

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.711.079>

Proteomic Analysis of a Compatible Interaction between Sorghum Downy Mildew Pathogen (*Peronosclerospora sorghi*) and Maize (*Zea mays* L.)

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ABSTRACT

Sorghum downy mildew (SDM) caused by the oomycete, *Peronosclerospora sorghi*, is one of the destructive diseases that afflict maize. *P. sorghi* infects a susceptible host, hindering its growth and altering its morphology. To understand the molecular basis of the compatibility interaction between *P. sorghi* and maize, a comparative proteomic approach (2D-PAGE) was employed between the mock-inoculated (control) and SDM-inoculated leaves in the susceptible genotypes of maize (UMI79 and CM500). Seventeen spots showed a significant difference in the abundance of proteins in control and inoculated samples were further analyzed with MALDI-TOF/MS. The resulting peptide mass fingerprint was subjected to MASCOT analysis and it was found that most were related to stress that includes lipoxygenase and DEAD-box ATP-dependent RNA helicases, microtubule-associated protein and probable protein disulphide isomerase. Additionally, proteins involved in the cell cycle/endoreplication such as DNA topoisomerase 6 subunit A and retinoblastoma-related proteins were differentially expressed during infection. The possible roles of these proteins in response to *P. sorghi* infection in maize are discussed.

Keywords

Maize, Sorghum downy mildew, 2D-PAGE, MALDI-TOF, Lipoxygenase, Cell cycle

Article Info

Accepted:
07 October 2018
Available Online:
10 November 2018

Introduction

Maize is the third most important crop worldwide after rice and wheat (Hoisington

and Melchinger 2004). It is affected by several pests and diseases. Among the diseases, sorghum downy mildew (SDM), caused by *Peronosclerospora sorghi* [(Weston and

Uppal) C. G. Shaw], an obligate oomycete, is one of the destructive diseases. Downy mildew has had a cosmopolitan distribution from 1960 in all maize growing areas of Indonesia, the Philippines, Thailand, Nepal, and India (Gerpacio and Pingali, 2007). In India, SDM is predominant in the southern states (Karnataka, Tamil Nadu and Andhra Pradesh), causing yield losses of 30% and higher (Yen *et al.*, 2004).

The SDM pathogen, *P. sorghi*, infects maize through oospores (sexual phase) and/or conidia (asexual phase). The primary inoculum, the oospore, enters through the roots and causes systemic infection, whereas the conidia act as the secondary source of inoculum. Conidia enter through leaf lamina at the onset of conducive weather conditions and cause local infection. It may also lead to systemic infection, if seedlings less than a week old are affected.

SDM infection leads to the development of characteristic symptoms, including the half-leaf stage in which half of the leaf lamina from the base becomes chlorotic and extends further at later stages of infection and finally covers the whole lamina (Safeulla, 1976; Williams, 1984). The infected leaf becomes narrow and erect. The tassel is replaced by a vegetative structure called 'crazy top' and pollen production is hampered. SDM infection at early developmental stages leads to shortening of the internode and a stunted appearance of the plant (Ajala *et al.*, 2003; Williams, 1984). Thus, SDM infection alters the growth and morphology of maize during compatible interaction. These alterations may likely be accounted for by the changes in plant proteome level. With the aid of proteomics, identifying and quantifying the proteins involved in plant - pathogen interaction has become easier, which may help to elucidate their relative importance in the process of disease development (Amey *et al.*, 2008). 2D-

PAGE and mass spectroscopy are powerful proteomic tools that have gained importance in plant pathology to characterize proteins from either the phytopathogen or the host plant or both (Kav *et al.*, 2007).

Understanding plant disease resistance mechanisms has been emphasized over the decades and numerous studies have been devoted to examine the differential expression of proteins involved in an incompatible interaction, *i.e.*, to evaluate resistant genotype response, but there are few reports on compatible interaction. However, studies on compatible interaction will ultimately help us to improve our understanding regarding plant disease susceptibility. The few proteomics studies that focused on compatible phyto - pathogen interaction includes *Pisum sativum-Peronospora viciae* (Amey *et al.*, 2008), *Triticum aestivum-Fusarium graminearum* (Zhou *et al.*, 2006), *Vitis vinifera-Plasmopara viticola* (Milli *et al.*, 2012), *Vigna mungo-Mungbean Yellow Mosaic virus* (Cayalvizhi *et al.*, 2015; Kundu *et al.*, 2013), *Fagus sylvatica-Phytophthora citricola* (Valcu *et al.*, 2009), and *Zea Mays L. - Rice Black-Streaked Dwarf Virus* (Li *et al.*, 2011).

An obligate biotrophic pathogen infects susceptible plants, establishes itself in the plant structures and makes it compatible to harvest nutrients without killing the host plant. Thus, understanding the molecular mechanism of compatible interaction would help elucidate novel protection strategies by interfering with the compatibility (Hok *et al.*, 2010).

Hence, a 2D-PAGE strategy was employed to understand differential protein abundance in control and SDM-inoculated samples of two susceptible genotypes of maize (UMI79 and CM500) and their relative importance in disease susceptibility, possible involvement in morphological and developmental changes in the host.

Materials and Methods

Plant materials

Two test genotypes were used in this study: UMI79 (origin - Pioneer 102), an elite well-adapted superior maize inbred, but it is susceptible to SDM infection; and CM500 (origin – Antigua Gr 1), an open pollinated variety that is a national check and spreader for all types of downy mildew studies. Though both genotypes are highly susceptible to SDM, the levels of susceptibility are different.

One study (Nallathambi *et al.*, 2010) reported that UMI79 was affected by SDM with 68% disease incidence and CM500 with 100% disease incidence. Another group in 2010 also reported that maximum disease incidence among 100 maize inbreds was registered in CM500 (Premalatha *et al.*, 2012).

Inoculation of maize seedling with a conidial suspension of *P. sorghi*

Seeds of SDM-susceptible genotypes (UMI79 and CM500) were sown in pots in a glasshouse at a controlled temperature (20°C) and humidity (>90). Nine days after sowing, SDM-infected maize leaves showing visible conidial growth were collected from infected fields in the evening. Leaves were wiped with cotton to remove mature conidiophores, and then placed overnight for sporulation in moist gunny bags under controlled temperature (20–22°C) and humidity (>90%).

