Co-Regulation of Long Non-Coding RNAs with Allele-Specific Genes in Wheat Responding to Powdery Mildew Infection

Weiguo Hu1,2, Guanghao Wang1, Siwen Wang1, Xiaojun Nie1, Changyou Wang1, Yajuan Wang1,3, Hong Zhang1,3,4 and Wanquan Ji1,3,*

1 State Key Laboratory of Crop Stress Biology for Arid Areas, College of Agronomy, Northwest A&F University, Yangling 712100, China; hnhuweiguo@163.com (W.H.); wgh90325@126.com (G.W.); wangsiwen2017@126.com (S.W.); small@nwafu.edu.cn (X.N.); chywang2004@126.com (C.W.); wangyj7604@163.com (Y.W.); zhangh1129@nwafu.edu.cn (H.Z.)
2 Institute of Wheat, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China
3 Shaanxi Research Station of Crop Gene Resources and Germplasm Enhancement, Ministry of Agriculture, Yangling 712100, China
4 China-Australia Joint Research Center for Abiotic and Biotic Stress Management, Northwest A&F University, Yangling 712100, China
* Correspondence: jiwanquan2008@126.com

Received: 8 May 2020; Accepted: 22 June 2020; Published: 24 June 2020

Abstract: Powdery mildew (caused by Blumeria graminis f. sp. tritici; Bgt) is an important fungal disease of wheat (Triticum aestivum) worldwide, and results in significant crop damage in epidemic years. Understanding resistance mechanisms could have undoubted benefits in controlling disease and minimizing crop losses. The recent explosion in genomic knowledge and the discovery of noncoding RNAs have led to the idea that long ncRNAs (IncRNAs) might be key regulators of protein-coding gene expression. However, in-depth functional analyses of IncRNAs in wheat remain limited. Here, we evaluated the possible role of IncRNAs in regulating functional genes in wheat responding to Bgt pathogen, using genome-wide transcriptome data and quantitative RT-PCR. Our results demonstrated that both long intron ncRNAs (linncRNA) and long intergenic ncRNAs (lincRNAs) play roles in regulating allele-specific genes, including transcription factors, both positively and negatively. The correlation of expression between lincRNAs and flanking functional genes increased as the spacing distance decreased. Co-expression of microRNAs, their target lncRNA and target functional genes showed that lncRNA interacted competitively with functional genes via miRNA regulation. These results will be beneficial for further dissecting molecular mechanisms of IncRNAs functions at the transcriptional and post-transcriptional levels in wheat.

Keywords: wheat; powdery mildew; IncRNAs; allele-specific functional genes; miRNA; co-regulation

1. Introduction

Long noncoding transcripts (IncRNA), defined as a group of RNA transcripts that exceed 200 nt in length with no apparent discernible coding potential, were previously seen as the ‘junk’ RNAs or ‘dark matter’ of the genome [1]. However, the recent explosion in genomic knowledge demonstrated that ncRNAs can play roles as key regulators of protein-coding gene expression, either directly or indirectly [2,3], such as competing with endogenous RNAs to regulate miRNA levels [4] and scaffolding ribonucleoprotein complexes [5]. Compared with mammalian systems, the functional dissection of plant IncRNAs is still in its infancy. Initial identification of plant IncRNAs was based on bioinformatic searches in cDNA databases for RNAs with poor coding capacity [6,7]. Fortunately, high-resolution
analyses of plant transcriptomes by RNA-sequencing allowed a more comprehensive view of IncRNAs in several plant species over the last few years, such as *Arabidopsis*, *Populus trichocarpa*, *Oryza sativa*, *Zea mays*, and *Triticum aestivum* [8–11]. Emerging evidence indicates that IncRNAs play key roles in diverse biological processes in plant development, including flowering [12], root organogenesis, seedling photomorphogenesis [13], reproduction, and defense against fungal infection [14]. Depending on their genomic location, long ncRNAs were classified into long intron ncRNAs, promoter lncRNAs, long intergenic ncRNAs (lincRNAs) and natural antisense transcripts (lncNATs) [15]. Natural antisense transcripts can form RNA dimers via complementary base pairing between the lncRNA and the target mRNA, and can block the binding sites of transcription factors in humans [16–18]. Some IncRNAs bind miRNAs and competitively inhibit the interaction between miRNAs and target mRNAs to modulate gene expression [19–21]. LincRNA transcription appears to positively or negatively affect the expression of nearby genes [1,22]. For example, lncRNA can directly bind to a protein mediator as a molecular decoy for regulating gene transcription, such as ELF18-induced long noncoding RNA1 (ELENA1), which can evict fibrillarin from a mediator subunit to enhance PR1 expression in *Arabidopsis* [23]. Similarly, a lincRNA (cold assisted intronic noncoding RNA, COLDAIR) is required for the vernalization-mediated epigenetic repression of FLOWERING LOCUS C [12]. Overexpressing lncRNA LAIR (LRK Antisense Intergenic RNA) was proved to increase grain yield and regulate neighboring gene cluster expression in rice [22]. Thus, IncRNAs in plants can be considered as essential elements of gene regulation, and the analysis and process of IncRNA regulation has become a research hotspot. So far, however, only a few detailed functional studies of plant IncRNA have been reported, especially in bread wheat.

Powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*; Bgt) is an important fungal disease of wheat (*Triticum aestivum*) worldwide, and results in significant crop damage in epidemic years [24,25]. Epidemics of cereal diseases are highly dependent upon environmental conditions, including weather conditions and cropping systems. Deployment of resistance genes is an effective way to control the disease, but the deployed resistances usually fail after some time because of changes in the pathogen virulence population [26,27]. Therefore, understanding resistance mechanisms could have undoubted benefits in controlling the disease and minimizing crop losses. Previously, we isolated several IncRNAs and analyzed the activation of expression in wheat responding to stripe rust (*Puccinia striiformis* f. sp. *Tritici*, Pst) stress, using qRT-PCR [7]. Furthermore, we employed a large-scale sequencing approach [14], and identified 283 IncRNA loci as differentially expressed in wheat that had been inoculated with *Bgt* and *Pst* pathogens, compared with non-inoculated leaves as the control. Among those genes, 254 of 283 DE-IncRNAs were detected in the *Bgt* test. Here, we identified allele-specific functional genes located near differentially expressed (DE) IncRNAs in wheat following infection by fungus. Furthermore, we evaluated the relationship of co-expression between them, especially IncRNAs with adjacent transcription factors (TFs), in wheat after infection with powdery mildew pathogen. In addition, we investigated the influence of IncRNAs on functional genes competitively targeted by the same miRNA, using qRT-PCR.

2. Materials and Methods

2.1. Plant Materials and Pathogen Stress Treatment

The winter wheat line N9134, developed by Northwest A&F University, China (NWAFU), shows high immune resistance to all *Bgt* races in China. This resistance is conferred by one all-stage resistance gene *PmA*846 located on chromosome 5BL bin 0.75–0.76 [28]. To obtain near-isogenic lines (NILs) differing only for *PmA*846, N9134 was crossed seven times with the recurrent susceptible parent Shaanyou 225. Contrasting homozygous lines were then selected using the marker and by powdery mildew tests in BC6F2, which was derived from one BC5F1 resistant plant (Shaanyou 225/6*PmA*846 heterozygous) and named as N9134R (resistant) and N9134S (susceptible). The *Bgt* race E09 was
Ten-day-old wheat seedlings were inoculated with Bgt conidia collected from sporulating seedlings of Shaanyou 225 pre-infected 20 days before.

2.2. Identifying Functional Genes Adjacent to Differentially Expressed Long Noncoding Transcripts (lncRNAs)

We identified lncRNAs of wheat line N9134, that were regulated in expression pattern after inoculation with Pst and Bgt separately, from the RNA-Seq database obtained in our previous study [14]. Briefly, after all annotated and pathway identified gene were removed, the lncRNAs were identified following four rigorous criteria (transcript length; encoding less than 50 aa; not any transposable elements (TEs); and no gap) as previous described. OrfPredictor was used to identify protein-coding regions in each strand. Differential gene expression analysis was performed with the bioconductor package DESeq, version 3.2. To identify genes neighboring lncRNAs, all 283 assembled DE-lncRNAs were mapped onto the reference genome [29] through alignment with BlastN at \( p \)-value \( < 1.0 \times 10^{-10} \), and the adjacent functional genes were predicted according to the annotation in URGI (Unité de Recherche Génomique Info).

2.3. Real-Time Quantitative PCR Analysis

LncRNA, adjacent functional genes and miRNA expression profiles in the contrasting NILs were analyzed by SYBR green-based real-time quantitative PCR (Q-PCR) with cDNA, after cDNA synthesis and RNA extraction from infected leaves sampled at 0, 6, 12, 24, 36, 48, 72, 120, 168, and 240 h post-inoculation (hpi). Three independent biological replications were performed for each time point. Q-PCR was performed on a QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies Corporation, USA) with the FastKing RT kit (with gDNase) (TIANGEN, Beijing). Sequence-specific primers of relevant genes (Supplemental Table S1) and \( \beta \)-actin-F/R were designed using the Primer Premier 5 Design Program (Premier Biosoft International, Palo Alto, CA, USA) and were used to quantify the accumulation of transcripts, and to normalize the amounts of cDNA in samples, respectively. To ensure the specificity of PCR amplification or eliminate the interference of homologues in the other subgenomes, primers were selected with mismatched bases to specific homologues by mapping to genome sequences (EnsemblPlants, http://plants.ensembl.org/Triticum_aestivum/Info/Index). The reverse primer for miRNA was according to the instructions for the miRcute Plus qPCR kit (TIANGEN, Beijing). PCR was conducted in a 20-µL volume containing 10 µL 2 × SYBR Green PCR Master Mix (Takara, Dalian, China), 0.2 µM each primer and 2 µL template (6× diluted cDNA from leaf samples). The amplification program was as follows: 95 °C for 10 s; 40 cycles of 95 °C for 5 s, and 60 °C for 31 s. For each sample, reactions were carried out in triplicate and three non-template negative controls were included. Products were analyzed by melt curves obtained at the end of amplification, while the \( 2^{-\Delta\Delta C_{T}} \) method was employed to quantify the relative gene expression. The correlation coefficients between co-expression genes were calculated with Pearson statistical method and t-test was used to test the statistical significance at the level of 0.05.

