SnRK1 Phosphorylates and Destabilizes WRKY3 to Enhance Barley Immunity to Powdery Mildew

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ABSTRACT

Plants recognize pathogens and activate immune responses, which usually involve massive transcriptional reprogramming. The evolutionarily conserved kinase, Sucrose non-fermenting-related kinase 1 (SnRK1), functions as a metabolic regulator that is essential for plant growth and stress responses. Here, we identify barley SnRK1 and a WRKY3 transcription factor by screening a cDNA library. SnRK1 interacts with WRKY3 in yeast, as confirmed by pull-down and luciferase complementation assays. Förster resonance energy transfer combined with noninvasive fluorescence lifetime imaging analysis indicates that the interaction occurs in the barley nucleus. Transient expression and virus-induced gene silencing analyses indicate that WRKY3 acts as a repressor of disease resistance to the Bgh fungus. Barley plants over-expressing WRKY3 have enhanced fungal microcolony formation and sporulation. Phosphorylation assays show that SnRK1 phosphorylates WRKY3 mainly at Ser83 and Ser112 to destabilize the repressor, and WRKY3 non-phosphorylation-null mutants at these two sites are more stable than the wild-type protein. SnRK1-overexpressing barley plants display enhanced disease resistance to Bgh. Transient expression of SnRK1 reduces fungal haustorium formation in barley cells, which probably requires SnRK1 nuclear localization and kinase activity. Together, these findings suggest that SnRK1 is directly involved in plant immunity through phosphorylation and destabilization of the WRKY3 repressor, revealing a new regulatory mechanism of immune derepression in plants.

Keywords: SnRK1, phosphorylation, WRKY transcription factor, immunity, powdery mildew fungus

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INTRODUCTION

As sessile organisms, plants are constantly exposed to biotic and abiotic stress conditions; therefore, it is crucial for plants to effectively activate and integrate distinct signaling pathways to cope with the stresses (Fujita et al., 2006). To ward off pathogens, plants have evolved a two-tiered innate immune system. The first layer of plant immunity relies on plasma membrane-localized pattern recognition receptors that detect pathogen-associated molecular patterns (PAMPs) and trigger PAMP-triggered immunity (PTI) (Zipfel, 2014). The second layer depends on plant intracellular nucleotide-binding site/leucine-rich repeat (NLR) receptors (also known as Nod-like receptors) that, directly or indirectly, recognize isolate-specific effector molecules and initiate effector-triggered immunity (ETI) (Dodds and Rathjen, 2010; Dou and Zhou, 2012; Jones et al., 2016). Plants employ distinct and common signaling pathways to trigger cellular defense responses in both PTI and ETI, such as reactive oxygen species bursts, Ca2+ spikes, mitogen-activated protein kinase activation, and phytohormone production (Tsuda and Katagiri, 2010; Cook et al., 2015). Central to plant immune responses is the capability of plants to integrate different signaling pathways.
Plant WRKY transcription factors (TFs) play important roles in the regulation of transcriptional processes to modulate pathogen-triggered cellular responses (Pandey and Somssich, 2009; Tsuda and Somssich, 2015). Some WRKY TFs, such as WRKY-IIa subgroup members, have been shown to act as repressors of plant immunity. Arabidopsis AtWRKY18, AtWRKY40, and AtWRKY60 have been shown to repress basal defenses against virulent hemibiotrophic Pseudomonas syringae and biotrophic Golovinomyces orontii (Xu et al., 2006; Shen et al., 2007; Pandey et al., 2010; Schon et al., 2013; Birkenbihl et al., 2017a). Rice OsWRKY28, OsWRKY62, and OsWRKY76 negatively regulated basal immunity against Xanthomonas oryzae pv. oryzae (Xoo) and/or Magnaporthe oryzae (Peng et al., 2008; Seo et al., 2011; Chujo et al., 2013; Liu et al., 2016). OsWRKY62 and OsWRKY76 were also shown to repress Xa21-mediated defense against Xoo (Peng et al., 2008; Seo et al., 2011). Barley WRKY1 and WRKY2 were shown to interact with mildew locus A (MLA) NLR receptors by yeast two-hybrid (Y2H) screening, and both TFs act as repressors of barley basal immunity against Blumeria graminis f. sp. hordei (Bgh), an important obligate biotrophic fungal pathogen (Shen et al., 2007). Significantly, activated MLA receptors were shown to interact with the two WRKYs in the nucleus to derepress barley immunity and potentiate ETI responses (Shen et al., 2007). Furthermore, WRKY1 was found to specifically interact with MYB6 and repress its function in transcriptional regulation of barley immunity (Chang et al., 2013). These examples suggest that members of the WRKY-IIa subgroup have a negative regulatory role in plant immunity that is conserved in both dicots and monocots. However, the mechanism by which plants regulate these TF repressors and derepress immune responses is not fully understood.

Plant SUCROSE-NONFERMENTING1 (SNF1)-related kinase 1 (SnRK1) has a central role in the maintenance of energy homeostasis for growth and survival. SnRK1 is functionally related to yeast SNF1 and mammalian AMP-activated protein kinase and acts as a metabolic/energy sensor and signal integrator in response to diverse stress and energy conditions (Baena-Gonzalez et al., 2007; Emanuelle et al., 2016). Emerging evidence suggests that SnRK1 has a key role in defense responses to viruses, bacteria, fungi, and oomycete pathogens, as well as herbivores (Hulsmans et al., 2016). For example, SnRK1 can directly target germivirus proteins for phosphorylation to alleviate viral infections, and its targets include the βC1 protein from the tomato yellow leaf curl China virus β-satellite (TYLCCNB) and the AL2/C2 protein from cabbage CaCuV and tomato ToMoV viruses (Shen et al., 2011, 2014; Zhong et al., 2017). Conversely, viral proteins such as AL2 from tomato germivirus TGMV and L2 from beet BCTV virus can target and inactivate SnRK1 to increase viral pathogenesis (Hao et al., 2003). Evidence also suggests that SnRK1 functions in interactions between plants and fungal pathogens (Yuan et al., 2008; Kim et al., 2015; Perochon et al., 2015). In rice, SnRK1b (OSK35) has a positive role in defense against M. oryzae blast fungus (Kim et al., 2015), and in wheat, TaSnRK1 interacts with TaFROG to positively regulate resistance against Fusarium head blight fungus (Perochon et al., 2015). These examples indicate an important role for SnRK1 in the regulation of plant disease resistance in diverse pathosystems. Given that a plant obligate biotrophic fungus obtains all of its nutrition from the host and is metabolically intertwined with host cells, it is an intriguing question whether SnRK1, as a metabolic sensor kinase, functions in plant defense responses to obligate biotrophs.

