Overexpression of TiERF1 enhances resistance to sharp eyespot in transgenic wheat

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Abstract

Wheat sharp eyespot, primarily caused by a soil-borne fungus Rhizoctonia cerealis, has become one of the most serious diseases of wheat in China. In this study, an ethylene response factor (ERF) gene from a wheat relative Thinopyrum intermedium, TiERF1, was characterized further, transgenic wheat lines expressing TiERF1 were developed, and the resistance of the transgenic wheat lines against R. cerealis was investigated. Southern blotting analysis indicated that at least two copies of the TiERF1 gene exist in the T. intermedium genome. Yeast one-hybrid assay indicated that the activation domain of TiERF1 is essential for activating the transcript of the reporter gene with the GCC-box cis-element. The TiERF1 gene was introduced into a Chinese wheat cultivar, Yangmai12, by biolistic bombardment. Results of PCR and Southern blotting analyses indicated that TiERF1 was successfully integrated into the genome of the transgenic wheat, where it can be passed down from the T0 to T4 generations. Quantitative reverse transcription-PCR analysis demonstrated that TiERF1 could be overexpressed in the stable transgenic plants, in which the expression levels of wheat pathogenesis-related (PR) genes primarily in the ethylene-dependent signal pathway, such as a chitinase gene and a β-1,3-glucanase gene, were increased dramatically. Disease tests indicated that the overexpression of TiERF1 conferred enhanced resistance to sharp eyespot in the transgenic wheat lines compared with the wild-type and silenced TiERF1 plants. These results suggested that the overexpression of TiERF1 enhances resistance to sharp eyespot in transgenic wheat lines by activating PR genes primarily in the ethylene-dependent pathway.

Key words: Defence-related gene, ethylene response factor (ERF), Rhizoctonia cerealis, sharp eyespot, Thinopyrum intermedium, transgenic wheat plants.

Introduction

Wheat sharp eyespot, mainly caused by Rhizoctonia cerealis, has become one of the most serious diseases of wheat (Triticum aestivum L.) in China since the 1980s. In infected wheat plants, R. cerealis may destroy the transport tissues and other tissues in stems and sheaths of host plants, leading to a block in the transportation of substances required for nutrition, to lodging, and even to dead spikes. In China, wheat production, with >6.00 million ha each year, was subjected to a sharp eyespot threat from 2005 to 2008, resulting in economic losses of >¥1 billion.

Breeding wheat varieties with resistance to sharp eyespot is the most promising and reliable means of wheat sharp eyespot control. However, there is very little basic theoretical research on wheat defence against R. cerealis, and no reliably resistant cultivars in different environment conditions have been developed by traditional breeding to date. Therefore, it is necessary and urgent for effective control of sharp eyespot to strengthen research on wheat defence mechanisms against R. cerealis and to develop genetic engineering to increase the disease resistance in wheat.

Plants have evolved a complex battery of defence mechanisms against attacks of various pathogens. The key to understanding plant defence responses lies in the elucidation of the signalling pathways involved in their regulation (McGrath et al., 2005). In the model plant Arabidopsis (Arabidopsis thaliana), the salicylic acid (SA)-dependent systemic acquired resistance (SAR) pathway, and jasmonic acid (JA) and ethylene (ET)-induced resistance pathways are well recognized (Thomma et al., 1998; Hammond-Kosack...
Transcription factors (TFs) are believed to play a crucial role in the transmission of pathogen-derived defence signals to either activate or suppress expression of downstream defence genes as well as in the regulation of cross-talk between different signalling pathways (Lorenzo et al., 1998; McGrath et al., 2005). Transactivation activity assay of TiERF1 in yeast cells

The reporter constructs, pGcc-LacZ and pmGcc-LacZ, were prepared according to the protocol of Zhang et al. (2007). These constructs contain three copies of the Gcc box (3×Gcc) or mutant Gcc (3×mGcc), respectively, fused to the Lac-Z reporter gene in the vector pLacZi (Clontech). The effector vectors pYTiERF1, containing the full open reading frame (ORF) sequence of TiERF1 (GenBank accession no. EF570121), and pYTiERF1AAD, in which the putative activation domain (from amino acid 51 to 75) in pYTiERF1 was deleted, were also prepared. All of these constructs possess activation and binding domains derived from TiERF1 rather than yeast, and can be expressed in yeast cells (Fig. 2A).

Using the methodology provided in the yeast one-hybrid manual (Clontech), the effector and reporter constructs were transformed into competent cells of the yeast strain YM4271 to form four kinds of combinations, namely Y-pYTiERF1/pGcc-LacZ, Y-pYTiERF1/pmGcc-LacZ, Y-pYTiERF1AAD/pGcc-LacZ, and Y-pYTiERF1/pLacZi. The transformants were selected by growth on SD medium minus Ura and Trp. Subsequently, a filter-lift assay on β-galactosidase (encoded by LacZ) activity was performed to determine the transcription activation character of the TiERF1 protein and TiERF1AAD protein according to the manual (Clontech).

