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Expression analysis of maize genes during *Bipolaris maydis* infection and assessing their role in disease resistance and symptom development

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Bipolaris maydis causing maydis leaf blight (MLB) is an aggressive fungal pathogen of maize. The present study focused on the responses of certain defence genes in the resistant and susceptible maize inbred lines *viz.*, SC-7 and CM 119, respectively, against MLB and symptom development in the host. Biochemical activity of the PR protein *β*-1, 3-glucanase and phenylalanine ammonia lyase (PAL) assay, with total chlorophyll content was recorded for both the inbred lines before and after pathogen inoculation. Gene expression was studied by quantitative polymerase chain reaction (qPCR) at different time intervals post inoculation. Differential expression pattern was observed even at the same time point in both the inbred lines. Enhanced expression of the pathogenesis related (PR) protein and phenylalanine ammonia lyase (PAL) enzyme at different time points in resistant lines indicated their association with infection stages of *B. maydis* and response of the resistant line against disease establishment. Down regulated gene expression of pheophytinase suggests reduced enzyme activity linked with less chlorophyll degradation in the resistant line compared to the susceptible line. This fact directly correlates with symptom development of MLB disease. The present study thus revealed that the expression of defence related genes is aligned with developmental stages of the pathogen to restrict its growth and gene expression of constitutive genes also changes differentially during the disease development in resistant and susceptible lines.

Keywords: Maize gene expression, maydis leaf blight, *Bipolaris maydis* infection, phenylalanine ammonia lyase, *β*-1, 3-glucanase, pheophytinase

Introduction

Maize is an important food for human being, feed for livestock and having a wide range of industrial uses. Its grains contain abundant starch (65%)[1]. Among the diseases of maize, maydis leaf blight (MLB) or southern corn leaf blight (SCLB) caused by *Bipolaris maydis* Shoemaker (Teleomorph *Cochliobolus heterostrophus* Drechs.) is a widespread foliar disease in many maize growing regions of the world. In India, it was first reported from Malda, West Bengal[2]. Losses in severe condition are as high as 70%[3]. Typically it causes tan, elliptical to rectangular lesions on the leaves and on under surface of foliage which later coalesce and gives blight appearance. The pathogenic fungus exists principally as race O (reconfirmed data unpublished yet) and, to a lesser extent, as race T which produces host selective toxin (HST) that is "T" toxin. So far most of the studies have been conducted on race "T" pathogen and host-pathogen interactions are being reported for maize and *B. maydis* (race T) pathogen which specifically affects cytoplasmic male sterile lines (CMS).

Plants interact with the pathogen on arrival of its inoculum during which compatible reaction successfully establish the disease and incompatible interaction show resistance by the plant. Presence of certain genes in plant that recognizes the pathogen effectors and activates defence mechanism, which hampers invading pathogen called effector triggered immunity (ETI)[4]. Plants defend themselves through a variety of mechanisms, among them a few are related to cell wall structural alterations, creating barriers to infection, antimicrobial metabolites production and antimicrobial protein expression[5]. Sometimes resistance is accompanied by the expression of diverse defense-related genes, most of which are referred to as pathogenesis-related (PR) proteins. Therefore in present study we selected certain genes such as *β*-1, 3- gulcanase is a class 2 PR protein and found in trace amount in plants before infection; its production increases as pathogens comes in contact[6]. It is also associated with basic functions in plants such as cell division, passing off material via

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plasmodesmata and flower formation[7]. Enzyme β-1, 3- glucanase as a defence protein and it regulates symplastic permeability and hydrolysis of callose[8], hydrolyses β-1, 3- linked glucans, a structural component of fungal cell wall. In addition, constitutive resistance often occurs in association with the over expression of defense-related genes. During infection pathogens triggers some metabolic pathways and signal transduction i.e. PAL enzyme is also known as the key enzyme for phenyl propanoid pathway and is associated with biosynthesis of salicylic acid which involves in induction of resistance in plants against pathogen[9]. Chlorophyll, the main green pigment of plants, is vital for photosynthesis, which helps plants get energy from light[10], therefore, another important gene pheophytinase (PPH) was selected which is associated with chlorophyll degradation pathway, because MLB disease symptom develops mainly due to chlorophyll degradation.

Bipolaris maydis is a highly aggressive and necrotrophic pathogen[11] and hypersensitive response (HR) is not sufficient to restrict the pathogen in susceptible lines, while in resistant lines certain proteins and enzymes play important roles in disease suppression. It is hypothesized in the present study that there are defence related genes in resistant inbred lines of maize derived through natural and artificial selection that independently or cumulatively provide disease resistance to plant. Further, certain constitutive genes might play role during disease development in susceptible lines, which differ in expression in case of resistance lines of maize.

Therefore present study was aimed to decipher the response of key genes responsible for disease resistance and symptom development in maize plants during infection with respect to development stages of the pathogen such as germination (at 24 h), penetration (at 48 h), disease phase (at 72 h) and symptom appearance (at or after 96 h). Maize SC-7 line shows excellent resistance under field condition against MLB and registered variety (INGR 07025) germplasm committee by ICAR, Therefore this variety was chosen to understand few aspects of resistance in this variety. Maize inbred line SC-7 was used which is a non CMS line developed through artificial and natural selection and is resistant against *B. maydis* race "O" pathogen. Race "O" can cause infection on both cytoplasmic male sterility (CMS) and non CMS lines[12]. In tropical Asian countries including India major yield loss in maize is due to the race O compared to temperate regions where major challenge is caused by the race "T" in CMS lines. Another aspect of this study is to understand the role of genes existed in the MLB resistant inbred lines in various developmental stages of the pathogen after inoculation, such as 24 h is as germination stage, 48 hr as germ tube formation and penetration stage, 72 h as diseased phase and post 96 h as symptoms expression.

