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H3K4/K9 acetylation and *Lr28*-mediated expression of six leaf rust responsive genes in wheat (*Triticum aestivum*)

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Abstract

Development of leaf rust-resistant cultivars is a priority during wheat breeding, since leaf rust causes major losses in yield. Resistance against leaf rust due to *Lr* genes is partly controlled by epigenetic modifications including histone acetylation that is known to respond to biotic/abiotic stresses. In the present study, enrichment of H3K4ac and H3K9ac in promoters of six defense responsive genes (*N-acetyltransferase, WRKY 40, WRKY 70, ASR1, Peroxidase 12* and *Sarcosine oxidase*) was compared with their expression in a pair of near-isogenic lines (NILs) for the gene *Lr28* following inoculation with leaf rust pathotype '77-5'; ChIP-qPCR was used for this purpose. The proximal and distal promoters of these genes contained a number of motifs that are known to respond to biotic stresses. The enrichment of two acetylation marks changed with passage of time; changes in expression of two of the six genes (*N-acetyltransferase* and *peroxidase12*), largely matched with changes in H3K4/H3K9 acetylation patterns of the two promoter regions. For example, enrichment of both the marks matched with higher expression of *N-acetyltransferase* gene in susceptible NIL and the deacetylation (H3K4ac) largely matched with reduced gene expression in resistant NIL. In *peroxidase12*, enrichment of H3K4ac and H3K9ac largely matched with higher expression in both the NILs. In the remaining four genes, changes in H3 acetylation did not always match with gene expression levels. This indicated complexity in the regulation of the expression of these remaining four genes, which may be controlled by other epigenetic/genetic regulatory mechanisms that need further analysis.

Keywords Bread wheat · Leaf rust resistance · Epigenetics · Histone modifications · Acetylation · Chromatin immunoprecipitation (ChIP)

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Introduction

In wheat, leaf rust, caused by *Puccinia triticina* is an important disease, which is responsible for major losses in yield (Huerta-Espino et al. 2011). The resistance for this disease can be either seedling resistance (SR) or adult plant resistance (APR), each caused by a resistance (R) gene. There are about 80 known *Lr* genes in wheat (McIntosh et al. 2017), which provide resistance in a race-specific manner, there being an avirulence (*Avr*) gene for each *Lr* gene in the corresponding race/pathotype of the fungus. In addition to the R gene providing resistance to the host, there are numerous genes, which either help the pathogen in the absence of R gene or facilitate cultivar's defense in the presence of R gene. The study of these genes (other than R genes in the host and Avr genes in the pathogen) involved in infection/defense has been an important area of research. The genes involved in infection or defense are also known to undergo

epigenetic modifications, which influence their expression. The types and levels of these epigenetic modifications and their effect on expression of specific genes are poorly understood. These epigenetic modifications mainly include DNA methylation and histone modifications (methylation, acetylation, sulphonation, uniquitination, etc.) although non-coding RNAs (e.g., miRNAs and lncRNAs) represent another modification. While DNA methylation includes cytosine methylation in CG, CHG and CHH contexts, the histone methylations/acetylations take place in different amino acid residues of each of the five histone proteins (H1, H2A, H2B, H3, H4) that occur in the form of histone octamers in the nucleosome subunits of the chromatin.

Histone modifications are mainly involved in chromatin-based gene regulation, which affects developmental processes and also respond to pathogen's attack. This contributes significantly to plant immunity against all kinds of bacterial and fungal pathogens (Ding and Wang 2015). Among all histone modifications, acetylation of specific lysine residues in H3 histone protein is an important mark that occurs in all eukaryotes ranging from yeast to higher plants and mammals. A simple model for the function of histone acetylation suggests that acetylation neutralizes the positive charge on lysine side chains and thereby reduces interaction with the negatively charged DNA backbone. The chromatin thus gets decompacted, and the associated DNA becomes accessible to a variety of binding proteins (including transcription factors) that are involved in basal transcription and its regulation (Imhof and Wolffe 1998). However, in plants infected with fungal pathogens, the importance of such modifications has only just begun to be appreciated, so that only a handful of studies are available, which examined the role of epigenetic modifications in the form of histone acetylation in bacterial/fungal pathogenesis (Gomez-Diaz et al. 2012; Ayyappan et al. 2015).

Histone acetylation/deacetylation is often necessary for induced expression /repression of genes that are spatially or temporally regulated. This is a reversible process, so that histone acetyltransferases (HATs) cause acetylation, whereas histone deacetylases (HDACs) remove acetyl groups. Recently, in wheat, a detailed profiling of lysine acetyl proteome partially elucidated the role of histone lysine acetylation in different physiological processes including photosynthesis and its associated Calvin Cycle, etc. (Zhang et al. 2016a). For instance, developmental and environmental signals have been shown to induce distinct histone modifications on distal and proximal promoter elements of the maize gene *C4-Pepc* encoding pep-carboxylase (Offermann et al. 2008).

Acetylation of lysine residues in H3 histone associated with promoter regions of target genes is relatively frequent and induces expression of associated genes. More important is the acetylation in H3 histone associated with the distal promoter, which plays an important role in transcriptional regulation in a quantitative manner. Acetylation of H3 associated with the proximal promoter and the coding region seems to be uncommon and occurs mostly in a selective manner in response to biotic and abiotic stresses (Latrasse et al. 2017). Among plants, the role of such acetylation of lysine residues in H3 has largely been documented in model plant *Arabidopsis*, particularly under abiotic stresses such as drought. In rice also, which is another model plant system, an increase in expression of four different HATs (a large number of HATs are known) was observed in response to drought stress (Fang et al. 2014). Relative to abiotic stresses, fewer reports are available, where histone modifications have been associated with response against a pathogen attack. One such example in rice includes association of monoubiquitinated H2A and H2B histones with the promoter regions targeted by gene *BRHIS1* (encoding SNF2 ATPase) leading to suppression of immunity against the fungal pathogen causing blast (Li et al. 2015).

Acetylation of histone H3 at lysine residues 4 and 9 (H3K4ac and H3K9ac) are two important epigenetic marks that are involved in chromatin organization and transcription of genes associated with plant acclimation and development (Zhou et al. 2010; Hu et al. 2011; Zhang et al. 2011). Genome-wide ChIP experiments in yeast, revealed that H3K4ac is generally associated with upstream promoter region of active genes (Guillemette et al. 2011), a pattern which has also been conserved in many human CD4^{+} T-cell promoters (Wang et al. 2008). Mutant analysis also revealed that promoters of highly transcribed genes are enriched with H3K4ac, which plays an active role in regulation of transcription (Guillemette et al. 2011; Jaskiewicz et al. 2011), although H3K4ac often functions in association with H3K4me3 (Guillemette et al. 2011).

H3K9ac is another well-characterized epigenetic chromatin mark which regulates expression of some genes which respond to stresses in plant systems. It is often associated with actively transcribed genes, thus influencing numerous biological processes in higher plants (Benhamed et al. 2006; Lang-Mladek et al. 2010; Zhou et al. 2010; Bilichak et al. 2012). In rice, an increase of H3K9 acetylation at the upstream promoter regions of *OsDREB1* was associated with response against cold treatment, as resolved through ChIP analysis (Roy et al. 2014). Sometimes, H3K9 acetylation associated with H3K4 methylation (H3K4me3/H3K9ac) also plays a positive role in gene activation/regulation, but this is certainly not the only combination that directly regulates gene expression. There are several other combinatorial histone modifications that fine-tune the gene expression during abiotic/biotic stresses. There are also reports, where association of promoter sequences with H3K9ac was not correlated with gene expression levels (Tian et al. 2005). Such discrepancies suggest that we do not fully understand the complex

interactions among various epigenetic marks that regulate gene expression (Zhou et al. 2010; Bilichak et al. 2012; Song and Noh 2012).