3. Results

3.1. Identification of Transcription Factor Genes Adjacent to Differentially Expressed Long Non-Coding RNA in Wheat Responding to Pathogen Infection

Since lncRNAs play a regulatory role in the expression of nearby protein-coding genes and even gene clusters [15,22], we identified 461 functional genes close to 249 DE-lncRNAs, by alignment of the DE-lncRNA sequences with the reference sequences of Chinese Spring (IWGSC RefSeq v1.1) [29] (Supplemental Table S2). Among the functional genes, 27 transcription factors (TFs) were identified close to the interesting DE-lncRNAs, as listed in Table 1. These TFs could be classified into 15 types or families, including WRKY, NAC, MYB, C2H2, MADs, bHLH (Basic Helix-Loop-Helix), bZIP (basic leucine zipper), AP2/ERF (Activating Protein 2/ethylene responsive factor), CSD (the cold-shock domain), NF-X1 (nuclear transcription factor, X-box binding 1), B3 (plant-specific B3 superfamily), BES
(BRI1-EMS suppressor), TUB (Tubby protein), GNAT (GCN5-related N-acetyltransferase), and mTERF (mitochondrial transcription termination factor). In addition, we similarly identified related resistance genes close to DE-lncRNAs (Table 2) (distance < 0.1 Mb) based on the annotation in IWGSC. The detailed information of locations was shown in the Supplemental Table S2. To further check the co-expression of DE-lncRNAs with these functional genes in response to pathogen stress, we used DESeq analysis, setting the threshold change at ≥2-fold and the false discovery rate (FDR) at 1.0%. The analysis identified 249 functional genes adjacent to 181 DE-lncRNAs as differentially expressed among pathogen-infected groups, compared with non-inoculated leaves as the control. This result suggested that about 75% of DE-lncRNAs might form DE-lncRNA–functional gene pairs.

### Table 1. The list of DE-lncRNAs with adjacent transcript factor.

| LncRNA ID          | Adjacent Functional Gene | TF Type | LncRNA Type |
|--------------------|--------------------------|---------|-------------|
| T4_Unigene_BMK.9130| Ta_TraesCS1A01G200500.1  | B3      | LincRNA     |
| T16_Unigene_BMK.1187| Ta_TraesCS1B01G146800.1  | C2H2    | LincRNA     |
| T10_Unigene_BMK.12768| Ta_TraesCS1B01G243100.1  | WRKY55L | LincRNA     |
| T10_Unigene_BMK.12768| Ta_TraesCS1B01G243200.1  | AP2/ERF-ERF | LincRNA |
| T4_Unigene_BMK.17456| Ta_TraesCS1B01G273100.1  | CSD     | LincRNA     |
| T4_Unigene_BMK.17456| Ta_TraesCS1D01G262500.1  | CSD     | LincRNA     |
| T19_Unigene_BMK.34110| Ta_TraesCS2A01G319700.1  | GNAT    | LincRNA     |
| T13_Unigene_BMK.49502| Ta_TraesCS3A01G421400.1  | bHLH    | LincRNA     |
| T16_Unigene_BMK.67438| Ta_TraesCS3A01G432900.1  | MADS-M-type | LincRNA |
| T4_Unigene_BMK.30836| Ta_TraesCS3D01G136600.1  | NF-X1   | LincRNA     |
| T10_Unigene_BMK.65297| Ta_TraesCS3D01G333100.1  | NAC68L/4L | LincRNA |
| T16_Unigene_BMK.92879| Ta_TraesCS3D01G365300.1  | B3      | LincRNA     |
| T4_Unigene_BMK.9309 | Ta_TraesCS4A01G211100.1  | MYB     | LincRNA     |
| T3_Unigene_BMK.19448| Ta_TraesCS4D01G172200.1  | WRKY64/70 | LincRNA |
| T3_Unigene_BMK.40522| Ta_TraesCS4D01G265400.1  | GNAT    | LincRNA     |
| T19_Unigene_BMK.49358| Ta_TraesCS5A01G312000.1  | AP2/ERF-ERF | LincRNA |
| T4_Unigene_BMK.45663| Ta_TraesCS5D01G279100.2  | NAC17L  | LincRNA     |
| T4_Unigene_BMK.47960| Ta_TraesCS6A01G085800.1  | BES1    | LincRNA     |
| T16_Unigene_BMK.71332| Ta_TraesCS6B01G192900.1  | mTERF   | LincRNA     |
| T13_Unigene_BMK.34604| Ta_TraesCS6B01G237700.1  | AP2/ERF-ERF | LincRNA |
| T9_Unigene_BMK.34604| Ta_TraesCS6B01G237700.1  | AP2/ERF-ERF | LincRNA |
| T9_Unigene_BMK.45663| Ta_TraesCS6B01G279100.2  | NAC17L  | LincRNA     |
| T16_Unigene_BMK.22544| Ta_TraesCS7A01G326400.1  | TUB     | LincRNA     |
| T16_Unigene_BMK.22544| Ta_TraesCS7B01G227000.1  | TUB     | LincRNA     |
| T16_Unigene_BMK.22544| Ta_TraesCS7D01G323100.1  | TUB     | LincRNA     |
| T13_Unigene_BMK.23889| Ta_TraesCS7D01G166500.1  | MYB     | LincRNA     |
| T13_Unigene_BMK.30347| Ta_TraesCS7D01G269300.1  | bZIP    | LincRNA     |

Note: The types of lncRNA were given in the symbols ‘LincRNA, linncRNA, lpncRNA, and luncRNA’ representing long intergenic ncRNAs, long intron ncRNAs, promoter lncRNAs, and untranslated region lncRNA. The star symbol mean that the functional genes were differential expressed in previously RNA-Seq profile.