Materials and Methods

Layout of Experiment, Plant Inoculation and Disease Scoring

An inbred line of maize SC-7-2-1-2-6-1 (SC-7 will be referred here after), highly resistant to MLB, and another inbred susceptible line CM 119 were sown in the net house. SC-7 and CM 119 having similar parental background and resistant and susceptible character identified based on phenotypic selection. Inoculation was done on 35 days old plants (stage 5 of maize growth stages) using sorghum grains culture of *B. maydis.* Inoculum was spread in the whorls on both resistant and susceptible inbred lines[13]. Un- inoculated plants of both inbred lines were maintained as control. Data of seed germination was recorded from 5th to 10th day after sowing (DAS) in net house for two seasons *viz.*, *kharif* (June - October) 2017 and 2018. Disease scoring for MLB was done following the standard method[14] adopted in the All India Coordinated Maize Improvement Project (Table 1).

Seed Germination Count

Germination percentage for both susceptible (CM119) and resistant (SC-7) maize inbred lines was calculated by using the formula mentioned below[15]. Average data of both seasons were taken under net house condition.

$$\text{Percent of seed germination} = \frac{\text{Average number of normal germinated seeds}}{\text{Total number of seeds sown}} \times 100$$

Starch Estimation

Leaf samples were collected before inoculation (control) and after inoculation (infected). The collected leaves were decolorized using 80% ethanol, later soaked in Lugol's iodine for 20 min and washed quickly and stained[16]. Transverse section were cut manually and mounted on slides with glycerol and observed under a binocular microscope for each sample from five replicates.

Total Sugar Estimation

Total sugars were estimated by anthrone method[17]. One hundred mg of leaf sample was crushed in liquid

Table 1 — Rating scale (1-9) of maydis leaf blight disease (AICRP, 2016)

Score	Degree of infection	Degree reaction
1	Nil to very slight infection (≤ 10%).	
2	Slight infection, a few lesions scattered on two lower leaves (10.1 - 20%).	Resistant (R) (Score: ≤ 3.0)
3	Light infection, moderate number of lesions scattered on four lower leaves (20.1 - 30%).	
4	Light infection, moderate number of lesions scattered on four lower leaves (20.1 - 30%).	
5	Moderate infection, abundant number of lesions scattered on lower leaves, moderate number of lesions scattered on middle leaves below the cob (40.1 - 50%).	Moderately resistant (MR) (Score: 3.1 - 5.0)
6	Heavy infection, abundant number of lesions scattered on lower leaves, moderate infection on middle leaves and a few lesions on two leaves above the cob (50.1 - 60%).	Moderately susceptible (MS) (Score: 5.1 - 7.0)
7	Heavy infection, abundant number of lesions scattered on lower and middle leaves and moderate number of lesions on two to four leaves above the cob (60.1 - 70%).	
8	Very heavy infection, lesions abundant scattered on lower and middle leaves and spreading up to the flag leaf (70.1 - 80%).	Susceptible (S) (Score: > 7.0)
9	Very heavy infection, lesions abundant, scattered on almost all the leaves, plant prematurely dried and dead (> 80%).	

nitrogen and immediately 5 ml of 80% ethanol was added and centrifuged at 4000 rpm for 10 min at 40ºC. Supernatant was collected and made-up to a volume of 10 ml with sterilized distilled water. In 1 ml of extract 4 ml of anthrone (dissolved in concentrated HCL) was added and heated at 100ºC for 10 min. It developed blue-green colour which was allowed to cool down. Optical density (OD) was recorded at 625 nm. Total sugar content was calculated using standard glucose curve.

Estimation of Chlorophyll Content

Chlorophyll content of the fresh leaves was recorded once from 35 days old plants of SC-7 and CM 119 before inoculation with *B. maydis*. Disease symptoms started appearing at 3rd day (96 h) of inoculation, and hence chlorophyll content was again recorded when disease symptoms were clearly visible on the plants, i.e. 5 days after inoculation (DAI). Chlorophyll was recorded using SPAD meter (Konica Minolta 502) based on optical density difference between two wave lengths. Data was recorded and average value was taken for 10 replicates from inoculated and un-inoculated plant leaves.

Biochemical and Gene Expression Study

Leaf samples were collected 35 DAS just before inoculation and at 5th DAI for biochemical estimation of PAL enzyme and PR protein β-1, 3- glucanase. For RNA extraction, leaf samples were collected at 0, 24, 48, 72 and 96 h after inoculation and also at the same time interval from un-inoculated control plants. Collected samples were immediately put into ice bags and stored at -80°C for future study.

Phenylalanine Ammonia Lyase (PAL) Assay

The activity of PAL was assayed by following standard method[18-19]. One gram of plant sample was homogenized in pre-cooled mortar and pestle using liquid nitrogen, thereafter 20% of polyvinyl pyrrolidone (PVP) and 2 ml of borate buffer (pH 8.5) were added and it was centrifuged at 14,000 g, at 4^{o}C for 20 min. The supernatant was used as enzyme extract for assay. The reaction (3 ml reaction mixture contained 1 ml of 4 mM L-phenylalanine, 1.5 ml of 0.05 mM borate buffer (pH 8.8), 0.1 ml of enzyme extract and 0.4 ml distilled water) was started by incubating test tubes without shaking at 38^{o}C in hot water bath for 1 h. The reaction was stopped by adding 0.1 ml of 5 M HCL. Absorbance was recorded in a UV-VIS spectrophotometer at 290 nm to know the amount of trans-cinnamic acid. A reaction mixture without phenylalanine was used as control. The activity of PAL was calculated by using standard curve of trans-cinnamic acid.

β-1, 3- Glucanase Assay

The activity of β-1, 3- glucanase was assayed by the following method[19]. One gram of plant tissue was ground in liquid nitrogen and transferred to a tube containing 3 ml of 0.05 M potassium acetate buffer (pH 5.0). The homogenate was filtered through a muslin cloth, centrifuged at 14,000 g at 4^{o}C for 10 min, and the supernatant (enzyme extract) was used for immediate assay. Enzyme extract of 0.5 ml was added in 0.5 ml of 2% laminarin solution. The mixture was incubated at 50^{o}C for 1 to 2 hrs. The reaction was stopped by adding 3 ml of dinitrosalicylic acid reagent. The contents were

diluted with distilled water and optical density was observed at 500 nm by using UV-visible nanodrop (Biorad). The activity of *β*-1, 3- glucanase was calculated by using the D-glucose standard curve.

