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TaWIR1 contributes to post-penetration resistance to *Magnaporthe oryzae*, but not *Blumeria graminis* f. sp. *tritici*, in wheat

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SUMMARY

Members of the *Wheat-Induced Resistance 1* (*TaWIR1*) gene family are highly induced in response to a wide range of pathogens. Homologues have been identified in barley, but not in *Brachypodium*, whereas, in rice, only distant *WIR1* candidates are known. Phylogenetic analysis placed *TaWIR1a* and *TaWIR1b* within a distinct clade of wheat transcripts, whereas *TaWIR1c* clustered with *HvWIR1* genes. Transcripts of all three *TaWIR1* genes were strongly induced by a wheat-adapted isolate of *Magnaporthe oryzae*. Virus-induced gene silencing of the *TaWIR1* gene family had no effect on the initial penetration of epidermal cells by *M. oryzae*. However, following the establishment of an infection site, the fungus was able to grow more extensively within the leaf tissue, relative to control leaves, indicating a role for the *TaWIR1* gene family in the cell-to-cell movement of *M. oryzae*. In contrast, the silencing of *TaWIR1* transcripts had no effect on epidermal cell penetration by a wheat-adapted isolate of *Blumeria graminis*, or on the subsequent growth of hyphae. Differential transcription of *TaWIR1* genes was also seen in epidermal peels, relative to the remaining leaf tissue, following inoculation with *M. oryzae*.

INTRODUCTION

In a world economy faced with global food insecurity, the demand for increased agricultural production has never been greater. Together with rice and maize, wheat provides a substantial proportion of the calorific intake of the human population, either directly or through livestock feed (http://faostat.fao.org). Disease presents a major constraint to wheat production, causing significant yield losses (Kosina *et al*., 2007). The most effective means of disease control is by the informed use of resistance genes through conventional breeding. A greater understanding of how individual genes contribute to resistance would advise breeders as to which combinations of resistance genes would prove to be most effective, providing long-term resistance.

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Plants have evolved elegant molecular defence mechanisms that can be broadly considered to operate at two stages. The first is an early, broad defence response that recognises potential pathogens through the detection of conserved molecules, termed microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) (Zipfel, 2009). This receptor-mediated recognition leads to PAMP-triggered immunity (PTI), which functions to limit the extent of initial pathogen penetration and invasion (Jones and Dangl, 2006). The second layer of defence involves plant resistance (R) proteins that have evolved to recognise specific pathogen-derived effector molecules that function to suppress PTI and promote pathogen colonisation. This effector-triggered immunity (ETI) is typically race specific and is often mediated by nucleotide-binding site leucine-rich repeat (NBS-LRR)-type *R* genes (Jones and Dangl, 2006). When a pathogen successfully evades both PTI and ETI, a compatible interaction is established.

In addition to the many race-specific *R* genes identified in wheat, resistance loci that confer race-nonspecific resistance have also been identified (Boyd, 2006; Singh *et al*., 2011). These genes often confer a partial resistance, and the recent cloning of the well-characterised leaf rust (causal agent *Puccinia triticina*) race-nonspecific resistance *Lr34* has identified a gene that encodes for a multidrug ABC transporter (Krattinger *et al*., 2009). Similarly, in barley, the *Rphq11* gene for partial, race-nonspecific resistance to leaf rust has been cloned, and encodes for a phospholipid hydroperoxide glutathione peroxidase (Chen *et al*., 2010). The mapping of defence-related genes in barley, including barley homologues of the *Wheat-Induced Resistance 1* (*TaWIR1*) genes, has shown co-localisation with partial resistance loci for leaf rust and powdery mildew, further supporting the value of these defence-related genes as candidates for partial resistance amenable to resistance breeding (Aghnoum *et al*., 2010; Douchkov *et al*., 2011; Marcel *et al*., 2007).

The *TaWIR1* gene family was first identified following inoculation of the wheat cv. Fidel with a nonadapted, barley isolate of the powdery mildew pathogen *Blumeria graminis* (Schweizer *et al*., 1989). Two sequences were discovered, *TaWIR1a* and *TaWIR1b* (Bull *et al*., 1992). Subsequently, a third sequence, *TaWIR1c*, was identified (Franck and Dudler, 1995). *TaWIR1* encodes for a small glycine- and proline-rich protein, the N-terminus of which has a potential signal peptide or membrane-spanning region. The

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negative charge difference between the amino acids in the C-terminal region and those flanking the N-terminal region suggests that the C-terminal region is extracytoplasmic. This, together with the abundance of glycine and proline residues, which may support cell wall contact, led Bull *et al*. (1992) to suggest that these proteins may play a role in enhancing the adhesion of the plasma membrane to the cell wall during pathogen attack.

In wheat, *WIR1* transcripts have been shown to steadily increase over time in response to a number of adapted and nonadapted isolates of fungal pathogens, including isolates of *Magnaporthe* spp. (Tufan *et al*., 2009), *Blumeria* spp. (Schweizer *et al*., 1989), *Puccinia striiformis* (Bozkurt *et al*., 2010; Coram *et al*., 2008a, b, 2010), *P. triticina* (Bolton *et al*., 2008) and *Fusarium* spp. (Desmond *et al*., 2008; Diethelm *et al*., 2011; Jia *et al*., 2009). Homologues of *TaWIR1* have only been identified in barley (*HvWIR1*; Douchkov *et al*., 2011) and rice (*OsRIR1*; Mauch *et al*., 1998). In barley, *HvWIR1* transcripts were induced in response to adapted and nonadapted isolates of *Blumeria* spp. (Jansen *et al*., 2005; Zierold *et al*., 2005), and a wheat-adapted isolate of leaf rust *P. triticina* (Neu *et al*., 2003). In rice, the OsRIR1 protein shows only approximately 30% amino acid identity to the predicted TaWIR1 protein (Mauch *et al*., 1998; Yuan *et al*., 2004). *OsRIR1* transcripts accumulate on inoculation with adapted *M. oryzae* isolates, the nonadapted bacterium *Pseudomonas syringae* pv. *syringae* and following treatment with the *Pseudomonas syringae* pv. *syringae* defence activator peptide syringolin A (Wäspi *et al*., 1998). *TaWIR1*- and *OsRIR1*-like genes have also been reported to respond to feeding by Hessian fly (*Mayetiola destructor*) larvae in wheat (Sardesai *et al*., 2005) and brown plant hopper (*Nilaparvata lugens*) in rice (Yuan *et al*., 2004), whereas *HvWIR1* transcripts have been found in barley phloem sap following bird-cherry oat aphid (*Rhopalosiphum padi*) feeding (Gaupels *et al*., 2008). Unlike *TaWIR1*, *OsRIR1* transcripts are also induced on wounding (Bull *et al*., 1992; Mauch *et al*., 1998; Schweizer *et al*., 1998; Wäspi *et al*., 1998). This induction of transcription in response to such a broad range of pests and pathogens suggests that *TaWIR1*, *HvWIR1* and *OsRIR1* genes are involved in basal resistance in cereals.

Blumeria graminis f. sp. *tritici*, the causal agent of powdery mildew, is a common field pathogen of wheat (Cunfer, 2002). More recently, wheat blast, causal agent *M. oryzae*, has occurred as a field disease of wheat in South America, appearing for the first time in Brazil in 1986 (Urashima *et al*., 1993, 2004). Both *B. graminis* f. sp. *tritici* and *M. oryzae* form appressoria on the leaf surface and enter an epidermal cell via an infection peg which breaches the plant cell wall (Boyd *et al*., 1995; Tufan *et al*., 2009). *Blumeria graminis* does not invade past the epidermis, haustoria being the only fungal structures formed within plant cells, with all subsequent hyphal growth occurring on the leaf surface (Boyd *et al*., 1995). *Magnaporthe oryzae* hyphae invade both epidermal and mesophyll tissue, spreading from the initial infected epidermal cell into neighbouring epidermal and mesophyll cells through plasmodesmata (Kankanala *et al*., 2007). An in-depth histopathological study of the interaction between wheat and *M. oryzae* has been reported by Tufan *et al*. (2009).