The next morning, at around 3.00 AM, fresh conidia were harvested from leaves in chilled water using a brush, and diluted to the desired 6×10^5 conidia per mL concentration. The resulting conidial suspension of *P. sorghi* was sprayed over ten-day-old seedlings using the “seedling spray inoculation” technique (Craig *et al.*, 1977), whereas the control was mock inoculated with sterile distilled water.

Phenotypic observation of SDM

The inoculated plants were observed for the occurrence of morphological and phenotypic changes due to SDM. As the reduction in total chlorophyll content is a common characteristic of plant fungal disease, the total chlorophyll content was recorded using a portable chlorophyll meter (Minolta SPAD-502 Plus; Konica Minolta Inc., Europe) at 20 dpi. Leaf narrowing is another symptom of SDM, so width of the mock/SDM inoculated leaves was measured and compared.

To understand the level of susceptibility in two susceptible genotypes, at 20 dpi, the leaf samples inoculated with SDM (20 dpi) were cut into 5 mm² pieces, and both the adaxial and abaxial surfaces were mounted on stubs and observed under an ICON-Quanta 200 Mark II Environmental Scanning Electron Microscope (ESEM) in low vacuum mode.

Sample collection and protein extraction

Three biological replicates of leaf tissue were collected at 20 dpi from each control and SDM-inoculated plant and stored in liquid nitrogen at –80°C until protein extraction. Frozen leaf tissues were finely powdered with liquid nitrogen in a pre-chilled mortar and pestle, suspended in 10 mL trichloro acetic acid (TCA) extraction buffer (10% (w/v) TCA in 10% (v/v) in acetone and 0.07% (w/v) dithiothreitol (DTT)), then the samples were incubated at –20°C for 1 h. Following incubation, the extract was centrifuged at 12,000 rpm for 20 min. The supernatant was discarded and the pellet was washed with ice-cold acetone containing (w/v) 0.07% DTT. Washing was repeated until the pellet was devoid of chlorophyll. Finally, the pellet was lyophilized for 2 h and stored at –80°C. About 15 mg of lyophilized powder was re-suspended in 200 µL of lysis buffer (9M urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1% (w/v)

ampholyte (pH 3–10), 35 mM Tris base) The samples were incubated at 37 °C for 1 h with intermittent vortexing followed by centrifugation. The supernatant served as the protein extract and the concentration of protein was determined using the Bradford method (Bradford 1976).

Two-dimensional electrophoresis (2-DE)

For the first dimension, 150 µg of proteins was rehydrated using 17 cm immobilized linear pH gradient (IPG) strips (Bio-Rad laboratories, USA), pH 4–7, in a rehydration buffer (8M urea, 2% (w/v) CHAPS, 0.07% (w/v) DTT and 50 µL of IPG buffer). Isoelectric focusing was performed at 20°C with a GE Healthcare Life-sciences Multiphor II kit. Electrophoresis was carried out at 500 V for 30 min, 1000 V for 30 min and 3000 V for 10 h. Prior to the second dimension, the IPG strips were equilibrated twice for 15 min each in 20 mL/strip of equilibration solution containing 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS, and 50 mM Tris-HCL. DTT (50 mM) was added to the first equilibration solution and 4% (w/v) iodoacetamide was added to the second. For the second dimension, equilibrated IPG strips were placed on top of vertical sodium dodecyl sulphate-polyacrylamide gels (12%) (Jagadish *et al.*, 2010). Electrophoresis was performed at 4°C in a 1 × SDS buffer at 30 mA/gel constant current. 2-DE gels were stained with silver nitrate (Blum *et al.*, 1987). 2-DE silver-stained gels were scanned for image visualization and for densitometrical analysis by using the ImageMaster 2D Platinum version 7, Scanner III (GE Healthcare, USA). Total number of spots, matched spots and differential expressed spots were counted separately to each replication and analysis of variance was done to find any significant difference. The abundance ratio (AR) of spots was calculated by the percentage of volume of spot under inoculation to the ratio of the percentage of

volume of spot under control. The criterion for differential expression of protein was based on the AR: when AR > 1, then the spot is said to be upregulated, when AR < 1 then the spot is said to be downregulated (Jagadish *et al.*, 2010).

Peptide mass fingerprinting (PMF)

Protein spots of different intensities and regions of the 2-DE gel were selected for PMF analysis. Samples were excised manually using a scalpel from a silver-stained 2-DE gel, dehydrated in 100% acetonitrile (ACN), dried by vacuum centrifugation, and subjected to trypsin digestion overnight at 37°C. Samples were dehydrated and mixed with an equal volume of matrix solution (α -acyano- 4-hydroxycinnamic acid, HCCA) and then subjected to MALDI TOF/MS (Shevchenko *et al.*, 1996) (matrix-assisted laser desorption/ionization time-of-flight/mass spectroscopy) on a Voyager-DE STR mass spectrometer (Applied Biosystems, Franklin Lakes, NJ, USA).

Identification of putative proteins by MASCOT analysis

Proteins were identified by correlative searching PMF against well-curated swissprot databases in *Oryza sativa* L. taxonomy, using the MASCOT program (<http://www.matrixscience.com>) (Cottrell and London 1999). The following parameters were applied during the MASCOT search: peptide fragment tolerance of 100 ppm, maximum number of missed trypsin cleavages one, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification were allowed. Decoy search was done automatically by Mascot on randomized database of equal composition and size. The peptide mixtures that produced the highest statistically significant ($P < 0.05$) match scores and accounted for the majority

of the peaks present in the mass spectra, were considered to be positively identified proteins. The identified proteins were categorized based on their probable biological function and discussed.

Results and Discussion

Phenotypic observation of SDM symptoms in the control and SDM-inoculated leaves

At 20 dpi, it was observed that the mock-inoculated controls of both genotypes were symptomless, whereas the SDM-inoculated leaves showed typical symptoms of chlorosis, downy growth and narrowing of leaves (Fig. 1). Depletion of chlorophyll was observed in the SDM-inoculated leaves over the control. Most of the SDM-inoculated leaves of the UMI79 genotype showed the half leaf-stage symptom, whereas at the same timeframe (20 dpi), the CM500 inoculated leaves showed full leaf chlorosis. The mean decrease in chlorophyll content was 53% for UMI79 and 61% for CM500 over its respective control (Table 1). SDM infection also led to narrowing of the leaves. Leaf width decreased by 23 and 30 % over control in UMI79 and CM500, respectively. The downy growth was evident on the adaxial and abaxial surfaces of the leaf.

