



# Complex relationship between DNA methylation and gene expression due to *Lr28* in wheat-leaf rust pathosystem

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## Abstract

Differential DNA methylation due to *Lr28* was examined in susceptible (S) wheat cv. HD2329 and its resistant (R) near isogenic line (NIL) (HD2329+*Lr28*) using two approaches: methylation sensitive amplified polymorphism (MSAP) and methylated DNA immunoprecipitation (MeDIP). S/R lines each had a large number of hypomethylated genes and relatively fewer hypermethylated genes at 96 hai (hours after inoculation) relative to 0 hbi (hours before inoculation), suggesting activation of many genes during the passage of time (96 hai), although identity of genes may differ in S and R lines. When R NIL was compared with S cultivar, there were many hypermethylated and fewer hypomethylated genes in R NIL relative to S cultivar, suggesting that many genes that are active in S cultivar are silenced in R NIL, both at 0 hbi and at 96 hai. Level of methylation was generally abundant in intergenic regions followed by that in promoters, transcription termination sites (TTSs) and exons/introns. Hypermethylation in promoter and gene body regions was not always associated with inhibition of gene expression and vice-versa, indicating that more than one regulatory mechanisms may control the expression of genes due to pathogen attack in presence and absence of *Lr28*. MSAP analysis also showed abundance of <sup>m</sup>CG methylation in S cultivar and that of <sup>m</sup>CCG methylation in R NIL (at 96 hai), suggesting differences in methylation context in NILs with and without *Lr28*. The results of the present study improved our understanding of the epigenetic control of leaf rust resistance in wheat.

**Keywords** Wheat · Leaf rust · *Lr28* · DNA methylation · Gene expression

## Introduction

Wheat is one of the most important crops world-wide; its production has shown remarkable growth during the last 50 years, both globally and in South-East Asia. However, a sharp decline in average annual growth in production from ~3% in the past to ~0.7% in recent decades has been witnessed. This is a matter of concern, keeping in view the projected global demand for substantial increase in wheat

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production by the year 2050 [1]. The decline in the growth of wheat production is attributed to a number of biotic and abiotic stresses [2]. Among biotic stresses, leaf rust of wheat (also described as brown rust) caused by *Puccinia triticina* is an important disease, which is responsible for major loss in grain yield [3]. As many as ~80 Lr genes (*Lr1–Lr80*) conferring resistance to leaf rust in wheat are known [4]; these genes include those conferring race-specific resistance and also those for non-race specific adult plant resistance (APR).

During the last two decades, significant progress has also been made in understanding the molecular mechanism of disease resistance including leaf rust in wheat. For leaf rust, four Lr genes (*Lr1*, *Lr10*, *Lr21*, *Lr34*) were cloned and characterized, providing a better understanding of the molecular mechanism of leaf rust resistance in wheat [5–8]. Recently, avirulence genes (*Avr*) interacting with two Sr genes in wheat (*Sr35* and *Sr50*) were also identified [9, 10], providing information about specific gene-for-gene interactions between Sr genes and Avr genes of the pathogen causing stem rust. However, no Avr gene against any Lr gene in wheat has so far been identified or cloned, which is therefore an important area for future research. In Arabidopsis, recent evidence suggested formation of a resistosome through oligomerization of nucleotide binding leucine rich repeat containing nod-like receptor (NLR), named Hopz Activated Resistance 1 (ZAR1) with other unique proteins during infection by the microbial pathogen *Xanthomonas campestris* bacteria [11]. Formation of such a resistosome may turn out to be a common feature in all pathosystems in crop plants including leaf rust in wheat. Another major recent finding is that a TIR (Toll/Interleukin receptor) domain in NLR protein functions as NADase for cleavage of NAD<sup>+</sup> and is responsible for cell death leading to hypersensitive reaction during infection by biotrophs like leaf rust pathogen [12, 13].

There is a growing realization that resistance to diseases in plants has an element of epigenetic control in addition to the classical genetic control through Mendelian R genes. One of these important epigenetic marks is cytosine methylation in DNA, which is known to play an important role during plant development by regulating gene expression [14, 15]. In plants, it occurs at CG, CHG and CHH islands (C and G indicate cytosine and guanine, respectively, and H indicates any nucleotide other than guanine) and differs from that in mammals, where it occurs mainly at CG sites and only rarely it occurs at non-CG sites [16, 17]. The regulation of gene expression by DNA methylation mainly occurs by silencing specific genes either at the transcriptional level or at the post-transcriptional level. The former involves inhibition of transcription due to hypermethylation in the promoter region, while the latter involves post-transcriptional degradation of mRNA using non-coding RNAs [18]. DNA

methylation occurs both in gene body and intergenic regions, but its relationship with gene expression is not well understood. Generally, gene body methylation is known to induce gene expression rather than its suppression, involving alternate splicing [19]. Similarly, the role of intergenic DNA methylation in prevention of aberrant gene transcription has been reported in Arabidopsis [20]. Thus, the relationship of DNA methylation with gene expression seems to be quite complex as shown in rice during drought stress [21]. Similar complexity was also reported in humans [22].

Differential DNA methylation in any plant system can be studied using several techniques including methylation sensitive amplified polymorphism (MSAP), methylated DNA immunoprecipitation (MeDIP) and bisulphite sequencing (BiS-Seq). MSAP is a modified amplified fragment length polymorphism (AFLP) technique [23], while MeDIP and BiS-Seq require DNA sequencing. MSAP makes use of a pair of isoschizomers like HpaII and MspI, which recognize the same restriction site (CCGG), but differ in their sensitivity to methylated cytosine residues [23]. MSAP is also relatively cost-effective, since no sequencing is involved, but it has low resolution and does not give information about cytosine methylation at individual nucleotide level.

In wheat, several reports of the study of differential DNA methylation using each of the above three approaches are available, both at the whole genome level and at specific gene level. For instance, MSAP was used in at least three studies in wheat [24–26]. One of these studies also reported methylation pattern due to two Lr genes, namely *Lr19* and *Lr41* [25]. MeDIP has also been used for exploring the epigenomic landscape along chromosome 3B under the Epi3B project that was launched at INRA in France [Joint EUCARPIA Cereal Section & International Triticeae Mapping Initiative (I-T-M-I) Conference, International Wheat Genome Sequencing Consortium (IWGSC) workshop 29-06-2014]; the results of this work on Epi3B were not published (personal communication). BiS-Seq has been used in at least three studies to examine differential methylation at the whole genome level [25, 27, 28]. In view of the large genome of wheat, sometimes genome complexity reduction techniques such as “sequence capture” was also used in combination with Methyl-Seq, a method based on ‘Agilent SureSelect Methyl-Seq’ [29]. Genome-wide differential methylation patterns in wheat have been examined for a variety of traits including resistance to powdery mildew [30], vernalization [31] and seed germination [32]. Studies on differential methylation of individual genes were also conducted, as in case of photoperiod insensitivity gene *Ppd-B1a* [33]. Studies were also conducted on methylation patterns due to aneuploidy/alien chromatin introgression [34, 35] and polyploidization [36,

37]. Obviously, interest in DNA methylation studies has increased in recent years.

The gene *Lr28* is an alien gene from *Aegilops speltoides* Tausch that confers resistance against the most prevalent Indian leaf rust pathotype 77-5. Therefore, this gene has been deployed for developing several leaf rust resistant Indian wheat cultivars (e.g. MACS6145), although the resistance imparted by *Lr28* has recently been overcome by a new pathotype (121R60-1 = 77-10) [38]. However, relatively fewer studies have been conducted to understand the molecular mechanism underlying leaf rust resistance due to *Lr28* [39–43]. In a recent study, the role of histone acetylation in regulating the expression of six genes during pathogen attack in the presence of *Lr28* was also examined [44]. However, no studies on the role of DNA methylation in defence pathway due to *Lr28* are available. The earlier methylation study on leaf rust involving two *Lr* genes (*Lr19* and *Lr41*) was based on low-resolution MSAP approach [25]. In the present study, we examined differential methylation patterns associated with leaf rust resistance gene *Lr28*. For this purpose, differential gene expression due to DNA methylation was examined in a wheat cv. HD2329 and its resistant NIL (HD2329+*Lr28*). The study provides useful information about the role of differential methylation in regulation of gene expression in wheat-leaf rust pathosystem.

## Material and methods

### Origin of plant material

Initially the leaf rust resistance gene *Lr28* was accidentally transferred from *Aegilops speltoides* into hexaploid wheat cv. Chinese Spring (CS) during attempts to transfer the gene *Yr8* (a yellow rust resistance gene) from *Aegilops comosa* to common wheat [45]. *Ae. speltoides* was used only for the purpose of inducing homoeologous recombination between the wheat and *Ae. comosa* chromosomes (*Ae. speltoides* suppresses the gene *Ph1* and thus induces pairing between homoeologous chromosomes). A segment of chromosome 7S#2S of *Ae. speltoides* carrying *Lr28* was translocated onto wheat chromosome 4AL resulting in a translocated chromosome T4AS.4AL-7S#2S [46]. Later, a donor stock CS2A/2M 4/2 containing *Lr28* from *Ae. speltoides* and *Yr8/Sr34* from *Ae. comosa* was used to transfer *Lr28* into several Indian wheat cultivars (including HD2329) by Dr. M.K. Menon, IARI, Regional Station, The Nilgiris, Tamil Nadu, India [47, 48]. The resistant NIL HD2329+*Lr28* was developed following seven backcrosses to a susceptible recurrent parent cv.

HD2329. The susceptible cv. HD2329 and its resistant NIL (HD2329+*Lr28*) were used in the present study.

