MPMI Vol. 33, No. 3, 2020, pp. 433–443. https://doi.org/10.1094/MPMI-09-19-0257-R

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WRKY Transcription Factors Shared by BTH-Induced Resistance and *NPR1*-Mediated Acquired Resistance Improve Broad-Spectrum Disease Resistance in Wheat

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Accepted 9 December 2019.

In *Arabidopsis*, both pathogen invasion and benzothiadiazole (BTH) treatment activate the *nonexpresser of pathogenesis-related genes 1* (*NPR1*)-mediated systemic acquired resistance, which provides broad-spectrum disease resistance to secondary pathogen infection. However, the BTH-induced resistance in Triticeae crops of wheat and barley seems to be accomplished through an *NPR1*-independent pathway. In the current investigation, we applied transcriptome analysis on barley transgenic lines overexpressing wheat *wNPR1* (*wNPR1-OE*) and knocking down barley *HvNPR1* (*HvNPR1-Kd*) to reveal the role of *NPR1* during the BTH-induced resistance. Most of the previously designated barley chemical-induced (*BCI*) genes were upregulated in an *NPR1*-independent manner, whereas the expression levels of several pathogenesis-related (*PR*) genes were elevated upon BTH treatment only in *wNPR1-OE*. Two barley *WRKY* transcription factors, *HvWRKY6* and *HvWRKY70*, were predicted and further validated as key regulators shared by the BTH-induced resistance and the *NPR1*-mediated acquired resistance. Wheat transgenic lines overexpressing *HvWRKY6* and *HvWRKY70* showed different degrees of enhanced resistance to *Puccinia striiformis* f. sp. *tritici* pathotype CYR32 and *Blumeria graminis* f. sp. *tritici* pathotype E20. In conclusion, the transcriptional changes of BTH-induced resistance in barley were initially profiled, and the identified key regulators would be valuable resources for the genetic improvement of broad-spectrum disease resistance in wheat.

Keywords: barley, broad-spectrum disease resistance, BTH-induced resistance, defense signaling pathways, pathogenesis-related proteins, plant antifungal responses, salicylic acid, systemic acquired resistance, wheat

Systemic acquired resistance (SAR) provides broad-spectrum disease resistance to secondary pathogenic invasions beyond the initial infection site. External treatments with salicylic acid (SA) or its chemical analog benzothiadiazole (BTH) can also stimulate SAR (Fu and Dong 2013). In *Arabidopsis*, the *nonexpresser of pathogenesis-related genes 1* (*NPR1*) is reported as the key regulator of SAR, during which it associates with the TGACG-binding factor (TGA) transcription factors to induce the expression of downstream pathogenesis-related (*PR*) genes (Dong 2004).

WRKY transcription factors played crucial roles in SAR in either NPR1-dependent or -independent ways. Using a microarray approach, *AtWRKY18*, *AtWRKY58*, and *AtWRKY70* were identified as key regulatory nodes of SAR pathways in *Arabidopsis* (Wang et al. 2006). The expression level of the rice homolog of *NPR1* (*rNH1*) was regulated by *OsWRKY3* and *OsWRKY71* (Liu et al. 2005; Liu et al. 2007), whereas *OsWRKY45* regulated the BTH-induced resistance in an *rNH1*-independent pathway (Shimono et al. 2007). The transcriptional regulatory network of OsWRKY45 during the BTH-induced resistance has been further profiled using RNA sequencing (RNA-seq) analysis (Nakayama et al. 2013).

In one of our previous investigations, wheat homolog of NPR1 (wNPR1) showed much conserved protein interactions with homologs of TGA transcription factors from both wheat and rice (Cantu et al. 2013), which are essential for the functioning of NPR1 in other plant species (Chern et al. 2001; Després et al. 2003). Although the protein interactome of

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Funding: This work was supported by the National Natural Science Foundation of China (31701776 and 31772150), the Provincial Natural Science Foundation of Hebei for Excellent Young Scholar (C2018204091), the China Agriculture Research System (CARS-3), the Open Project Program of State Key Laboratory of Crop Stress Biology for Arid Areas (CSBAAKF2018009), Shaanxi Innovation Team Project (2018TD-004), and the Supporting Program of Hebei Agricultural University for Oversea Talents (ZD201603).

*The *e*-Xtra logo stands for "electronic extra" and indicates that supplementary figures and supplementary tables are published online.

The author(s) declare no conflict of interest.

wNPR1 seems to be conserved, SAR in wheat and barley exhibited several unique features (Wang et al. 2018).

Systemic immunity, a SAR-like response in barley to secondary infection of *Xanthomonas translucens* pv. *cerealis*, could be induced in systemic uninfected leaves by infection of *X. translucens* pv. *cerealis* or *Pseudomonas syringae* pv. *japonica* in the elder leaves (Dey et al. 2014). However, the systemic immunity was not associated with local or systemic accumulation of SA but with a moderate local induction of jasmonate acid (JA) and, especially, abscisic acid (ABA). By applying RNA-seq analysis on the barley transgenic line knocking down *HvNPR1* (*HvNPR1-Kd*), the systemic immunity seemed to be regulated by several WRKY and ERF transcription factors in an *NPR1*-independent manner.

In the region adjacent to the infiltration area of *P. syringae* pv. *tomato* DC3000 in barley leaf, another form of SAR-like response to the secondary fungal pathogen *Magnaporthe oryzae* could be induced (Colebrook et al. 2012). The designated acquired resistance shared several common features with SAR in *Arabidopsis*, including induction of *PR* genes and elevation of SA level. In our previous investigation, we observed significant associations between the inductions of *PR* genes and the expression level of *NPR1* during the *P. syringae* DC3000-triggered acquired resistance (Wang et al. 2016). The results of our recent study showed that the acquired resistance to *M. oryzae* in barley was directly controlled by *NPR1* (Gao et al. 2018). In the same investigation, the transcriptome analysis results revealed the downstream genes of *NPR1* and key *WRKY* transcription factors during the acquired resistance response.

In several earlier studies, BTH treatment of Triticeae crops of wheat and barley induced broad-spectrum disease resistance to multiple fungal diseases, including leaf rust and powdery mildew (Beßer et al. 2000; Görlach et al. 1996; Hafez et al. 2014). Different from BTH-induced SAR in *Arabidopsis*, few *PR* genes were sensitive to BTH treatment, whereas the expression levels of another group of genes, including *wheat chemical-induced* (*WCI*) and *barley chemical-induced* (*BCI*) genes, were highly expressed (Beßer et al. 2000; Molina et al. 1999; Vallélian-Bindschedler et al. 1998). Interestingly, a faster activation of disease resistance to Fusarium head blight was observed in a wheat transgenic line overexpressing *Arabidopsis AtNPR1*, and the expression of *TaPR1* became more sensitive to the BTH treatment (Makandar et al. 2006). Nevertheless, the regulatory network of the BTH-induced resistance, as well as the specific role of NPR1 during such SAR-like responses, was largely unknown.

In the present investigation, transcriptome sequencing was performed on barley transgenic lines overexpressing wheat *wNPR1* (*wNPR1-OE*) and *HvNPR1-Kd* to reveal the role of *NPR1* during the BTH-induced resistance. Most of the *BCI* genes were regulated independently from *NPR1*, whereas few *PR* genes became more sensitive to BTH only in *wNPR1-OE*. Two differentially expressed *WRKY* transcription factors, *HvWRKY6* and *HvWRKY70*, were selected for further functional characterization and both of them showed great potential in the improvement of broad-spectrum disease resistance in wheat.

RESULTS

Transcriptome analysis of *wNPR1-OE* and *HvNPR1-Kd* during the BTH-induced resistance.