Here, we report that barley SnRK1 kinase interacts with and phosphorylates WRKY3, a repressor of barley disease resistance to the Bgh fungus. Phosphorylation of WRKY3 at two sites destabilizes the protein. SnRK1 positively regulates barley disease resistance, and this function probably requires its nuclear localization and kinase activity. Our data shed new light on the function of SnRK1 in plant immunity to an obligate biotrophic fungus, revealing a regulatory mechanism of immune derepression through the destabilization of an immune repressor in planta.

RESULTS

WRKY3 Phosphorylation Enhances Barley Immunity against Bgh

To test the function of WRKY3 in barley disease resistance, we first employed a single-cell transient gene expression assay (Shen et al., 2003). A plasmid for WRKY3 expression driven by the ubiquitin promoter and a β-glucuronidase (GUS) reporter were codelivered into barley leaf epidermal cells by particle

Barley WRKY3 Interacts with WRKY1 and WRKY2

Barley WRKY1 and WRKY2 have been identified as repressors of PAMP-triggered basal defense against the fungus B. graminis (Shen et al., 2007). To identify additional components of WRKY1/2-mediated immune suppression, we used WRKY1/2 as baits to screen a prey library of Bgh-infected barley leaves. WRKY3 was identified as a candidate interactor by both WRKY1/2-containing baits. Targeted Y2H analysis further confirmed that WRKY1/2 interacted with WRKY3, probably through the C-terminal fragment of 40 amino acids, WRKY3(292–331) (Supplemental Figures 1A, 1B, and 3). We conducted a maltose-binding protein (MBP) pull-down assay to confirm the interactions, using purified fusion proteins from Escherichia coli. Our results showed that MBP-WRKY3 specifically pulled down WRKY1-3xHA and WRKY2-3xHA fusion proteins (Supplemental Figure 1C). A bimolecular fluorescence complementation (BiFC) assay indicated that WRKY3 interacted with WRKY1/2 inside the nucleus of Nicotiana benthamiana but did not interact with WRKY28, a member of group III and a positive regulator of MLA-triggered immunity (Supplemental Figure 1D) (Meng and Wise, 2012). Interestingly, WRKY3 could self-associate in yeast and in the nucleus of N. benthamiana, as shown by the BiFC assay (Supplemental Figure 2).

We performed sequence alignment of full-length WRKY1/2/3 and found that the three WRKYs share a highly conserved domain and motif structure, with ~60% amino acid similarity (Supplemental Figure 3A), and possibly belong to the subgroup IIa family. Our results indicate that WRKY3 interacts with two closely related WRKY TFs, WRKY1 and WRKY2, and also self-associates in the nucleus of barley cells.

WRKY3 Acts as a Suppressor in Barley Immunity against Bgh

To test the function of WRKY3 in barley disease resistance, we first employed a single-cell transient gene expression assay (Shen et al., 2003). A plasmid for WRKY3 expression driven by the ubiquitin promoter and a β-glucuronidase (GUS) reporter were codelivered into barley leaf epidermal cells by particle
WRKY3 phosphorylation enhances barley immunity

(A) Transient overexpression of WRKY3 in barley epidermal cells represses basal and MLA1-mediated isolate-specific disease resistance against powdery mildew fungus. Haustorial index was scored at 48 h after spore inoculation in leaf epidermal cells expressing the empty vector (EV) or the indicated plasmids in the MLA1-containing near-isogenic line P01. Bgh isolates A6 and K1 were used for inoculation. At least 50 cells were analyzed in each experiment, and the data show average values calculated from three independent experiments. Data represent the means ± SD from three independent replicates. \( **P < 0.01 \), significant difference between the EV control and WRKY3 using Student’s t-test.

(B) Silencing of WRKY3 using BSMV-VIGS enhances basal disease resistance against powdery mildew fungus in barley. Bgh microcolony index was scored at 48 h after spore inoculation with isolate A6. The ±SD analyses and error bars reflect the means of three independent experiments. At least 2000 interaction sites were analyzed under the microscope in one experiment. Data represent the means ± SD from three independent replicates. \( ^*P < 0.05 \), significant difference using Student’s t-test.

(C) Stable overexpression of WRKY3 in transgenic barley compromises disease resistance to powdery mildew fungus. Representative images show the effects of WRKY3 overexpression in barley against powdery mildew infection at the seedling stage. Western blotting confirms the presence of WRKY3-HA accumulation in transgenic GP plants. Scale bar, 5 mm.

(D) Statistical analyses of microcolony formation rate in transgenic GP lines overexpressing WRKY3. Bgh microcolony index was scored at 48 h after inoculation with isolate K1. The ±SD analyses and error bars reflect the means of three independent experiments. At least 2000 interaction sites were analyzed under the microscope in one experiment. Data represent the means ± SD from three independent replicates. \( ^*P < 0.05 \), significant difference using Student’s t-test.

(E) Representative images showing microcolony formation on the leaf surface of transgenic plants and recipient GP plants. Scale bar, 200 µm.

WRKY3 overexpression suppressed basal and MLA1-mediated immunity against Bgh fungus in barley.

