TaEIL1, a wheat homologue of AtEIN3, acts as a negative regulator in the wheat–stripe rust fungus interaction

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SUMMARY

Transcription factors (TFs) play crucial roles in the transcriptional regulation of plant development and defence responses. Increasing evidence has implicated ETHYLENE INSENSITIVE3 (EIN3) in the plant defence response to pathogen infection and environmental stimuli. However, the role of EIN3 in wheat resistance to *Puccinia striiformis* f. sp. *tritici* (*Pst*) is not clear. Here, *TaEIL1* was isolated by rapid amplification of cDNA ends (RACE) based on a sequence fragment from a wheat–*Pst* interaction cDNA library. The *TaEIL1* protein contains a typical EIN3-binding domain, and transient expression analyses indicated that *TaEIL1* is localized in the nucleus. Yeast one-hybrid assay revealed that *TaEIL1* exhibits transcriptional activity, and its C-terminus is necessary for the activation of transcription. *TaEIL1* transcripts were regulated by environmental stress stimuli and were decreased under salicylic acid (SA) treatment. When wheat leaves were challenged with *Pst*, the transcript level of *TaEIL1* in the compatible interaction was approximately three times higher than that in the incompatible interaction. Knocking down *TaEIL1* through the *Barley stripe mosaic virus* (BSMV) virus-induced gene silencing (VIGS) system attenuated the growth of *Pst*, with shortened hyphae and reduced hyphal branches, haustorial mother cells and colony size. Moreover, enhanced necrosis was triggered by the *Pst* avirulent race CYR23, indicating that the hypersensitive response was strengthened in *TaEIL1*-silenced wheat plants. Thus, the up-regulation of defence-related genes and increased sucrose abundance might contribute to the enhanced disease resistance of wheat to the virulent race CYR31. Taken together, the results suggested that the suppression of *TaEIL1* transcripts could enhance the resistance of wheat to stripe rust fungus.

INTRODUCTION

Defence responses are activated on pathogenic infection in plants. The complex response mechanisms of plants are regulated through a large number of genes that encode important metabolic (functional) or regulatory proteins, such as transcription

factors (TFs) (Huang *et al.*, 2005; Umezawa *et al.*, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Extensive research has revealed that the transcriptional regulation of gene expression is an important part of the plant defence response to pathogen infection (Guo *et al.*, 2004; Xiong *et al.*, 2005; Yang *et al.*, 2009). Generally, the expression of pathogen-responsive genes is largely governed through the activities of specific TFs, which bind to specific regulatory sites upstream of constituent genes in a regulatory network through direct physical interactions or in combination with other proteins. In plants, more than 50 families of TFs have been identified based on sequence analysis in *Arabidopsis* and rice (Riaño-Pachón *et al.*, 2007; Xiong *et al.*, 2005). Among these TFs, members of the NAC, WRKY, C2H2-type zinc finger, APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN (AP2/EREBP) and MYB families have been well characterized for their regulatory roles in plant stress or defence responses (Agarwal *et al.*, 2010; Buchanan-Wollaston *et al.*, 2005; Guo *et al.*, 2004; Lin and Wu, 2004; Yang *et al.*, 2009). However, the studies of ETHYLENE INSENSITIVE 3 (EIN3) family genes have primarily focused on the regulation of plant development. In recent years, increasing evidence has implicated EIN3 family genes in defence responses to pathogen attack and environmental stimuli (Asensi-Fabado *et al.*, 2011; Ohme-Takagi *et al.*, 2000; Shi *et al.*, 2012; Umezawa *et al.*, 2006).

The EIN3 proteins are a family of small plant-specific TFs that contain one EIN3 protein and five other EIN3-like (EIL) proteins in *Arabidopsis* (Alonso *et al.*, 2003; Chao *et al.*, 1997). Structurally, the EIN3 family proteins contain a special, highly conserved EIN3-binding domain at the N-terminus. Some EIN3/EILs have a DNA-binding activity site, A(A/C)G(A/T)A(A/C)CT, which has also been identified in the promoters of many *pathogenesis-related* (*PR*) genes, indicating the potential direct activation of basic *PR* promoters (Kosugi and Ohashi, 2000). In tobacco, TEIL (TOBACCO EIN3-LIKE) binds directly to the promoter of the tobacco *PR1a* gene, which is a putative negative transacting factor. EIN3 is also involved in a regulatory cascade for the modulation of *PR* gene expression. EIN3 activates ETHYLENE RESPONSE FACTOR1 (ERF1) TFs, which act as activators or repressors of *PR* genes by binding to their promoter region (Alonso *et al.*, 2003; Boutrot *et al.*, 2010; Chen *et al.*, 2009; Ohme-Takagi *et al.*, 2000; Zhang *et al.*, 2011b). In the *Arabidopsis ein3* mutant, the transcript levels of *ERF1* and of a basic chitinase gene were reduced, and the overexpression

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of *EIN3* resulted in the constitutive expression of this basic chitinase gene, *ERF1*, and the defence marker gene *PLANT DEFENSIN1.2* (*PDF1.2*) (Chao *et al.*, 1997; Solano *et al.*, 1998).

The EIN3 TF family participates in the regulation of various plant responses through various pathways. Previous reports have demonstrated that EIN3/EILs mediate the synergy of methyl jasmonate (MeJA) and ethylene (ETH) phytohormone signalling, which play crucial roles in plant development and defence responses (Browse, 2009; Dong, 1998). The role of ETH in the hormonal regulation of plant development has been well established. Furthermore, ETH functions as a signalling compound in disease resistance and as a virulence factor for several plant fungal and bacterial pathogens, improving the ability of these infectious agents to colonize plant tissue (Chagué *et al.*, 2006). EIN3 is a key TF in the ETH signalling pathway, which functions downstream of EIN2 and acts as a positive regulator (Chao *et al.*, 1997; Yoo *et al.*, 2008). EIN3 and EIL1 are positive regulators of a subset of jasmonic acid (JA) responses, including *PR* gene expression, plant resistance to necrotrophic fungi and root development. These results suggest that EIN3/EILs participate in stress responses not only via ETH signalling, but also via jasmonate signalling (Lorenzo *et al.*, 2003; Zhu *et al.*, 2011). Recently, it has been reported that EIN3/EILs are also involved in the regulation of plant innate immunity via the salicylic acid (SA) signalling pathway (Chen *et al.*, 2009). SA is a major plant defence hormone central to the activation of a range of defences, including the induction of systemic acquired resistance (SAR) and hypersensitive responses (HRs) (Durrant and Dong, 2004; Mishina and Zeier, 2007; Tsuda *et al.*, 2007). EIN3 and EIL1 target directly the promoter of *SID2* which controls SA biosynthesis, negatively regulating *SID2* expression and SA biosynthesis to repress plant immunity (Chen *et al.*, 2009; Wildermuth *et al.*, 2001).

