Targeting the pattern-triggered immunity pathway to enhance resistance to *Fusarium graminearum*

SUJON SAROWAR¹,¹*, SYEYA T. ALAM¹,²#, RAGIBA MAKANDAR¹,³, HYEONJU LEE⁴, HAROLD N. TRICK⁴, YANHONG DONG⁵ AND JYOTI SHAH ¹,²,⁎

¹Department of Biological Sciences, University of North Texas, Denton, TX 76201, USA
²BioDiscovery Institute, University of North Texas, Denton, TX 76201, USA
³Department of Plant Sciences, University of Hyderabad, Gachibowli, Hyderabad 500046, India
⁴Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, USA
⁵Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA

SUMMARY

Fusarium head blight (FHB) is a disease of the floral tissues of wheat and barley for which highly resistant varieties are not available. Thus, there is a need to identify genes/mechanisms that can be targeted for the control of this devastating disease. *Fusarium graminearum* is the primary causal agent of FHB in North America. In addition, it also causes Fusarium seedling blight. *Fusarium graminearum* can also cause disease in the model plant *Arabidopsis thaliana*. The Arabidopsis–*F. graminearum* pathosystem has facilitated the identification of targets for the control of disease caused by this fungus. Here, we show that resistance against *F. graminearum* can be enhanced by flg22, a bacterial microbe-associated molecular pattern (MAMP). flg22-induced resistance in Arabidopsis requires its cognate pattern recognition receptor (PRR) FLS2, and is accompanied by the up-regulation of WRKY29. The expression of WRKY29, which is associated with pattern-triggered immunity (PTI), is also induced in response to *F. graminearum* infection. Furthermore, WRKY29 is required for basal resistance as well as flg22-induced resistance to *F. graminearum*. Moreover, constitutive expression of WRKY29 in Arabidopsis enhances disease resistance. The PTI pathway is also activated in response to *F. graminearum* infection of wheat. Furthermore, flg22 application and ectopic expression of WRKY29 enhance FHB resistance in wheat. Thus, we conclude that the PTI pathway provides a target for the control of FHB in wheat. We further show that the ectopic expression of WRKY29 in wheat results in shorter stature and early heading time, traits that are important to wheat breeding.

Keywords: *Arabidopsis thaliana*, flg22 peptide, Fusarium head blight, microbe-associated molecular pattern, PTI, wheat, WRKY29.

INTRODUCTION

The ascomycetous fungus *Fusarium graminearum* (hereafter referred to as *Fg*) is an important phytopathogen. In wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), *Fg* is the primary causal agent of Fusarium head blight (FHB) disease which affects floral tissues (Bai and Shaner, 2004; McMullen et al., 1997a; Xu and Nicholson, 2009). In addition, it also causes Fusarium seedling blight. FHB epidemics in the past have resulted in $0.3–3$ billion in losses (Bai and Shaner, 2004; Johnson et al., 2003; Wilson et al., 2017). FHB adversely impacts grain yield and quality. Mycotoxins, for example deoxynivalenol (DON), which accumulate in infected grains, further limit grain acceptability for human and animal consumption (Bai and Shaner, 2004; McMullen et al., 1997b; Wilson et al., 2017). Monogenic gene-for-gene-type resistance is not available for FHB. In many cultivated wheat varieties, resistance to FHB is derived from the cultivar Sumai 3 and its derivatives (Bai and Shaner, 2004). Sumai 3-derived resistance is a quantitative trait that limits fungal spread from the infection site. The non-availability of highly resistant wheat and barley cultivars, the practical difficulties with the timing of fungicide application during anthesis and the high humidity conditions when disease threat is the highest further constrain efforts to control FHB (McMullen et al., 1997b; Pirgozliev et al., 2003).

The genes and mechanisms that contribute to the basal resistance to *Fg* offer targets for molecular breeding and genetic engineering of FHB resistance. For example, salicylic acid (SA) signalling, which contributes to basal resistance to FHB in wheat and barley (Diethelm et al., 2014; Hao et al., 2018; Makandar et al., 2006, 2012, 2015), is a target for enhancing FHB resistance. FHB resistance in wheat was enhanced by the constitutive expression of *NPR1* (*NON-EXPRESSOR OF PR GENES 1*), which is a key regulator of SA signalling, and *NPR1*-like genes in wheat (Gao et al., 2013; Makandar et al., 2006; Yu et al., 2017). Furthermore, natural variations at two homeologous *NPR1*-like genes located on the long arm of chromosomes 2A and 2D were associated with resistance to FHB in winter wheat (Diethelm et al., 2014).
FHB resistance was also enhanced in transgenic wheat that accumulated higher levels of SA as a result of the constitutive expression of PAD4, a positive modulator of SA accumulation (Makandar et al., 2015). FHB resistance was also enhanced in barley plants that overexpressed ICS, a gene that encodes an isochorismate synthase, which synthesizes SA (Hao et al., 2018). In contrast, RNA interference (RNAi)-mediated repression of ICS in barley compromised FHB resistance (Hao et al., 2018).

