HvWRKY10, HvWRKY19, and HvWRKY28 Regulate *Mla*-Triggered Immunity and Basal Defense to Barley Powdery Mildew

Yan Meng¹ and Roger P. Wise^{1,2}

¹Department of Plant Pathology and Microbiology, Center for Plant Responses to Environmental Stresses, Iowa State University, Ames, IA 50011–1020, U.S.A.; ²Crop and Insect Genetics, Genomics, and Informatics Research Unit (CIGGIR), United States Department of Agriculture–Agricultural Research Service, Iowa State University

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WRKY proteins represent a large family of transcription factors (TF), involved in plant development and defense. In all, 60 unique barley TF have been annotated that contain the WRKY domain; 26 of these are represented on the Barley1 GeneChip. Time-course expression profiles of these 26 HvWRKY TF were analyzed to investigate their role in mildew locus *a* (*Mla*)-mediated immunity to *Blumeria graminis* f. sp. *hordei*, causal agent of powdery mildew disease. Inoculation-responsive, *Mla*-specified interactions with *B. graminis* f. sp. *hordei* revealed that 12 HvWRKY were differentially expressed: 10 highly upregulated and two significantly downregulated. Barley stripe mosaic virus-induced gene silencing of HvWRKY10, HvWRKY19, and HvWRKY28 compromised resistance-gene-mediated defense to powdery mildew in genotypes harboring both *Rar1*-dependent and *Rar1*-independent *Mla* alleles, indicating that these WRKY TF play key roles in effector-triggered immunity. Comprehensive yeast two-hybrid analyses, however, did not reveal a direct interaction between these three nuclear-localized WRKY TF and *MLA*. Transient overexpression of all three WRKY TF in single cells expressing *Mlo*, which encodes a negative regulator of penetration resistance, significantly decreased susceptibility. Taken together, these loss- and gain-of-function studies demonstrate that HvWRKY10, HvWRKY19, and HvWRKY28 positively regulate the barley transcriptome in response to invasion by *B. graminis* f. sp. *hordei*.

Nucleotide sequence data for HvWRKY48, HvWRKY49, HvWRKY53, HvWRKY55, and HvWRKY61 are available in the GenBank database under accession numbers JQ806389, JQ806390, JQ806391, JQ806392, and JX216840, respectively. Microarray data are available as PLEXdb datasets BB2 (ArrayExpress E-TABM-82) and BB10 (ArrayExpress E-TABM-142).

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Corresponding author: R. P. Wise;
Fax: +1.515.294.9420; E-mail: rpwise@iastate.edu

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Plants have evolved a complex regulatory system to temporally and spatially manipulate response to disease and environmental stress. Transcriptional reprogramming of gene expression is a crucial part of this regulatory process (Chen et al. 2002; Singh et al. 2002). To achieve this, a significant portion of a genome’s capacity is dedicated to the regulation of transcription; for example, over 5% of the *Arabidopsis* (*Arabidopsis thaliana*) genome encodes greater than 1,500 transcription factors (TF) (Riechmann et al. 2000). Plants deploy this vast TF network to rapidly and effectively limit the growth of pathogens by cell death at and surrounding the infection site or to regulate basal defense to avoid unnecessary damage because only harmless or helpful molecules are recognized (Dangl 2007).

The WRKY family of TF comprises 74 members in *Arabidopsis* and 109 members in rice (*Oryza sativa*) (Eulgem and Somssich 2007; Mao et al. 2007). The existence of one or two WRKY domains is the main feature of WRKY proteins, which are approximately 60 amino acids long, containing a conserved motif of WRKYGQK or its variants at the N terminus and a zinc finger motif (CX₄₋₇CX₂₂₋₂₃HXH/C) at the C terminus. Based on the number of WRKY domains and particular features of zinc-finger motif, the WRKY family has been classified into three groups (I, II, and III). Group II has been further divided into five subgroups (IIa to IIe), which are classified according to additional short conserved structural motifs (Wu et al. 2005). To carry out its regulatory function, the WRKY domain generally binds to a W-box in the promoter region of its target gene, a DNA-element with the consensus sequence of (C/T)TGAC(C/T). W-boxes have been found in the promoter or the 5′ untranslated regions of many target genes involved in plant disease defense, abiotic stress, and seed development as well as leaf senescence (Yu et al. 2001).

WRKY proteins play essential roles in the transcriptional regulation of innate immunity. Gain- or loss-of-function experiments illustrate that many WRKY proteins are key regulators of two interdependent branches of the plant immune system: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Bhattarai et al. 2010; Chen et al. 2010; Peng et al. 2008; Xing et al. 2008). In recent years, such studies have been extended from model plants (e.g., *Arabidopsis*) to crop species (Dellagi et al. 2000; Park et al. 2006). A recent study in soybean revealed that three novel WRKY TF (GmWRKY36, GmWRKY40, and GmWRKY45) are involved in regulating *Rpp2*-mediated resistance response to Asian soybean rust *Phakopsora pachyrhizi*. *Rpp2*-mediated resistance was compromised when these

three *WRKY* genes were silenced individually via *Bean pod mottle virus*-induced gene silencing (Pandey et al. 2011). In rice, OsWRKY13 is involved in the regulation of resistance to bacteria blight and the rice blast fungus, *Magnaporthe grisea* (Qiu et al. 2007). Additionally, OsWRKY31 and OsWRKY45 are also implicated in resistance to rice blast (Tao et al. 2009; Zhang et al. 2008a). OsWRKY71 regulates rice resistance to the bacteria pathogen *Xanthomonas oryzae*, and overexpression of OsWRKY71 results in an enhanced resistance response (Liu et al. 2007). Further, tomato SIWRKY72 contributes to basal defense against root-knot nematodes and potato aphids, as well as *Mi-1*-mediated gene-for-gene resistance to these pests (Bhattarai et al. 2010).

In barley (*Hordeum vulgare* L.), mildew-resistance locus *a* (*Mla*) confers isolate-specific resistance to the powdery mildew *Blumeria graminis* f. sp. *hordei*, which requires the recognition of a corresponding effector (avirulence) protein in the cytoplasm. *MLA* was revealed to associate with HvWRKY1 and HvWRKY2, two negative regulators of PAMP-triggered basal defense in the nucleus; during this interaction, WRKY acts as a repressor to regulate resistance against *B. graminis* f. sp. *hordei* (Shen et al. 2007). Hence, this study indicates that *WRKY* genes are involved in mechanistically connecting and balancing PTI and ETI. HvWRKY1, also designated HvWRKY38 (Marè et al. 2004), likewise functions in transcriptional reprogramming of barley genes against cold and drought stresses, indicating its regulatory role in barley abiotic stress response.