Although only a handful of studies are available, which are aimed at understanding an epigenetic control of fungal pathogenesis, some recent studies have suggested that histone acetylation/deacetylation is a crucial regulatory mechanism during onset of specific plant diseases (Jeon et al. 2014). Some reports on the role of epigenetic modifications are also available in wheat, which include the following: (i) Difference in DNA methylation pattern between Thatcher and its NIL for *Lr41* gene as examined through MSAP (Fu et al. 2009). (ii) tae-miR159-mediated regulation of the expression of *taMyb3* during infection of wheat with Chinese race of *Puccinia striiformis* f. sp. *tritici* (Pst) (Feng et al. 2013). Reports are also available for histone modifications in wheat although these do not involve biotic or abiotic stresses. In one of these studies, increased level of H3K9me2 and decreased level of H3K4me3 and H3K9ac were found to be involved in silencing of expansin genes (*TaEXPA1*) in roots which were responsible for regulation of cell size as well as organ size (Hu et al. 2013a). In another study, ChIP was performed to confirm the binding of the transcription factors NAC2A to the promoter regions of genes encoding nitrate transporter and glutamine synthase (He et al. 2015). Similarly, high expression of the gene *TaGS2-B* encoding glutamine synthase (involved in ammonium assimilation) in leaves was found to be correlated with increased H3K4me3 levels (Zhang et al. 2017) It is thus obvious that much remains to be learnt to fully understand the role of epigenetic modifications in imparting resistance against specific diseases in wheat.

The present study was undertaken with the aim to study the epigenetic changes associated with onset of leaf rust disease in a pair of wheat NILs (HD2329 and HD2329 + *Lr*28) differing for the gene *Lr28* for leaf rust resistance. We hoped that this study will provide information that will contribute to a better understanding of the mechanism involved in the complex process of resistance against leaf rust. In this connection, we earlier conducted transcriptome analysis through RNA-Seq and identified a large number of differentially expressed defense responsive genes (Sharma et al. 2018). Six differentially expressed genes that were selected for this study encode the following proteins: (i) N-acetyltransferase, (ii) WRKY 40, (iii) sarcosine oxidase, (iv) WRKY 70, (v) peroxidase 12, and (vi) Asr1 (ABA-stress-ripening). These six genes were selected on the basis of their differential expression in our earlier transcriptome study, and also keeping in mind that all the six genes are known to respond to biotic/abiotic stresses; availability of full genomic sequences of all the six genes in the database was another reason for their selection. These six genes were used for ChIP-qPCR following chromatin immunoprecipitation

(ChIP) with antibodies against the two histone acetylation marks (H3K4ac and H3K9ac). The study provided information about the possible role of these marks in expression of each of the six genes in response to leaf rust infection in the presence and absence of the leaf rust resistance gene *Lr28*.

Materials and methods

Wheat genotypes and leaf rust pathotype

Two bread wheat (*Triticum aestivum* L.) genotypes including a leaf rust susceptible cv. HD2329 and its resistant near-isogenic line (NIL), HD2329 + *Lr*28, carrying a dominant seedling leaf rust resistance gene *Lr28*, were used for ChIP-qPCR analysis. Single spore derived inoculum of the most prevalent and virulent pathotype 77-5 (syn. 121R63-1, avirulent against *Lr28*) of the leaf rust pathogen *Puccinia triticina* Erikss in South Asia, was procured from Regional Station, ICAR-Indian Institute of Wheat and Barley Research, Flowerdale, Shimla.

Inoculation of wheat seedlings and collection of leaf samples

The seedlings of the two NILs (as above) were raised in growth-chamber under controlled conditions of 16 h light (240 µmol m^{-2} s^{-1} light)/25 °C and 8 h dark/18 °C at the National Phytotron Facility, ICAR-Indian Agricultural Research Institute (IARI), New Delhi. Two-week-old seedlings were inoculated with leaf rust inoculum (spore and talc powder in 1:10 ratio) of the pathotype 77-5 with the help of a CAMEL hair brush (0 size) (Michigan Brush Manufacturing Company Inc., New Delhi, India); the inoculum was evenly spread on moistened open leaves of each seedling. The pots carrying the seedlings were well watered and covered with water saturated polythene to maintain relative humidity (RH > 95%). Following inoculation, the seedlings were kept in a dark humid chamber for 36 h at the optimal temperature (23 ± 2 °C) for pathogen's growth. Thereafter, normal conditions were restored. Leaf samples from seedlings of the susceptible and resistant NILs were collected in three biological replicates at 0 h before inoculation (hbi) and 96 h after inoculation (hai); 96 hai was chosen since in our earlier study abundance of differentially expressed transcripts was observed between the resistant and susceptible NILs at this time point (Dhariwal et al. 2011).

Sample fixation and cross-linking

Leaf samples (1–2 g) were cut into small pieces and fixed in 37 ml of cross-linking buffer [1% formaldehyde, 0.4 M sucrose, 10 mM Tris–HCl (pH 8), 1 mM EDTA, 1 mM

PMSF], mixed properly and kept on ice. Samples were then placed under vacuum for 10 min for cross-linking, which was stopped by adding 2.5 ml of 2M glycine (final concentration 0.125 M) and by keeping the samples in vacuum for another 5 min. Buffer was removed and samples were washed three times using sterile distilled water. Excess water was removed using paper towel and samples were quickly frozen in liquid nitrogen and stored at − 80 °C till further use.

Chromatin isolation and shearing

Chromatin isolation was undertaken following Gendrel et al. (2005) with minor modifications. Following steps were involved: (i) Fixed and frozen leaf tissue was ground into fine powder with the help of mortar and pestle in liquid nitrogen. (ii) 30 ml of pre-chilled extraction buffer #1 [0.4M sucrose, 10 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 5 mM β-ME, 0.1 mM PMSF, 1% protease inhibitor cocktail (Sigma-Aldrich)] was added to fine powder in a 50-ml falcon tube. Tube was vortexed, and the contents mixed properly and kept on ice for 5 min. (iii) The solution was filtered twice through double layers of miracloth (Millipore) and transferred to a fresh 50-ml falcon tube and filtrate was centrifuged at 3000g at 4 °C for 20 min. (iv) The supernatant was discarded, and pellet was re-suspended in 1 ml extraction buffer # 2 [0.25M sucrose, 10 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 5 mM β-ME, PMSF (0.1 mM), 1% Triton X-100, 1% protease inhibitor cocktail (Sigma-Aldrich)]. (v) The re-suspended pellet was then transferred into a new 1.5-ml micro-centrifuge tube and centrifuged at 12,000g at 4 °C for 10 min; a white pellet appeared at the bottom. (vi) Supernatant was removed, and pellet was re-suspended in 300 μl extraction buffer #3 [1.7 M sucrose,10 mM Tris–HCl (pH 8), 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-ME, 1% protease inhibitor cocktail (Sigma-Aldrich)]. (vii) In a fresh 1.5-ml micro-centrifuge tube, 300 μl of extraction buffer #3 was carefully used to over-lay the re-suspended pellet from step (vi) and centrifuged at 16,000g at 4 °C for 1 h. (viii) Supernatant was removed and chromatin pellet was re-suspended in 300 μl nuclei lysis buffer [50 mM Tris–HCl (pH 8), 10 mM EDTA, 1% SDS, 1% protease inhibitor cocktail (Sigma-Aldrich)]. (ix) Sonication of the re-suspended chromatin was standardized for different time intervals (2 min, 5 min, 8 min, and 10 min) using covaris S-220 (Covaris, Inc., USA) with the following settings: duty cycle—2%, intensity—3, peak incident power—105 w, cycle per burst—200, sample volume—130 μL, water level—12 and temperature 4 °C, time—2 to 10 min. (x) The sonicated (sheared) chromatin was collected and centrifuged for 10 min at 12,000g at 4 °C; supernatant was collected and stored in 50 μl aliquot at − 80 °C.