In this study, we investigate the genomic complexity of the *TaWIR1* gene family in the hexaploid wheat cv. Renan, and the relationship of members of the *TaWIR1* gene family to *WIR1* homologues in other plant species. The role of the different members of the *TaWIR1* gene family in restricting fungal pathogen infection in wheat is addressed through transient gene silencing using the *Barley stripe mosaic virus* (BSMV)-mediated virus-induced gene silencing (VIGS) system and microscopy to follow pathogen development. The fungal pathogens causing wheat blast, *M. oryzae*, and powdery mildew, *B. graminis* f. sp. *tritici*, are examined to compare the influence of *TaWIR1* gene expression on the initial penetration of an epidermal cell by the fungus, a stage common to both *M. oryzae* and *B. graminis* f. sp. *tritici*, and on the cell-to-cell movement of the fungal pathogen, for *M. oryzae* only.

RESULTS

Expression of *TaWIR1* transcripts in response to fungal pathogen inoculation

Transcript analysis of wheat inoculated with *Magnaporthe* spp. indicated that transcripts of *TaWIR1* genes were induced significantly by both adapted and nonadapted isolates of *Magnaporthe* spp. (Tufan *et al*., 2009). Published wheat Affymetrix GeneChip microarray data generated from pathogen-inoculated wheat tissue were used to investigate the expression pattern of *TaWIR1* transcripts in other wheat–pathogen interactions. Analysis included species of the fungal pathogens *Magnaporthe* and *Blumeria*, *P. striiformis* and *P. triticina*, and *Fusarium pseudograminearum*. Nine probe sets with significant similarity to the *TaWIR1a*, *TaWIR1b* and *TaWIR1c* coding sequences, based on low *E*-value scores (<E-30), were identified on the Affymetrix GeneChip (Table S1, see Supporting Information). Meta-analysis indicated that, in most cases, *TaWIR1* transcripts were up-regulated from 24 to 48 h after pathogen inoculation in compatible, incompatible and nonhost interactions, reflecting a peak of *TaWIR1* gene transcription during the initial stages of attempted pathogen colonisation (Fig. 1).

Genetic complexity of the *TaWIR1* gene family in wheat

Transcript cloning, sequencing and single-strand conformation polymorphism (SSCP) analysis were used to investigate the genetic complexity of the *WIR1* gene family in wheat cv. Renan, together with phylogenetic analyses of all *WIR1*-like sequences retrieved *in silico*. Polymerase chain reaction (PCR) primers

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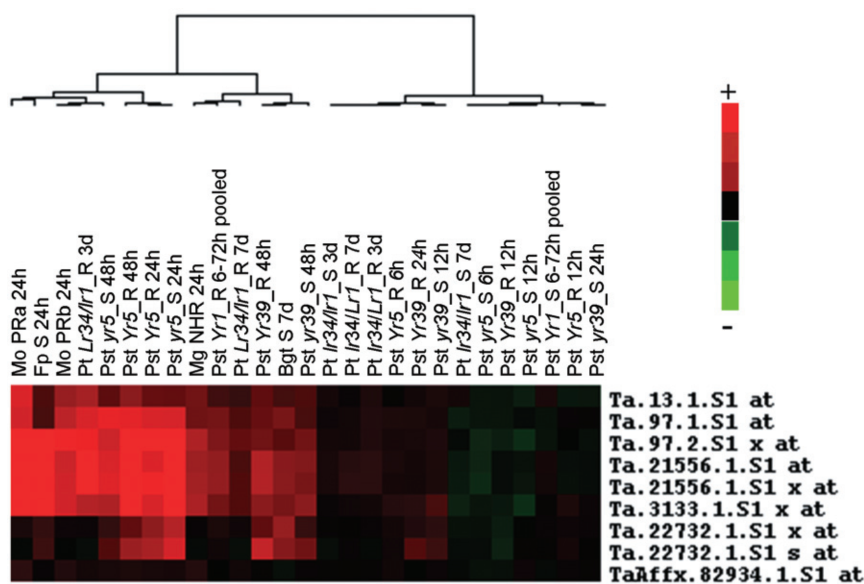

Fig. 1 Meta-analysis of *TaWIR1* probe sets on the Affymetrix Wheat GeneChip differentially transcribed in response to fungal pathogen inoculation. Experiment descriptions are shown at the top of the figure, with the probe set names listed on the right of the figure. Experiments included are *Magnaporthe oryzae* (Mo) inoculations with isolate BR32 (a) or BR37 (b), partial resistance (PR) interactions, with *M. grisea* (Mg) isolate BR29, a nonhost interaction (NHR) (Tufan *et al*., 2009), incompatible and compatible interactions with *Puccinia striiformis* f. sp. *tritici* (Pst) (*Yr5*, *Yr39*, *Yr1*; Coram *et al*., 2008a, b and Bozkurt *et al*., 2010, respectively), incompatible and compatible interactions with *P. triticina* (Pt) (*Lr34* and *Lr1*; Bolton *et al*., 2008), and compatible *Blumeria graminis* f. sp. *tritici* (Bgt; Chain *et al*., 2009) and *Fusarium pseudograminearum* (Fp; Desmond *et al*., 2008) inoculations. The time points sampled post-inoculation are shown alongside the pathogen labels. Red blocks indicate transcript up-regulation and green blocks indicate transcript down-regulation in pathogen-inoculated tissue relative to controls. Samples represent leaf-inoculated tissues, except Fp, which is from inoculated stem bases.

designed specifically to the coding sequences of *TaWIR1a* (GenBank accession number M95500), *TaWIR1b* (M94959) and *TaWIR1c* (X87686; Table S2, see Supporting Information) were used to amplify full-length cDNAs from cv. Renan post-inoculation with an isolate of *M. oryzae* to which Renan shows partial resistance. SSCP analysis of these cDNAs indicated that *TaWIR1a* and *TaWIR1b* each produced a single transcript in Renan, whereas at least two transcripts were amplified using the primers for *TaWIR1c* (Fig. 2A).

Comparative analysis of the DNA sequences of clones of *TaWIR1a*, *TaWIR1b* and *TaWIR1c* indicated that *TaWIR1a* and *TaWIR1b* transcripts from Renan were identical to the published nucleotide sequences from wheat cv. Fidel. Although SSCP analysis identified two possible transcript sequences, only a single *TaWIR1c* sequence was cloned from Renan. The reason for this was not clear, but may indicate alternative transcript splicing that was not detected by cloning and sequencing. The sequence differed from the published *TaWIR1c* sequence by a three-nucleotide insertion (CGG after position 116) and a single nucleotide substitution (T→C position 186). The three-nucleotide insertion resulted in a single amino acid insertion in Renan (G after position 35), whereas the nucleotide substitution at position 186 was silent. The full-length coding sequences of *TaWIR1a*, *TaWIR1b* and *TaWIR1c* from wheat cv. Renan have been deposited in the GenBank database under the accession numbers HQ337015, HQ337016 and HQ337017.