Aside from HvWRKY1 and HvWRKY2, the functions of other *WRKY* genes in barley and powdery mildew interactions remain unclear. Bioinformatic data mining has shown that transcript accumulation of numerous HvWRKY genes are altered upon *B. graminis* f. sp. *hordei* infection (Mangelsen et al. 2008). However, to our knowledge, no detailed functional analysis has been published. In this report, we performed a comprehensive functional analysis of WRKY TF in barley. Loss- and gain-of-function studies indicate that HvWRKY10, HvWRKY19, and HvWRKY28 act as positive regulators in a multifaceted network to fine-tune the response of barley to *B. graminis* f. sp. *hordei* invasion.

RESULTS

Informatic identification of HvWRKY TF.

Based on early expressed sequence tag (EST) collections (Close et al. 2004), initial computational analysis identified 45 HvWRKY genes (Mangelsen et al. 2008). In recent years, however, sequencing of the 5.3-gigabase barley genome has made rapid progress (Mayer et al. 2011; Schulte et al. 2009); therefore, we undertook an effort to identify a more complete

HvWRKY gene family. Using the rice annotations described by Xie and associates (2005), we first used tBLASTn to interrogate all OsWRKY TF against HarvEST Barley Assembly 21, which contains the barley EST contigs used for probe set selection for the Barley1 GeneChip array (Close et al. 2004). In total, 74 nonredundant alignments were identified, of which 45 sequences represented 35 previously annotated HvWRKY genes (Mangelsen et al. 2008). The remaining 29 sequences were then further compared with the newly published 24,783 barley full-length cDNAs (Matsumoto et al. 2011) to obtain extended sequence information. As a result, 19 sequences were removed, because they did not include the WRKY signature domain. The 10 novel HvWRKY proteins all contain the conserved heptapeptide WRKYQGK or a slight variation, WKKYGQK. Additionally, these 10 additional WRKY proteins also possess the unique zinc-finger motif (CX₄₋₇CX₂₂₋₂₃HXH/C), which is the second characteristic feature of WRKY proteins. These HvWRKY proteins were designated HvWRKY47 to HvWRKY56, respectively.

Similar strategies were used to compare the OsWRKY sequences to the more comprehensive HarvEST Barley Assembly 36 to complete the available HvWRKY gene set, beyond what was represented on the Barley1 GeneChip. Five additional HvWRKY proteins, HvWRKY57 to HvWRKY61, were identified that contained the conserved heptapeptide WRKY domain and the zinc-finger motif. HvWRKY47 to HvWRKY61 are presented in Table 1, along with their classifications into different groups or subgroups, and aligned to their predicted orthologs in the model monocot rice and dicot *Arabidopsis*.

Experimental concept and design: Identification of *B. graminis* f. sp. *hordei*-responsive HvWRKY TF.

In most cases, transcript accumulation will be affected by particular biotic and abiotic stresses, and the response pattern can be utilized to predict its functional contribution to the interaction (Caldo et al. 2004; Chen et al. 2002; Dong et al. 2003; Ramamoorthy et al. 2008; Ryu et al. 2006). We utilized a GeneChip-based approach, combined with a comprehensive set of fast neutron- and ethyl methane sulfonate-derived loss-of-function *m1a* mutants (Meng et al. 2009; Moscou et al. 2011; Xi et al. 2009; Zhou et al. 2001) to aid in the identification of HvWRKY genes that are differentially expressed in response to powdery mildew. Homozygous *m1a* mutants manifest in a partially or fully susceptible phenotype to *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{al}*, *AVR_{ab6}*, and *AVR_{al2}*), however, display an otherwise normal growth habit.

In all, 26 of the 60 putative HvWRKY TF are represented by probe sets on the 22K Barley1 GeneChip (Table 2), including four of the newly annotated HvWRKY TF listed in Table 1.

Table 1. Newly identified HvWRKY transcription factors and their putative orthologs in *Arabidopsis* and rice

Gene number	Accession number	WRKY group	AtWRKY ortholog	OsWRKY ortholog ^a
HvWRKY47	AK364800	IIb	6, 42	99
HvWRKY48	JQ806389	IIc	71	60
HvWRKY49	JQ806390	III	70	64
HvWRKY50	AK357671	I	33	24
HvWRKY51	AK366837	IIc	71	3
HvWRKY52	AK368514	IIa	18, 40, 60	71
HvWRKY53	JQ806391	III	67	22
HvWRKY54	AK368994	IIe	35	37
HvWRKY55	JQ806392	III	30	19
HvWRKY56	AK374747	IId	74	*
HvWRKY57	AK250089	I	4	1
HvWRKY58	AK248640	IId	39, 74	*
HvWRKY59	AK376765	I	2	*
HvWRKY60	AK248555	IIb	72	73
HvWRKY61	JX216840	I	3, 4	1

^a Asterisk (*) indicates that ortholog could not be assigned with significance.

We utilized these probe sets to interrogate Barley1 GeneChip datasets BB2 (ArrayExpress E-TABM-82) and BB10 (ArrayExpress E-TABM-142) (Caldo et al. 2006; Meng et al. 2009; Moscou et al. 2011) in PLEXdb (Dash et al. 2012). In the experiments represented by these two datasets, RNA was extracted from 7-day-old seedling first leaves (PO:0007094) harvested at 0, 8, 16, 20, 24, and 32 h after inoculation (HAI) with *B. graminis* f. p. *hordei* isolate 5874 (*AVR_{ab}*) (Moscou et al. 2011). Non-inoculated plants at these six time points were used as controls. The analysis presented here is based on six genotypes × six time points × two inoculation treatments × three biological replications for a total of 216 GeneChip hybridizations. False discovery rate (FDR) was calculated using the histogram-based technique as described by Nettleton and colleagues (2006). Using an FDR of 0.01%, 12 of these HvWRKY probe sets exhibited significant differential expression between inoculated (INOC) and noninoculated (NON-INOC) plants for at least one time point among wild-type (CI 16137 [*Mla1*], CI 16151 [*Mla6*], and Sultan-5 [*Mla12*]) and loss-of-function (*m1a1-m508*, *m1a6-m9472*, and *m1a12-m66*) mutants.

In total, 10 genes were highly induced upon pathogen infection (Figs. 1 and 2) whereas two genes displayed reduced transcript accumulation when compared with the NON-INOC control. Transcript accumulation of genes represented by Contig 4386_at, Contig 23697_at, Contig 21110_at, Contig 12005_at, Contig 10167_at, Contig 10168_at, Contig 4387_at, and Contig 16040_at displayed the largest differential expression at 16 HAI, the initial time point for establishment of the periaustorial interface between fungal haustoria and host epidermal cells (Caldo et al. 2004). However, transcript accumulation represented by Contig 7517_at and Contig 15957_at steadily increased from 20 to 32 HAI, suggesting that different WRKY TF might function at different stages of the defense response (Ryu et al. 2006). All 12 of these genes exhibited expression patterns in the *m1a1-m508*, *m1a6-m9472*, and *m1a12-m66* loss-

of-function mutants similar to those in lines containing *Mla1*, *Mla6*, and *Mla12* wild-type alleles, respectively (data can be visualized at the PLEXdb website). Among the rice homologues of these 12 predicted HvWRKY genes, three genes (Contig 7243_at, Contig 12033_at, and Contig 23697_at) belong to group I, six genes (Contig 4386_at, Contig 4387_at, Contig 7517_at, Contig 10167_at, Contig 10168_at, and Contig 16040_at) belong to group II, and three genes (Contig 12005_at, Contig 15957_at, and Contig 21110_at) belong to group III (Table 2). In contrast, the remaining 14 HvWRKY genes showed little change in transcript levels.