Magnetic immunoprecipitation

For each immunoprecipitation reaction, 20 μl DiaMag Protein A-coated magnetic beads (Diagenode, cat no: C03010020-660) were transferred to fresh 1.5-ml micro-centrifuge tubes and washed four times with 1 ml of ice-cold ChIP dilution buffer [1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, 167 mM NaCl and 1% protease inhibitor cocktail (Sigma-Aldrich)] on magnetic rack (DynaMag-2, cat no: 12321-D, Invitrogen). Following the last wash, the beads were re-suspended in 100 μl of ChIP dilution buffer and 5 μg of anti-H3K4ac antibody (Millipore, cat no: 07-539) or anti-H3K9ac antibody (Millipore, cat no: 07-352) was added and kept overnight, on rotating wheel at 4 °C. In a separate reaction tube, an equal amount of anti-IgG antibody (Abcam, cat no: ab171870) was used for immunoprecipitation and proceeded in similar way to be used as negative control. Next day, the antibody-coated beads were washed three times with 1 ml ChIP dilution buffer and after the last wash, supernatant was discarded, and beads were re-suspended in the original volume of beads as done earlier (i.e., 20 ul); 50 μl of sheared chromatin (aliquot kept earlier at − 80 °C) was diluted in ratio 1:5 by adding 200 μL of ice-cold ChIP dilution buffer and mixed thoroughly. For each immunoprecipitation reaction, 1% of diluted chromatin (i.e., 2.5 μl) was kept aside at − 20 °C to be served as 'input DNA control'. In a fresh 1.5-ml micro-centrifuge tubes, ChIP reaction mix was prepared using 250 μl diluted chromatin, 20 μl antibody-coated magnetic beads and 1% protease inhibitor cocktail (Sigma-Aldrich). Tubes were incubated overnight on a rotating wheel at 4 °C. Then immunoprecipitated complexes were sequentially washed on rotating wheel (at 4 °C) with 2 × 1 ml of ice-cold low-salt wash buffer (20 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl); 2 × 1 ml of ice-cold high-salt wash buffer (20 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 500 mM NaCl); 2 × 1 ml of ice-cold LiCl wash buffer (0.25M LiCl,10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40) and 1 × 1ml TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA).

Elution, de-crosslinking and DNA isolation

To elute the immune complex, 400 μl elution buffer 1 (0.1M $NaHCO_3$, 1% SDS) was added to the washed beads and incubated for 30 min in a thermo-shaker at 65 °C at 1300 rpm. Tubes were spinned briefly and placed on magnetic rack for 1 min and supernatant was transferred to a fresh tube. Cross-link reversal was done by adding 16 μl of 5M NaCl to the tubes and incubated overnight on thermo-shaker at 65 °C at 1300 rpm. In addition, 2.5 μl of input DNA control kept earlier was processed in similar way for cross-link reversal by adding 397.5 μl elution buffer 1 and 16 μl of 5M NaCl. Each

immunoprecipitated sample of elute and input DNA control from previous steps were treated with proteinase K (Thermo Fisher scientific) by adding 10 µl of 0.5M EDTA, 20 µl 1M Tris–HCl, pH 6.5, and 1 µl of 20 mg/ml proteinase K to elute and incubated for 1 h at 45 °C and also further treated with RNase A (Sigma-Aldrich). Immunoprecipitated DNA was extracted using phenol/chloroform method, recovered and precipitated by adding 0.3 M sodium acetate (pH 5.2), 2 µl of glycogen (20 mg/ml, Thermo Fisher scientific), and 1 ml of 100% cold ethanol. The DNA pellets were washed with 70% ethanol and re-suspended in 20 µl of sterile distilled water. Immunoprecipitated DNA in each sample was quantified using Qubit dsDNA HS Assay Kits (Life technologies Cat. No. Q32851) using the Qubit Fluorometer.

Selection of genes for ChIP-qPCR

Six genes were selected on the basis transcriptome data available from our earlier study (Sharma et al. 2018). The details of treatments in transcriptome data and the corresponding treatments in the present study are mentioned in Supplementary Table S1. All the six genes are known to be involved in stress responses in plants (Table 1) and their full gene sequences were also available in the database. The results in the present study involving ChIP-qPCR were compared with those of our earlier transcriptome study (Sharma et al. 2018).

Table 1 List of studies showing the role of the six genes during biotic or abiotic stresses in different plant species

Gene	Function	Plant system	References
Enzymes			
N-Acetyltransferase	Gene inactivation and plant development	Arabidopsis	Servet et al. (2010)
Sarcosine oxidase	ROS production; downregulates due to fungal attack	Plants, Arabidopsis	Goyer et al. (2004), Sandalio et al. (2013)
Peroxidase 12	Lignification, ROS production during leaf rust disease	Wheat	Bindschedler et al. (2006), Dmochowska-Boguta et al. (2013)
Transcription factor			
WRKY70	SA/JA signaling, osmotic stress tolerance, resistance against *P. Syringae** and stripe rust disease	Arabidopsis, wheat	Li et al. (2004, 2006, 2013), Jiang et al. (2016); Wang et al. (2016)
WRKY40	Upregulated during *Fusarium* infection, tolerance against ABA, and *P. syringae*, interacts with WRKY18 and WRKY60	Wheat, Arabidopsis	Erayman et al. (2015), Xu et al. (2006), Chen et al. (2010)
Asr1	Plant metabolism, ROS homeostasis, abiotic stress upregulation due to *Fusarium oxysporum* infection	Banana, rice, tobacco, tomato, wheat	Liu et al. (2010), Hu et al. (2013b), Joo et al. (2013), Golan et al. (2014), Dominguez and Carrari (2015)

Pseudomonas syringae is a gram-negative bacterium, which is used as a model pathogen