Wheat stripe rust (or yellow rust), caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive wheat (*Triticum aestivum*) diseases worldwide (Hau and de Vallavieille-Pope, 2006; Saari and Prescott, 1985; Villaréal *et al.*, 2002). The fungus is strictly biotrophic and completely dependent on living host cells for reproduction, which has largely hindered the deciphering of the mechanism of the wheat–*Pst* interaction. Therefore, we constructed a cDNA library with RNA from wheat infected by the virulent race CYR31 to analyse the transcripts (Ma *et al.*, 2009). We isolated a novel wheat *EIN3* homologue, *TaEIL1*, which was induced in wheat challenged by CYR31. Subcellular localization and transcriptional activation assays revealed that *TaEIL1* functions as a transcriptional activator in the nucleus. The transcript level of *TaEIL1* was up-regulated in wheat leaves challenged by *Pst* race CYR31 and reduced under SA treatment. Knocking down *TaEIL1* through the *Barley stripe mosaic virus* (BSMV) virus-induced gene silencing (VIGS) system enhanced the resistance of wheat to *Pst*. Our results indicate that *TaEIL1* might serve as a negative regulator in the defence response during the early stages of the wheat–*Pst* interaction through the suppression of the SA pathway.

RESULTS

Cloning and comparative analyses of *TaEIL1*

Based on the isolated sequence from a wheat cDNA library constructed previously in our laboratory (Ma *et al.*, 2009), primers *TaEIL1-F* and *TaEIL1-R* were designed to amplify a 1014-bp partial coding sequence. Both 5' and 3' rapid amplification of cDNA ends (RACE) products were amplified and sequenced to obtain the upstream and downstream sequences of the partial cDNA, and a 2393-bp cDNA sequence, *TaEIL1*, was finally assembled, which contained an open reading frame (ORF) encoding a polypeptide of 650 amino acids with a molecular weight of 71.76 kDa and a *pI* of 4.93.

The *TaEIL1* protein possesses an amino-terminal acidic domain, a proline-rich domain and five small clusters of basic amino acids dispersed throughout the protein, which are typical features of EIN3/EILs. The acidic domain is generally variable among EIN3/EILs, although the acidic nature of this domain is maintained. The N-terminus of *TaEIL1* contains the DNA-binding domain, which shares high homology among EIN3/EILs of different species (Fig. S1, see Supporting Information). The phylogenetic analysis of *TaEIL1* and other EIN3/EILs revealed that *TaEIL1* was most similar to *BdEIL* (GenBank accession number XP_003558012) and *AtEIN3* (GenBank accession number AtEIN3NP_188713), which play important roles in stress responses (Fig. S2, see Supporting Information).

Transcriptional responses of *TaEIL1* to *Pst*, abiotic stresses and hormone elicitors

The results of quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that the *TaEIL1* transcript in the compatible interaction increased as early as 12 h post-inoculation (hpi), reached a maximum level that was approximately six-fold higher than the initial level (near 0 hpi) at 24 hpi, and subsequently diminished to the basal level at 72 hpi (Fig. 1). In the incompatible interaction, the relative transcript level of *TaEIL1* was reduced as early as 12 hpi, exhibited a small increment that was much lower than that in the compatible interaction between 18 and 24 hpi, and subsequently diminished to basal levels at 48 hpi (Fig. 1).

We also assayed the responses of *TaEIL1* to various environmental stresses. Wheat seedlings were treated with low temperature, wounding and polyethylene glycol 4000 (PEG4000). Although wounding induced an increase in transcript level at 6 h post-treatment (hpt), the low-temperature treatment resulted in a significant decrease in transcript level at 2 hpt. No significant accumulation of the *TaEIL1* transcript was detected in wheat leaves after PEG4000 treatment (Fig. 2A). Wheat seedlings were also treated with the exogenous plant hormones MeJA, abscisic

Fig. 1 Quantitative real-time polymerase chain reaction (PCR) analysis of the relative transcript levels of *TaEIL1*. The leaves of one-leaf-stage wheat were inoculated with *Puccinia striiformis* f. sp. *tritici* (Pst) CYR23 (incompatible) or CYR31 (compatible) and sampled at 0, 12, 18, 24, 48, 72 and 120 h post-inoculation (hpi). The relative gene quantification was calculated using the comparative threshold ($2^{-\Delta\Delta C_T}$) method and was relative to that at the 0-hpi time point. Mean and standard deviation were calculated from three independent biological replicates. The different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)]. The developmental stage of *Pst* is shown beneath each time point. CF, colony formed; HMC, haustorial mother cell; PH, primary haustoria; PIH, primary infection hypha; SH, secondary haustoria; SIH, secondary infection hypha; SV, substomatal vesicle.

acid (ABA), SA and ETH (Fig. 2B). The up-regulation of the *TaEIL1* transcript was observed on exogenous application of MeJA and ABA. However, SA treatment reduced significantly the induction of the *TaEIL1* transcript immediately following treatment (2 hpt).

TaEIL1 encodes a nuclear-localized protein

The deduced *TaEIL1* protein sequence contains a putative nuclear localization signal (Fig. S1), indicating the nuclear localization of *TaEIL1*. To confirm this result, we performed an *in vivo* targeting experiment in which the fusion construct *pCaMV35S::TaEIL1-GFP* and the control vector *pCaMV35S::GFP* were transformed into onion epidermal cells. Fluorescence microscopy revealed that the *TaEIL1*-GFP fusion protein was exclusively localized to the nucleus of transformed cells, whereas the control green fluorescent protein (GFP) was uniformly distributed throughout the cell (Fig. 3).