HvWRKY10, HvWRKY19, and HvWRKY28 are required for *Mla*-mediated resistance to *B. graminis* f. sp. *hordei*.

Next, we were interested to test whether the 12 differentially expressed HvWRKY genes are phenotypically associated with *Mla*-specified resistance. We utilized a DNA-bombardment-mediated *Barley stripe mosaic virus* (BSMV) gene-silencing system to analyze these HvWRKY genes in response to powdery mildew (Meng et al. 2009). GenBank accession numbers and primer sequences are shown in Supplementary Table S1. We designed primers to target unique regions of each gene, as well as the highly conserved WRKY domain. However, to prevent off-target silencing, the sequence-specific region from each gene was used for the functional analysis presented here. Inserts were amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) and cloned into the *PacI* and *NorI* sites downstream of the γ b stop codon in the BSMV: γ subgenome, driven by the *Cauliflower mosaic virus* 35S promoter (Meng et al. 2009). Constructs were co-bombarded with BSMV: α and BSMV: β into 7-day-old barley 'Black Hull-less' (CIho 2277) seedlings, which are susceptible to BSMV infection. Approximately 7 days after bombardment, typical infected first leaves display brown striping adjacent to the middle vein, and the second leaf will exhibit stripe mosaic or necrotic symp-

Table 2. Total of 26 probe sets representing HvWRKY transcription factors on the Barley1 GeneChip

Gene number	WRKY group	Barley1 GeneChip probe set ID	AtWRKY ortholog ^a	OsWRKY ortholog ^a
HvWRKY46 ^b	I	Contig7243_at	20	78
HvWRKY41	I	Contig12033_at	*	*
HvWRKY6	I	Contig14308_at	1	82
HvWRKY42	I	Contig15657_at	2	80
HvWRKY50 ^c	I	Contig23697_at	33	24
HvWRKY1	IIa	Contig4386_at	18, 40, 60	28, 71
HvWRKY2 ^d	IIa	Contig4387_at	18, 40, 60	28, 71
HvWRKY52 ^c	IIa	EBro02_SQ004_H10_at	18, 40, 60	71
HvWRKY47 ^c	IIb	Contig7517_at	6, 42	99
HvWRKY19	IIc	Contig10167_at	50	7
HvWRKY20	IIc	Contig10168_at	50	7
HvWRKY13	IIc	Contig13268_at	56	23
HvWRKY5	IIc	Contig18462_at	50	77
HvWRKY7	IId	Contig7798_at	11, 17	68
HvWRKY10	IId	Contig16040_at	*	51
HvWRKY9	IId	Contig22226_at	7	*
HvWRKY8	IId	Contig23011_at	39, 74	*
HvWRKY39	IId	Contig13375_at	65	13
HvWRKY45	IId	HVSMEa0001M23r2_at	69	14
HvWRKY54 ^c	IId	EBro08_SQ008_D15_at	35	37
HvWRKY34	III	Contig10471_at	*	46
HvWRKY28	III	Contig12005_at	30, 41, 53	74
HvWRKY33	III	Contig15869_at	*	46
HvWRKY32 ^c	III	Contig15957_at	54, 70	45
HvWRKY4	III	Contig20450_at	54, 70	47
HvWRKY30	III	Contig21110_at	30, 41, 53	69

^a Adapted from Mangelsen and colleagues (2008), except the newly identified HvWRKY47, HvWRKY50, HvWRKY52, and HvWRKY54. Asterisk (*) indicates that ortholog could not be assigned with significance.

^b Additional Barley1 probe set that represents this gene is rbaal15j13_s_at.

^c Newly annotated HvWRKY TFs in this study.

^d Additional Barley1 probe sets that represent this gene are HB25K10r_at and HB25K10r_s_at.

^e Additional Barley1 probe set that represents this gene is S0001000055P18F1_s_at.

toms. Accordingly, we utilized second leaf sap, which harbors the amplified recombinant virions, to mechanically infect 7-day-old seedlings of CI 16137 (*Mla1*; incompatible), CI 16151 (*Mla6*; incompatible), and Sultan5 (*Mla12*; incompatible). These three genotypes were also susceptible to BSMV infection, although slightly less than the original incubator, Black Hull-less. After 12 days of silencing, plants were then inoculated with *B. graminis* f. sp. *hordei* 5874 (*AVR_{al}*, *AVR_{ab}*, *AVR_{al2}*, and *avr_{al3}*). At least three independent biological replications were performed for each of the 12 candidate HvWRKY TF (discussed below).

After challenge with *B. graminis* f. sp. *hordei* 5874, we assayed the relative penetration index by fixing and staining the leaves, followed by recording the number of secondary hyphae (as an indicator of functional haustoria) in each treatment (Hu et al. 2009). At 4 days after inoculation (DAI), a significant increase in the number of secondary hyphae was observed for three of the 12 constructs (BSMV:WRKY10, BSMV:WRKY19, and BSMV:WRKY28) compared with BSMV:00 and non-BSMV-inoculated control plants (mock) for all three genotypes (Fig. 3; Table 3). CI 16137 (*Mla1*) and Sultan-5 (*Mla12*) backgrounds were more difficult to quantify due to the vigorous growth of mycelia tangling together on silenced leaves. We further tested silencing of HvWRKY10, HvWRKY19, and HvWRKY28 in a compatible interaction when Clansman (*Mla13*) was used as a host; however, no significant differences

were seen in the *Mla13*–*avr_{al3}* specified interactions. Therefore, based on the experiments presented here, HvWRKY10, HvWRKY19, and HvWRKY28 appear to act as positive regulators in defense signaling to *B. graminis* f. sp. *hordei*.

