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Comparative gene expression analysis of wheat with special emphasis on myeloblastosis protein transcript in *Bipolaris sorokiniana*-wheat interaction

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Spot blotch disease (pathogen: *Bipolaris sorokiniana*) is one of the major constraints for wheat production in parts of India and the globe. The disease could be managed by growing resistant cultivars as one of the feasible options. Determination of variable expression of different defense-associated genes post-inoculation by *B. sorokiniana* in the resistant (UBW-9, BHU-35) and susceptible (Sonalika) wheat genotypes was targeted during 12-72 hours post-inoculation (hpi) using conventional reverse transcriptase-PCR and qRT-PCR analyses. The variable manifestation of the candidate genes in resistant and susceptible genotypes were scrutinized using Analysis of Variance, Dunn's post hoc analysis, heat map analysis and Principal Component Analysis Biplot. Among the twelve genes, four transcripts were up-regulated during 12-24 hpi whereas the eight genes including myeloblastosis protein gene were manifested through 48-72 hpi in the resistant (UBW-9, BHU-35) genotypes matching the highly susceptible Sonalika. To our knowledge this study could confirm the identity of genotype UBW-9 as a new resistant source against the Spot blotch of wheat caused by *B. sorokiniana*, which also could work as valuable tool for comparative functional genomic studies to unravel the molecular devices of pathogenesis of *B. sorokiniana* in wheat.

Key words: *Bipolaris sorokiniana*, defense-related genes, Myeloblastosis protein, Spot blotch disease, *Triticum aestivum*

INTRODUCTION

Wheat is a key contributor to the agricultural economy of India with an estimated annual production of 109.52 x 10^6 tons (GoI 2021a) from 30.6 x 10^6 ha of cultivated land (GoI 2021b) with a mean yield of 3.46 t/ha. In spite of considerable increase in production, disparities exist and the average yield in some geographic regions in India remains consistently low. Among the reasons for yield inconsistency is ineffective management of diseases, particularly the foliar blight of wheat. The disease is a severe menace in the eastern India. Among the different types of foliar blights affecting wheat, Spot blotch triggered by *Bipolaris sorokiniana* has been reported as early as 1914 (Herbarium Cryptogamae Indiae Orientalis = HCIO). However, its significance has been recognized only after the Green Revolution, when a majority of semi-dwarf wheat cultivars were noted to be susceptible to the disease (Gupta *et al*. 2017). Globally approximately 25 million ha is affected by Spot blotch (Chowdhury 2021), about 40% of which is in the Indian subcontinent (Joshi *et al*. 2007). The disease caused 15-20% grain loss in commercial farms in the early nineties. In the northern parts of West Bengal, the crop damage caused by the disease has been reported up to 43% in susceptible varieties (Chowdhury *et al*. 2008).

Foliar disease like Spot blotch can be managed by developing resistant varieties. Inheritance of its resistance has been noted in many genotypes of wheat and the resistance is governed by a few

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genes (Gupta *et al*. 2017). Although the use of disease-resistant genotypes is a key to environment-friendly and economically sustainable disease management in modern crop production, the employment of genetic resistance to minimize yield losses induced by *B. sorokiniana* remains largely unexplored in wheat (Gupta *et al*. 2017; McDonald *et al*. 2018).

In conventional breeding for resistance, the introgression of resistance genes from donor genotypes into the popular cultivars by repeated backcrossing is a long-term process that takes many generations before the backcrossing occurs (Gururani *et al*. 2012). Therefore, identifying the existing genotypes with resistant genes and studying the underlying genetics of defense to *B. sorokiniana* could potentially expedite the development of resistant varieties in breeding programs of wheat. Though a few genes for defense against *B. sorokiniana* are noted, as of now no commercial variety of wheat resistant to *B.sorokiniana* Spot blotch has been developed (Gupta *et al*. 2017; McDonald *et al*. 2018; Kang *et al*. 2021). For instance, comparative expression analysis of eight resistance-associated genes (Glucanase, Chitinase, Lipid transfer protein, PR-1, UDP-glycosyl transferase, Serine palmitoyl transferase, Translational control of tumor protein and dnaJ-like) were reported by Gurjar *et al*. (2018), that were highly expressed in resistant genotype in relation to susceptible cultivar. Wei *et al*. (2017) also reported that Myeloblastosis proteins (TaPIMP2) positively regulate defense in wheat against *B. sorokiniana* infection by modifying expression of a few Pathogenesis-Related (PR) genes. However, this investigation is the first of its kind to provide information regarding different levels of expression of genes in one susceptible and two high yielding resistant genotypes of wheat post-inoculation with *B. sorokiniana*.

With the objective of exploring the biological processes associated with the resistance mechanism of wheat against *B. sorokiniana*, we chose to analyze the levels of expression of fourteen defense-related genes in selected genotypes of wheat that possibly contribute towards developing defense against Spot blotch. Therefore, the three wheat genotypes that phenotypically exhibited resistance (UBW-9, BHU-35) and susceptibility (Sonalika) to Spot blotch, were used to scrutinize the transcriptional activity of Chitinase, Glucanase, Lipid Transfer Protein, PR-1 protein, UDP-glycosyltransferase, Translationally controlled tumor protein, Defensins, PR-10 protein, Myeloblastosis protein, Thaumatin-like protein, PR-2 protein, PR-5 protein, Lr34sus and Lr34res genes during infection caused by *B. sorokiniana*. This report provides the knowledge regarding different levels of gene expression in a hitherto unknown resistant wheat genotype against *B. sorokiniana* infection and unraveled the array of expression of the Myeloblastosis protein gene in resistant genotypes in relation to the susceptible genotype.

