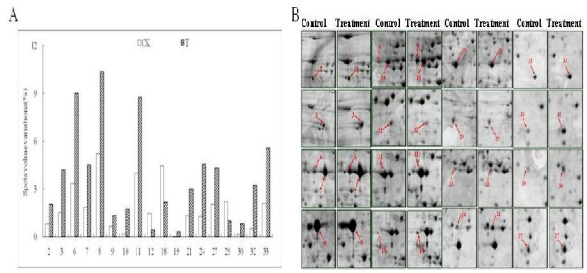

Figure. 3 Quantification of differentially expressed proteins. A) The volume variation of the eighteen identified protein spots. The horizontal axis represents the spots according to the spots labeled in 2-DE map. B) Enlarged images of eighteen identified proteins.

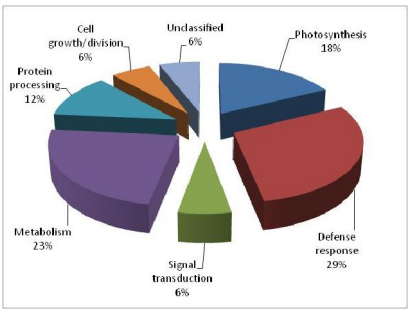

Figure. 4 Functional classification of the 18 identified protein spots. The relative percentages of proteins in each category are shown.

4. Discussion

Anatomical, physiological and biochemical defense in sugarcane responding to *Ustilago Scitaminea* Syd. infection have been described previously (Singh et al., 2004; Piñon et al., 1999; De Armas et al., 2007). An inventory of candidate genes preferentially expressed during sugarcane-smut interaction were identified at molecular level (LaO et al., 2008). However, it has not been reported that sugarcane in response to smut inoculation in proteomic level at the early stage of pathogen penetration. Plant diseases could be managed most effectively if the control measures are introduced at the early stage of disease development. Reliance on symptoms often is not adequate since the disease is well established before symptoms first appear (Singh et al., 2004). In the present study, though there was no difference between smut-inoculated and control plantlets in external morphology, a 459 bp fragment of smut DNA was detected by PCR in smut inoculation seedling after 3 d. The sequence of the fragment was identical to the *b* East mating-type gene in GenBank. This result confirmed the plant material was eligible for the proteomic analysis since smut pathogen had begun to colonize in the smut-inoculated plantlets. The differentially expressed protein spots were detected from both smut inoculated and control plantlets by 2-DE gels. The expression level for almost all proteins was not altered and more proteins were upregulated, at the early stages of infection, than down-regulated. Protein identification was an analytical challenge because of lack of the genome sequence information for sugarcane. Tandem mass spectrometry (MS/MS) has been reported to be the most successful technique to identify proteins correctly from organisms that DNA sequence is unknown (Jouber et al., 2001). In the present study, eighteen protein spots with significant change in intensity were successfully identified using MALDI-TOF/TOF analysis (Table 1). These proteins could be classified into 7 categories according to their putative biological functions (Figure 4): defense response, metabolism photosynthesis, unclassified, cell

growth/division, protein processing and signal transduction. Their functional significance was discussed below.

Table 1 The list of eighteen identified proteins

Spot No.[a)]	NCBI Accession No.	Protein	Organism	Protein sequence		Mr (kDa)/pI
				score	coverage(%)	
2	gi|15231255	chaperonin	Arabidopsis thaliana	345	22	63.3/5.6
3	gi|145388994	chloroplast heat shock protein 70	Pennisetum glaucum	654	38	73.0/5.23
6	gi|164565025	ribulose-1,5-bisphosphate carboxylase /oxygenase large subunit	Sorghum bicolor	634	42	49.4/6.44
7	gi|164565025	ribulose-1,5-bisphosphate carboxylase /oxygenase large subunit	Sorghum bicolor	522	38	49.4/6.44
8	gi|100801600	S-adenosylmethionine synthase	Oryza rufipogon	442	37	42.5/5.74
9	gi|195621752	pyruvate dehydrogenase E1 component subunit beta	Zea mays	166	28	39.9/5.46
10	gi|4582787	adenosine kinase	Zea mays	146	27	36.0/5.23
11	gi|195619530	oxygen-evolving enhancer protein 1	Zea mays	484	60	34.5/5.59
12	gi|226530526	isoflavone reductase IRL	Zea mays	343	27	32.9/5.52
18	gi|195628708	malate dehydrogenase	Zea mays	334	58	35.4/7.63
19	gi|46805452	putative inorganic pyrophosphatase	Oryza sativaJaponica Group	225	58	31.8/5.8
21	gi|162457709	ascorbate peroxidase 2	Zea mays	346	57	27.3/5.28
24	gi|15667623	drought inducible 22 kD protein	Saccharum officinarum	150	45	15.9/5.78
27	gi|226493193	abscisic stress ripening protein	Zea mays	176	63	14.9/6.15
29	gi|148927396	cysteine proteinase	Elaeis guineensis	116	32	39.5/6.03
30	gi|242058321	thioredoxin-dependent peroxidase	Sorghum bicolor	580	78	17.4/5.01
32	gi|164698715	chloroplast ribulose-1,5-bisphosphate carboxylase /oxygenase small subunit	Miscanthus x giganteus	329	45	14.2/5.14
33	gi|242070055	hypothetical protein SORBIDRAFT	Sorghum bicolor	429	62	19.0/8.77