We also documented whether conidiation occurred on second or third leaves of silenced plants at 7 DAI. On CI 16151 (*Mla6*) and CI 16137 (*Mla1*) backgrounds, colonization of *B. graminis* f. sp. *hordei* was not detected on the leaf surface of either WRKY-gene silenced plants or the BSMV:00 control plants whereas, on Sultan-5 (*Mla12*) plant leaves, a few colonies were observed on both silenced plants and control plants. In general, the *Mla6* and *Mla1* alleles confer rapid and absolute resistance, whereas *Mla12* mediates intermediate resistance to *B. graminis* f. sp. *hordei* isolate 5874. Therefore, we assume that the sporulation on Sultan-5 was not due to silencing of HvWRKY10, HvWRKY19, and HvWRKY28. Thus, these HvWRKY TF likely influence the early stages of *B. graminis* f. sp. *hordei* proliferation in host plants, because silencing in an *Mla*-specified incompatible interaction triggers haustoria formation and growth of elongated secondary hyphae but not the subsequent colonization on the leaf surface. These observations are consistent with results previously observed for BSMV-induced silencing of HvCS (*Chorismate synthase*), HvAsa2 (*Anthranilate synthase 2*), and HvCMI (*Chorismate mutase1*) (Hu et al. 2009). The observed expression profiles of HvWRKY10, HvWRKY19, and HvWRKY28 also correlate with

Fig. 1. Time-course expression profiles of 26 predicted barley WRKY transcription factors in barley–*Blumeria graminis* f. sp. *hordei* interactions. Data displayed are derived from PLEXdb accession BB10 (shown in the left four panels) and BB2 (shown in the right two panels) (Caldo et al. 2006; Meng et al. 2009; Moscou et al. 2011). Barley plants were either inoculated with *B. graminis* f. sp. *hordei* 5874 (INOC) or without inoculation (NON-INOC). Natural log values of the mean signal intensities at each time point in CI 16137, CI 16151, and Sultan-5 were used in the cluster analysis. A data matrix was constructed with genes represented by corresponding probe sets in rows and time points of all barley genotype–*B. graminis* f. sp. *hordei* treatment combinations in columns. A Pearson correlation was used to measure similarities of transcript accumulation. Hierarchical clustering was performed with HCE 3.5 software. Probe sets marked with asterisks represent the differentially expressed genes after challenge with *B. graminis* f. sp. *hordei* (in all three genotypes); these genes were used for *Barley stripe mosaic virus* virus-induced gene silencing in this study.

Fig. 2. Reciprocal expression profiles of 12 differentially expressed barley WRKY transcription factors in different genotype–*Blumeria graminis* f. sp. *hordei* combinations. The natural logarithm of signal intensities in CI 16137 (*Mla1*), CI 16151 (*Mla6*), and Sultan-5 (*Mla12*) with (solid line) or without (dashed line) *B. graminis* f. sp. *hordei* isolate 5874 treatment were plotted in graphs. Standard errors were calculated based on the mixed linear model used for analysis.

these data, in that transcripts were highly induced up to 16 HAI (Figs. 1 and 2), an early time point coinciding with conidiospore germination and appressorial germ tube growth.

To monitor the level of target gene silencing, relative transcript accumulation for HvWRKY10, HvWRKY19, and HvWRKY28 were assayed via quantitative (q)RT-PCR. Third

leaves (same stage as for phenotyping) of BSMV-treated plants were sampled at 24 HAI with *B. graminis* f. sp. *hordei* and RNA was isolated. Barley *Actin* mRNA was used as an internal quantitative control for all samples. A significant reduction in target transcripts was observed in infected leaves as compared with BSMV:00 control plants in all three genotypes

Fig. 3. Infection structures for *Blumeria graminis* f. sp. *hordei* in *Barley stripe mosaic virus* (BSMV) virus-induced gene silencing (VIGS) and control barley leaves. CI 16137 (*Mla1*), CI 16151 (*Mla6*), and Sultan-5 (*Mla12*) plants infected with BSMV-VIGS constructs and inoculated with *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{a1}*, *AVR_{a6}*, and *AVR_{a12}*). Barley leaves were fixed at 96 h after inoculation (HAI). Mock was included as a non-BSMV-infected control and BSMV:00 designates the BSMV empty vector control; BSMV-VIGS constructs and controls are designated at the left lane of each row of the pictures. Horizontal bars (inset) represent 100 μm.

Table 3. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) in CI 16151 (*Mla6*) treated with *Barley stripe mosaic virus* (BSMV) constructs

Construct	Total spores ^a	With ESH ^b	PE ^c	<i>P</i> value ^d
BSMV:00	1,012	3	0.30	n.a.
BSMV:HvWRKY10	843	126	14.95	<0.001
BSMV:HvWRKY19	954	67	7.02	<0.001
BSMV:HvWRKY28	895	106	11.84	<0.001

^a Total spores in the silenced region indicate the combined total of three independent experiments.
^b ESH = elongated secondary hyphae.
^c PE is shown as the mean of the PE from three independent replications.
^d *P* values (BSMV:00 versus others) were obtained using a generalized linear mixed model to test for a significant difference of the PE by comparing the test constructs versus the BSMV:00 control. A *P* value < 0.05 indicates that the PE are significantly different; n.a. = not applicable.

(Fig. 4). qRT-PCR was not performed for the nine WRKY proteins that did not produce a hyphal growth phenotype.

Subcellular localization

of HvWRKY10, HvWRKY19, and HvWRKY28.

As putative TF, HvWRKY10, HvWRKY19, and HvWRKY28 are likely to be localized in the nucleus. To test this, we fused

Fig. 4. Barley stripe mosaic virus (BSMV) virus-induced gene silencing-mediated degradation as evidenced by quantitative reverse-transcriptase polymerase chain reaction analysis on transcript accumulation of **A**, HvWRKY10; **B**, HvWRKY19; and **C**, HvWRKY28. RNA isolated from silenced third leaves of barley CI 16137 (*Mla1*), CI 16151 (*Mla6*), and Sultan-5 (*Mla12*)-treated BSMV:00 and BSMV silencing constructs. The amount of RNA in each reaction was normalized using primers specific for *Actin*. The $2^{-\Delta CT}$ method was used to calculate the target gene expression for each individual silencing construct- as compared with BSMV:00-treated plants (Schmittgen and Livak 2008). Fold change due to silencing is calculated by dividing the expression mean value for the targeted WRKY gene in silenced plants by the mean value measured in BSMV:00-treated plants.

the HvWRKY10, HvWRKY19, and HvWRKY28 open reading frames (ORF) with *Aequorea victoria* green fluorescent protein (GFP), and the maize ubiquitin promoter was used to drive the expression of the fusion proteins. Constructs were individually bombarded into barley epidermal cells. All three transiently expressed fusion proteins localized exclusively to the nuclei of barley epidermal cells (Fig. 5). By contrast, the GFP protein was found in both the nucleus and cytoplasm due to its small size (Grebenok et al. 1997). Nuclear localization of the three WRKY protein supports their roles as transcriptional regulators.

Yeast two-hybrid interactions with MLA.