MATERIAL AND METHODS

Plant material and pathogenesis assay

The selected *B. sorokiniana* strain UBS-11 (Accession number: MW854818) was maintained in the Department of Plant Pathology and the Microbiology Laboratory, Directorate of Research, Uttar Banga Krishi Viswavidyalaya, West Bengal, was cultured on wheat leaf extract and potato dextrose agar (PDA) slants at 27$\pm$1°C. Based on a multi-locational experiment conducted by the authors (Chattopadhyay *et al*. 2021) it was established that, genotypes UBW-9 and BHU-35 showed highly resistant response and genotype Sonalika was susceptible against the different strains of *B. sorokiniana*. Ten-day old seedlings of UBW-9, BHU-35 and Sonalika genotypes were inoculated with UBS-11 isolate of *B. sorokiniana* following the methodology of Gurjar *et al*. (2018). Upon inoculation, the wheat plants were kept in growth chamber (VTECH make; Model: VTPGC-1; at 25±1°C; 100% relative humidity for 2 days followed by 25±1°C temp; 80% relative humidity for 7 days under 16 h light of *ca* 1200 lx and 8 h darkness alteration per day; Patsa *et al*. 2018). Leaves from uninoculated plants of the same genotypes served as controls. Samples of leaves from all genotypes were collected at 0, 12, 24, 26, 48 and 72 h post inoculation (hpi) using sterilized forceps and were kept in RNAlater stabilization solution [GCC Biotech (I) Pvt. Ltd.]. The samples were immediately stored at -20°C until used for RNA extraction.

Primer designing

Fifteen genes including fourteen genes associated with fungal disease of wheat/maize/sorghum and one housekeeping gene (Actin gene) were derived

from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) database. The primer pairs were designed using Primer 3.0 (Koressaar and Remm 2007). List of primers is provided in Table 1.

RNA extraction, quantification and cDNA synthesis

To determine the level of expression of pathogenesis-related protein genes in three genotypes, the leaf samples sourced from three individual plants of each genotype were used for extraction of total ribonucleic acid (RNA) using GSure® RNA isolation kit (GCC Biotech, India). RNA was estimated using Nanodrop and preserved at -20°C. The complementary Deoxyribonucleic acid (cDNA) was synthesized using R2DFast 1st strand cDNA synthesis kit (GCC Biotech, India) and stored at -80°C.

Targeted gene amplification and sequencing

Initially, total RNA isolated from genotype UBW-9 was employed for optimization of primer annealing temperature for amplification of the targeted genes through PCR (only 12 genes plus the house keeping actin gene amplified). The PCR was performed in a 25 μl reaction mixture that contained 2.5 μl of 10X PCR buffer (Thermo Fisher Scientific), 1 μl of 2.5 mM dNTPs mixture, 1 μl of each forward and reverse primers (100ng/ μl), 0.2 μl of 5U/μl Taq DNA polymerase (Thermo Fisher Scientific), 0.5 μl of cDNA, and 16.8 μl of water of HPLC grade (Fisher Scientific). The thermal profiles for the PCR included an initial heating for 2 min at 94°C, followed by 35 cycles of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 55°C to 65°C depending on the primers (Table 1), and extension for 0.5 min at 72°C with a final extension for 5 min at 72°C. The PCR amplicons were resolved on a 1.5% agarose gel by staining with ethidium bromide (0.05 mg/ml) and visualized followed by documentation under UV Gel Documentation Imaging System (QUANTUM-ST4, France). After confirmation of gene amplification, PCR products were purified with the help of GSure® PCR Purification kit (GCC Biotech, India) and were sent for sequencing to AgriGenome Labs Pvt. Ltd.

Quantitative Real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR was accomplished employing an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific) to examine the expression of twelve defense-related genes (except Lr34sus and Lr34res in the listed 14 genes due to no amplification of those two) isolated from three wheat genotypes viz., UBW-9, BHU-35 and Sonalika. Thermal cycle was accomplished using 0.5 μl first strand cDNAs and SYBR Green PCR Master Mix (GCC Biotech, India) with these conditions: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 15 sec, and extension at 72°C for 45 sec. Relative quantitation was calculated [ΔCt = Ct (Target) – Ct (Housekeeping); $\Delta\Delta$Ct = (ΔExp.) - (ΔControl) and fold change = $2^{-(\Delta\Delta Ct)}$] and normalized to housekeeping Actin gene.

Data analysis

The qRT-PCR results were scrutinized by a completely randomized factorial design with three replications and analysis of data was accomplished using the R Software version 3.6.1. Analysis of Variance (ANOVA) means comparison ($p<0.05$), heat map analysis and Principal Component Analysis Biplot were performed by Past 3 software.

RESULTS

Confirmation for presence of defense-associated genes against B. sorokiniana, causal organism of Spot blotch

Among the 14 target genes, 12 defense-related genes (except Lr34sus and Lr34res) were successfully amplified by conventional reverse transcriptase-PCR using RNA derived from the genotype UBW-9 of wheat. The amplicons were sequenced for confirming the identity of the genes. The nucleotide sequences of 12 genes were deposited in the NCBI-EST database. Basic Local Alignment Search Tool **(**BLAST) depicted that the queried genes had significant similarity with the genes of known function in the GenBank databases. Classification of the homology search and gene function indicated that these genes represented those that are related to intracellular metabolism and development, responses to biotic stress, transcription and signal transduction responses (Table 2).