Table 2 List of primers used for ChIP-qPCR analysis for six selected genes

Gene	Forward primer (F)	Reverse primer (R)
N-Acetyltransferase	P1_F-CGCAGTGAATCAGCCCATAA	P1_R-TCCCCTTCCCGGTTTCTG
	P2_F-AGCCTTGGAGATCTGGGAGAA	P2_R-CCTGTAGAGCTGCGTTTTGAATC
WRKY 40	P1_F-GAAGGGCGGTGGTTGTTG	P1_R-GTCGTTGTGCTAAGGCCCATA
	P2_F-CAGCTCGCTTTTGTGATTGTAGTT	P2_R-TTTCAATTATAACCACCAACACAACA
WRKY 70	P1_F-CATGTGGCTTGTTTATACCTGAAAA	P1_R-CGAGCCCGAGTGTTCCAT
	P2_F-TGAAGAAACCACGGTGTGAAGT	P2_R-GCGTGGACCCAGTTTGGTA
Asr1	P1_F-GGCACATCTGAGTCTAATAGTTTTGG	P1_R-GTTTGATGCCTTTGGTAAATGAGA
	P2_F-TCCTCATCGCCTTCGACATC	P2_R-TCGGCAACCTTCTTTTTGGA
Peroxidase 12	P1_F-CGTAGGACAGCTTCCGTGAAA	P1_R-TGTAAGTGTAGCATTACTCGGAGAATG
	P2_F-GCCACATCAGTTCGTAGGACAA	P2_R-GAGAACGAAATTGCTCCACTTACA
Sarcosine oxidase	P1_F-CGTCGAAGATTTGAGCTGGAT	P1_R-TTTAAACTGGCCCTATATGTTTGGA
	P2_F-CAAGGTGCGGTCCGAGAA	P2_R-TGTCGCTGCCCCAGATGT

ChIP-qPCR

DNA samples obtained from immunoprecipitated chromatin were utilized for analysis of H3K4ac/H3K9ac enrichment at 0 hbi and 96 hai in susceptible and resistant NILs for each of the six selected genes by quantitative real-time PCR (qRT-PCR). Primers for selected genes were designed from upstream promoter regions, designated as P1 (600–900 bp) and P2 (900–1200 bp) using Primer Express (v3.0) software (Table 2). The P1(proximal) and P2 (distal) promoter regions were selected during the present study, since these are widely used and are known to differ in motifs, which are used as binding sites for regulatory proteins (Zhang 2007). Equal amounts of immunoprecipitated DNA were used for ChIP-qPCR, and the reactions were performed using KAPA SYBR FAST Mastermix (Kapa Biosystems, cat no: KK4600), according to the manufacturer's protocol. ChIP-qPCR was performed in three replicates using ABI 7500 fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The data were normalized using input DNA and relative enrichment was calculated by taking IgG as negative control. Relative enrichment levels for both H3K4ac and H3K9ac marks on target regions (promoter regions P1 and P2) of selected genes were analyzed by $^{\Delta\Delta}$Ct method (Yuan et al. 2006; Livak and Schmittgen 2001).

Preparation of heat maps

Heat map for the six selected genes, showing the differential expression was generated using the online tool clustvis (http://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo 2015) from the available transcriptome data. For this purpose, log2 transformed fpkm values were used for all the six selected genes in both susceptible and resistant NIL at 0 hbi and 96hai.

## Motif analysis in the promoter regions of six selected genes

Biotic stress in plants activates several signaling pathways such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), which consequently change the transcription level of associated genes and ultimately protein post-processing (Chen et al. 2012). Keeping this in view, using PlantCARE database, 1.5 kb upstream promoter region for each selected gene was analyzed for the presence of biotic stresses responsive motifs (Lescot et al. 2002). The motifs on the sense strand showing a matrix value of ≥ 5 were selected following the criteria suggested by Chen et al. (2007). Only those motifs falling in the regions P1 and P2 of the six genes were considered for further analysis.

Results

Optimization of chromatin sonication and establishment of ChIP protocol

ChIP protocol for DNA sequences associated with H3K4ac and H3K9ac was standardized using a pair of NILs for *Lr28* in the background of HD2329. A flowchart of the standardized protocol is given in Figure S1 and described in the section on Material and Methods. Briefly, the protocol included isolation of chromatin from seedlings followed by shearing, immunoprecipitation and de-crosslinking of chromatin. To maximize the efficiency of the ChIP, duration of shearing as well as de-crosslinking of the chromatin were standardized. Briefly, chromatin shearing to obtain chromatin fragments in the optimum size range of 200–500 bp (Lin et al. 2012) was found to be 5 min (Fig. 1a). This sheared chromatin was subjected to immunoprecipitation (IP) with the help of antibodies against H3K4ac and H3K9ac separately. ChIP was

Fig. 1 Agarose gel image showing shearing and de-cross-linking of isolated chromatin from seedling leaf tissues of bread wheat. **a** Chromatin sheared for different durations in different lanes. M: 100 bp DNA ladder. **b** De-crosslinking and RNAse treatment of sheared chromatin for 5 min. Lane 1: sheared chromatin, RNAse treated (no de-crosslinking); lane 2: Sheared chromatin, de-crosslinking and RNAse treatment; lane 3: sheared chromatin with no de-crosslinking and no RNAse treatment; M: 100 bp DNA ladder
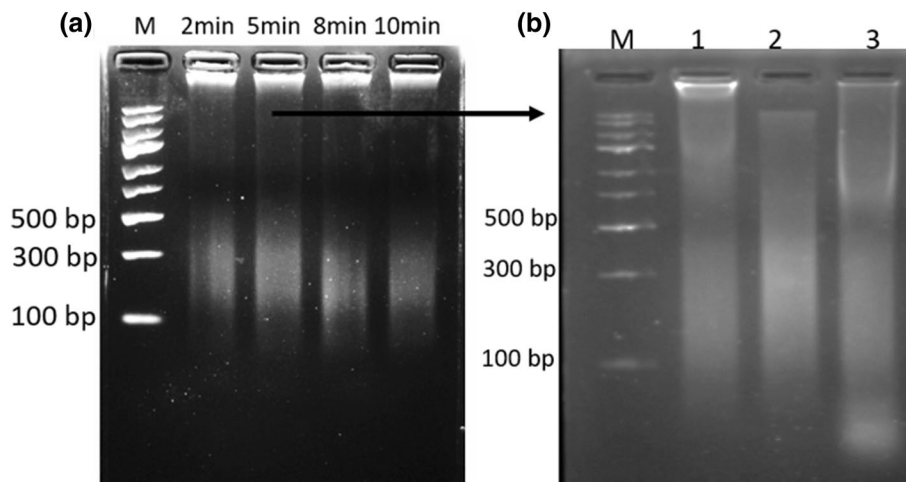
also performed with the antibody against IgG to account for any non-specific enrichment of the chromatin. These three chromatin samples containing immunoprecipitated complexes were subjected to de-crosslinking using an optimized de-crosslinking duration of 30 h (Fig. 1b). Isolated DNA was purified and used for qRT-PCR.

ChIP results for six genes using H3K4ac and H3K9ac

ChIP-qPCR was conducted for all the six genes in the given pair of NILs (samples collected at two time-points: 0 hbi; 96 hai) using antibodies for H3K4ac and H3K9ac (IgG antibodies were used as control). DNA samples from immunoprecipitated chromatin of NILs and control (IgG) were used for qRT-PCR using suitably designed primers for two promoters (P1 and P2). Relative abundance of immunoprecipitated DNA (relative to IgG) for different samples was worked out. The results are summarized in Fig. 2a, b.

Fold changes in promoter sequences of six genes associated with H3K4ac/H3K9ac

The ChIP-qPCR results presented in Fig. 2a, b were utilized for calculation of fold changes that occurred at 96 hai relative to 0 hbi in each case. This allowed assessment of DNA sequences associated with H3K4ac and H3K9ac marks during the period of 96 hai. The mean enrichment of two upstream promoter regions [P1 (600–900 bp) and P2 (900–1200 bp)] for each of the six genes due to two histone acetylation marks is presented in Table 3 (data for biological replicates are presented in Tables S2a and S2b). It was observed that the pathogen attack (inoculation) led to changes in the relative enrichment of promoter DNA sequences for the two acetylation marks (H3K4ac and H3K9ac).