3.2. Co-Expression of Long Non-Coding RNAs with Adjacent Functional Genes

By DESeq analysis of adjacent functional genes, we identified 6 DE-lncRNA–TF pairs using RNA-Seq data (Table 2). Considering the hysteresis quality of the time-points in RNA-Seq data and the lack of comparable samples, we reassessed the relationship between the expression of DE-lncRNA and their nearby TFs. We randomly selected five predicted pairs of genes and tested the expression in resistant/susceptible NILs N9134R/S under powdery mildew pathogen stress. The gene expression pattern of lincRNA T10_unigene_BMK.12768 (hereafter abbreviated as T10.12768) was very similar to that of the adjacent gene TraesCS1B01G243100 (annotated as WRKY55-like), as shown in Figure 1. The co-expression of TraesCS3D01G333100 (NAC68L) vs. linncRNA T4.45663 and TraesCS1B01G146800 (C2H2) vs. lincRNA T16.1187 showed parallel expression trends in the resistant line N9134R. The correlation coefficient values were 0.810, 0.922, 0.653, and 0.940, respectively (Supplemental Table S3, Value for significance at p = 0.05 and 8 df.
was down-regulated at 6 hpi, then increased stepwise to a peak at 36 hpi, followed by a rapid decline
was also exhibited between T13.19448 and TraesCS4D01G172200.1 (homologous to WRKY64
Agronomy 2020 10, 896
transcription factor at early stages. This suggests that the lincRNA T13.19448 might negatively regulate the WRKY64/
but the expression of
Strikingly, the expression of lincRNA T13.19448 in the susceptible genotype increased progressively
back to a minimum at 120 hpi, but then increased again, reaching maximum accumulation at 240 hpi.
expression of WRKY64/70 was down-regulated at 6 hpi, then increased stepwise to a peak at 36 hpi, followed by a rapid decline
inoculation. The inconformity of expression
inoculation. The inconformity of expression
T13.19448 and TraesCS4D01G172200.1 (homologous to WRKY64/70) in both compatible and incompatible lines. The expression of T13.19448 fluctuated only slightly in the resistant genotype. In contrast, the TF gene showed marked fluctuation: expression of WRKY64/70 transcription factor at early stages.

Table 2. The list of DE-lncRNAs with adjacent functional gene in chromosome 1B and 5B.

| LncRNA ID | Adjacent Functional Gene | Definition | LncRNA Type |
|-----------|--------------------------|------------|-------------|
| T16.92960 | TraesCS5B01G062400       | uncharacterized protein LOC109734965 | lncRNA |
| T13.33010 | TraesCS5B01G036800       | chloroplast stem-loop binding protein of 41 kDa | lincRNA |
| T16.65990 | TraesCS5B01G076100       | Putative lipid transfer protein DRE1 | lncRNA |
| T13.22353 | TraesCS5B01G098000       | peptidyl-prolyl cis-trans isomerase G | lncRNA |
| T13.42814 | TraesCS5B01G117000       | uncharacterized protein LOC109768056 isoform X1 | lincRNA |
| T4.63565  | TraesCS5B01G121400       | 2-oxoisovalerate dehydrogenase | lincRNA |
| T19.46503 | TraesCS5B01G34500        | uncharacterized protein LOC109774113 | lincRNA |
| T19.46503 | TraesCS5B01G34600        | uncharacterized protein LOC109774111 isoform X1 | lincRNA |
| T16.29097 | TraesCS5B01G177300       | mediator complex subunit 25 | lincRNA |
| T16.6266  | TraesCS5B01G208100       | cytochrome c oxidase subunit 1 | lncRNA |
| T7.1464   | TraesCS5B01G232600       | 1-aminocyclopropane-1-carboxylate oxidase 1-like | lncRNA |
| T16.68333 | TraesCS5B01G300100       | uncharacterized protein LOC10973149 isoform X2 | lncRNA |
| T16.68333 | TraesCS5B01G30200        | CDGSH iron-sulfur domain-containing protein NEET | lincRNA |
| T10.61842 | TraesCS5B01G302500       | GDP-mannose 3,5-epimerase 2 | lincRNA |
| T1.39963  | TraesCS5B01G404600       | subtilisin-like protease SBT1.7 | lncRNA |
| T1.37489  | TraesCS5B01G453800       | Lr10 disease-resistance locus receptor-like protein kinase 1.5 | lncRNA |
| T10.43063 | TraesCS5B01G478100       | uncharacterized protein LOC109760008 | lncRNA |
| T13.36635 | TraesCS5B01G488300       | protein synthesis inhibitor II-like | lncRNA |
| T13.38179 | TraesCS5B01G535800       | pirin-like protein isoform X1 | lncRNA |
| T13.40997 | TraesCS5B01G547000       | cinnamoyl-CoA reductase 1-like | lncRNA |
| T16.26398 | TraesCS5B01G565100       | MAP kinase kinase | lncRNA |
| T16.83333 | TraesCS5B01G69300        | uncharacterized protein LOC109765977 | lncRNA |
| T16.13852 | TraesCS5B01G110200       | Alanyl-RNA synthetase | lncRNA |
| T16.13852 | TraesCS5B01G113000       | hypothetical protein BRAD1_2g36145v3 | lncRNA |
| T6.53265  | TraesCS5B01G113500       | glutathione S-transferase 4-like | lncRNA |
| T16.1187  | TraesCS5B01G146700       | uncharacterized protein LOC109759591 | lncRNA |
| T19.42425 | TraesCS5B01G163000       | uncharacterized protein LOC100846051 isoform | lncRNA |
| T19.42425 | TraesCS5B01G163100       | uncharacterized protein LOC109772407 | lncRNA |
| T16.3724  | TraesCS5B01G174600       | uncharacterized protein LOC109772407 | lncRNA |
| T16.16515 | TraesCS5B01G200700       | endonuclease MutS2 isoform X1 | lncRNA |
| T16.16515 | TraesCS5B01G203000       | polyprotein/resin-transposon protein, unclassified | lncRNA |
| T16.98558 | TraesCS5B01G226800       | putative proteinase inhibitor-related protein | lncRNA |
| T7.4304   | TraesCS5B01G269600       | wali/Ai-inducible genes | lncRNA |
| T3.33369  | TraesCS5B01G289100       | tankyrase-1-like isoform X4 | lncRNA |
| T3.32410  | TraesCS5B01G289600       | guanylyl cyclase | lncRNA |
| T13.23192 | TraesCS5B01G384100       | chaperone protein dnaJ 10-like | lncRNA |
| T3.32192  | TraesCS5B01G384200       | unnamed protein product | lncRNA |
| T10.79431 | TraesCS5B01G394900       | Lr10 disease-resistance locus receptor-like protein kinase 1.2 | lncRNA |
| T3.31457  | TraesCS5B01G410300       | tropinone reductase homolog At5g06060-like isoform X3 | lncRNA |
| T16.7005  | TraesCS5B01G410300       | tropinone reductase homolog At5g06060-like isoform X3 | lncRNA |
| T16.23559 | TraesCS5B01G416400       | Pre-mRNA-processing-splinting factor SA | lncRNA |
| T16.23559 | TraesCS5B01G416500       | ferredoxin-3, chloroplastic-like isoform X2 | lncRNA |
| T13.36624 | TraesCS5B01G433300       | Chlorophyll a-b binding protein WACB precursor | lncRNA |
| T16.538  | TraesCS5B01G433300       | Chlorophyll a-b binding protein WACB precursor | lncRNA |