HvWRKY10, HvWRKY19, and HvWRKY28 were also highly expressed after *B. graminis* f. sp. *hordei* infection on *Mla* mutants *m1a1-m508*, *m1a6-m9472*, and *m1a12-m66* (Fig. 6). In the absence of corresponding resistance (*R*) gene, these three mutants are susceptible to *B. graminis* f. sp. *hordei* isolate 5874. This may suggest that these three HvWRKY genes function in an *R*-gene-independent manner (i.e., they might not be regulated directly by *Mla*). To test this hypothesis, we checked for potential interactions between HvWRKY10, HvWRKY19, and HvWRKY28 and the three *Mla* alleles using the Gal4 yeast co-transformation system (Clontech, Mountain View, CA, U.S.A.). HvWRKY10, HvWRKY19, and HvWRKY28 were cloned into the prey vector and *Mla1*, *Mla6*, and *Mla12* were cloned into the bait vector appropriately, and each pair of plasmids was co-transformed into yeast strain Y2HGold. HvWRKY10, HvWRKY19, and HvWRKY28 were also co-transformed with constructs encoding the *MLA1*, *MLA6*, and *MLA12* coiled coil (CC; amino acids 1 to 46) and CC-nucleotide binding (CC-NB; amino acids 1 to 578) domains. Specific interactions were observed in control tests with HvWRKY1 and *MLA* CC domain, consistent with Shen and associates (2007). However, in side-by-side assessments with the HvWRKY1 and *MLA* CC controls, no interaction was detected among HvWRKY10, HvWRKY19, and HvWRKY28 prey constructs and *MLA*, *MLA* CC, or *MLA* CC-NB encod-

Fig. 5. Subcellular localization of HvWRKY10, HvWRKY19, and HvWRKY28. HvWRKY10, HvWRKY19, and HvWRKY28 were independently fused with green fluorescent protein (GFP). Chimeric proteins localized exclusively to the nucleus of barley epidermal cells whereas GFP alone was observed in both the nucleus and the cytoplasm due to its small size. Horizontal bars (inset) represent 100 μm.

ing baits in three independent experiments (Fig. 7). These results suggest that HvWRKY10, HvWRKY19, and HvWRKY28 either do not directly interact with MLA1, MLA6, or MLA12 or that, alternatively, these genes and gene fragments do not function correctly in a yeast two-hybrid system.

HvWRKY10, HvWRKY19, and HvWRKY28 are required for *mlo*-mediated basal defense.

The barley *Mlo* gene encodes a seven-transmembrane protein and acts as a negative defense regulator in barley-*B. graminis* f. sp. *hordei* interactions (Büschges et al. 1997). Loss-of-function *mlo* derivatives mediate a broad-spectrum resistance to *B. graminis* f. sp. *hordei* (Jørgensen 1992). Thus, barley MLO is required by powdery mildew fungi to infect host plants; this suggests that it might be recruited by the pathogen to suppress basal defense responses (Panstruga 2005).

We overexpressed HvWRKY10, HvWRKY19, and HvWRKY28 in barley epidermal cells via an established single-cell-transient assay (Hu et al. 2009; Shirasu et al. 1999). The full-length HvWRKY10, HvWRKY19, and HvWRKY28 ORF were cloned into the vector pUbi:Nos to create the expression constructs pUbi:HvWRKY10, pUbi:HvWRKY19, and pUbi:HvWRKY28. Individual WRKY constructs were co-bombarded with the V26UMUG vector, which encodes both the β -glucuronidase (GUS) and *Mlo* genes, into epidermal cells of barley *mlo*-5 BC7 Ingrid seedlings and, subsequently, challenged with *B. graminis* f. sp. *hordei* isolate 5874. Control bombardments were performed with the V26UMUG reporter construct alone. In this

test, GUS-marked epidermal cells are transformed to susceptibility as a result of transient wild-type *Mlo* expression, whereas surrounding nontransfected cells retain *mlo*-5 resistance. Fungal penetration efficiency (PE) was calculated as the ratio of GUS-marked cells exhibiting elongating secondary hyphae (ESH) to the total transformed cells attacked by *B. graminis* f. sp. *hordei* (Halterman and Wise 2004; Hu et al. 2009).

Transient overexpression of pUbi:HvWRKY10, pUbi:HvWRKY19, and pUbi:HvWRKY28 significantly decreased fungal PE compared with the V26UMUG (GUS-*Mlo*) control (*P* value < 0.001) (Table 4). Co-expression of *Mlo* and WRKY genes in *mlo*-5 compromised fungal colony growth in *Mlo*-transformed cells, suggesting that pUbi:HvWRKY10, pUbi:HvWRKY19, and pUbi:HvWRKY28 also play a role in barley basal defense response to *B. graminis* f. sp. *hordei*.

DISCUSSION

Regulation of barley defense to *B. graminis* f. sp. *hordei*.

WRKY proteins comprise a superfamily of TF implicated in regulating various physiological processes, including defense response to pathogens (Eulgem 2006; Eulgem and Somssich 2007; Eulgem et al. 2000; Rushton et al. 2010; Singh et al. 2002; Zhang and Wang 2005). Here, we screened 26 barley HvWRKY TF for their roles in a defense network to *B. graminis* f. sp. *hordei*, the causal agent of barley powdery mildew. Among them, silencing constructs targeting HvWRKY10, HvWRKY19, and HvWRKY28 resulted in *Mla* resistance-break-

Fig. 6. Reciprocal expression profiles of three differentially expressed barley WRKY transcription factors in various genotype-*Blumeria graminis* f. sp. *hordei* treatment combinations. The natural logarithm of signal intensities in CI 16137 (*Mla1*), CI 16151 (*Mla6*), and Sultan-5 (*Mla12*) and their mutants with (solid line) or without (dashed line) *B. graminis* f. sp. *hordei* isolate 5874 treatment were plotted in graphs. Standard errors were calculated based on the mixed linear model used for analysis.

ing phenotypes; consistently, overexpression of these genes suppressed *Mlo*-mediated susceptibility.

HvWRKY10, HvWRKY19, and HvWRKY28 belong to three different WRKY groups. HvWRKY10 is in the IIc subgroup and has been shown to interact with calmodulin (CaM), a ubiquitous Ca^{2+} -binding protein regulating diverse cellular functions by amending the activity of various target proteins in *Arabidopsis* (Park et al. 2005). HvWRKY19 is a member of the IIc subgroup while HvWRKY28 fits in group III (Table 2). In *Arabidopsis*, the majority of the WRKY group III members are responsive to both pathogen infection and salicylic acid stimulation (Kalde et al. 2003). The observation that HvWRKY10, HvWRKY19, and HvWRKY28 belong to different subgroups may point to their roles in different plant processes (Eulgem et al. 2000). Nevertheless, results reported here suggest that all three have similar roles as activators in reprogramming the defense-signaling pathway.