From the data presented in Table 3, it is obvious that there is a wide range (-50.00 to $+9.92$) of fold changes in the proportion of promoter DNA sequences of six genes associated with H3K4ac/H3K9ac following rust inoculation (at 96hai relative to 0hbi). Generally, both promoters of a gene showed the same trend in fold change except in two genes in susceptible NIL HD2329 involving H3K4ac (P2 in case
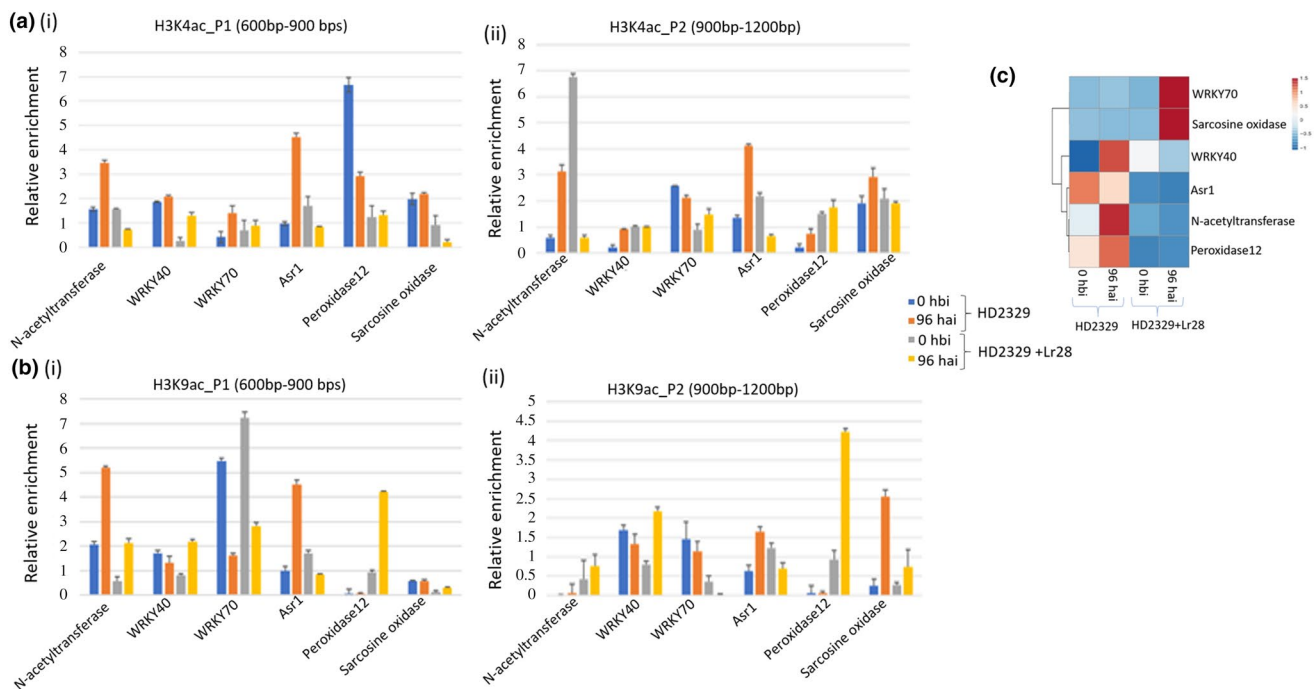

Fig. 2 ChIP-qPCR results showing relative enrichment of promoter sequences [P1(600–900 bp) and P2 (900–1200 bp)] of six selected genes associated with each of the **a** H3K4ac and **b** H3K9ac marks. In each case, estimates of immunoprecipitated DNA from seedlings of susceptible (HD2329) and resistant (HD2329 + Lr28) NILs of bread wheat were made at 0 h before inoculation (0 hbi) and 96 h after inoculation (96 hai) with the pathotype 77-5. Data were normalized using input DNA; relative enrichment was calculated by taking IgG as neg-

ative control. The values on the *y*-axis represent the relative enrichment of DNA sequences associated with acetylation marks; *x*-axis carries the names of genes. **c** Heat map representation for differential expression of six selected genes in susceptible (HD2329) and resistant (HD2329 + *Lr28*) NILs of bread wheat. 0 hbi: at 0 h before inoculation and 96 hai; 96 h after inoculation. Heat map was generated using log 2 transformed RPKM values

Table 3 Fold changes based on ChIP-qPCR enrichment data of studied acetylation marks (H3K4ac and H3K9ac) and RNA-Seq data of six selected genes in the leaf rust susceptible (HD2329) and resistant (HD2329 + *Lr28*) NILs of bread wheat

Gene name	H3K4ac				H3K9ac				RNA-Seq	
	HD2329		HD2329 + *Lr28*		HD2329		HD2329 + *Lr28*		HD2329	HD2329 + *Lr28*
	P1	P2	P1	P2	P1	P2	P1	P2		
N-Acetyltransferase	2.20	5.39	− 2.17	− 11.11	2.51	2.20	3.66	1.76	2.11	− 1.36
WRKY 40	1.12	4.01	4.85	1.00	− 1.27	− 1.27	2.69	2.69	14.74	− 1.66
WRKY 70	3.22	− 1.22	1.23	1.62	− 3.45	− 1.27	− 2.56	− 50.00	1.17	10.19
Asr 1	4.65	3.05	− 2.00	− 3.44	4.65	2.59	− 2	− 1.75	− 1.33	− 20.00
Peroxidase 12	− 2.27	3.38	1.08	1.15	1.21	1.21	4.57	4.57	1.47	1.44
Sarcosine oxidase	1.10	1.52	− 4.17	− 1.10	1.00	9.92	2.24	2.57	− 1.75	50.95

Fold changes were calculated at 96 hai (relative to 0hbi) in both the ChIP-qPCR and RNA-Seq analyses

of *WRKY70* and P1 in case of *peroxidase 12*, both showing negative fold change; in each case, the corresponding fold change value for the other promoter is positive).

A closer look at the data presented in Table 3 indicates that for the two epigenetic marks (H3K4ac and H3K9ac), 30 of the 48 gene-promoter combinations represented varying levels of positive fold changes (> 1.00). These 30 gene-promoter combinations included 15 out of 24 combinations for each of the two acetylation marks.

The most important fold changes available in the present study involved a comparison of the two NILs; in this context, the results for H3K4ac and H3K9ac differed and will be described separately. In case of H3K4ac, the resistant NIL (relative to susceptible NIL) generally exhibited either a reduction in the overall proportion of DNA sequences associated with H3K4ac with the passage of time (fold change < − 1) or only a minor and insignificant increase, when 96 hai is compared with 0 hbi, but still lower than the increase observed in the susceptible genotype. This is true for almost all the six analyzed genes (except P1 of the gene *WRKY40* and P2 of the gene *WRKY70*) (Table 3). However, in the susceptible NIL at 96 hai, proportion of DNA sequences associated with H3K4 acetylation usually increased following infection. The exceptions were *WRKY70* (P2 region) and *peroxidase 12* (P1 region), where a decline was noticed. If we assume that histone acetylation always leads to activation of genes, the results of the present study suggest that relatively more genes are perhaps activated in susceptible than in the resistant NIL.