Pattern-triggered immunity (PTI) is another process that can be targeted to promote disease resistance. PTI, which involves a complex set of physiological and molecular responses in the plant, including reactive oxygen species (ROS) accumulation and callose deposition, is induced in response to the recognition of conserved microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) located on the plant cell surface (Bigeard et al., 2015; Li et al., 2016). PTI is an important contributor to non-host resistance in plants (Bigeard et al., 2015). Some well-studied MAMPs include the bacterial flagellar protein flagellin and elongation factor EF-Tu, which are perceived by the cognate PRRs FLS2 (FLAGELLIN-SENSITIVE 2) and EFR (EF-Tu RECEPTOR), respectively (Bigeard et al., 2015). A 22-amino-acid long region of flagellin, epitomized by flg22 from Pseudomonas aeruginosa, is sufficient for the activation of PTI via FLS2 (Gomez-Gomez and Boller, 2000), whereas an 18-amino-acid long epitope of EF-Tu, represented by elf-18 from Escherichia coli, is sufficient for PTI activation through EFR (Zipfel et al., 2006). The polysaccharide chitin, which is a major component of fungal cell walls, is another MAMP (Sánchez-Vallet et al., 2015). In Arabidopsis thaliana, LysM (extracellular lysin motifs)-containing receptor-like kinases have been implicated in chitin signalling and resistance against fungal pathogens (Sánchez-Vallet et al., 2015; Wan et al., 2008). Similarly, in rice (Oryza sativa, Os), chitin fragments are perceived by the LysM domain-containing OsCEBiP (chitin elicitor binding protein) and OsCERK1 (Kaku et al., 2006; Shimizu et al., 2010). In barley, the LysM domain-containing HvCERK1 (Chitin Elicitor Receptor Kinase 1) is required for plant response to chitin (Karre et al., 2017). Wheat leaves are also responsive to chitin and flg22, both of which induce the expression of wheat homologues of chitin- and flg22-responsive Arabidopsis genes, including TaPUB23-like and TaWRKY23-like (Schoonbeek et al., 2015). In addition, the expression of Arabidopsis EFR is sufficient to confer elf-18 recognition and to enhance resistance in wheat against the bacterial pathogen Pseudomonas syringae pv. oryzae (Schoonbeek et al., 2015), therefore suggesting the conservation of PTI signalling mechanisms between Arabidopsis and wheat.

There is significant overlap in the genes that are up-regulated by different MAMPs (Gust et al., 2007; Wan et al., 2008; Zipfel et al., 2006), thus signifying the activation of convergent signalling pathways by these discrete MAMPs, which control the expression of a common set of PTI-associated genes, although with different dynamics and amplitudes (Li et al., 2016). In Arabidopsis, WRKY29, which encodes a WRKY family transcription factor, is one such gene that is up-regulated by both flg22 and chitoooligosaccharide (Asai et al., 2002; Wan et al., 2008). This convergence of signalling associated with different MAMPs has led to the suggestion that MAMPs are perceived as general danger signals and that plants do not distinguish between different microbes via the defence signalling induced by different MAMPs (Zipfel et al., 2006). Therefore, it is expected that PTI activation should confer cross-protection against pathogens in different kingdoms. Indeed, ectopic application of the bacterial MAMP, flg22, enhances resistance in Arabidopsis to the fungal pathogen Botrytis cinerea (Ferrari et al., 2007; Galletti et al., 2011). Moreover, the application of chitoooligosaccharide promotes resistance in Arabidopsis to the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Wan et al., 2008). Cross-protection also extends to Fg. Chaturvedi et al. (2012) showed that prior treatment with a bacterial pathogen promotes resistance against Fg in Arabidopsis, which has been utilized in several studies as a model plant to characterize the physiological and molecular aspects of plant defense against Fg (Chen et al., 2006, 2009; Cuzick et al., 2008; Makandar et al., 2006, 2010, 2015; Nalam et al., 2015; Savitch et al., 2007; Skadsen and Hohn, 2004; Urban et al., 2002; Van Hemelrijck et al., 2006). Fg can infect leaves and inflorescences of Arabidopsis.

The PTI pathway has been implicated as a major player in the resistance to Fusarium ear rot in the maize inbred line BT-1 (Wang et al., 2016). Similarly, basal resistance to FHB in barley requires HvCERK1 (Karre et al., 2017), thus suggesting that the PTI pathway is engaged during Fg infection. The aim of this study was to determine whether PTI can be targeted to enhance resistance against Fg. We show that Arabidopsis can be protected against Fg infection by flg22-mediated induction of PTI via FLS2. This resistance to Fg infection conferred by flg22 requires WRKY29 which, when constitutively expressed in Arabidopsis, confers a high level of resistance to Fg. We further demonstrate that flg22 application and constitutive expression of Arabidopsis WRKY29 confer enhanced resistance to FHB in wheat, which is accompanied by stronger expression of PTI-associated genes, thus supporting our suggestion that the PTI pathway is a target for enhancing resistance to FHB.

**RESULTS**

*Fg infection induces WRKY29 expression in A. thaliana*

The expression of WRKY29 was used as a molecular marker of PTI to test whether Fg infection induces a PTI-like mechanism in Arabidopsis. Fg was infiltrated into Arabidopsis leaves and WRKY29 expression was monitored by real-time reverse transcription-polymerase chain reaction (RT-PCR). flg22
peptide-treated leaves provided the positive control for WRKY29 expression. In addition, expression of the Fg- and flg22-responsive PATHOGENESIS-RELATED 1 (PR1) (Asai et al., 2002; Makandar et al., 2006; Yi et al., 2014) was monitored as a positive control for the two treatments. Expression of PR1, which encodes a cysteine-rich secretory protein, has been used as an excellent molecular marker for the activation of SA signalling in plants. As shown in Fig. 1A, PR1 and WRKY29 expression were up-regulated in Fg- and flg22-treated leaves compared with the untreated and mock-inoculated controls, thus confirming the activation of downstream signalling by these treatments. Fg infection also resulted in the accumulation of hydrogen peroxide (H$_2$O$_2$), another hallmark of PTI (Fig. 1B). Taken together, these results suggest that Arabidopsis responds to Fg infection by stimulating a PTI-like response.