### Inoculation of seedlings with *P. triticina* inoculum

Inoculation of seedlings with *P. triticina* inoculum was carried out following Sharma et al. [39]. For this purpose, the seedlings of the susceptible cv. HD2329 and resistant NIL HD2329+*Lr28* were raised at the National Phytotron Facility, ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi, in growth-chambers under controlled conditions of 16 h day (25 °C) and 8 h night (18 °C) with 240  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light. *P. triticina* inoculum (urediniospores and talcum powder in 1:10 ratio) suspended in water (30 urediniospores per 10X microscopic area) was evenly spread on moistened open leaves of each of the 14-day old seedlings (two-leaf stage) with the help of a camel hair brush (0 size) (Michigan Brush Manufacturing Company Inc., New Delhi, India). The pots carrying the seedlings were well-watered and covered with water saturated polythene to maintain the desired level of relative humidity (RH > 95%). After inoculation, the seedlings were incubated in a humid chamber maintained at  $23 \pm 2$  °C in the dark for a period of 36 h; normal conditions were restored thereafter

### DNA isolation

Leaf samples for DNA isolation were collected from 14-day old seedlings of the two NILs at 0 hbi and 96 hai. The 96 hai stage was chosen on the basis of our earlier findings showing pale halo islands at the sites of infection on the leaves of the above susceptible cultivar and also due to our earlier observation of the abundance of differentially expressed transcripts between the susceptible cultivar and resistant NIL at 96 hai [40]. Genomic DNA was isolated using modified CTAB method [49] from leaf samples collected from two biological replicates of the following four treatments: (i) HD2329 (susceptible cultivar) at 0 hbi (S0); (ii) HD2329 (susceptible cultivar) at 96 hai (S96); (iii) HD2329+*Lr28* (resistant NIL) at 0 hbi (R0), and (iv) HD2329+*Lr28* (resistant NIL) at 96 hai (R96).

### Methylation sensitive amplified polymorphism (MSAP)

MSAP analysis was carried out using two pairs of restriction endonucleases (EcoRI + HpaII and EcoRI + MspI) (New England Biolabs Inc.) for restriction digestion of DNA of the above four samples. HpaII and MspI represent a pair of isoschizomers; both can cleave the un-methylated restriction site 5'-CCGG-3'; HpaII also cleaves hemi-methylated

external cytosine, <sup>m</sup>CCGG [50, 51], while MspI cleaves hemi- or fully methylated internal cytosine (C<sup>m</sup>CGG) but not hemi- and fully methylated external cytosine (<sup>m</sup>CCGG) or both external and internal cytosines (<sup>m</sup>C<sup>m</sup>CGG) of the restriction site CCGG.

Aliquots (500 ng) of DNA were digested for 2 h at 37 °C with 10 U each of EcoRI + MspI and EcoRI + HpaII to ensure complete digestion of DNA. This was followed by addition of 5 pmol EcoRI and 50 pmol of HpaII/MspI adaptors (Xcelris Genomics, India) to the ligation mixture containing 1 unit of T4 DNA ligase and 1 × ligation buffer (New England Biolabs Inc.). The mixture was incubated at 37 °C for 3 h. The ligation mixture was then diluted 1:10 with TE buffer, and was used as a template for the pre-amplification with single nucleotide selective EcoRI-A primer (GACTGC GTACCAATTCA) and MspI/HpaII-T primer (ATCATG AGTCCTGCTCGGT). The PCR reaction was performed for 30 cycles of 45 s denaturation at 94 °C, 40 s annealing at 56 °C, and 40 s extension at 72 °C. The PCR amplified products were further diluted to 20-fold with TE buffer to use it as a template for selective amplification with each of the 27 pairs of selective primer combinations carrying three selective nucleotides (Supplementary Table 1). Subsequently, a mix of Gene scan 500ROX (range 35–500 bp; used as size

standard) was added to the selective amplification product. The resulting mixture was used for capillary electrophoresis on ABI3730 DNA analyzer, Applied Biosystems, Life Technologies Pvt. Ltd., California, USA for resolving the amplified DNA fragments as peaks in the electropherograms. The results were analysed for four different MSAP profiles (Types I to IV) that are expected from the EcoRI + HpaII and EcoRI + MspI combinations (Table 1).

### Scoring and analysis of electropherograms following MSAP

The amplified DNA fragments were visualized as peaks on electropherograms; fragment size in each case was determined with the help of the size standards using the software GeneMarker version 1.80. The presence and absence of peaks (amplified fragments) in each treatment were scored as 1 (for presence of peak) and 0 (for absence of peak) and methylation patterns of the restriction site 5'-CCGG-3' were inferred as shown in Table 1 [51]. Whole genome level methylation was inferred on the basis of the total number of peaks (fragments) obtained in each treatment. The differential 5-methylcytosine peaks in two different treatment

**Table 1** Methylation status of restriction sites (first two columns) and four types of patterns (last column) due to individual peaks observed in MSAP profiles

Methylation status of restriction site		MSAP profile										
		EcoRI + HpaII	EcoRI + MspI	Type								
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr></table>	C	C	G	G	C	C	G	G	No methylation	+	+	I
C	C	G	G									
C	C	G	G									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Full-methylation of internal cytosine	–	+	II
C	C	G	G									
G	G	C	C									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Hemi-methylation of internal cytosine	–	+	II
C	C	G	G									
G	G	C	C									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Hemi-methylation of external cytosine	+	–	III
C	C	G	G									
G	G	C	C									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Full-methylation of external cytosine	–	–	IV
C	C	G	G									
G	G	C	C									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Full-methylation of both cytosine	–	–	IV
C	C	G	G									
G	G	C	C									
<table border="1"><tr><td>C</td><td>C</td><td>G</td><td>G</td></tr><tr><td>G</td><td>G</td><td>C</td><td>C</td></tr></table>	C	C	G	G	G	G	C	C	Hemi-methylation of both cytosine	–	–	IV
C	C	G	G									
G	G	C	C									
M	Mutation	–	–	IV								

The first column shows the restriction site with four base pairs (CCGG). HpaII and MspI are methylation sensitive restriction enzymes and EcoRI is a rare cutter. Methylated cytosines at restriction sites in the first column are indicated in grey shades. Four different types of methylation patterns (shown in last column) are obtained due to the differential sensitivities of two restriction enzymes that are isoschizomers. Full methylation indicates methylation of both strands and hemi-methylation indicates methylation in either of the two strands

+ Presence of peak

– Absence of peak

combinations were inferred on the basis of presence/absence of peaks.

### Methylated DNA immunoprecipitation (MeDIP)

For MeDIP analysis, DNA of two biological replicates was pooled in equal concentrations for each of the four treatments as detailed above i.e. S0, S96, R0 and R96. MeDIP sequencing of the pooled immunoprecipitated DNA of each sample was undertaken to decipher the genome-wide DNA methylome. The sequencing data was obtained through outsourcing the sequencing work to SciGenome Pvt. Ltd., India. The protocol adapted by Li et al. [52] with few modifications was followed for preparing MeDIP-Seq libraries. Briefly, the following steps were involved in MeDIP sequencing: (i) 1 µg of the genomic DNA was sheared independently for each of the four treatments and end repaired to obtain sheared fragments with blunt ends. (ii) A single 'A' nucleotide was then added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation. (iii) The multiple indexing adapters were then ligated to the ends of the DNA fragments to prepare them for hybridization on to the flow cell. (iv) This was followed by immunoprecipitation using MeDIP buffer and <sup>5</sup>mC specific monoclonal antibody (Methylamp Methylated DNA capture kit, Epigentek, USA). This immunoprecipitated DNA is supposed to be enriched for methylated DNA fragments, which had adapter molecules on both ends for amplification of the DNA in the library. (v) The PCR was performed with the PCR Primer Cocktail that annealed to the ends of the adapters. The number of PCR cycles was kept to the minimum to retain library representation. (vi) Qubit concentration of library was checked followed by quality check of the library; this was done by loading 1 µl of sample on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip (e.g., Agilent DNA 1000 or DNA HS chip) and (vii) MeDIP library obtained thus was sequenced and the data were obtained in the form of FASTA files and reads quality checked using the following parameters: Base/sequence quality score: Phred score > 30; average base content per read: 100 bp; GC distribution: > 40% and over-represented sequences. Based on the quality of sequence reads, trimming was done, wherever necessary, to retain only high-quality sequence.

The pre-processed reads were aligned to the reference genome of wheat (*Triticum aestivum*) downloaded from [https://urgi.versailles.inra.fr/download/iwgsc/IWGSC\\_RefSeq\\_Assemblies/v1.0/](https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/). The alignment was performed using Bowtie program V.2 with default parameters. These alignment files (\*.bam file) were used as input (i) for

identification of methylated peaks using MACS2.0 (Model Based Analysis of ChIP-Seq data) software [21, 53] in all the four individual treatments (S0, R0, S96 and R96) and (ii) for extracting the differential methylated regions (DMRs) in the four different pairs of treatments (S0 vs S96, R0 vs R96, S0 vs R0 and S96 vs R96) using MEDIPS (R) package [21, 54, 55]. In all these four treatment comparisons, the second treatment was compared to first. The above-mentioned wheat genome reference sequence along with the bam files were used in MEDIPS to calculate the differential methylation between the two treatments of a pair at a genome-wide window of regular width of 500 bps. The DMRs following FDR < 0.05 and FC ≥ 2 or ≤ -2 (log FC ≥ 1 or ≤ -1) [21, 54] were considered to be differentially methylated and were used for further detailed analysis. The methylated peaks in individual treatments and also the DMRs between the four different pairs of treatments were assigned to different genomic regions [introns, exons, promoters (- 1 kb to + 100 bp), transcription termination sites (TTS) (- 100 bp to + 1 kb) and intergenic regions] using Homer software [56] and latest version of wheat reference sequence annotation v1.1 available at IWGSC ([https://urgi.versailles.inra.fr/download/iwgsc/IWGSC\\_RefSeq\\_Annotations/v1.1/](https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.1/)). The pipeline followed for analysis of data is available in Supplementary Fig. 1.

