Systemic acquired resistance (SAR) in Arabidopsis is established beyond the initial pathogenic infection or is directly induced by treatment with salicylic acid or its functional analogs (SA/INA/BTH). NPR1 protein and WRKY transcription factors are considered the master regulators of SAR. Our previous study showed that NPR1 homologs in wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) regulated the expression of genes encoding pathogenesis-related (PR) proteins during acquired resistance (AR) triggered by Pseudomonas syringae pv. tomato DC3000. In the present examination, AR induced by P. syringae DC3000 was also found to effectively improve wheat resistance to Puccinia triticina (Pt). However, with more complex genomes, genes associated with this SAR-like response in wheat and barley are largely unknown and no specific WRKYs has been reported to be involved in this biological process. In our subsequent analysis, barley transgenic line overexpressing wheat wNPR1 (wNPR1-OE) showed enhanced resistance to Magnaporthe oryzae isolate Guy11, whereas AR to Guy11 was suppressed in a barley transgenic line with knocked-down barley HvNPR1 (HvNPR1-Kd). We performed RNA-seq to reveal the genes that were differentially expressed among these transgenic lines and the wild-type barley plants during the AR. Several PR and BTH-induced (BCI) genes were designated as downstream genes of NPR1. The expression of few WRKYs was significantly associated with NPR1 expression during the AR events. The transient expression of three WRKY genes, including HvWRKY6, HvWRKY40, and HvWRKY70, in wheat leaves by Agrobacterium-mediated infiltration enhanced the resistance to Pt. In conclusion, a profile of genes associated with NPR1-mediated AR in barley was drafted and WRKYs discovered in the current study showed a substantial potential for improving wheat resistance to Pt.

Keywords: WRKY transcription factors, NPR1, acquired resistance, barley, wheat, Puccinia triticina
INTRODUCTION

Systemic acquired resistance (SAR) is an inducible form of plant defense that confers broad-spectrum immunity to secondary infections beyond the initial infection site. In Arabidopsis, SAR is associated with accumulation of the plant hormone salicylic acid (SA) and transcriptional activation of pathogenesis-related (PR) genes (Zheng and Dong, 2013). The Arabidopsis NPR1 protein (Non-expressor of PR genes 1, also known as NIM1 and SA11) is a master regulator required for SAR. Upon pathogen infection or treatment with SA or its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), NPR1 translocates from the cytoplasm into the nucleus, where it interacts with the TGA2 transcription factor to promote expression of multiple PR genes (Cao et al., 1994; Delaney et al., 1995; Ryals et al., 1997; Shah et al., 1997; Mou et al., 2003). Overexpression of Arabidopsis NPR1 (AtNPR1) in other plant species (e.g., rice and wheat) enhances their resistance against multiple pathogens (Chern et al., 2001; Makandar et al., 2006; Quilis et al., 2008; Gao et al., 2013; Xu et al., 2017). In Arabidopsis and rice, several WRKY transcription factors (TFs) have been suggested to play important roles in the NPR1-mediated SAR. A previous genomic approach has identified several WRKYs, including AtWRKY18, AtWRKY58, and AtWRKY70, as regulatory nodes in the transcriptional network of SAR in Arabidopsis (Wang et al., 2006). On the other hand, OsWRKY3 and OsWRKY71 in rice were reported as upstream genes of the rice NPR1 homolog (NH1) (Liu et al., 2005, 2007). Another WRKY transcription factor in rice, OsWRKY45, was established as an independent regulator in the SA/BTH signaling pathway (Shimono et al., 2007; Nakayama et al., 2013).

Our previous analysis showed that the wheat NPR1 homolog (wNPR1) interacts with four members of the basic-region leucine zipper (bZIP) transcription factor family (also known as TGA) (Cantu et al., 2013). The interactions between NPR1 and TGA5s are critical for NPR1 functioning in Arabidopsis and rice (Chern et al., 2001; Després et al., 2003). However, SAR in wheat and barley somewhat differs from that described in model plants of Arabidopsis and rice (Wang et al., 2018). In early investigations, SA/INA/BTH treatment of wheat and barley induced a SAR-like response, BTH-induced resistance (BIR), to various pathogens, including Blumeria graminis and Puccinia triticina (Pt) (Görlich et al., 1996; Beßer et al., 2000; Hafez et al., 2014). Other studies established that most of the PR genes were not induced by such treatments (Vallérianbindschleder et al., 1998; Molina et al., 1999). Moreover, another group of BTH-inducible genes, such as the wheat chemical-induced (WCI) and barley chemical-induced (BCI) genes, may be responsible for the enhanced resistance to various pathogens during BIR.