SDM-inoculated leaf sample analysis with ESEM

ESEM was adapted to analyze the abaxial and adaxial surfaces of SDM-inoculated leaf samples at 20 dpi. The conidiophore was consistently observed to be exiting through the stomata from both surfaces of the leaf. The progression of the emergence of conidiophore was found to be slower in the UMI79 genotype than CM500 (Fig. 2). At 20 dpi, the unbranched conidiophores found emerging from the stomata was observed in the UMI79 genotype, while at the same timeframe,

matured conidiophores bearing conidia were observed on both surfaces of CM500. CM500 had a greater amount of conidiophores on the leaf surface. Moreover, two or more conidiophores emerging from single stomata was often seen in CM500.

Effect of SDM on host protein expression

Proteomics is a powerful tool for studying plant response to different stress factors, including plant - pathogen interactions, plant herbivore interactions and wounding (Butt and Lo 2007). Of the methods employed in recent proteomics studies, 2-DE provides reasonably good resolution and coverage of the proteome. 2-DE gel analysis was executed for the control and SDM-inoculated samples. ImageMaster 2D Platinum version 7 software detected an average of 1066 ± 37 and 1211 ± 22 spots in silver stained gels of control and inoculated samples of UMI79. Whereas, 676 ± 3 and 915 ± 26 spots were evident in the gels of control and inoculated samples of CM500. Approximately 330 spots were matched among all the gels. Four upregulated, nine downregulated and two newly induced proteins spots after infection in both genotypes along with the one newly induced spot and one downregulated spot exclusively in the UMI79 were chosen for analysis (Fig. 3).

Figure 4 represents the seventeen protein spots that showed variation in expression after infection. Of these 17 protein spots, the abundance of ten proteins was low (depicted as D1–D10), four were high (depicted as U1–U4) and three protein spots were present only in the *P. sorghi* inoculated samples (depicted as N1–N3). Thus, the 17 spots used for trypsin digestion and subjected to MALDI TOF/MS, and further identified and characterized using the MASCOT program, are shown in Table 2. Of these 17 protein spots, the protein spots corresponding to D1 and D2 were found to be phosphatase and pentatricopeptide repeat-

containing protein OTP51, respectively. D3, D4 and N3 were acyl-[acyl-carrier-protein] desaturase with different isoforms. D5 and D6 were found to be DNA topoisomerase 6 subunit A and microtubule-associated protein, respectively. DEAD-box ATP-dependent RNA helicase was represented by more than one protein spot. The protein spots D7, D9 and U3 corresponded to DEAD-box ATP-dependent RNA helicase 50, 52B and 16, respectively. D8 and N2 were found to be lipoxygenase 7 and probable linoleate 9 S-lipoxygenase 4, respectively. The PMF of spots corresponding to D10 and U2 did not match any protein in the MASCOT analysis.

Further, biological annotation was done for the proteins, which were classified into six categories based on their putative functions in the UniProtKB database (<http://www.uniprot.org/>) and previous literature (Table 2). The categories include signal transduction, organeller gene expression, stress/defense, fatty acid metabolism, cell cycle/endoreplication, and energy. The probable role of each protein in compatible interaction is discussed with hypothetical network (Fig. 5).

The downy mildew of maize remains an important constraint in establishing sustainable crop production worldwide. Severe yield losses have been reported during the twentieth century from warm and moist tropical and subtropical areas (Singburadom and Renfro 1982). Metalaxyl is an effective fungicide for all downy mildews, but Metalaxyl resistance developed by some of the downy mildew pathogens, including *P. parasitica* and *P. sorghi*, has limited its widespread use (Isakeit *et al.*, 2003). Hence, it is necessary to find an alternative strategy to control downy mildew. Studying molecular events associated with compatible phyto-pathogen interaction at the proteomic level will provide a way to elucidate the molecular

mechanism of disease establishment and symptom development, and thus to develop novel control strategies.

This study was conducted to help understand host proteins altered in abundance after infection in both the genotypes during maize - *P. sorghi* interaction. Fifteen overlapping proteins spots between UMI79 and CM500 and two UMI79 genotype-specific protein spots that are differentially expressed after infection were subjected to PMF and MASCOT analysis. Further, the probable functions of the proteins were identified (Table 2) and their probable role in compatible plant - pathogen interaction is discussed according to their functional categories.

Signal transduction

Protein kinases and phosphatases are key signal transducers during plant - pathogen interaction that leads to plant defense responses (Xing *et al.*, 2002). Probable protein phosphatase 2C 77 (PP2C) was found to be downregulated (D1) in both the genotypes after infection, but the extent of downregulation was higher in the CM500 genotype ($AR=0.11 \pm 0.03$) than UMI79 ($AR=0.28 \pm 0.06$). The protein phosphatase is involved in numerous cellular processes like plant growth, development, abscisic acid (ABA) signaling and response to environmental stresses.

Upregulation of PP2C has been reported previously in incompatible MYMV-*Vigna mungo* interaction (Kundu *et al.*, 2013), and it was thought to be involved in pathogen effector recognition and the induction of the resistance response by triggering PR protein production in the resistant genotype. Transgenic tobacco was successfully produced against tobacco virus with overexpression of the rice PP2C2 gene that improved disease resistance (Hu *et al.*, 2009).

PP2C is a negative regulator of ABA signaling pathways (Schweighofer *et al.*, 2004; Sheen 1998). ABA is widely known for its role in abiotic stress but it also plays a significant role in biotic stress. In the initial stage of pathogen infection, ABA induces stomata closure and callose deposition thereby restricting pathogen entry.