A potential obstacle with gene silencing is that genes sharing identity for as few as 11 contiguous nucleotides may be unintentionally silenced (Jackson et al. 2003). To avoid off-target silencing, inserts in the silencing constructs were designed on the gene specific region. Pairwise comparison of the nucleotide sequences of HvWRKY10, HvWRKY19, and

HvWRKY28 showed that they do not share longer than 6-bp stretches of identity, which renders off-target silencing unlikely (Xu et al. 2006). Further analysis via comparative RNA gel blot analysis also revealed no RNA degradation for the off-target WRKY transcripts. For example, if HvWRKY10 was silenced as the target gene, HvWRKY19 and HvWRKY28 remained intact. This was consistent for all three combinations (data not shown).

In addition to HvWRKY10, HvWRKY19, and HvWRKY28, nine HvWRKY genes are also differentially expressed upon *B. graminis* f. sp. *hordei* infection (Figs. 1 and 2). However, these plants retained a wild-type phenotype after silencing. We hypothesize that these genes might be co-expressed with other HvWRKY genes and act in a co-regulatory networks (Berri et al. 2009); in this case, silencing of one specific gene will have only a small effects on the resistance of the plants. Nevertheless, HvWRKY10, HvWRKY19, and HvWRKY28 might occupy the pivotal branch points on the defense signaling pathway and work autonomously as defense regulators.

Layers of the plant immune system.

Two interdependent branches of the plant immune system, PTI and ETI, sense different protein receptors and trigger subsequent defense signaling networks in response to microbes (Jones and Dangl 2006). ETI is stronger and faster than PTI, when employed by the plant to counter specific effector proteins, by means of a corresponding *R*-gene-mediated defense-signaling pathway. However, PTI and ETI seem to share common downstream signaling machinery. A largely overlapping set of plant cellular responses is triggered in both branches, including transcriptional reprogramming of gene expression (Caldo et al. 2004; Tsuda and Katagiri 2010). During this process, WRKY TF have been demonstrated to be involved in regulating both basal and *R*-gene-mediated defense response against pathogens (Bhattarai et al. 2010; Caldo et al. 2004; Kishor et al. 2010; Knoth et al. 2007; Peng et al. 2008).

Our interpretation of HvWRKY10, HvWRKY19, and HvWRKY28 function is illustrated in Figure 8. Silencing of HvWRKY10, HvWRKY19, and HvWRKY28 creates a threshold by which recognition of certain defense proteins is inhibited, thus lessening the output of an *R*-gene-mediated defense response. This would result in effector-triggered susceptibility to *B. graminis* f. sp. *hordei*. Unlike HvWRKY1 and HvWRKY2, HvWRKY10, HvWRKY19, and HvWRKY28 have no physical interaction with MLA (Shen et al. 2007). How they are associated with MLA to activate the plant immune system remains unresolved. Moreover, overexpression of HvWRKY10, HvWRKY19, and HvWRKY28 in lines carrying *Mlo*, which encodes a negative regulator of penetration resistance, significantly reduced plant susceptibility. In several European countries, *mlo*-mediated broad-spectrum resistance, which is effec-

Fig. 7. Results of yeast two-hybrid assays between bait fusion-containing MLA1/6/12, MLACC, and MLA1/6/12 coiled-coil nucleotide-binding (CCNB) domains and prey fusion-comprising HvWRKY1, HvWRKY10, HvWRKY19, and HvWRKY28. Positive interaction between MLA CC (1-46) and HvWRKY1 (control) shown by α -galactosidase (α -Gal) activity (blue color) on selective media (SD/-Leu/-Trp/X- α -Gal/AbA). No positive interactions were observed from all the other combinations (white colonies); n.d. = not determined.

Table 4. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) with overexpression of HvWRKY10, HvWRKY19, HvWRKY28 and β -glucuronidase (GUS)-*Mildew resistance locus o* (*Mlo*) in *mlo*-5 plants

Construct	Number of cells ^a		PE ^d	P value ^e
	Attacked ^b	With ESH ^c		
GUS- <i>Mlo</i> ^f	698	463	66.33	n.a
GUS- <i>Mlo</i> + HvWRKY10	951	482	50.68	<0.001
GUS- <i>Mlo</i> + HvWRKY19	1,669	729	43.68	<0.001
GUS- <i>Mlo</i> + HvWRKY28	1,106	551	49.82	<0.001

^a Raw numbers indicate the combined total of three independent experiments.

^b Attacked with *B. graminis* f. sp. *hordei*.

^c ESH = elongated secondary hyphae.

^d PE is shown as the mean of the PE from three independent replications.

^e P values (GUS-*Mlo* versus others) were obtained using a generalized linear mixed model to test for a significant difference of the PE by comparing the test constructs versus the GUS-*Mlo* control. A P value < 0.05 indicates that the PE are significantly different; n.a. = not applicable.

^f Control GUS-*Mlo* construct was co-bombarded with the overexpression empty vector to obtain a more equivalence with other treatments.

tive against all isolates of *B. graminis* f. sp. *hordei*, has been cultivated extensively (Jørgensen 1992). In the absence of gene-for-gene interactions, overexpression of HvWRKY10, HvWRKY19, and HvWRKY28 can still affect Mlo-mediated susceptibility. This implies that overexpression of HvWRKY10, HvWRKY19, and HvWRKY28 might boost the output of defense gene expression and, thus, halt fungal growth. Because PTI is more ancient than ETI, the involvement of HvWRKY10, HvWRKY19, and HvWRKY28 in R-gene-mediated resistance may have been recruited from their original function in basal defense (Bhattarai et al. 2010; Jones and Dangl 2006).

Potential roles in other biological processes.

WRKY TF might have functional roles in controlling several seemingly disparate processes. For instance, HvWRKY1/38 has regulatory roles in both biotic and abiotic stress responses. HvWRKY1/38 negatively regulates basal defense against barley powdery mildew; however, it is also involved in the cold and drought stress response (Eckey et al. 2004; Marè et al. 2004; Shen et al. 2007). The putative ortholog of HvWRKY10 in rice is OsWRKY51, which shares 79% amino acid identity. Abscissic-acid (ABA) inducible OsWRKY51 physically interacts with OsWRKY71, both involved in gibberellin (GA)-regulated MYB class transcription factor (GAMYB) signaling in aleurone cells and, thus, play roles in ABA and GA signaling and cross-talk (Xie et al. 2006). Interestingly, the putative ortholog of OsWRKY71 in barley is HvWRKY1, which is a negative regulator in barley defense to *B. graminis* f. sp. *hordei* (Shen et al. 2007). This poses the question: is it possible that HvWRKY10 and HvWRKY1 physically interact in the process of regulating the defense response? This question remains to be tested.

OsWRKY7 is the closest ortholog of HvWRKY19 in rice; they share 64% identity at the amino acid level. OsWRKY7 is highly induced upon infection by the fungal pathogen *M. grisea*, the causal agent of rice blast disease. After challenging with bacterial pathogen *X. oryzae* pv. *oryzae*, OsWRKY7 is also differentially expressed (Ryu et al. 2006). The closest HvWRKY28 ortholog in *Arabidopsis* is AtWRKY41 which, upon overexpression, displayed enhanced resistance to wild-type *Pseudomonas syringae* pv. *tomato* DC3000 but increased susceptibility to *Erwinia carotovora* EC1 (Higashi et al. 2008).