The flg22 peptide induces resistance against Fg infection in A. thaliana and wheat

To determine whether the PTI pathway can be targeted to enhance resistance against Fg, we tested whether pretreatment of Arabidopsis leaves with the flg22 peptide is capable of augmenting resistance to Fg. Leaves of wild-type (WT) Arabidopsis accession Columbia plants were infiltrated with flg22 peptide to activate PTI; 24 h later, the same leaves were inoculated with Fg and disease severity was scored at 5 days post-inoculation (dpi). As shown in Fig. 2A, Fg disease severity was significantly lower in flg22-treated leaves than in mock-treated leaves, thus suggesting that an flg22-activated mechanism can enhance resistance against Fg. We further tested basal resistance to Fg in transgenic Arabidopsis engineered to express a chimeric PR1-flg22 construct that expresses flg22 fused to the C-terminus of PR1. As mentioned above, PR1 is a secretory protein that accumulates in the apoplast (Gu and Innes, 2012; Pečenková et al., 2017; Watanabe et al., 2013). Furthermore, the activation of SA signalling promotes the export of PR1 into the apoplast (Wang et al., 2005). Thus, the PR1-flg22 fusion is expected to deliver flg22 into the apoplast, where it should be perceived by FLS2 to activate PTI. As shown in Fig. 2B, Fg disease severity was lower in leaves of two independently derived PR1-flg22-expressing transgenic lines compared with the WT control (Figs 2B and S1, see Supporting Information). Disease severity was also lower in the inflorescence of PR1-flg22 lines compared with the WT control (Fig. 2B). These results confirm that an flg22-activated mechanism can confer resistance to Fg infection in Arabidopsis.

We further tested whether flg22 application was capable of promoting FHB resistance in wheat. Varying amounts of the flg22 peptide dissolved in 10 µL of water were applied with a syringe to two central spikelets of each spike of the spring wheat cultivar Bobwhite; 24 h later, these spikelets were inoculated with Fg and FHB disease severity was monitored 21 days later. A Ubi:NPR1 wheat line, which is in the Bobwhite background and constitutively expresses the Arabidopsis NPR1 gene from the maize Ubiquitin promoter to increase FHB resistance (Makandar et al., 2006, 2012), provided the disease-resistant control for this experiment. As shown in Fig. 2C, pretreatment with flg22 peptide enhanced FHB resistance in the wheat cultivar Bobwhite. The resistance-promoting effect of flg22 exhibited a dose-dependent response. At the highest level of 200 ng, the FHB resistance-promoting effect of flg22 was comparable with that observed in the Ubi:NPR1 line. Taken together, these experiments with Arabidopsis and wheat signify the potential for targeting PTI to enhance resistance against Fg.

**FLS2 is required for flg22-induced resistance to Fg infection in A. thaliana**

To confirm that the flg22-induced resistance to Fg was indeed a result of the activation of PTI, the ability of flg22 to enhance...
Resistance to Fusarium graminearum resistance to Fg in the fls2 mutant was studied. WRKY29 expression was monitored as a molecular marker for the activation of PTI. As shown in Fig. 3A, although flg22 treatment, compared with mock treatment, was effective in inducing WRKY29 expression in the WT plant, flg22 was unable to induce WRKY29 expression in the fls2 mutant, thus confirming the requirement of FLS2 for the flg22-induced expression of WRKY29. Compared with the WT, the Fg resistance-promoting effect of flg22 was not observed in the fls2 mutant (Fig. 3B). The Fg disease severity in leaves of the flg22-treated fls2 mutant was comparable with that in the mock-treated fls2 mutant and significantly higher than that in flg22-treated WT plants. Experiments with the PR1-flg22 chimera also confirmed the importance of FLS2 to flg22-induced resistance to Fg, which was lacking in the fls2 mutant background compared with the FLS2 background (Fig. 3C).

Although the leaf disease index, which reflects the average disease severity across the different disease categories (see Experimental procedures), was not significantly different between the WT and fls2 plants that were not treated with flg22 (Fig. 3B,C), we repeatedly observed significant differences (P < 0.05; χ² test) in the distribution of the four disease categories in the fls2 mutant compared with the WT. We therefore suggest that there is a subtle influence of the FLS2 allele on the basal resistance to Fg in Arabidopsis.

flg22-induced resistance to Fg in A. thaliana requires NPR1 and WRKY29

SA signalling has an important function in Arabidopsis and wheat defence against Fg (Diethelm et al., 2014; Gao CS et al., 2013; Makandar et al., 2006, 2010, 2012, 2015; Yu et al., 2017). In Arabidopsis, SA signalling is also induced in response to flg22 (Tsuda et al., 2008; Yi et al., 2014). Furthermore, SA stimulates FLS2 expression and SA analogues prime the induction of flg22-triggered responses, including flg22-triggered up-regulation of WRKY29 expression (Pick et al., 2012; Yi et al., 2014). In contrast, flg22-triggered responses, including WRKY29 expression, are attenuated in the SA biosynthesis sid2 mutant (Yi et al., 2014). To determine whether SA signalling is critical for the flg22-conferred resistance to Fg, we tested the ability of flg22 to promote resistance to Fg in the SA-insensitive npr1 mutant. 35S: NPR1 plants, which constitutively express NPR1 from the Cauliflower mosaic virus 35S promoter (Cao et al., 1998; Makandar et al., 2006), provided the Fg-resistant control for this experiment. As shown in Fig. 4A and reported previously (Makandar et al., 2010), Fg disease severity was higher in leaves of the npr1 mutant than in the WT plant. Furthermore, flg22 was unable to enhance resistance to Fg in the npr1 mutant compared with the WT, thus confirming that flg22-induced PTI cannot bypass the need for SA signalling in defence against Fg. Similarly, WRKY29 function was required for defence against Fg (Fig. 4A). Compared with the WT, Fg disease severity was higher in the wrky29 mutant and comparable with
that in the npr1 mutant. Furthermore, unlike in the WT, flg22 was unable to promote resistance in the wrky29 mutant. These results provide further confirmation that the flg22-conferred resistance to \( F_g \) is mediated through genetic components that function downstream of the FLS2/flg22 receptor/ligand pair. The higher level of disease in the wrky29 mutant compared with the WT further indicates that a \( WRKY29 \)-dependent defence mechanism(s) is critical for basal resistance to \( F_g \).