The wheat and barley NPR1 homologs seemed to be very conserved with previously reported rice NPR1 protein (rNH1, GenBank accession AAX18700.1) (Supplementary Fig. S1). The similarities between barley HvNPR1 and wheat NPR1 homologs deduced from A, B, and D subgenome copies were 92.72, 91.68, and 91.03%, respectively, indicating a very conserved role of NPR1 in these relative plant species. To explore the role of *NPR1* during BTH-induced resistance in barley, we applied transcriptome analysis on samples harvested from BTH-sprayed third leaves of barley transgenic lines *wNPR1-OE-E1-T3* (wheat *wNPR1* overexpressing line, independent transgenic event number 1, T3 generation) and *HvNPR1-Kd-E1-T6* (barley *HvNPR1* knockdown line, independent transgenic event number 1, T6 generation), as well as the wild-type plants. Samples from the water-sprayed wild-type plants served as a control. The transcript abundance of the *NPR1* transgene was determined by quantitative reverse-transcription (qRT)-PCR assay. The barley elongation factor 1a (*HvEF1a*, GenBank accession Z50789) was used as an internal reference gene. Compared with the wild-type plants, approximately 4.3-fold more and 0.4-fold less *NPR1* transcripts were detected in the *wNPR1-OE-E1-T3* and *HvNPR1-Kd-E1-T6*, respectively (Supplementary Fig. S2). Each of the materials included four biological replicates and, in total, 16 samples were subjected to a 12-Gb Illumina sequencing (Supplementary Table S1). For each of the samples, more than 58 million 150-bp pair-end reads were collected. The genome of *Hordeum vulgare* from Ensembl Genomes was utilized for the assembly of the transcriptome. Briefly, in total, 32,247 genes were mapped on the genome sequence (Supplementary Table S2). We observed significant ($R^2 > 0.92$) correlations of the overall gene expression levels between biological replicates (Supplementary Fig. S3). The fragments per kilobase of transcript per million mapped reads (FPKM) value was employed to estimate the abundance of transcripts. Raw reads for the transcriptome were stored at NCBI BioProject PRJNA509975.

The expression patterns of *PR* and *BCI* genes during the BTH-induced resistance in barley.

The expression patterns of all 18 *PR* gene families were initially profiled by searching their FPKM values in our transcriptome database (Fig. 1). We found that BTH treatment was sufficient to elevate the expression levels of *HvPR1*, *HvPR2*, *HvPR3* (*Chit2a* and *Chit2b*), *HvPR5* (*TLP6*, *TLP7*, and *TLP8*), *HvPR14*, and *HvPR17b*. The sensitivities of all such genes to BTH treatment were dramatically increased in *wNPR1-OE*. On the other hand, most of the *BCI* genes were highly induced upon BTH treatment in an *NPR1*-independent manner (Fig. 1).

We then performed a qRT-PCR assay to validate the gene expression patterns of *PR* and *BCI* genes determined by FPKM values. Two independent lines for each of the transgenic materials were included. The transcriptional abundances of *NPR1* transgene, *HvPR1*, *HvPR2*, *HvBCI1*, *HvBCI3*, and *HvBCI7* were monitored and expressed as linearized fold *HvEF1a* levels (Fig. 2; Supplementary Fig. S2). The expression levels of *HvBCI1* (** $P = 0.0021$), *HvBCI3* (** $P = 0.0008$), and *HvBCI7* (** $P = 0.0054$) were significantly induced by the BTH treatment in an *NPR1*-independent manner. Of note, the expression levels of *HvPR1* (*** $P < 0.0001$) and *HvPR2* (*** $P < 0.0001$) were significantly upregulated only in *wNPR1-OE* upon the BTH treatment.

Regulatory network of *NPR1* during the BTH-induced resistance.

DESeq2 was applied to identify significant upregulated (q value < 0.05 and $\log_2$-fold change > 1) differentially expressed genes (DEGs) in the comparisons of WT_BTH versus WT_CK, OE_BTH versus WT_BTH, and Kd_BTH versus WT_BTH. In total, 847 genes in the wild-type plants were significantly upregulated upon the BTH treatment. Among these DEGs, the expression levels of 104 genes were much more highly elevated

in the *wNPR1-OE* than those in the wild-type plants, which were designated as type I DEGs (Supplementary Fig. S4; Supplementary Table S3). We speculated that the type I DEGs were downstream genes of the *NPR1*-mediated signaling pathway, triggered by the BTH treatment. DEGs encoding WRKY transcription factors, glucan endo-1,3-β-glucosidase, and various protein kinases were enriched in this group. Gene ontology (GO) analysis showed that the type I DEGs were annotated with "catalytic activity" and "binding" in molecular function category and with "metabolic process" in biological process category (Supplementary Fig. S5A). The remaining 743 upregulated genes were predicted to be *NPR1*-independent BTH-sensitive genes, and designated as type II DEGs (Supplementary Figs. S4 and S5B; Supplementary Table S4). Interestingly, we also detected, in total, 107 genes more highly expressed in the *HvNPR1-Kd* than those in the wild-type plants upon BTH treatment. Among these DEGs, 73 genes seemed to be uniquely induced in *HvNPR1-Kd*, which were designated as type III DEGs (Supplementary Figs. S4 and S5C; Supplementary Table S5).

HvWRKY6 and *HvWRKY70* were predicted as key transcriptional regulators shared by BTH-induced resistance and the *NPR1*-mediated acquired resistance.

In the present transcriptome database, 46 *WRKY* genes were identified, roughly half of which were significantly induced upon BTH treatment (Fig. 3). All of the identified *WRKY* genes were temporarily designated based on their closest homologs in relative plant species. Most of the induced *WRKY* genes seemed to be regulated in an *NPR1*-independent manner. However, among the differentially expressed *WRKY* genes, the induction levels of two genes, *MLOC_66134* and *MLOC_78461*, were higher in *wNPR1-OE* than those in the wild-type plants (Supplementary Fig. S6A). We then double-checked the expression profiles of these two genes in one of our previous transcriptome databases referring to *P. syringae* DC3000-triggered acquired resistance (Gao et al. 2018). Based on their FPKM values, we found that *HvWRKY6* and *HvWRKY70* were also more highly induced in *wNPR1-OE* than in the wild-type plants upon *P. syringae* DC3000 treatment (Supplementary Fig. S6B). Based on the results, we speculated that these two *WRKY* genes might serve as key transcriptional regulators shared by BTH-induced resistance and *NPR1*-mediated acquired resistance.

The closest homologs of the deduced proteins of *MLOC_66134* and *MLOC_78461* in other plant species, including *Triticum aestivum* (Ta), *Hordeum vulgare* (Hv), *Aegilops tauschii* (Att), *T. uratu*, *Oryza sativa*, and *Arabidopsis thaliana* (At), were collected from GenBank and wheat genome databases. Furthermore, a neighbor-joining tree was generated (Supplementary Fig. S7). We temporarily designated the *MLOC_78461* gene as the barley *HvWRKY6* based on its closest homolog, AttWRKY6 in *Aegilops tauschii*, and *MLOC_66134* as barley *HvWRKY70*, referring to AttWRKY70 in *A. tauschii.* The closest homolog of HvWRKY6 protein in a common wheat genome database (TRIAE_CS42_2DS_TGACv1_179236_AA0605510) was clustered with another protein, AttWRKY72 from *A. tauschii*, and the closest homolog of HvWRKY70 in common wheat (TRIAE_CS42_7DL_TGACv1_602597_AA1962010) was clustered with a recently identified TaWRKY146 protein (Ma et al. 2017).

To investigate the subcellular localization of HvWRKY6 and HvWRKY70 proteins, a green fluorescent protein (GFP) tag was fused to each end of the HvWRKY6 and HvWRKY70 proteins, and the recombinant transcripts were transiently expressed in *Nicotiana benthamiana* using *Agrobacterium*. Clear fluorescence for HvWRKY6-GFP, GFP-HvWRKY6, and GFP-HvWRKY70 was observed in the nucleus (Supplementary Fig. S8). On the other hand, for HvWRKY70-GFP, GFP signals were detected in both the nucleus and the cytoplasm, indicating a block of nuclear localization signal of HvWRKY70 by GFP tag at the C-terminal end.

Wheat transgenic line overexpressing the *HvWRKY6* gene exhibited promoted resistance to *M. oryzae* in a *PR* gene-associated manner.

To explore the potential application of the *HvWRKY6* and *HvWRKY70* genes, we generated wheat transgenic lines overexpressing the *HvWRKY6* or *HvWRKY70* gene under the maize *Ubiquitin* promoter (*HvWRKY6-OE* and *HvWRKY70-OE*) in the genetic background of spring common wheat JW1. The expression levels of the *HvWRKY6* transgene were from 2.3- to 7.2-fold of the levels of the *TaActin* endogenous control (Supplementary Fig. S9A), and those of *HvWRKY70* transgene were from 0.4- to 1.4-fold (Supplementary Fig. S9B). We did not detect any signals of *HvWRKY6* and *HvWRKY70* in the wild-type plants.