ANOVA, Dunn's Post Hoc test and PCA-Biplot

Single factor Analysis of Variance showed that significant differences existed in the levels of expression of genes between resistant (UBW-9,

Table 1: Details of Expressed Sequence Tags (ESTs) derived primers used in this study

Primer Id	Sequence (5'-3')	EST accession no.	T_a(^oC)
PR-1 F	GCCAGCTACTACTCTCTCCG	HM489878.1	62
PR-1 R	AGGTATCCCATGCACGACTC		
TLP F	TTCCTCCTCCTGGCTGTTTT	KJ764822.1	54
TLP R	ATATCCTCCCGGCTTTGGTG		
CHI F	ACGGCGATATGGTTCTGGAT	AK457167.1	55
CHI R	TAGCGCTTGTAGAACCCGAT		
GLC F	CTACAGGTCCAAGGGCATCA	LR792829.1	55
GLC R	GCGGCGATGTACTTGATGTT		
DFN F	TGTCCAATAAGAACTGCGCG	AK446776.1	55
DFN R	TGGTTCCATGGGCTAGCTAG		
ACT F	GAGAAGCTCGCATATGTGGC	AK457930.1	55
ACT R	TCCAGCAGCTTCCATACCA		
LTP F	TGCTCTAAGATTCGCTGATCG	AK455582.1	55
LTP R	TGTGACCTCAACGTACTAAGC		
TPG F	TTCTTGTCAAAAGCAGGTTGC	XM_037571404.1	62
TPG R	AAGGAGCAGTTGATGTGGAC		
UPP F	CAAATGGTTTCTTGGAGGCTTC	HQ391124.1	55
UPP R	TCAATGGATGAGGCACAGTG		
Lr34res F	GGGAGCATTATTTTTTTCCATCATG	KU711572.1	55
Lr34resR	ACTGGCAGAAGAACCTTGAAACA		
Lr34sus F	GGGAGCATTATTTTTTTCCATCTT CAT	FJ436986.1	55
Lr34sus R	ACTGGCAGAAGAACCTTGAAACA		
TaPIMP2 F	GCATTGTACGGCCAGTTCG	KX683396.1	55
TaPIMP2 R	CGAGGAGGCTCTGTTCTTGG		
PR-2 F	CCGCACAAGACACCTCAAGATA	AK446628.1	55
PR-2 R	CGATGCCCTTGGTTTGGTAGA		
PR-5 F	ACAGCTACGCCAAGGACGAC	CP018168.1	55
PR-5 R	CGCGTCCTAATCTAAGGGCAG		
PR-10 F	CGTGGAGGTAAACGATGAG	LR778317.1	55
PR-10 R	GCTAAGTGTCCGGGGTAAT		

BHU-35) and susceptible (Sonalika) genotypes at 24 hpi, 48 hpi and 72 hpi but not at 12 hpi (Table 3). When pairwise differences of gene expression were tested using Dunn's Post Hoc analysis (Table 4), the data showed Sonalika and UBW-9 have significant differences at all time-points whereas, Sonalika and BHU-35 showed significant differences in respect of all the twelve gene expressions at 48 hpi and 72 hpi. Genotypes UBW-9 and BHU-35 showed significant differences at 24 hpi, 48 hpi and 72 hpi. Overall, based on the expression of the twelve genes of interest, Sonalika was established as the susceptible genotype whereas UBW-9 and BHU-35 were resistant genotypes.

Expression analysis by qRT-PCR

The transcript levels of the gene Chitinase increased (12, 24, 48 and 72 hpi) in leaves of the resistant genotypes, higher in UBW-9 than BHU-