Similarity searches using the DNA sequences of the three published *TaWIR1* genes identified up to 15 *TaWIR1*-like transcripts in wheat (Table S3, see Supporting Information). A number of barley homologues (Douchkov *et al*., 2011) and distant rice *WIR1* candidates (Mauch *et al*., 1998; Schaffrath *et al*., 2000) have been described; however, no *WIR1*-like transcripts were identified in the *Brachypodium* genome using either the BLASTN or TBLASTX search tools (http://blast.brachypodium.org/). Phylogenetic analysis, using the predicted amino acid sequences of the *WIR1*-like transcripts found in wheat and barley, indicated that *TaWIR1a* and *TaWIR1b* were distinct from the barley *HvWIR1* transcripts (Douchkov *et al*., 2011; Fig. 2B). The *WIR1*-like transcripts were initially divided into two clades. The first clade contained the TaWIR1a-like and TaWIR1b-like proteins, which were further subdivided into two separate groups. The second clade contained all the other TaWIR1 proteins, including TaWIR1c and all of the HvWIR1 proteins, except HvWIR1m, which formed an outgroup together with the rice protein RIR1b. Using SignalP, all TaWIR1 proteins were predicted to contain a signal peptide, although the probability of cleavage was lower for the TaWIR1a group of proteins (Table S4, see Supporting Information). Subcellular localisation predictions using TargetP indicated that TaWIR1b (clade I) and

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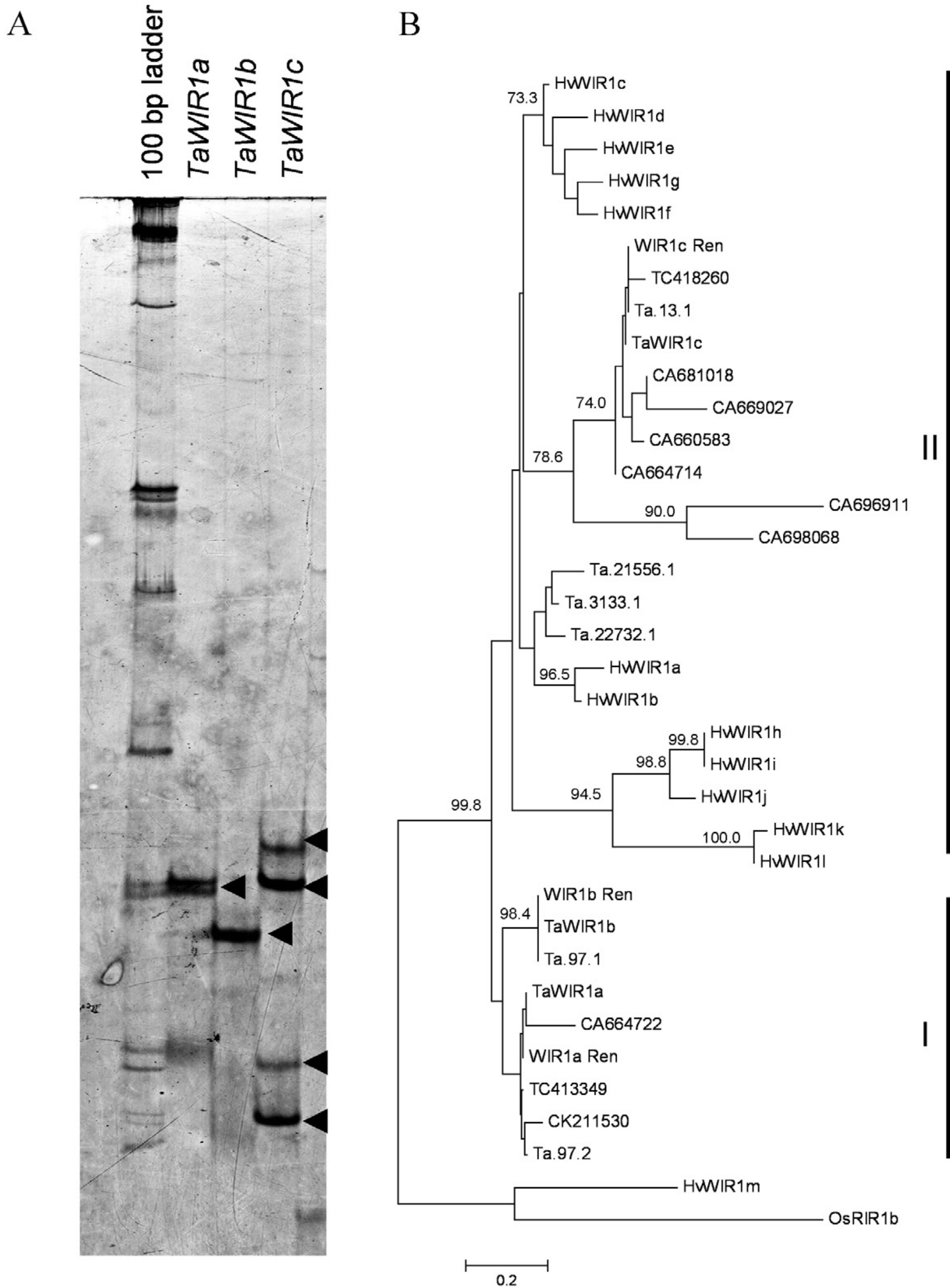

Fig. 2 Molecular analysis of the *TaWIR1* gene family in wheat. (A) Single-strand conformation polymorphism (SSCP) analysis of *TaWIR1a*, *TaWIR1b* and *TaWIR1c* transcripts amplified from the wheat cv. Renan. (B) Phylogenetic analysis of TaWIR1 and HvWIR1 proteins using the neighbour-joining method in PHYLIP. Confidence levels are given above the nodes, obtained from a 1000 bootstrap analysis. The rice WIR1-like protein, OsRIR1b, was used as an outlier. The sequences shown represent the wheat Affymetrix probe sets (Ta), the Dana Farber Cancer Institute (DFCI) tentative consensus (TC) sequences and GenBank accessions. The GenBank accession numbers for the published TaWIR1 and OsRIR1b sequences are: TaWIR1a (M95500), TaWIR1b (M94959), TaWIR1c (X87686) and OsRIR1b (Y14824).

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almost all of the TaWIR1 proteins in clade II, including TaWIR1c, were predicted to be secreted (Table S5, see Supporting Information). Transmembrane helix prediction (TMHMM) analysis predicted a single transmembrane domain for all of the TaWIR1 proteins analysed (Table S6, see Supporting Information). However, the probability of N-terminus localisation on the cytoplasmic side of the membrane was low ($P < 0.15$) for all of the TaWIR1a-like proteins (TaWIR1a, Ta.97.2, CK211530, CA664722, TC413349), suggesting that the C-termini of these proteins are likely to be found on the cytoplasmic side of the membrane. Together, the low probability scores for cleavage site and cytoplasmic N-terminus localisation for the TaWIR1a proteins support the likelihood that these proteins may be retained in a membrane.

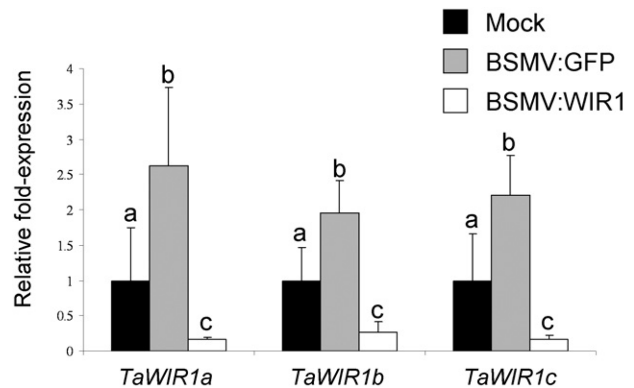

Fig. 3 Transcript levels of the *TaWIR1* genes in *Barley stripe mosaic virus* (BSMV)-mediated virus-induced gene silenced wheat seedlings of cv. Renan. Mean transcript levels of *TaWIR1a*, *TaWIR1b* and *TaWIR1c*, relative to mock-inoculated controls, are shown for two independent experiments, consisting of three biological replicates, inoculated with BSMV:WIR1 or the BSMV:GFP control. Relative transcript levels which do not differ significantly from each other are represented by the same lower case letter. Error bars show standard errors.