Plant transformation vector and wheat transformation

The pAHC25 plasmid (Christensen and Quail, 1996; a gift from Dr Peter Quail, USDA-ARS, Albany, CA, USA) contains the uidA [encoding the β-glucuronidase (GUS)] and bar [encoding the phosphinothricin acetyltransferase (PAT)] genes, respectively, driven by the maize ubiquitin (Ubi) promoter and terminated by the 3′-non-transcribed region of the Agrobacterium tumefaciens nopalin synthase (nos) gene. PAT activity confers resistance to phosphinothricin-containing herbicides, such as Bialaphos.

The full ORF sequence of the TiERF1 gene with Smad and SacI was cloned into the Smal and SacI sites of pAHC25 to form the transformation vector pAHCTiERF1, in which the TiERF1 replaced the uidA gene of the original pAHC25 vector (Fig. 3A). The orientation and the ORF integrity of TiERF1 in the construct were confirmed by sequence analysis.

The wheat cv. Yangmai12 was used as the host of the TiERF1 transformation. All aspects of the transformation protocols were conducted according to the protocol described by Xu et al. (2001). Briefly, the immature embryos of Yangmai12 were incubated to form embryogenic calli. The plasmid DNA (20 μg) of the TiERF1 expression vector pAHCTiERF1 was packed onto gold powder at 1,100 psi bombardment pressure, a 25 pA vacuum, and a bombardment distance of 6 cm. The bombarded wheat calli were regenerated and selected using Bialaphos. When the plantlets had grown to 7–8 cm, the seedlings with roots were transferred to soil pots and grown in an environmentally controlled greenhouse at ~70% relative humidity for 16 h in the light at 23–25 °C and 8 h in the dark at ~13–16 °C.
PCR detection of TiERF1 in the transgenic wheat plants

The presence of the TiERF1 gene in the transgenic wheat plants was monitored by PCR using the gene-specific primers TiE-406F (GAGCCCAACGGGTTCATG) and TiE-746R (GACCGGAC-GAGGAAGAG). The PCR was performed in a total volume of 25 μl containing 1× GC buffer I (TaKaRa), 100–200 ng of genomic DNA, 187.5 μM of each dNTP (TaKaRa), 0.25 μM of each primer, and 1 U of Taq polymerase (TaKaRa), and subjected to a program of initial denaturation at 94 °C for 4 min, followed by 10 cycles of 45 s at 94 °C, 45 s at 62 °C, and 1 min at 72 °C, then 25 cycles of 45 s at 94 °C, 45 s at 58–60 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The amplified product (341 bp) specific to the TiERF1 gene was resolved on a 1% (w/v) agarose gel and visualized by ethidium bromide staining.

Southern blotting analysis

Total genomic DNAs were isolated according to the extraction protocol reported previously (Zhang et al., 1999). Southern blotting was conducted according to the modified protocol of Sharpe et al. (1989). The genomic DNAs (~20 μg each) of T. intermedium and wheat cv. Yangmai12, digested by the restriction enzymes BglIII, EcoRV, HindIII, and BamHI, or 20 pg of pAHC-TiERF1 plasmid DNA (positive control) were resolved on a 0.8% agarose gel, and then were depurinated in 0.25 M HCl, and transferred to a nylon DNA (positive control) were resolved on a 0.8% agarose gel, and then were depurinated in 0.25 M HCl, and transferred to a nylon membrane (Hybond N+, Amersham BioScience) using alkalinity transfer buffer (0.4 M NaOH plus 1 M NaCl). A specific fragment of TiERF1 (nucleotides 406–746) labelled by [α-32P]dCTP was used as the probe. Hybridization was carried out at 65 °C for 16 h in Denhardt’s buffer (5× SSC, 5× Denhardt’s solution, 0.1% SDS). The hybridized membrane was washed twice (2× SSC, 0.1% SDS) at 65 °C for 5 min, twice (1× SSC, 0.1% SDS) at 65 °C for 5 min, and then in 0.1× SSC, 0.1% SDS at 65 °C for 5 min 2–4 times based on the radioactivity intensity. Blots were exposed on a phosphor screen (Kodak-K) for 2 d, and the signals were captured using a Molecular Imager FX System (Bio-Rad).

RNA extraction and transcript analysis

Total RNA of each sample was extracted using the TRIZOL reagent according to the manufacturer’s protocol (Invitrogen). A 2 μg aliquot of RNA per sample was used to synthesize the first-strand cDNA using an RNA PCR Kit (TaKaRa).