Isolation of RNA

Total RNA was isolated from the leaf samples using Pure Link™ RNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) as per the manufacturer's instructions.

Complementary DNA (cDNA) Synthesis

First strand cDNA synthesis reaction was carried out in a DEPC-treated and autoclaved 0.2 ml PCR tubes by using ImProm-II™ reverse transcription system kit (Promega, USA) following manufacturer's instruction. The 20 µl reaction mixture containing 4.5 µl nuclease-free water, 4 µl ImProm-II™ 5X reaction buffer, 4 µl $MgCl_2$, 1 µl dNTP mix, 0.5 µl (40 U/µl) recombinant RNase in ribonuclease inhibitor, 1 µl ImProm-II reverse transcriptase, 1 µl random primers and 4 µl (50 ng/µl) total RNA (samples) was prepared and the reaction mixture was centrifuged for a while. Polymerase chain reaction (PCR) condition was followed as per the manufacturer's instruction.

Primer Designing

Primers were designed (Table 2) using genomic data from with National Centre for Biotechnology Information (NCBI) reference sequence: NC_024462.2, NC_024461.2, NC_024465.2 for three maize genes *viz.*, PAL, PR protein *β*-1, 3-glucanase and chlorophyll degrading enzyme pheophytinase, respectively using the primer designing tool Primer-3 software.

Performance of qRT-PCR

qRT- PCR assay was performed to assess the expression of three maize genes. The amounts of cDNA in the samples were balanced employing actin as house-keeping gene and used as templates. qRT PCR, amplification was carried out with the primer pairs as detailed (Table 2). Quantification of gene expression was performed through MJ Miniopticon-48 wells real time PCR detection system (Bio-Rad Labs Inc.) in triplicate. The PCR mixture contained 100 ng of cDNA template, 10 µl of 2X dynamo colour flash SYBR green mix dye (Thermo Scientific USA), 0.5 µl (10 µM/µl) of forward and reverse primer together in a final volume of 20 µl. Thermal cycling conditions were maintained as follows: 95°C for 10 min followed by 35 cycles of 95°C for 15 sec and 53°C for 30 sec and a melt curve from 65°C to 95°C. Melt curve and threshold data was observed during each cycle. Actin gene was used as internal control along with NTC (non template control). Relative gene expression levels were expressed as the number of cycles (Ct) required for amplification to reach a threshold fixed in the exponential phase of PCR reaction. The level of gene expression was normalized as that of house-keeping genes for each repetition of samples in every run to provide ΔCt value. Mean of ΔCt values for each target gene was then normalized to the expression of treated samples with control samples to find ΔΔCt[20]. Comparison of relative gene expression among all treatments was determined according to $2^{-\Delta\Delta Ct}$ method in terms of fold changes using the formula recited below. Each of the samples along with a NTC was used in triplicate.

Formula, $\Delta\Delta CT = (C_{T.}\text{Target} - {}_{CT.}\text{Actin})_{\text{Timex}} - (C_{T.}\text{Target} - C_{T.}\text{ Actin})_{\text{Time 0}}$

Time x = Any time point, Time 0 = 1 × expression of the target gene normalized to β-actin. Here, Time x = 24, 48, 72 and 96h, Time 0 = at 0 h for susceptible (SC00) and resistant control (RC00) (Table 4, 5 & 6).

Gene expression was studied in CM119 and SC-7 at different time intervals *viz.*, 0, 24, 48, 72 and 96 h after inoculation to obtain response of the maize genotypes. Quantification was carried out in resistant inoculated (RI) and susceptible inoculated (SI) inbred lines and in their corresponding resistant control (RC) and susceptible control (SC) plants at different time points as mentioned earlier.

Statistical Analyses

Statistical analysis was done for all replicated samples by using SPSS version 16. Replications were maintained in a completely randomized design and all experiments were repeated twice. Data are expressed

Table 2 — Primers used for gene expression studies along with maize housekeeping gene actin

Gene	Forward primer	Reverse primer
PAL	$^{5'}$AGCCGTGTTCAGTTTGGATT$^{3'}$	$^{5'}$ATCCCGTGAACAGCTAGCAC$^{3'}$
β-1, 3-glucanase	$^{5'}$ATG GCG AGG CAG GGT GTC$^{3'}$	$^{5'}$ACG CCG ATG GAT TGG ACT C$^{3'}$
Pheophytinase	$^{5'}$GAAATGGAGGGGCTACAACA$^{3'}$	$^{5'}$AGTGCACCGGTAGCTAAGGA$^{3'}$
Actin	$^{5'}$TTTGTCTTCGCTTGCTTCCT$^{3'}$	$^{5'}$TACCGGACAACACCAGTCAA$^{3'}$

as mean of 3–5 independent replications with ± standard deviations. The treatment mean values were compared by Duncan's multiple range test at $p \leq 0.05$ significance level. Statistical significance between the inoculated and the control was analysed by student's t test (* $p<0.05$) for qRT-PCR data using SPSS software.

Results

Seed Germination Count and Disease Scoring

Average disease 8 and 2 was scored (Table 3) during two *kharif* seasons for both susceptible (CM 119) and resistant (SC-7) lines respectively. Resistant line SC-7 score indicated that it belongs to resistant line whereas CM 119 fell under the susceptible category. The germination was slow in CM 119 compared to SC-7.

Starch Content and Total Sugar Content in Resistant and Susceptible Plants

Iodine staining which marked the presence of starch was done to observe the starch granules accumulation pattern in the guard cells of leaves of both susceptible (CM 119) and resistant (SC-7) inbred lines. Dark area of bundle sheath in transverse section (TS) of leaves indicates densely stained areas (Fig. 1). In CM 119, starch content was reduced after infection whereas in SC-7 starch content was not changed even after infection compared to susceptible line's starch content which was high in resistant line. On comparing resistant control (RC), starch content was found still higher in resistant than the susceptible control (SC). Further total sugar content was estimated to validate the result of starch content accumulation in maize leaves. Total sugar content in RI recorded significantly high compared SC and RC (before inoculation). Susceptible inoculated (SI) (after inoculation) estimated significant reduction compared to its control (SC) and resistant line RI and RC (Fig. 2).