We then utilized the *M. oryzae* isolate P131 to investigate the degree of acquired resistance induced by *P. syringae* DC3000 in *HvWRKY6-OE* and *HvWRKY70-OE* (Fig. 4A). The third leaves of wheat seedlings were infiltrated with either *P. syringae* DC3000 or sterile water as a control. *M. oryzae* isolate P131 was inoculated as the secondary pathogen in the region adjacent to the *P. syringae* DC3000 infiltration area at 2 days post-infiltration (dpi). In the wild-type plants, acquired resistance triggered by *P. syringae* DC3000 significantly (** $P < 0.01$)

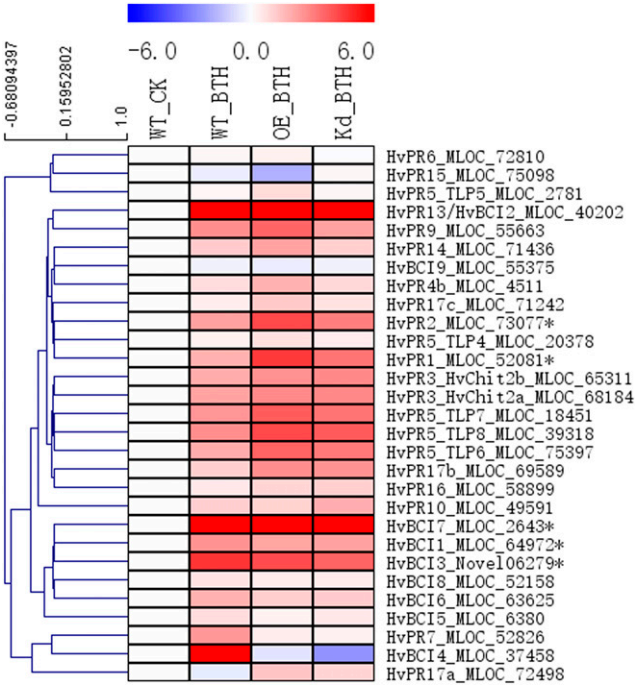

Fig. 1. Expression patterns of *pathogenesis-related* (*PR*) and *barley chemical-induced* (*BCI*) genes during benzothiadiazole (BTH)-induced resistance were initially profiled. Fragments per kilobase of transcript per million mapped reads values for each of the transcripts in WT_BTH, OE_BTH, and Kd_BTH were relative to that in WT_CK. Data were transformed into $\log_2$-fold change format using Microsoft Excel software. A heatmap was created using MeV software. The function "Hierarchical Clustering" in MeV software was employed to cluster genes with similar expression patterns. Five genes were selected (labeled with an asterisk [*]) for further quantitative reverse-transcription PCR validation assay. OE = overexpressing wheat *wNPR1* (*wNPR1-OE*), Kd = knocking down barley *HvNPR1* (*HvNPR1-Kd*), WT = wild type, CK = mock-inoculation with water, and *NPR1 = nonexpresser of pathogenesis-related genes 1*.

decreased the lesion size of *M. oryzae* isolate P131 (Fig. 4A). Compared with the wild-type plants, we observed more pronounced (* $P < 0.05$) resistance to *M. oryzae* in *HvWRKY6-OE*, even in the mock control (Fig. 4A; Supplementary Table S6), which indicated that *HvWRKY6* improved the basal resistance of wheat. On the other hand, we did not observe any elevated resistance to *M. oryzae* during the acquired resistance in the wheat transgenic line *HvWRKY70-OE* (Fig. 4A).

To investigate the possible regulatory downstream genes of *HvWRKY6* during the *NPR1*-mediated acquired resistance, a series of qRT-PCR assays was performed. *P. syringae* DC3000 was applied to induce the acquired resistance in *HvWRKY6-OE* and *HvWRKY70-OE*, as well as the wild-type plants. Water infiltration served as a mock control. RNA samples were harvested from the region adjacent to the *P. syringae* DC3000 infiltration area at 2 dpi. The expression levels of *TaPR1a*, *TaPR2*, and *TaPR4b*, were determined by qRT-PCR assay. The wheat *TaActin* gene served as a reference gene. Significant inductions of *TaPR1a* (**$P = 0.0001$), *TaPR2* (*$P = 0.0106$), and *TaPR4b* (**$P = 0.0066$), were detected during the acquired resistance in the wild-type plants (Fig. 4B; Supplementary Fig. S10). Interestingly, we found that the expression levels of such *PR* genes in the mock control of the wheat transgenic line *HvWRKY6-OE* were significantly (*$P < 0.05$) higher than that in the wild-type plants (Fig. 4B). The whole experiment was repeated twice with two independent lines of *HvWRKY6-OE*,

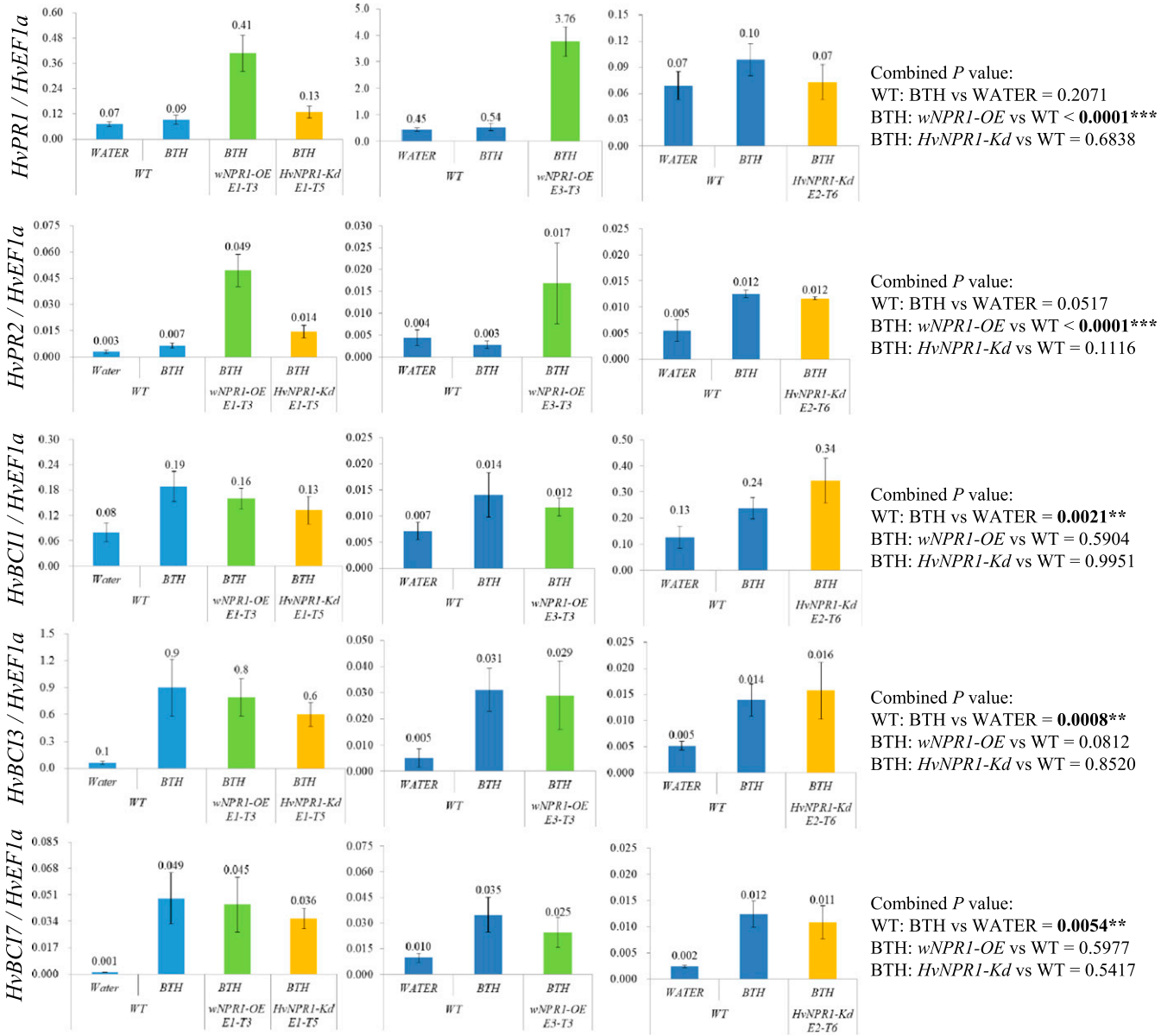

Fig. 2. Expression levels of selected *pathogenesis-related* (*PR*) and *barley chemical-induced* (*BCI*) genes in barley transgenic lines overexpressing wheat *wNPR1* (*wNPR1-OE*) and knocking down barley *HvNPR1* (*HvNPR1-Kd*) during the benzothiadiazole (BTH)-induced resistance were validated by a quantitative reverse-transcription PCR assay. Third leaves of *wNPR1-OE* and *HvNPR1-Kd*, as well as wild-type (WT) plants, were sprayed with 0.1 mM BTH. Water-sprayed WT plants served as a control. RNA samples were harvested at 48 h posttreatment. The whole experiment was systemically repeated three times using independent transgenic lines (left, middle, and right panels) and each transgenic line consisted of six to 10 biological replicates. The relative transcript abundance was expressed relative to that of the internal reference *HvEF1a* following the $2^{-\Delta Ct}$ method. Mean and standard error for the relative expression levels were calculated using Microsoft Excel software. A general linearized model analysis of variance (*, **, and *** indicate $P < 0.05$, 0.01, and 0.0001, respectively) was performed using SAS v9.4 software. OE = overexpressing transgenic line, Kd = knockdown transgenic line, E = independent transgenic event, and T = generation of the transgenic material.

and each repeat included six to eight biological replicates. On the other hand, we did not observe any significant differences in the expression levels of the selected *PR* genes between the wheat transgenic line *HvWRKY70-OE* and the wild-type plants (Supplementary Fig. S10).