However, after successful entry and establishment of the pathogen (later stage of the infection) in the susceptible plant, it negatively regulates disease resistance at the later stage of infection by suppressing ROS production, which was evident in tomato and *Botrytis cinerea* interaction (Asselbergh *et al.*, 2007), and also negatively regulates the salicylic acid pathway thus hampering systemic acquired resistance (SAR) (Mohr and Cahill 2007; Yasuda *et al.*, 2008). In this present compatible interaction study (*P. sorghi*-maize), ABA might have induced upon the downregulation of PP2C, which thereby suppressed the SAR response and helped in the colonization of the pathogen.

Regulation of organelle gene expression

Pentatricopeptide repeat-containing protein (OTP51) regulates organeller gene expression through post-transcriptional control such as intron splicing, RNA editing, RNA processing, RNA stability and RNA cleavage (Schmitz-Linneweber and Small 2008). In chloroplasts, it is also involved in photosystem I assembly. The loss of this OTP51 deleteriously affects photosystem I and II of the *Arabidopsis thaliana* mutant (Longevialle *et al.*, 2008). In the present study, pentatricopeptide repeat-containing protein OTP51 (spot D2) was found to be downregulated in the SDM-inoculated samples of both the susceptible genotypes of the maize. The downregulation was higher in the CM500 (AR=0.22 ± 0.03) than the UMI79 genotype (AR=0.57 ± 0.09). Similarly,

depletion of chlorophyll content measured by SPAD was higher in CM500 than UMI79. Thus, both proteomics and SPAD reading suggest that the pathogen affected the photosynthesis process and its effect was high on the CM500.

Stress/defense

The DEAD-box RNA helicases comprise the largest subfamily of RNA helicases. DEAD-box RNA helicases are prominent candidates for RNA chaperones because these proteins can use energy derived from nucleotide triphosphate hydrolysis to actively disrupt misfolded RNA structures so that correct folding can occur. Both upregulation (U3) and downregulation (D7, D9) of DEAD-box RNA helicase was noticed after infection. Different isoforms like 50, 52B (upregulated) and 16 (downregulated) may eventually play different roles during pathogen infection. A similar phenomenon of opposite expression for some of the proteins was observed in *Sugarcane mosaic virus* and maize interaction indicating complex regulatory mechanisms of plants in response to pathogen infections.

The upregulation of DEAD-box ATP-dependent RNA helicase 16 was much higher in the CM500 than the UMI79 (variance of AR=15.38 thus speculating the CM500 is being undergone higher stress condition which requires higher amount of RNA chaperone specifically DEAD-box ATP-dependent RNA helicase 16 to deal with the stress condition.

The plant cytoskeleton is a highly dynamic scaffold comprising microtubules (polymer of α/β tubulin heterodimer) and microfilaments (polymer of actin monomer). It is versatile in its function and plays a role in growth and development, cell division, cell expansion, intracellular organization and organelle motility (Wasteneys and Galway 2003).

Fig.1 Phenotypic symptoms of SDM in the control and inoculated samples of the UMI79 and CM500 genotypes. SDM symptoms such as chlorosis, leaf narrowing and white growth on the leaf surface was observed in the SDM-inoculated leaves at 20 dpi. (A) UMI79 leaf: left, control; right, SDM inoculated and (B) CM500 leaf: left, control; right, SDM inoculated

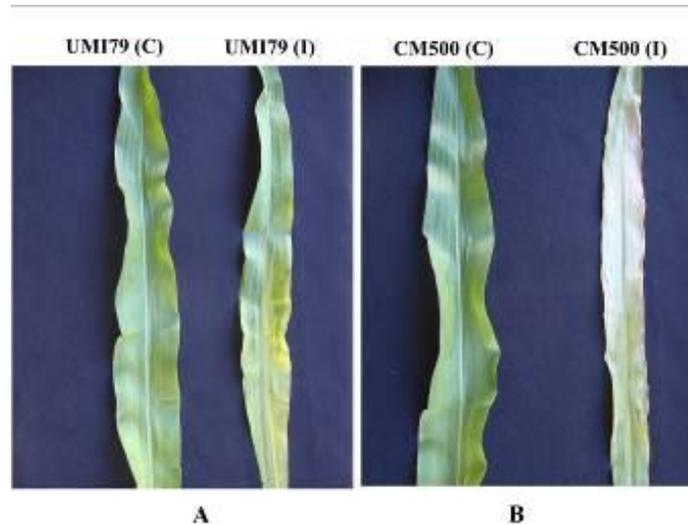


Fig.2 Environmental scanning electron microscope images of the adaxial and abaxial surfaces of SDM-inoculated leaves at 20 dpi in the two susceptible genotypes of the maize. An unbranched conidiophore (Cph) emerging from stomata (S) was observed from the adaxial and abaxial leaf surfaces of the genotype UMI79. In CM500, branched and profused growth of conidiophores on the adaxial surface bearing conidia (C) on the highly susceptible genotype CM500 and two conidiophore emerging from single stomata on the abaxial surface were observed

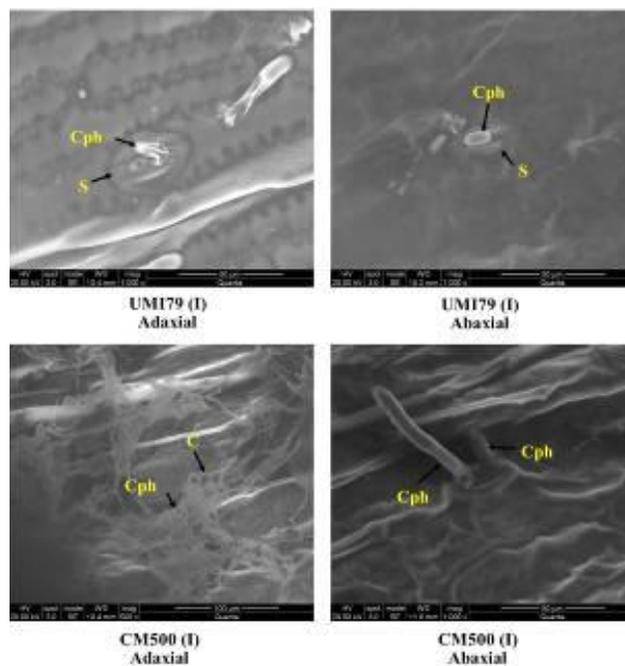


Fig.3 Representative images of two-dimensional gel electrophoresis of leaf proteins from the control and SDM-inoculated susceptible maize genotype UMI79 and CM500. Downregulated protein spots were numbered D1–D10, upregulated protein spots as U1–U4, newly induced spots as N1–N3