**Constitutive expression of \( WRKY29 \) enhances resistance to \( F_g \) in \( A.\ thaliana \)**

We further tested the feasibility of engineering the PTI pathway to enhance resistance against \( F_g \) by developing plants that constitutively express \( WRKY29 \), which encodes a transcription factor that is common to PTI induced by flg22 and chitin (Asai et al., 2002; Wan et al., 2008). The Cauliflower mosaic virus 35S promoter was used to constitutively express \( WRKY29 \) in Arabidopsis (Fig. 4B). Compared with the WT plant, \( F_g \) disease severity and fungal accumulation, which was monitored by comparing the accumulation of \( F_g NahG \) gene DNA, were significantly lower in leaves of all three independently derived 35S:WRKY29 plants (Fig. 4B). Similarly, \( F_g \) disease severity was also lower in the inflorescence tissues of 35S:WRKY29 plants compared with the WT (Fig. 4C). These results provide further proof-of-concept that the PTI pathway is amenable for engineering resistance to \( F_g \). Compared with the WT, basal expression of the SA- and flg22-responsive \( PR1 \) gene was not altered in 35S:WRKY29 plants (Fig. 4D). However, fungal infection resulted in significantly stronger induction of \( PR1 \) in 35S:WRKY29 plants than in the WT (Fig. 4D), therefore indicating that constitutive expression of \( WRKY29 \) promotes robust activation of defence responses. In contrast, constitutive expression of \( WRKY29 \) did not result in stronger accumulation of \( \text{H}_2\text{O}_2 \) in response to fungal infection (Fig. S2A, see Supporting Information).

**Wheat engineered to express \( WRKY29 \) exhibits enhanced resistance to FHB and seedling blight**

To study the feasibility of targeting \( WRKY29 \) expression for the engineering of FHB resistance in wheat, we developed transgenic wheat plants containing a chimeric \( Ubi:WRKY29 \) construct, which constitutively expresses the Arabidopsis \( WRKY29 \) coding sequence (CDS) from the maize Ubiquitin promoter. Three independently derived \( Ubi:WRKY29 \) transgenic lines that stably express \( WRKY29 \) were identified (Fig. 5A). All three lines showed significantly higher level of resistance to FHB compared with the control cv. Bobwhite. Disease spread and fungal growth were restricted in the \( Ubi:WRKY29 \) plants compared with the non-transgenic Bobwhite (Fig. 5A,B). In addition, the accumulation of the mycotoxin DON was also significantly lower in the transgenic \( Ubi:WRKY29 \) lines than in the control cv. Bobwhite (Fig. 5C). Basal expression of the SA-responsive \( TaPR1.2 \), as well as the PTI marker genes \( TaWRKY70 \) and \( TaPUB-23 \)-like (Kage et al., 2017; Schoonbeek et al., 2015), was very low and not altered in the \( Ubi:WRKY29 \) plants compared with the non-transgenic Bobwhite.
Resistance to Fusarium graminearum

Thus suggesting that, as in Arabidopsis, constitutive expression of *WRKY29* is not sufficient to constitutively activate PTI. However, in response to *Fg* infection, *WRKY29* expression conferred strong expression of *TaPR1.2*, *TaWRKY70* and *TaPUB23*-like in the *Ubi:WRKY29* relative to non-transgenic Bobwhite plants (Fig. 6A). In contrast, as in Arabidopsis, constitutive expression of *WRKY29* in wheat did not promote stronger *H₂O₂* accumulation in response to fungal infection (Figs 6B and S2B).

The *Ubi:WRKY29* plants also demonstrated elevated resistance to Fusarium seedling blight disease (Fig. 7). Taken together, the above results validate our suggestion that the PTI mechanism provides an excellent target for enhancing plant resistance against *Fg*.

**Impact of constitutive WRKY29 expression on agronomic and growth parameters of wheat**

As shown in Fig. 8A, the *Ubi:WRKY29* wheat plants were 25% shorter than the non-transgenic Bobwhite plants. This was paralleled by an earlier heading time in the *Ubi:WRKY29* plants compared with Bobwhite (Fig. 8B). No significant impact on other agronomic parameters, such as the number of spikes produced per plant, the number of seeds produced per spike and seed yield per plant, was observed in *Ubi:WRKY29* wheat compared with the control Bobwhite plants (Fig. 8A).