### Gene ontology (GO) analysis and identification of transposable elements

GO analysis was carried out using Blast2GO [57] and differentially methylated genes (DMGs) (introns, exons, promoters and TTS) were assigned to cellular localisations, molecular functions and biological processes. For identifying transposable elements (TEs) in differentially methylated regions (DMRs), the nucleotide sequences of all the DMRs distributed in different genomic regions (exons, introns, promoters, TTS and intergenic) were used as input in the online tool Censor [58] (<https://www.girinst.org/censor/>). The TEs (including LTR = long terminal repeats and non-LTRs) and simple repeats were identified in the DMRs of all the four treatment pairs.

### Relationship between methylation and gene expression

The DMGs identified using MeDIP analysis were compared with differentially expressed genes (DEGs) to identify the genes undergoing both methylation and differential expression and to establish the relationship between them. For this purpose, RNA-Seq data were available for all the four treatment pairs; differentially expressed genes were also available

for the four treatment pairs that were used in MeDIP. The fold changes in methylation of the genic regions (intron, exon, promoter and TTS) of DEGs were compared to the fold changes in expression of the corresponding DEGs. The fold change in methylation levels of genes showing differential methylation in more than one out of the above four genic regions were merged; average fold change in methylation was calculated in each case for comparison with corresponding fold change in expression that was available from RNA-Seq data.

### RNA isolation and qRT-PCR analysis: validation of DMGs

Quantitative real time PCR (qRT-PCR) analysis was performed for comparing abundance of gene-specific transcripts (based on RNA-Seq data) with methylation patterns. For this purpose, first, total RNA was isolated from the 14-days old seedlings of susceptible cv. HD2329 and its resistant NIL (HD2329+*Lr28*) at 0 hbi and 96 hai using the TRIzol Reagent (Ambion) as per the manufacturer's specifications. Then, cDNA synthesis was performed from the total RNA using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with RT enhancer, to remove any contaminating DNA, according to the manufacturer's instructions.

For conducting qRT-PCR, 18 DMGs were selected, which included 10 DMGs for which RNA-Seq data was

also available (Table 2); these 18 genes were selected such that they contained domains involved in biotic/abiotic stress tolerance and they had more than twofold difference in methylation level. Primers for these DMGs were designed using Primer Express (ver. 3.0) software (Applied Biosystems; for details of primers see Supplementary Table 2). The qRT-PCR was performed using ABI 7500 Real-Time PCR System (Applied Biosystems). All reactions were performed using three biological and three technical replicates and the target gene expression (fold change) between two treatments was calculated using  $2^{-\Delta\Delta CT}$  method by using actin as a reference gene for normalisation [59]. Heat maps were generated using online tool Clustvis (<http://biit.cs.ut.ee/clustvis/>) and were used to depict the fold changes in qRT-PCR results and differential methylation patterns following MeDIP analysis.

## Results

### MSAP analysis

#### Global methylation patterns using MSAP

The number of MSAP fragments amplified with 27 individual primer pairs in four individual treatments ranged from 4 (E-AGG/H-TTC) to 24 (E-ACG/H-TCA); the size of fragments ranged from 61 to 294 bp. The details of amplified

**Table 2** Details of DMGs selected for qRT-PCR analysis

Treatment pairs	Protein domains	Methylation status	RNA-seq status
S0 vs S96	Ribosomal protein L20	Hyper	Low
	ATP synthase	Hyper	Low
	LRR	Hyper	–
	NB-ARC	Hypo	–
	UDP glucosyltransferase	Hypo	–
R0 vs R96	Cytochrome c oxidase subunit 3 like	Hypo	Low
	Cytochrome b6/f complex	Hypo	High
	Photosystem 1	Hypo	High
	ATPase	Hypo	–
	Cytochrome C oxidase	Hypo	Low
S0 vs R0	Transferase	Hyper	–
	Tetratricopeptide repeat1	Hyper	Low
	NADH:quinone oxidoreductase	Hyper	High
	Protein kinase	Hypo	–
S96 vs R96	Aspartic acid peptidase	Hyper	–
	Myb	Hyper	Low
	MADS box	Hyper	High
	NB-ARC	Hyper	–

S0 susceptible cultivar HD2329 at 0 hbi, S96 susceptible genotype HD2329 at 96 hai, R0 resistant NIL HD2329+*Lr28* at 0 hbi, R96 resistant NIL HD2329+*Lr28* at 96 hai, Hyper/Hypo indicate hypermethylation and hypomethylation in second treatment relative to first. Similarly, high/low indicate high and low expression in second treatment relative to first

**Table 3** Distribution of four types of MSAP profiles in four different treatments

MSAP profile	Number of amplified fragments in four treatments			
	S0	S96	R0	R96
Type I	18	18	22	8
Type II	74	160	149	56
Type III	32	47	49	46
Type IV	292	204	220	237
Total amplified fragments	416	429	440	347
Total methylated fragments (%)	398 (95.67)	411 (95.80)	418 (95.0)	339 (97.70)

Methylation patterns Type I, II, III and IV shown in column 1 are based on patterns shown in Table 1. Total methylated fragments at the bottom of the table are calculated by subtracting the fragments classified as Type I pattern (which indicate no methylation) from total amplified fragments (416, 429, etc.). S0:HD2329 at 0 hbi; S96:HD2329 at 96 hai; R0:HD2329+*Lr28* at 0 hbi; R96:HD2329+*Lr28* at 96 hai

fragments belonging to four different methylation patterns (Types I to IV in Table 1) are summarized in Table 3 (for details of primer pairs, see Supplementary Table 1).

### Differential methylation detected using MSAP

Four treatments were compared in pairs (S0 vs S96; R0 vs R96; S0 vs R0; S96 vs R96) using four MSAP types (Type I to Type IV). A summary of this comparison is available in Table 4, where differences in methylation patterns with passage of time (0 hbi to 96 hai) and also between susceptible cultivar and its resistant NIL at the same time can be noticed. The results suggest that the number of differentially methylated fragments is almost the same (> 200) in all the four comparisons, suggesting that there are large-scale differences in methylation patterns in different pairs of comparisons. The data also suggest that there are differences in methylation patterns not only over time (0–96 h), but also due to presence or absence of *Lr28* gene at the same time

point. For instance, in the susceptible cultivar, presence of more sites show demethylation in S96, suggesting a general pattern that many more genes are activated (demethylated) than the number of genes, which may undergo repression during progression of infection in the susceptible cultivar. The trend seems to be opposite in the resistant NIL carrying *Lr28* (R0 vs R96), suggesting that in resistant NIL, methylation increases due to the presence of *Lr28*, thus bringing about repression of many genes in the resistant NIL. However, the sites of methylation reduction in S96 and R0 with respect to S0 are different, suggesting that the genes that are activated in susceptible and resistant lines should be different.

MSAP data also give some information about the methylation context, as evident from differences between T1 and T2 shown in Table 4. Major differences include <sup>m</sup>CCG to <sup>m</sup>CG in S0 vs S96 and in S0 vs R0 and <sup>m</sup>CG to <sup>m</sup>CCG in R0 vs R96 and S96 vs R96.

In summary, infection seems to be facilitated by demethylation (activation of more genes) and increased <sup>m</sup>CG, while

**Table 4** Frequency of methylated and demethylated sites inferred from pairs of MSAP patterns in four treatment comparisons (methylation status in column 3 is inferred on the basis of patterns explained in Table 1)

T1	T2	Methylation status	S0 vs S96	R0 vs R96	S0 vs R0	S96 vs R96
Type I	Type II, III, IV	Methylation	15	13	15	11
Type III	Type IV		22	31	24	29
Total			37	44	39	40
Type III,II,IV	Type I	Demethylation	14	6	19	7
Type IV	Type III		31	33	33	36
Total			45	39	52	43
Type II	Type III	( <sup>m</sup> CG) to ( <sup>m</sup> CCG)	4	7	6	4
Type IV	Type II	( <sup>m</sup> CCG) to ( <sup>m</sup> CG)	115	30	118	35
Type III	Type II	( <sup>m</sup> CCG) to ( <sup>m</sup> CG)	0	3	3	2
Type II	Type IV	( <sup>m</sup> CG) to ( <sup>m</sup> C CG)	34	94	40	98
Total			235	217	258	222

S0:HD2329 at 0 hbi; S96:HD2329 at 96 hai; R0:HD2329+*Lr28* at 0 hbi; R96:HD2329+*Lr28* at 96 hai. T1 and T2 respectively represents first and second treatments in all the four pairs of comparisons

resistance is facilitated by methylation (repression of gene activity) associated with increased <sup>m</sup>CCG.

## MeDIP analysis

### MeDIP sequence data and methylated regions in four different treatments

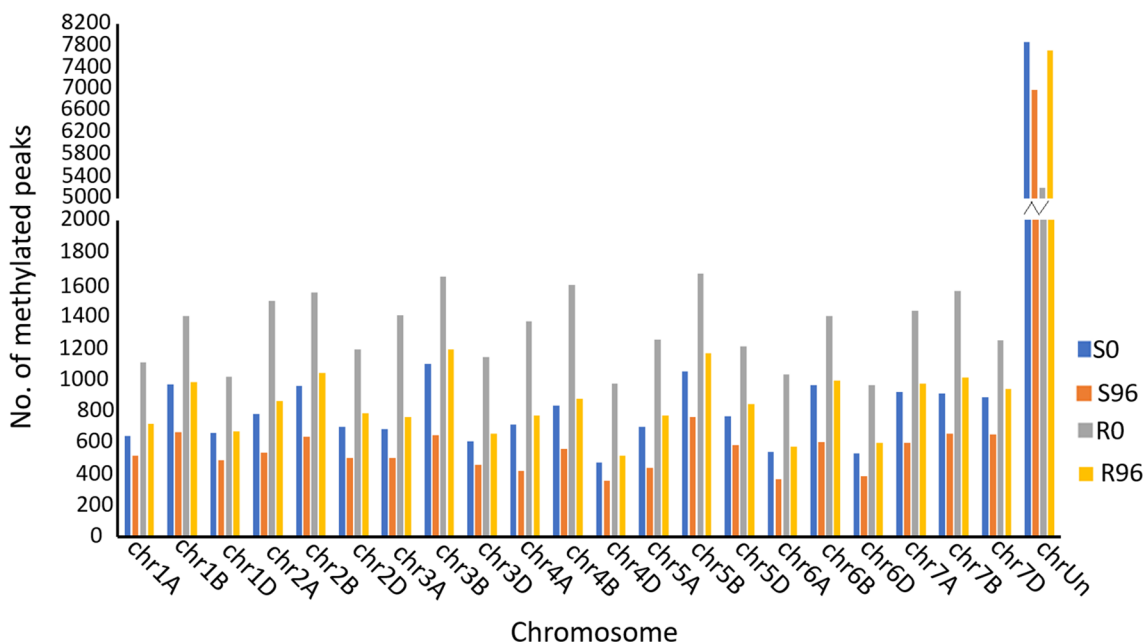
The total number of sequence reads was 504, 428, 562; the proportions of sequences aligned to reference genome of wheat ranged from 80.12% (R0) to 93.12% (S96) with an overall value of 87.94% (Supplementary Table 3).