TaEIL1 shows transcriptional activation in yeast

To examine the transcriptional activity of *TaEIL1*, the fusion plasmids pBD-*TaEIL1*₁₋₆₅₀, pBD-*TaEIL1*₁₋₃₃₉ and the negative control pGBKT7 were separately transformed into yeast strain AH109. Transformants grew well on selective dropout/tryptophan (SD/-Trp) medium, indicating that the three vectors were successfully transformed into the yeast strain (Fig. 4). However, only yeast

Fig. 2 The relative transcript level of *TaEIL1* in wheat leaves after treatment with abiotic stresses and hormone elicitors. (A) Abiotic stresses: low temperature, 4 °C; wounding, cutting wheat leaves with a pair of sterilized clean scissors; drought, 20% polyethylene glycol 4000 (PEG4000). (B) Hormone elicitors: ABA, abscisic acid; ETH, ethylene; MeJA, methyl jasmonate; SA, salicylic acid. The mock control was treated with 0.1% (v/v) ethanol solution. Three independent biological replicates were performed. The relative gene quantification was calculated using the comparative threshold ($2^{-\Delta\Delta C_T}$) method and was relative to that at the 0-h post-treatment (hpt) time point. The different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)].

cells containing pBD-*TaEIL1*₁₋₆₅₀ vector grew well on the selective dropout/tryptophan-histidine-adenine (SD/-Trp-His-Ade) medium, whereas yeast cells containing pBD-*TaEIL1*₁₋₃₃₉ or negative control pGBKT7 vector did not grow. In the α -galactosidase activity assay, transformants containing pBD-*TaEIL1*₁₋₆₅₀ vector turned blue on SD/-Trp-His-Ade medium containing 5-bromo-4-chloro-3-indoxyl- α -D-galactopyranoside (X- α -Gal) (Fig. 4), suggesting that *TaEIL1* effectively activated the transcription of *His3* and *LacZ* reporter genes. However, the C-terminal deletion mutant of *TaEIL1* did not positively activate the transcription of the reporter genes.

Suppression of *TaEIL1* reduces the susceptibility of wheat to *Pst*

To further characterize the function of *TaEIL1* during the wheat-stripe rust fungus interaction, *TaEIL1* was silenced in wheat

Fig. 3 Subcellular localization of the *TaEIL1* protein. The control and fusion vectors were transformed into onion epidermal cells by the particle bombardment method. All images were observed using a fluorescence microscope. (A) Onion epidermal cells expressing the green fluorescent protein (GFP) alone driven by the 35S promoter. (B) Onion epidermal cells expressing the *TaEIL1*-GFP fusion protein. Bars, 50 μ m.

Fig. 4 Transactivation analysis of the *TaEIL1* protein in yeast. The fusion and control vectors were separately transformed into the yeast strain AH109. (A) The diagram indicates the corresponding vector for the assay. (B) The transformants were selected through growth on selective dropout/–tryptophan (SD/–Trp) medium at 30 °C for 3 days. (C) Transformants were streaked onto selective dropout/–tryptophan–histidine–adenine (SD/–Trp–His–Ade) medium. (D) The α -galactosidase assay was started on SD/–Trp–His–Ade medium containing 5-bromo-4-chloro-3-indoxyl- α -D-galactopyranoside (X- α -Gal).

seedlings using the BSMV VIGS system, which is an effective reverse genetics tool in barley and wheat (Holzberg *et al.*, 2002; Scofield *et al.*, 2005; Wang *et al.*, 2011). To confirm that the VIGS system functioned correctly, we tested the silencing of the wheat

phytoene desaturase (*PDS*) gene by inoculating the second leaf of a two-leaf wheat seedling with the recombinant virus BSMV:TaPD-Sas. The mild chlorotic mosaic symptoms were visible on the new leaves at 9 days post-inoculation (dpi), and photobleaching

Fig. 5 Phenotypes of wheat leaves in *TaEIL1* knockdown plants inoculated with *Puccinia striiformis* f. sp. *tritici*. (A) Mock wheat leaves treated with Fes buffer; mild chlorotic mosaic symptoms were observed on the fourth leaves of wheat at 9 days post-inoculation (dpi) with BSMV: γ , BSMV:TaEIL1-1as and BSMV:TaEIL1-2as; photobleaching was evident on the fourth leaves of plants infected with BSMV:TaPDSas at 15 dpi. (B) Mock wheat leaves treated with Fes buffer, followed by challenge with *Pst* CYR23 (avirulent) or CYR31 (virulent); BSMV: γ , BSMV:TaEIL1-1as, BSMV:TaEIL1-2as, phenotypes of wheat leaves inoculated with BSMV: γ , BSMV:TaEIL1-1as or BSMV:TaEIL1-2as, followed by challenge with *Pst* CYR23 or CYR31. Typical leaves were observed and photographed at 15 dpi.

phenotypes were observed at 15 dpi. Wheat inoculated with Fes buffer developed normal new leaves under the same conditions, indicating that the silencing of *TaPDS* occurred specifically in BSMV:TaPDSas-infected plants (Fig. 5A). These results showed that the BSMV-VIGS system could be used to assess the potential effects of *TaEIL1* after silencing its transcript in wheat.

All BSMV-inoculated plants displayed mild chlorotic mosaic symptoms at 9 dpi and no obvious defects during subsequent leaf growth. The fourth leaves of wheat plants were inoculated with the avirulent *Pst* race CYR23 or the virulent race CYR31. A typical HR was elicited by CYR23 on all wheat leaves (Fig. 5B). Wheat leaves of *TaEIL1* knockdown plants that were inoculated with CYR31 exhibited normal disease development. However, the level of uredia in the leaves of *TaEIL1* knockdown plants was lower than that in the mock-inoculated plants (Fig. 5B). Meanwhile, knockdown plants with BSMV carrying TaEIL1-1as and TaEIL1-2as

as two different fragments showed a similar result, which proved that the silencing was specific.

To determine the efficiency of VIGS, qRT-PCR was performed to examine the relative transcript level of *TaEIL1* in the fourth leaves of infected plants. Compared with BSMV: γ -infected wheat leaves, the *TaEIL1* transcript in BSMV:TaEIL1-1as-infected leaves was reduced by 68%, 73%, 78% and 74% at 0, 24, 48 and 120 hpi with CYR23, respectively; in leaves inoculated with BSMV:TaEIL1-2as, the *TaEIL1* transcript was reduced by 78%, 69%, 81% and 65%, respectively (Fig. 6). Under similar conditions, wheat leaves infected with BSMV:TaEIL1-1as, followed by CYR31 challenge, resulted in 68%, 70%, 78% and 74% decreases in the abundance of *TaEIL1* transcripts at 0, 24, 48 and 120 hpi, respectively, compared with that of the plants infected with BSMV: γ . Similarly, BSMV:TaEIL1-2as-inoculated leaves caused 79%, 77%, 70% and 67% reductions in *TaEIL1* transcript, respectively (Fig. 6).