**DISCUSSION**

In the absence of monogenic gene-for-gene resistance, current control measures for FHB in wheat and barley involve the use of partially resistant varieties combined with fungicide application and management practices (Bai and Shaner, 2004; Wilson et al., 2017). Limited knowledge of plant mechanisms that can be targeted to enhance FHB resistance has constrained progress on the development of FHB-resistant wheat and barley varieties. Previously, using an approach that utilized the
interaction between Arabidopsis and Fg to identify genes and mechanisms that contribute to defence against Fg, and transgenic validation of the ability of these genes/mechanisms to control Fg infection in Arabidopsis and wheat, we showed that the SA signalling pathway provides a target for enhancing FHB resistance (Makandar et al., 2006, 2010, 2015, 2012). Recent studies have confirmed that genes associated with SA biosynthesis in barley and alleles at NPR1-like genes in winter wheat are associated with basal resistance to FHB (Diethelm et al., 2014; Hao et al., 2018). Utilizing a similar approach, here we demonstrate that the PTI pathway provides a target for enhancing resistance against Fg. We show that resistance against Fg can be enhanced in wheat and Arabidopsis by the bacterial MAMP flg22. In Arabidopsis, the flg22-conferred resistance to Fg required the PTI-associated PRR FLS2. Resistance against Fg was also enhanced in wheat and Arabidopsis by the constitutive expression of WRKY29, which encodes a transcription factor that is associated with PTI in Arabidopsis. Expression of the Arabidopsis WRKY29 CDS also restricted DON accumulation in Ubi:WRKY29 transgenic wheat lines. Compared with the non-transgenic Bobwhite, the Ubi:WRKY29 wheat plants responded to Fg infection with stronger expression of TaPR1.2 and the PTI marker genes TaWRKY70 and TaPUB-23-like, thus suggesting stronger activation of PTI responses. These results, when considered together with the results of Kage et al. (2017), who showed that the TaWRKY70 gene contributes to basal resistance to FHB, demonstrate that the PTI pathway can be targeted to enhance resistance against Fg.

The fact that Fg infection stimulates WRKY29 expression to comparably high levels in the WT and fls2 mutant suggests that another pathway, presumably involving another PRR and its cognate ligand, stimulates PTI leading to WRKY29 expression in Fg-inoculated Arabidopsis. The Fg infection-derived elicitor that induces WRKY29 could potentially be chitin. Previously, a chitooligosaccharide was shown to induce WRKY29 expression in Arabidopsis and to promote resistance against fungal and bacterial pathogens (Wan et al., 2008). Chitosan treatment also promoted resistance to seed-borne Fg infection in wheat (Bhaskara Reddy et al., 1999). Chitosan promoted the accumulation of lignin precursors and phenolics that have antimicrobial activity and could potentially contribute to resistance (Bhaskara Reddy et al., 1999). Chitin also induced the expression of wheat homologues of PTI-associated genes (Schoonbeek et al., 2015).
More recently, a metabolol-transcriptomic approach in barley identified HvCERK1, a predicted chitin elicitor receptor kinase encoding gene, to be involved in defence against FHB (Karre et al., 2017). In Arabidopsis, WRKY29 expression is also induced by the Fusarium T-2 toxin and other type A trichothecenes (Nishiuchi et al., 2006). These toxins, or derived metabolites, could also potentially function as elicitors of PTI in plants infected with Fg.

**WRKY genes** include a large family of plant-specific DNA-binding proteins that contain the conserved WRKYQGK sequence together with a zinc-finger-like motif (Eulgem et al., 2000; Pandey and Somssich, 2009). Several of these WRKY proteins are involved in the stress response, including plant defence against pathogens. Although some are positive regulators, others are negative regulators of the stress response (Pandey and Somssich, 2009). In Arabidopsis, WRKY29 is associated with PTI and defence against pathogens (Asai et al., 2002; Wan et al., 2008). Our results indicate an important role for WRKY29 in the control of Fg infection. Not only was WRKY29 expression up-regulated in response to Fg infection, but, compared with the WT, Fg disease severity was higher in the wrky29 mutant and lower in plants constitutively expressing WRKY29. As Arabidopsis WRKY29 was capable of similarly enhancing disease resistance in wheat, we propose that wheat contains the downstream machinery that is regulated by WRKY29. WRKY homologues are present in wheat and barley, and some of these WRKYS have been shown to confer stress tolerance when constitutively expressed in heterologous systems (Liu et al., 2014; Niu et al., 2012; Wang et al., 2017, 2015). Recently, a wheat WRKY gene, TaWRKY70 (also known as TaWRKY49), was shown to be required for basal resistance to FHB (Kage et al., 2017). TaWRKY70 is associated with QTL-2DL, which limits FHB severity by the control of fungal spread from the site of initial infection (Kage et al., 2017). TaWRKY70 expression is up-regulated in FHB-resistant near-isogenic lines (NILs) compared with susceptible NILs. TaWRKY70 expression is also up-regulated in response to infections with Puccinia triticina, which causes leaf rust, and the powdery mildew fungus Blumeria graminis (Bahrini et al., 2011b). Wheat plants over-expressing TaWRKY70 exhibit enhanced resistance to FHB, powdery mildew and leaf rust (Bahrini et al., 2011a, b), thus suggesting that TaWRKY70 is involved in defence against a variety of fungal infections. TaWRKY70 has been suggested to regulate the expression of genes involved in the synthesis of metabolites that are associated with resistance to fungi (Kage et al., 2017). Our results indicate that Arabidopsis WRKY29, when expressed in wheat, promotes the stronger activation of the PTI pathway, leading to the expression of TaWRKY70.

**FLS2** is not required for the Fg-induced up-regulation of WRKY29 or for the flg22-induced resistance to Fg in Arabidopsis. Furthermore, the Arabidopsis leaf disease index, which reflects the average of disease severity across the different leaf disease categories, was not significantly different between the
Fg-inoculated WT and fls2 mutant, thus suggesting that FLS2 does not have a major contribution to basal resistance to Fg in Arabidopsis. However, a significant difference ($P < 0.05$; $\chi^2$ test) was observed between the WT and fls2 mutant for the relative distribution of leaves over the four disease categories, suggesting a subtle impact of FLS2 on basal resistance to Fg.