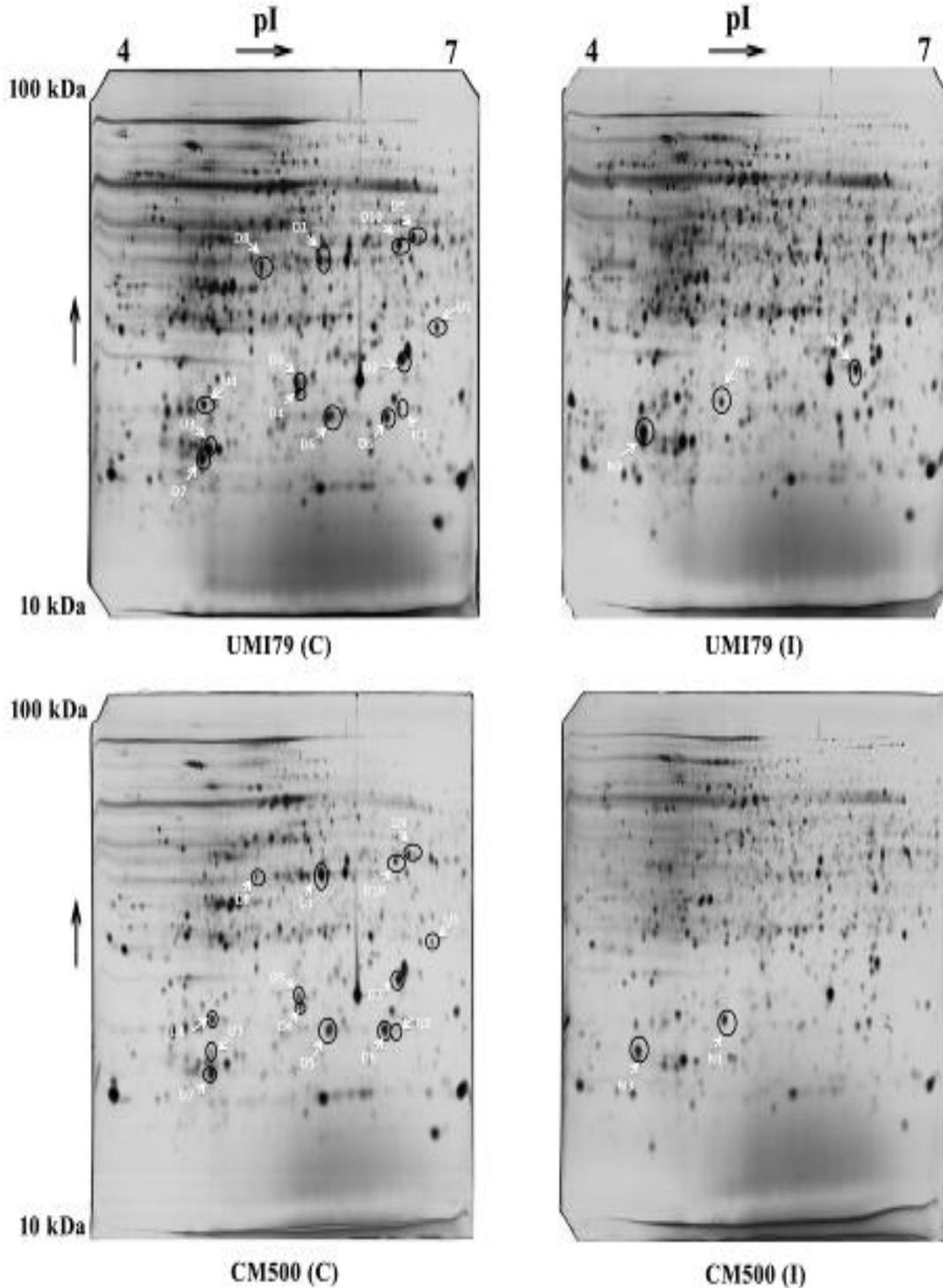


Fig.4 Magnified view of differentially expressed proteins in two-dimensional gel in the compatible interaction between *P. sorghi* and the two maize genotypes (UMI79 and CM500). Labeled spots show significant changes in the control and SDM-inoculated samples. (I) denotes inoculated samples; (C) denotes control samples