Peak-calling gave 100,452 peaks with the minimum number of peaks (18,249) in R0, and the maximum number of peaks (32,722) in S96 (Table 5). The number of peaks on 21 individual chromosomes also varied (Fig. 1), but the pattern in four treatments seems to be the same (Fig. 1). The maximum number of peaks were available in the intergenic regions followed by promoters, TTSs and either exons or introns (for details see Table 5). A large number of peaks could not be assigned to any of the 21 chromosomes. The MeDIP raw reads of susceptible cv. HD2329 and resistant NIL HD2329 + *Lr28* at 0 hbi and 96 hai were deposited in Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI)

**Table 5** A summary of methylated peaks identified in four different treatments using the software MACS 2.0

Treatment	Total peaks	Distribution of peaks in genome				
		Exon	Intron	Promoter	TTS	Intergenic region
S0	24,166	163	236	485	409	22873
S96	32,722	483	317	522	398	31002
R0	18,249	172	149	449	378	17101
R96	25,315	160	239	498	388	24030
Total	100,452	978	941	1954	1573	95006

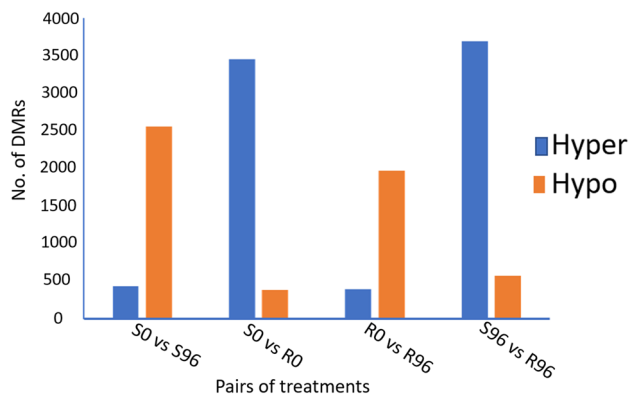
Methylated peaks in individual treatments were assigned to different genomic regions [exon, intron, promoter, transcription termination sites (TTSs) and intergenic region]. S0: HD2329 at 0 hbi; S96: HD2329 at 96 hai; R0: HD2329+*Lr28* at 0 hbi; R96: HD2329+*Lr28* at 96 hai (in each case, the second treatment is compared with the first)



**Fig. 1** Variation in the number of methylated peaks identified in four different treatments and mapped to 21 chromosomes (chr1A to chr7D) of wheat using MACS 2.0 software. A large number of peaks could

not be assigned to chromosomes; these are designated as chrUn (Un for unassigned). S0=HD2329 at 0 hbi; S96=HD2329 at 96 hai; R0=HD2329+*Lr28* at 0 hbi; R96=HD2329+*Lr28* at 96 hai





**Fig. 2** Variation in the number of differentially methylated regions (DMRs) identified using MeDIP in four different comparisons (S0 vs S96, S0 vs R0, R0 vs R96 and S96 vs R96). DMRs were identified following the criteria  $FDR < 0.05$  and  $FC \geq 2$  or  $\leq -2$  ( $\log FC \geq 1$  or  $\leq -1$ ) (Wang et al. [21]; Hu et al. [54]) where  $FC \geq 2$  indicates hypermethylated (hyper) regions and  $FC \leq -2$  indicates hypomethylated (hypo) regions. S0=HD2329 at 0 hbi; S96=HD2329 at 96 hai; R0=HD2329+Lr28 at 0hbi; R96=HD2329+Lr28 at 96 hai (in each case, the second treatment is compared with the first)

and can be accessed through Bioproject Database under the accession number PRJNA517568.

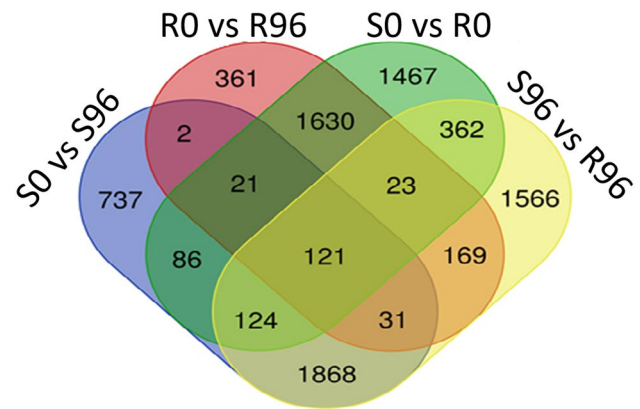
### Differentially methylated regions (DMRs)

The data on DMRs are summarised in Fig. 2. It is apparent that the frequency of hypomethylated DMRs increases with passage of time after inoculation both in susceptible cultivar and its resistant NIL, as apparent from comparison of S96 with S0 and that of R96 with R0. However, the frequency of hypermethylated DMRs is higher in resistant NIL relative to susceptible cultivar at both the time-points (0 hbi, i.e. S0 vs. R0 and 96 hai, i.e. S96 vs. R96) (Fig. 2).

The number of unique DMRs were substantially higher in S0 vs R0 (1467) and S96 vs R96 (1566) when compared to the remaining two treatment pairs. It is also apparent that there are more unique DMRs in susceptible cultivar (737) than in the resistant NIL (361) (Fig. 3).

### Identification of genes in DMRs and distribution of methylated regions in coding and non-coding regions

DMRs were subjected to identification of individual genes; 1320 genes (common genes between different treatment pairs were counted only once) were identified. These genes belonged to diverse classes and encoded proteins including the following: proteins with NBS-LRR domain, protein kinases, serine threonine kinases, NADH ubiquinone/quinone reductase, glutathione-S-transferase, ferredoxin, cytochrome b6/f, transcription factors like WAK, Myb; transporters like



**Fig. 3** Venn diagram showing distribution of the number of DMRs in four pairs of different treatments (S0 vs S96; R0 vs R96, S0 vs R0 and S96 vs R96). S0=HD2329 at 0 hbi; S96=HD2329 at 96 hai; R0=HD2329+Lr28 at 0 hbi; R96=HD2329+Lr28 at 96 hai (in each case, the second treatment is compared with the first)

citrate transporter, ABC transporter, methyltransferases like SAM dependent carboxyl methyltransferase, etc. Some of the genes were unique to an individual pair of treatments; others occurred in more than one treatment (Supplementary Table 4).

The DMRs and individual genes were also subjected to study of methylation in five different components of the genomic regions (introns, exons, promoters, TTS and intergenic regions). The results of this analysis are presented in Table 6 and further details are provided in Supplementary Tables 5–12. The pattern of methylation in DMRs did not differ, when examined at the level of individual genes. These results also corresponded with the overall genome-wide representation of hypermethylated and hypomethylated DMRs (Fig. 2).

### Transposable elements and SSRs in DMRs

Transposable elements (TEs) and simple sequence repeats (SSRs) were also identified in the DMRs. TEs mainly included DNA transposons, EnSpm/CACTA, Harbinger, Mariner/Tc1, Hat, MuDR, LTR and non-LTR transposons (Table 7). Highest number of TEs and SSRs were identified in the DMRs of S96 vs R96, and the least were identified in R0 vs R96, suggesting that in the resistant NIL, the frequency of TEs in DMRs decreased with passage of time.

### Functional annotation of genes associated with DMRs

GO terms were available for 1130 of 1320 genes and were classified into cellular components, molecular function and biological processes. Proteins encoded by some of these genes also belonged to photosynthetic membranes, membrane protein complexes and cell organelles. When all the

**Table 6** The distribution of DMRs in DMGs (exon, intron, promoter, transcription termination site i.e. TTS) and the intergenic regions

Genomic region	DMR/ genes	S0 vs S96			S0 vs R0			R0 vs R96			S96 vs R96		
		Hr <sup>*</sup>	Ho <sup>#</sup>	T	Hr	Ho	T	Hr	Ho	T	Hr	Ho	T
Introns	DMR	4	42	46	71	1	72	3	32	35	69	12	81
	Genes	4	20	22	48	1	41	3	25	26	37	9	53
Exons	DMR	7	26	33	83	1	84	1	34	35	55	12	67
	Genes	5	17	23	65	1	65	1	29	32	34	10	45
Promoter	DMR	5	29	34	429	3	432	31	157	188	256	5	261
	Genes	5	22	27	297	3	289	29	109	109	181	5	191
TTS	DMR	17	36	53	270	3	273	15	85	100	184	19	203
	Genes	14	27	41	188	3	201	15	65	75	132	15	154
Intergenic	DMR	404	2420	2824	2600	373	2973	344	1656	2000	3124	528	3652

Each DMR is of 500 bp. Contiguous DMRs were merged to identify DMGs. These DMGs were annotated using biomaRt tool available in Ensembl plants database. S0:HD2329 at 0 hbi; S96:HD2329 at 96 hai; R0:HD2329+*Lr28* at 0 hbi; R96:HD2329+*Lr28* at 96 hai (in each case, the second treatment is compared with the first). \*Hypermethylated; #Hypomethylated

three categories of GO terms were compared, maximum GO terms were found for biological processes. In molecular functions, protein binding, heterocyclic ion binding and organic cyclic compound binding were the most predominant terms (Figs. 4 and 5).