We next employed the barley stripe mosaic virus (BSMV)-mediated gene-silencing system to knock down WRKY3 expression (Hein et al., 2005; Yuan et al., 2011). Two weeks after BSMV infection, leaves were inoculated with a virulent Bgh isolate. The frequency of fungal microcolonies on the leaf surface (microcolony index [MI%]) was scored under the microscope at 2 days post inoculation (dpi) (Shen et al., 2007). A BSMV vector containing a WRKY3 antisense fragment, BSMV-WRKY3as, specifically reduced WRKY3 expression by ~80% in the cultivar Golden Promise (GP) (Supplemental Figure 4).

Interestingly, silencing of WRKY3 markedly reduced Bgh MI% by almost one half compared with the BSMV EV control (Figure 1B). Thus, silencing of WRKY3 expression increased barley disease resistance to Bgh fungus.

To validate the data obtained in the transient expression assays, we generated stable transgenic barley lines overexpressing a WRKY3-3xHA fusion with GP as the recipient. Two homozygous T2 transgenic lines, #13 and #14, were selected, and their expression of WRKY3-3xHA was confirmed by western blotting (Figure 1C, bottom panel). Detached leaves of seedlings from these two lines were inoculated with the compatible isolate BghK1, and the disease phenotype of typical leaves was evaluated at 7 dpi. Greater Bgh sporulation was observed at 7 dpi on leaf surfaces of the two transgenic lines expressing the WRKY3 fusion than on the surfaces of GP leaves (Figure 1C).

To obtain an earlier evaluation of Bgh infection, we scored Bgh microcolony index at 2 dpi under the microscope. MI% in leaves of the WRKY3 overexpression lines was ~64%, significantly higher than that of GP (~46%) (Figure 1D). Development of fungal colonies was visualized by Coomassie blue staining at 2 dpi, and the secondary hyphae typically appeared longer in leaves of the WRKY3 overexpression lines than in GP leaves (Figure 1E). Together, these data suggest that WRKY3 overexpression in barley transgenic lines...
increased barley susceptibility to Bgh, again confirming that WRKY3 acts as a suppressor of barley immunity against Bgh.

SnRK1 Interacts with WRKY3 in the Barley Nucleus

To understand how WRKY3 represses barley immunity, we performed Y2H using WRKY3 baits to screen the prey library mentioned above. From $2 \times 10^7$ independent clones screened, we independently identified one candidate interactor six times, a barley SNF1-related kinase a subunit, hereafter designated barley SnRK1. A truncated fragment of this SnRK1 was previously reported as BKim2, one of two isoforms found in barley (Hannappel et al., 1995; Slocombe et al., 2002). Interestingly, a BLAST search using the SnRK1 sequence identified at least five more closely related sequences from the barley genome database, suggesting that there are more SnRK1 isoforms in the barley genome (Mascher et al., 2017). Furthermore, we confirmed the SnRK1–WRKY3 interaction by targeted Y2H analysis. Our results indicated that the N-terminal sequence of WRKY3 mediated the interaction and that SnRK1 specifically interacted with WRKY3 but not WRKY1/2 (Figure 2A and Supplemental Figure 5), despite the fact that the three WRKYs share a conserved domain structure with relatively high sequence relatedness (Supplemental Figure 3). An in vitro pull-down assay was performed to test their physical interaction. MBP-WRKY3 fusions were purified from E. coli and incubated with MBP pull-down analysis. MBP alone served as a negative control. The interacting protein was revealed by anti-HA immunoblotting. The experiment was repeated three times with similar results.

Next, a luciferase complementation imaging assay was performed in N. benthamiana following agroinfiltration of an nLUC/cLUC fusion of SnRK1 and WRKY3. A strong luminescence signal was detected only in the area coexpressing WRKY3-nLUC and SnRK1-cLUC but not in areas coexpressing the control pairs, i.e., WRKY1-nLUC and SnRK1-cLUC, WRKY2-nLUC and SnRK1-cLUC.
SnRK1 phosphorylates WRKY3 mainly at S83 and S112

Because SnRK1 interacted with WRKY3, we wondered whether SnRK1 phosphorylated WRKY3. We first predicted the potential phosphorylation sites in WRKY3 using the NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos-3.1/). Among several dozen predicted sites, S83 and S112 were of particular interest because S83 had the highest score and S112 resided in a consensus sequence recognized by SnRK1 (Figure 3A) (Halford and Hardie, 1998). We then performed an in vitro phosphorylation assay using the recombinant proteins WRKY3-His, GST-SnRK1-kinase domain (KD), and GST-GRIK1, followed by proteolytic digestion and liquid chromatography–tandem mass spectrometry (LC–MS/MS). A GST–SnRK1-KD fusion containing the SnRK1 KD and a UBA domain was used here because SnRK1 full-length protein was insoluble (Shen et al., 2011). Because Arabidopsis GRIK1 was reported to be the upstream kinase for activating AtSnRK1 (Shen et al., 2009), a GST–GRIK1 fusion containing barley GRIK1 was also included. The recombinant proteins purified from E. coli were incubated in different combinations and then subjected to Phos-tag analysis (Figure 3B). Following digestion with trypsin, GluC, and LysC, four residues likely to be phosphorylated by SnRK1 were identified: S83, S112, T160, and T167 (Figure 3A; Supplemental Figures 7 A and 7C; Supplemental Table 2). S83 was the most abundant site identified by trypsin and GluC digestions. S112 was only identified by LysC digestion, very probably because the upstream K or R residue is sensitive to trypsin digestion (Figure 3A; Supplemental Figure 7A; Supplemental Table 2). In addition, T160 and T167 were also identified by trypsin and LysC digestion (Figure 3; Supplemental Figure 7B and 7C; Supplemental Table 2). Together, these results suggest that SnRK1 can phosphorylate WRKY3 in vitro, most likely at S83, S112, T160, and T167.