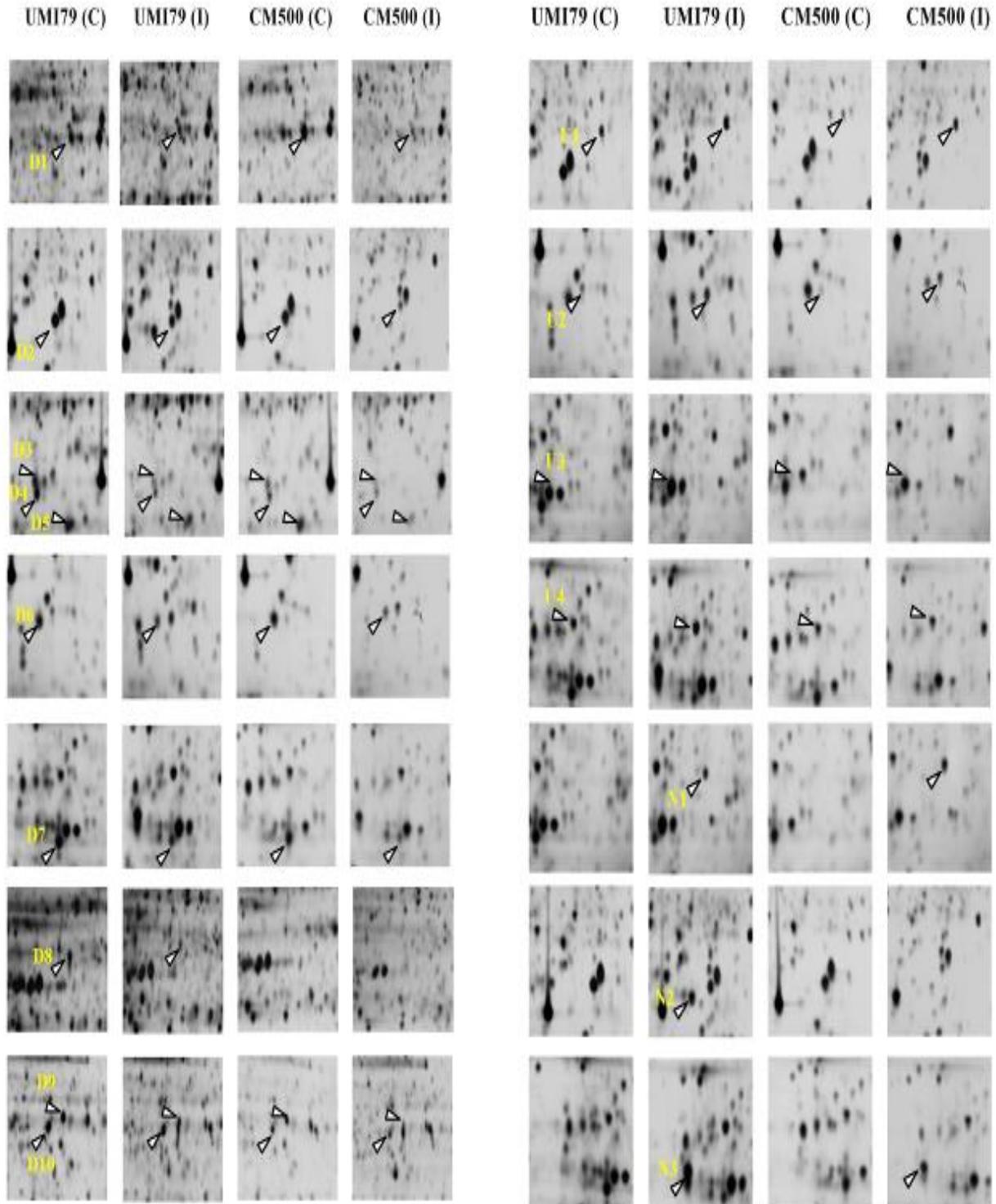


Fig.5 Hypothetical regulatory network of compatible interaction between *P. sorghi* and maize

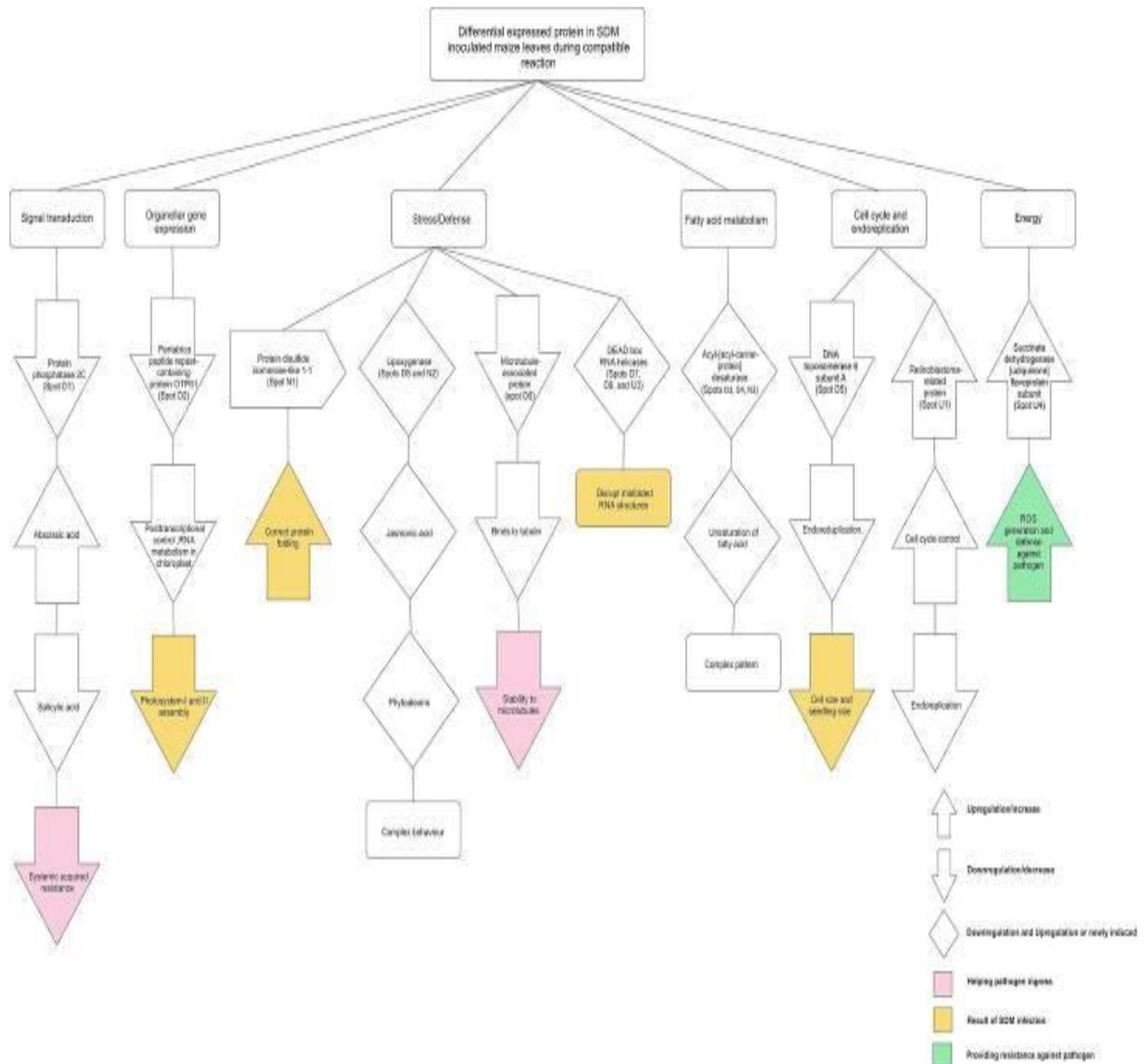


Table.1 Estimation of chlorophyll content and leaf width in control and inoculated samples of two SDM-susceptible genotypes of maize