#### Relationship of gene expression and methylation

The MeDIP samples were also used for RNA-Seq, the data of which was used for a comparison with DMRs. Only 214 of the 1320 genes identified from DMRs were available in RNA-Seq data for comparison (Table 8). A proportion of these 214 genes were exclusively methylated in exons (7), introns (3), promoters (75) and TTS (46), whereas the remaining genes were methylated in more than one genomic region.

Out of the 214 genes, methylation in 88 genes showed negative relationship with gene expression, whereas remaining 126 genes showed positive relationship. A representative heat map showing the relationship of gene expression with methylation in S96 vs R96 is shown in Fig. 6; data for all the four treatment pairs is given in Supplementary Tables 13–16. Genes in four treatment pairs encoded mainly for the following proteins: ribosomal proteins, photosystem genes, cytochrome genes, ATPase, and NADH oxidoreductase, heat shock proteins, etc.

#### Quantitative real time PCR analysis

qRT-PCR was conducted for 18 selected DMGs, which differed in methylation patterns in different treatments as detailed in Table 1. For 10 of these 18 DMGs, RNA-Seq data was also available. qRT-PCR results for only four of these 10 DMGs matched the expression pattern inferred

from RNA-Seq data; these genes included genes containing domains for MADS box, NADH-quinone oxidoreductase, cytochrome b6/f complex and photosystem I. These genes showed abundance in RNA-Seq as well as qRT-PCR data and were hypomethylated. The remaining six genes showed opposite pattern during qRT-PCR and RNA-Seq (Fig. 7).

For the remaining eight of the 18 DMGs, for which RNA-Seq data was not available, qRT-PCR results were compared with only methylation patterns. For only three of these eight DMGs, qRT-PCR results matched with methylation patterns assuming that methylation leads to repression of gene expression; these three genes included genes encoding ATPase, UDP glucosyl transferase and NBS-LRR (Fig. 7)

#### Discussion

Molecular mechanism involved in disease resistance in plants (including leaf rust in wheat) has been a subject of intensive study. A two-layer system of plant immunity involving PTI and ETI has been widely accepted. Several earlier studies and our own studies (including the present study) involving *Lr28* gene for leaf rust resistance in wheat suggest that the presence of the pathogen is perceived by the host cells with the help of receptor molecules of some kind [38, 39, 41]. This is apparently followed by activation of many genes and repression of perhaps fewer genes, which start operating in more than one defence pathways that are interconnected in a complex manner to provide protection. Among these genes, the most extensively studied genes are R genes which generally encode proteins containing nucleotide-binding site leucine-rich repeat i.e. NBS-LRR or nod-like receptors (NLR) domains, although a variety of following kinases are also known to be involved: receptor like kinases (RLKs), mitogen associated protein (MAP)

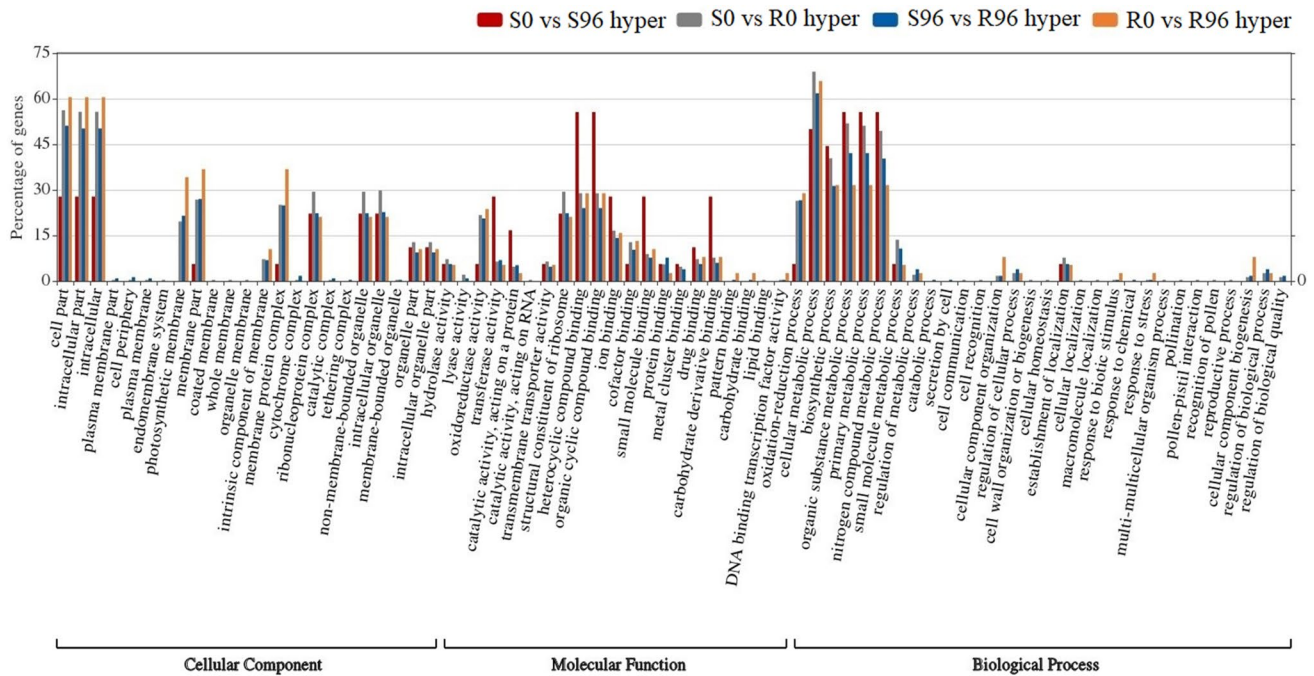
**Table 7** Details of SSRs and transposable elements (TE) identified in the DMRs of all the four treatment pairs

SSRs/TEs	Treatment pairs											
	S0 vs S96			S0 vs R0			R0 vs R96			S96 vs R96		
	Hr*	Ho <sup>#</sup>	Hr	Ho	Hr	Ho	Hr	Ho	Hr	Ho	Hr	Ho
1. SSRs	59	83	131	0	1	11	190	63				
2. Transposable element (TE) (DNA transposon + LTR retrotransposon + Non-LTR Retrotransposon)	242	834	1234	228	303	127	1658	339				
(a) DNA transposon ( <i>EnSpm/CACTA + Har-binger + Mariner/ Tc1 + hAT + MuDR</i> )	89	429	565	181	252	23	946	123				
(b) LTR retrotransposon ( <i>Copia and Gypsy</i> )	144	394	610	46	50	92	663	209				
(c) Non-LTR retrotransposon ( <i>LI, RTE and SINE</i> )	9	11	59	1	1	12	49	7				
Total (SSRs + TEs)	59 + 242 = 301	83 + 834 = 917	131 + 1234 = 1365	0 + 228 = 228	1 + 303 = 304	11 + 127 = 138	190 + 1658 = 1848	63 + 339 = 402				

Each DMR sequence belonging to different genomic regions was used as an input in online tool Censor (Kohany et al. [57]; <https://www.girinst.org/censor/>) to identify the SSRs and transposable elements (TEs), S0:HD2329 at 96 hai; S96:HD2329+Lr28 at 0 hbi; R0:HD2329+Lr28 at 0 hbi; R96:HD2329+Lr28 at 96 hai

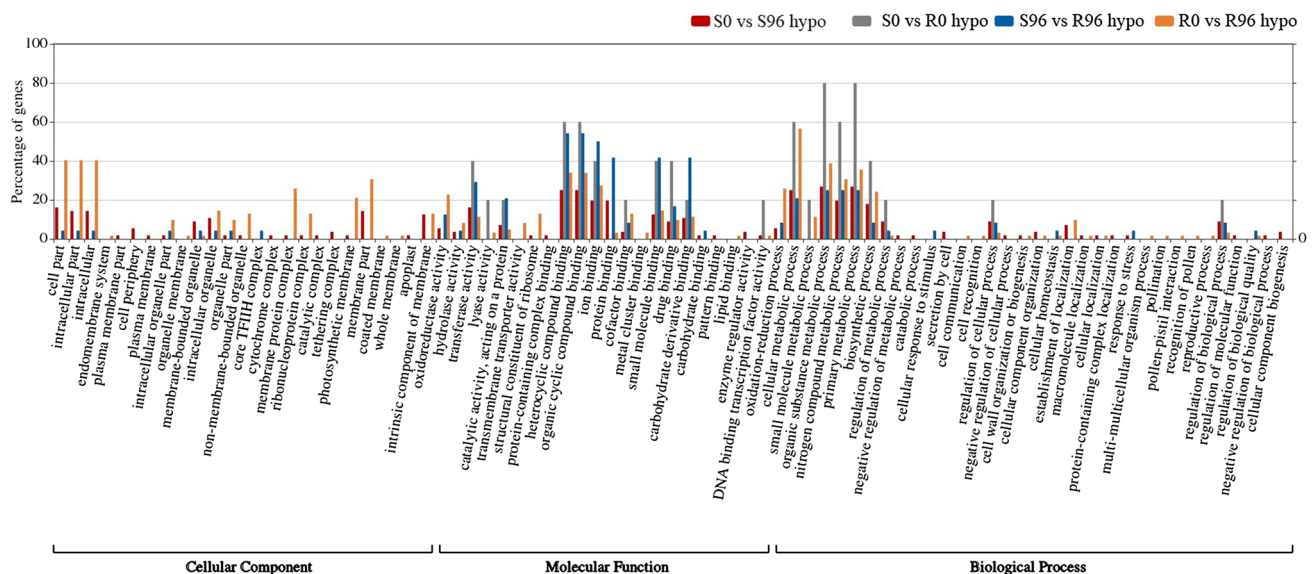
SSR simple sequence repeat, LTR long terminal repeats, LI line1, RTE retro transposon like element, SINE short interspersed nucleotide elements, TE transposable element