Fig. 6 Relative transcript levels of *TaEIL1* in knocked down wheat leaves. RNA samples were isolated from the fourth leaves of wheat infected with BSMV:γ, BSMV:TaEIL1-1as or BSMV:TaEIL1-2as at 0, 24, 48 and 120 h post-inoculation (hpi) with *Pst* CYR23 or CYR31. CK, wheat leaves inoculated with BSMV:γ and sampled at 0 h. Error bars represent the variations among three independent replicates. The different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)]. Relative gene quantification was calculated using the comparative threshold ($2^{-\Delta\Delta C_T}$) method and was compared with that in CK.

To determine whether the transcript levels of defence-related genes in *TaEIL1* knockdown wheat leaves were affected after infection with *Pst*, we selected certain *PR* genes and genes involved in secondary metabolic pathways for qRT-PCR analysis. As shown in Fig. 7, the transcript levels of *TaPR1*, *TaPR2*, *TaPR5* and wheat phenylalanine ammonia lyase (*TaPAL*) were up-regulated in *TaEIL1* knockdown leaves at 24, 48 and 120 hpi. The accumulation of *TaPAL* transcript was significantly induced as early as 24 hpi, and the increase reached almost five-fold that of the BSMV:γ-infected plants. These results suggested that the knockdown of *TaEIL1* affected the transcripts of a number of plant defence-related genes.

Silenced wheat plants accumulate higher levels of sucrose in leaf tissues

Transgenic tobacco plants expressing *PRms* accumulate higher levels of sucrose in leaves (Murillo *et al.*, 2003). In the present study, we investigated whether the accumulation of sucrose was higher in the leaves of *TaEIL1* knockdown plants. The sucrose content was determined from wheat leaves separately sampled from *TaEIL1* knockdown plants and control plants at the fourth-leaf stage. The sucrose content in leaves of silenced plants was higher than that in leaves of control wheat (Fig. 8).

Histological observation of the fungal growth and host response in *TaEIL1* knockdown plants

Leaf segments from at least three plants inoculated with CYR23 or CYR31 were sampled and examined microscopically to observe the detailed histological changes associated with the reduced susceptibility of *TaEIL1* knockdown plants to *Pst* (Fig. S3, see Supporting Information). Necrotic cells at the infection sites of

Fig. 7 Transcript levels of defence-related genes in response to *Puccinia striiformis* f. sp. *tritici* (*Pst*) infection in *TaEIL1* knockdown wheat seedlings. Wheat leaves infected with recombinant Barley stripe mosaic virus (BSMV) were sampled at 0, 24, 48 and 120 h post-inoculation (hpi) with *Pst*. *TaPR1*, pathogenesis-related protein 1; *TaPR2*, β-1,3-glucanase; *TaPR5*, thaumatin-like protein; *TaPAL*, phenylalanine ammonia lyase; *TaACC*, 1-aminocyclopropane-1-carboxylase. Error bars represent the variations among three independent replicates. The different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)]. Relative gene quantification was calculated using the comparative threshold ($2^{-\Delta\Delta C_T}$) method and was relative to that at the 0-hpi time point.

Fig. 8 Sucrose content in the fourth leaves of wheat seedlings. Wheat leaves were infected with BSMV:γ, BSMV:TaEIL1-1as or BSMV:TaEIL1-2as and sampled at 0, 24, 48 and 120 h post-inoculation (hpi) with *Puccinia striiformis* f. sp. *tritici* (*Pst*). CK, wheat leaves inoculated with BSMV:γ. The sucrose content was analysed using high-performance liquid chromatography (HPLC). Error bars represent variations among three independent replicates. The different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)]. BSMV, Barley stripe mosaic virus.

leaves inoculated with CYR23 were observed through autofluorescence, and the proportion of necrotic area was calculated using DP-BSW software (Table 1, Fig. S3). At 48 hpi, the average necrotic area per infection site in BSMV:TaEIL1-2as-inoculated leaves was larger than that in BSMV:γ-inoculated leaves, but the BSMV:TaEIL1-1as-inoculated leaves did not show a significant difference. In addition, the proportions of necrotic areas in both BSMV:TaEIL1-1as-inoculated and BSMV:TaEIL1-2as-inoculated

Table 1 Histological observations in *TaEIL1* knockdown wheat leaves during the interaction between wheat and the stripe rust fungus.

Treatment*	Hyphal length (µm)†		Hyphal branches‡		Percentage of occurrence of haustorial mother cells‡		Colony size per infection site (µm²)‡		Necrotic area per infection site (µm²)‡	
	24 hpi	48 hpi	24 hpi	48 hpi	24 hpi	48 hpi	120 hpi	120 hpi	48 hpi	120 hpi
BSMV:γ/CYR23	4.17 ± 0.56 ^A	5.59 ± 0.26 ^A	1.23 ± 0.21 ^A	2.57 ± 0.36 ^A	87.5% ± 0.08 ^A	95.1% ± 0.04 ^A	2098.90 ± 196.32 ^A	6676.20 ± 279.27 ^B	1497.94 ± 41.73 ^B	9320.56 ± 260.42 ^A
BSMV:TaEIL1-1as/CYR23	4.02 ± 0.50 ^A	5.42 ± 0.44 ^A	1.18 ± 0.35 ^A	2.61 ± 0.52 ^A	83.6% ± 0.06 ^A	84.8% ± 0.02 ^B	1602.80 ± 454.22 ^{AB}	1639.65 ± 55.80 ^B	1639.65 ± 55.80 ^B	11 913.60 ± 570.8.1 ^A
BSMV:TaEIL1-2as/CYR23	3.93 ± 0.23 ^A	4.47 ± 0.53 ^B	1.26 ± 0.28 ^A	2.53 ± 0.31 ^A	85.3% ± 0.06 ^A	92.5% ± 0.03 ^A	1439.28 ± 451.71 ^B	2534.68 ± 76.71 ^A	2534.68 ± 76.71 ^A	—
BSMV:γ/CYR31	4.72 ± 0.26 ^A	6.17 ± 0.53 ^A	1.15 ± 0.37 ^A	2.81 ± 0.62 ^A	90.8% ± 0.03 ^A	92.7% ± 0.05 ^A	2760.39 ± 289.82 ^A	—	—	—
BSMV:TaEIL1-1as/CYR31	3.87 ± 0.25 ^B	5.82 ± 0.43 ^A	1.13 ± 0.31 ^A	2.18 ± 0.64 ^B	87.7% ± 0.04 ^{AB}	91.3% ± 0.03 ^A	2008.08 ± 403.65 ^B	—	—	—
BSMV:TaEIL1-2as/CYR31	4.56 ± 0.56 ^A	4.79 ± 0.44 ^B	1.06 ± 0.23 ^A	2.23 ± 0.54 ^B	79.1% ± 0.03 ^B	89.6% ± 0.06 ^A	2108.21 ± 294.33 ^B	—	—	—