These studies imply that HvWRKY10, HvWRKY19, and HvWRKY28 might have other roles in plant biological processes. Indeed, analysis of Barley1 GeneChip data (accessions BB49, BB9 and BB61) (PLEXdb) revealed that all three HvWRKY genes were highly induced after challenge with the stem rust fungus *Puccinia graminis* f. sp. *tritici* (Zhang et al. 2008b) whereas only HvWRKY19 was highly induced when infected with *Fusarium graminearum*, causal agent of Fusarium head blight of barley (Boddu et al. 2006). When wild barley (*H. vulgare* subsp. *spontaneum*) accession 'Shechem 12-32' was inoculated with *P. hordei* and *Cochliobolus sativus*, causal agents of leaf rust and spot blotch, respectively (Millett et al. 2009), all three HvWRKY genes were also highly induced. Nevertheless, the comprehensive function of HvWRKY10, HvWRKY19, and HvWRKY28 in these specific fungus–barley interactions remains to be characterized.

Conclusions.

A balanced defense response prevents plants from overexpression of specific outputs, thus limiting damage to target cells (Dangl 2007). This appropriate response requires a deli-

Fig. 8. Model for the functional prediction of HvWRKY10, HvWRKY19, and HvWRKY28 in the barley immune system. In this model, HvWRKY10, HvWRKY19, and HvWRKY28 act as transcriptional activators by regulating the output of defense gene expression in basal defense; overexpression of HvWRKY10, HvWRKY19, and HvWRKY28 can significantly reduce the Mlo-mediated susceptibility (left). Unlike HvWRKY1 and HvWRKY2, HvWRKY10, HvWRKY19, and HvWRKY28 do not appear to directly interact with MLA; however, they are essential in resistance-gene-mediated signaling, because gene silencing resulted in increased susceptibility to *Blumeria graminis* f. sp. *hordei*, which presents pathogen-associated molecular patterns (PAMPs) or avirulence effectors (right). In both cases, HvWRKY10, HvWRKY19, and HvWRKY28 are essential in a delicate regulation pathway to potentiate the expression of defense genes to achieve a balanced defense.

cate regulatory system; many WRKY TF are involved in regulating this process in the nucleus, and exhibit a vital function in fine-tuning the defense response upon pathogen invasion. HvWRKY10, HvWRKY19, and HvWRKY28 are such examples, which have precise roles in integrated plant defense networks.

MATERIALS AND METHODS

Plant material, growth conditions, and fungal isolates.

Seedlings of barley lines CI 16151 (*Mla6*), CI 16137 (*Mla1*), Sultan-5 (*Mla12*), Clansman (*Mla13*), Clho 2277 (Black Hull-less), and *mlo-5* BC7 Ingrid were grown in a temperature-controlled greenhouse with supplemental lighting. Following BSMV virus-induced gene silencing (VIGS) bombardment or mechanical inoculation, plants were transferred to a temperature-controlled growth chamber with a 16-h photoperiod, with light intensity ranging from 400 to 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and day and night temperatures 24 and 20°C, respectively. *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{al}*, *AVR_{ab}*, and *AVR_{al2}*) was propagated on *H. vulgare* ‘Manchuria’ (CI 2330) in a controlled growth chamber at 18°C (16 h of light and 8 h of darkness).

Expression profiling and analysis.

Datasets BB2 (ArrayExpress E-TABM-82) and BB10 (ArrayExpress E-TABM-142) in PLEXdb were used to analyze the 26 HvWRKY genes in response to powdery mildew (Caldo et al. 2006; Dash et al. 2012; Meng et al. 2009; Shen et al. 2005). RNA was extracted from 7-day-old seedling first leaves (PO:0007094) harvested at 0, 8, 16, 20, 24, and 32 HAI with *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{ab}*) (Moscou et al. 2011). Noninoculated plants at these six time points were used as controls. The analysis presented here was based on six genotypes $\times$ six time points $\times$ two inoculation treatments $\times$ three biological replications for a total of 216 GeneChip hybridizations.

FDR was calculated using the histogram-based technique as described by Nettleton and colleagues (2006). FDR was controlled at 0.01% among inoculated and noninoculated plants for at least one time point throughout all six genotypes, respectively. Hierarchical clustering was performed with HCE 3.5 software (University of Maryland, College Park). A Pearson correlation was used to measure similarities of transcript accumulation.

BSMV-mediated gene silencing.

Total RNA was extracted from CI 16151 (*Mla6*) plants challenged at 20 HAI with *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{ab}*) using Trizol agent (Caldo et al. 2004). First-strand cDNA was synthesized using 2 μg of total RNA, oligo(dT)₂₀ primer, and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Subsequently, first-strand cDNA was used as the template to amplify each silencing targeted gene fragment. Amplified PCR fragments each contained an introduced *PacI* and *NotI* recognition sites at the 5′ and 3′ ends, respectively. PCR products then were digested with *PacI* and *NotI* and ligated into the *PacI* and *NotI* sites of BSMV:γ. DNA bombardment and subsequent virion mechanical infection was performed according to Meng and associates (2009).

Twelve days after mechanical infection, plants displayed a BSMV infection phenotype (i.e., a brown stripe on the first leaf and stripe mosaic or necrosis on the second and third leaves). Whole plants were then inoculated with fresh *B. graminis* f. sp. *hordei* conidiospores and placed in an 18°C growth chamber (16 h of light and 8 h of darkness). *B. graminis* f. sp. *hordei* infection types were scored at 7 DAI. Although it is possible that an overlap in general defense gene functions induced by BSMV may interfere with *B. graminis* f. sp. *hordei* development in barley, a recent investigation has shown that it

does not affect the infection of *B. graminis* f. sp. *tritici*, the causal agent of powdery mildew in wheat (Tufan et al. 2011).

Along with the whole-plant inoculation, segments from the bottom half of five second leaves were placed on 0.6% phyto agar (Duchefa, Haarlem, The Netherlands) medium and then inoculated with fresh *B. graminis* f. sp. *hordei* conidiospores. For the convenience of counting the conidiospores, inoculum was applied to give approximately 2,000 spores/cm². Detached leaves were stained and scored microscopically at 4 DAI. The staining process was performed according to Hein and associates (2005), with minor modifications. Leaves were fixed for 24 h on filter paper soaked with 1:1 (vol/vol) ethanol/acetic acid and for 48 h on filter paper soaked with lactoglycerol (1:1:1 [vol/vol] lactic acid/glycerol/H₂O) and stained with 0.05% (wt/vol) Coomassie brilliant blue R-250 stain in 50% methanol and 10% acetic acid. A Zeiss Axio Imager M.1 microscope (Zeiss, Inc., Thornwood, NY, U.S.A.) was used for observation.