\*Hypermethylated; <sup>#</sup>Hypomethylated



**Fig. 4** Bar diagram showing results of GO annotation in the form of proportions of differentially hypermethylated genes (DMGs) in four different comparisons shown in the form of bars with four different colours (S0 vs S96 hyper, etc.) belonging to three categories of functions: cellular component, molecular functions and biological pro-

cesses of hypermethylated genes (in each case, the second treatment was compared with first treatment) in four different treatment pairs. S0=HD2329 at 0hbi; S96=HD2329 at 96 hai; R0=HD2329+*Lr*28 at 0hbi; R96=HD2329+*Lr*28 at 96 hai (in each case, the second treatment is compared with the first)



**Fig. 5** Bar diagram showing results of GO annotation in the form of proportions of differentially hypomethylated genes (DMGs) in four different comparisons shown in the form of bars with four different colours (S0 vs S96 hyper, etc.) belonging to three categories of functions (cellular component, molecular function and biological process)

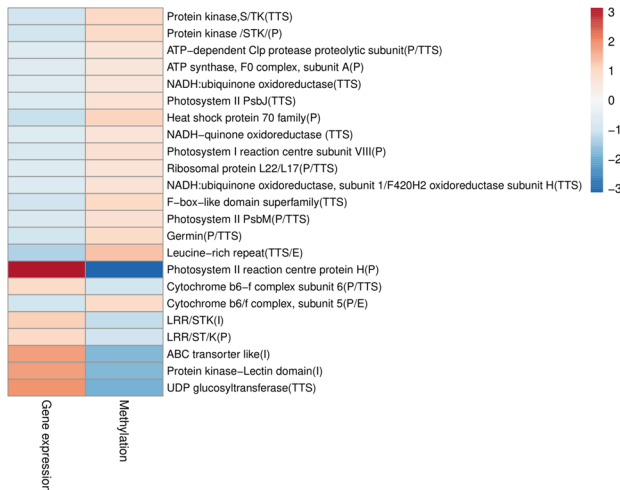
(in each case the second treatment is compared to first treatment). S0=HD2329 at 0 hbi; S96=HD2329 at 96 hai; R0=HD2329+*Lr*28 at 0 hbi; R96=HD2329+*Lr*28 at 96 hai (in each case, the second treatment is compared with the first)

**Table 8** Frequencies of hypermethylated (Hyper) and hypomethylated (Hypo) DMGs and their relationship with expression level

Relationship	S0 vs S96		R0 vs R96		S0 vs R0		S96 vs R96		Total
	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	
Positive	–	–	2	10	67	–	45	2	126
Negative	3	5	–	28	28	–	21	3	88

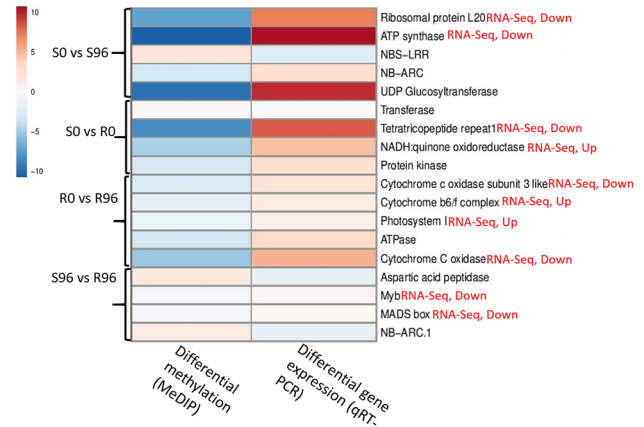
Positive relationship indicates hypermethylation accompanied by higher expression or hypomethylation accompanied by lower expression. On the contrary, negative relationship indicates hypermethylation accompanied by lower expression and hypomethylation accompanied by higher expression which is generally expected situation

S0: HD2329 at 0 hbi; S96: HD2329 at 96 hai; R0: HD2329 + *Lr28* at 0 hbi; R96: HD2329 + *Lr28* at 96 hai, (–) indicates absence of genes in that particular situation



**Fig. 6** Heatmap showing the relationship between methylation and gene expression for some important genes (relevant to disease resistance) in S96 vs R96 (for each row, name of genes given on the right of the heatmap). The scale on the extreme right shows the levels of methylation/gene expression (dark red shade indicates maximum change in expression/methylation whereas dark blue shade indicates minimum change in methylation/gene expression). Both for methylation and gene expression, DMGs and DEGs showing  $FC \geq 2$  ( $\log FC \geq 1$ ) were considered as hypermethylated/upregulated and  $FC \leq -2$  ( $\log FC \leq -1$ ) were considered as hypomethylated/downregulated, respectively. The letters mentioned in parenthesis at the end of the name of each gene indicates genomic regions as follows: *P* promoter, *TTS* transcription termination site, *E* exon, *I* intron. S96 = HD2329 at 96 hai; R96 = HD2329 + *Lr28* at 96 hai.

kinases, wall associated kinases (WAK), etc. Calcium ion fluxes, generation of ROS and oxidative burst have also been suggested to be involved to enforce physical barriers by cell wall appositions and causing hypersensitive response (HR). An oligomerization of NLR with other proteins leading to the formation of a resistosome/inflammasome has also been suggested in a recent study [11]. More recently, presence of NLR-TIR domain carrying NADase activity for cleavage of  $NAD^+$  has also been suggested to play a role in cell death and HR [12, 13]. Expression of all these genes/protein domains needs to be regulated in an intricate but precise manner to provide defence on pathogen infection. We



**Fig. 7** Heatmap showing the methylation and gene expression changes in a set of genes in all the four treatment pairs (S0 vs S96, S0 vs R0, R0 vs R96 and S96 vs R96). The genes indicated with RNA-Seq are the common differentially expressing genes available in transcriptome data and Up and Down indicate the change in the level of expression i.e. upregulated and downregulated. The scale shown at the top left corner indicates the methylation/expression fold changes. The heat map is drawn using online tool Clustvis with each row subjected to univariate scaling in order to represent the fold changes in each treatment pair. In each treatment pair, the expression/methylation fold change is indicated in second treatment relative to first treatment

believe that this regulation is partly achieved through epigenetic modifications including reversible DNA methylation and demethylation, histone modifications and non-coding RNAs. The present study is focussed on DNA methylation as a component of epigenetic modifications, which was examined using MSAP and MeDIP.

### Resolution of DNA methylation using MSAP

MSAP allowed us to analyse differentially methylated sites (DMSs) and also provided some information about methylation at CG and CCG (CHG) contexts; a similar analysis was earlier conducted in tobacco [60]. MSAP results also allowed us to study changes in methylation contexts from  $^mCG$  to  $^mCCG$  and vice versa in both S and R lines during

96 hai. Alteration of  $^m\text{CCG}$  to  $^m\text{CG}$  seems to be the most conspicuous change followed by the reverse methylation contexts, i.e.  $^m\text{CG}$  to  $^m\text{CCG}$  (Table 4). This is also in agreement with earlier study in wheat where CG methylation was reported to be most abundant followed by CHG (H=C in the present study) and CHH methylation [24]. Methylation in intergenic regions was particularly observed in TEs (LTR and non-LTR retrotransposons, DNA transposons) and SSRs (Table 7). In earlier studies, the role of non-CG methylation in addition to CG methylation was also observed in case of Arabidopsis [61], rice [62] and wheat [24]. In Arabidopsis, it was also shown that gene body methylation mainly occurs in CG, although it also occurs at CHH and CHG in intergenic regions.

One of the limitations of MSAP is that exact methylation changes (methylation or demethylation) cannot be inferred due to the difficult interpretation of Type IV methylation pattern [63, 64]. Some researchers earlier considered type IV pattern as informative [65, 66], while others considered it to be uninformative and hence ignored it for inferring methylation from MSAP [63, 64]. However, in the present study, type IV was most abundant followed by type II, type III and type I patterns in that order (Table 3). Therefore, type IV results were critically examined. Further analysis revealed that during the passage of time after inoculation, type IV was changed to type II in majority of CCGG sites, suggesting demethylation of external cytosine or conversion of  $^m\text{CCGG}$  to  $\text{C}^m\text{CCGG}$ ; this change occurred in S cultivar during 96 hai (Table 4). A reverse situation leads to methylation of at least some CCGG sites or conversion of  $\text{C}^m\text{CCGG}$  to  $^m\text{CCGG}$  (Table 4). Based on the available information of four different methylation patterns, changes from types III, II and IV to type I or type IV to type III can be classified as demethylation and the opposite situations may be classified as methylation. This indicated occurrence of demethylation during the passage of 96 hai both in S (in S0 vs S96) and R lines (in S96 vs R96) (Table 4). Similar pattern in S cultivar was also observed with MeDIP, which also helped us to identify the DMRs and the corresponding genes (DMGs), which are involved in susceptibility or resistance. Methylation changes due to both the techniques are discussed later in this “Discussion” section.

### Resolution of DNA methylation using MeDIP

MeDIP allowed us to identify differentially methylated regions (DMRs). These DMRs also included those in the body of the genes, which are then designated by us as differentially methylated genes (DMGs); we also assume that in most DMRs/DMGs, methylation should be reversible thus playing a role in regulation of gene expression. However, it was not possible to identify individual methylated cytosines in specific genes, which requires bisulphite-sequencing [67].