Effect of BSMV-mediated VIGS of *TaWIR1* on resistance to *M. oryzae* and *B. graminis* f. sp. *tritici*

To silence transcripts of the *TaWIR1* gene family, a 159-bp fragment was designed to a region of the *TaWIR1b* coding sequence which was relatively conserved in all of the *TaWIR1* sequences identified in this study (Fig. S1, see Supporting Information). The silencing fragment exhibited approximately 80% identity to all of the identified *TaWIR1* sequences, except Ta.21556.1, Ta.3133.1, Ta.22732.1, CA669027 and CA696911 (Table S7, see Supporting Information). The 159-bp *TaWIR1* VIGS fragment was cloned in antisense orientation, 3′ of the γb gene (BSMV:WIR1; Holzberg *et al*., 2002). The silencing target prediction software si-Fi v1.4.0 (http://labtools.ipk-gatersleben.de/) was used to determine which wheat sequences would be potential targets for silencing by the BSMV:WIR1 construct. Wheat sequences with similarity to *TaWIR1a* and *TaWIR1b* (Fig. 2, clade I) were predicted to be efficiently silenced by the BSMV:WIR1 construct (Table S8, see Supporting Information). The only transcript from clade II predicted to be potentially silenced was CA698068. No other wheat sequences were predicted to be silenced by the BSMV:WIR1 construct, indicating that the 159-bp VIGS fragment was specific to the *TaWIR1* gene family.

The first and second leaves of 10-day-old Renan seedlings were rub inoculated with BSMV:WIR1 or BSMV:GFP (GFP, green fluorescent protein), or mock inoculated with FES buffer (sodium-pyrophospate 1% w/v; macaloid 1% w/v; celite 1% w/v; 0.5 M glycine; 0.3 M K2HPO4, pH 8.5). *TaWIR1* transcript levels were then monitored, 14 days post-inoculation (dpi), by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using primer sets specific for *TaWIR1a*, *TaWIR1b* and *TaWIR1c*. The transcript levels for all three *TaWIR1* genes were reduced in BSMV:WIR1-inoculated plants (Fig. 3). Although the BSMV:GFP VIGS control was shown to induce *TaWIR1* genes, in seedlings inoculated with BSMV:WIR1, the VIGS mechanism still effectively reduced transcript levels to well below the levels seen in mock-inoculated leaf tissue. Furthermore, transcripts of Ta.21556.1, Ta.3133.1 and Ta.22732.1, which showed less than 80% nucleotide identity to the BSMV:WIR1 VIGS insert, also exhibited suppressed transcript levels in BSMV:WIR1-inoculated plants (Fig. S2, see Supporting Information).

The effects of silencing of the *TaWIR1* gene family on resistance in wheat cv. Renan was analysed for the fungal pathogens *M. oryzae* and *B. graminis* f. sp. *tritici*. For both pathogens, analysis of the infection process was carried out in BSMV:WIR1-inoculated seedlings in which *TaWIR1* transcript levels were confirmed to be reduced by qRT-PCR in the adjacent leaf. *M. oryzae* isolate BR32 was spray inoculated onto Renan seedlings at 14 dpi with the VIGS construct BSMV:WIR1 or the viral construct control BSMV:GFP. The interaction between Renan and isolate BR32 was assessed 96 h after fungal inoculation by comparing the proportion of infection sites at three different stages of the wheat–*Magnaporthe* interaction: HALO, HYPFLUO and MULTIFLUO (see Experimental procedures) (Fig. 4; Tufan *et al*., 2009). In BSMV:WIR1-inoculated plants, there were significantly more infection sites at which *M. oryzae* hyphae had invaded multiple cells (MULTIFLUO; *t*-test probability, 0.038) and significantly fewer infection sites at which hyphae were restricted to the first invaded epidermal cell (HYPFLUO; *t*-test probability, 0.057), relative to BSMV:GFP-inoculated plants. No significant differences (*t*-test probability, 0.362) were found in the number of infection sites halted at the HALO stage (Fig. 4A). This suggests that *TaWIR1* does not play a role in pre-penetration resistance to *M. oryzae*, but that silencing of the *TaWIR1* genes enabled increased intracellular spread of isolate BR32.

The *B. graminis* f. sp. *tritici* isolate JIW2 was inoculated onto Renan seedlings at 14 dpi with the VIGS construct BSMV:WIR1 or the viral control BSMV:GFP. The effects of silencing of the *TaWIR1*

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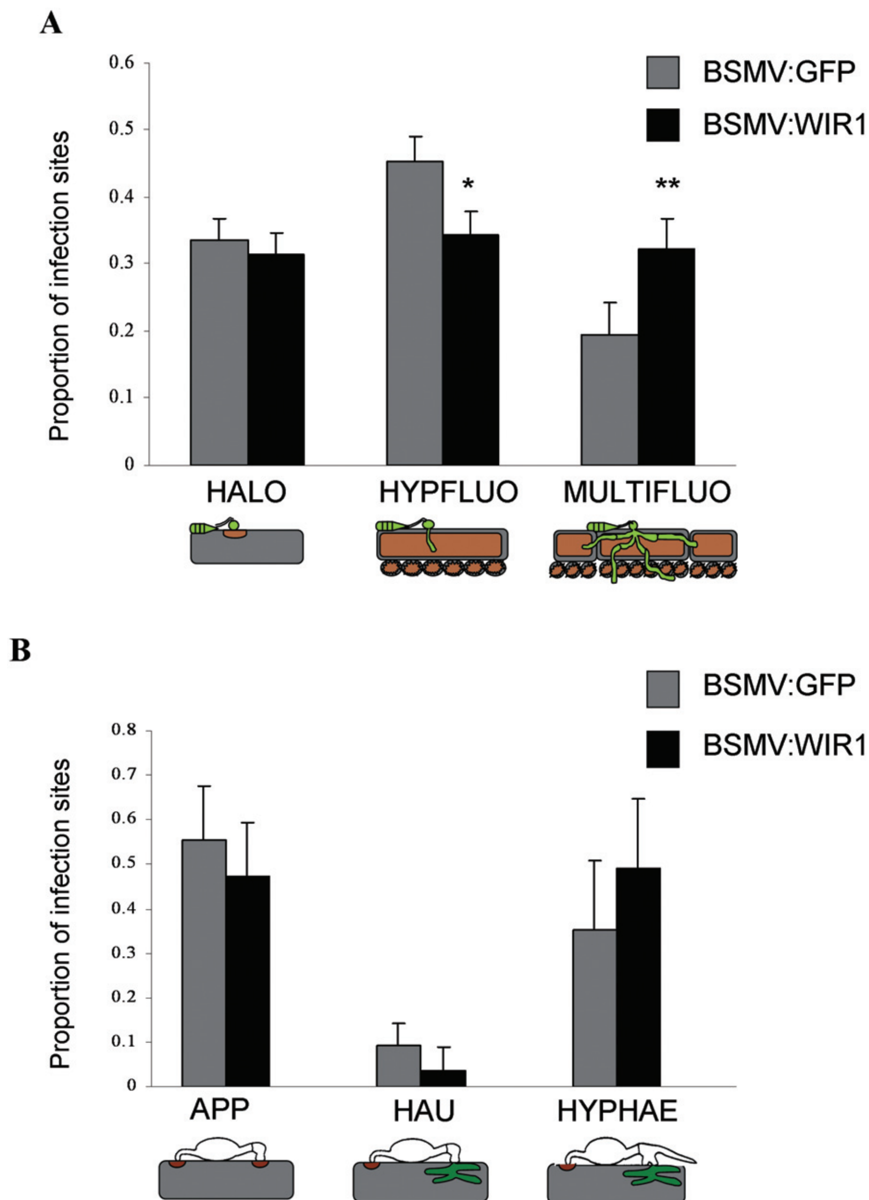