*Treatment: leaves inoculated with BSMV:γ, BSMV:TaEIL1-1as or BSMV:TaEIL1-2as, followed by inoculation with *Puccinia striiformis* f. sp. *tritici* (Pst) race CYR23 and CYR31. hpi, hours post-inoculation.

†Average distance from the base of subterminal vesicles to hyphal tips calculated from at least 50 infection sites (measured in 10-µm units using DP-BSW software).

‡All data are the average number calculated from at least 50 infection sites.

—, Necrotic cells were not observed.

The values followed by the different letters represent significant differences [$P \leq 0.05$ according to analysis of variance (ANOVA)].

leaves were significantly ($P \leq 0.05$) increased compared with that in BSMV:γ-inoculated leaves at 120 hpi (Table 1).

In addition, we examined the hyphal length, hyphal branches, haustorial mother cells at 24 and 48 hpi, and the colony size at 120 hpi. In plants inoculated with CYR23, the hyphae in BSMV:TaEIL1-2as-inoculated leaves were significantly shorter at 48 hpi, and the colony size per infection site was significantly reduced at 120 hpi, than that observed on BSMV:γ-infected leaves. The percentage of occurrence of haustorial mother cells in BSMV:TaEIL1-1as-inoculated leaves was reduced significantly at 48 hpi. In plants inoculated with CYR31, the hyphae in BSMV:TaEIL1-1as- and BSMV:TaEIL1-2as-inoculated leaves were significantly shorter than those observed in BSMV:γ-infected leaves at 24 and 48 hpi, respectively. In both *TaEIL1* knockdown wheat leaves, the number of hyphal branches and the colony size were reduced significantly compared with that in BSMV:γ-infected leaves at 48 hpi and 120 hpi, respectively. The percentage of occurrence of haustorial mother cells in BSMV:TaEIL1-2as-inoculated leaves was significantly reduced at 24 hpi. These results indicated that the knockdown of the *TaEIL1* transcript diminished hyphal growth, hyphal branching, the occurrence of haustorial mother cells and colony size, but enhanced host cell death.

DISCUSSION

The EIN3 family of TFs has been implicated in various biological functions in plant development and stress responses (Asensi-Fabado *et al.*, 2011; Ohme-Takagi *et al.*, 2000; Shi *et al.*, 2012). In this study, we isolated a novel *TaEIL1* from wheat leaves infected with Pst CYR31. *TaEIL1* is homologous with other EIN3 proteins, indicating a high sequence conservation of EIN3 TFs among different plant species, which is important for interactions with regulators and effectors. The deduced *TaEIL1* protein was predicted to contain a nuclear localization signal and a plant-specific EIN3-binding domain in the N-terminal region, which was verified through subcellular localization in onion cells. The yeast one-hybrid assay further showed that *TaEIL1* has transcriptional activity and the C-terminal region of amino acids 340–650 is necessary for transactivation (Fig. 4). These data suggest that *TaEIL1* is a novel wheat EIN3 TF, which functions as a transcriptional activator in the plant nucleus.

EIN3/EILs are regulated through environmental factors, such as wounding and cold (Shi *et al.*, 2012; Zhu *et al.*, 2011). Hence, we determined the transcript level of *TaEIL1* using qRT-PCR. Consistent with results from previous studies, the *TaEIL1* transcript was up-regulated in wounding and down-regulated at low temperature. Thus, the results suggest that the ubiquitously expressed *TaEIL1* gene in wheat functions as a basal regulator in response to various abiotic stresses.

Currently, EIN3/EILs are proposed to represent a branching point for the cross-talk between ETH, MeJA and SA signalling

pathways and participate in plant defence through these signalling pathways (Anderson *et al.*, 2004; Chen *et al.*, 2009). In wheat, the expression of *TaEIL1* was induced by the exogenous application of MeJA and ABA, and was reduced by SA treatment during the early stages of treatment; consistent with the reported regulation of EIN3/EIL expression by exogenous ETH, no significant changes in *TaEIL1* transcript level were detected under ETH treatment. ETH has also been shown to inhibit EIN3 proteolysis and to rapidly increase EIN3 protein levels without significant changes in mRNA levels (Chao *et al.*, 1997; Lee and Kim, 2003; Tieman *et al.*, 2001). Cross-talk between SA, MeJA and ETH signalling pathways has emerged as an important regulatory mechanism in plant immunity (Pieterse *et al.*, 2009; Verhage *et al.*, 2010), facilitating the selection of appropriate defence strategies in plants when attacked by pathogens of different lifestyles (Adie *et al.*, 2007). SA signalling is often effective against biotrophic pathogens, such as *Erysiphe orontii* and *Peronospora parasitica*, whereas MeJA/ETH signalling is required for effective resistance to necrotrophic pathogens, such as *Botrytis cinerea* and *Erwinia carotovora* (Rojo *et al.*, 2003; Thomma *et al.*, 2001). Recent publications have shown that jasmonate signalling plays a dichotomous role in wheat interaction with *Fusarium graminearum*, suppressing the activation of SA signalling during the early stages of infection and promoting resistance during the later stages of infection (Makandar *et al.*, 2012). It has also been reported that EIN3/EILs negatively regulate SA biosynthesis to repress plant immunity in *Arabidopsis*, which is uncoupled from ETH-induced plant development. Thus, EIN3/EIL1 possesses both transcriptional activator and repressor activities depending on the target genes, and represses plant immunity through the negative regulation of SA biosynthesis in *Arabidopsis* (Chen *et al.*, 2009; Wildermuth *et al.*, 2001). Therefore, it is reasonable to assume that *TaEIL1* negatively participates in basal defences in the early stage of the wheat–*Pst* interaction, mainly through the SA-mediated signalling pathway. However, there is limited insight into the role of the SA signalling pathway in the wheat–*Pst* interaction and the regulatory mechanisms underlying the cross-talk between different signalling pathways.