PAL Enzyme Assay

PAL assay showed significant increase in its activity, both in RI and SI lines after infection as compared to the RC and SC, respectively. In RI increase in PAL activity observed significantly more than RI. Overall PAL activity was high in the resistant line than that of susceptible inbred lines, both before and after infection (Fig. 3).

β-1, 3 Glucanase Assay

The level of PR protein *β*-1, 3-glucanase activity increased significantly in case of resistant line (SC-7), whereas activity decreased significantly after infection in case of susceptible line (CM 119) which was estimated highest in resistant inoculated (RI) inbred line compared to susceptible inoculated (SI), susceptible control (SC) and resistant control (RC) lines (Fig. 4).

Table 3 — Germination of seeds and maydis leaf blight (MLB) disease score at 30-35 days after inoculation

Maize inbred line	Seed germination %			MLB disease rating (1- 9 scale)		
	2017	2018	Pool	2017	2018	Pool
CM 119	65	67	66	8	8	8
SC-7	78	82	80	2	2	2

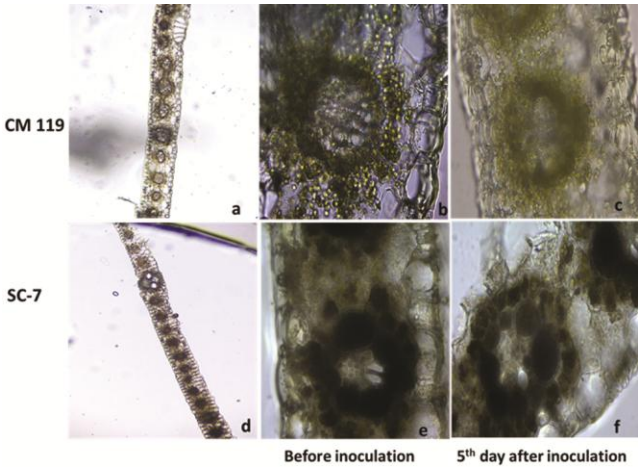

Fig. 1 — Staining of starch in maize leaves before and after inoculation with *Bipolaris maydis* rac 'O' in resistant (SC-7) and susceptible (CM 119) maize inbred lines. [View at 10x (a and d) and at 40x (b, c, e and f)]

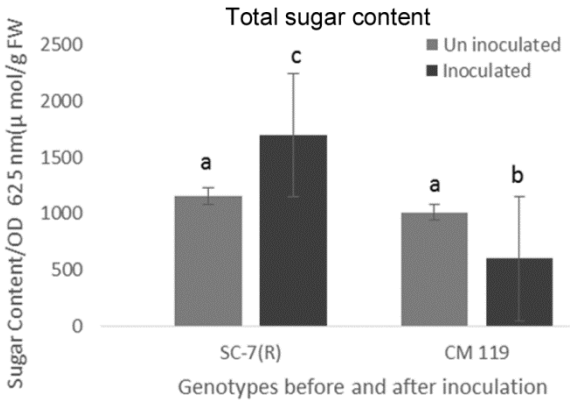

Fig. 2 — Total sugar content in maize leaves before and after inoculation of *Bipolaris maydis* race 'O' resistant (SC-7) and susceptible (CM119) inbred lines

Chlorophyll Content

The value of chlorophyll absorbance difference revealed that before infection chlorophyll content was nearly same in both resistant (SC-7) and susceptible (CM 119) inbred lines. But at 5th DAI, chlorophyll

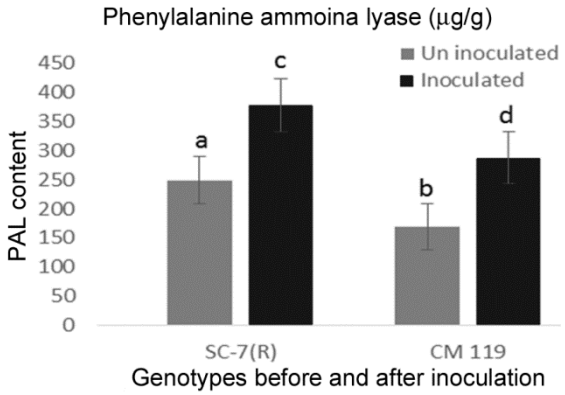

Fig. 3 — Enzymatic activity of PAL in maize leaves before and after inoculation of *Bipolaris maydis* race 'O' in resistant (SC-7) and susceptible (CM119) inbred lines

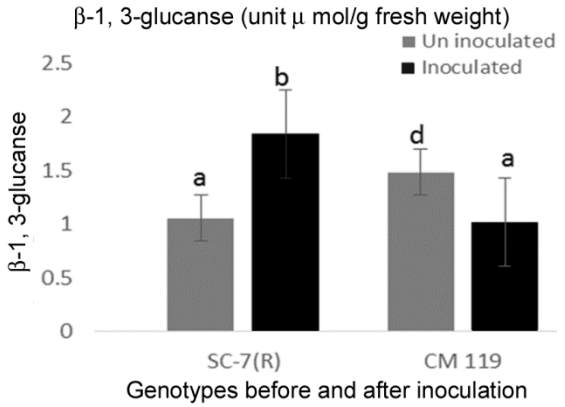

Fig. 4 — Activity of *β*-1, 3-glucanase in maize leaves before and after inoculation with *Bipolaris maydis* race 'O' in resistant (SC-7) and susceptible (CM119) inbred Lines

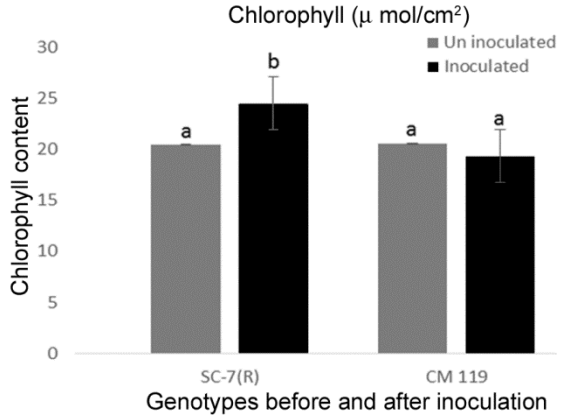

Fig. 5 — Non-destructive chlorophyll content in maize leaves before and after inoculation with *Bipolaris maydis* race 'O' in resistant (SC-7) and susceptible (CM 119) inbred lines

content was significantly high in resistant line as compared to the susceptible line. Even in SI, chlorophyll content recorded significantly lesser compared to the SC line (Fig. 5).