We further analyzed the migration pattern of WRKY3-His in a Phos-tag mobility shift assay. In the absence of either one or both GST-GRIK1 and SnRK1-KD, WRKY3-His was detected as a single band of non-phosphorylated form, indicating that both kinases are necessary for the phosphorylation of WRKY3.
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WRKY3 Phosphorylation Enhances Barley Immunity

We used the Arabidopsis leaf protoplast transfection assay to measure the transcriptional activity of WRKY3 (Liao et al., 2008). We transfected a luciferase (LUC) reporter together with any phosphorylation at T160 and T167 in this assay, although the phosphorylation level may have been too low for detection. Together, these results indicate that, upon activation by GRIK1, SnRK1 directly phosphorylates WRKY3 in vitro, mainly at S83 and S112.

Phosphorylation of WRKY3 at S83 and S112 Promotes Its Degradation

Phosphorylation of a TF may affect its stability, activity, or subcellular localization (Yoo et al., 2008; Kaneda et al., 2009; Zhai et al., 2013). To understand the effect of phosphorylation on WRKY3, we conducted an agroinfiltration-mediated transient expression and phosphorylation assay in N. benthamiana and compared the stability of wild-type WRKY3 with that of phosphorylation-null mutants. GRIK1-Myc and SnRK1-YFP fusions were coexpressed with WRKY3-3xHA, WRKY3S83A-3xHA, WRKY3S112A-3xHA, and WRKY3S83A/S112A-3xHA by agroinfiltration in N. benthamiana (Figure 4A). The protein levels of the GRIK1, SnRK1, and WRKY3 fusion proteins were analyzed by western blotting at 72 h after agroinfiltration using N. benthamiana actin as a loading control. Remarkably, the wild-type fusion WRKY3-3xHA accumulated at the lowest level, the single-mutant fusions WRKY3S83A-3xHA and WRKY3S112A-3xHA accumulated at 1.35- to 1.40-fold higher levels, and the double-mutant fusion WRKY3S83A/S112A-3xHA accumulated at the highest level, 3.29-fold higher than the wild-type fusion (Figure 4A, panel 3, lanes 1–4). These results suggest that WRKY3 is destabilized by phosphorylation at S83 and S115 by SnRK1 in planta.

To determine whether WRKY3 was degraded through the proteasome, we applied MG132 approximately 12 h before harvesting N. benthamiana samples. The level of GRIK1-Myc was not significantly affected, whereas the level of SnRK1-YFP increased slightly after the MG132 treatment (Figure 4A, panels 1 and 2). Notably, the levels of WRKY3 wild-type and mutant fusions increased by ~1.5- to 2.0-fold after the MG132 treatment compared with their levels after the dimethyl sulfoxide (DMSO) treatment (Figure 4A, panel 3, lanes 5–8). These data indicate that WRKY3 degradation is dependent on the 26S proteasome.

To further investigate WRKY3 protein stability in barley, we first examined the pattern of WRKY3 phosphorylation in the transgenic line WRKY3-3xHA-#14 by Phos-tag mobility shift analysis using crude protein extract. Phosphorylated forms of WRKY3 were detected in leaves infected with BghK1 for 0, 12, and 24 h, and more proteins had accumulated at 24 h after inoculation. Interestingly, phosphorylated forms of WRKY3 declined and the non-phosphorylated form accumulated to a much higher level after the MG132 treatment (Supplemental Figure 9). Similarly, the MG132 treatment significantly increased the accumulation of WRKY3-3xHA in healthy barley and in barley infected with BghK1 for 24 h (Figure 4B). These data suggest that WRKY3 is phosphorylated in barley plants and is degraded through the proteasome.

WRKY3S83A and WRKY3S112A Mutants Retain Repressor Functions

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**Figure 4. Phosphorylation at S83 and S112 Destabilizes WRKY3.**

(A) 3xHA-tagged WRKY3 with or without S/A substitution at S83 or S112 was transiently coexpressed with YFP-tagged SnRK1 and Myc-tagged GRIK1 in N. benthamiana leaves by agroinfiltration. Protein accumulation was examined by western blotting at 3 days after infiltration. Numbers below the blots indicate the relative protein levels calculated with ImageJ software. MG132 was applied 12–14 h before sample harvesting. DMSO was used as a mock control, and actin served as a loading control. (B) Accumulation of WRKY3-3xHA was detected in transgenic barley lines in the absence or presence of Bgh infection. The addition of MG132 inhibited the degradation of WRKY3 in vitro.

In the presence of both GST-GRIK1 and SnRK1-KD, two phosphorylated forms of WRKY3-His were detected (Figure 3B, lane 5), indicating that only a few major sites in WRKY3 are phosphorylated by SnRK1. S83 and S112 may be the two major sites because the former was most abundantly detected in the assay and the latter resides in a consensus motif recognized by SnRK1. To test this possibility, we generated three fusions of phosphorylation-null mutants, WRKY3S83A-His, WRKY3S112A-His, and WRKY3S83A/S112A-His, for Phos-tag mobility shift analysis (Figure 3B, lanes 6–11). We found that the single mutants WRKY3S112A-His and WRKY3S83A-His each retained only one phosphorylated form that was identified as phosphorylated S83 (pS83) or pS112, respectively (Figure 3B, lanes 7 and 9). Consistent with this finding, the two phosphorylated forms were eliminated in the double mutant WRKY3S112A/S83A-His (Figure 3B, lanes 10 and 11). These results suggested that S83 and S112 were the two major phosphorylation sites. Interestingly, we did not detect any phosphorylation at T160 and T167 in this assay, although the phosphorylation level may have been too low for detection. Together, these results indicate that, upon activation by GRIK1, SnRK1 directly phosphorylates WRKY3 in vitro, mainly at S83 and S112.