[a)] Spot numbers correspond to 2-D gels in Fig. 1

4.1 Defense response proteins

Defense response proteins were identified as the largest differentially expressed protein group in this research which were Hsp70s, isoflavone reductase, abscisic stress ripening protein, ascorbate peroxidase and thioredoxin-dependent peroxidase. Heat shock proteins (Hsps) are a conserved and ubiquitous protein family that plays important roles in maintaining cellular functions when plants are subject to a variety of biotic and abiotic stresses (Vierling 1991). Hsp70s distribute in the nucleus, cytoplasm, endoplasmic reticulum, mitochondria, and chloroplasts and play essential roles in repairing misfolded proteins, transporting nascent proteins across membranes into organelles, folding newly translated proteins, and helping target damaged proteins for degradation (Brandalise et al., 2009). Previous studies found that Hsp70s were upregulated under stress conditions. They could participate in the refolding of denatured proteins, maintaining cell homeostasis and protecting organisms from damage (Shoji et al., 2002). In this research, Hsp70 (spot 3) was up-regulated in the smut inoculated plantlets and its expression was related to sugarcane defense response to smut invasion although its role in disease resistance needs further defined.

Protein spot 12 was identified as isoflavone reductase, an NAD(P)H-dependent reductase that involves in the biosynthesis of plant defense metabolites such as lignans and isoflavonoids (Brandalise et al., 2009). Several isoflavone reductase-like genes have been reported to be regulated or induced in response to biotic or abiotic stresses in plants, such as *Zma mays ZmIRL* (Petrucco et al., 1996), *Oryza saliva OsIRL1* (Kim et al., 2003) and *Ginkgo biloba GbIRL1* (Hua et al., 2013), but their function in plants is known very little. The result of the present study suggests the infection of smut pathogen destroyed the balance of IRLs metabolism. We speculated IRLs actively participated in response to smut although their expression was suppressed by colonization of *U. Scitaminea* Syd.

Protein spot 27 was identified as abscisic stress ripening protein (ASR). ASRs are highly hydrophilic and intrinsically unstructured proteins with molecular masses generally less than 18 kDa (Wang et al., 2013). They are presumed to respond to different abiotic stress factors, including salt (Jha et al., 2012), drought (Liu et al., 2013), and cold (Hsu et al., 2011), and also act as part of a transcription-regulating

complex involved in plant development processes such as fruit ripening (Igamberdiev and Roussel 2012), pollen maturation (Whitney et al., 1999) and glucose metabolism (Saumonneau et al., 2012). Acosta-Muniz et al. (2012) reported that two ASRPs were up-regulated in response to infection of the *oomycete Phytophthora cinnamomi*. Liu et al. (2010) also found plantain *Asr* gene *MpAsr* might be integral in response to *Fusarium oxysporum f. sp. Cubense* attack. In the present study, we also found sugarcane ASR was responsive to pathogen *U. Scitaminea* Syd.

Peroxidases played significant roles in defense mechanisms and could be induced by several stresses (Passardi et al., 2005). In this research, protein spots no. 21 and no. 30 were identified as ascorbate peroxidase and thioredoxin-dependent peroxidase, respectively. It has been demonstrated that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl free radical (OH) and superoxide anion (O^{2-}) were strikingly increased in plants in the early resistance response to plant pathogen attack. Hückelhoven et al. (2001) reported that after inoculation with *Blumeria graminis* f. sp. *hordei*, the protein of ascorbate peroxidase was up-regulated in barley leaf tissues. Do et al. (2003) found pepper ascorbate peroxidase and thioredoxin peroxidase genes might function as regulators of H_2O_2 level and total peroxidase activity in the oxidative burst during the hypersensitive response to *Xanthomonas campestris pv. vesicatoria* in *Capsicum annuum*. We speculated that ascorbate peroxidase and thioredoxin-dependent peroxidase could scavenge the excessive ROS and protect the sugarcane plants from smut pathogen infection.