Table 2: Details of sequenced target genes

Target gene name	Sequence	Similarity %	E value	Reference gene name accession	Function
Chitinase	GGTGAAATAC GGTTCGAACACGACGTGATCACAGGCC CTGTGGACCCCTACGCCGCCGACACGCAGCGGGGAG GTGCTGGTACGGCGTGATCACCAACATCATCAACGGC GGGATCGAGTGCGGCGCGGGCAGGACAACCGTGTTG CCGACCGGATCGGGTTCTACAAGCGCTAA	87.57	5×10^{-44}	Chitinase gene from *Triticum aestivum* (KR049250)	Protein Id: AKQ09033.1 Biological role: Chitinases are enzymes that catalyze the hydrolysis of the beta-1,4-N-acetyl-D glucosamine linkages in chitin polymers.
Defensin	GGGGGGGTAGGGCAAGGCAGGATGGGGCCGGCGGTA CTGCCAACGGGACCTTCCGCCGTGCAAGTGCATCAGG CAGTGCTGATCATATGTGCTACGGCTAGTTGGTCTTCG TCGTACGTCAGCTAGTTGGTCCATGAAACCAACAAAAA	87.22	2×10^{-31}	*Triticum dicoccoides* defensin-like protein 1 (XM_037582357)	Protein ID: XP_037438254.1 Biological role: Defense response to fungus and killing of cells of other organism
Glucanase	CAGCTGGGGATCGATCGTCGACGCGAGGACCTGTACG CGCTCCGCGGCTCCGGGATCGACCTCATCCTCGACAT CGGTACGACCACCTCTCCAACATCGCCGCCAGCACCT CCACGCGGCGTCCTGGGTCCAGAACAAAGTGAGGCCC TACTAGGACCTTGTGATAAAAAGAACATCGCAGCATCA ATTACTCAC ATGGCATTTTTCATCTTTTTATTCGTGCATC GTGAGGACTTTTTCTCGCAGAATATAGTTTGGCTGGAC GAGTAGATACATA TGCAACACCTAACGCGATGCAGAAG ACTGAGCACATCTTGGGATTGTCCCCGTTGTCGGACGA AAAATGACATGAGTTATAAATCGCTCAAAATCAAGTACA TCAGCCGCA	87.00	9×10^{-37}	*Triticum dicoccoides* glucan endo-1,3-beta-glucosidase GII-like (XM_037552426)	Protein ID: XP_037408323.1 Biological role: (1->3)-beta-D-glucan catabolic process and defense response to fungus
Myeloblastosis protein	GCGGCAGTCAGAGTATGGTTCCAGCTCCAAGTTGAAAT CGAACTTGCGAGGAGGCTCTGTTA	97.00	3×10^{-15}	*Triticum dicoccoides* myb-related protein (XM_037568725)	Protein ID: XP_037424622.1 Biological role: Transcription, Transcription regulation
UDP-glycosyltransferase	GGGGGTGTTCAGTCTCACATGCGAGTTGAGGTGTAGT GCAAGCGAGGCTTCCAAAACTCACAAAGATCACTGAGC CTGGCTTCCTTGAATCAAGCCATTG CAAGCACTGTGCC TCATCCATTGAA	100	4×10^{-43}	*Aegilops tauschii* subsp. *tauschii* UDP-glycosyltransferase (XM_020345200)	Protein ID: XP_020200789.1 Biological role: Para-aminobenzoic acid metabolic process and response to salicylic acid
LTP: Lipid Transfer Protein gene	CTAGCTATTTCATCATCTCTGCCTGAGCTCACCACCACT ACTAGTGCTAGCTAGCTTGATCGTGATGGCCCATTC TG CTGTTGCTCAGGTCGTGCTCGTCGCCGTGGTGGCCGC TATGCTCCTCGCAGCCACGGAGGCGGCCGTATCGTGC GGTCAGGTGAGCTCTGCCTTGAGCCCCTGCATCTCCTA TGCACGCGGCAACGGCGCCAACCCGTCTGCGGCCTGC TGCAGCGGCGTTAGGAGTCTAGC CAGCTCAGCCCGGA GCACCGCTGACAAG	100	0.00	*Triticum aestivum* lipid transfer protein precursor (AJ852555)	Protein ID: CAH69206.1 Biological role: They are small proteins characterized by a tunnel-like hydrophobic cavity, which makes them suitable for binding and transporting various lipids. The LTPs are abundantly expressed in most tissues.
TPG: Translationally controlled tumor protein gene	GTCTTCGTCGCCTGCTCCTGCGATGAGCTTCTGTCGGA TTCCTTCCCGTACAGGGAGTTGGAGAACGGCGTGCTCT GGGAAGTCGATGGCCATTGGGTCGTTCAAGGAGCAGT TGATGTGGA CATTGGAGCCAATCCCTCTGCTGAGGGTG GTGGTGACGATGAGGGTGTCGATGACCAGGCCGTGAA GGTGGTTGACATTGTTGACACCTTCCGTCTTCAGGAGC AACCTGCTTTTGACAAGAAGCAGTTTATCTCTCACATGA AGCGCTACATCAA	100	0.00	*Triticum dicoccoides* translationally-controlled tumor protein homolog (XM_037571404)	Protein ID: XP_037427301.1 Biological role: Calcium ion transport, cellular calcium ion homeostasis, negative regulation of apoptotic process, negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage, regulation of apoptotic process and response to virus
TLP: Thaumatin -like protein gene	AGCGCGGCCACCTTCTACATCAAGAACAACTGCGGCTC CACAATTTGGCCGGCGGGCATCCCGGTGGGTGGGGG CTTCGCGCTGGGCTCAGGCCAGACGGCCAGCATCAAC GTGCCCGCGGGCACCAAAGCCGGGAGGATATGGGCC CGCACCGGGTGCTCCTTCAATGGCGGTAGCGGGAGCT GCCAGACCGGCGACTGCGGAGGCCAGCTGTCCTGCTC CCTCTCCGGGCGGCCACCGGCAA	100	0.00	*Triticum aestivum* thaumatin-like protein (KJ764822)	Protein ID: AIG62904.1 Biological role: Defense response, response to bacterium and response to ethylene
Pathogenesis -Related protein 1	CCTCCATTTCCTAAGGCATCATTAGCGATGGAGTACTC GCCAAAGCTAGCGGCGGCACTGCTCTTAGCTCTCGCG TCCGCCATGATCGTCA CCGCCCAGAACGGGGCCGATG ACATGCTGAACGCCCACAATGAAGTGCGCGCCGCCGT CGGTGTGGGGCCAGTGACGTGGGACCCCATAGTGGCG GCGTACGCGCAGTCGTACGCGGAGAAGCGCCGCGCC GACTGCCAGCTACTACTCTCTCCGGAGGTGCGCCCATA CGGAGAGAACCTTTTT	100	0.00	*Triticum aestivum* pathogenesis-related protein 1 (HM489878)	Protein ID: AEI28607.1 Biological role: Defense response, response to vitamin B1, response to water deprivation and systemic acquired resistance
Pathogenesis -Related prote in 2	CGGCGCCGAGCGACGTCGTGAAGCTCTACCAAACCAA GGGCATCGATGCCATGCGGATCTACGCGCCGGAGAGC AACGTCCTCAAGGCGCTCAGCGGCACGGGCATCAGCC TCCTCATGGACGTCGGCAACGGCGCGCTAACCAGCCT CGCAAACGACCCCTCCGCCGCGCCCGCCTGGGTCAAG GCCAACGTGCAGCCCTTCCCGGGCGTCTCCTTCCGCT ACATCGCCGTCGGCAACGAGGTCACGGACAGCGCCGG CCAGAAGACCATCCTCCCGGCCATAAA	100	0.00	*Triticum aestivum* pathogenesis-related protein 2 (AK446628)	Protein ID: BAJ89985.1 Biological role: Abscisic acid-activated signaling pathway, defense response and response to biotic stimulus
Pathogenesis -Relat ed protein 5	TGTGGCCGCGCACCGGGTGCACCTTCGACGGCAGCG GCCGCGGCCGGTGCATCACTGGCGACTGCGGCGGCG CGCTGGCCTGCAGGGTGTCCGGCCAGCAGCCCACCAC GCTGGCCGAGTACACCCTTGGCCAGGGCGGAAACAAG GACTTCTTCGACCTGTCCGTCATCGACGGGTTCAACGT GCCCATGAGCTTCGAGCCCGTCGGCGGTTCGTGCCGC GCTGCGCGCTGCAGCACCGACATCACCAAGGAGTGCC TCAAGGAGCTGCAGGTGCCGGGAGGGTGCGCGAGCG CGTGCGGCAAGTTCGGCGGCGACACCTACTGCTGCCG GGGCCAGTTCGAGCACAA	100	0.00	*Triticum* aestivum pathogenesis-related protein 5 (PR-5) (AK332672)	Protein ID: XP_037439078.1 Biological role: Defense response, regulation of anthocyanin biosynthetic process, response to cadmium ion, response to UV-B, response to virus and systemic acquired resistance
Pathogenesis -Related protein 10	CTGGCACACACTGGCCCCTAAGCTCGCACCGCACATC GTCGCCAGCGCCCACCCTGTTGAGGGCGAAGGCGGCA TCGGCAGCGTCAGGCAGTTCAACTTCACCTCAGCCATG CCCTTCAGCCTCATGAAA	100	0.00	*Triticum aestivum* pathogenesis related protein 10 (EU908212)	Protein ID: ACG68733.1 Biological role: Abscisic acid-activated signaling pathway, defense response to fungus, regulation of protein serine/threonine phosphatase activity, response to abscisic acid, response to absence of light, response to auxin, response to bacterium, response to cold, response to copper ion, response to cytokinin, response to fungus, response to gibberellin, response to hydrogen peroxide, response to salicylic acid, response to salt stress and response to wounding