Previous studies have demonstrated that EIN3 and EIL1 negatively regulate disease resistance through the suppression of the pathogen-associated molecular pattern (PAMP)-triggered transcriptional programme in *Arabidopsis*, and that EIN3-overexpressing plants are compromised in defence and show enhanced disease susceptibility (Chen *et al.*, 2009). In the present study, we used a knockdown approach to determine the role of *TaEIL1* in the wheat–*Pst* interaction. The reduced disease symptoms in wheat seedlings infected with virulent *Pst* suggested that the suppression of *TaEIL1* transcripts could partially reduce the susceptibility of wheat to virulent *Pst*. In addition, the average necrotic area per infection site, representing the level of HR, was increased significantly in the incompatible interaction, suggesting that the HR was enhanced during the wheat–*Pst* interaction

when *TaEIL1* expression was knocked down. BSMV:TaEIL1-2as-inoculated leaves showed a much greater increase, suggesting a more successful knockdown genotype. In addition, the hyphal length was dramatically shortened, and the number of hyphal branches, occurrence of haustorial mother cells and colony size were reduced, implying that disease resistance was enhanced in *TaEIL1* knockdown wheat plants. Furthermore, the histological observations were consistent with the results of *TaEIL1* gene expression profiles in wheat leaves inoculated with CYR23 or CYR31. Taken together, these results indicate that *TaEIL1* might play a positive role in pathogen recognition and penetration.

To confirm this supposition, we analysed the expression profiles of some well-documented defence genes, including those encoding PR proteins (PR1, PR2, PR5) and enzymes for phytoalexin biosynthesis, such as PAL, in *TaEIL1* knockdown wheat plants infected with the *Pst* virulent pathotype CYR31. The PR proteins are generally considered as marker genes in HRs and are necessary for resistance (Schaffrath *et al.*, 1997; Van Loon and Van Strien, 2002). PAL genes, which are involved in SA synthesis, have been shown to accumulate transcripts in several different incompatible host–pathogen combinations and in response to elicitors (Mauch-Mani and Slusarenko, 1996). The accumulation of the transcripts of PR protein genes and secondary metabolites of PAL in *TaEIL1* knockdown plants might contribute to the reduced susceptibility of wheat to the virulent pathotype. The results obtained in the present study are consistent with previous reports that, in *Arabidopsis*, the *eil1-1* mutant and *ein3-1 eil1-1* double mutant exhibited constitutive PR1 expression, and the *ein3-1* mutant showed elevated PR1 expression, when inoculated with *Pseudomonas syringae* DC3000 (*avrB*) and *hrpL* mutant bacteria (Chen *et al.*, 2009). Based on the results obtained in the present study, we conclude that *TaEIL1* knockdown wheat plants show reduced susceptibility to *Pst*.

The susceptibility of plants to a number of diseases is often dependent on the sugar levels in leaf tissues (Vargas *et al.*, 2009). The increased level of endogenous sucrose could be responsible for the earlier and stronger expression of rice defence genes, thereby protecting rice plants from *Magnaporthe oryzae* infection. In the present study, leaf tissue of *TaEIL1* knockdown wheat plants accumulated higher levels of sucrose than did controls. Thus, we propose that *TaEIL1* knockdown wheat plants develop enhanced resistance to stripe rust fungus. This hypothesis is consistent with previous findings that resistance is associated with sucrose signalling, and the level of sugar in tissues increases during the infection of viruses (Gómez-Ariza *et al.*, 2007; Shalitin and Wolf, 2000). Plants might have evolved a mechanism to modulate their sucrose levels in response to pathogen attack, and through altered sucrose levels to activate defence reactions (Gómez-Ariza *et al.*, 2007). Sucrose functions as a signalling molecule for the induction of defence-related gene expression. SA is an important signal for SAR development and the activation of defence responses. However, SA does not appear to be a long-distance mobile signal

in SAR (Murillo *et al.*, 2003). It is likely that sucrose can act as an endogenous molecular signal in SAR. Increasing concentrations of sucrose induce the expression of tobacco *PR-1a*, *PR-5* and chitinase (Gómez-Ariza *et al.*, 2007; Murillo *et al.*, 2003). Thus, sucrose accumulation is coincident with the expression profiles of defence-related genes in *TaEIL1* knocked down wheat plants. Therefore, we presume that the sucrose accumulation in silenced wheat plants is associated with the enhanced resistance of wheat to stripe rust fungus.

It is interesting to note that sucrose prevents the up-regulation of senescence-associated genes through an inhibition of the up-regulation of the EIN3/EIL protein, which is the master switch for the expression of ETH response genes (Hoeberichts *et al.*, 2007). Thus, it is reasonable to assume that sucrose mediates the defence response through the regulation of the *TaEIL1* transcript level. However, it is not clear how these events are related, and further studies of sucrose-modulated wheat resistance to *Pst* are needed. Nevertheless, based on the results of the present study, we conclude that *TaEIL1* might negatively regulate the defence response during the wheat–*Pst* interaction.

EXPERIMENTAL PROCEDURES

Plant materials, inoculations and treatments

The wheat (*Triticum aestivum*) cultivar Suwon 11 (Su11 contains *YrSu*) and *Pst* pathotypes CYR23 (avirulent on Su11) and CYR31 (virulent on Su11) were used in this study. Su11 shows a typical HR to CYR23, but is highly susceptible to CYR31. Wheat was grown, inoculated and maintained according to previously described procedures and conditions (Kang and Li, 1984). For RNA isolation, leaves were sampled at 0, 12, 18, 24, 48, 72 and 120 hpi with *Pst*. Control wheat plants were inoculated with sterile distilled water in the same manner. The time points were selected based on the microscopic study of the interactions between Su11 and CYR23 or CYR31 (Kang *et al.*, 2002; Wang *et al.*, 2007). Disease symptoms were scored at 15 dpi.