4.2 Metabolism related proteins

S-adenosylmethionine (SAM) synthase (spot 8) catalyzes the SAM biosynthesis using methionine (Met) and ATP as substrates. Almost all organisms are able to synthesise SAM de novo. SAM is the main methyl donor and plays a central role in most methylation reactions, such as methylation of nucleic acids, proteins, lipids, and polysaccharides, and acts as a precursor of poly-amines (PAs). SAMs play an important role in tolerance to salt stress in *Lycoris radiate* (Li et al., 2013). The accumulation of polyamine transgenic tomato could increase tolerance against *Fusarium oxysporum* and *Alternaria solani*, and tolerance to multiple abiotic stresses such as drought, salinity, cold and high temperature (Hazarika and Rajam 2011). We speculated the up-regulated SAMs could promote the accumulation of PAs to respond the invading of smut pathogen.

Pyruvate dehydrogenase E1 (spot 9) is a α2β2 heterodimer, a component of pyruvate dehydrogenase complex (PDC) which locates at the intersection of several metabolic pathways and plays an important role for regulation of carbon metabolism (Johnston et al., 2000). PDC plays a critical role in response of plants to low O_2 stress (Agarwal et al., 2007). Expression of PDC E1 subunit beta was significantly higher in the smut-inoculated plantlets than in the control plants, suggesting this protein involved in reaction to smut pathogen. However, the molecular mechanism is not known.

Adenosine kinase (ADK, spot 10) catalyzes the transfer of gamma-phosphate from adenosine triphosphate (ATP) to adenosine (ADO) leading to formation of adenosine monophosphate (AMP) and is also important for sustaining S-adenosylmethionine dependent methyltransferase activity and cytokinin homeostasis *in vivo* (Baliji et al., 2010). Wang et al. (2003) found ADK is targeted by viral pathogens and might be a part of host defense responses. Cytokinin is involved in cellular division, chloroplast biogenesis, programmed senescence and pathogen resistance (Novak et al., 2013). The phosphorylation of cytokinin ribosides via ADK could be a way by which plants modulate intracellular CKs (Baliji et al., 2010). The upregulated adenosine kinase in smut inoculated sugarcane was speculated to regulate the cytokinin levels to defend against smut pathogen attack.

The malate dehydrogenase (MDH, spot 18) is TCA cycle enzyme involved in several cellular processes including nutrient uptake, plant development and oxidative stress (Menckhoff et al., 2013). MDH also plays an significant role in response to pathogen attack. Subramanian et al. (2005) reported the expression of MDH in resistant *Brassica carinata* was elevated upon pathogen challenge. Vincent et al. (2012) also found the MDH of wheat was up-regulated in response to SnToxA effector protein, which was secreted by the fungal pathogen *Stagonospora nodorum* during infection. In the present study, MDH was down-regulated in sugarcane seedlings in response to *U. Scitaminea* Syd., which might suggest that the penetration of smut pathogen had disrupted carbon metabolism in sugarcane.

4.3 Photosynthesis related proteins

Rubisco is a key enzyme in photosynthesis and photorespiration. The Rubisco holoenzyme is a heteropolymer consisting of eight large subunits (RbCLs) and eight small subunits (RbCSs). It has been reported that the expression or activity of Rubisco was affected by many biotic and abiotic factors including drought and climatic conditions (Carmo-Silva et al., 2010). Zhao et al. (2013) found *Nicotiana benthamiana* Rubisco small subunit also played a vital role in tobamovirus movement and plant antiviral defense. Photorespiration is the major source of intracellular hydrogen peroxide (H_2O_2) in

plants and peroxisomal metabolism plays multi-faceted roles in pathogen defence (Sorhagen et al., 2013). Qiang et al. (2007) also found photorespiration played an important role in *Rosa roxburghii tratt* resistance to powdery mildew infection, and as a member of the path Rubisco was up-regulated significantly. In the present study, two Rubisco large subunits (spot 6 and spot 7) and one Rubisco small subunit (spot 32) were all upregulated after smut pathogen inoculation. The increase in Rubisco activity may enhance photorespiration and the production of ROS, which is an essential component of the hypersensitive defence response. The accumulation of these toxic compounds impairs cell death containment and counteracts the effectiveness of the plant defenses to restrict pathogen infection (Moreno et al., 2005)

Oxygen-evolving enhancer proteins (OEE) are nuclear-encoded chloroplast proteins, and peripherally bound to photosystem II (PSII) on the luminal side of the thylakoid membrane. OEE1 is the most important protein for oxygen evolution and PSII stability as OEE plays an important role in several stresses including selenium (Ning et al., 2013), virus (Diaz-Vivancos et al., 2008) and drought (Gazanchian et al., 2007). The expression of OEE1 (spot 11) increased in the present study indicated that smut pathogen affected the photosynthesis of sugarcane.