Table 3 : Single factor Analysis of Variance based on the level of gene expression in three wheat genotypes

Sources of variation	df	Mean of Sum Square				F value				P value			
		12hpi	24 hpi	48 hpi	72 hpi	12 hpi	24 hpi	48 hpi	72 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Among the genotype	2	0.27	0.33**	0.86**	0.62**	3.11	6.3	121.8	25.96	0.06	0.005	5.83×10^{-16}	1.68×10^{-7}
Within genotype	33	0.09	0.05	0.007	0.02								
Total	35	0.06	0.005	1×10^{-5}	1×10^{-5}								

**Significant at 1% level; df: degrees of freedom; hpi: hours post-inoculation

Table 4 : Dunn's Post hoc tests based on the level of gene expression in three wheat genotypes

Time	12 hpi			24 hpi			48 hpi			72 hpi		
Genotype	Sonalika	BHU-35	UBW-9	Sonalika	BHU-35	UBW-9	Sonalika	BHU-35	UBW-9	Sonalika	BHU-35	UBW-9
Sonalika		0.38	0.03*		0.46	0.002**		0.006**	2.69×10^{-8}**		0.03*	4.39×10^{-6}**
BHU-35			0.17			0.02*			0.005**			0.01**
UBW-9	0.03*	0.17		0.002**	0.02*		2.69×10^{-8}**	0.005**		4.39×10^{-6}**	0.01**	