In another study, Pseudomonas syringae pv. japonica (PsJ) or Xanthomonas translúcens pv. cerealis (Xtc) induced systemic immunity (SI) against secondary infection of Xtc in uninoculated barley leaves (Dey et al., 2014). The findings of the same study also indicated that SI was not associated with barley HvNPR1, or local or systemic accumulation of SA, but with several WRKY and ERF transcription factors.

As a third form of SAR-like response in barley, acquired resistance (AR) to the secondary pathogen Magnaporthe oryzae (Mo) was induced in the area adjacent to the initial infection of P. syringae pv. tomato DC3000 in a PR genes-induced manner, but such resistance was not systemic (Colebrook et al., 2012). The levels of both free and conjugated SA were significantly upregulated in barley leaves infiltrated with P. syringae pv. syringae (Vallérianbindschleder et al., 1998). In our previous research, the induction of several barley PR genes, including HvPR1b, HvPR2, HvPR3_Chit2a, and HvPR5_TLP6, was significantly associated with the expression level of NPR1 in transgenic lines overexpressing wheat NPR1 (wNPR1-OE) or suppressing barley NPR1 (HvNPR1-Kd) during the P. syringae DC3000-triggered AR (Wang et al., 2016). However, genes associated with these biological processes are largely unknown and no specific WRKYs has been reported to be involved in this SAR-like response in barley.

In the present study, we established that AR induced by P. syringae DC3000 also improved wheat resistance to P. triticina (Pt). In addition, the AR responses of the barley transgenic lines wNPR1-OE and HvNPR1-Kd to the Mo isolate Guy11 were evaluated. Further, transcriptome analysis of these transgenic lines during AR response was carried out. The downstream genes of NPR1 were identified based on their expression profiles. The inductions of several transcription factors displayed significant association with the expression of NPR1 during AR. Three differentially expressed WRKY genes, identified in our transcriptome database, showed a considerable potential for improving wheat resistance to Pt.

MATERIALS AND METHODS

Plants and Pathogens

A transgenic line of barley overexpressing wheat wNPR1 (wNPR1-OE) and a transgenic line with suppressed barley HvNPR1 (HvNPR1-Kd) under maize Ubiquitin promoter in the background of cultivar “Golden Promise” were derived from previous studies (Dey et al., 2014; Wang et al., 2016). The wild-type plants served as control. The fully expanded third leaves from the experimental plants were used for P. syringae pv. tomato DC3000 infiltration and subsequent M. oryzae (Mo) inoculation. Brieilly, P. syringae DC3000 was grown in KB medium with Rif antibiotics and was then diluted to OD600 = 0.5 in sterile water. Third leaves were inoculated with a 1-ml needle syringe by pressure infiltration of bacterial suspensions through the leaf abaxial surface. The borders of the infiltrated region were marked using a marker pen. Control seedlings were infiltrated in the same way with sterile water. After bacterial inoculation, seedlings were maintained at a constant temperature of 23°C to facilitate bacterial growth. Samples for RNA extraction were collected from regions adjacent to the infiltration from the transgenic lines and the wild-type plants 48 h post-inoculation (hpi) after a clear cell death phenotype triggered by P. syringae DC3000 infection was observed (Colebrook et al., 2012; Wang et al., 2016).
The same adjacent regions were also used for *Mo* inoculation to evaluate the degree of acquired resistance in the transgenic lines and the wild-type plants. *Mo* isolate Guy11 was grown on complete medium for 11 days at 25°C under a 16/8-h light-dark cycle. Ten microliters of the conidia suspension (5.0 × 10^5 spores per milliliter) containing 0.05% Tween-20 was dropped to the press-injured spots on the adjacent region and then wrapped with cellophane tape. The plants were kept in a mist chamber at 25°C in the dark for the first 24 hpi and then transferred to a growth chamber at 23°C and 80% humidity under a 16/8-h (light/dark) photoperiod. The inoculated leaves were photographed 5 days later. The size of the lesion caused by *Mo* on each leaf was measured. The assay for each treatment and phenotype combination consisted of at least six biological replicates. Data were transformed to restore normality and Dunnett’s test was performed using SAS software v9.4 (SAS Institute, Cary, NC, United States).

A similar approach was employed to induce AR in wheat using *P. syringae* DC3000 infiltration. Water infiltration served as a negative control. Fully expanded secondary leaves from seedlings of the wheat susceptible line "Thatcher" were used in the experiments. Urediospores of highly virulent *Pt* pathotype THTT were sprayed on the region adjacent to the *P. syringae* DC3000 infection area 2 days post-infiltration. Inoculated wheat plants were maintained in a moist chamber at 18°C for 16 h in the dark and were next transferred into a growth chamber with 16 h light at 23°C and 8 h darkness at 18°C. The phenotype of leaf rust was recorded at 10 dpi. The percentage of *Pt* sporulation area in the corresponding region for each leaf was determined using ASSESS (version 2.0) image analysis software for plant disease quantification from the American Phytopathology Society (Lamari, 2008; Zhang et al., 2017). The whole experiment was repeated twice and each repeat consisted of 11–14 biological replicates. The data were transformed to restore normality and general linearized model (GLM) ANOVA was conducted using SAS software version 9.4 (SAS Institute, Cary, NC, United States).