In the present study, the distribution of methylation in different genomic regions (like introns, exons, promoter, TTS, intergenic) was also found to be important, since the effect of methylation in promoter sequences differs from the effect of methylation in other parts of the genome. In the present study, 75–94% DMRs were present in regions outside the promoter regions (1 kb upstream of TSS). Similar results were reported earlier in the Arabidopsis-*Pseudomonas syringae* pathosystem [68]. Such DMRs have also been shown to have a role in limiting transcription and in providing stability to the TEs [69]. Hypermethylated DMRs containing TEs were particularly conspicuous in R NIL relative to S cultivar (S96 vs R96) (Table 7), which may be associated with the repression of neighbouring genes, as also reported earlier [70]. Differential methylation in promoters and TTS in the present study also seem to be in agreement with the results of earlier studies in maize [71] and rice [21].

### Methylation versus gene expression

The DMGs were also compared with differentially expressed genes (DEGs) identified in the RNA-Seq data. Only 16% (214 out of 1320 genes) DMGs were available in RNA-Seq data indicating only a partial role of DNA methylation in regulation of genes involved in defense against leaf rust (Table 8). Obviously, several other genetic and epigenetic factors should be involved in regulating leaf rust resistance in wheat. Further comparison revealed that for 88 of the 214 DMGs (Supplementary Tables 13–16), the expression pattern matched with methylation results assuming that hypermethylation was associated with repression of gene expression and hypomethylation was associated with enhanced gene expression. In 20 of these 88 genes, exclusive methylation of the promoter region suppressed the gene expression, which is in agreement with earlier observations [72, 73]. In the remaining 68 DMGs, besides the promoters, specific regions of gene body were also differentially methylated. Therefore, specific role of the promoter methylation in regulation of gene expression seems to be limited.

For the remaining 126 of the 214 DMGs, hypermethylation was associated with higher gene expression, while hypomethylation was associated with repression of gene expression. This was contrary to the generally expected pattern. Similar results, however, were also reported in rice under drought stress [21], in maize under salt stress [71] and in other plant systems like *Populus trichocarpa* [74] and apple [75]. Overall, we conclude that resistance to leaf rust is influenced by expression of a number of genes partly mediated by DNA methylation. This methylation may occur exclusively in promoter region or in promoter as well as other genomic regions (introns, exons and TTS).

On the basis of a comparison of RNA-Seq data (earlier data and the data obtained in the present study) and

the methylation data obtained from MeDIP samples used in the present study, we believe that the widely accepted belief that hypermethylation leads to repression and hypomethylation leads to activation of genes is an oversimplification, and that this generalization is applicable only in a small fraction of DMGs. qRT-PCR results also support this observation since only four out of the eight DMGs showed the expected negative relationship of methylation with gene expression. Role of some of these DMGs in response to biotic stresses in wheat or other cereals is known from published literature (summarized later in Table 9).

The details of the complexity associated with infection and immunity processes and involvement of DNA methylation in these processes have also been widely discussed in published literature and are summarized in a recent review [76]. We believe that differential methylation of a number of genes in the two lines used in the present study, is largely due to the presence or absence of *Lr28* gene, although some differences in the genetic background between the two lines and the associated downstream pathways may also be responsible. However, we believe that the leaf rust resistance gene *Lr28* had no harmful effect due to possible linkage drag as revealed in an earlier report where the two lines used in the present study were compared for yield related and bread making quality traits and no deleterious effects due to the resistance gene *Lr28* were observed [77]. A summary of the earlier studies that are relevant to the discussion is presented in Table 9; the results of the present study are discussed in the following text.

In the past a role of differential methylation in incompatible/compatible interactions has been reported in

different host-pathogen systems. First, hypomethylation in the presence of powdery mildew resistance gene was reported in wheat [30]. Second, MSAP revealed a role of hyper-methylation in bacterial blight adult plant resistance in rice [15]. Third, in Arabidopsis, hyper/hypomethylation was shown to be associated with compatible/incompatible interactions involving a number of pathogens including *P. syringae*, *Plectosphaerella cucumerina*, *Alternaria brassicicola*, *Hyaloperonospora arabidopsidis* and *Fusarium oxysporium* [78, 79]; for review, see [76] Deleris et al.).

### Hypomethylation of many and hypermethylation of few but different sets of genes involved in susceptibility (S0 vs S96) and resistance (R0 vs R96)

The results of the present study seem to suggest that both hypomethylation and hypermethylation of different sets of genes occur during S0 to S96 and R0 to R96. Our results also suggest that in both susceptible cultivar and resistant NIL, many genes get hypomethylated during passage of time after inoculation. Interestingly, only eight DMGs were common between the susceptible cultivar and resistant NIL indicating that resistance and susceptibility are mainly governed by activation of different sets of unique genes (160 in resistant NIL and 56 in susceptible cultivar). Assuming that hypermethylation leads to repression and hypomethylation leads to activation of genes, these results can be used to infer that activation of different sets of unique genes is involved in resistant and susceptible reactions. However, this inference needs to be treated with caution as hypomethylation does not always lead to gene activation and similarly, hypermethylation does not always leads to suppression of gene expression as reported earlier

**Table 9** Functions of different DMGs in biotic/abiotic stresses in wheat and other cereal crops

Protein	Crop	Function	References
Clp protease	Wheat	High expression due to fungal, bacterial and insect infections	[103]
Clp protease and HSP70	All crops	Clp protease and HSPs required for protection against oxidative stress	[104]
GSTs	Wheat	Upregulation in <i>Lr28</i> resistant reaction during leaf rust infection	[39]
Glutathione S transferase	Wheat	Enzyme activity increased in Thatcher NILs carrying <i>Lr19</i> , <i>Lr33</i> and <i>Lr26</i> genes	[105]
	Rice	Overexpression improves salinity and oxidative stress tolerance in Arabidopsis	[106]
F-Box (TaFBA1)	Wheat	Involved in tolerance to drought and heat and oxidative stress tolerance	[107, 108, 109]
Terpene synthase	Wheat	Genes involved in terpene synthesis were highly expressed in response to Fusarium crown rot infection	[110]
	Rice	Overexpression leads to enhanced resistance to blast fungus <i>Magnaporthe oryzae</i>	[111]
LRR repeats and S/TPKs	Wheat	Upregulation due to leaf rust and powdery mildew infection	[112, 113]
Myb TF	Wheat	Transgenic wheat overexpressing Myb TF shows enhanced resistance to take-all disease caused by <i>Gaeumannomyces graminis</i>	[114]

and also revealed by comparison of methylation with gene expression during the present study [21, 71, 74, 75].

### Unique hypomethylated genes in susceptible S0 vs S96 and resistant R0 vs R96 reactions

The unique DMGs in the susceptible cultivar perhaps facilitate pathogen infection (assuming hypomethylation leads to gene activation) (Supplementary Table 4). Except PR6 (Protease inhibitor Birk I) and dirigent protein coding genes, which showed up-regulation in expression data, no other gene was available in differential RNA-Seq expression data. Some of these unique DMGs were earlier reported to show induced expression in susceptible genotypes and thus help the pathogen for survival and multiplication on the host. For instance, PR6 (protease inhibitor) was highly expressed in susceptible wheat cultivars when infected with *Fusarium graminearum* [80]. Similarly, a dirigent protein coding gene had high expression during compatible interaction between *Vitis vinifera* and *Erysiphe necator*, causing powdery mildew [81]; dirigent proteins are also widely known to be associated with resistance against biotic and abiotic stresses [82, 83]. Overexpression of the gene encoding WRKY7 TF was also shown to repress the expression of defence related genes encoding PR1 proteins during compatible interaction of *P. syringae* in Arabidopsis [84].

The unique DMGs which were identified in the resistant NIL with progress of infection are perhaps involved in the defence response in the resistant NIL. The details of such unique DMGs are provided in Supplementary Table 4. Some of these DMGs like ribosomal protein L2, photosystem I and ATPase were also available in RNA-Seq data where they showed upregulation. Some DMGs which showed hypomethylation mediated up-regulation as examined by qRT-PCR analysis included cytochrome C oxidase, photosystem I, and cytochrome b6/f complex. Some of these genes were earlier reported to be involved in defence response against different biotic stresses. Following are two such examples: (i) In Arabidopsis, *Attrxh5* which is a type of cytosolic thioredoxin was shown to be induced due to wounding, abscission, senescence as well as during incompatible interaction between Arabidopsis and bacterial pathogen *P. syringae* [85]. The role of thioredoxins in oxidative stress mediated redox signalling in plant immunity is reviewed in detail by Perez and Spoel [86]. (ii) Bulb type lectin domain containing genes (B-Lec) belong to a class of lectin like receptor kinases (Lec-RLKs) which are known to play a critical role during stress conditions, besides plant growth and development. In wheat, a number of different types of Lec-RLKs were characterised and about 120 different types of B-Lec encoding genes were characterised and their expression was found to be affected due to inoculation with *Blumeria*

*graminis* and *Puccinia striiformis* [87]. The roles of some other unique DMGs in resistance or susceptibility are summarised in Table 9.

### Unique hypermethylated genes in susceptible cultivar (S0 vs S96) and resistant NIL (R0 vs R96)

Assuming that the hypermethylation leads to repression of gene activity, it seems that the genes which get hypermethylated in susceptible cultivar or resistant NIL perhaps negatively regulate susceptibility or resistance. Majority of the hypermethylated DMGs in susceptible cultivar and resistant NIL were unique (28 in S0 vs S96 and 43 in R0 vs R96) to either of the two treatment pairs except four DMGs, which were common to both. At least some of these genes (for details, refer to Supplementary Table 4) are known to provide resistance when over expressed indicating that their repression will lead to susceptibility. For instance, (i) RRBI2-3 which is a type of Bowman Birk trypsin inhibitor when overexpressed in rice, provided resistance against fungal pathogen *Pyricularia oryzae* indicating that its repression would lead to susceptibility in rice [88]. (ii) In Arabidopsis, the mutants of MAX2 (a type of F-box protein), showed increased susceptibility to bacterial pathogen like *Pectobacterium carotovorum*, and fungal hemi-biotrophic pathogen like *P. syringae* [89].