*Significant at 5% level; **Significant at 1% level; hpi: hours post-inoculation

35 (Fig. 1A). Although the relative manifestation of Chitinase in Sonalika was significant at different hpi, but the manifestation level was lower than that of UBW-9 and BHU-35. Expression of Chitinase in UBW-9 and BHU-35 was up-regulated by about 14.8 and 6.4 folds in the leaves, respectively, in relation to the respective controls (Fig. 2A). Matching the observation in leaves, levels of transcript of Glucanase were notably elevated in leaves of UBW-9 and BHU-35 during 12-48 hpi compared to that in the respective controls that declined at 72 hpi (Fig. 1B). Glucanase transcript levels were considerably lower in the leaves of Sonalika in relation to UBW-9 and BHU-35. So, the resistant UBW-9 showed over seven-fold enhancement of Glucanase transcription at 48 hpi which was the strongest as compared to controls (Fig. 2B). The Lipid Transfer Protein gene had high expression at 12 hpi in Sonalika, which reduced at 24 hpi and further at 72 hpi. In contrast, the general manifestation remained down-regulated in the resistant genotypes (UBW-9, BHU-35) when matched with the susceptible one (Sonalika). The resistant genotypes showed substantial difference in expression of gene of Lipid Transfer Protein transcripts in different hours post-inoculation in wheat (Fig. 1C). In UBW-9 and BHU-35, PR-1 protein transcript level was highly up-regulated as early as 12 hpi (1.8 and 1.2 folds, respectively), which steadily rose to a peak at 72 hpi (10.56 and 8.0 folds, respectively) (Fig. 1D). Strikingly, highly susceptible Sonalika showed low expression of PR-1 protein consistently from 12 hpi to 72 hpi. The expression of PR-2 protein gene notably increased in *B. sorokiniana* -infected leaves of all genotypes at 12, 24 and 48 hpi while the general manifestation remained lower in Sonalika in relation to UBW-9 to BHU-35 at all the time points. PR-2 protein transcript was progressively up-regulated thereby peaking at 48 hpi and declined thereafter at 72 hpi (Fig. 2A). The PR-5 protein was up-regulated in *B. sorokiniana* -infected leaves of all genotypes in the first 48 hpi followed by a decrease at 72 hpi; expression of the gene remained lower in the highly susceptible genotype (Sonalika) in relation to the resistant ones (UBW-9, BHU-35). The manifestation level of PR-5 protein in the leaves of UBW-9 and BHU-35 increased by 5.6 folds and 4.5 folds, respectively at 48 hpi in relation to the respective controls (Fig. 2B). The PR-10 protein transcript showed up-regulation at 12 hpi in the resistant genotypes that peaked to 10.2 and 7.3 folds, respectively in UBW-9 and BHU-35 at 72 hpi compared to the respective controls (Fig. 2C). The level of expression of PR-10 protein gene remained low in the susceptible genotype at all the time points. Up-regulation of the Defensin gene (Fig. 2D) was highest at 72 hpi compared to control (3.0 and 2.5 folds in UBW-9 and BHU-35, respectively); however the level of manifestation remained low in Sonalika. In relation to the respective controls, the Thaumatin-like protein gene had increased

expression levels during 12-72 hpi in UBW-9 and BHU-35 that was higher than the expression in Sonalika (Fig. 3A). Expression of the Thaumatin-like protein transcript in the leaves of UBW-9 was 6.2 folds higher at 72 hpi compared to the control (Fig. 3A). The Translationally controlled tumor protein gene was strongly up-regulated in leaves of the three genotypes at 12 hpi compared to respective controls that decreased markedly during 24-72 hpi; expression in UBW-9 > BHU-35 >

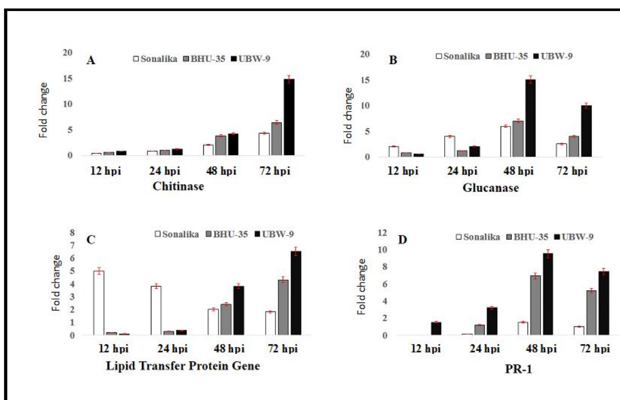

Fig. 1 : Expression patterns of defense-related genes (A: Chitinase; B: Glucanase; C: Lipid transfer protein; D: PR-1 protein) in wheat genotypes (Sonalika, BHU-35, UBW-9) over hours post-inoculation (hpi).

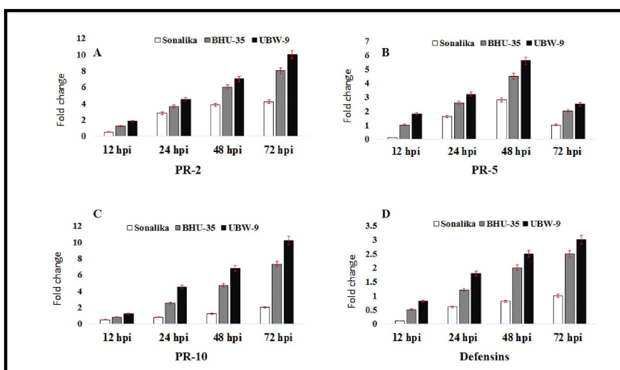

Fig. 2: Expression patterns of defense-related genes (A: PR-2 protein; B: PR-5 protein; C: PR-10; D: Defensins) in wheat genotypes (Sonalika, BHU-35, UBW-9) over hours post-inoculation (hpi).