***PR* and *BCI* genes in wheat transgenic line *HvWRKY70-OE* became more sensitive to the BTH treatment.**

To investigate the roles of *HvWRKY6* and *HvWRKY70* genes during the BTH-induced resistance, we further performed qRT-PCR assays on *HvWRKY6-OE* and *HvWRKY70-OE.* Water treatment served as a mock control. Two days posttreatment (dpt) with BTH, the expression levels of *TaPR1a*, *TaPR2*, *TaPR4b*, *TaBCI1*, and *TaBCI3* were monitored by qRT-PCR assay (Fig. 5). In the wild-type plants, only the expression levels of *TaBCI1* (***$P < 0.0001$) and *TaBCI3* (***$P < 0.0001$) genes were significantly induced upon the BTH treatment. However, the expression levels of all of the tested *PR* and *BCI* genes were significantly (**$P < 0.01$) higher in the *HvWRKY70-OE* than those in the wild-type plants. The whole experiment was systemically repeated twice with two independent lines of *HvWRKY70-OE*, and each line included five to seven biological replicates. On the other hand, we did not observe any significant differences in the expression levels of the selected *PR* and *BCI* genes between the *HvWRKY6-OE* and the wild-type plants after the BTH treatment (Fig. 5).

The genomic regions of *TaPR1a*, *TaPR2*, *TaPR4b*, *TaBCI1*, and *TaBCI3* genes were collected from the wheat genome database (Chinese Spring, TGACv1 version) and were subjected to analysis of the transcription factor-binding site in their promoter regions (Supplementary Table S7). We did not detect any WRKY transcription factor-binding sites in the tested *PR* and *BCI* genes, which indicated that *HvWRKY6* and *HvWRKY70* might regulate these genes indirectly.

***HvWRKY6* and *HvWRKY70* improved wheat resistance to stripe rust and powdery mildew.**

We hypothesized that the identified *HvWRKY6* and *HvWRKY70* might improve broad-spectrum disease resistance in wheat. The generated wheat transgenic lines were then subjected to inoculation with both wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) and powdery mildew (*Blumeria graminis* f. sp. *tritici*), both of which cause severe fungal diseases threatening global wheat production. For the stripe rust inoculation, the wheat seedlings of the wild-type JW1 plants showed phenotypes highly susceptible (full sporulation) to the virulent *P. striiformis* f. sp. *tritici* pathotype CYR32 at 15 dpi (Fig. 6). The proportion of *P. striiformis* f. sp. *tritici* sporulation area on each of the collected leaves was recorded using ASSESS software. The sporulation of the rust fungi was significantly (*$P < 0.05$ and **$P < 0.01$) diminished or delayed in the wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE* compared with the wild-type plants (Fig. 6). On the other hand, for the powdery mildew inoculation, the wild-type plants exhibited phenotypes highly susceptible (full sporulation) to the prevalent *B. graminis* f. sp. *tritici* isolate E20 at 15 dpi (Fig. 7). Compared with the wild-type plants, the proportion of *B. graminis* f. sp. *tritici* sporulation area on each of the collected leaves was significantly (*$P < 0.05$ and **$P < 0.01$) lower in the wheat transgenic line *HvWRKY70-OE* (Fig. 7). We did not observe any enhanced resistance to *B. graminis* f. sp. *tritici* in the wheat transgenic line *HvWRKY6-OE.*

DISCUSSION

SAR triggered by initial pathogen infection or BTH treatment is considered to confer plant broad-spectrum resistance to secondary pathogenic invasions (Klessig et al. 2018). However, due to the large genome size and the complex gene homolog network, to date, SAR in Triticeae crops (wheat and barley) has been poorly explored (Wang et al. 2018). The acquired resistance induced by *Pseudomonas syringae* DC3000 shared several key features with the SAR described in *Arabidopsis* (e.g., inductions of *PR* genes, elevation of endogenous SA, and involvement of *NPR1*) (Colebrook et al. 2012; Gao et al. 2018). Meanwhile, BTH-induced resistance was sufficient to improve broad-spectrum disease resistance in wheat and barley to multiple pathogens in a *PR* gene-independent manner (Beßer et al. 2000; Görlach et al. 1996; Hafez et al. 2014; Molina et al. 1999; Vallélian-Bindschedler et al. 1998). The regulatory

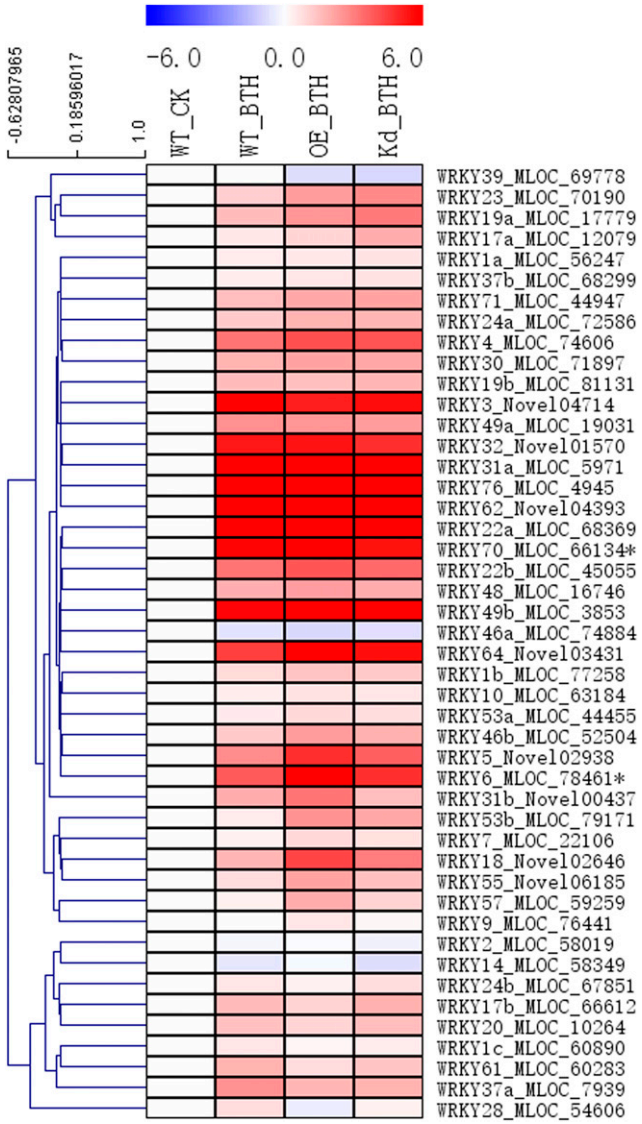

Fig. 3. Genome-wide expression profiles of *WRKY* genes during benzothiadiazole (BTH)-induced resistance. Fragments per kilobase of transcript per million mapped reads values for each of the transcripts in WT_BTH, OE_BTH, and Kd_BTH were relative to that in WT_CK. Data were transformed into $\log_2$-fold change format using Microsoft Excel software. A heatmap was created using MeV software. The function "Hierarchical Clustering" in MeV software was employed to cluster genes with similar expression patterns. Names of the *WRKY* genes were temporally designated based on their closest homologs in relative plant species. *HvWRKY6* and *HvWRKY70* were chosen (labeled with an asterisk [*]) for functional exploration. OE = overexpressing wheat *wNPR1* (*wNPR1-OE*), Kd = knocking down barley *HvNPR1* (*HvNPR1-Kd*), and CK = mock inoculation with water.

networks of BTH-induced resistance in wheat and barley were largely unknown.