Plant-associated microbes are known to prime plant defences (Conrath et al., 2015; Pieterse et al., 2014). This priming of FLS2-dependent defences by random plant-associated microbes may influence basal resistance to Fg in Arabidopsis.

The results presented here, taken together with the knowledge that TaWRKY70 and the LysM domain-containing HvCERK1 are required for basal resistance to FHB in wheat and barley, respectively (Kage et al., 2017; Karre et al., 2017), lead us to propose that genes associated with the PTI pathway are good candidates for the development of FHB-resistant wheat and barley. Alternatively, factors that can induce the PTI pathway could also promote FHB resistance. However, the impact of PTI pathway activation needs to be tested on additional biotypes of Fg to determine whether it is effective against the different chemotypes of Fg, as well as other FHB-causing Fusarium species. Ubi:WRKY29 wheat also exhibits reduced plant height and faster heading time, without any detrimental effects on yield. Height and heading time are traits that are important to wheat breeding (Hedden, 2003; Wilhelm et al., 2013). Thus, the pathway targeted by WRKY29 has the potential to influence additional beneficial traits for wheat breeding.

**EXPERIMENTAL PROCEDURES**

**Cultivation of Arabidopsis and wheat**

A peat-based soil mix (Fafard #2, Sungro, Agawam, MA) was used to cultivate Arabidopsis and wheat. Arabidopsis was cultivated as described previously (Nalam et al., 2016) in growth chambers programmed for 22 ºC and a 14-h light (80–100 µE/m²/s) and 10-h dark regime. The soil was autoclaved for 1 h prior to use. Arabidopsis npr1-1, wrky29 (CS3024690) and fls2-101 mutants, and the 35S:NPR1 transgenic lines and the wheat Ubi:NPR1 transgenic line in the cultivar Bobwhite, have been described previously (Cao et al., 1998; Li et al., 2017; Makandar et al., 2006; Pfund et al., 2004). Generation of the Arabidopsis 35S:WRKY29 and 35S:PR1-flg22 lines and wheat Ubi:WRKY29 lines is described below. Wheat was cultivated in a glasshouse in which natural sunlight was supplemented with halogen lamps to provide a minimum of 14 h exposure to light. The glasshouse was programmed for day/night-time temperatures of 21 ºC and 18 ºC, respectively.

**Pathogen strains, culture conditions and plant infection**

Half-strength potato dextrose medium (Difco Laboratories, Detroit, MI, USA) was used for the growth and maintenance of Fg isolate Z-3639, and carboxymethylcellulose (CMC) medium was used to promote sporulation, as described previously (Nalam et al., 2016). The fungus was cultivated at 28 °C. Fungal inoculation of Arabidopsis leaves involved infiltration of a suspension of fungal mycelial fragments through the abaxial surface with a needleless syringe (Nalam et al., 2016). Approximately 4-week-old Arabidopsis plants were used for leaf assays and 6–7-week-old plants for inflorescence assays. Disease severity was scored at 5 dpi, unless stated otherwise. Depending on the extent of chlorosis, leaves were grouped into four categories: Category I, chlorosis covering <25% of leaf area; Category II, chlorosis covering 25%–50% of leaf area; Category III, chlorosis covering 50%–75% of leaf area; Category IV, chlorosis covering 75%–100% of leaf area. A minimum of 50 leaves of each genotype/treatment were analysed for each experiment. The
percentage of leaves in each category was used to calculate the leaf disease index, as described previously (Nalam et al., 2016). Inoculations of Arabidopsis inflorescences with Fg macroconidia and the disease rating [expressed as the Fusarium Arabidopsis Disease (FAD) score] were conducted as described previously (Nalam et al., 2016).

Inoculations of wheat spikelets with Fg macroconidia and disease evaluation were performed as described previously (Makandar et al., 2006, 2012). Briefly, at the anthesis stage, two central spikelets were inoculated with 10 µL of a suspension containing 300 fungal macroconidia. High humidity was maintained for 3 days by covering the inoculated spikes with a moistened zip-lock bag. Over time, the fungal infection spread out to the other spikelets within each spike. Disease spread was monitored at periodic intervals. The final reading was taken at 21 dpi and the disease severity was calculated as the percentage of diseased spikelets.

To study the effect of wheat genotypes on Fusarium seedling blight, seeds of the indicated lines were soaked with an Fg macroconidial suspension for 24 h. In addition, after germination, seedlings were spray inoculated with a macroconidial suspension (100 000 macroconidia/mL) and covered for 3 days. The percentage of surviving seedlings was determined at 14 dpi.

**flg22 peptide treatment**

A needleless syringe was used to infiltrate 50 ng of flg22 peptide (QRLSTGSRINSKDDAAGLQIA; Alpha Diagnostic, International Inc., San Antonio, TX; Cat# FLG22-p-1) dissolved in 20 µL of water through the abaxial surface of Arabidopsis leaves. Water-infiltrated leaves provided the controls. After 24 h, the leaves were harvested for RNA isolation or treated with Fg mycelial fragments to monitor the impact of PTI activation on fungal disease. For experiments with wheat, flg22 peptide (50, 100 and 200 ng), dissolved in 10 µL of water, and water as control, were applied with a Hamilton syringe to the central spikelet of each spike.

**Mycotoxin analysis**

DON content in wheat grains was determined as described previously (Fuentes et al., 2005; Mirocha et al., 1998).