Sonalika (Fig. 3B). The UDP-glycosyltransferase gene was up-regulated in *B. sorokiniana* -infected leaves of all genotypes in the first 12 hpi compared to respective controls followed by a consistent decrease from 24 hpi to 72 hpi; expression was highest in leaves of UBW-9 at 12 hpi compared to other genotypes (Fig. 3C). The transcript levels of TaMPI2A increased in leaves of wheat genotypes during 12-24 hpi compared to respective controls that decreased from 48 hpi to 72 hpi (Fig. 3D). The levels of expression of TaMPI2A in leaves of UBW-9 at 12 hpi and 24 hpi was up-regulated by 38 and 65 folds, respectively, in BHU-35 by 25 and 50 folds, respectively, in relation to the controls. Interestingly, the TaMPI2A gene expression rose gradually with time, being highest in UBW-9 followed by BHU-35 and Sonalika at all the time points. The higher level of manifestation of genes PR-5, TaPIMP2 and Chitinase in UBW-9 and BHU-35 suggests that they are responsible for a large part of defense in wheat against *B. sorokiniana*

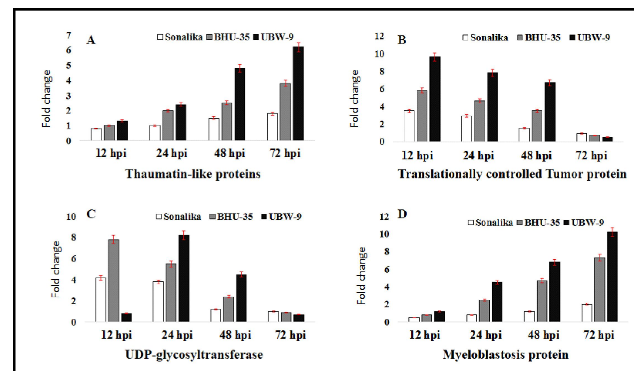

Fig. 3: Expression patterns of defense-related genes (A: Thaumatin-like proteins; B: Translational control of tumor protein; C: UDP-glycosyltransferase; D: Myeloblastosis protein) in wheat genotypes (Sonalika, BHU-35, UBW-9) over hours post-inoculation (hpi).

infection. The differential manifestations of these genes may lead to the difference in resistance levels among the three different genotypes.

DISCUSSION

Arrays of expression of genes can provide important clues for the analysis of defense-related genes associated with the process of resistance. It is believed that genes that exhibit similar pattern of expression are expected to be associated in relation to their functionality (Qi *et al*. 2018). Moreover, relative analysis of the array of expression of these genes in the three different wheat genotypes with varying degrees of resistance in response to *B. sorokiniana*, helps in better understanding of host-pathogen interaction in the specific case. The manifestation of the UDP-glycosyltransferase, Translationally Controlled Tumor Protein, Thaumatin-Like Protein and PR-5 protein genes at the early stage (12 -24 hpi) and up-regulation of Chitinase, Glucanase, PR-1, PR-10, PR-2, Defensin, Lipid Transfer Protein and Myeloblastosis Protein Transcripts at the later stage (48-72 hpi) in challenged UBW-9 and BHU-35 in relation to Sonalika may explain the high level

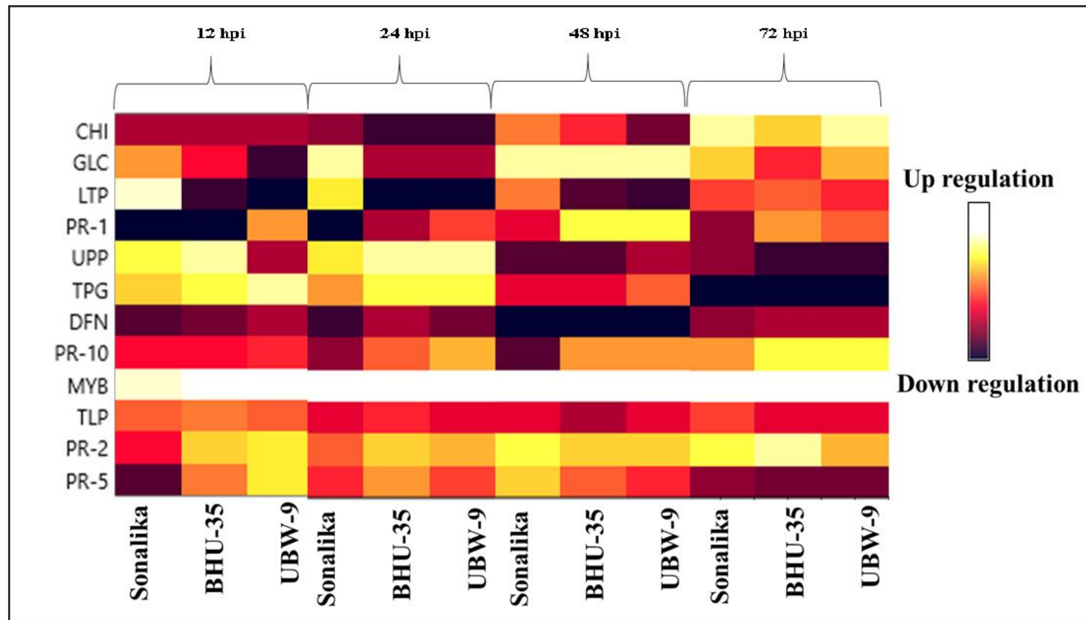

Fig. 4: Comparison of expression pattern of twelve defense-related genes (CHI: Chitinase gene; GLC: Glucanase gene; LTP: Lipid Transfer Protein gene; PR-1 Protein; UPP: UDP-glycosyltransferase gene; TPG: Translationally controlled tumor protein gene; DFN: Defensins gene; PR-10 protein; MYB: Myeloblastosis protein; TLP: Thaumatin-like protein gene; PR-2 protein; B: PR-5 protein) in three wheat genotypes (Sonalika, BHU-35, UBW-9) over hours post-inoculation (hpi).