**RNA samples isolation and qRT-PCR**

RNA samples for RNA-seq and qRT-PCR assays were isolated using a plant total RNA extraction kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The first-strand cDNA was synthesized using a reverse transcription kit (Clontech, Mountain View, CA, United States). Then, gene expression was quantified as described before (Wang et al., 2016), using the barley elongation factor 1-a (*HvEF1a*, GenBank accession number Z50789) and actin (*HvActin*, GenBank accession number AK362208) as internal references. The qRT-PCR primers, designed for the selected PR genes (*HvPR1b*, *HvPR2*, and *HvPR3*, *Chit2a*, derived from our previous study), BCI genes (*HvBCI1*, *HvBCI3*, and *HvBCI7*), and NPR1 gene, are listed in **Supplementary Table S1**. Further, the amplification efficiency for each pair of primers was calculated using five fourfold cDNA dilutions (1:1, 1:4, 1:16, 1:64, and 1:256). To ensure amplification specificity, dissociation curves for the temperature range from 60 to 94°C were generated for each reaction. The threshold values (Ct) generated from the Roche LightCycler 96 were used to quantify the relative gene expression using the Delta-Ct method as described earlier (Wang et al., 2016). Two independent transgenic lines for each of the wNPR1-OE and HvNPR1-Kd were utilized. Each experiment consisting of 4–11 biological replicates was considered as a block. Calculations of the mean and standard error were performed using Microsoft Excel (Microsoft, Redmond, WA, United States). The data were transformed to restore normality and GLM ANOVA was conducted using SAS software version 9.4 (SAS Institute, Cary, NC, United States).

**RNA-seq**

RNA preparation and RNA-seq were performed as described in the Illumina TruSeq RNA Sample Preparation Version 2 Guide, the Illumina HiSeq 1000 System User Guide (Illumina), and the KAPA Library Quantification Kit-Illumina/ABI Prism User Guide (Kapa Biosystems) by Novogene Co., Ltd. The sequencing run was performed on a HiSeq 1000 instrument. Then, the sequence reads were mapped on the Ensembl Genomes *Hordeum vulgare* genome sequence (International Barley Genome Sequencing Consortium, 2012) using TopHat 2.0.8 (Trapnell et al., 2009) with default parameter settings and an expected mean insert size of 150 bp. The assembled contigs that were not aligned with the reference genome were annotated as “Novel” transcripts. Further, HTSeq was used to assemble the mapped RNA-seq reads into transcripts to quantify their relative abundance (Trapnell et al., 2010). Differentially expressed genes were identified using the default settings of DESeq2 (Love et al., 2014) and filtered for an FDR-adjusted *P* < 0.05. Statistically significant over-representation of GO categories within differentially expressed genes was determined using the GOseq package (Young et al., 2010). Statistically significant GO terms tested by conditional hypergeometric tests (*P* < 0.05) were considered enriched. Next, heatmaps were generated by MeV software using FPKM values from the RNA-seq database. Hierarchical clustering analysis was performed by the MeV software, based on which the genes with similar expression patterns were clustered. A summary of GO annotation categories was generated using the GO level2 Counter in the TBtools software.

**Transient Expression Assay**

RNA isolated from barley leaves was utilized for cDNA synthesis using the reverse transcription kit (Clontech, Mountain View, CA, United States). Subsequently, the cDNA sequences of 10 selected WRKY genes were PCR-amplified using the primers listed in **Supplementary Table S1**. Initially, the PCR products were cloned into a pGEM-T easy vector (Promega, Madison, WI, United States) and then into a wheat transgenic vector pLGY02, which contained the maize (*Zea mays*) ubiquitin 1 promoter and T-DNA insertion site. The recombinant constructs were transformed into the *Agrobacterium* strain AGL1, and the wheat leaves were subjected to transient gene expression assays as previously described (Lu et al., 2016). Fresh *Agrobacterium* was grown overnight in yeast extract broth (YEB) medium supplemented with Rifampicin and Kanamycin. The bacterial
pellets obtained after centrifugation were resuspended in an infiltration buffer containing 10 mM MES, 10 mM MgCl₂, and 400 µM acetosyringone to an optical density of OD₆₀₀ = 2.0. For wheat infiltration, the fully expanded secondary leaf of wheat seedlings was infiltrated using a 1-mL syringe without a needle. The border of the infiltration area was marked with a mark pen. Urediniospores of highly virulent Pt pathotype THTT were spray-inoculated 4 days post-infiltration. The inoculated leaves were photographed at 10 days post-inoculation. The percentage of Pt sporulation area in the infiltration region for each leaf was calculated using ASSESS software v2.0 (American Phytopathology Society) (Lamari, 2008; Zhang et al., 2017). The assay for each gene was repeated at least twice, and each repeat consisted of 5–18 biological replicates. Data were transformed to restore normality and Dunnett’s test was carried out using SAS software v9.4 (SAS Institute, Cary, NC, United States). Raw data for all these conducted experiments were archived in Supplementary File S1.