To investigate the role of *NPR1* during BTH-induced resistance, we established a transcriptome database by sequencing RNA samples collected from BTH-sprayed barely transgenic lines (*wNPR1-OE* and *HvNPR1-Kd*) and wild-type plants. Based on the expression profiles generated from the transcriptome database and subsequent qRT-PCR validation (Figs. 1 and 2), most of the *BCI* genes were highly expressed independently from *NPR1*. It is noteworthy that *PR* genes became

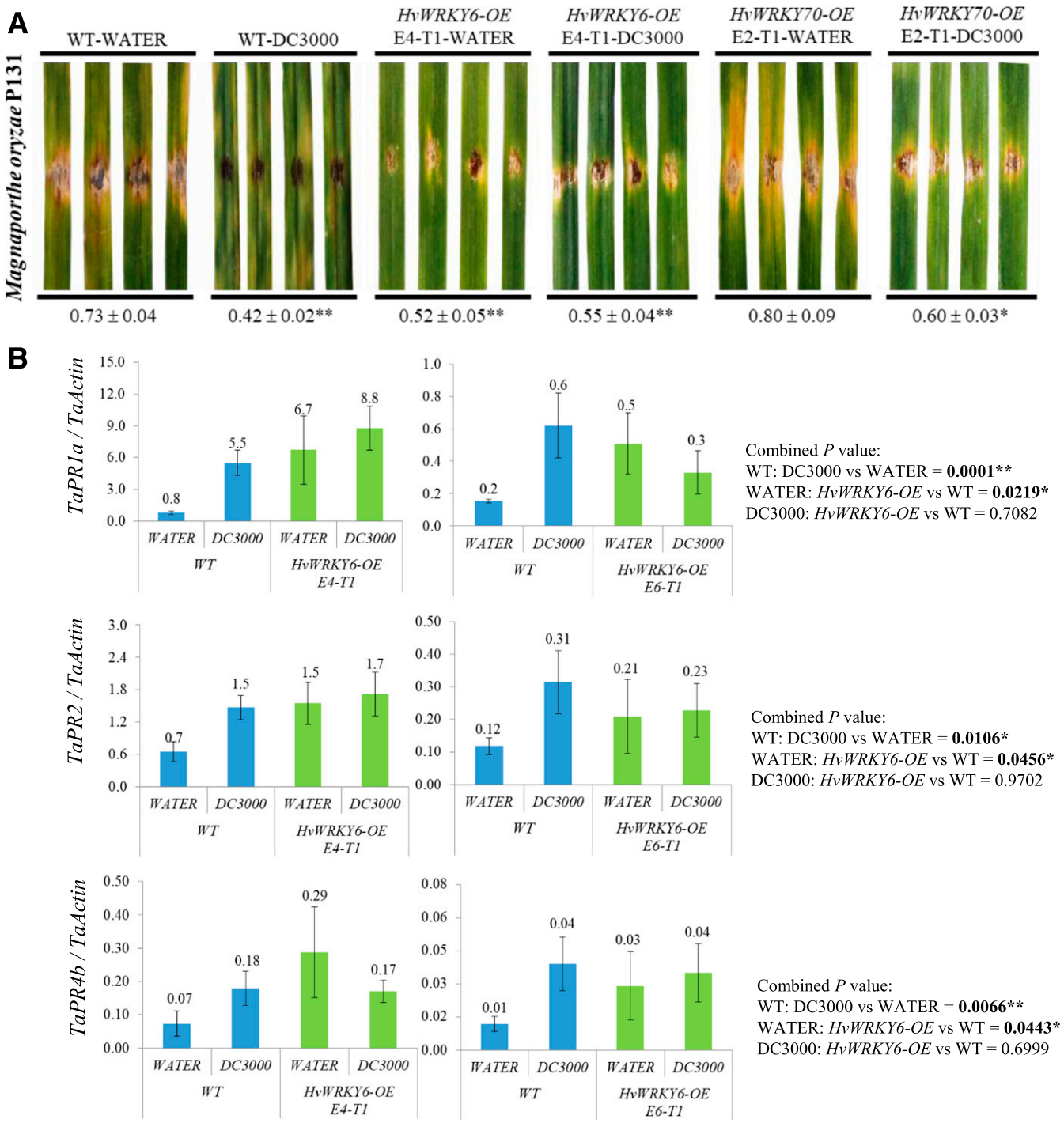

Fig. 4. Wheat transgenic line overexpressing the *HvWRKY6* gene showed promoted resistance to *Magnaporthe oryzae* in a *pathogenesis-related* (*PR*) gene-associated manner. Third leaves of wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE*, as well as wild-type (WT) plants, were infiltrated with either *Pseudomonas syringae* DC3000 or water as a control. **A,** *M. oryzae* isolate P131 was inoculated as the secondary pathogen in the region adjacent to the *P. syringae* DC3000 infiltration area at 48 h postinfiltration (hpi). Numbers represent the average lesion size of *M. oryzae* P131. The whole experiment was repeated twice with two independent lines of *HvWRKY6-OE* and each line included six to eight biological replicates (Supplementary Table S6). A Dunnett's test (* and ** indicate $P < 0.05$ and 0.01, respectively) was performed using SAS v9.4 software. **B,** Transcript levels of *TaPR1*, *TaPR2*, and *TaPR4b* in the wheat transgenic line *HvWRKY6-OE* during acquired resistance. RNA samples were harvested from the region adjacent to the *P. syringae* DC3000 infiltration area at 48 hpi. The experiment consisted of five to six biological replicates. The relative transcript abundance was expressed relative to that of the internal reference *TaActin* following the $2^{-\Delta Ct}$ method. Mean and standard error for the relative expression levels were calculated using Microsoft Excel software. A general linearized model analysis of variance (* and ** indicate $P < 0.05$ and 0.01, respectively) was performed using SAS v9.4 software.

more sensitive to BTH treatment only in *wNPR1-OE*, which was consistent with previously reported higher expression of the *TaPR1* gene in a wheat transgenic line overexpressing *AtNPR1* after the BTH treatment (Makandar et al. 2006). A large number of DEGs were identified in our subsequent analysis; especially, most of the BTH-sensitive genes (type II DEGs) seemed to have been induced in an *NPR1*-independent manner (Supplementary Fig. S4; Supplementary Table S4). Based on this evidence, we