of resistance in the resistant genotypes (Fig. 4). Evidences in this investigation suggest a critical role of the Chitinase transcript in regulating defense-associated genes for resistance against spot blotch (Gurjar *et al*. 2018). The antifungal activity of chitinases seem to be linked with the function of β-1,3-glucanases that result in weakening of fungal cell walls (Kasprzewska, 2003). For instance, Chitinase genes are up-regulated during early infection of wheat and barley spikes by *F. graminearum* and *B. sorokiniana* (Theis and Stahl 2004; Aradhika *et al*. 2013; Manghwar *et al*. 2018; Gurjar *et al*. 2018). In this investigation, the expression of Glucanase gene from wheat was induced 48 hpi by *B. sorokiniana* and high level of expression of the gene was observed in UBW-9 and BHU-35 that is in accordance with previous reports (Theis and Stahl, 2004; Gurjar *et al*. 2018; Manghwar *et al*. 2018). So the plants constitutively expressing Glucanase gene were resistant to Spot blotch. The Lipid Transferase Protein (LTP) gene was up-regulated in BHU-35 and UBW-9 at 72 hpi with *B. sorokiniana* in comparison to Sonalika. The mechanism accountable for the antifungal activity of the LTPs remains unidentified, although it was proposed that these proteins insert themselves into the fungal cell membrane allowing efflux of intracellular ions that lead to fungal cell death (Selitrennikoff 2001). The present finding is in accordance with previous studies that demonstrated the association of LTP genes in response to resistance against *B. sorokiniana* (Gurjar *et al*. 2018; Manghwar *et al*. 2018). Thus, LTPs may be linked with spot blotch disease resistance in UBW-9 and BHU-35 by systemic acquired resistance (Maldonado *et al*. 2002; Ferreira *et al*. 2007). Genes of different PR proteins like PR-1, PR-2, PR-5 and PR-10 were highly up-regulated in UBW-9 and BHU-35 at early stages of infection by *B. sorokiniana* in relation to Sonalika, which indicated that these proteins have important function in defense mechanism against *B. sorokiniana* in wheat; these observations are in tune with previous findings (Anzlovar and Dermastia, 2003; Aradhika *et al*. 2013; Rather *et al*. 2015; Gurjar *et al*. 2018; Manghwar *et al*. 2018). The higher manifestation of Defensin (DFN) gene was recorded in the resistant genotypes in difference to the susceptible one at 12, 24, 48 and 72 hpi with *B. sorokiniana* as compared to controls that match previous reports (Stotz *et al*. 2009; Manghwar *et al*. 2018). In this investigation, higher manifestation of the Thaumatin-Like Proteins (TLPs) was noted in UBW-9 and BHU-35 during 12-72 hpi against *B. sorokiniana* infection in comparison to controls. Previously, expression of TLP genes in plants including wheat were reported to provide enhanced tolerance to fungal pathogens (Velazhahan and Muthukrishnan 2004;

Manghwar *et al*. 2018). The TPG (Translational control of tumor protein) gene expressed at higher levels in UBW-9 and BHU-35 in relation to Sonalika at 12 hpi; however, its expression declined at 72 hpi. The manifestation of the TPG gene was earlier demonstrated to decrease in response to infection by *B. sorokiniana* (Fabro *et al*. 2008; Gurjar *et al*. 2018), which was higher in the resistant genotypes in the initial hours that possibly indicated its possible role in resistance of UBW-9 and BHU-35 against *B. sorokiniana*. The UDP-glycosyltransferase (UPP) gene was highly manifested at 12 hpi in BHU-35 and at 24 hpi in UBW-9 followed by a decline at 48 and 72 hpi. Expression of UPP gene was highly prompted in resistant genotype of wheat in response to challenge by *Fusarium* (head blight) and *B. sorokiniana* which indicated the rapid response of this genotype to fungal attack (Kosaka *et al*. 2015; Gurjar *et al*. 2018). The transcripts of TaPIMP2 gene (encoding a pathogen-induced myeloblastosis protein in wheat) was highly manifested in resistant wheat genotypes at all the time-points in relation to the susceptible one that matched the observation of Wei *et al*. (2017).

The qRT-PCR was accomplished to examine the manifestation of the fourteen defense-related genes against *B. sorokiniana* in the three wheat genotypes. Among the expression profiles of fourteen transcripts in resistant genotypes, TaPIMP2 played a key role in different responses of wheat to challenge by *B. sorokiniana.* The findings from this study on wheat *B. sorokiniana* interaction are useful in understanding the defense mechanism of wheat against spot blotch disease such as the role of TaPIMP2 gene encoding myeloblastosis (MYB) protein in wheat for disease resistance. To our knowledge, this report is the first on examining the mRNA expression profiles of candidate defense-associated genes in wheat in response to challenge by *B. sorokiniana.* The study also could confirm the identity of genotype UBW-9 as a new resistant source against the spot blotch of wheat caused by *B. sorokiniana*, which also has potential of a commercial variety and unraveled the array of expression of the MYB gene in resistant genotypes in relation to the susceptible genotype.

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