RESULTS

AR Triggered by *P. syringae* DC3000 in Wheat Reduces the Severity of *Pt*

AR triggered by *P. syringae* DC3000 in barley was considered as a SAR-like response providing broad-spectrum protection against subsequent pathogen challenge (Colebrook et al., 2012). To elucidate whether such AR can be utilized to improve the resistance of other *Triticaceae* crops, we examined its effect in the defense reaction of wheat to leaf rust, which is a severe fungal disease in wheat. A highly virulent Pt pathotype THTT was used to inoculate the region adjacent to *P. syringae* DC3000 or water infiltration area in the wheat leaves of the susceptible line “Thatcher.” Cell death triggered by *P. syringae* DC3000 was observed 2 days post-infiltration. The susceptible phenotypes of leaf rust were identified in both *P. syringae* DC3000 and water mock treatments 10 days post-inoculation (Figure 1). The percentage of *Pt* sporulation area for each leaf was calculated using ASSESS software. Significantly more ($P < 0.0001$) promoted resistance to *Pt* was observed in the region adjacent to the *P. syringae* DC3000 infection area than in the mock control (Figure 1).

Barley AR to *Mo* Isolate Guy11 Is Mediated by *NPR1*

The *Mo* isolate Guy11 was used to determine the extent of AR triggered by *P. syringae* DC3000 in a barley transgenic line overexpressing wheat wNPR1 (wNPR1-OE), a transgenic line with knocked-down barley HvNPR1 (HvNPR1-Kd), and wild-type plants (Figure 2). The third leaf of barley plants was infected by infiltration with *P. syringae* DC3000 or treated with sterile water as a mock control. *Mo* isolate Guy11 was inoculated in the region adjacent to *P. syringae* DC3000 infiltration area when a cell death was observed 2 days post-infiltration (dpi). Strong AR against *Mo* isolate Guy11 was established in the region adjacent to *P. syringae* DC3000 infection area in the wild-type plants (Figure 2A). The size of the lesions caused by the *Mo* isolate Guy11 in the region adjacent to *P. syringae* DC3000 infection area was significantly ($P < 0.05$) lower than that in the mock control (Figure 2B). The wNPR1-OE transgenic line had more pronounced resistance to *Mo* infection than the wild-type plants, even in the mock control (Figure 2). The AR triggered by *P. syringae* DC3000 in the HvNPR1-Kd transgenic line, was suppressed but not fully eliminated (Figure 2), possibly because *NPR1* was not completely removed from the HvNPR1-Kd line. These results indicate that the AR to the *Mo* isolate Guy11, triggered by *P. syringae* DC3000, is mediated by *NPR1*.

RNA-Seq Analysis Was Applied on wNPR1-OE and HvNPR1-Kd Barley Transgenic Lines During AR Triggered by *P. syringae* DC3000

To explore the gene regulation network during the *NPR1*-mediated AR in barley, we performed RNA-seq analysis on...
Expression Profiles of PR and BCI Genes in the NPR1-Mediated AR in Barley