Similarly, the unique hypermethylated genes in the resistant reaction (Supplementary Table 4) which were earlier reported to induce resistance through negative regulation include genes involved in photosynthesis like cyt b6/f and photosystem II which exhibited decreased expression in response to *P. syringae* infection in soybean leading to hypersensitive reaction associated resistance. This resistance was induced due to damage in the photosynthesis PSII system which leads to oxidative burst leading to expression of other defence related genes [90]. Many of the domains contained in hypermethylated genes are similar to the domains of the genes which underwent hypomethylation, indicating similar function although the actual genes differ in two cases

### Hypermethylation of many and hypomethylation of few genes due to the presence of *Lr28* (S0 vs R0 and S96 vs R96)

Our results also suggest that methylation status of the DMRs differ between resistant NIL and susceptible cultivar merely due to the presence of *Lr28* in the resistant NIL (Fig. 2). Thus, a large number of DMGs showed hypermethylation (423 in S0 vs R0 and 298 in S96 vs R96) and few DMGs showed hypomethylation (9 in S0 vs R0 and 167 in S96 vs R96) in the resistant NIL. Further, between



the two treatment pairs, there were 167 common hypermethylated DMGs and only one common hypomethylated DMG in the resistant NIL.

### Hypermethylated genes due to the presence of *Lr28*

The hypermethylated DMGs perhaps may act as negative regulators of leaf rust resistance assuming that they get repressed due to the presence of *Lr28*. The 177 common hypermethylated DMGs involving the treatment pairs S0 vs R0 and S96 vs R96 as well as the unique DMGs identified in R0 (relative to S0) may be perhaps involved in preparing the host for future attack by pathogen; these may act as constitutive barrier as also reported earlier in wheat involving powdery mildew resistance gene [91]. Ninety six DMGs identified in resistant NIL in the absence of infection (R0) also showed association with DEGs in RNA-Seq data. Some of these genes contained domains for tetratricopeptide repeat 1, ribosomal proteins L23/L25, ATPase, ATP synthase, clp protease, etc. (Supplementary Table 4).

The unique DMGs that are hypermethylated in resistant NIL in the treatment comparison S96 vs R96 may be actually involved in defence response against leaf rust disease. These DMGs contain the domains for lipid transfer protein, AP2/ERF, clp protease proteolytic subunit B3 domain binding domain, a cysteine peptidase, wall associated receptor kinase, etc. (Supplementary Table 4). These DMGs perhaps act as negative regulators of leaf rust resistance assuming that hypermethylation of these genes leads to silencing of genes. For instance, (i) in rice, the loss of function mutant of *WAK112d* was shown to induce resistance against *M. oryzae* [92] (Table 9); (ii) in rice, RNAi knockdown of TFs with AP2/ERF domain (e.g. *OsERF109*) resulted in increased drought tolerance [93]; this effect seems to be due to altered ethylene biosynthesis. In Arabidopsis also, *AtERF014* negatively regulate resistance against necrotrophic fungus *Botrytis cineria* where the knockdown of this gene gave increased resistance [94]; (iii) in wheat, a lipid transfer protein (*TaDIR1-2*) was shown to confer resistance against *Puccinia striiformis*, since knockdown of this gene has been reported to enhance disease resistance by modulating ROS and/or SA signalling [95].

### Hypomethylated genes due to the presence of *Lr28*

There were eight DMGs in R0 but 39 DMGs in R96; only one DMG being common. Some unique genes in R96 which also showed upregulation in RNA-Seq data include those encoding glutathione transferase or GST, glutaredoxin, NBS-LRR (NLR), ABC transporter, lectin domain containing protein kinase, UDP-glucosyltransferase, etc. (Supplementary Tables 13–16). Activation of these genes also seems to provide enhanced resistance. Several examples are

available from published literature, where some of these genes were shown to be involved in providing resistance against plant pathogens. Following are some examples: (i) the role of GSTs in providing resistance against bacterial, fungal and viral diseases is widely known (for details see review by Gullner et al. [96]; also see Table 9). GSTs perhaps act as ROS scavenging enzymes and thus help in enhancing *Lr28* mediated leaf rust resistance. (ii) Glutaredoxins represent another group of oxidoreductases that are involved in controlling ROS. Genes from both these classes exhibited higher expression in the incompatible interaction in our earlier study involving transcriptome analysis [39], thus indicating that ROS homeostasis is an important mechanism imparting leaf rust resistance in wheat. (iii) Genes encoding proteins with LRR repeats are also important components of receptor like kinases and are reported to exhibit higher expression during incompatible interaction involving the wheat-leaf rust pathosystem involving *Lr28* [11, 39, 41, 42]. (iv) Adult plant leaf rust resistant gene *Lr34* is also known to encode for ABC transporter [97]. (v) Genes encoding proteins containing lectin domain were found to be strongly upregulated in resistant genotypes for fusarium head blight [98]. Thus, although the role of all these genes in imparting disease resistance was known, but perhaps it is for the first time, that evidence of regulation of the expression of all these genes through hypomethylation is being reported in the present study.

### DMGs involved in biochemical and physiological pathways

We discussed above the differential methylation of some specific genes that are apparently involved in resistance (F-box, glutaredoxin, kelch repeats, S/TPK, etc.). However, there were other differentially methylated genes that are known to be involved in physiological (photosynthesis and electron transport) and biochemical pathways (oxidative stress, growth hormone response) and also in plant development. For instance, genes encoding PS1, PSII and NADH-Ubiquinone Plastoquinone Oxidoreductase that were found to be differentially methylated are known to be involved in photosynthesis and electron transport. In earlier studies, expression of these genes was shown to be drastically affected during biotic stresses [99] but also during abiotic stresses like cold, salt, etc. [100]. In case of rice also, many genes involved in photosynthesis, oxidative stress, transport and other metabolic processes have been reported to be differentially methylated due to drought stress [21]. Similarly, oxidative stress response genes like glutathione S transferase encoding gene was also differentially methylated in resistant reaction during the present study. This gene helps in the protection of cells from oxidative damage in plants during various abiotic (reviewed in [101]) and biotic stresses (reviewed

in [96]). Examples of methylation of genes involved in plant development were also available. For instance, a hormone regulated gene *KNOX1*, that is known to be involved in leaf development [102] was hypermethylated during incompatible interaction, so that its silencing may perhaps impair plant development, thereby rendering the plant susceptible.

### Some specific features related to resistance due to *Lr28*

Overall, the results of DNA methylation in resistant NIL (R) and susceptible cultivar (S) suggest some specific features which may be mainly related to resistance conferred by *Lr28* gene. Some of these specific features include the following: (a) Alteration of <sup>m</sup>CG to <sup>m</sup>CCG which is more conspicuous during resistance as revealed through MSAP analysis in treatment pairs R0 vs R96 and S96 vs R96. (b) Hypomethylation of different sets of genes seems to be involved in imparting susceptibility and resistance. Some important DMGs which are specifically involved in imparting leaf rust resistance are those carrying domains for ATP synthase, ATPase, NBS-LRR, bulb type lectin, clp protease, cytochrome c oxidase, isoprenoid synthase, cytochrome C oxidase, photosystem I, and cytochrome b6/f complex, etc. (Supplementary Table 4). Other DMGs that may also be responsible for resistance include those which get uniquely hypermethylated in resistant NIL in the treatment pair S96 vs R96 which include genes containing domains for ABC transporter, glutathione S transferase, glutaredoxin, lectin domain containing receptor kinases, etc. (Supplementary Table 4). Even the hypermethylated DMRs containing SSRs and transposable elements are more abundant in R96 (relative to S96) (Table 7).

### Conclusions

The following important conclusions were made, based on the present study: (i) Both MSAP and MeDIP approaches can be used to identify specific DMRs involved in wheat-leaf rust pathosystem. However, complete information about the role of different methylation contexts (CG, CHG and CHH) could not be deciphered, although MSAP provided some information about CCG and CG contexts. Only future studies involving bisulfite sequencing (BiS-Seq) may give better insights into the role of different methylation contexts in wheat-leaf rust interaction. BiS-Seq may also provide validation for the role of differentially methylated genes that were related with rust resistance during the present study (such BiS-Seq using the same genotypes is currently underway in our lab). (ii) Only some DMGs could be related with resistance/susceptibility, since only 16% of DMGs were available in expression data (RNA-Seq). Some of these genes seem

to have a direct role in disease resistance, while some others were apparently involved in physiological or biochemical pathways that may indirectly influence disease resistance/susceptibility. (iii) Differential methylation was identified in both intergenic and intragenic regions, but methylation in intergenic region is more abundant followed by promoter/TTS/intron and exon. (iv) Since only 16% DMGs match the RNA-Seq based gene expression data, it was concluded that other epigenetic factors like histone modifications, non-coding RNA (miRNA and lncRNA) may also be involved in partial epigenetic control of leaf rust infection. (v) Since DMRs carried a large number of TEs, it is possible that a large fraction of DMRs be involved in maintaining genome stability, probably through silencing the TEs. (vi) Since DNA methylation was found to be associated with positive (unexpected) as well as negative (expected) gene expression, we inferred that DNA methylation may not always repress gene expression during leaf rust resistance

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**Author contributions** PKG and HSB conceived the experiment. GS with the help of CS, KS and TG conducted data analysis. GS and CS also prepared the first draft of the manuscript which was edited and finalised by PKG, HSB and PKS. PP provided inoculum for leaf rust pathotype 77-5 and NJ, KVP and JBS raised and provided the seedlings tissues. JKR assisted GS and CS in conducting MSAP analysis.

### 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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