In case of H3K9ac, at 96 hai, an increase in the frequency of P1 and P2 DNA sequences associated with H3K9 acetylation was witnessed in 15 out of 24 combinations. Out of the 15 combinations, 11 combinations included both P1 and P2 in three of the six genes, namely *N-acetyltransferase, peroxidase 12* and *sarcosine oxidase*. The remaining four combinations showing increased association with H3K9ac included both P1 and P2 of *WRKY40* gene in resistant NIL, and those of *Asr1* gene in susceptible NIL. Eight of the

remaining nine combinations (out of total 24 combinations) were such where a negative fold change was observed, and these included the following: (i) all the four combinations for the gene *WRK70*, (ii) both P1 and P2 for the gene *WRKY40* in the susceptible NIL, and (iii) both P1 and P2 for the *Asr1* gene in the resistant NIL. In the remaining combination (P1 of *sarcosine oxidase* in susceptible NIL), no change (FC = 1.0) was observed for the H3K9ac-associated DNA sequences. It is thus obvious that the two promoters (P1 and P2) of the six genes differ for their association with H3K9ac. This suggested that although the extent of H3K9 acetylation differed in different cases, the different genes differed not only among themselves but also between the two NILs.

Comparison of histone acetylation levels (ChIP-qPCR data) with gene expression levels (RNA-Seq data)

The RNA-Seq (transcriptome) data for the differential expression of six selected genes were available from our earlier study (Sharma et al. 2018) and are included in Table 3 for a comparison. The corresponding heat map is available as Fig. 2c. These data were compared with ChIP-qPCR data on acetylation fold change (96 hai compared with 0 hbi). It is apparent from the summary presented in Table 4 that in only 24 comparisons out of all possible 48 comparisons, changes in acetylation match with changes in gene expression in P1 and P2. While in 22 of 24 remaining comparisons following observations were recorded: (i) higher acetylation matched with lower expression in both the promoters for either for H3K9ac (each for *N-acetyltransferase* and *WRKY40* in resistant NIL) or both the acetylation marks in case of *sarcosine oxidase* and *Asr1* in susceptible NIL. (ii) Lower acetylation matched with higher expression in both the promoters for H3K4ac (*sarcosine oxidase* in resistant NIL) and H3K9ac (*WRKY40* in susceptible NIL and *WRKY70* in both the NILs). (iii) Low acetylation matched with high expression for H3K4ac in P1 (*peroxidase 12*) and

Table 4 Summary of data representing relationship between the RNASeq data and ChIP-qPCR data for acetylation mark (H3K4ac and H3K9ac) in analyzed promoter regions (P1 and P2) of six selected genes in susceptible (HD2329) and resistant (HD2329 + Lr28) NILs of bread wheat

Gene	H3K4ac				H3K9ac			
	HD2329		HD2329 + Lr28		HD2329		HD2329 + Lr28	
	P1	P2	P1	P2	P1	P2	P1	P2
N-Acetyltransferase	Y	Y	Y	Y	Y	Y	N	N
WRKY 40	Y	Y	N	NC	N	N	N	N
WRKY 70	Y	N	Y	Y	N	N	N	N
Asr 1	N	N	Y	Y	N	N	Y	Y
Peroxidase 12	N	Y	Y	Y	Y	Y	Y	Y
Sarcosine oxidase	N	N	N	N	NC	N	Y	Y

Data represented here are based on fold changes observed at 96 hai (relative to 0hbi), for both RNA-Seq and ChIP-qPCR data

Y represents the similar trend of fold changes in RNA-Seq and ChIP qPCR; *N* represents opposite trends; NC represents no fold changes observed at 96 hai (relative to 0hbi) for analyzed acetylation mark

in P2 (*WRKY70*) in susceptible NIL. For the remaining two comparisons (H3K4ac in case of P2 for *WRKY40* in resistant NIL and H3K9ac in case of P1 for *sarcosine oxidase* in susceptible NIL), the expression data could not be compared with status of acetylation because there was no change in acetylation (FC = 1.0).

In summary, gene expression data generally correlated with H3K4ac mark. However, in two of the genes, only one of the two promoters shows this agreement. Gene for *sarcosine oxidase* is the only exception. With H3K9ac mark, generally the correlation between acetylation and high expression was largely missing for all the six genes, except for *peroxidase12*.

In silico identification of biotic stress responsive motifs in the upstream promoter regions of six selected genes

In plants, several signaling pathways including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) pathway operate in response to biotic stresses. This would involve activation of some and repression of other associated genes (Chen et al. 2012). Considering these facts, for each of the six selected genes, promoter regions were analyzed for the presence of motifs that respond to biotic stress (Fig. 3). The P1s of *Asr1* contained motifs for defense and stress responsive elements and SA responsive element and *peroxidase 12* contained fungal elicitor responsive element. None of the remaining genes had a motif in P1 region. In P2 region, only three of the six genes contained a single motif each.
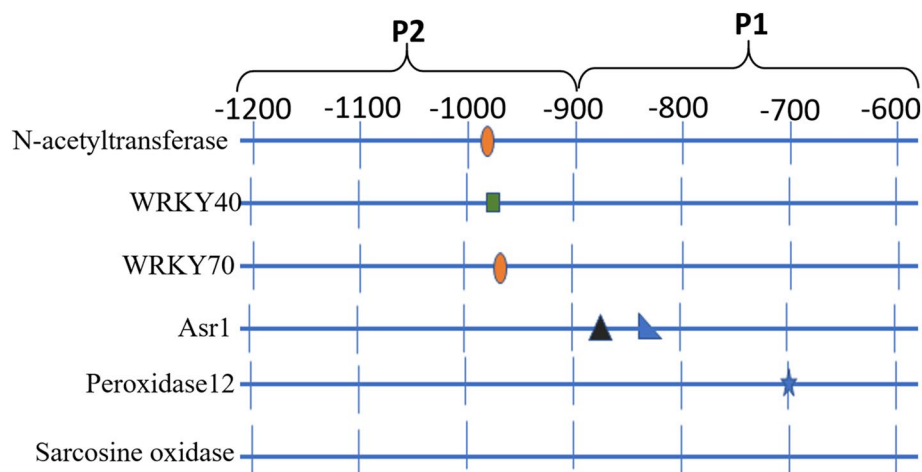

Fig. 3 Five different motifs related to biotic stress response identified in P1 and P2 regions of all the six genes in susceptible NIL (HD2329) and resistant NIL (HD2329 + Lr28). Different symbols shown in the figure indicate different motifs. Filled ellipse—MeJA responsive element. Filled rectangular—Cis-acting defense and stress-responsive element. Filled scalene—Elicitation; wounding and pathogen responsiveness. Filled triangle—Cis-acting element involved in salicylic acid responsiveness. Filled star—Fungal elicitor-responsive element

These were MeJA-responsive element (*N-acetyltransferase* and *WRKY70*) and elicitation, wounding and pathogen-responsive element (*WRKY40*).

Discussion

Leaf rust in wheat caused by *P. triticina* Erikss. and Henn is a dreadful disease which leads to more than 50% loss in grain yield worldwide (Huerta-Espino et al. 2011). Therefore, it is necessary to fully understand the mechanism underlying the leaf rust pathogenesis in wheat. A fairly good idea about the general molecular basis of complex plant immunity system is now available (Wu et al. 2018). However, not many studies like the present study are available, where an effort has been made to understand the molecular mechanism of leaf rust resistance involving a specific *Lr* gene like *Lr28* (Chandra et al. 2016; Singh et al. 2017; Sharma et al. 2018). In our earlier study, a large number of genes were found to be differentially expressed following inoculation by the pathogen (pathotype 77-5) (Sharma et al. 2018). The present study is an extension of this earlier transcriptome study, using the same plant material (a pair of NILs: HD2329, HD2329 + *Lr28*). In the present study, we tried to understand the extent to which the differential expression of six selected genes (from a large number of genes) is regulated through two specific histone acetylation marks (H3K4ac and H3K9ac) as a component of epigenetic control. Effort was also made to understand how this H3 acetylation-mediated differential expression of each of the six genes modulates the level of disease resistance.