Fig. 4 Proportion of fungal growth stages on *Barley stripe mosaic virus* (BSMV)-mediated virus-induced gene silenced seedlings of the wheat cv. Renan at 96 h post-inoculation (hpi) with *Magnaporthe oryzae* (A) and 48 hpi with *Blumeria graminis* f. sp. *tritici* (B). Each growth stage is shown relative to the number of infection sites. For *M. oryzae*, growth stages were defined as sites that had failed to establish hyphae, attempted penetration being associated with the accumulation of autofluorescent compounds beneath the appressorium (HALO), as sites at which hyphae had invaded the first epidermal cell, being associated with plant cell autofluorescence (HYPFLUO), and as sites of multiple cell invasion associated with autofluorescence (MULTIFLUO). For *B. graminis* f. sp. *tritici*, growth stages were defined as appressoria that had failed to establish a haustorium (APP), as haustoria within epidermal cells, but lacking surface hyphae (HAU), and where hyphae had formed on the leaf surface (HYPHAE). Mean values are shown from three independent experiments. Significant differences between growth stages are shown at *t*-test probabilities of >0.01 (**) and >0.1 (*). Error bars show standard errors.

genes on isolate JIW2 development was assessed 48 h after fungal inoculation by measuring the proportion of infection sites at three different stages of the wheat–powdery mildew interaction: APP, HAU and HYPHAE (see Experimental procedures) (Fig. 4B; Tufan *et al*., 2011). There were no significant differences between BSMV:WIR1-silenced plants and plants inoculated with BSMV:GFP with regard to the proportion of infection sites at each of the three stages: APP (*t*-test probability, 0.643), HAU (*t*-test probability, 0.485) or HYPHAE (*t*-test probability, 0.555). In addition, no significant differences were found in the number of powdery mildew colonies observed on BSMV:WIR1- (*t*-test probability, 0.779) or BSMV:GFP- (*t*-test probability, 0.661) inoculated

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Renan seedlings at 10 dpi with *B. graminis* f. sp. *tritici* isolate JIW2, relative to mock-inoculated control leaves (data not shown). This suggests that silencing of the *TaWIR1* genes has no effect on the early development of *B. graminis* f. sp. *tritici* on wheat cv. Renan, or on subsequent colony development.

Spatial expression of *TaWIR1* transcripts in wheat leaf tissue following inoculation with *M. oryzae*

TaWIR1 transcript levels were measured in epidermal peels, in the remaining leaf tissue minus the abaxial epidermis (referred to as the mesophyll region) and in intact leaves at 48 h post-inoculation (hpi) with *M. oryzae* isolate BR32 or 0.25% gelatine solution, the mock-inoculated control. The 48 h time point was chosen as previous studies have shown *TaWIR1* transcripts to be highly induced at this time point in intact leaves inoculated with the *M. oryzae* isolate BR32 (Fig. S3, see Supporting Information; Tufan *et al*., 2009). Following inoculation with BR32, all three *TaWIR1* genes were induced in both regions of the leaf, relative to the same region in the mock-inoculated control (Fig. S4, see Supporting Information). Comparison of the transcript levels of *TaWIR1a*, *TaWIR1b* and *TaWIR1c* in epidermal peels and in the mesophyll region, relative to the transcript levels in the intact leaf, in three independent experiments, provided a measure of the relative expression of each gene in these two regions of the leaf (Table 1). Transcript levels of *TaWIR1a* and *TaWIR1c* in BR32-inoculated leaves were higher in the mesophyll region of the leaf relative to the abaxial epidermal tissue at 48 hpi, whereas transcript levels of *TaWIR1b* were not significantly different between the two regions of the leaf. In the mock-inoculated control, transcript levels of all three genes were similar across the leaf (Table 1). *TaRUBISCO* (RUBISCO, ribulose-1,5-bisphosphate carboxylase oxygenase) expression was not induced by *M. oryzae* inoculation, and confirmed the quality of the epidermal peel tissue, with significantly less *TaRUBISCO* transcripts being found in the epidermis relative to the remaining leaf tissue (Table 1).

Table 1 Gene transcript levels in leaf tissues inoculated with *Magnaporthe oryzae* isolate BR32 or mock inoculated with FES buffer, relative to the transcript levels in the intact leaf.

Tissue	*TaWIR1a*‡	*TaWIR1b*	*TaWIR1c*	*TaRUBISCO*
Mock				
Epidermis*	0.69[a]	1.06[a]	0.89[a,b]	0.28[b]
	(0.44)	(0.47)	(0.24)	(0.09)
Mesophyll†	1.13[a]	0.80[a]	1.35[a]	1.12[a]
	(0.46)	(0.45)	(0.57)	(0.23)
BR32				
Epidermis	0.28[b]	0.59[a]	0.32[c]	0.24[b]
	(0.15)	(0.26)	(0.16)	(0.03)
Mesophyll	0.80[a]	0.82[a]	0.86[b]	1.29[a]
	(0.58)	(0.34)	(0.59)	(0.18)

*Epidermal peels of the abaxial leaf layer.
†The remaining leaf tissue minus the abaxial epidermal layer.
‡The mean relative transcript levels from three replicate experiments are shown for each gene. For each gene, mean values showing the same letter do not differ significantly at a *t*-test probability of <0.001.
Standard deviations of the means are shown in parentheses.

DISCUSSION

WIR1 was first described in wheat, being induced by the fungal pathogen responsible for powdery mildew, *B. graminis* (Schweizer *et al*., 1989). Homologues of *TaWIR1* have been found in barley, as well as a distant candidate in rice, *OsRIR1*. No candidate for *WIR1* was found in the *Brachypodium* genome sequence. *WIR1* transcripts are induced in wheat and barley following inoculation with a diverse range of pathogenic organisms (Bolton *et al*., 2008; Bozkurt *et al*., 2010; Bull *et al*., 1992; Coram *et al*., 2008a, b, 2010; Desmond *et al*., 2008; Jia *et al*., 2009; Schweizer *et al*., 1989; Tufan *et al*., 2009), but not by abiotic stress (Douchkov *et al*., 2011). In barley, *HvWIR1* transcripts have also been found to accumulate in noninoculated leaves of adult plants (Douchkov *et al*., 2011), which may indicate a link with the developmentally regulated, broad-spectrum, partial resistance often seen as plants mature (Boyd, 2006). Four *HvWIR1* loci have been shown to co-locate with quantitative trait loci conferring partial resistance to *B. graminis* f. sp. *hordei* in barley (Aghnoum *et al*., 2010; Douchkov *et al*., 2011). This association was further supported using single nucleotide polymorphisms (SNPs) within *HvWIR1* genes in a genetic association analysis of resistance towards *B. graminis* f. sp. *hordei* (Comadran *et al*., 2009; Douchkov *et al*., 2011). In rice, overexpression of the *TaWIR1* homologue, *OsRIR1b*, has also been shown to enhance resistance to *M. oryzae* (Schaffrath *et al*., 2000). The *WIR1* genes are therefore good candidates for a resistance mechanism which supports broad-spectrum resistance. However, the mechanism by which the WIR1 proteins deliver this resistance remains to be determined.

Although similarity searches identified up to 15 *TaWIR1*-like transcripts, phylogenetic analysis grouped the *TaWIR1a* and *TaWIR1b* gene sequences into a distinct clade, whereas the *TaWIR1c* sequence clustered together with the barley *HvWIR1* homologues. A Southern analysis in wheat cv. Fidel identified up to six bands detected by the *TaWIR1* sequence (Bull *et al*., 1992), whereas cloning of the *TaWIR1a*, *TaWIR1b* and *TaWIR1c* transcripts from cv. Renan only identified three sequences. The *TaWIR1a* and *TaWIR1b* gene sequences from Renan were also identical to the published sequences cloned from cv. Fidel. Mapping of the *TaWIR1a* and *TaWIR1b* sequences in wheat identified a single locus for both genes, located on chromosomes 7BS and 5DS, respectively (Diethelm *et al*., 2011). Collectively, these data suggest a relatively simple genomic structure for the *TaWIR1* genes, with a high degree of sequence conservation. The diversity of *TaWIR1*-like sequences found in database searches probably reflects gene sequence variation between different wheat varieties. Subsequent analysis of the wheat 454 genomic

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sequence data, variety Chinese Spring (http://www.cerealsdb.uk.net/CerealsDB/Documents/DOC_CerealsDB.php), supported our observations, indicating that a single locus of each *TaWIR1* gene probably exists on each of the three homoeologous genomes, whereas, between Chinese Spring and cv. Fidel, two to three potential varietal SNPs were identified within the *TaWIR1* genes.