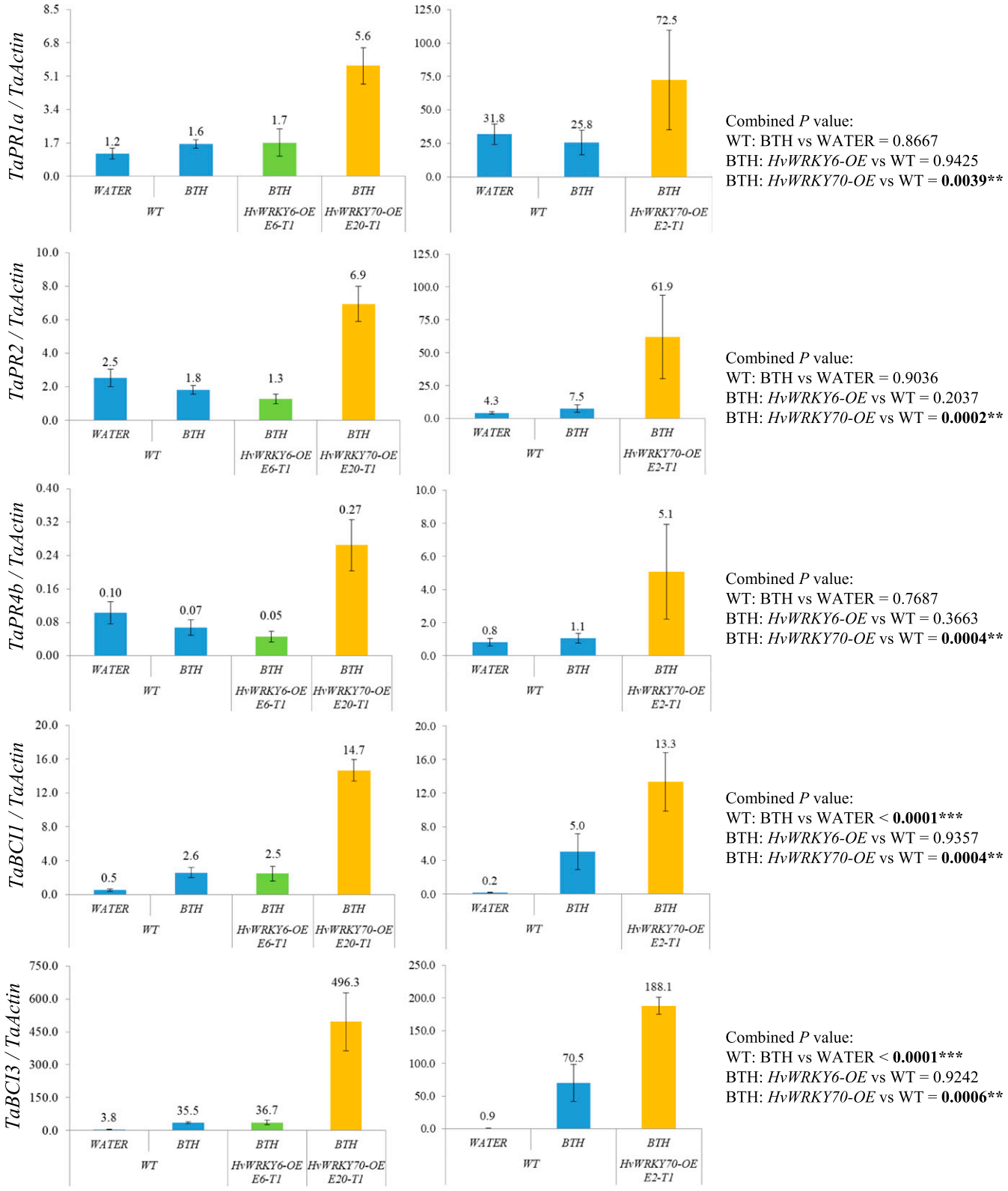

Fig. 5. *Pathogenesis-related* (*PR*) and *barley chemical-induced* (*BCI*) genes became more sensitive to benzothiadiazole (BTH) treatment in *HvWRKY70-OE*. Third leaves of wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE*, as well as wild-type (WT) plants, were sprayed with 0.1 mM BTH. Samples from water-sprayed WT plants served as a control. RNA samples were harvested at 48 h posttreatment. The whole experiment was systemically repeated twice with two independent transgenic lines, and each line included five to seven biological replicates. The relative transcript abundance was expressed relative to that of the internal reference *TaActin* following the $2^{-\Delta Ct}$ method. Mean and standard error for the relative expression levels were calculated using Microsoft Excel software. A general linearized model analysis of variance (* and ** indicate $P < 0.05$ and 0.01, respectively) was performed using SAS v9.4 software.

speculated that *NPR1* and its regulated *PR* genes were partially involved in the BTH-induced transcriptional response in wheat and barley. In *Arabidopsis*, roughly half of the BTH-sensitive genes were elevated in an *NPR1*-dependent manner, some of which were further regulated by *WRKY18* transcription factor (Wang et al. 2006).

WRKY transcription factors have been considered to be among the most important gene families in SAR (Eulgem and Somssich 2007). However, exceedingly few *WRKY* genes controlling either BTH-induced resistance or *NPR1*-mediated acquired resistance in Triticeae crops (wheat and barley) have

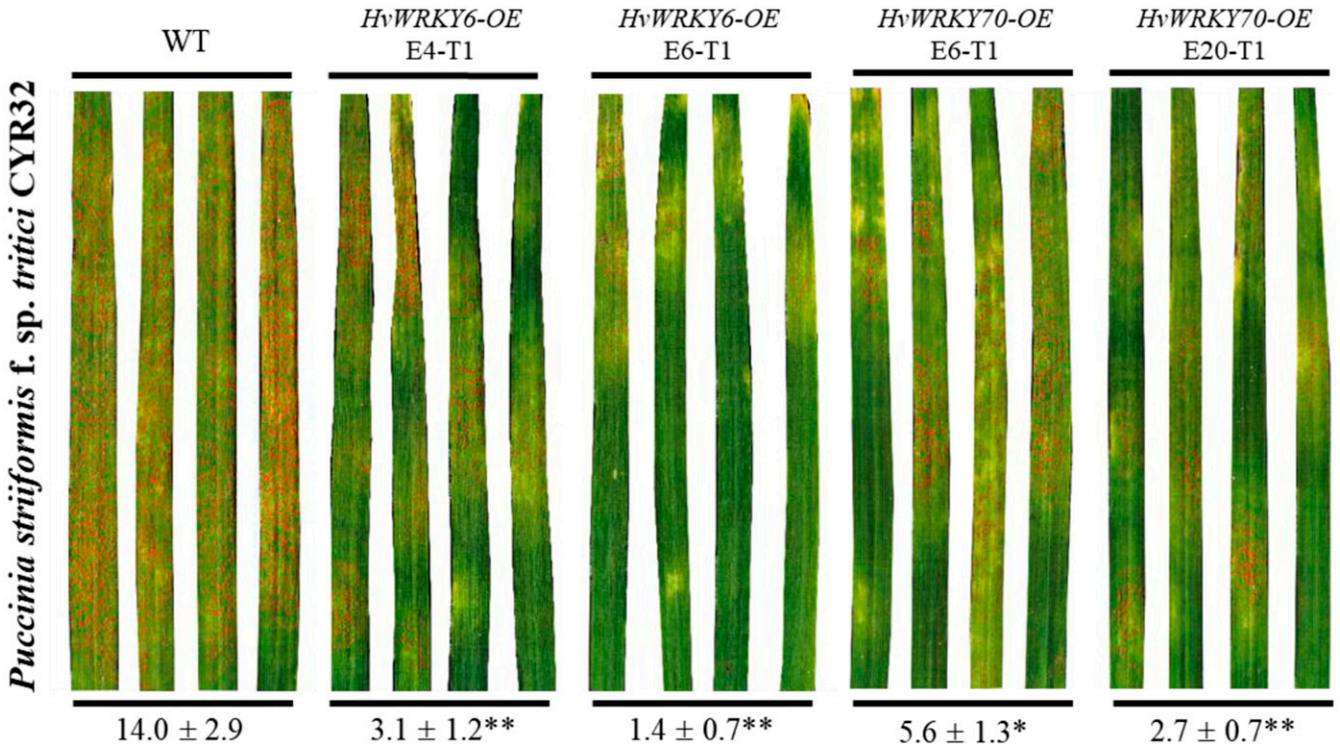

Fig. 6. Wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE* showed enhanced resistance to *Puccinia striiformis* f. sp. *tritici*. The third leaves of *HvWRKY6-OE* and *HvWRKY70-OE*, as well as the wild-type (WT) plants, were spray inoculated with urediniospores of the highly virulent *P. striiformis* f. sp. *tritici* pathotype CYR32. The phenotype of the stripe rust was photographed at 15 days postinfiltration. A susceptible phenotype (*P. striiformis* f. sp. *tritici* sporulation) was exhibited in all of the genotypes. Numbers represent the average proportion of the *P. striiformis* f. sp. *tritici* sporulation area in each of the leaves. Two independent transgenic lines consisting of five to nine biological replicates were employed. Asterisks indicated the significance of the differences between the transgenic lines and the WT plants established using a Dunnett's test (* and ** indicate $P < 0.05$ and 0.01, respectively).