Note: The types of lncRNA were given in the symbols ‘LincRNA, linncRNA, lpncRNA, and luncRNA’ representing long intergenic ncRNAs, long intron ncRNAs, promoter lncRNAs, and untranslation region lncRNA. The star symbol mean that the functional genes were differential expressed in previously RNA-Seq profile.
Figure 1. Expression patterns of long noncoding transcripts (lncRNAs) and their nearby transcription factors in the near isogenic lines N9134R and N9134S induced by powdery mildew infection at 0, 6, 12, 24, 36, 48, 72, 120, 168, 168, and 240 hpi. Gene expression levels were assessed by Q-PCR and data were normalized to the actin expression level in wheat. The mean expression value was calculated using three independent replicates. The vertical bars represent the standard deviation in three repeats. The letters ‘R’ and ‘S’ represent resistance and susceptible lines, respectively. Blue and orange lines represent the expression pattern of lncRNAs and TFs separately.

Similarly, we compared the expression of DE-lncRNAs with that of several functional genes. The expression of lincRNA T10.79431 was nearly synchronized with the nearby gene TraesCS1B01G394900 (annotated as leaf rust 10 disease-resistance locus receptor-like protein kinase-like protein, R-RLK) in N9134R (Figure 2), while the expression of lincRNA T16.26398 and functional gene TraesCS5B01G565100 (a MAPK kinase) followed similar patterns in both resistant and susceptible lines. The correlation coefficient values reached to 0.998 and 0.968 in N9134R, respectively (Supplemental
Table S3). The lncRNA T16.21355 and T13.23192 are long intergenic ncRNAs (lincRNAs). The former is located in the interval between TraesCS1B01G416400 (pre-mRNA-processing-splicing factor 8A, PRPF) and TraesCS1B01G416500 (ferredoxin-3 protein), while the latter is flanked by TraesCS1B01G384100 (TaDNAJ 10) and TraesCS1B01G384200 (unnamed protein). The physical distances from lincRNA T13.23192 to its two flanking functional genes are 31,632 and 50,821 bp, respectively. The distance between T16.21355 and TraesCS1B01G416400 is 11,232 bp, which is far less than the 232,840 bp distance from the lncRNA to TraesCS1B01G416500. These results showed that the expression patterns of lncRNAs were not totally coincident with functional genes, although similar expression patterns could be seen between them at several time-points (Figure 2). For example, both T16.21355 and TraesCS1B01G416400 (splicing factor 8A) were upregulated at 36 and 120 hpi in the susceptible line, and at 168 hpi in the resistant line. T13.23192 and TaDNAJ 10 were induced at 168 hpi in the resistant N9134R, while T13.23192 and the unnamed protein gene TraesCS1B01G384200.1 were induced at 36 hpi in the susceptible N9134S. In addition, the unnamed protein gene expression peak at 36 hpi was accompanied by the other two peaks at 6 and 120 hpi, which showed an on-and-off pattern.

3.3. Co-Expression of Long Non-Coding RNAs and Allele-Specific Genes

Figure 2. Expression patterns of lncRNAs and their nearby functional genes in N9134R and N9134S induced by powdery mildew infection at 0, 6, 12, 24, 36, 48, 72, 120, 168, 168, and 240 hpi. Gene expression levels were assessed by Q-PCR as aforementioned similarly. Blue lines represent the expression pattern of lncRNAs while red and green lines represent the flanked functional genes.
These results showed that the lncRNAs are generally co-expressed with adjacent protein-coding genes, but not in all cases. It is noted that lncRNAs T10.65297, T4.45663, T10.79431, and T16.26398 are long intron ncRNAs, while T16.1187, T10.12768, T13.19448, T13.23192, and T16.21355 belong to long intergenic ncRNAs. The long intron ncRNAs (linncRNAs) seem to have closer co-expression relationships with their nearby functional genes. Taking the distance between lincRNAs with their adjacent functional genes into consideration, we noted that the distance from lincRNA T13.19448 to TraesCS4D01G172200 is 98,620 bp (r = 0.555), from T16.1187 to TraesCS1B01G146800 is 28,814 bp, and from T10.12768 to TraesCS1B01G243100 is 6,625 bp. Thus, the strength of correlation of expression maybe increased as the distance decreased. This hints that two types of lncRNAs both play regulatory roles, in both positive and negative ways. However, the degree of regulation via lincRNA was influenced by the distance.