Genotype	Sample	SPAD (Mean ± SD)	Decrease over control (%)	Leaf width (cm) (Mean ± SD)	Decrease over control (%)
UMI79	Control	31.64 ± 1.37	-	4.86 ± 0.61	-
	Inoculated	14.68 ± 2.22	53.60	3.70 ± 0.57	23.87
CM500	Control	33.20 ± 1.02	-	4.94 ± 0.34	-
	Inoculated	12.80 ± 1.31	61.45	3.42 ± 0.38	30.77

*Mean of five replications

Table.2 Differentially expressed proteins in SDM-inoculated leaves of two maize genotypes identified by 2-DE/MALDI-TOF/MS analysis. D - Downregulated, U - Upregulated, N - Newly induced proteins

Spot ID	Abundance ratio		Protein accession no.	Protein Name	MASCOT score	Sequence coverage (%)	Mr/pI	
	UMI79	CM500					Theoretical	Experimental
Signal transduction								
D1	0.28 ± 0.06	0.11 ± 0.03	P2C77_ORYSJ	Probable protein phosphatase 2C 77	49	27	44.21/6.99	46/5.82
Gene expression								
D2	0.57 ± 0.09	0.22 ± 0.03	OTP51_ORYSJ	Pentatricopeptide repeat-containing protein OTP51	55	21	89.94/6.04	26/6.5
Stress/defense								
D7	0.46 ± 0.11	0.49 ± 0.11	RH50_ORYSJ	DEAD-box ATP-dependent RNA helicase 50	59	58	71.44/9.25	15/4.84
D9	0.72 ± 0.03	0.16 ± 0.04	RH52B_ORYSJ	DEAD-box ATP-dependent RNA helicase 52B	61	37	67.30/8.29	53/6.59
U3	2.11 ± 0.73	7.66 ± 2.01	RH16_ORYSJ	DEAD-box ATP-dependent RNA helicase 16	56	67	74.74/8.61	15/4.9
D6	0.59 ± 0.22	0.62 ± 0.04	MP702_ORYSJ	Microtubule-associated protein 70-2	57	65	66.473/8.4	19/6.37
D8	0.54 ± 0.04	0.40 ± 0.09	LOXC1_ORYSJ	Lipoxygenase 72	50	29	103.15/5.91	45/5.33
N2	+	-	LOX4_ORYSJ	Probable linoleate 9S-lipoxygenase 4	56	46	98.69/6.82	25/6.33
N1	+	+	PDI11_ORYSJ	Protein disulfide isomerase-like 1-1	67	76	57.04/5.01	21/5.21
Fatty acid metabolism								
D3	0.35 ± 0.10	0.54 ± 0.14	STAD4_ORYSJ	Acyl-[acyl-carrier-protein] desaturase 4	57	44	45.679/7.77	23/5.62
D4	0.33 ± 0.10	0.62 ± 0.05	STAD6_ORYSI	Acyl-[acyl-carrier-protein] desaturase 6	65	43	47.049/7.16	22/5.64
N3	+	+	STAD6_ORYSJ	Acyl-[acyl-carrier-protein] desaturase 6	85	61	46.52/7.65	18/4.56
Cell cycle/Endoreplication								
U1	2.39 ± 0.23	4.21 ± 0.10	RBR1_ORYSI	Retinoblastoma-related protein	49	47	112.69/8.84	31/6.79
D5	0.28 ± 0.04	0.11 ± 0.02	TOP6A_ORYSI	DNA topoisomerase 6 subunit A	48	45	48.61/9.36	19/5.88
Energy								
U4	2.07 ± 0.09	1.19 ± 0.13	DHSA_ORYSJ	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	54	20	69.49/6.61	20/4.86
Not significant								
D10	0.77 ± 0.05	0.83 ± 0.10	-	Not significant	-	-	-	51/6.47
U2	3.18 ± 0.05	3.85 ± 0.07	-	Not significant	-	-	-	20/6.47

Microtubule-associated protein binds to tubulin molecules and provides stability to microtubules. The actin microfilaments are consistently observed to target the infection site in both compatible and incompatible interaction, while microtubule response to pathogen attack was found to be variable in different plant-microbe interactions (Takemoto and Hardham, 2004). In the present study, the microtubule-associated protein 70-2 (spot D6) was found to be downregulated, which might induce instability in the microtubule and thus might have helped pathogen ingress.

Plant lipoxygenase may be involved in a number of diverse aspects of plant physiology including growth and development, pest resistance and senescence or responses to wounding. It catalyzes hydroperoxidation by introducing molecular oxygen exclusively into the C-9 and C-13 position of linoleic and linolenic acids in the octodecanoic pathway through which jasmonic acid is synthesized (De Domenico *et al.*, 2007). In this study, probable linoleate 9S-lipoxygenase 4, a newly expressed protein (spot N2), was exclusively found in the UMI79 inoculated samples in which disease susceptibility was found to be lower than in CM500. Spot D8 (lipoxygenase 7) was observed only in the UMI79 gels and it was downregulated after infection. These differences may be the reason for differential susceptibility or resistance reaction.

Upregulation of lipoxygenases was observed in the *rice black-streaked dwarf virus* infection to maize. It was suggested that the increase in lipoxygenase content may result in phytoalexins and other cytotoxic products, which could improve plant resistance against pathogens (Royo *et al.*, 1996). The decrease/downregulation of LOX due to colonization of the pathogen *Sclerospora graminicola* in pearl millet during compatible reaction was observed (Babitha *et al.*, 2006).

During stress conditions, protein damage occurs because of an imbalance in cellular homeostasis. Probable protein disulphide isomerase (spot N1), a newly expressed protein after infection, facilitates correct disulphide cross-linking between and within the damaged polypeptide by rapid reshuffling of disulfide bonds until the correct set is achieved, thereby stabilizing tertiary and quaternary structure conformation, which might have been distorted because of an oxidative environment during compatible plant - pathogen interaction. Nwugo *et al.*, (2013) reported that this protein is upregulated 1.52 times in lemon plants in response to Las infection.