Since many of the PR genes were reported as downstream genes of NPR1 during the P. syringae DC3000-triggered AR in our previous study (Wang et al., 2016), in the present investigation, we initially checked the expression levels of all the PR gene families in our RNA-seq database (Figure 3). We noticed that the transcript levels of HvPR1, HvPR2, HvPR3_Chit2a, HvPR5 (TLP6, TLP7, and TLP8), HvPR9, and HvPR13, were significantly associated with the expression of NPR1 as either significantly \( (P < 0.05) \) higher induced in the wNPR1-OE transgenic line or lower induced in the HvNPR1-Kd transgenic line (Figure 3). In contrast, the expression levels of PR5 (TLP1), PR15, PR16, PR17a, and PR17b showed induction by P. syringae DC3000 but in a NPR1-independent manner. Since a group of BCI genes were previously reported to be responsible for the BTH-induced resistance in barley (Beßer et al., 2000), we examined the expression patterns of all barley BCI genes in our database and found that HvBCI2 (the same gene as HvPR13) and HvBCI7 were regulated by NPR1 during the P. syringae DC3000-triggered AR (Figure 3). qRT-PCR assay was carried out to validate the gene expression data obtained from the RNA-seq database. Two independent transgenic lines for each of the wNPR1-OE and HvNPR1-Kd were used and the barley elongation factor HvEF1a (GenBank accession number Z50789) were employed as a reference gene. We found that the expression level of NPR1 in the wNPR1-OE and HvNPR1-Kd transgenic lines was significantly \( (P < 0.01) \) higher and lower, respectively, than that in the wild-type plants (Supplementary Figure S2).
addition, the expression levels of six selected genes, including HvPR1, HvPR2, HvPR3_Chit2a, HvBCI1, HvBCI3, and HvBCI7, were measured by qRT-PCR. In the wild-type plants, significant (P < 0.01) inductions of HvPR1, HvPR2, HvPR3_Chit2a, and HvBCI1, were observed upon the P. syringae DC3000 treatment (Figure 4). Furthermore, the expression levels of HvPR1, HvPR2, HvPR3_Chit2a, and HvBCI7, were significantly (P < 0.05) upregulated in the wNPR1-OE transgenic lines by the P. syringae DC3000 treatment (Figure 4), which indicated their roles as downstream components of NPR1 during the AR response. On the other hand, the inductions of such downstream genes during the P. syringae DC3000-triggered AR were suppressed in the HvNPR1-Kd transgenic lines (Figure 5). Additionally, we used another reference gene, HvActin (GenBank accession number AK362208), to validate the results of our qRT-PCR assay for HvPR1 and found similar expression patterns (Supplementary Figure S3).

Differentially Expressed Genes (DEGs) Associated With NPR1 Expression During AR

DEGs in different comparisons, including “WT_PST vs. WT_CK,” “OE_PST vs. OE_CK,” “OE_PST vs. WT_PST,” “Kd_PST vs. Kd_CK,” and “Kd_PST vs. WT_PST,” were identified by DESeq2 (q-value < 0.05 and | log2foldchange| > 1, with gene annotation). Three types of DEGs were manually classified based on their possible roles in the NPR1-mediated AR (Supplementary Figure S4). Type I DEGs were highly upregulated genes in the wNPR1-OE transgenic line after P. syringae DC3000 induction. A total of 24 Type I DEGs were designated from significantly upregulated genes based on the comparisons “OE_PST vs. OE_CK” and “OE_PST vs. WT_PST” (Supplementary Figure S4 and Table 1). Several PR and BCI genes were annotated as Type I DEGs, including probable glucan 1,3-beta-glucosidase A (HvPR2), peroxidase A2-like (HvPR9), and thionin BTH7-like (HvBCI7). GO annotation for the Type I DEGs showed that the majority of these possible downstream genes of NPR1 during AR were annotated with “binding” and “catalytic activity” in the molecular function category, and with “metabolic process” and “response to stimulus” in the biological process category (Supplementary Figure S5A).

Type II DEGs were obtained and categorized into groups from significantly upregulated genes based on the comparisons “Kd_PST vs. Kd_CK” and “Kd_PST vs. WT_PST” (Supplementary Figure S4 and Supplementary Table S3).
A total of 64 genes were classified into this group, including several genes encoding transcription factors, such as *transcription factor JUNGBRUNNEN 1*-like, probable WRKY transcription factor 48, and probable WRKY transcription factor 50. Compared with GO annotations for the Type I DEGs, more genes in this group were annotated with “cellular process” but not “response to stimulus” (Supplementary Figure S5B). A third group of Type III DEGs, including only nine genes, were shared DEGs from Type I and Type II groups (Supplementary Figures S4, S5C and Supplementary Table S4).

The Transient Expression of *HvWRKY6*, *HvWRKY40*, and *HvWRKY70* Enhanced Wheat Resistance to *Pt*

A total of 46 WRKY genes were annotated in our RNA-seq database, and, based on their expression patterns, 10 of them were found to be involved in the NPR1-mediated AR (Figure 6). Since the naming system for the WRKY genes in the genera of *Triticeae* was confusing, a polygenic tree was generated using sequences of all these 10 WRKY proteins and their homologs from *Hordeum vulgare* (Hv), *Aegilops tauschii* (At), *Brachypodium distachyon* (Bd),
FIGURE 5 | Transcript levels of selected PR and BCI genes in the HvNPR1-Kd transgenic lines. Third leaves of HvNPR1-Kd barley transgenic lines and wild-type plants were infiltrated with water (control) or P. syringae pv. tomato DC3000. Then, samples for qRT-PCR assays were collected from the leaf region adjacent to the infection 48 h after inoculation, after a cell death phenotype observed. The transcript levels are expressed relative to those of the endogenous control HvEF1α using the $2^{-\Delta CT}$ method. Two independent transgenic lines for the HvNPR1-Kd were used, and, each experiment, consisting of 4–11 biological replicates, was considered a block. Calculations of the mean and standard error were performed using Microsoft Excel software. Data were transformed to restore normality and general linearized model (GLM) ANOVA ($^* P < 0.05$, $^{**} P < 0.01$) was conducted using SAS software version 9.4.