For the low-temperature treatment, 8-day-old wheat seedlings were transferred to a chamber and incubated at 4 °C. Wounding treatment was applied by cutting wheat leaves with a pair of sterilized scissors. For the drought stress treatment, roots of wheat seedlings were soaked in 20% PEG4000 (causing water deficit stress). For chemical treatment, the leaves of 8-day-old wheat seedlings were sprayed separately with 100 mM MeJA, 100 mM ETH, 2 mM SA and 100 mM ABA using the method of Zhang *et al.* (2004). Control wheat seedlings were treated with 0.1% (v/v) ethanol. Wheat seedlings treated with various abiotic stresses and hormone elicitors, together with control plants, were sampled at 0, 2, 6, 12, 24 and 48 hpt.

All samples were immediately frozen in liquid nitrogen and stored at –80 °C. Three independent biological replicates were performed for each treatment.

RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA from wheat was extracted using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The

potential contaminating DNA was digested with *DNase I* (Promega, Madison, WI, USA). First-strand cDNA was synthesized using the GoScript Reverse Transcription System (Promega). Oligo(dT)₁₈ was used as a primer, and the reverse transcription reaction was incubated at 42 °C for 1 h in a total volume of 20 µL. After a 1:10 dilution, 2 µL of the synthesized cDNA was used for qRT-PCR. The primers for qRT-PCR were designed based on the cDNA sequence (Table S1, see Supporting Information), and the qRT-PCR was conducted as described previously (Wang *et al.*, 2009). To standardize the data, the wheat translation elongation factor 1 α subunit gene (*TaEF-1a*, GenBank accession number M90077.1) was used as an internal reference for the qRT-PCR analysis. The quantification of gene expression was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Dissociation curves were generated for each reaction to ensure specific amplification. All reactions, including the nontemplate control, were performed three times. The threshold values (*C_T*) generated from the ABI PRISM 7500 software tool (Applied Biosystems) were employed to quantify the relative gene expression using the comparative threshold ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001). Three independent biological replicates were performed for each experiment.

Isolation and cloning of *TaEIL1*

To clone the *TaEIL1* gene, we isolated a partial sequence of the *TaEIL1* gene from the wheat–*Pst* interaction cDNA library previously constructed in our laboratory (Ma *et al.*, 2009). Based on this sequence, RACE primers were designed to clone the missing 5' and 3' terminal sequences of *TaEIL1* (Table S1). RACE PCR amplification was performed using a Su11-CYR31-compatible cDNA sample as a template. The PCR products were purified, cloned into the pGEM-T vector (Promega) and sequenced.

Sequence analysis, alignment and domain prediction

The cDNA sequences were analysed using DNASTAR (<http://www.dnastar.com>) and BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) to search the Non-Redundant (NR). Pfam (<http://pfam.sanger.ac.uk/>), InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) and PROSITE Scan (<http://prosite.expasy.org/scanprosite/>) were used to predict conserved domains or motifs. Target P (<http://www.cbs.dtu.dk/services/TargetP/>) was used to predict the subcellular localization of *TaEIL1*.

Multiple sequence alignments were performed using CLUSTALW and DNAMAN6.0. Phylogenetic trees of *TaEIL1* and *EIN3/EIL* members were constructed by MEGA4 software.

Plasmid construction

To verify the subcellular localization of *TaEIL1*, the coding region sequences without stop codons were PCR amplified (the primers are shown in Table S1) and subcloned into the plant binary expression vector *pCaMV35S::GFP* in frame with the GFP sequence, resulting in the vector *pCaMV35S::TaEIL1-GFP*, under the control of the *CaMV35S* promoter.

To investigate the transcriptional activity of the *TaEIL1* protein, the ORF and C-terminal deletion sequence of the *TaEIL1* gene were PCR amplified using gene-specific primers (Table S1) and fused in frame with the GAL4 DNA-binding domain in the vector pGBKT7 (Clontech, Palo Alto, CA, USA), resulting in two reporter plasmids pBD-*TaEIL1*_{1–650} and pBD-*TaEIL1*_{1–339}.

The plasmids used for gene silencing were based on previously described constructs (Holzberg *et al.*, 2002). To maximize the specificity of *TaEIL1* gene silencing, stringent efforts were made to select the fragments. First, all nucleotide sequences of the EIN3 gene family from Arabidopsis and rice were downloaded and alignments were made. The results showed that the 5' and 3' terminal regions of the EIN3 gene family exhibited high diversity among the EIN3 gene families of Arabidopsis and rice (Fig. S4, see Supporting Information), and the identity was less than 55%. Previous studies have reported that sequences with 81% identity or less do not induce targeted gene silencing (Holzberg *et al.*, 2002). Based on the results of alignment, a 238-bp cDNA fragment was derived from the 5' untranslated region and partial coding sequence for silencing, and the other 180-bp fragment was derived from the 3' untranslated region. In addition, a BLAST search of the two fragments in the GenBank database did not show similarities with any other wheat genes, indicating the specificity of the fragments. Subsequently, these two fragments, in an antisense orientation, were used to replace the GFP coding sequence in the original BSMV:GFP vector to generate BSMV:TaEIL1-1as and BSMV:TaEIL1-2as vectors (primers for all plasmid constructions are given in Table S1).

Subcellular localization analysis

To examine the subcellular localization of *TaEIL1*, we performed a transient expression analysis using onion epidermal cells. Onion inner peels were incubated inside out on Murashige and Skoog (MS) medium plates for 4–8 h before bombardment. The plasmid *pCaMV35S:TaEIL1-GFP* and the control plasmid *pCaMV35S:GFP* were separately transformed into onion epidermal cells through particle bombardment at a helium pressure of 7.6 MPa (1100 psi) using the Bio-Rad He/1000 single-barrelled particle delivery system (Bio-Rad, Hercules, CA, USA) (Eady *et al.*, 1996). The transformed onion epidermal cells were incubated in the dark at 24 °C for 18–24 h in MS solid medium. The GFP signals were detected with a fluorescence microscope (Olympus BX-51; Olympus, Tokyo, Japan).