3.3. Co-Expression of Long Non-Coding RNAs and Allele-Specific Genes

Since lncRNAs, as miRNA targets or target mimics, competitively inhibited the interaction between miRNAs and target mRNAs to modulate gene expression, we identified five potential miRNA-targeted lncRNAs and seven mimic lncRNAs. Detailed information about the targets and target types of the lncRNAs are shown in Table 3. Intriguingly, all of the predicted target functional genes were identified as DE genes in the RNA-Seq profile. Among them, the lincRNA T16.13521 and phospholipid hydroperoxide glutathione peroxidase gene TraesCS6A02G246400 both are targeted and cleaved by tae-miR1137a. The lincRNAs T13.17661 and lincRNA T13.21716 are target mimics of miR339b and mir156d-3p, respectively, which were predicted to target TraesCS1B02G415800 (putative ubiquitin-conjugating enzyme E2) and TraesCS2D02G400500 (pentatricopeptide repeat-containing protein) for cleavage and translation inhibitor activity, respectively.

Table 3. The list of DE-lncRNAs/lincRNA mimics with allele-specific gene targeted by the same miRNA.

| miRNA       | lncRNA Mimic | lncRNA    | Functional Gene     |
|-------------|--------------|-----------|---------------------|
| tae-miR1137a| T16.13521    | TraeCS6A02G246400.1 |
| ath-miR414  | T13.33064    | TraesCS1D02G123200.1|
| ath-miR5658 | T19.34869    | 1B: 392147777-392149747 |
| osa-miR1439 | T1.46244     | TraeCS1B02G377700.1 |
| hvu-miR5049f| T1.37489     | TraeCS6D02G428200.1 |
| bdi-miR394  | T10.71969    | 7B:267192134-267192333 |
| tae-miR167a | T10.3513     | TraeCS6D02G360600.1 |
| ath-miR390a-3p| T19.51118    | TraeCS1B02G808500.1 |
| ata-miR160a-3p| T13.34604    | 1D:247796039-247796286 |
| ata-miR395c-5p| T13.17661    | TraeCS1B02G415800.1 |
| ath-miR399b | T13.21716    | TraeCS2D02G400500.1 |

The Q-PCR results showed that the gene expression level of lncRNA T13.17661 and TraeCS1B02G415800.1 (both targeted by miR399b) were low and stable after Bgt-inoculation, but the expression of miRNA399b was upregulated 4–7 fold compared to 0 hpi in N9134R resistant background (Figure 3). The expression of lncRNA T13.17661 and Ub-enzyme E2 were both induced at 12 hpi in N9134S susceptible background. However, their expression levels were lower than that of miR399b at subsequent time-points, especially at 36 and 48 hpi. As for miRNA1137a and its targets, the expression of lincRNA T16.13521 and functional gene TraesCS6A02G246400.1 were significantly repressed at all tested time-points, compared with 0 hpi, and accompanied by slight fluctuation, while the expression of miRNA1137a was slightly induced at 12 and 48 hpi. From Figure 3, the highest expression level of T16.13521 was observed at 24 hpi and was half that in the control. Interestingly, the trend of lncRNA T16.13521 was very similar to the functional gene TraesCS6A02G246400.1 (encoding glutathione peroxidase-like protein) at 0, 12, 24, 36, 48, and 72 hpi, while similar patterns could be seen comparing the
expression of T13.17661 with TraesCS1B02G415800.1 in N9134R (Figure 3). Furthermore, the expression patterns were also similar between lncRNA T13.17661 and TraesCS1B02G415800.1 in N9134S, although the miR399b was induced at different time-points from the pattern in N9134R. The expression of TraesCS2D02G400500.1 (pentatricopeptide repeat-containing protein) steeply increased, with two peaks at 12 and 36 hpi in both N9134R and N9134S, while the lincRNA T13.21716 was upregulated at 24 hpi in N9134R. For miRNA156d as a translation inhibitor, the expression was generally stable after Bgt inoculation in both genotypes apart from slight down-regulation at 36 hpi in N9134R. Taken together, these results substantiated the view that lincRNA could competitively interact with functional genes via miRNA regulation.

Figure 3. Expression patterns of lncRNAs, the functional genes and their paired miRNAs in N9134R and N9134S induced by powdery mildew infection at 0, 12, 24, 36, 48, and 72hpi. Gene expression levels were assessed by Q-PCR as aforementioned similarly. Blue lines represent the expression pattern of lncRNAs, while green and red lines represent the expression of miRNAs and its targeted functional genes, respectively.

4. Discussion

Plants are sessile and must continuously integrate both abiotic and biotic environmental signals for development and defense responses. Because plants lack circulating cells, they rely on systemic signals emanating from infection sites to trigger the innate immunity response [30]. In this process, thousands of genes have been involved, including noncoding RNAs (ncRNAs) encoded by specific genomic regions [31]. Large-scale sequencing analyses have revealed that most of the eukaryotic genome is transcribed to RNAs, including short and long ncRNAs [5,6,14], not just functional genes. In plants, analysis of over 200 Arabidopsis transcriptome data sets identified about 40,000 putative
lncRNAs, including over 30,000 natural antisense transcripts (NATs) and over 6000 lincRNAs [10,13,15]. However, in-depth functional analysis of lncRNAs in wheat remains limited, although some preliminary reports have been given [7,14,32]. Here, we identified 461 neighboring genes with 249 DE-lncRNA in wheat after fungal infection, and then investigated the co-expression relationship between lncRNA and the adjacent functional genes. Regretfully, because of missing the direction of cDNA in the second-generation RNA-Seq, here we were not able to detect possible co-regulation relationships between lncNATs and their target genes. There is no doubt that further lncRNA regulators will be detected using strand-specific RNA-Seq [33]. In any case, as far as we know, this is the first genome-wide study on the possible role of lncRNAs in regulating functional genes. Our results provide a powerful foundation for future functional and molecular research on wheat–fungus interactions.