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a WRKY3 effector and a control plasmid, then measured reporter LUC activity quantified as luminescence. The Gal4 DNA-binding domain (DBD), which binds the 5xGal4 upstream activating sequences (UAS) in the promoter, was used to measure basal LUC activity, and the DBD-VP16 fusion was used as a transcriptional activator control (Sadowski et al., 1988). In contrast to the DBD-VP16 activator, the DBD-WRKY3 fusion suppressed LUC activity by ~65% (Figure 5A, lanes 1–3), and the DBD fusion with WRKY3S83A or WRKY3S112A suppressed LUC activity to a similar extent (Figure 5A, lanes 4 and 5). These data indicate that WRKY3 acts as a transcriptional repressor and that the phosphorylation-null mutants retain this repressor activity. We further asked whether the WRKY3S83A and WRKY3S112A mutants also retained a suppressor function in barley disease resistance. Using a transient gene expression assay, we overexpressed WRKY3 wild type or the phosphorylation-null mutants in barley and found that both increased fungal HI% to ~80%, compared with ~60% for the EV control in a compatible interaction (Figure 5B). To examine the subcellular localization of phosphorylation-null mutants, we expressed WRKY3-YFP, WRKY3S83A-YFP, and WRKY3S112A-YFP fusions in barley cells and found that their nuclear localization was unaltered in every case (Supplemental Figure 10). Together, these data indicate that WRKY3 is a transcriptional repressor and that the phosphorylation-null mutants retain repressor activity in transcription and disease resistance.

SnRK1 Positively Regulates Disease Resistance to the B. graminis Fungus

The role of SnRK1 in barley disease resistance was investigated by expressing an SnRK1-YFP fusion in single barley cells and stable barley transgenic lines. The mutant fusion SnRK1K139R-YFP, in which the K139R mutation of the catalytic site eliminates the kinase activity, was generated as a control (Cho et al., 2012). Both SnRK1-YFP and SnRK1K139R-YFP were localized to the cytoplasm and nucleus upon transient expression in barley and N. benthamiana (Supplemental Figure 11). Stable barley transgenic plants overexpressing SnRK1-YFP or SnRK1K139R-YFP were generated and selected based on western blotting. We obtained lines #5 and #9 for expressing SnRK1-YFP and lines #11 and #13 for expressing SnRK1K139R-YFP (Figure 6A, bottom panels). Detached leaves of barley seedlings were inoculated with BghK1, then phenotyped at 7 dpi or scored for MI% at 2 dpi. Compared with GP wild type, the lines expressing SnRK1-YFP significantly suppressed fungal sporulation, whereas the lines expressing SnRK1K139R-YFP marginally increased fungal sporulation (Figure 6A, top panel). Consistent with this finding, Bgh MI% was significantly reduced in SnRK1-YFP-expressing lines but markedly increased in SnRK1K139R-YFP expressing lines in comparison with GP plants (Figure 6B). Fungal secondary hyphal development was assessed in leaves of different lines at 2 dpi (Figure 6C). These data suggest that SnRK1 has a positive role in barley resistance to Bgh and that this function may require SnRK1 kinase activity.

The Nuclear Localization of SnRK1 Is Important for Its Function

To determine where SnRK1 functions in the barley cell, we fused a nuclear localization signal (NLS) or a nuclear export signal (NES) to the C terminus of SnRK1-YFP, using the mutated non-functional signals “nls” and “nes” as controls (Lanford and Butel, 1984; Shen et al., 2007). Following expression in N. benthamiana, SnRK1-YFP-NLS was exclusively localized to the nucleus, whereas SnRK1-YFP-NES was excluded from the nucleus and mainly localized to the cytoplasm. As expected, SnRK1-YFP-nls and SnRK1-YFP-nes were localized to both the nucleus and the cytoplasm (Figure 7A). Similarly, SnRK1-YFP was localized to both the nucleus and the cytoplasm when it was expressed in barley cells (Supplemental Figure 6). We further examined the function of these SnRK1 fusions using a transient expression assay in an MLA1-containing line during a compatible interaction (Figure 7B). Significantly, the expression of SnRK1-YFP-NES in barley cells resulted in an HI% of ~55%, comparable to that of the EV control, whereas the expression of SnRK1-YFP-NLS and other control fusions all markedly reduced HI% to ~22%–25% (Figure 7B). This indicates that...
SnRK1 nuclear localization is essential for its function in disease resistance.

DISCUSSION

Previous studies have indicated that SnRK1 functions as a master regulator that integrates diverse signals in response to internal and external cues, thereby coordinating plant growth and stress responses (Baena-Gonzalez et al., 2007; Emanuelle et al., 2016; Hulsmans et al., 2016). In recent years, an increasing number of studies have suggested a key role for SnRK1-mediated signaling in plant defense responses to different pathogens. SnRK1 has been shown to positively regulate immunity to biotrophic and hemibiotrophic pathogens. SnRK1 is involved in immunity against geminivirus in *N. benthamiana*, *Arabidopsis*, and tomato (Hao et al., 2003; Shen et al., 2011, 2014; Zhong et al., 2017). Interestingly, SnRK1 has also been implicated in hypersensitivity-associated immune responses against bacterial pathogens in pepper, sweet orange, and rice (Devaranne et al., 2006; Cernadas et al., 2008; Ek-Ramos et al., 2010; Szczesny et al., 2010; Seo et al., 2011; Avila et al., 2012; Sharma et al., 2013). Moreover, the overexpression of rice SnRK1a confers broad-spectrum disease resistance against bacterial and fungal pathogens, irrespective of pathogen lifestyles and/or infection strategies (Filipe et al., 2018). In the present study, overexpression of SnRK1 in barley transgenic plants or in epidermal cells enhanced basal and/or penetration resistance against powdery mildew fungus. Our data extend the function of SnRK1 to include the regulation of immunity against an obligate biotrophic fungal pathogen in a monocot crop. Nevertheless, the mechanisms by which SnRK1 regulates immunity and hypersensitivity-associated responses in plants remain largely elusive.