Transcriptional activation analysis in yeast

According to the yeast transformation protocol, the reporter plasmids pBD-*TaEIL1*_{1–650} and pBD-*TaEIL1*_{1–339} and the negative control plasmid pGBKT7 were individually transformed into the yeast strain AH109 (Clontech). The transformed strains were grown on SD/-Trp and SD/-Trp-His-Ade media at 30 °C for 3 days, and subsequently the growth status was evaluated. The α -galactosidase activity assay was performed using X- α -Gal as a substrate, according to the manufacturer's instructions.

BSMV-mediated *TaEIL1* gene silencing

Capped *in vitro* transcripts were prepared from linearized plasmids containing the tripartite BSMV genome (Petty *et al.*, 1990) using the mMessage mMachine T7 *in vitro* transcription kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Three independent sets of plants were used, with a total of 144 seedlings inoculated for each of the three BSMV viruses (BSMV: γ , BSMV:TaEIL1-1as, BSMV:TaEIL1-2as). BSMV-TaPDSas (*TaPDS*: wheat *PDS* gene) was used as a positive control. As a negative control, another 48 seedlings were mock inoculated with 1 $\times$ Fes buffer (Pogue *et al.*, 1998) devoid of BSMV transcripts. The second leaf of

a two-leaf wheat seedling was inoculated with BSMV transcripts by gently rubbing the surface with a gloved finger (Holzberg *et al.*, 2002; Scofield *et al.*, 2005), followed by incubation at 23 $\pm$ 2 °C, and subsequent examination for symptoms at regular intervals. At 9 days after virus inoculation, the virus phenotypes were observed and photographed. The fourth leaves were further inoculated with *Pst* pathotypes CYR23 and CYR31, and sampled at 0, 24, 48 and 120 hpi for RNA isolation, sucrose content quantification and histological observation (Wang *et al.*, 2007). The *Pst* infection phenotypes were recorded and photographed at 15 dpi.

qRT-PCR and histological analyses of silenced plants inoculated with *Pst*

qRT-PCR was performed to determine the changes in the transcript levels of *TaEIL1* and resistance-related genes in response to *Pst* infection in *TaEIL1* knockdown wheat plants.

The leaf segments sampled for histological observation were fixed and decolorized according to Wang *et al.* (2007). The cleared leaf segments were observed using an Olympus BX-51 microscope (Olympus) for infection sites. Only infection sites in which substomatal vesicles had formed in the stomata were considered to have successful penetration. The autofluorescence of mesophyll cells in infected leaves was indicative of necrotic death using epifluorescence microscopy (excitation filter, 485 nm; dichromatic mirror, 510 nm; barrier filter, 520 nm). The samples were stained with 0.1% (w/v) Calcofluor M2R (Sigma-Aldrich, St. Louis, MO, USA) and observed for the formation of substomatal vesicles, infection hyphae, hyphal branches, haustorial mother cells and colony size (Zhang *et al.*, 2011a). At least 50 infection sites were examined for each of the five randomly selected leaf segments per treatment.

Determination of sucrose content

A total of 0.2 g fresh weight of leaves from the control and *TaEIL1* knockdown plants was separately pulverized in the presence of liquid nitrogen. This sample was added to 2.5 mL of extraction buffer (chloroform : methanol, 1.5:3.5, v/v) according to Ralf's method (Voegelé *et al.*, 2005), homogenized through vortexing, and kept on ice for 30 min. This mixture was subsequently added to 1.5 mL of water and mixed through vortexing for 1 min. The samples were centrifuged at 13 000 g for 5 min at room temperature and the supernatant was collected. The centrifugation step was repeated, and the resulting supernatant was collected and combined with the supernatant from the first spin. The solution was evaporated to 300 μ L in a rotary evaporator, filtered through a 0.22- μ m Millipore filter and stored at -80 °C until further analysis.

The sucrose content was analysed using high-performance liquid chromatography (HPLC/Waters 600 controller, Milford, MA, USA) with an NH₂ (5 μ L; 4.6 mm $\times$ 250 mm) column (Inertsil® HPLC Column). The mobile phase (acetonitrile : filtered water, 4:1, v/v) was pumped through the column at a flow rate of 1.4 mL/min. The temperature of the oven was adjusted to 70 °C, whereas that of the cuvette was maintained at 40 °C. A refractive index detector (Waters 2414) was used.

Data analysis

Analysis of variance (ANOVA) and Tukey's test for statistical analysis were performed using SAS software 9.13.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Multiple alignment of the amino acid sequences of ETHYLENE INSENSITIVE 3/EIN3-like (EIN3/EIL) proteins. Amino acid residues conserved in all five sequences are indicated in black and those conserved among four sequences are indicated in light grey. The acidic (AD), basic (BDI–V) and proline-rich (PR) domains are boxed. The asterisk refers to the lysine residue. The putative nuclear localization signals are indicated with full lines.

Fig. S2 Phylogenetic analysis of *TaEIL1* and selected ETHYLENE INSENSITIVE 3 (EIN3) family proteins. At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Cs, *Citrus sinensis*; Gm, *Glycine max*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Zm, *Zea mays*. GenBank accession numbers are provided after the gene names.

Fig. S3 Histological observation of fungal growth and host cell death in *TaEIL1* knockdown wheat leaves. Leaf segments were sampled at 24, 48 and 120 h post-inoculation (hpi) with the *Puccinia striiformis* f. sp. *tritici* (*Pst*) avirulent race CYR23 and virulent race CYR31. (A, B) Observation of the infection site and *Pst* growth under differential interference contrast optics (light microscopy). (C, D) Observation of necrotic cells under differential interference contrast optics and fluorescence microscopy. (E, F) Observation of colony area under differential interference contrast optics and fluorescence microscopy. (C–F) Photographs were captured at the same infection sites. HC, haustorial cell; HMC, haustorial mother cell; IH, infection hypha; NC, necrotic cell; SV, substomatal vesicle. (A–D) Bar, 20 μ m. (E, F) Bar, 50 μ m.

Fig. S4 Multiple alignment of nucleotide sequences of *TaEIL1* gene for gene silencing. (A) Multiple alignment of the nucleotide sequences of *TaEIL1* and *OsEILs*. Two virus-induced gene silencing (VIGS) sites were designed on both sides of *TaEIL1*. The knockdown fragments are indicated with full lines; the initiation codon and termination codon are boxed. (B) Multiple alignment of the nucleotide sequences of *AtEILs*. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ta, *Triticum aestivum*. GenBank accession numbers are provided after the gene names.

Table S1 Primers designed for *TaEIL1* research.