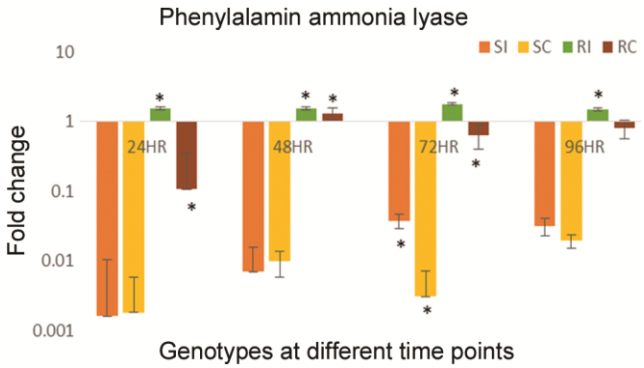

Fig. 6 — PAL gene expression pattern during 24 to 96 hr compared to 0 hr in maydis leaf blight resistant (SC-7) and susceptible (CM 119) inbred lines of maize. SI-susceptible inoculated, SC- susceptible control, RI-resistant inoculated RC-resistant control, *- Significance ($p < 0.05$)

Expression Pattern of Genes through qRT-PCR

The *PAL* gene showed up-regulation at 24, 48, 72 and 96 h after inoculation, and its gene expression was highest at 72 h in RI plants. It was more prominent than RC which was down regulated. In contrast, SI and SC continuously showed down regulation of gene expression (Fig. 6, Table 4). There is no significance difference between SC and SI for 24, 48 and 96 h but they significantly differ with the relative expression of RI and RC for the same time intervals. SI and SC showed significant difference in down regulation of *PAL* gene at 72 h where SC is highly down regulated.

β -1, 3 Glucanase

β-1,3- glucanase showed enhanced expression in RI plants at 48, 72 and 96 h. The expression was highest at 96 h followed by 48 h. It was higher than its corresponding check RC and both susceptible SI and SC plants (Fig. 7, Table 5). SI recorded maximum down regulation of gene at 24 h followed by 72 h even its corresponding check SC also showed down regulation of gene expression.

Pheophytinase

Pheophytinase gene expression was lowest at 72 h. It was significant when compared with other time points, but within the same time period the expression was non-significant. Among all four combinations lowest pheophytinase gene expression was recorded in RC at 96 h and at 24 h for RI and RC plants. Maximum up-regulation was recorded for SI plants at 48 h followed by at 24 h which is significantly more as compared to SI of 72 and 96 h (Fig. 8, Table 6).

Table 4 — Relative expression 0 to 96 h of PAL gene in inoculated resistant and susceptible maize inbred lines

Treatments	Ct value* mean (Actin)	Ct value* mean (β-1, 3-glucanase)	Δ Ct	ΔΔ Ct	Fold change
SI (24 h)	28.97	29.92	0.95	9.27	0.001615
SC (24 h)	29.85	30.63	0.78	9.10	0.001817
RI (24 h)	29.57	27.64	-1.94	-0.62	1.535814
RC(24 h)	29.32	31.22	1.90	3.22	0.1075
SI (48 h)	31.86	30.68	-1.18	7.14	0.007068
SC(48 h)	31.34	29.68	-1.66	6.66	0.009858
RI(48 h)	30.09	27.94	-2.15	-0.83	1.535814
RC(48 h)	31.62	29.93	-1.69	-0.37	1.294508
SI (72 h)	29.89	26.30	-3.59	4.74	0.037521
SC (72 h)	29.04	29.06	0.02	8.34	0.003076
RI(72 h)	28.04	30.08	2.04	3.36	1.78065
RC(72 h)	27.49	26.82	-0.67	0.65	0.638954
SI (96 h)	30.83	27.47	-3.36	4.96	0.03203
SC (96 h)	31.74	29.11	-2.63	5.69	0.019311
RI (96 h)	29.49	27.59	-1.9	-0.58	1.497342
RC (96 h)	29.15	28.14	-1.01	0.30	0.807987
SC00	32.60	24.28	-8.32	0.00 (NA)	NA
RC00	24.28	22.96	-1.32	0.00 (NA)	NA

* Data of the table are mean of three replications, SC00/RC00= susceptible/ resistant control at "0" h for comparison

Table 5 — Relative expression 0 to 96 h of β-1, 3-glucanase gene in inoculated resistant and susceptible maize inbred lines

Treatments	Ct value* mean (Actin)	Ct value* mean (β-1, 3-glucanase)	Δ Ct	ΔΔ Ct	Fold change
SI (24 h)	29.42	24.25	-5.17	3.15	0.112399
SC (24 h)	30.68	24.40	-6.27	2.05	0.24091
RI (24 h)	29.70	24.73	-4.96	1.10	0.467335
RC(24 h)	29.24	24.56	-4.67	1.38	0.38393
SI (48 h)	28.81	20.59	-8.22	0.10	0.925049
SC(48 h)	28.88	21.41	-7.47	0.85	0.556296
RI(48 h)	28.82	21.39	-7.42	-1.37	2.581914
RC(48 h)	28.00	21.56	-6.43	-0.38	1.300402
SI (72 h)	28.32	21.33	-6.99	1.34	0.396299
SC (72 h)	29.61	24.42	-8.19	0.14	0.907897
RI(72 h)	29.65	23.15	-6.50	-0.44	1.35348
RC(72 h)	28.63	22.65	-5.99	0.07	0.952219
SI (96 h)	29.67	21.47	-8.20	0.13	0.916327
SC (96 h)	30.10	22.12	-7.98	0.35	0.786156
RI (96 h)	30.09	21.99	-8.10	-2.04	4.747617
RC (96 h)	29.92	21.61	-8.31	-2.25	0.516837
SC00	24.57	31.72	-8.32	0.00 (NA)	NA
RC00	25.08	30.44	-6.06	0.00 (NA)	NA