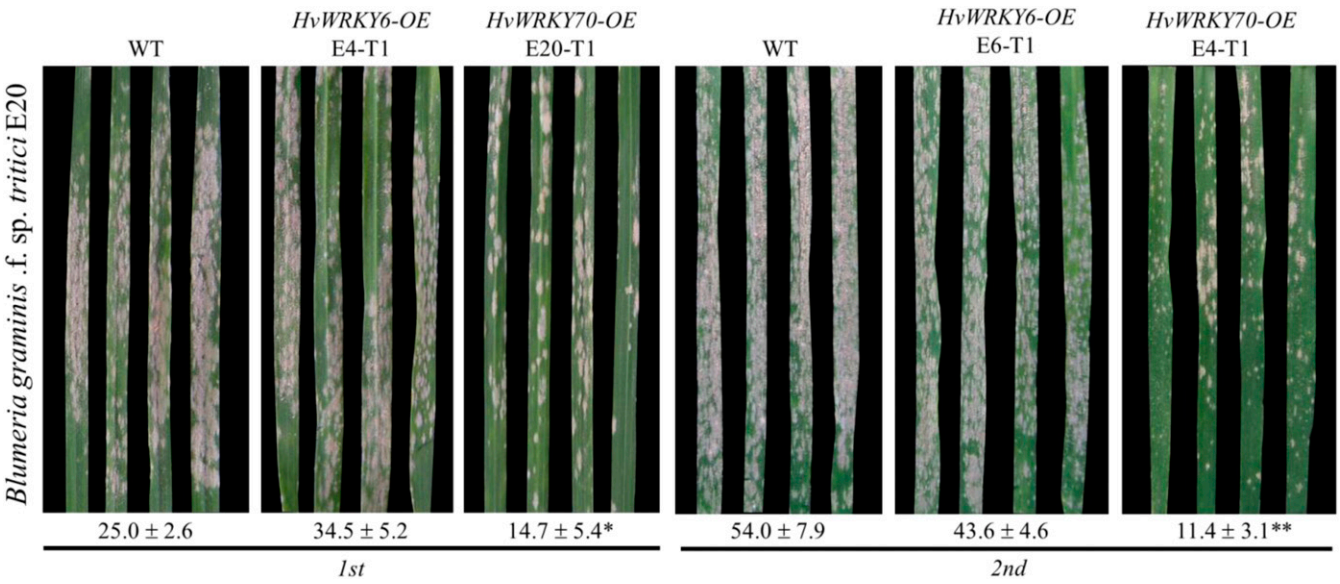

Fig. 7. Wheat transgenic line *HvWRKY70-OE* showed enhanced resistance to *Blumeria graminis* f. sp. *tritici*. The third leaves of the wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE*, as well as the wild-type (WT) plants, were inoculated with conidia spores of the prevalent *B. graminis* f. sp. *tritici* isolate E20. The phenotype of the powdery mildew was photographed at 15 days postinfiltration. A susceptible phenotype (*B. graminis* f. sp. *tritici* sporulation) was observed in all of the genotypes. Numbers represent the average proportion of the *B. graminis* f. sp. *tritici* sporulation area in each of the leaves. Two independent transgenic lines consisting of 9 to 30 biological replicates were employed. A Dunnett's test (* and ** indicate $P < 0.05$ and 0.01, respectively) was performed using SAS v9.4 software.

been identified. In total, 46 and 171 *WRKY* genes were annotated in barley and wheat, respectively (Mangelsen et al. 2008; Ning et al. 2017). To explore the key transcriptional regulators of BTH-induced resistance in these two relative plant species, we profiled the expression patterns of all 46 barley *WRKY* genes in the transcriptome (Fig. 3). Approximately half of the *WRKY* genes were highly induced upon BTH treatment, indicating that a broad range of plant defense was activated by this chemical reagent. In one of our previous investigations, we profiled the regulatory network of *NPR1* during acquired resistance induced by *P. syringae* DC3000 using a sequencing approach similar to the one employed in the current study (Gao et al. 2018). Two barley *WRKY* genes, *MLOC_78461* and *MLOC_66134*, were significantly induced by both BTH treatment and *P. syringae* DC3000 infection, with even higher expressions in barley transgenic line *wNPR1-OE* (Supplementary Fig. S6). We temporally designated these two genes as *HvWRKY6* and *HvWRKY70*, respectively, based on their closest homologs in other plant species (Supplementary Fig. S7). However, because the designating system of the *WRKY* genes seemed to be confused, homologous analysis provided extremely limited information on the functional prediction.

Nevertheless, the generated wheat transgenic lines overexpressing *HvWRKY6* and *HvWRKY70* showed distinct improvements of acquired resistance, BTH-induced resistance, and resistance to biotrophic fungal pathogens of *Puccinia striiformis* f. sp. *tritici* and *B. graminis* f. sp. *tritici*. Although the specific roles of *BCI* genes in plant defense response remain to be characterized, they have been widely considered to be downstream of the BTH treatment and involved in barley resistance to powdery mildew (Beßer et al. 2000; Jansen et al. 2005; Kogel and Langen 2005). Interestingly, all of the tested *PR* and *BCI* genes became more sensitive to BTH treatment only in *HvWRKY70-OE* (Fig. 5). Along with the observed improvement of wheat resistance to *B. graminis* f. sp. *tritici* in transgenic material (Fig. 7), we hypothesized that BTH treatment could activate the *HvWRKY70* pathway, which was positively involved in resistance to powdery mildew by indirectly regulating *PR* and *BCI* genes. On the other hand, the basal resistance of wheat transgenic line *HvWRKY6-OE* to *M. oryzae* was enhanced in association with higher expression of *PR* genes (Fig. 4), which was similar to our previous observations of the barley transgenic line *wNPR1-OE* (Gao et al. 2018). We predicted that *HvWRKY6* was an endogenous SA-responsive gene that acted as a partner of *NPR1* and indirectly regulated *PR* genes during acquired resistance. Moreover, the sporulation of *P. striiformis* f. sp. *tritici* was significantly diminished or delayed in both wheat transgenic lines *HvWRKY6-OE* and *HvWRKY70-OE* (Fig. 6). SA and its responsive genes are considered key components of wheat resistance to rust infections. Recent investigation showed that the endogenous SA level during wheat resistance to stripe rust was significantly elevated accompanying the hypersensitive response triggered by the *YrSu* resistance gene (Wang et al. 2017). Several *PR* genes seemed to be shared downstream genes involved in wheat resistance to various rust diseases (e.g., *Sr13*-mediated high-temperature resistance to stem rust, multiple *Yr* gene-mediated resistances to stripe rust, and the *Lr47*-mediated resistance to leaf rust) (Farrakh et al. 2018; Wu et al. 2019; Zhang et al. 2017). We speculated that wheat resistance to rust infections recruited a broad range of plant defense mechanisms, including both the *HvWRKY6* and *HvWRKY70*-mediated regulatory pathways.

In conclusion, based on the present results and those of our previous studies, we drafted the possible regulatory networks of BTH-induced resistance and acquired resistance in wheat and barley (Fig. 8). Briefly, the *Pseudomonas syringae* DC3000-triggered acquired resistance was controlled by *NPR1* with several features of SAR in model plant *Arabidopsis*, including elevation of endogenous SA and involvement of *PR* genes. For the BTH-induced resistance, a large number of genes were induced by the BTH treatment in an *NPR1*-independent manner. Most of the *PR* genes became sensitive to BTH treatment only in barley transgenic line *wNPR1-OE*. Overexpression of two differentially expressed *WRKY* genes in wheat improved wheat resistance to fungal pathogens, possibly through distinct regulatory pathways. The *HvWRKY70* gene seemed to be the key regulator of BTH-induced resistance, whereas *HvWRKY6* might act as the partner of *NPR1* during acquired resistance.

MATERIALS AND METHODS

Plants, BTH treatment, and pathogen inoculations.

The barley transgenic lines *wNPR1-OE* and *HvNPR1-Kd* were generated in cultivar Golden Promise background in earlier investigations (Dey et al. 2014; Wang et al. 2016). The fully expanded third leaves of barley seedlings were sprayed with 0.1 mM BTH (acibenzolar-S-methyl, CAS number 135158-54-2; Aladdin Co. Ltd., Shanghai, China). RNA samples were harvested from the BTH-sprayed leaves at 2 dpt. Wild-type plants and a mock treatment with water severed as controls. A similar protocol was applied to generate the BTH-induced resistance in wheat plants.

Seedlings of the wheat transgenic lines and wild-type plants were inoculated with stripe rust following a previous described

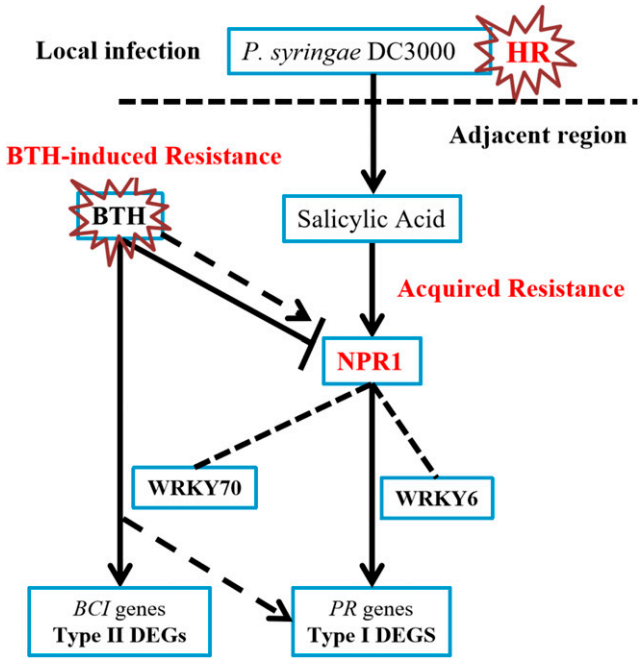

Fig. 8. Predicted regulatory network of benzothiadiazole (BTH)-induced resistance and the *nonexpresser of pathogenesis-related genes 1* (*NPR1*)-mediated acquired resistance. The *Pseudomonas syringae* DC3000-induced acquired resistance was controlled by *NPR1* with several features of systemic acquired resistance in model plant *Arabidopsis*, including elevation of endogenous salicylic acid and involvement of *PR* genes. However, for the BTH-induced resistance, large number of genes, including *barley chemical-induced* (*BCI*) genes, were induced by the BTH treatment in an *NPR1*-independent manner. Most of the *PR* genes became sensitive to BTH treatment only in barley transgenic line overexpressing wheat *wNPR1* (*wNPR1-OE*). Overexpression of two differentially expressed *WRKY* genes was sufficient to improve wheat resistance to fungal pathogens, possibly through different regulatory pathways. The *HvWRKY70* gene seemed to be a key regulator of BTH-induced resistance, whereas *HvWRKY6* might act as a partner of *NPR1* during acquired resistance. HR = hypersensitive response and DEGs = differentially expressed genes.