As an evolutionarily conserved energy sensor kinase, SnRK1 triggers extensive transcriptional reprogramming to restore energy homeostasis and promote cell survival and plant adaptation under stress and sugar-deprivation (Baena-Gonzalez and Sheen, 2008). SnRK1 acts as a central transcriptional integrator of stress and energy signaling by interacting with and phosphorylating diverse types of TFs, e.g., bZIP, AP2, and MYB type TFs (Baena-Gonzalez et al., 2007; Mair et al., 2015; Hulsmans et al., 2016; Zhai et al., 2017). Moreover, data from several recent studies suggest that the SnRK1-TOR (target of rapamycin) module has an important role in the regulation of plant growth–immunity tradeoffs, and two hormones, salicylic acid and jasmonic acid, may signal through this module to link growth and defense (Nukarinen et al., 2016; Baena-Gonzalez and Hanson, 2017; Song et al., 2017; De Vleesschauwer et al., 2018; Filipe et al., 2018). Molecular models have been proposed to explain the role of SnRK1 in the regulation of growth–immunity tradeoffs, whereby hormonal crosstalk and metabolic regulation are crucial for the redirection of cell-cycle function from growth to immunity during biotic stress (Eichmann and Schafer, 2015; Hulsmans et al., 2016; Margalha et al., 2019). In the present study, we identify barley WRKY3 as a suppressor of basal immunity against the Bgh fungus, supported by both overexpression and BSMV silencing data. Similar to WRKY1/2 of the WRKY-IIa subgroup (Shen et al., 2007), WRKY3 may function primarily to restrict the output of PTI below a harmful threshold for growth and development. WRKY3 may also dampen the amplitude of PTI during the early stages of *Bgh* infection. In support of this hypothesis, *WRKY3* expression is induced at early stages of *Bgh* infection, e.g., 0.5, 4, 12, and 48 h after inoculation (Supplemental Figure 12), roughly corresponding to the formation of the primary germ tube, secondary germ tube, haustorium, and secondary hyphae, respectively (Both et al., 2005). Significantly, our data suggest that barley SnRK1 is an upstream kinase of WRKY3 and it may directly control growth–immunity tradeoffs by regulating the turnover of WRKY3 and promoting defense gene expression during *Bgh* infection. Whether
the SnRK1-mediated defense response also involves hormone signaling crosstalk during Bgh fungal infection of barley remains to be investigated.

WRKY3 and WRKY1/2 share a conserved domain and motif structure and exhibit high amino acid identity in key subdomains, findings that are also reflected in their functional conservation during the barley–Bgh interaction (Shen et al., 2007; Chang et al., 2013; Liu et al., 2014). Functional data suggest that WRKY1/2 and WRKY3 act as repressors of barley basal immunity against pathogens. If so, what are the functional relationships between them during barley interactions with pathogens? In this context, it is worth noting that homo- and heterointeractions have been observed for several WRKYs from different families, implicating a complex mode of functional regulation among WRKY TFs (Chi et al., 2013). In Arabidopsis, AtWRKY18, AtWRKY40, and AtWRKY60, the homologs of barley WRKY1/2/3, form both homodimers and heterodimers, which significantly affect DNA binding activity, downstream target gene expression, and the resistance phenotype and spectrum of defense responses to pathogens such as P. syringae and Botrytis cinerea (Xu et al., 2006; Chen et al., 2010; Liu et al., 2012). AtWRKY18 and AtWRKY40 are also functionally redundant in repressing basal immunity to G. orontii powdery mildew in Arabidopsis (Shen et al., 2007). In the current study, WRKY3 was found to associate with itself and with WRKY1 and WRKY2, suggesting that these three WRKYs may also dynamically form homo- and heterocomplexes in barley. It remains to be investigated whether these WRKYs antagonize or cooperate with each other in modulating PTI defense transcription in barley.

Interestingly, our data also reveal that WRKY3 differs from WRKY1/2 in domain function and interactors. The C termini of WRKY3 and WRKY1/2 appear to interact with different proteins, i.e., with WRKY1/2 and MLA, respectively (Supplemental Figure 1) (Shen et al., 2007; Jordan et al., 2011). This may be due to sequence divergence in the C-terminal ~40 amino acids of WRKY3 and WRKY1/2 (Supplemental Figure 3). Furthermore, WRKY3 but not WRKY1/2 interacts with SnRK1 (Figure 2 and Supplemental Figure 5). By contrast, WRKY1/2 but not WRKY3 interacts with the MLA N terminus (Supplemental Figure 1) (Shen et al., 2007). These differences between WRKY1/2 and WRKY3 imply that they may be regulated differently during barley–pathogen interactions. We speculate that WRKY1/2 and WRKY3 may be targeted for phosphorylation by different upstream kinases. Future investigations will shed more light on the interaction dynamics and regulation mechanisms of these WRKY repressors.

Post-translational modification has a critical role in the generation and/or rapid alteration of cellular defense signaling in plant immune responses (Withers and Dong, 2017). Emerging evidence suggests that phosphorylation is often coupled with ubiquitination to alter the stability and/or activity of transcriptional regulators and defense signaling components in plants (Lu et al., 2011; Saleh et al., 2015; Swaney et al., 2015; He et al., 2017). For example, AtMPK3 phosphorylates AtWRKY46 in Arabidopsis, and OsMKP6 phosphorylates OsWRKY45 in rice, leading to degradation and/or change in activity of the respective WRKY proteins (Matsushita et al., 2013; Ueno et al., 2015, 2017; Sheikh et al., 2016). Similarly, a recent study has demonstrated that Arabidopsis KIN10 (SnRK1.1) phosphorylates WR1, a member of the APETALA2 (AP2) class of TFs, leading to rapid WR1 degradation through the proteasome (Zhai et al., 2016). In line with these findings, our results indicate that SnRK1 targets WRKY3 for phosphorylation and degradation. Future investigations will determine whether SnRK1 directly phosphorylates WRKY3 to promote its degradation during the barley–Bgh interaction.

In conclusion, our data suggest that SnRK1 phosphorylates WRKY3 to promote its proteasomal degradation, leading to at least partial derepression of barley immunity against Bgh. The SnRK1–WRKY3 regulatory module suggests a molecular mechanism for growth–immunity tradeoffs during the barley–pathogen interaction.