* Data of the table are mean of three replications, SC00/RC00= susceptible/ resistant control at "0" h for comparison

Table 6 — Relative expression 0 to 96 h of pheophytinase gene in inoculated resistant and susceptible maize inbred lines

Treatments	Ct value* mean (Actin)	Ct value* mean (β-1, 3-glucanase)	Δ Ct	ΔΔ Ct	Fold change
SI (24 h)	24.22	29.63	5.42	-1.73	3.84777
SC (24 h)	26.01	31.21	5.21	-1.94	3.327642
RI (24 h)	24.35	30.24	5.89	0.52	0.695373

(*Contd*.)

Table 6 — Relative expression 0 to 96 h of pheophytinase gene in inoculated resistant and susceptible maize inbred lines (*Contd*.)

Treatments	Ct value* mean (Actin)	Ct value* mean (*β*-1, 3-glucanase)	Δ Ct	ΔΔ Ct	Fold change
RC(24 h)	24.94	31.07	6.13	0.77	0.586847
SI (48 h)	24.76	30.43	5.67	-1.49	3.991114
SC(48 h)	25.09	30.25	5.16	-2.00	2.803207
RI(48 h)	25.33	29.7	4.37	-0.99	1.984775
RC(48 h)	25.16	30.71	5.55	0.19	0.87912
SI (72 h)	23.99	30.82	6.83	-0.32	0.796789
SC (72 h)	24.11	31.59	7.48	0.33	1.250296
RI(72 h)	23.73	29.32	5.59	0.23	0.854764
RC(72 h)	24.45	29.87	5.42	-0.17	1.125058
SI (96 h)	24.39	30.84	6.45	-0.70	1.39694
SC (96 h)	25.11	31.78	6.67	-0.48	1.627063
RI (96 h)	25.35	30.76	5.41	0.05	0.968348
RC (96 h)	24.59	30.88	6.29	0.93	0.526169
SC00	24.57	31.72	7.15	0.00 (NA)	NA
RC00	25.08	30.44	5.36	0.00 (NA)	NA

* Data of the table are mean of three replications, SC00/RC00= susceptible/ resistant control at "0" h for comparison

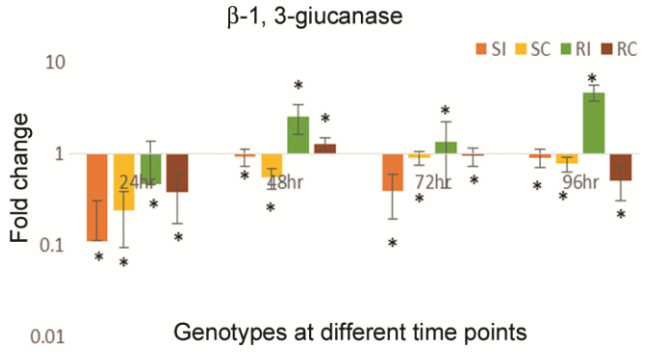

Fig. 7 — *β*-1, 3-gulcanase gene expression pattern during 24 to 96 hr compared to 0 hr in maize in resistant (SC-7) and susceptible (CM119) inbred lines. SI-susceptible inoculated, SC- susceptible control, RI-resistant inoculated and RC-resistant control, *- significance ($p < 0.05$)

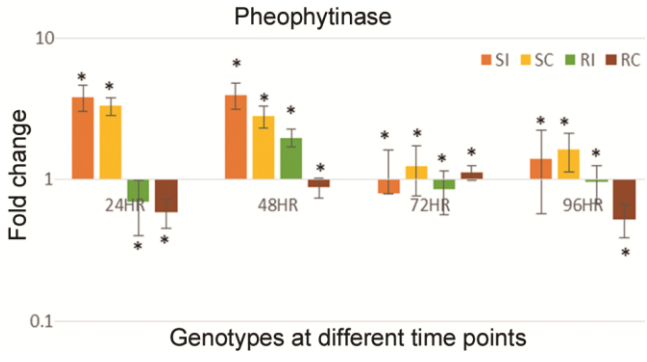

Fig. 8 — Pheophytinase gene expression pattern at 24 to 96 hr of inoculation against 0 hr control in maize inbred lines in SC-7 and CM119. SI-susceptible inoculated, SC-susceptible control, RI-resistant inoculated and RC-resistant control, *- significance ($p < 0.05$)

***In silico* Study of Protein- Protein Interaction**

In silico study conducted using STRING software version 11 for genes selected in present study to get a broad idea of genes of present study and their neighboring proteins (genes) which provide clue for their predicted interactions and functions among them (Fig. 9). It was illustrated to understand that which are the neighboring genes of selected genes (present study) that may get influence due to change in expression pattern.