procedure (Wan and Chen 2014). Briefly, fully expanded third leaves of wheat seedlings were inoculated with urediniospores of highly virulent *Puccinia striiformis* f. sp. *tritici* pathotype CYR32. The inoculated plants were maintained in relatively low-temperature conditions: 10°C for the first 10 h in the dark, followed by 16 h in the light at 16°C and 8 h in the dark at 8°C. The disease symptom of stripe rust was photographed at 15 dpi. The proportion of *P. striiformis* f. sp. *tritici* sporulation area on each of the leaves was measured using ASSESS v2.0 software (Lamari 2008; Zhang et al. 2017). Two independent lines with five to nine biological replicates were used for each of the transgenic materials. A Dunnett's test was performed using SAS software (v9.4; SAS Institute, Cary, NC, U.S.A.).

For the powdery mildew inoculation, a prevalent *B. graminis* f. sp. *tritici* isolate E20 was maintained in the asexual phase in a greenhouse and used for inoculation following the procedure specified in an earlier investigation (Geng et al. 2016). Briefly, seedlings at the third-leaf stage were inoculated with conidia spores and kept in a high-humidity environment at 18 and 12°C (day and night, respectively) with a photoperiod of 14 h of light per day. The phenotype of powdery mildew was photographed at 15 dpi. The proportion of *B. graminis* f. sp. *tritici* sporulation area on each of the leaves was measured using ASSESS software. The whole experiment was systemically repeated twice with two independent lines, and each line included 9 to 30 biological replicates. A Dunnett's test was conducted using SAS v9.4 (SAS Institute).

Acquired resistance in local leaves was induced by *Pseudomonas syringae* DC3000 following a previous description (Colebrook et al. 2012; Gao et al. 2018). Generally, *P. syringae* DC3000 was cultivated in Luria-Bertani liquid medium with rifamycin antibiotic for 2 days. The bacteria were collected by a brief centrifugation and then diluted to an optical density at 600 nm = 0.5 in distilled water. Third leaves of wheat seedlings were infiltrated with bacterial suspensions with a 1-ml needless syringe. The borders of the infiltration area were marked. Mock infiltration with distilled water served as a control. Inoculated seedlings were then transferred into a 23°C growth chamber. The region adjacent to the infiltration area of *P. syringae* DC3000 at 2 dpi was further used for *M. oryzae* inoculation or qRT-PCR assay.

M. oryzae isolate P131 was cultivated on tomato oat medium for 10 days at 25°C under a photoperiod of 16 h of light and 8 h of darkness. The conidia were suspended in 0.05% Tween-20 (5.0×10^5 spores/ml). The region adjacent to the infiltration area (approximately 1 cm from the border) was press injured and further inoculated with 10 µl of the *M. oryzae* conidia suspensions. The inoculated leaves were transferred into a growth chamber at 23°C and 80% humidity under a cycle of 16 h of light and 8 h of darkness. The disease symptom was photographed at 5 days postinoculation and the lesion size of *M. oryzae* on each of the leaves was measured. The whole experiment was systemically repeated twice with two independent lines of *HvWRKY6-OE*, and each line included six to eight biological replicates. A Dunnett's test was conducted using SAS v9.4 (SAS Institute).

RNA extraction and qRT-PCR assay.

Samples were rapidly ground into fine powders in liquid nitrogen. Total RNAs for transcriptome sequencing and qRT-PCR assay were extracted using a QIAGEN plant RNA extraction kit (QIAGEN, Hilden, Germany). The first-strand cDNA was generated using a Takara reverse-transcription kit (Takara, Dalian, China). The barley elongation factor 1a (*HvEF1a*, GenBank accession Z50789) was applied as an internal reference gene. The qRT-PCR primers for *HvPR1b*, *HvPR2*, *HvBCI1*, *HvBCI3*, and *HvBCI7* were derived from our previous study (Supplementary Table S8) (Gao et al. 2018). For qRT-PCR assays performed using wheat samples, *TaActin* (GenBank accession AB181991.1) was applied as an internal reference. The qRT-PCR primers for *TaPR1a*, *TaPR2*, *TaPR4b*, *TaBCI1*, and *TaBCI3* were designed and genome-specific amplification information for these wheat primers was summarized (Supplementary Table S8). Preliminary amplifications in six twofold wheat cDNA dilutions (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) were utilized to determine the efficiency for each pair of the primers. Melting curves for the temperatures from 60 to 94°C were drawn to ensure the specificity. The threshold values (Ct) were recorded by the Roche LightCycler 96 qRT-PCR instrument (Roche, Basel, Switzerland). The relative transcript abundance was calculated following the $2^{-\Delta Ct}$ method (Chen et al. 2014; Schmittgen and Livak 2008). Mean and standard error for the relative expression levels were analyzed using Microsoft Excel software (Microsoft, Redmond, WA, U.S.A.).

RNA-seq and bioinformatics.

KAPA library preparation and transcriptome sequencing were conducted by Novogene Co., Ltd. following the default protocols. The HiSeq 1000 system was utilized for the 12-Gb sequencing for each of the samples. The Ensembl Genomes *H. vulgare* genome (The International Barley Genome Sequencing Consortium 2012) was employed for the transcriptome assembly using TopHat 2.0.8 (Trapnell et al. 2009). Then, the relative abundance for each of the transcripts assembled from mapped reads was determined by HTSeq (Trapnell et al. 2010). Comparing different groups, genes with a false discovery rate-adjusted P value < 0.05 were filtered and designated as DEGs by DESeq2 (Love et al. 2014). Enrichment of GO categories for each of the DEGs were annotated with the GOseq (Young et al. 2010). Heatmaps for the gene expression patterns were generated based on the FPKM values for each of the transcripts by MeV software. The neighbor-joining polygenetic tree was generated by MEGA software using protein sequences aligned using the MUSCLE method.

Subcellular localization of GFP-tagged HvWRKY6 and HvWRKY70 proteins.

The full-length open reading frames (ORF) of *HvWRKY6* and *HvWRKY70* genes were cloned from cDNA synthesized using RNA samples from leaves of barley cultivar Golden Promise (primers in Supplementary Table S8). They were then constructed into both pGWB5 (*35S::gene-GFP*) and pMDC43 (*35S::GFP-gene*) using Gateway LR Clonase II Enzyme (Life Technologies, Carlsbad, CA, U.S.A.). The recombinant constructs were transformed into *Agrobacterium* strain GV3101. Leaves from 4- to 6-week-old *N. benthamiana* plants were infiltrated with transformed *Agrobacterium*. An empty pGWB5 vector expressing only the GFP was used as a control. Green fluorescence was detected 48 h after infiltration using fluorescence microscopy (Ti-2 microscopy with fluorescent accessories; Nikon, Tokyo, Japan).

Wheat transgenic lines overexpressing *HvWRKY6* and *HvWRKY70*.

The full-length ORF of *HvWRKY6* and *HvWRKY70* genes were cloned into the wheat transgenic vector pLGY02 (*Ubi::gene*). The recombinant vectors were then transformed into the *Agrobacterium* strain EHA105. Wheat transgenic lines were generated using wheat embryos of the spring common wheat cultivar JW1 by Jinan Bangdi Bio Co. Ltd., Shandong, China. Transgenic lines were initially validated by amplifying the insertion of transgene in the genomic DNA using a PCR assay (primers in Supplementary Table S8). The expression levels of *HvWRKY6* and *HvWRKY70* transgenes in corresponding wheat transgenic lines were determined by qRT-PCR assay (primers in Supplementary Table S8).

ACKNOWLEDGMENTS

We thank G. Li (Shandong Academy of Agricultural Sciences, China) for excellent support in generating wheat transgenic materials.

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