Triticum aestivum (Ta), Triticum urartu (Tu), and Sorghum bicolor (Sb) from the GenBank nr database (Supplementary Figure S6). All WRKYs were temporally designated according to their closest homologs in relative plant species, such as HvWRKY4, HvWRKY6, HvWRKY17, HvWRKY19, HvWRKY20, HvWRKY31, HvWRKY40, HvWRKY64, HvWRKY70, and HvWRKY76.

To test the potential of the differentially expressed WRKYs for improving wheat resistance to Pt, the open reading frame (ORF) of each WRKY gene was cloned and incorporated into a pLGY02 vector (Ubiquitin promoter, with T-DNA insertion site). The recombinant vector was transformed into the Agrobacterium strain AGL1. The transformed Agrobacterium was infiltrated into the secondary leaves of wheat seedlings of the susceptible line “Thatcher,” and the infiltration area was marked with a mark pen. Urediniospores of the highly virulent Pt pathotype THTT were spray-inoculated 4 days post-infiltration. The phenotype of leaf rust was recorded 10 days
# List of Type I DEGs.

| Gene_id       | WT_CK | WT_PST | OE_CK | OE_PST | Kd_CK | Kd_PST | Fold Change padj log2 | Gene annotation information                                                                 |
|---------------|-------|--------|-------|--------|-------|--------|------------------------|------------------------------------------------------------------------------------------------|
| MLOC_65675    | 6.35  | 18.98  | 1.50  | 17.51  | 0.72  | 2.59   | 1.91                   | UDP-glycosyltransferase 74E1-like                                                                  |
| Nove05826     | 0.04  | 0.23   | 0.03  | 2.49   | 0.00  | 0.04   | 1.85                   | (3S,6E)-nerolidol synthase 1                                                                      |
| MLOC_40202    | 0.37  | 0.44   | 0.44  | 36.86  | 0.09  | 0.20   | 1.83                   | Thionin BTH7-like                                                                                   |
| MLOC_13045    | 2.34  | 5.92   | 0.83  | 6.21   | 1.40  | 1.82   | 1.63                   | Multprotein-bridging factor tc                                                                       |
| Nove05366     | 1.82  | 6.27   | 1.03  | 8.15   | 0.60  | 1.54   | 1.60                   | Extradiol ring-deavaging deoxigenase-like                                                            |
| MLOC_64594    | 0.72  | 2.98   | 0.68  | 8.25   | 0.68  | 0.99   | 1.38                   | Bowman-Birk type trypsin inhibitor-like                                                               |
| MLOC_56924    | 0.13  | 0.33   | 0.06  | 6.87   | 0.12  | 0.14   | 1.36                   | 23 kDa jasmonate-induced protein-like                                                                  |
| MLOC_81846    | 0.00  | 0.20   | 0.01  | 9.57   | 0.01  | 0.00   | 1.36                   | Subtilisin-chymotrypsin inhibitor-2A-like                                                               |
| Nove06256     | 0.00  | 1.07   | 0.35  | 6.66   | 0.00  | 0.12   | 1.21                   | Subtilisin inhibitor OLSI-I-like                                                                     |
| MLOC_76389    | 1.20  | 5.14   | 1.60  | 6.11   | 0.41  | 0.63   | 1.20                   | Patatin-like protein 2                                                                               |
| MLOC_60806    | 4.18  | 9.71   | 1.92  | 7.20   | 1.72  | 2.65   | 1.20                   | ABC transporter B family member 11-like                                                               |
| Nove03795     | 8.30  | 17.72  | 5.15  | 17.08  | 5.56  | 8.00   | 1.12                   | Alcohol dehydrogenase 1                                                                               |
| MLOC_22702    | 26.71 | 59.93  | 11.80 | 36.61  | 9.88  | 17.82 | 1.12                   | Flavonol 3-sulfotransferase-like                                                                      |
| MLOC_44543    | 18.33 | 42.15  | 17.45 | 43.89  | 18.65 | 38.20 | 1.04                   | Probable LRR receptor-like                                                                             |
| MLOC_2643     | 1.39  | 2.44   | 0.36  | 112.85 | 0.10  | 0.27   | 1.03                   | Subtilisin-chymotrypsin inhibitor-2A-like                                                               |
| Nove00269     | 69.17 | 106.80 | 26.96 | 74.38  | 31.19 | 44.56 | 1.01                   | Probable glucan 1,3-beta-glucosidase A                                                                |
| MLOC_34615    | 0.08  | 0.03   | 0.03  | 3.51   | 0.00  | 0.02   | 0.84                   | Subtilisin-chymotrypsin inhibitor-2A-like                                                               |
| MLOC_60316    | 0.12  | 0.90   | 0.40  | 5.01   | 0.02  | 0.12   | 0.64                   | Endopeptidase 1                                                                                      |
| MLOC_70788    | 0.86  | 0.59   | 0.67  | 2.58   | 0.36  | 0.61   | 0.33                   | Probable calcium-binding protein CML45                                                                |
| MLOC_39951    | 5.20  | 4.91   | 4.33  | 16.60  | 0.22  | 3.43   | 0.11                   | Tricetin 3',4',5'-O-trimethyltransferase-like                                                           |
| MLOC_79167    | 11.75 | 15.52  | 6.15  | 15.22  | 7.29  | 4.95   | 0.02                   | Peroxidase A2-like                                                                                   |
| MLOC_10292    | 2.19  | 0.91   | 2.62  | 2.37   | 1.99  | 0.85   | 0.02                   | F-box protein PP2-A1-3-like                                                                           |
| MLOC_15925    | 4.22  | 3.75   | 11.63 | 9.21   | 9.32  | 3.33   | -0.24                  | Cytochrome P450 86B1-like                                                                              |
| MLOC_56051    | 16.08 | 7.45   | 84.69 | 24.27  | 47.07 | 16.87 | -0.25                  | Chlorophyll a-b binding protein of LHCI type 1                                                          |
| MLOC_39951    | 5.20  | 4.91   | 4.33  | 16.60  | 0.22  | 3.43   | 0.11                   | Tricetin 3',4',5'-O-trimethyltransferase-like                                                           |