Discussion

Response of the genes and compounds associated with MLB disease symptom development during susceptible and resistance reactions were investigated in two maize inbred lines of genetically different background after inoculation with the MLB pathogen *B. maydis*. The entire study was initially started with checking the ability of seeds to germinate, in which the resistant maize inbred line showed better germination ability than the susceptible inbred line under same environmental condition. Previous findings suggested a relation of seed germination capacity correlated with the stress response, since successful germination along with seedling establishment are the crucial aspects for maintenance of plant populations[21]. This physiological factor also helps in recovering from biotic and abiotic stresses during early stages of plant growth. This fact denoted that the seeds of resistant inbred lines possess inbuilt high vigour and germination capacity. Further, lowest

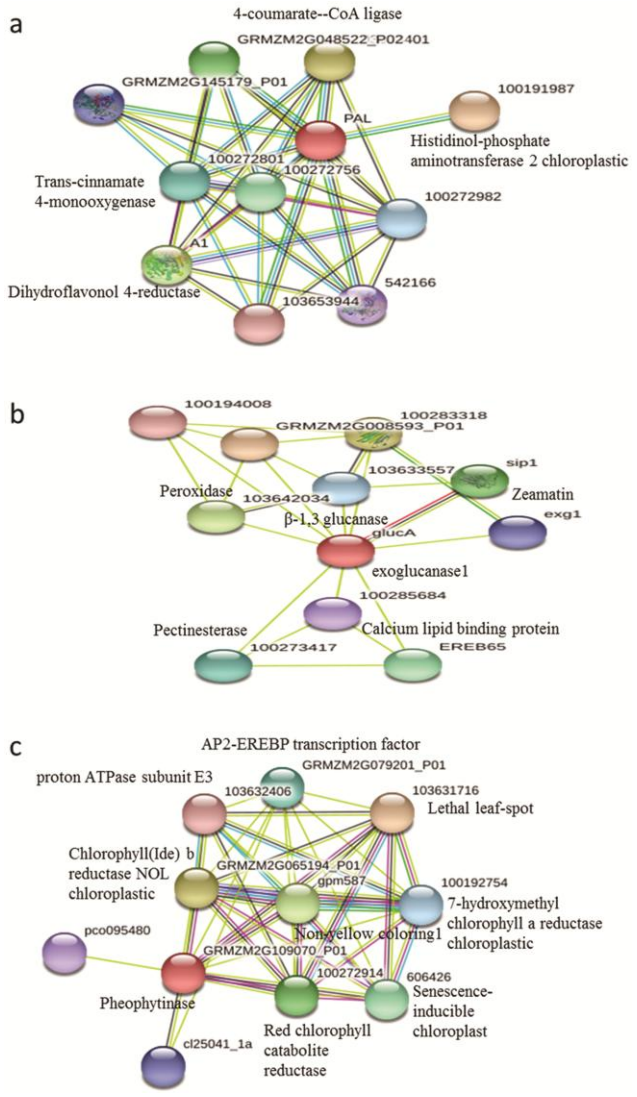

Fig. 9 — Protein-protein interaction network generated using STRING software version 11 which illustrate neighbouring gene (protein) of a) β 1-3, glucanase b) Phenylalanine ammonia lyase C) Pheophytinase. Green bond = neighbourhood, Red = gene fusions, black = coexpression

disease score confirmed SC-7 line as a highly resistant inbred line while CM 119 as the susceptible inbred line to the MLB disease based on the score of foliar disease symptoms. Earlier also grouped SC-7 and CM 119 in the MLB resistant and susceptible categories, respectively[22].

Sugar accumulated in plants in the form of starch has profound role in plant physiology. Changes in sugar accumulation can alter the response of plants towards biotic and abiotic stress[23]. It is a strong physiological determinant of plant fitness under stress condition[24]. In our study, resistant line (SC-7) was found to have higher starch content even under un-inoculated condition as compared to the susceptible lines (CM 119) (Figs. 1 & 2). On the other hand starch content was reduced after inoculation. It indicated degradation or probably less production of starch and sugar after infection. Interestingly ample amount of starch detected in resistant line even after infection suggested about having the capacity to maintain its starch content under stress condition too which provides vigour to plant and helps to resist disease or symptom development of MLB. High starch content is also an important factor in obtaining better yield in maize crop.

Phenylalanine ammonia lyase (*PAL*) gene is crucial for synthesis of many secondary metabolites that plays important role in growth and development of plants as well as provide resistance to plants against biotic and abiotic stresses[25]. In case of pepper infected by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), *PAL1* gene silencing showed high disease incidence due to suppression of phenyl propanoid pathway and confirmed the association of *PAL* gene with salycyclic acid (SA) dependent defence pathway. On the other hand, during interaction between *Fusarium oxysporum* f. sp. *ciceris* and chickpea applied with biocontrol agent *Trichoderma viride* enhanced expression of *PAL* gene along with other genes associated with phenyl propanoid pathway that denoted induction of *PAL* gene in presence of biocontrol agent[26]. In the present investigation, PAL enzyme activity was induced and enhanced in PAL content in resistant line after inoculation and indicated gene expression in non CMS maize host during infection of *B. maydis* race O at different time points. The fold change in the expression of PAL gene at different time intervals was also compared and found significantly high in the resistant inoculated line (RI) and its control (RC) plants as compared to susceptible inoculated (SI) and susceptible control (SC) lines. Plants of resistant line inoculated with pathogen showed more expression of the PAL gene than in the un-inoculated condition. This fact suggested that *B. maydis* is able to induce PAL activity at higher level in resistant line by means of which the host triggers defence through phenyl propanoid pathway to restrict the pathogen. This fact is supported in case of *Bipolaris zeicola* in maize[27] and *B. sorghicola* in sorghum[28], association of PAL gene was reported with respect to the disease severity and infection stages. The expression of *PAL* gene was maximum at 72 h in the inoculated resistant line SC-7 than in all

other time points, which suggests the activity of *PAL* gene is linked with the pathogen's developmental stages more specifically with disease phase when the histopathological and biochemical changes are likely to occur at cellular level. Hence it is opined that the PAL activity was highly functional in diseased phases which might have profusely contributed biosynthesis of secondary metabolites against this foliar pathogen *B. maydis*. PAL protein is neighbour of important proteins which may be directly and indirectly related to plant defence (Fig. 9a), i.e. 4- coumarate--CoA ligase and dihydroflavonol 4-reductase is related with phenylpropanoid derivative and it takes part in lignin and flavonoids synthesis[29]. Another important gene is trans-cinnamate 4-monooxygenase (putative cytochrome P450) which produces phenylacetaldoxime and indole-3- acetaldoxime and might contribute towards defence and auxin production[30].