**METHODS**

**Plant and Fungal Materials**

The barley (Hordeum vulgare) cultivars GP, P01 (a near-isogenic line containing Mla1 in a Pallas background), and “110” (a near-isogenic line containing Mla12 in an Ingrid background) were grown in a growth chamber at 18°C–20°C with a 16-h/8-h light/dark cycle. Nicotiana benthamiana plants were grown in a greenhouse at 22°C–25°C with a 16-h/8-h light/dark cycle.
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The *B. graminis* f. sp. *hordei* (Bgh) isolates K1 (AvrMla1; virMla6, virMla10, virMla12) and A6 (AvrMla6, AvrMla10, AvrMla12; virMla11) used in this study were multiplied on plants of barley cultivars I10 and P01, respectively, and maintained at 18°C–20°C in a growth chamber with 70% relative humidity and a 16-h/8-h light/dark cycle.

Yeast Two-Hybrid Assay

Y2H assays were performed according to the manufacturer’s protocol (Clontech). For Y2H, screening, full-length coding sequences of barley WRKY1, WRKY2, WRKY3, and SnRK1 were cloned into the binary vector CTAPi-YN (fused to nYFP) or CTAPi-YC (fused to cYFP) through an LR reaction (Invitrogen). The indicated YN/YC construct pairs were coexpressed in *N. benthamiana* leaves by agroinfiltration, and the infiltrated plants were then maintained in darkness for 48 h. The leaves were infiltrated with 2 μg/ml DAPI (4’,6-diamidino-2-phenylindole) solution for nuclei staining 2 h before microscopic observation. Fluorescence signals were detected using a confocal laser microscope (Carl Zeiss 710).

Agrobacterium-Mediated Transient Expression Assay (Agroinfiltration Assay)

Constructs harboring different genes were transformed individually into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cultures were harvested and resuspended in infiltration buffer (0.5% Murashige and Skoog, 2% sucrose, 100 μM acetosyringone, and 10 mM 2-(N-morpholino)ethanesulfonic acid [MES]) to an ultimate concentration of OD_{600} = 1.0, then infiltrated into leaves of *N. benthamiana*. For coexpression assays, equal amounts of the indicated constructs were mixed thoroughly before infiltration. The plants were maintained in darkness for 48 h after infiltration and before microscopic observation or immunoblotting.

For the protein degradation assay, indicated *Agrobacterium* cultures were resuspended to an ultimate concentration of OD_{600} = 1.5, and equal amounts of the indicated strain suspensions were mixed completely before infiltration. Three days after infiltration, samples were collected for immunoblotting. MG132 was applied 12–14 h before sampling.

Recombinant Protein Expression and Purification

The pMAL-c2x, pGEX4T-1, and pET32a constructs carrying the indicated genes were transformed into *Escherichia coli* strain BL21 (DE3) or Rosetta (DE3). Purification of the recombinant proteins was performed according to the manufacturer’s instructions (New England Biolabs for MBP-tagged proteins, GE Healthcare for GST-tagged proteins, and Qiagen for Histagged proteins).

In Vitro Protein–Protein Interaction

The *in vitro* protein–protein interaction assay was performed as previously described (Qi et al., 2011; Chang et al., 2013). In brief, 5 μg of purified MBP-tag fused proteins or MBP protein alone was incubated with 150 μl of amylose resin (New England Biolabs) for 3 h at 4°C. After incubation, the resin was washed five times with RB buffer (100 mM NaCl, 50 mM Tris–HCl [pH 7.8], 25 mM imidazole, 0.1% Tween 20, 10% glycerol, EDTA-free complete mini protease inhibitor cocktail, and 20 mM β-mercaptoethanol) and then incubated for 2 h with 150 μl of concentrated total proteins extracted from *N. benthamiana* leaves expressing the indicated constructs by agroinfiltration. After incubation, the resulting proteins were washed five times with RB buffer, resuspended in 2× SDS sample buffer, and boiled for 5 min. The resulting proteins were separated on SDS–PAGE gels and detected using an anti-HA antibody.

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In Vitro Phosphorylation Assays

In vitro phosphorylation assays were performed as previously described (Shen et al., 2011) with minor modifications. Wild-type or mutated WRKY3 protein fused with a mutated Trx tag at Thr-78 (a putative phosphorylation site for SnRK1) were coincubated with GST-tagged GARK1 and SnRK1-KD proteins at a ratio of 3:2:3 in a total volume of 30 μl of kinase reaction buffer (20 mM Tris–HCl [pH 7.5], 5 mM MgCl₂, 0.1 mM CaCl₂, 50 μM ATP, and 2 mM dithiothreitol) at 30°C for 30 min. The reaction was stopped by adding one volume of 2× SDS sample buffer. After boiling at 95°C for 5 min, proteins were separated on SDS–PAGE gels followed by Coomassie brilliant blue R250 staining or western blotting analysis using anti-His antibody (CWBio).

Phos-Tag Mobility Shift Assays

The Phos-tag gels were prepared according to the manufacturer’s instructions (Wako Chemicals). Phosphorylated proteins from the *in vitro* kinase assays were separated on 10% SDS–PAGE gels with 50 μM Phos-tag and 100 μM MnCl₂ followed by Coomassie brilliant blue R250 staining or western blotting analysis. WRKY3-HA was dephosphorylated by treating the total protein isolated from transgenic barley plants with lambda protein phosphatase (lPPase, NEB) according to the manufacturer’s instructions. The samples were then mixed with SDS sample buffer, boiled for 5 min, and analyzed on 8% SDS–PAGE gels supplemented with 20 μM Phos-tag and 20 μM MnCl₂ before use in western blotting analysis.

LC–MS/MS Analysis

Phosphorylated proteins were separated on 12% SDS–PAGE gels. After Coomassie brilliant blue R250 staining, the corresponding band was cut for further analysis. LC–MS/MS analysis was performed using a Triple-TOF 5600+ system (AB Sciex) after trypsin digestion, followed by analysis with Mascot version 2.3.02 (Matrix Science), or using a nanoLC-LTQ-Orbitrap XL system (ThermoFinnigan) after LysC digestion, followed by analysis with Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific).