**Arabidopsis and wheat transgenics**

The 35S:NPR1 Arabidopsis and Ubi:NPR1 wheat plants used in this study have been described previously (Cao et al., 1998; Makandar et al., 2006). Three PCR steps were used to generate
the PR1-flg22 chimera, such that the flg22 peptide fused to the apoplast-localized PR1 protein could be delivered to the extracellular space. In the first PCR, cDNA prepared from Arabidopsis leaves was used to amplify the PR1 CDS (At2g14610) with the primers PR1-CDS-F (BamHI) and flg22-R (ClaI) to give a 513-bp amplicon containing the PR1 CDS, without the stop codon, followed by the coding information for the first seven amino acids of flg22. In a second PCR, the flg22-F and flg22-R 66-mer oligos, which contain the coding information for the flg22 peptide (QRLSTGSRINSKDDAALQIA), were mixed with the PR1(21)-flg22(12)-F and flg22-R(ClaI) primers (Table S1, see Supporting Information) which, on PCR, yielded a 99-bp product which, at one end, contained the information for the last seven amino acids of PR1 fused to flg22 with a stop codon included at the end of the flg22 CDS. The products of the previous two PCRs were mixed in equal proportions and used in PCR with the primers PR1-CDS-F (BamHI) and flg22-R (ClaI) (Table S1) to yield a 570-bp product that includes the PR1 CDS fused at its C-terminal end to the flg22 CDS with a stop codon included. This final product was cloned into the pCR8/GW/TOPO entry vector (Life Technologies, Carlsbad, CA; www.lifetechnologies.com) from which the insert was mobilized into the destination binary vector pMDC32 with the Gateway LR clonase system (Life Technologies, Carlsbad, CA; www.lifetechnologies.com) from which the insert was mobilized into the destination binary vector pMDC32 with the Gateway LR clonase system (Life Technologies; www.lifetechnologies.com). The resultant pMDC32:PR1-flg22 plasmid with the Gateway LR clonase system (Life Technologies; www.lifetechnologies.com) was mobilized into the destination binary vector pMDC32 from which the in-cloned into the pCR8/GW/TOPO entry vector (Life Technologies, Carlsbad, CA; www.lifetechnologies.com) from which the insert was mobilized into the destination binary vector pMDC32 with the Gateway LR clonase system (Life Technologies; www.lifetechnologies.com). The resultant pMDC32:PR1-flg22 plasmid contains the PR1-flg22 chimera between the Cauliflower mosaic virus 35S promoter and Agrobacterium tumefaciens nos (nopaline synthase) gene terminator. This construct was transformed into Arabidopsis accession Columbia and the fls2 mutant by the floral dip method (Zhang et al., 2006). Hygromycin-resistant transformants containing the 35S:PR1-flg22 chimera were selected in the presence of hygromycin (25 mg/L). The presence of the insert was confirmed by PCR and expression was tested by RT-PCR.

The WRKY29 (At4g23550) CDS was amplified from cDNA prepared from flg22-inoculated leaves with the primers WRKY29-CDS-F (BamHI) and WRKY29-CDS-R (ClaI) (Table S1). The resultant amplicon was cloned into the pMD32 vector via the pCR8/GW/TOPO intermediate, as described above for 35S:PR1-flg22. The resultant plasmid pMD32-WRKY29 contains the WRKY29 CDS flanked on its 5’ end by the 35S promoter and at the 3’ end by the nos terminator. This construct was transformed into Arabidopsis accession Columbia by the floral dip method (Zhang et al., 2006). Hygromycin-resistant transformants were selected as described above for 35S:PR1-flg22. The presence of the 35S:WRKY29 transgene in Arabidopsis was monitored by PCR conducted with 35S-F and WRKY29-R (ClaI) primers (Table S1).

The Ubi::WRKY29 chimeric gene was generated by cloning the WRKY29 CDS amplicon, generated as described above, into the pJS406 backbone (Makandar et al., 2006). The resultant pSS:Ubi::WRKY29 plasmid contains the WRKY29 CDS flanked on the 5’ end by the maize Ubi gene promoter plus intron (Christensen and Quail, 1996) and on the 3’ end by the nos terminator. To generate transgenic wheat, the pSS: Ubi::WRKY29 plasmid and the bar selectable marker containing plasmid pAH20 (Christensen and Quail, 1996) were co-bombarded into embryos from the spring wheat cv. Bobwhite, and wheat plants were regenerated as described previously (Anand et al., 2003). Glufosinate (Liberty; Bayer Crop Sciences, Research Triangle, NC, USA) resistance conferred by the bar gene was used as the selectable marker for transgenic wheat. The presence of the Ubi::WRKY29 transgene was monitored by PCR using the WRKY29-F and WRKY29-R primers (Table S1). PCR conditions included a 5-min denaturation at 95 ºC, followed by 50 cycles of 95 ºC for 45 s, 55 ºC for 45 s and 72 ºC for 60 s, with a final extension of 72 ºC for 7 min.

DNA and RNA isolation

DNA for PCR and genotyping was extracted from leaf tissue as described previously (Nalam et al., 2016). RNA was extracted from frozen tissues using an acidic guanidinium thiocyanate–phenol–chloroform mix (Chomczynski and Sacchi, 1987).

RT-PCR and real-time PCR

After removal of DNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA), the purified RNA was used for cDNA synthesis with oligo-dT 18-mer primer (New England Biolabs, Ipswich, MA, USA) and GoScript™ reverse transcriptase (Promega). The cDNA was subsequently utilized for RT-PCR and quantitative real-time RT-PCR.