LncRNAs, like functional genes, have key functions in transcriptional, post-transcriptional, and epigenetic gene regulation [2]. In transcriptional level, transcription factors (TFs) are important regulators of gene expression in plants when responding to abiotic and biotic stress [34,35]. The NAC, WRKY, AP2/ERF, and C2H2 TFs have all been reported as being involved in plant responses to pathogen attack [36]. In this study, we analyzed the co-expression of lncRNAs with NAC17L, NAC68L, WRKY55L, C2H2, and WRKY64/70. The results supported the idea that lncRNAs have a co-regulation relationship with neighboring functional genes, although some exhibited opposite and/or positive expression patterns in different genetic backgrounds, and others showed no relation at all. WRKY transcription factors play important and well substantiated roles in plant immunity responses at both transcriptional and post-translational regulation levels [37,38]. Several NAC and C2H2 have been identified with roles in antioxidant defense mechanisms in plants [39], in particular a natural allele of a C2H2 transcription factor in rice that was shown to confer broad-spectrum rice blast resistance [40]. Here, our results demonstrated that lncRNAs maybe play the role of regulating the expression of TFs, especially in incompatible interaction. This means that lncRNAs could indirectly regulate the downstream functional genes via TFs. Taking co-regulation of lncRNAs with allele-specific functional genes directly together, our results will be helpful for improved understanding of the regulatory mechanisms of TFs in plant immune responses to disease. Comparing lincRNA with lincRNA, the lincRNA seemed to have closer co-expression relationships with their nearby functional genes, while the correlation of lincRNA expression with their respective genes increased as the intervening distance decreased. These results suggest that lncRNAs might have regulatory functions for neighboring functional genes, including transcription factors, in both positive and negative ways.

Conversely, MicroRNAs are endogenous short ncRNAs (21–24 nucleotides) that play important regulatory roles by repressing gene translation or degrading target mRNAs at the post-transcriptional levels [6,41]. Some lncRNAs have been predicted to be targets of miRNA [9,14,32,42]. To further clarify the relationship between lncRNA and miRNA, here we analyzed the co-expression of miRNAs, lncRNAs and their targeted functional genes, and substantiated the view that lncRNAs, including lncRNA targets and target mimics, are able to interact competitively with functional genes via miRNA regulation at the post-transcriptional levels [20]. However, further work is required to determine whether lncRNAs could be immune resistance markers as functional genes. Taking the regulation of miRNA with lncRNA together, the regulatory network of functional gene expression will be probably complex and flexible. Although it is not yet clear how lncRNAs are involved in regulation and with which effectors they interact, the knowledge and resources gathered here will provide useful insights into the mechanisms that regulate defense pathways against fungi, and these results should facilitate future investigating into epigenetic resistance in wheat, as well as understanding the plant–pathogen interactions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/896/s1, Supplemental Table S1. PCR primers used for Q-PCR amplification of lincRNA and functional genes. Supplemental Table S2. List of the identified adjacent functional genes nearby DE-lncRNAs regulated by fungi. Supplemental Table S3. List of the detailed relative expression values used to infer the correlation.
Author Contributions: H.Z. and W.J. designed the study. H.Z. and W.H. analyzed data and wrote the article. W.H., G.W., S.W. and X.N. performed the research. C.W. and Y.W. contributed by collecting the samples and the development of material. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the National Key Research and Development Program of China (grant no. 2017YFD0100701).

Conflicts of Interest: The authors declare no competing financial interest.

Abbreviations
DE differentially expressed
ncRNA non-coding RNA
lincRNAs long intergenic non-coding RNA
linncRNAs long intron non-coding RNA