Arabidopsis Protoplast Transactivation Assays

Transcriptional activity assays in *Arabidopsis* protoplasts were performed as previously described (Liao et al., 2008). In brief, assays were established by cotransfection of the effector plasmids, the reporter plasmid, and internal control into *Arabidopsis* mesophyll protoplasts. The pPTRL plasmid containing a CaMV 35S promoter and a luciferase gene was used as the internal control. The plasmids and the *Arabidopsis* protoplasts were cultured at 23°C for 16 h. LUC activity was measured using a Promega dual-LUC reporter assay system and the GloMax20/20 Lumino-meter with luciferase substrate. The experiments were repeated independently three times.

Single-Cell Transient Gene Expression Assays

Single-cell transient gene expression assays were performed as described previously (Shen et al., 2003). In brief, a plasmid expressing β-glucuronidase (GUS) reporter under the control of the maize ubiquitin promoter was coexpressed together with plasmids expressing genes of interest at a 1:1 molar ratio and delivered into barley leaf epidermal cells by particle bombardment (Bio-Rad, Model PDS-1000/He). The bombarded leaf segments were challenged with powdery mildew fungus *Bgh* at 4 h after bombardment. Transformed cells were stained with GUS staining solution, and the fungal haustorium index was scored using a light microscope at 48 h after *Bgh* spore inoculation.

Barley Stripe Mosaic Virus-Induced Gene Silencing Assays

A detailed protocol for the BSMV virus-induced gene silencing (VIGS) assay was previously described (Yuan et al., 2011). In brief, the constructed plasmids pCaBS-α, pCaBS-β, and pCa-γ-dLic with target gene fragments were transformed individually into *Agrobacterium* strain EHA105. The *Agrobacterium* cells were collected and resuspended in...
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infiltration buffer (10 mM MgCl₂, 100 μM acetoxyringone, and 10 mM MES) to an ultimate concentration of OD₅₆₀ = 0.8. For agroinfiltration, equal amounts of bacteria harboring pCaBS-α, pCaBS-β, and pCa-γ-LUC (or its derivatives) were mixed thoroughly and infiltrated into N. benthamiana leaves. After growing for 9 days after agroinfiltration, the BSMV-infected N. benthamiana leaves were ground in 20 mM Na phosphate buffer (pH 7.2), and the sap was mechanically inoculated onto the first two emerging leaves of barley cultivar I10 or GP. The newly grown upper leaves with virus symptoms that appeared about 14 days after BSMV infection were collected and inoculated with powdery mildew fungal spores (Bgh isolate A8). Leaves were fixed in 50% ethanol/50% glacial acetic acid overnight, destained in a 1:1:1 ratio of glacial acetic acid/glycerol/water for 48 h, and stained with 0.6% Coomasie brilliant blue R250 to visualize the fungal haustorium structure at 48 h after conidia spore inoculation.

Protein Subcellular Localization Analysis

For protein subcellular localization in N. benthamiana, Agrobacterium strain GV3101 harboring proper constructs was infiltrated into N. benthamiana leaves. For protein subcellular localization in barley, plasmids containing genes of interest were delivered into barley epidermal cells by particle bombardment (Bio-Rad, Model PDS-1000/He). The fluorescence signal of YFP was observed at 48 h after infiltration or bombardment using a confocal laser scanning microscope (Leica TCS SP8).

FLIM–FRET Analyses in Barley Epidermal Cells

The indicated proteins were fused to CFP and YFP to form donor and receptor proteins, respectively. CFP fluorescence was excited with the 458-nm line of the argon laser and recorded with the confocal channel band-pass filter set to 480–520 nm. YFP fluorescence intensities in the ROI were averaged and plotted as a function of time. The TCSPC computer module was used to build up a three-dimensional histogram of photon density over spatial and temporal coordinates. The actual position of the scanning beam was generated from the FrameSync, LineSync, and PixelSync signals of the laser scanning microscope. A commercial software package (SPCImage v2.8, Becker & Hickl) was employed to analyze the TCSPC. The iterative reconvolution method was used to recover the fluorescence lifetime from fluorescence decay profiles obtained for each pixel. A modified Levenberg–Marquardt algorithm was used to calculate the best parameters of a given decay. The quality of a fit was determined by the value of a reduced χ². If χ² exceeded a value of 1.5, the model was excluded. Repetitive measurements were carried out to exclude artifacts that might have been caused by photoinduced reactions. Fluorescence decay curves obtained in two subsequent experiments could be superimposed (data not shown), excluding any photoinduced changes that might have affected the lifetime measurement.

Real-Time PCR Analysis

Total RNA extraction and cDNA synthesis were carried out as previously described (Chang et al., 2013). Real-time PCR was performed using the GoTaq qPCR Master Mix (Promega) and the ABI StepOne real-time PCR system according to the manufacturer’s instructions. The expression of ACTIN was used as an internal control for normalization.

ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: HvWRKY1 (AJ536667); HvWRKY2 (AJ853838); HvWRKY3 (AK359706.1); MLA1 (AY009939); HvSnRK1 (AB910929); HvGRIK1 (AK368039.1); AtWRKY18 (NP_567882); AtWRKY40 (NP_178199); AtWRKY60 (NP_180072); OsWRKY28 (DA05093); OsWRKY62 (DA05127); OsWRKY71 (DA05136); OsWRKY76 (DA05141).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

X.H., L. Zhang, and Q.-H.S. conceived and designed the project; X.H., L. Zhang, L. Zhao, P.X., C.Z., T.Q., and L. Zhou performed the experiments; H.Y. helped data analysis; J.Q., D.W., and Q.-H.S. supervised students; Q.-H.S., L. Zhao, X.H., and L. Zhang wrote the paper.

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