Fatty acid metabolism

Fatty acids and their derivatives play important roles in plant defense responses. Acyl-[acyl-carrier-protein] desaturase is a protein found to be involved in fatty acid metabolism. It converts stearyl-ACP to oleoyl-ACP by introducing a *cis* double bond.

It was represented by more than one spot with slightly different Mr and pI values, suggesting that these changes in the proteome may be attributed to post-transcriptional modification (PTM) (small shift in the pI) or degradation products (*i.e.*, significant differences between theoretical and experimental Mr values).

For example, the downregulated spots D4, and the newly induced spot N3 were identified as being different forms of acyl-[acyl-carrier-protein] desaturase 6, each with slight differences in pI and Mr values and could probably reflect some PTMs occurring *in vivo* or be the consequence of artifactual modifications such as deamidation in the proteins during sample preparation and proteolytic processing or the existence of different genes encoding the same protein (Jorge *et al.*, 2005).

Cell cycle/endoreplication

Tumor suppressor protein, a retinoblastoma protein, is a negative regulator of cell cycles, a well-studied example of cell cycle regulator in mammals. It acts during the G1 to S phase transition checkpoint. In a dephosphorylated state, RB binds to E2F transcription factor, thereby suppressing it, which in turn terminates transcription of the genes encoding enzymes required for DNA replication machinery during the S phase. Retinoblastoma-related protein (RBR) (Spot U1) is a plant homologue of the retinoblastoma protein, found to be upregulated in both genotypes after infection. The extent of upregulation was much higher in the CM500 than UMI79 (variance=1.65; supplementary table S4) Besides cell cycle control, its role in histone deacetylase chromosome modelling increases in heterochromatinisation, thereby suppressing the transcription as was observed in previous reports.

RBR interacts with the geminiviridae replication-associated proteins and favors viral DNA replication (Ascencio-Ibáñez *et al.*, 2008; Desvoyes *et al.*, 2013). Similarly, RBR may play a role in pathogen proliferation; however, further studies are needed to confirm the actual mechanism. Endoreduplication (duplication of a genome without further mitotic division) is observed in many compatible biotrophic plant-pathogen interaction studies to mitigate metabolite demand of successful invaded and established pathogens (Chandran *et al.*, 2010; Chandran *et al.*, 2013; de Almeida Engler and Gheysen 2013). RBR plays a key role in regulating endoreduplication. Suppression of endogenous NbRBR1 in tobacco by virus-induced gene silencing (VIGS) was found to increase endoreduplication in tobacco leaf (Park *et al.*, 2005), whereas studies on RBR overexpression in its own domain revealed

suppression of both endoreplication and cell proliferation (Magyar *et al.*, 2012). The phosphorylated, dephosphorylated or hyperphosphorylated state of RBR also determines its activity, for instance, hyperphosphorylation of RBR was found to increase polyploidization of maize endosperm. Thus, the complex nature and diverse function of RBR needs further study to gain an insight into the maize - *P. sorghi* pathogen interaction.

DNA topoisomerase 6 subunit A is a component of the DNA topoisomerase VI, which is involved in chromatin organization and progression of endoreduplication cycles. As endoreduplication increases, cell size increases, whereas mutants with a loss of function of DNA topoisomerase VI subunit A leads to dwarf seedlings, reduced root hairs, and small/under-branched trichomes (Hartung *et al.*, 2002; Sugimoto-Shirasu *et al.*, 2002; Yin *et al.*, 2002). Downregulation extent is high in the CM500 (0.11 ± 0.02) than UMI79 ($AR=0.28 \pm 0.04$). Similarly, the CM500 had high decrease in the leaf width in infected samples over control (30%) than the UMI79 (23%).

Energy

Excess food demand by an established pathogen in a susceptible host is mitigated by higher respiration and oxidative phosphorylation. The succinate dehydrogenase [ubiquinone]-flavoprotein (FP) subunit of succinate dehydrogenase (SDH) was found to be upregulated in the present study (spot U4). SDH is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q). Mitochondria are both a source of ATP and a site for reactive oxygen species (ROS) production. It has been established that SDH of the electron transport

chain contributes to localized mitochondrial ROS and hence regulates plant stress and defense responses (Gleason *et al.*, 2011).

Our study has provided the first insights into the maize - *P. sorghi* compatible interaction, revealing that the biotrophic pathogen *P. sorghi* induced downregulation of PP2C and thereby suppressed systemic acquired resistance. *P. sorghi* infection directly or indirectly affects organellar gene expression, and photosystem I and II and modulate host cell cycle, which was corroborated by the phenotypic symptoms such as chlorosis and leaf narrowing. Although we used both susceptible hosts in this study, it was evident from proteomics studies that the host tries to sustain itself during infection through some mechanism such as RNA chaperon and protein disulphide isomerase to withstand an oxidative environment during compatible plant - pathogen interaction. Overall, this study gives cues to understand the changes in protein abundance levels that occur upon infection by SDM in maize. It also serves as a basic prelude to comprehend maize-SDM interaction, which needs to be validated through functional genomics approaches.

Competing interests

We declare that there are no conflicts of interests.

Author contributions

Conceived and designed the experiments: NS, KNG, JR; performed the 2D experiment: MR, RVR, KPJ; MALDI-TOF/MS analysis: NA, GSK; downy mildew inoculation and disease establishment: PMT, KS, VP.

Acknowledgments

The financial support from the Department of Biotechnology, Government of India, under

for scheme no. BT/PR/108910/GBD/27/111/2008 provided to NS is kindly acknowledged. We thank the UGC for providing a fellowship to KPJ to participate in the doctoral research program.

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How to cite this article:

Jadhav, K.P., R. Veera Ranjani, N. Senthil, N. Arulkumar, P.M. Tamilarasi, K. Sumathi, K.N. Ganesan, V. Paranidharan, M. Raveendran, Gon Sup Kim and Ramalingam, J. 2018. Proteomic Analysis of a Compatible Interaction between Sorghum Downy Mildew Pathogen (*Peronosclerospora sorghi*) and Maize (*Zea mays* L.). *Int.J.Curr.Microbiol.App.Sci.* 7(11): 653-670. doi: <https://doi.org/10.20546/ijcmas.2018.711.079>