**Note:** The above table represents a list of Type I differentially expressed genes (DEGs) with their corresponding gene identifiers, fold changes, and annotations. The table includes gene identifiers, fold changes, and annotations for each gene. The annotations provide insights into the functions and roles of these genes in plant biology, particularly in response to environmental stress or other biological processes.
post-inoculation (Figure 7). The percentage of Pt sporulation area in the infiltration region of each leaf was calculated using ASSESS software. Wheat with transient expression of TaPR1b (GenBank accession number HQ541962) was utilized as a positive control, whereas the untransformed Agrobacterium strain AGL1 was employed as a negative control. Although susceptible phenotypes were observed in all treatments, Pt sporulation in the leaves of the wheat with transient expression of TaPR1b and three WRKYs, including HvWRKY6, HvWRKY40, and HvWRKY70, were significantly (P < 0.001) reduced or delayed (Figure 7 and Supplementary Table S5). The transient expression of the other seven WRKY genes, including HvWRKY4, HvWRKY17, HvWRKY19, HvWRKY20, HvWRKY31, HvWRKY64, and HvWRKY76, in wheat leaves exerted no positive effect on wheat resistance to Pt (Supplementary Table S5).

**DISCUSSION**

*NPR1* has been reported as the master regulator of SAR in model plants of *Arabidopsis* and rice. Recent studies on *NPR1* homologs in wheat and barley have provided the initial clue to understand the mechanism of SAR in these two plant species (Cantu et al., 2013; Dey et al., 2014; Wang et al., 2016). Compared with SAR in *Arabidopsis*, three different SAR-like responses can be induced by either pathogens or SA/INA/BTH treatment in wheat and barley (Wang et al., 2018). In the current study, the observed beneficial effect of acquired resistance, which led to the reduction of Pt infection in wheat (Figure 1), suggested a potential use of *NPR1*-mediated AR in improving the resistance of *Triticaceae* crops. The diminished AR to *M. oryzae* observed in the HvNPR1-Kd transgenic line indicated a key role of *NPR1* during the AR triggered by *P. syringae* DC3000 infection (Figure 2). Finally,
the resistance of the wNPR1-OE transgenic line to Mo was enhanced (Figure 2), providing valuable evidence that NPR1 can be utilized in improving barley resistance to Mo, possibly also to the recently emerged wheat blast disease (Inoue et al., 2017).

So far, a total number of 18 PR gene families have been designated from plant species, many of which showed involvement in wheat and barley resistance to various pathogens (Loon et al., 2006; Wang et al., 2018). Several barley PR genes, including HvPR1, HvPR2, HvPR3_Chit2a, HvPR4b, and HvPR5_TLP6, were validated as downstream genes of NPR1 during the P. syringae DC3000-triggered AR (Wang et al., 2016). Another group of BCI genes seems to be responsible for the enhanced resistance in barley induced by BTH treatment (Beßer et al., 2000). In the present study, the expression profiles of all the PR and BCI genes were generated using FPKM values from the RNA-seq assay. The NPR1-regulated genes, including HvPR1, HvPR2, HvPR3_Chit2a, HvPR5_TLP6/7/8, HvPR13/BCI2, and HvBCI7, during the P. syringae DC3000-triggered AR, were established (Figures 3, 8). Further studies on HvPR13/BCI2 and HvBCI7 may provide initial evidence for understanding the relationship between AR and BIR. Interestingly, the expression levels of several PR genes, including HvPR3_Chit2b, HvPR5_TLP1, HvPR15, HvPR16, HvPR17a, and HvPR17b, were induced in a NPR1-independent manner, which indicated that other unknown regulators were functioning during the AR triggered by P. syringae DC3000.