A fairly detailed knowledge about the molecular events involved in resistance against biotrophs in wheat and other cereals is now available, where it was shown that a variety of cell surface receptors (including RLKs and RLPs) and cytoplasmic receptors (including NLRs, also known as NBS-LRR) may be involved. These receptors sometimes make complexes, initiating a downstream network of signal transduction pathways that are involved in defense. A large number of receptors and the components of downstream pathways are encoded by genes, which may be subject to differential expression (Wu et al. 2018). The six differentially expressed genes studied in the present study belong to this immune system and seem to be involved in the complex network of downstream signal transduction pathways. Of the six genes studied, apparently none encodes a receptor. Three of the six genes encode transcription factors (*WRKY40, WRKY70* and *Asr1*), which should be directly involved in binding DNA sites, thus bringing about induction or repression of gene expression (perhaps as the last step of a signaling pathway). The remaining three genes encode enzymes (*N-acetyltransferase, peroxidase12* and *sarcosine oxidase*), which should take part indirectly in enzymatic reactions and

facilitate progression of one or more signaling pathways. The differential expression of a large number of genes in response to biotic stress (as in the present case) is controlled not only through a variety of regulatory DNA sequences such as promoters and enhancers (Dmochowska-Boguta et al. 2015; Singh et al. 2017; Sharma et al. 2018) but also through epigenetic modifications in response to the attack by the pathogen (Pan et al. 2012; Ayyapan et al. 2015). While some information is available about the genetic regulation, very little is known about the epigenetic regulation, which in its turn may involve several mechanisms mainly including DNA methylation, histone modifications and regulation through non-coding RNAs (Ayyapan et al. 2015; Wang et al. 2016; Sun et al. 2018).

Histone modifications involve a multitude of modifications in each of a large number of amino acid residues of each histone protein that take part in chromatin constitution and organization (Fan et al. 2015). The most commonly studied histone modifications include methylation and acetylation of a number of lysine residues in H3 histone protein; the effect of only H3K4ac and H3K9ac from a large number of possible histone modifications was examined during the present study, since acetylation is known to induce expression of associated genes. The role of H3K9ac was earlier studied in rice (Roy et al. 2014), maize (Li et al. 2014) and wheat (Hu et al. 2013b); similar reports involving H3K4ac, however, are not available. The present study demonstrates that histone acetylation marks, H3K4ac and H3K9ac in the two 5′ upstream promoter regions do play a role in regulations of the expression of genes participating in wheat leaf rust pathogenesis involving the resistance gene *Lr28*.

The two promoter regions examined for the H3 acetylation during our study included a proximal (−600 to −900 bp) and a distal (−900 to −1200 bp) promoter (i.e., P1 and P2) for each of the six genes. In the past, H3 acetylation of the proximal or distal promoters has been shown to play a role in transcription of C_4-*Pepc* (*C4-phospoenolpyruvate carboxylase*) involved in C acquisition in maize (Offermann et al. 2008) and *DREB1b* involved in cold response in rice (Roy et al. 2014). H3 acetylation of the distal promoter was more closely related to the expression of C_4-*Pepc* in maize. In case of *DREB1b*, the H3 acetylation of proximal promoter (containing ABRE elements, bZIB binding sites and MYc like HLH binding sites) as well as upstream elements is closely associated with TF binding and transcription. Similarly, in peanut, expression of *oleosin* genes (*AhOleo17.8* and *18.5*) was attributed to change in H3 acetylation levels at proximal promoter regions (which contain RY elements and G-box motifs for seed-specific expression) in maturing embryos (Li et al. 2009). We attempted to analyze the H3 acetylation (H3K4ac and H3K9ac) in the promoters P1 (proximal) and P2 (distal) keeping in view the five important biotic response motifs, of which only one is present in each

of the six genes, except *Asr1*, which carries two motifs in the P1 promoter, but none in the P2 promoter. The results will, therefore, be discussed for each of the six genes also in the context of the specific response motif carried by each gene.

The fold changes in acetylation in the promoters P1 and P2 of six genes associated with H3K4ac and H3K9ac differed in the two NILs at two different time points (i.e., 0 hbi and 96 hai). Since, the extent of histone acetylation in the chromatin associated with promoters should be generally correlated with expression levels of associated genes (Hu et al. 2013a, b; Brusslan et al. 2015), the extent of two acetylation marks in two promoters for each of the six genes was examined and related with their role in progression of the disease in HD2329 and the absence of disease in HD2329 + *Lr28*.

Assuming that acetylation leads to enhanced expression, in 24 of the 48 comparisons involving six genes and eight treatments, the variation in the extent of acetylation was found to match with the gene expression levels (based on RNA-seq based transcriptome study conducted earlier), (Table 3). The levels of acetylation enrichment exhibited positive significant correlation (r = 0.474) with gene expression levels, when 22 of the 24 comparisons (excluding two outliers, which appeared exceptions) were used for a test of significance. In the remaining two cases, involving *sarcosine oxidase*, small changes in acetylation (FC = 2.24 and 2.54) were associated with a large fold change (50.95) in the level of gene expression; this nonlinear relationship between the acetylation and gene expression levels suggest that other genetic/epigenetic factors may also be involved in regulation of gene expression. Obviously, H3 acetylation is not the only mechanism, and there must be other epigenetic/genetic mechanisms operating for regulating expression of these six genes. Histone acetylation data from the present study and the expression data from our earlier study (Sharma et al. 2018) were utilized to examine the extent of regulation of each of the six genes due to H3K4ac and H3Kac. These six genes are already known to be involved in responses against biotic or abiotic stresses in plants such as *Arabidopsis*, rice and wheat (Table 1). The results of each individual gene will be discussed, outlining the correlation of associated H3 acetylation enrichment of one or both the promoter sequences not only with resistance to leaf rust, but also with gene expression due to *Lr28* (studied by us earlier).

N-Acetyltransferase

The gene '*N-acetyltransferase*' (*NAT*) used in the present study is one of the *Gcn5* genes and is a member of GNAT family (Gcn5-related *N*-acetyltransferases). It has four functional domains, including an N-terminal domain, a highly conserved catalytic (HAT) domain, an Ada2 interaction domain, and a C-terminal bromodomain. The enzyme is known to be responsible for most of the cytoplasmic HAT activity in yeast, and it binds strongly to histone H4 by its association with an additional subunit, Hat2.