4.4 Signal transduction related protein

Drought inducible 22 kD protein (spot 24) is a member of ABA stress and ripening-inducible (ASR) protein family. It has been reported that the functions of Sodip22, a drought-inducible gene which encodes a 22 kD protein, is to adapt to drought stress in the bundle sheath cell of sugarcane, and involves in the signaling pathway induction mediated by ABA (Sugiharto et al., 2002). Desclos et al. (2008) found the drought inducible 22 kD protein possesses dual functions (water-soluble chlorophyll-binding protein and trypsin inhibitor) that lead to the protection of younger tissues from adverse conditions by maintaining metabolism (i.g., protein integrity and photosynthesis). ASR proteins are typically upregulated by a wide range of factors, including drought, cold, salt, abscisic acid, and injury. It was reported *MpAsr* expression was upregulated in *Fusarium oxysporum* f. sp. *cubense* infected plantain (Liu et al., 2010). The drought inducible 22 kD protein might act as a signal transduction protein in response to smut pathogen attack in sugarcane seedling.

4.5 Cell growth/division related protein

Protein spot 19 was identified as inorganic pyrophosphatase and the expression level was increased significantly after smut pathogen inoculation. Inorganic pyrophosphatase catalyzes the hydrolysis of pyrophosphate (PPi). It was reported that AVP1, the H^+-pyrophosphatase, was involved in the development of shoots, roots, and flowers and fertility in *Arabidopsis thaliana* (Li et al., 2005). Overexpression of vacuolar H^+-PPases was reported to enhance abiotic stress tolerance (specifically, high salinity and drought stressors), likely associated with increased photosynthesis, proline production, relative water content, and fiber yield in transgenic plants, such as Arabidopsis and cotton (Zhang et al., 2011). Although the molecular mechanism of inorganic pyrophosphatase mediated smut pathogen resistance is not clear, we speculated it might play an important role in plant-pathogen interactions.

4.6 Protein processing related proteins

Cysteine proteinase played a role in many processes of plants, including protein turnover, proprotein processing, programmed cell death (PCD) and senescence (Salas et al., 2008). In addition, they are involved in signaling pathways and in the response to biotic and abiotic stresses (Hao et al., 2006; Fu et al., 2007). Protease inhibitors in plants have been involved in defense mechanisms against pests and pathogens (Popovic et al., 2012; Martinez et al., 2003). Solomon et al. (1999) found the activity poised between the cysteine proteases and the cysteine protease inhibitors could regulate plant PCD. In the present study, spot 29 was identified as cysteine proteinase and down-regulated post-inoculation. We concluded sugarcane seedling could mediate the balance of cysteine proteinase and cysteine proteinase inhibitor to defense against the smut pathogen invasion at the early stage.

Conclusion

To the best of our knowledge, the present research firstly reveals the responses of sugarcane seedlings to smut inoculation at the early stage of pathogen invasion with proteomics. Although the plants showed no difference in morphology between control and smut pathogen inoculation treatment, 18 differentially expressed proteins were successfully identified and their function related with defense response, metabolism, photosynthesis, signal transduction, protein processing and cell growth/division according to bioinformatics analysis. The expression of 15 proteins were induced and 3 proteins were suppressed by the smut pathogen invasion. Compared with the results of previous research who used sugarcane with obvious smut symptom as material, it elucidates the response mechanism of sugarcane to *U. scitaminea* syd. is significant difference at different developmental stages. The present results indicate that the protein regulatory network is complicated during the

response of sugarcane seedlings to smut pathogen invasion at the early stage. The molecular mechanism of sugarcane seedling resistance to *U. scitaminea* Syd. was according to the gene for gene hypothesis. Smut pathogen attack could lead to the reactive oxygen burst in sugarcane seedlings at the early stage, which induced the expressions of related proteins. Then the cells around the pathogen infection sites might begin to programmed cell death to inhibit the growth of smut pathogens. Sugarcane seedlings also may resist the pathogen infection through changing the content of PAs, inhibitors, fiber, ASR, IRLs, and so on. We also deduced that photorespiration might play an important role in sugarcane seedling resistance to smut pathogen at the early stage of penetration. In this study, some interesting proteins were not unambiguously identified because of lacking full sugarcane genomic sequences. Further work is need to focus on functional characterization of these candidate genes in order to develop better control strategies for *U. scitaminea* Syd. infection.

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