References
1. Wilusz, J.E.; Sunwoo, H.; Spector, D.L. Long noncoding RNAs: Functional surprises from the RNA world. *Genes Dev.* 2009, 23, 1494–1504. [CrossRef] [PubMed]
2. Chekanova, J.A. Long non-coding RNAs and their functions in plants. *Curr. Opin. Plant Biol.* 2015, 27, 207–216. [CrossRef] [PubMed]
3. Kim, E.D.; Sung, S. Long noncoding RNA: Unveiling hidden layer of gene regulatory networks. *Trends Plant Sci.* 2012, 17, 16–21. [CrossRef]
4. Kornienko, A.E.; Guenzl, P.M.; Barlow, D.P.; Pauler, F.M. Gene regulation by the act of long non-coding RNA transcription. *BMC Biol.* 2013, 11, 59. [CrossRef]
5. Wang, K.C.; Chang, H.Y. Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 2011, 43, 904–914. [CrossRef] [PubMed]
6. Ariel, F.; Romero-Barrios, N.; Jegu, T.; Benhamed, M.; Crespi, M. Battles and hijacks: Noncoding transcription in plants. *Trends Plant Sci.* 2015, 20, 1–10. [CrossRef]
7. Zhang, H.; Chen, X.; Wang, C.; Xu, Z.; Wang, Y.; Liu, X.; Kang, Z.; Ji, W. Long non-coding genes implicated in response to stripe rust pathogen stress in wheat (*Triticum aestivum* L.). *Mol. Biol. Rep.* 2013, 40, 6245–6253. [CrossRef]
8. Liu, J.; Jung, C.; Xu, J.; Wang, H.; Deng, S.; Bernad, L.; Arenas-Huertero, C.; Chua, N.H. Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. *Plant Cell* 2012, 24, 4333–4345. [CrossRef]
9. Shuai, P.; Liang, D.; Tang, S.; Zhang, Z.; Ye, C.Y.; Su, Y.; Xia, X.; Yin, W. Genome-wide identification and functional prediction of novel and drought-responsive lincRNAs in *Populus trichocarpa*. *J. Exp. Bot.* 2014, 65, 4975–4983. [CrossRef]
10. Yuan, J.; Li, J.; Yang, Y.; Tan, C.; Zhu, Y.; Hu, L.; Qi, Y.; Lu, Z.J. Stress-responsive regulation of long non-coding RNA polyadenylation in *Oryza sativa*. *Plant J.* 2018, 93, 814–827. [CrossRef] [PubMed]
11. Heo, J.B.; Sung, S. Vernalization-Mediated Epigenetic Silencing by a Long Intronic Noncoding RNA. *Science* 2010, 331, 76–79. [CrossRef] [PubMed]
12. Wang, Y.; Fan, X.; Lin, F.; He, G.; Terzaghi, W.; Zhu, D.; Deng, X. Arabidopsis noncoding RNA mediates control of photomorphogenesis by red light. *Proc. Natl. Acad. Sci. USA* 2014, 111, 10359–10364. [CrossRef] [PubMed]
13. Zhang, H.; Hu, W.; Hao, J.; Lv, S.; Wang, C.; Tong, W.; Wang, Y.; Wang, Y.; Liu, X.; Ji, W. Genome-wide identification and functional prediction of novel and fungi-responsive lincRNAs in *Triticum aestivum*. *BMC Genomics* 2016, 17, 238. [CrossRef] [PubMed]
14. Laurent, S.G.; Wahlestedt, C.; Kapranov, P. The Landscape of long noncoding RNA classification. *Trends Genet.* 2015, 31, 239–251. [CrossRef] [PubMed]
16. Pandey, R.R.; Mondal, T.; Mohammad, F.; Enroth, S.; Redrup, L.; Komorowski, J.; Nagano, T.; Mancini-Dinardo, D.; Kanduri, C. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol. Cell* 2008, 32, 232–246. [CrossRef]  
17. Beltran, M.; Puig, I.; Pena, C.; Garcia, J.M.; Alvarez, A.B.; Pena, R.; Bonilla, F.; de Herreros, A.G. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. *Genes Dev.* 2008, 22, 756–769. [CrossRef]  
18. Faghihi, M.A.; Wahlestedt, C. Regulatory roles of natural antisense transcripts. *Nat. Rev. Mol. Cell Biol.* 2009, 10, 637–643. [CrossRef]  
19. Franco-Zorrilla, J.M.; Valli, A.; Todesco, M.; Mateos, I.; Puga, M.I.; Rubio-Somoza, I.; Leyva, A.; Weigel, D.; Garcia, J.A.; Paz-Ares, J. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.* 2007, 39, 1033–1037. [CrossRef]  
20. Wu, H.J.; Wang, Z.M.; Wang, M.; Wang, X.J. Widespread long noncoding RNAs as endogenous target mimics for microRNAs in plants. *Plant Physiol.* 2013, 161, 1875–1884. [CrossRef]  
21. Luo, Y.; Chen, J.; Lv, Q.; Qin, J.; Huang, Y.; Yu, M.; Zhong, M. Long non-coding RNA NEAT1 promotes colorectal cancer progression by competitively binding miR-34a with SIRT1 and enhancing the Wnt/beta-catenin signaling pathway. *Cancer Lett.* 2019, 440–441, 11–22. [CrossRef] [PubMed]  
22. Wang, Y.; Luo, X.; Sun, F.; Hu, J.; Zha, X.; Su, W.; Yang, J. Overexpressing lncRNA LAIR increases grain yield and regulates neighbouring gene cluster expression in rice. *Nat. Commun.* 2018, 9, 3516. [CrossRef] [PubMed]  
23. Seo, J.S.; Diloknavarit, P.; Park, B.S.; Chua, N.H. ELF18-INDUCED LONG NONCODING RNA 1 evicts fibrillarin from mediator subunit to enhance PATHOGENESIS-RELATED GENE 1 (PR1) expression. *New Phytol.* 2019, 221, 2067–2079. [CrossRef] [PubMed]  
24. Bourras, S.; McNally, K.E.; Ben-David, R.; Parlange, F.; Ro...
37. Ishihama, N.; Yoshioka, H. Post-translational regulation of WRKY transcription factors in plant immunity. Curr. Opin. Plant Biol. 2012, 15, 431–437. [CrossRef]
38. Pandey, S.P.; Somssich, I.E. The role of WRKY transcription factors in plant immunity. Plant Physiol. 2009, 150, 1648–1655. [CrossRef]
39. Nuruzzaman, M.; Sharoni, A.M.; Kikuchi, S. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front. Microbiol. 2013, 4, 248. [CrossRef]
40. Li, W.; Zhu, Z.; Chern, M.; Yin, J.; Yang, C.; Ran, L.; Cheng, M.; He, M.; Wang, K.; Wang, J.; et al. A Natural Allele of a Transcription Factor in Rice Confers Broad-Spectrum Blast Resistance. Cell 2017, 170, 114–126. [CrossRef]
41. Taft, R.J.; Pang, K.C.; Mercer, T.R.; Dinger, M.; Mattick, J.S. Non-coding RNAs: Regulators of disease. J. Pathol. 2010, 220, 126–139. [CrossRef] [PubMed]
42. Juan, L.; Wang, G.; Radovich, M.; Schneider, B.P.; Clare, S.E.; Wang, Y.; Liu, Y. Potential roles of microRNAs in regulating long intergenic noncoding RNAs. BMC Med. Genomics 2013, 6, S7. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).