H3K4ac-mediated abundance of *N-acetyltransferase* promoter sequences only in the susceptible NIL and their positive correlation with expression indicates that the enzyme may be involved in leaf rust pathogenesis. However, the results of H3K9ac differ, since no deacetylation was observed in the resistant line, so that it may be difficult to visualize how acetylation at H3K4 and H3K9 coordinate in regulating the expression of this gene. Although there is no direct evidence showing the involvement of *N-acetyltransferase* in biotic stress response, there is a report in *Arabidopsis* where the role of this gene in plant development was reported in priming inducible gene activation (Servet et al. 2010). Thus, in case of susceptible NIL, this gene may be causing acetylation-mediated activation of other genes that are involved in leaf rust pathogenesis in susceptible NIL. In our earlier transcriptome study involving the same experimental material, several genes including *WRKY53* TF were upregulated in susceptible NIL due to pathogen infection (Sharma et al. 2018). In Arabidopsis, *WRKY53* is known to be regulated through histone acetylation because the promoters of this gene are enriched with acetyl groups (Luna et al. 2012). Further, in *Arabidopsis, AtGCN5* is known to interact with several other chromatin proteins and DNA binding transcription factors (Servet et al. 2008). Therefore, such as *AtGCN5, N-acetyltransferase* gene may target a large number of promoters and may be involved in acetylation of several H3 lysine residues in wheat. There are few other studies where the role of GNAT domain containing genes has been implicated in abiotic stresses such as heat and drought in plants (see Table 1). The P2 promoter region in this gene also contained MeJA (Methyl Jasmonate)-responsive motif which is known to possess multifunctional roles in stress tolerance including biotic as well as abiotic stresses in plants (Ahmed et al. 2016).

Abscisic acid stress ripening 1 (Asr1)

As a member of ASR gene family, *Asr1* was the first gene to be cloned from tomato. This gene and also its orthologues in wheat and other monocots are known to be involved in abiotic stress responses including drought and salinity (for review see Dominguez and Carrari 2015). In the present study, this gene shows a pattern similar to that of *N-acetyltransferase*, except that similar patterns were observed with both H3K4 and H3K9, suggesting that the gene is upregulated in susceptible genotype and downregulated in resistant genotype; however, high acetylation in susceptible genotype does not match with low expression observed by us earlier in transcriptome study. Such lack of correlation between acetylation and expression has also been reported in some

earlier studies (Tian et al. 2005), where change in acetylation levels was not correlated to gene repression. However, in banana, *Asr1* was shown to undergo upregulation due to *F. oxysporum* infection. In resistant NIL, a correlation was observed between low *Asr1* gene expression with deacetylation of both the H3K4ac and H3K9ac marks. This indicates that resistance is achieved by suppression of the activity of this gene, and activation of this gene perhaps helps the pathogen in causing the disease. This suppression of gene activity through deacetylation may also be mediated by HDACs as observed in case of rice (Zhang et al. 2016b) and cotton. Deacetylation of H3K9 in cotton is caused due to *GhHDA5* (a type of histone deacetylase) causing fiber initiation (Kumar et al. 2018), and in rice due to *OsSRT1* (also a type of histone deacetylase) facilitating starch accumulation and seed development.

The results of the present study also indicate that *Asr1*-mediated resistance may also be regulated by feedback inhibition in the following manner: As a transcription factor and chaperone protein, *Asr1* is involved in synthesis of amino acids and sugar in maize, tomato and grapes. In wheat, it is also implicated in ROS homeostasis and transcription of stress-associated genes (Table 1). Thus, low expression (fold change −20.00) of *Asr1* in resistant NIL in the present study may actually lead to reduced accumulation of amino acid/sugars that are involved in promoting pathogenesis. In fact, the motif analysis in P1 promoter region also indicated two motifs (defense and stress responsive and cis-SA responsive) that are involved in disease response.

WRKY40 and *WRKY70*

Out of the two WRKY TFs (*WRKY70* and *WRKY40*), *WRKY70* is known to act as a common component of SA and JA pathways, where its expression is generally associated with JA and repression associated with SA (Li et al. 2004; Ren et al. 2008; Ulker et al. 2007; Knoth et al. 2007). In wheat, the role of *WRKY70* has been earlier implicated in biotic stresses such as leaf rust, stripe rust and infection due to *Fusarium graminearum* (Table 1). Broadly, it seems that only H3K4ac and not H3K9ac may be involved, in regulation of both these transcription factors. The acetylation pattern of H3K4 indicates that acetylation associated with promoters of *WRKY40* matches with higher expression in the susceptible NIL, and the acetylation associated with promoters for *WRKY70* matched with higher expression in the resistant NIL. In the past, several other WRKY TFs have been shown to be upregulated during leaf rust infection in wheat, suggesting their general role in disease response (Satapathy et al. 2014; Kumar et al. 2014; Sharma et al. 2018). The *WRKY* TFs regulate the expression of a variety of defense-related genes through binding to the W-box

elements in the promoter regions of these genes (for references, see Xu et al. 2006).

The higher expression of *WRKY40* in susceptible NIL may actually repress the expression of defense-related genes. For example, the overexpression of *GhWRKY40* was shown to enhance susceptibility to *Ralstonia solanacearum* in transgenic *Nicotiana benthamiana* through suppression of defense-related genes such as *PR1a, PR2*, ethylene responsive gene *ACS6* (Wang et al. 2014). In contrast, the higher expression of *WRKY70* in resistant NIL may be mediating leaf rust resistance through regulation of genes such as peroxidases (involved in ROS homeostasis) and carbohydrate and amino acid metabolism (see Sharma et al. 2018). *WRKY70* has also been reported to have a role in mediation of resistance against *Hyaloperonospora parasitica* through *RPP4* gene in Arabidopsis (Knoth et al. 2007).

Peroxidase 12 and *sarcosine oxidase*

Both peroxidases as well as sarcosine oxidase are known to be involved in oxidative stress response and ROS homeostasis in plants during biotic as well as abiotic stresses. For instance, expression of peroxidases such as *peroxidase12* has been shown to be associated with response to different stimuli, including pathogenic challenge (resistance to infection), oxidative burst, and diverse physiological and developmental processes such as cell wall formation, lignification and production of ROS (Bindschedler et al. 2006). In wheat, peroxidases have been implicated in resistance-related oxidative burst response against brown rust disease (Dmochowska-Boguta et al. 2013). Similarly, sarcosine oxidase is a source of peroxisomal H_2O_2 in plants. It catalyzes the oxidation of sarcosine, *N*-methyl amino acids and l-pipecolate, producing formaldehyde, glycine and H_2O_2 (Sandalio and Romero-Puertas 2015). In the present study, *peroxidase 1*2 was upregulated (due to acetylation) in both resistance as well as susceptible NIL. This suggests that the expression of these may not be directly involved in resistance. However, the peroxidase 12 is mainly involved in production of ROS, which is an immediate response of pathogen attack and activates defense-related genes (Ramu et al. 2016). Sarcosine oxidase showed association with resistance for one of the acetylation marks (H3K9ac), where increased enrichment of the sequences (fold change 2.24 and 2.57) showed association with 50.95-fold change in expression, suggesting that other regulatory mechanisms may also be involved.

In conclusion, the present study suggests that H3 acetylation at lysine residues 4 and 9 (H3K4 and H3K9) at least partly regulates the expression of six leaf rust responsive genes in wheat. For two (*N-acetyltransferase* and *peroxidase12*) of the six genes, H3 acetylation/deacetylation was largely correlated with upregulation/downregulation of gene expression. For the remaining four genes, changes in

H3 acetylation did not always match with changes in gene expression. These results suggested that besides H3 acetylation, other combinatorial epigenetic/genetic factors may have a role in fine-tuning the gene expression in wheat leaf rust pathosystem. Histone/DNA methylation and miRNA/lncRNA may also play a role, which is the subject of our future studies using whole genome ChIP-Seq, bisulphite sequencing and the analysis of non-coding RNA to answer some of the outstanding questions regarding the regulation of gene expression during leaf rust pathogenesis in wheat.

Compliance with ethical standards

Conflict of interest All the authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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