Silencing of the *TaWIR1* genes had no effect on the initial penetration of epidemal cells by the adapted *M. oryzae* isolate BR32, but facilitated the subsequent intracellular spread of the fungus, resulting in a greater number of infection sites colonizing multiple mesophyll cells by 96 hpi. The predicted protein structure of *TaWIR1* indicates a hydrophobic N-terminus which, in the case of *TaWIR1a*, has a low probability of cleavage and a greater probability of being membrane bound (Tables S4 and S6). This opens up the possibility that the TaWIR1a protein is membrane anchored, with the hydrophilic C-terminus being exposed to the acidic apoplast. In the cowpea rust system, expression of early, cell wall-related defence responses was associated with the maintenance of adhesion between the plant cell wall and the plasma membrane (Mellersh and Heath, 2001). *TaWIR1a* may therefore be required to maintain cell wall–plasma membrane contact during wheat–*M. oryzae* infection. As *M. oryzae* is believed to pass from cell to cell via plasmodesmata (Kankanala *et al*., 2007), *TaWIR1a* may play a role in maintaining the cell wall membrane integrity of these structures. The TaWIR1b and TaWIR1c proteins are predicted to contain signal peptides and are probably secreted. Analysis of OsRIR1a in rice protoplasts indicated that this protein was secreted and was probably ionically bound to cell walls (Mauch *et al*., 1998). It is therefore possible that TaWIR1b and TaWIR1c proteins are also involved in cell wall strengthening, enhancing the physical barrier that helps restrict pathogen colonization. However, further experimentation is required to determine definitively the functions of the TaWIR1 proteins in plant defence.

In contrast, BSMV-mediated VIGS of *TaWIR1* transcripts had no effect on *B. graminis* f. sp. *tritici* colonization, despite inoculation with this organism resulting in increased levels of *TaWIR1* transcripts (Fig. 1; Bull *et al*., 1992; Schweizer *et al*., 1989). In barley, transient induced gene silencing of *HvWIR1a* reduced the number of *B. graminis* f. sp. *hordei* haustoria seen in barley epidermal cells, whereas silencing of the *HvWIR1h* gene increased haustoria establishment; silencing of *HvWIR1m* had no effect on haustorial establishment (Douchkov *et al*., 2011). However, in wheat, transient overexpression of *TaWIR1a* had no effect on haustoria formation in the wheat–*B. graminis* f. sp. *tritici* interaction (Schweizer *et al*., 1999). This may indicate that the effects of *WIR1* on *B. graminis* are gene specific, and that the global silencing of the *TaWIR1* transcripts in Renan cancelled out any *WIR1* gene-specific effects on *B. graminis* development.

The initial BSMV:WIR1 construct was designed to the *TaWIR1b* transcript sequence. The si-Fi software predicted that this construct would silence the *TaWIR1b* and *TaWIR1a* sequences from phylogenetic clade I (Fig. 2A), as well as a single transcript (CA698068) from clade II (Table S8). However, qRT-PCR analysis indicated that *TaWIR1* transcripts within clade II, with less than the previously suggested 80% sequence similarity required for successful VIGS (Holzberg *et al*., 2002), were also suppressed (Table S7; Fig. S2). The reduced level of sequence similarity required for VIGS, together with the optimal size of 120–500 bp for VIGS fragments (Bruun-Rasmussen *et al*., 2007; Holzberg *et al*., 2002), meant that we were unable to effectively silence specific *TaWIR1* transcripts.

Following *M. oryzae* inoculation, different patterns of spatial expression were observed across the leaf for *TaWIR1a/c* and *TaWIR1b*. Similarly, in barley, most *HvWIR1* transcripts analysed accumulated to greater levels in the intact leaf relative to epidermal tissue; the exception being *HvWIR1a*, which accumulated almost as strongly in pathogen-attacked epidermal tissue as in the intact leaf (Douchkov *et al*., 2011; Zierold *et al*., 2005). The differences in spatial expression of the *TaWIR1* transcripts may therefore facilitate the role of this gene family in defence against pathogens with different lifestyles (biotrophic, hemibiotrophic or necrotrophic) and inhabiting different plant tissues (Fig. 1; Bolton *et al*., 2008; Bozkurt *et al*., 2010; Bull *et al*., 1992; Coram *et al*., 2008a, b, 2010; Desmond *et al*., 2008; Schweizer *et al*., 1989; Tufan *et al*., 2009).

Although we can only speculate on the function of the TaWIR1 proteins in pathogen defence, accumulating evidence indicates a significant role for this family of genes in cereal disease resistance. The conservation of the *WIR1* gene family in wheat and barley indicates a preserved function very specific to these cereal species. However, when comparing wheat with barley, the role of individual *WIR1* genes may have diverged, with different members of the gene family specifically targeting different pathogens.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of the winter wheat cv. Renan (*Triticum aestivum* L.) were provided by Jean-Benoit Morel (INRA, Montpellier, France). Plants were grown in a peat and sand (1:1) mix at 23 °C in a Fitotron growth cabinet (Sanyo Gallenkamp PLC, Loughborough, UK) with a 16-h/8-h light/dark cycle.

Meta-analysis of *TaWIR1* transcripts

The predicted amino acid sequences of the TaWIR1a (Q01482, Bull *et al*., 1992), TaWIR1b (Q01481, Bull *et al*., 1992) and TaWIR1c (Q41581, Franck and Dudler, 1995) proteins were retrieved from the National Center for Biotechnology Information (NCBI). Probe sets representing *TaWIR1* genes on the Affymetrix Wheat GeneChip were retrieved using the coding sequences of the three TaWIR1 proteins and BLASTN to query the microarray (Wise *et al*., 2007). Probe sets with high similarity scores (<E-30) and defined as not potentially unreliable (Schreiber *et al*., 2009) were selected for meta-analysis (Table S1). Wheat Affymetrix datasets for experiments investigating wheat–pathogen interactions were downloaded from

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PlexDB: TA9 (Coram *et al*., 2008b), TA11 (Coram *et al*., 2008a), TA24 (Tufan *et al*., 2009), TA25 (Bozkurt *et al*., 2010), TA31 (Desmond *et al*., 2008), TA32 (Bolton *et al*., 2008) and TA34 (Chain *et al*., 2009). Data were analysed in R using the package AffylmGUI (Wettenhall *et al*., 2006) following Robust Multichip Average (RMA) normalization (Irizarry *et al*., 2003). Differential expression was calculated using linear models and an empirical Bayes-moderated *t*-statistic (Smyth, 2004). Contrasts between pathogen-inoculated and mock-inoculated control samples were tested. Differential regulation of probe sets was assessed based on expression, the data being exported into a tab-delimited file. Expression patterns of *WIR1* probe sets were analysed using Cluster 3 (Eisen *et al*., 1998) with a Euclidean distance matrix and complete-linkage clustering. Data were visualized with Treeview v.1.0.13.