Our study on induction of this PR protein *β*-1, 3-glucanase after fungal infection in maize host supports the previous reports on association of PR protein to govern host plant defence against invading pathogen. In several of host-pathogen interactions, activation of plant defence was reported[31]. Apart from maize-*B. maydis* system, *β*-1, 3-glucanase had been reported to be significantly enhanced upon infection with *Exserohilum turcicum* leading to northern blight in maize. Through various transcriptome studies, the noticeable role of *β*-1, 3 -glucanase had been proved to provide disease resistance (Liu *et al*. 2015, Yazawa *et al*. 2013). PR protein content was found high in the resistant line SC-7 after infection with *B. maydis* as compared to susceptible CM 119 line and un-inoculated control of both resistant (RC) and susceptible (SC) maize inbred lines.

Time course evaluation of *β*-1, 3-glucanase gene revealed increased expression in infected resistant line compared to 0 h. *β*-1, 3-glucanase activity increased at 48 h then slightly decreased at 72 h and regained highest expression at 96 h. It showed that the activity of PR protein changes with time and send the signal in host to resist the attack of *B. maydis*. Data also suggest that the infection on plants induces PR protein even in the susceptible line but the magnitude is less than that of the resistant line. High content of PAL enzyme and PR protein denoted the response of resistant plant to pathogen invasion and enhanced expression of the genes of PR protein and PAL at different time points in resistant lines. It indicated their association with the infection stages of *B. maydis* and thus the resistant lines were able to suppress disease establishment. Enzyme β 1-3, glucanase, a neighbour of peroxidase and calcium lipid binding protein (Fig. 9b) which mainly governs defence activity and signaling in plants during biotic stress[32]. Other important neighbour genes are related with cell wall synthesis and maintenance which is also an essential part of plant defence.

Photosynthesis is greatly affected by foliar pathogen[33]. In case of *B. maydis*, during the course of pathogen development, chlorophyll content is drastically affected, this subsequently turns into blight symptoms. Many genes such as Mg chelatase, glutamyl-tRNA reductase, glutamate-1-semialdehyde 2, 1-aminotransferase, 5-aminolevulinate dehydrogenase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase, protoporphyrinogen oxidase, Mg-protoporphyrin IX methyltransferase, Mg-protoporphyrin IX monomethyl ester cyclase, protochlorophyllide oxidoreductase and divinyl chlorophyllide 'a' 8-vinyl-reductase are involved in chlorophyll biosynthesis[34]. On other hand there are certain genes that are involved in chlorophyll degradation such as stay green gene, pheophytinase (PPH), chlorophyllase, hydroxymethyl chlorophyll a reductase, pheophorbide 'a' oxygenase and red chlorophyll catabolite reductase[35]. PPH is main enzyme in chlorophyll degradation pathway and senescence. PPH initiates the cleavage activity. PPH performs phytol-cleavage activity by converting pheophytin a substrates into phytol-free pigment pheophorbide 'a' after that chlorophyllase and 'Mg dechelatase act upon pheophorbide 'a' and produces colourless substance[36]. Total chlorophyll estimation showed high chlorophyll content in resistant plants SC-7 which suggests less chlorophyll degradation in plants, Further this was confirmed through time course evaluation of PPH gene which indicated decrease in activity of PPH enzyme in resistant maize inbred line and its un-inoculated control at 96 h when disease symptoms were appeared clearly in susceptible infected plant CM 119 where highest activity of PPH gene was detected. In contrast least activity was found in resistant control, it indicated least degradation of chlorophyll due to reduced chlorophyllase enzyme activity in resistant line (SC- 7) as compared to susceptible inbred line (CM 119), whereas PPH enzyme activity was higher in inoculated and non-inoculated susceptible plants.

It suggested that resistant plants have a tendency to resist chlorophyll degradation by influencing genes involved in the chlorophyll degradation pathway. Previous studies also demonstrated that in resistant varieties after recognition of pathogen, there was increased synthesis of certain metabolites, re-localization of sugars and pigments which involve increased biosynthesis of chlorophyll[37]. Our study added an additional information regarding genes of chlorophyll degradation pathway down regulated in resistant cultivar with respect to time after inoculation with *B. maydis*. Pheophytinase gene expression also indicated least degradation of chlorophyll due to reduced PPH enzyme activity in resistant line as compared to susceptible inbred line, whereas activity of PPH was more in inoculated and non-inoculated susceptible plants. This fact was directly correlated with the development of MLB disease symptom in maize plants. Pheophytinase protein's (gene) important neighbouring genes are lethal leaf spot 1 of maize in which mutation results in enhanced disease resistance to fungal pathogen *Cochliobolus heterostrophus* which reconfirms the fact that in resistant and susceptible plants differential expression of pheophytinase and associated genes are responsible for symptom development (Fig. 9c)[38]. Another pheophytinase neighbouring gene non-yellow coloring is related and senescence-inducible chloroplast stay-green protein 1 are associated thylakoid membrane degradation during senescence which shows their vital role in symptom development. Protein- protein interaction study gives a broad idea that genes of defence and susceptibility influence expression of neighbouring genes differentially in resistant and susceptible plants during biotic stress. Understanding the genetics of disease resistance in host plants is an important aspect to develop best and non-chemical based management strategy for aggressive pathogen like *B. maydis*. Application of fungicides harms the environment and simultaneously damage of soil health. Under this situation biological control and use of resistant varieties can offer eco- friendly management ways against various pathogens. Plant diseases which are difficult to manage using bioagents or for which no effective biological control microbes are reported, the host resistance approach is only left as a suitable eco-friendly option.

Conclusion

Non CMS maize lines where no direct resistance gene is reported against MLB pathogen race 'O' shows disease resistance as a result of interaction of genes at molecular (cellular) as well as biochemical levels. The resistant genotype was found to have tendency to restrict the pathogen at various stages of the pathogen's growth and development and finally inhibiting the disease establishment. It also suggests that before selection of a variety for cultivation, one should prefer the variety which is having strong physiological features such as high germination rate, chlorophyll, starch contents and products of phenyl propanoid pathway as biochemical marker. The present study leads to the next course of investigation on whole transcriptome analysis of the various stages of host-pathogen interactions to identify more genes and their role in providing disease resistance of the non-CMS maize host.

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