In this investigation, we classified three DEG groups based on their expression patterns. Several PR and BCI genes were categorized into Type I and Type III DEGs (Table 1 and Supplementary Table S4). The possible downstream genes of NPR1 during the P. syringae DC3000-triggered AR were mainly identified as Type I DEGs. Interestingly, we also discovered a large group of Type II DEGs (Supplementary Table S3) which were highly induced only in the HvNPR1-Kd transgenic line after P. syringae infection. We speculated that some of them were negative regulators or upstream genes of NPR1 during the AR. Furthermore, the regulator network of NPR1 in barley during the P. syringae DC3000-triggered AR was initially established in our investigation (Figure 8).

In addition, we checked the involvement of barley WRKY transcription factors in the NPR1-mediated AR. In model plants of Arabidopsis and rice, several WRKYs, including AtWRKY18, AtWRKY58, AtWRKY70, OsWRKY03, OsWRKY71, and OsWRKY45, have been suggested to play important roles in the SAR (Liu et al., 2005, 2007; Wang et al., 2006; Shimono et al., 2007; Nakayama et al., 2013). A total of 171 TaWRKYs were identified in wheat, whereas 45 HvWRKYs were detected in barley (Chistri et al., 2008; Pan et al., 2017). It was rather difficult to determine the key WRKY genes during SAR in these two crop species. By checking the expression patterns of all the 46 WRKYS identified in our RNA-seq assay (Figure 6), a total of 10 HvWRKY genes with relatively higher induction in either the wNPR1-OE or HvNPR1-Kd transgenic line were selected for further functional validation.

Three differentially expressed HvWRKYs, including HvWRKY6, HvWRKY40, and HvWRKY70, exerted positive effects on wheat resistance to Pt (Figure 7).
FIGURE 8 | Possible regulatory network of the NPR1-mediated AR in barley. The transient expression of three barley WRKY genes, including HvWRKY6, HvWRKY40, and HvWRKY70 (bold-labeled), in wheat leaves by Agrobacterium-mediated infiltration enhanced the resistance to \textit{Pt}.

HvWRKY6_MLOC_78461 was a barley homolog of AtWRKY6_XP_020181741 (Supplementary Figure S6), which had been reported to be associated with both senescence- and defense-related processes (Robatzek and Somssich, 2001). HvWRKY40_NOVEL04527 was clustered with AtWRKY40_EMT21551 in our polygenetic analysis (Supplementary Figure S6). In an earlier study, AtWRKY40 was found to be a repressor of antimycin A-induced mitochondrial retrograde expression and high-light-induced signaling (Aken et al., 2013). In addition, our data evidenced that HvWRKY70_MLOC_66134 was clustered with \textit{Arabidopsis} AtWRKY70_XP_020165252 (Supplementary Figure S6), which exerted dual roles as negative regulators of SA biosynthesis and positive regulators of SA-mediated gene expression and resistance in \textit{Arabidopsis} (Wang et al., 2006). We speculated that these three \textit{HvWRKYs} were key regulators during the \textit{NPR1}-mediated AR and may represent valuable transgenic resources for resistance improvement of wheat plants.

CONCLUSION

In conclusion, \textit{NPR1} acts as a key regulator during the \textit{P. syringae} DC3000-triggered AR in barley (Figure 8). In the present study, we have identified genes associated with the \textit{NPR1}-mediated AR. Several \textit{PR} and \textit{BCI} genes were confirmed to be the downstream genes of \textit{NPR1}. The expression levels of several \textit{WRKY} transcription factors were significantly associated with \textit{NPR1} expression, which might be key components in the \textit{NPR1}-mediated AR. Furthermore, it is noteworthy that three
differentially expressed WRKYs identified in the current study showed potential for resistance improvement of wheat plants.

**AUTHOR CONTRIBUTIONS**

XW conceived the original screening and research plans. XW and DL supervised the experiments. JG and WB performed most of the experiments. HL, JW, and XY provided technical assistance to JG. XW designed the experiments and analyzed the data. XW conceived the project and wrote the article with contributions of all the authors. DL supervised and complemented the writing.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01486/full#supplementary-material
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