Sequence and phylogenetic analysis of *TaWIR1*

Primers were designed to the published sequences of *TaWIR1a*, *TaWIR1b* and *TaWIR1c* to amplify the entire coding sequence of each gene from Renan. RNA was extracted from leaves of Renan at 48 hpi with *M. oryzae* isolate BR32, processed and reverse transcribed as described by Tufan *et al*. (2009). Transcripts were amplified using Hotstar master mix (Qiagen, Hilden, Germany), following the manufacturer's recommendations. A G-Storm GS1 thermocycler was used for target amplifcation (GRI, Braintree, Essex, UK), with an initial activation step at 95 °C for 15 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C for *TaWIR1a* and *TaWIR1b* or 68 °C for *TaWIR1c*, 1 min at 72 °C and a final 10-min extension cycle. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy (Promega, Madison, WI, USA). Plasmid DNA was isolated from more than 10 independent clones for each *TaWIR1* gene using a QIAprep spin Miniprep kit (Qiagen) and sequenced by Genome Enterprise Ltd (http://orders2.genome-enterprise.com/).

Using BLASTN, the published *TaWIR1* coding sequences were used to query the Dana Farber Cancer Institute (DFCI) gene indices database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) to recover similar wheat tentative consensus (TC) sequences, as defined by low *E*-value scores (<E-30). Sequences that had identical coding sequences to the published *TaWIR1* sequences, to each other or that showed incomplete open reading frames, i.e. no start codon or multiple stop codons, were excluded. Selected TC sequences and Affymetrix GeneChip probe sets representing *TaWIR1* genes were translated and aligned using PRANKSTER (Löytynoja and Goldman, 2008) to the published amino acid sequences of TaWIR1a, TaWIR1b and TaWIR1c, together with the predicted protein sequences of these genes from Renan. Amino acid sequences of barley *HvWIR1* transcripts (Douchkov *et al*., 2011) and the rice *OsRIR1b* gene (Mauch *et al*., 1998) were included in the alignment.

Phylogenetic analysis was performed using PHYLIP (http://evolution.genetics.washington.edu/phylip.html), and a phylogenetic tree was constructed using the neighbour-joining method, with OsRIR1b as an outgroup. The tree was visualized in MEGA (Kumar *et al*., 2008). Percentage confidence levels were obtained after 1000 bootstraps. Signal peptide and subcellular targeting predictions were made using SignalP and TargetP, respectively (Emanuelsson *et al*., 2007). Transmembrane predictions were made using TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

SSCP analysis

Sequence polymorphisms in the *TaWIR1a*, *TaWIR1b* and *TaWIR1c* coding sequences recovered from Renan were examined by SSCP analysis based on the method of Martins-Lopes *et al*. (2001) with the following modifications. *TaWIR1* coding sequences were amplified by PCR as described above, 4 μL of the product was mixed with loading dye [98% formamide; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.1% (w/v) bromophenol blue; 0.1% (w/v) xylene cyanol] and denatured at 95 °C for 2 min. Following incubation on ice for 1 min, 6 μL of each sample were loaded onto the SSCP gel (390 mm × 300 mm × 0.3 mm) containing 12.5 mL of 2 × MDE (Lonza, Rockland, ME, USA), 1.5 mL of 20 × 1.78M Tris-base, 0.57 M taurine, 10 mM Na2-EDTA (TTE), 9 mL of 50% glycerol and 27 mL of water, and polymerized by adding 303 μL of 10% (w/v) ammonium persulphate and 27 μL N, N, N′, N′-tetramethylethylenediamine (TEMED). Samples were electrophoresed in a 0.6 × TTE running buffer for at least 16 h.

Silver staining of DNA was performed by passing the polyacrylamide gels through the following steps: fixation for 30 min in 10% acetic acid; washing in double-distilled H_2O for 10 min; staining in silver nitrate solution [5.7 mM silver nitrate plus 0.15% (v/v) formaldehyde] for 30 min; and placing in developer [0.73 M sodium carbonate, 0.15% (v/v) formaldehyde plus 15 mM sodium thiosulphate], chilled to 4 °C, until bands were clearly visible. The reaction was stopped by the addition of 10% acetic acid to the developer.

BSMV-mediated VIGS of *WIR1* genes in wheat cv. Renan

A 159-bp fragment corresponding to a relatively conserved region between *TaWIR1* gene sequences was amplified from Renan cDNA using gene-specific primers (Table S2), incorporating *Not*I and *Pac*I restriction enzyme sites at their terminal ends. The *phytoene desaturase* (PDS) fragment was removed from the BSMVγ.bPDSas vector by digestion with *Not*I and *Pac*I, and replaced with the *TaWIR1* PCR fragment, generating the construct BSMVγ:WIR1as (BSMV:WIR1; Hein *et al*., 2005; Holzberg *et al*., 2002).

Purified plasmids containing the BSMV α, β and γ genomes were linearized using endonucleases *Mlu*I for α, *Spe*I for β, *BssH*II for γWIR1 and *Swa*I for γGFP. Viral RNA was synthesized from linearized plasmids using an mMessage mMachine T7 *in vitro* transcription kit (Ambion, Austin, TX, USA). BSMV inoculum was prepared by adding 1 μL of RNA of each viral genome to 27 μL of FES buffer (Pogue *et al*., 1998). First and second leaves of 10-day-old Renan seedlings were rub inoculated with 30 μL of this mixture (Scofield *et al*., 2005). Plants were rinsed with water to remove excess inoculum and transferred to a 25 °C growth chamber. Secondary pathogen inoculations were performed at 14 dpi with BSMV. Three independent VIGS experiments, each consisting of three biological replicates, were carried out.

si-Fi analysis to predict potential off-target silencing effects

To test for potential off-target silencing effects of the *TaWIR1* VIGS fragment, si-Fi software v1.4.0 (http://labtools.ipk-gatersleben.de/) was used.

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The *TaWIR1* VIGS fragment was queried against databases downloaded from HarvEST v. 1.56 (http://harvest.ucr.edu/) and *Triticum aestivum* transcript assembly release 2 (ftp://ftp.tigr.org/pub/data/plantta/Triticum_aestivum/) using default settings. Any returned sequence that had hits to the *TaWIR1* VIGS fragment was considered to be a potential off-target for gene silencing, although some of the hits were not scored as efficient, suggesting that silencing in these cases may not be complete (Douchkov *et al*., 2011).

qRT-PCR analysis

Transcript levels were determined as described by Tufan *et al*. (2009) using sequence-specific primers (Table S2). Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) and genomic DNA contamination was removed with TURBO DNAse I treatment (Ambion). cDNA was prepared from 1 μg of RNA using the Superscript III reverse transcription system (Invitrogen, Carlsbad, CA, USA), primed with random hexamers. Transcripts were amplified with Sybr Green JumpStart™ Taq Ready mix (Sigma, St. Louis, MO, USA) using a DNA engine Opticon2 Continuous Fluorescence Detector (M.J. Research Inc., Alameda, CA, USA), with an initial activation step at 95 °C for 2 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Melt curve analysis was performed at the end of each reaction to monitor primer–dimer formation and the amplification of gene-specific products. Expression levels were normalized using normalization factors derived from geNorm analysis (Vandesompele *et al*., 2002), employing two or three reference genes (Table S2; Bozkurt *et al*., 2010; Tufan *et al*., 2009) that were shown to be stably expressed under the experimental conditions tested. Transcript levels are shown relative to mock-inoculated control samples, unless stated otherwise.

Magnaporthe oryzae and *B. graminis* inoculations of BSMV-VIGS seedlings of cv. Renan

Magnaporthe oryzae isolate BR32 was obtained from Didier Tharreau (CIRAD, Montpellier, France) and was cultured as described previously (Tufan *et al*., 2009). Wheat powdery mildew (*B. graminis* f. sp. *tritici*) isolate JIW2 was obtained from James Brown (John Innes Centre, Norwich, UK). The *B. graminis* f. sp. *tritici* isolate was maintained on detached leaf segments of the susceptible wheat cv. Cerco (Boyd *et al*., 1994).