The primer pairs WRKY29-qRT-F plus WRKY29-qRT-R, and EF-qRT-F plus EF-qRT-R (Table S1), were used for RT-PCR to monitor the expression of Arabidopsis WRKY29 and At1g07940, respectively. At1g07940, which encodes an elongation factor related to EF-1α, was previously identified as a gene that is very suitable for the normalization of gene expression (Czechowski et al., 2005). The PCR conditions for WRKY29 included a 3-min denaturation at 95 ºC, followed by 25 cycles of 95 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 30 s, with a final extension of 72 ºC for 5 min. The PCR conditions for At1g07940 included a 3-min denaturation at 95 ºC, followed by 25 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, with a final extension of 72 ºC for 5 min. RT-PCR was used to monitor the expression of PR1-flg22 from the 35S:PR1-flg22 construct in Arabidopsis. The primer pairs PR1-CDS-F (BamHI) and flg22-R (ClaI) (Table S1) were used in the PCR. Expression of the Arabidopsis ACT8 gene was monitored as control with the primer pair ACT8-RT-F plus ACT8-RT-R (Table S1). The PCR conditions included a 3-min denaturation at 95 ºC, followed by 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 45 s, with a final extension of 72 ºC for 7 min. To monitor WRKY29 expression from the Ubi::WRKY29 construct in wheat, the primer pairs WRKY29-F plus WRKY29-R (Table S1) were used for amplification in the
RT-PCRs. Expression of the wheat TaTUBB2 (Tubulin beta-2) gene was used as control for RT-PCR. The primer pair TaTUBB2-F and TaTUBB2-R (Table S1) was used in the PCR. The PCR conditions for WRKY29 expression derived from the Ubi:WRKY29 chimera in wheat included a 3-min denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, with a final extension of 72 °C for 5 min. The PCR conditions for TUBB2 included a 3-min denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min.

Quantitative real-time RT-PCR was performed with Sybr Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) following amplification conditions: 10-min polymerase activation and denaturation at 95 °C, 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s. This was followed by a product melt to confirm a single PCR product. The level of individual gene expression was normalized to that of At1g07940 by subtracting the cycle threshold value for At1g07940 from the cycle threshold value of the test genes. Fold induction, when calculated, was relative to expression in the mock-treated plants. The primer pairs WRKY29-qRT-F plus WRKY29-qRT-R, and EF-qRT-F plus EF-qRT-R (Table S1), were used for real-time RT-PCR to monitor the expression of Arabidopsis WRKY29 and At1g07940, respectively. For qRT-PCR analysis of wheat TaPR1.2, TaWRKY70 and TaPUB-23-like, the primer pairs TaPR1.2-F plus TaPR1.2-R, TaWRKY70-F plus TaWRKY70-R, and TaPUB-23-F plus TaPUB-23-R, respectively, were used (Table S1). The expression of these genes was normalized to that of TaTUBB2.

**Histological examination for H$_2$O$_2$ accumulation**

*In situ* accumulation of H$_2$O$_2$ was monitored by staining leaves with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St Louis, MO, USA) using a protocol developed by Thordal-Christensen et al. (1997) as modified by Gao X et al. (2013). Briefly, Arabidopsis leaves were infiltrated with Fg, whereas wheat leaves were first pierced with a needle and Fg spores were left in the growth room until ready for harvest. On harvest, leaves were boiled in 95% ethanol for 20 min, whereas wheat leaves were boiled for 30 min. Destained leaves were stored in 70% ethanol. A similar process was used for staining wheat spikes, except that the destaining utilized 70% ethanol and was conducted overnight in a shaker at 70 °C. The destained leaves were observed under a light microscope.

**Statistical analysis**

Two-tailed Student’s *t*-test was used to determine the significance of variance (*P* < 0.05) when comparing two treatments or genotypes. The $\chi^2$ test was used to determine whether the differences between disease categories on Arabidopsis leaves were significantly different (*P* < 0.05) between two genotypes and treatments. Analysis of variance (ANOVA) following the General Linear Model, followed by Tukey’s multiple comparison test, was used to determine the significance of variance (*P* < 0.05; Minitab v15; www.minitab.com) when comparing multiple genotypes and/or treatments with each other.

**Accession numbers**

At4g23550 (Arabidopsis WRKY29), At5g46330 (Arabidopsis FLS2), At1g07940 (Arabidopsis GTP-binding Elongation factor Tu family), At2g14610 (Arabidopsis PR1), At1g49240 (Arabidopsis ACT8), U76745 (wheat TUBB2), AJ007349 (wheat PR1.2), BQ743320 (wheat PUB-23-like), AB603890 (wheat WRKY70), FGSG_0811 (Fg NahG).

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**CONFLICTS OF INTEREST**

The authors declare that there are no conflicts of interest.

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reaction (RT-PCR) analysis of the PR1-flg22 chimeric transcript and, as control, the Arabidopsis ACT8 gene in the wild-type (WT) accession Columbia and two independent PR1-flg22 lines #2 and #5 in the FLS2 background. Bottom: PR1-flg22 and ACT8 expression in the fls2 mutant and two independent PR1-flg22 lines #2 and #5 that are in the fls2 mutant background.

**Fig. S2** 3,3′-Diaminobenzidine (DAB) staining for H₂O₂ accumulation in *Fusarium graminearum*-inoculated Arabidopsis and wheat leaves. (A) H₂O₂ accumulation in *F. graminearum*-inoculated leaves of wild-type (WT) accession Columbia and three independent 35S:WRKY29 transgenic Arabidopsis lines. (B) H₂O₂ accumulation in *F. graminearum*-inoculated leaves of wheat cv. Bobwhite (Bw) and two independent Ubi:WRKY29 transgenic lines #317 and #1081. In (A) and (B), leaves were harvested for DAB staining at 2 and 6 h post-inoculation.

**Table S1** Primers used in this study.