Three treatments of interest—BSMV:HvWRKY10 (Trt-1), BSMV:HvWRKY19 (Trt-2), and BSMV:HvWRKY28 (Trt-3)—as well as the BSMV:00 control were applied to leaves to assay their effect on the growth of secondary hyphae. In each of three replications, five leaves were exposed under each of the four treatments. On each leaf, the number of secondary hyphae and the total number of spores were counted. A generalized linear mixed model was used, fit to the data. In the model, we assume that the number of secondary hyphae on each leaf has a binomial distribution, with number of trials equal to the total number of spores on the leaf. The logit of each binomial success probability was modeled as a linear function of fixed treatment effects and normally distributed replication effects. An overdispersion parameter was included to model variation among leaves within each treatment and replication.

Quantitative real-time PCR.

Each individual construct-treated plant was considered a silencing event; the third leaves of three individual BSMV-VIGS-treated and three BSMV:00 vector control treated seedlings were collected after 24 h of *B. graminis* f. sp. *hordei* inoculation. Total RNA was extracted using Trizol-like reagent (Caldo et al. 2004); RNase-free DNase I (Ambion, Austin, TX, U.S.A.) then was utilized to remove the genomic DNA. First-strand cDNA was synthesized using 2 μg of total RNA oligo(dT)₂₀ primer by using SuperScript III reverse transcriptase (Invitrogen). To ensure that only the endogenous gene was amplified, gene-specific primers were designed independently beyond the insert fragment region in the silencing constructs. PCR reactions were run in an ICycler (Bio-Rad, Hercules, CA, U.S.A.) using PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences, Gaithersburg, MD, U.S.A.). Cycling conditions were as follows: 3 min at 95°C; followed by 40 cycles of 15 s at 95°C and 1 min at 60°C; then followed by 80 cycles of 10 s at 55°C, with increasing set point temperature after cycle 2 by 0.5°C for collection and analysis of melting curve data. The PCR program and quantification of the relative changes in gene expression were conducted according to the protocol described by Schmittgen and Livak (2008). Barley *Actin* was used as internal control. The 2^{-ΔCT} method was used to calculate the target gene expression in individual silencing construct-treated and BSMV:00-treated plants; the fold change due to silencing was presented by dividing the expression mean value for targeted WRKY gene in silenced plants by the mean value measured in BSMV:00-treated plants (Schmittgen and Livak 2008).

Subcellular localization.

HvWRKY-GFP fusion constructs were synthesized by amplifying the respective HvWRKY ORF from the transient overex-

pression vectors. The amplified PCR product contained the HvWRKY gene ORF without a stop codon and with an introduced *Hind*III and *Bam*HI recognition sites at the 5' and 3' ends, respectively. The PCR product was then digested with *Hind*III and *Bam*HI and was ligated to the N terminus of GFP within the Ubi:GFP vector (Nielsen et al. 1999). The HvWRKY-GFP chimeric gene constructs were introduced into barley epidermal cells by using biolistic PDS-1000/he system (Bio-Rad). Gold particles (Bio-Rad) were coated with plasmid and delivered into 7-day-old seedling detached leaves of barley CI 16151 (*Mla6*) using 900-psi rupture disks. As a negative control for the localization of the HvWRKY-GFP fusion protein, Ubi:GFP vector alone was also independently transformed into barley leaves. The GFP protein was visualized with a Zeiss Axioplan II fluorescence microscope (Zeiss, Inc.).

Yeast two-hybrid analysis.

The BD Matchmaker library construction and screening kit (Clontech) was adapted to detect the protein in vitro interaction between HvWRKY10, HvWRKY19, and HvWRKY28 and MLA. CI 16151 (*Mla6*), CI16137 (*Mla1*), and Sultan-5 (*Mla12*) cDNA were synthesized from RNA extracted from plant seedling leaves at 20 HAI with *B. graminis* f. sp. *hordei* isolate 5874 by using SuperScript III reverse transcriptase (Invitrogen). The full-length ORF of HvWRKY10, HvWRKY19, HvWRKY28, and HvWRKY1 and barley *Mla1*, *Mla6*, and *Mla12* were amplified from barley cDNA. The CC (amino acids 1 to 46) and CC-NB (amino acids 1 to 578) domains from MLA1, MLA6, and MLA12 were also amplified from barley cDNA. PCR products from HvWRKY were subsequently cloned into GAL4 activation domain of pGADT7 vector and PCR products from *Mla* were subsequently cloned into DNA binding domain (BD) of pGBKT7 vector. Co-transformation to yeast strain and subsequent analyses were performed as described in Clontech protocols.

Transient overexpression.

The full-length ORF of HvWRKY10 (GenBank accession DQ840409), HvWRKY28 (GenBank accession DQ863112), and HvWRKY19 (GenBank accession DQ840418) were amplified from barley CI 16151 cDNA, which was synthesized from RNA extracted from seedlings at 20 HAI with *B. graminis* f. sp. *hordei* isolate 5874. *Eco*RV and *Hind*III were used to double digest the PCR product, which was then inserted into the expression vector pUbi:Nos, which was also digested with the same enzymes. The newly constructed vector, pUbi:WRKY, was co-bombarded with V26UMUG into barley *mlo-5* BC7 Ingrid epidermal cells in three independent experiments (Halterman and Wise 2004; Shirasu et al. 1999). GUS and Coomassie blue staining were performed as described previously (Hu et al. 2009). Scoring for susceptibility was performed under light microscopy by calculating the ratio of GUS-marked cells to the total GUS cells with ESH by *B. graminis* f. sp. *hordei*.

In this transient overexpression assay, three treatments of interest—Ubi:HvWRKY10 + GUS/Mlo (Trt-1), Ubi:HvWRKY19 + GUS/Mlo (Trt-2), and Ubi:HvWRKY28 + GUS/Mlo (Trt-3)—and a control treatment were applied to leaves in order to demonstrate their effect on the growth of secondary hyphae. In each of three replications, five to six leaves were exposed under each of the four treatments. On each leaf, the number of secondary hyphae and the total number of spores were counted. A generalized linear mixed model was fit to the data. In the model, we assume that the number of secondary hyphae on each leaf has a binomial distribution, with number of trials equal to the total number of spores on the leaf. The logit of each binomial success probability was modeled as a linear function of fixed treatment effects and normally distributed replication

effects. An over-dispersion parameter was included to model variation among leaves within each treatment and replication.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

HarvEST database: harvest.ucr.edu
 Hierarchical Clustering Explorer software: www.cs.umd.edu/hcil/hce
 International Barley Sequencing Consortium webpage: barleygenome.org
 PLEXdb, Plant Expression database: www.plexdb.org/index.php