Magnaporthe oryzae inoculations were carried out as described by Tufan *et al*. (2009). Wheat seedlings were spray inoculated with 10^5 spores/mL inoculum. After inoculation, plants were kept at 25 °C and the final symptoms were assessed at 96 hpi. *Blumeria graminis* f. sp. *tritici* inoculations were carried out as detached leaf tests (Boyd *et al*., 1994). Leaf tissue was cut into 3–4-cm segments and placed, adaxial side up, on water agar (6 g/L) plates supplemented with benzimidazole (0.1 g/L). Spores were blown onto the leaf segments and incubated at 15 °C. Final symptoms were assessed at 10 dpi.

Microscopic and histopathologic analyses of *M. oryzae* and *B. graminis*

Clearing, fixing and staining with the fluorescent stain Uvitex-2B (Ciba-Geigy, Basle, Switzerland) of *M. oryzae*-inoculated leaf tissue were carried out as described by Tufan *et al*. (2009). Leaf tissue was immersed in chloral hydrate solution (300 mL of 95% ethanol, 125 mL of 90% lactic acid, 800 g of chloral hydrate, made up to 1 L with chloroform), periodically refreshing the solution, for up to 5 days. Leaf samples were stored in lactoglycerol (1:1:1, v/v/v, lactic acid : glycerol : water) at 4 °C. Leaf samples were mounted onto glass slides in 40% glycerol and stored in the dark.

Fungal and plant autofluorescent cellular structures were observed on a Zeiss LSM 510 META confocal microscope (Jena, Germany) using 25× LD LCI Plan-Apochromat (numerical aperture, 0.8) or 40× EC Plan-Neofluar (numerical aperture, 1.3) oil immersion objective lenses. Spectral data were collected by excitation with 488-nm argon (30 mW) and 405-nm diode (30 mW) lasers. Uvitex-2B-stained fungal structures and plant autofluorescing cellular structures were differentiated using a Uvitex-2B-specific filter (band pass, 420–480 nm) and an autofluorescence-specific filter (long pass, 530 nm), respectively.

For microscopic observation of *B. graminis* f. sp. *tritici* development, infected leaf segments were cleared and fixed by heating at 70 °C for 1 h in 3:1 ethanol : water (v/v), followed by staining with aniline blue (0.5% w/v in lactoglycerol). Pathogen development was observed using a bright-field microscope (Nikon 800 Eclipse; Nikon Precision Europe GmbH, Langen, Germany).

Magnaporthe oryzae infection sites were defined by the formation of an appressorium. Fungal growth stages were then defined by the proportion of infection sites: (i) that failed to establish hyphae within the invaded epidermal cell, this attempted penetration usually being associated with the accumulation of autofluorescent compounds beneath the appressorium (HALO); (ii) where hyphae had invaded the first epidermal cell and were associated with epidermal and mesophyll autofluorescence (HYPFLUO); and (iii) that had invaded multiple cells, being associated with epidermal and mesophyll cell autofluorescence in colonised as well as adjacent, noncolonised cells (MULTIFLUO). From each independent silencing experiment, 3–4 cm of leaf tissue, from at least two seedlings, were prepared for analysis. Approximately 100 infection sites were scored in each experiment.

Blumeria graminis f. sp. *tritici* infection sites were defined by the formation of an appressorium off the appressorium germ tube (Boyd *et al*., 1995). Fungal growth stages were then defined by the proportion of infection sites: (i) that had failed to establish a haustorium in the underlying epidermal cell, this attempted penetration occasionally being associated with the formation of a papilla beneath the appressorium (APP); (ii) had formed a haustorium within the epidermal cell (HAU); and (iii) had formed hyphae on the leaf surface (HYPHAE) (Boyd *et al*., 1995). Infection sites associated with long cells were excluded from the analysis. From each independent silencing experiment, 3–4 cm of leaf tissue, from at least two seedlings, were prepared for analysis. Approximately 60 infection sites were scored in each experiment. *Blumeria graminis* f. sp. *tritici* colonies were counted at 10 dpi on leaf segments from mock-inoculated seedlings or seedlings inoculated with BSMV:GFP or BSMV:WIR1, in each of the three independent silencing experiments.

Tissue-specific expression of wheat *TaWIR1* genes

Ten-day-old seedlings of cv. Renan were inoculated on both the abaxial and adaxial sides of the leaf with *M. oryzae* isolate BR32 or mock

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inoculated with 0.25% gelatine solution. The abaxial epidermis was removed at 48 hpi (Zellerhoff *et al*., 2010) and total RNA was extracted separately from this epidermal peel tissue, from the remaining leaf tissue and from inoculated whole leaves. Transcript levels of *TaWIR1a*, *TaWIR1b*, *TaWIR1c* and the *TaRUBISCO* genes were measured by qRT-PCR using the primers listed in Table S2. *TaRUBISCO* (accession number AB042066.1) was used to assess mesophyll contamination of the epidermal peels (Eichmann *et al*., 2010). Three independent epidermal peel experiments, each consisting of at least 100 Renan seedlings per treatment, were carried out.

Statistical analysis

Histopathological analysis of pathogen development was analysed using generalized linear mixed modelling (GLMM; Welham, 1993). A binomial distribution with a logit transformation was used to compare the ratio of the growth stages HALO, HYPFLUO and MULTIFLUO, and APP, HAU and HYPHAE, with the total number of infection sites observed. The model fitted compared replicate experiments and BSMV construct or mock treatments for each pathogen and growth stage. Differences between BSMV treatments significant at an *F*-value probability of $P < 0.05$ were further compared by *t*-test analysis.

qRT-PCR transcript levels were compared using general linear regression. Each *TaWIR1* gene was analysed independently. Differences between BSMV VIGS treatments or leaf tissues significant at an *F*-value probability of $P < 0.05$ were further compared by *t*-test analysis. All analyses were carried out using GENSTAT for Windows, 12th edition (GenStat Release 12 Committee, 2009).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 CLUSTALW nucleotide alignment of BSMV:WIR1 and *TaWIR1* sequences showing conservation of the target region between *TaWIR1* genes. Shading indicates 100% (black), 75% (dark grey) and 50% (light grey) conservation between sequences.

Fig. S2 *Barley stripe mosaic virus* (BSMV)-mediated virus-induced gene silencing (VIGS) of *TaWIR1* reduces the expression of transcripts corresponding to probe sets Ta.21556.1, Ta.22732.1 and Ta.3133.1 on the Affymetrix Wheat GeneChip, despite these sequence having less than 80% similarity to BSMV:WIR1. Transcript levels were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and shown relative to mock-inoculated controls. The mean values of three independent experiments are shown with standard error bars.

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Fig. S3 Transcript levels of *TaWIR1* genes in intact leaves of cv. Renan measured after inoculation (hours post-inoculation, hpi) with *Magnaporthe oryzae* isolate BR32, relative to mock-inoculated controls. The mean values of two independent experiments are shown with standard error bars.

Fig. S4 Transcript levels of *TaWIR1* genes in cv. Renan leaf tissues inoculated with *Magnaporthe oryzae* isolate BR32, relative to the same region of the leaf in the mock-inoculated control. The mean values of three independent experiments are shown with standard error bars.

Table S1 *TaWIR1* transcripts present as probe sets on the Affymetrix Wheat GeneChip.

Table S2 Primer sequences.

Table S3 *TaWIR1* genes identified from database similarity searches.

Table S4 SignalP analysis of TaWIR1 proteins.

Table S5 TargetP analysis of TaWIR1 proteins.

Table S6 Transmembrane prediction for TaWIR1 proteins.

Table S7 Nucleotide identities of *TaWIR1* sequences to BSMV:WIR1.

Table S8 BSMV:WIR1 silencing targets predicted by si-Fi analysis.

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