In the transgenic and wild-type wheat plants, and wild-type T. intermedium plants, the expression levels of TiERF1 and defence-related genes, including PR genes, a PR3-chitinase I gene (ChiI), a PR2 β-1,3-glucanase gene (D-GLU), PR10, and PR1, were investigated using RT-PCR and Q-RT-PCR methods with the specific primers listed in Table 1. Q-RT-PCR was performed with SYBR® Green I dye (TaKaRa) using an ABI PRISM® 7000 sequence detection system, where reactions were subjected to the following program: 95 °C for 1 min, 41 cycles of 95 °C for 10 s, and 60 °C for 31 s. For each sample, the Ct value of each target gene was normalized to the Ct value of the actin gene. The relative value of gene expression was derived from 2^-ΔΔCT (Livak and Schmittgen, 2001). The relative expression of the target gene in the transgenic wheat plants was present relative to average wild-type levels.

Inoculation with R. cerealis and the disease rating in wheat

At peak tillering stage, the bases of the first to second sheaths of the wheat plants were inoculated with small toothpick fragments harbouring the well-developed mycelia of R. cerealis according to the cultured toothpick method described by Cai et al. (2006). The disease ratings of wheat plants were evaluated and photographed at the maturation stage of wheat following the scoring standard described by Cai et al. (2006), where infection type 1 (IT:1) indicates a mini-lesion (<1%) on the first–second sheaths rather than stems; IT:2 indicates that the width of a disease lesion is less than half that of the infected stem perimeter; IT:3 indicates that the width of a disease lesion is more than half that of the infected stem perimeter; IT:4 indicates that the width of a disease lesion is >75% that of the infected stem perimeter; and IT:0 indicates no disease on the shoot and stem. The disease index = Σ(di×li)×100/ (L×dmax), where di indicates the plant numbers of each infection type, li indicates the plant numbers of each infection type, and L indicates the total numbers of plants in the disease investigation. Plants with a disease index of 0 were considered as immune, those with a disease index <20.0% as having high resistance, those with a disease index of 20.01–40.00% as being resistant, those with a disease index of 40.01–60.00% as susceptible, and those with a disease index of 60.01–100.00% as highly susceptible.

Results

At least two copies of TiERF1 exist in the T. intermedium genome

Southern blotting analysis is necessary to identify the size of the TiERF1 family since the genome of T. intermedium has not been sequenced. The result of Southern hybridization with the specific TiERF1 sequence showed that two clear hybridization bands were present in T. intermedium genomic DNA digested by BglII, EcoRV, HindIII, and BamHI (Fig. 1), indicating that at least two copies of TiERF1 should exist in the T. intermedium genome since the sequence of TiERF1 corresponding to the probe does not contain the above enzyme sites. One clear hybridization band was present in the genomic DNA of wheat Yangmai12 digested by BglII, and a weak hybridization band was present in the wheat genomic DNA digested by

### Table 1. Sequences of the gene-specific primer pairs used in the study

| Gene     | GenBank accession no. | Forward and reverse primers | Fragment (bp) |
|----------|-----------------------|-----------------------------|---------------|
| TiERF1   | EF570121              | F: 5'-GAGGCCACGCGTCATG-3'   | 341           |
| actin    | BE425627              | F: 5'-CAGTGAATGGTCAGG-3'    | 211           |
| Chil     | CK207575              | F: 5'-ACCTCCTTTGGCAGT-3'    | 173           |
| D-GLU    | DQ090946              | F: 5'-GCTGGAAGTTGTTG-3'     | 352           |
| PR10     | CV778999              | F: 5'-ACGAGCGGAGTG-3'       | 269           |
| PR1      | AF384143              | F: 5'-ATAACCTCGGCCT-3'      | 201           |

The presence of the TiERF1 gene in the transgenic wheat plants was monitored by PCR using the gene-specific primers TiE-406F (GAGCCCAACGGGTTCATG) and TiE-746R (GACCGGAC-GAGGAAGAG). The PCR was performed in a total volume of 25 μl containing 1× GC buffer I (TaKaRa), 100–200 ng of genomic DNA, 187.5 μM of each dNTP (TaKaRa), 0.25 μM of each primer, and 1 U of Taq polymerase (TaKaRa), and subjected to a program of initial denaturation at 94 °C for 4 min, followed by 10 cycles of 45 s at 94 °C, 45 s at 62 °C, and 1 min at 72 °C, then 25 cycles of 45 s at 94 °C, 45 s at 58–60 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The amplified product (341 bp) specific to the TiERF1 gene was resolved on a 1% (w/v) agarose gel and visualized by ethidium bromide staining.
BamHI and EcoRV, respectively (Fig. 1), suggesting that the wheat genome contained at least one homologous sequence of the TiERF1 gene.

**TiERF1 harbours a transactivation domain functioning as a transcriptional activator**

Sequence analysis revealed that the TiERF1 protein contains a putative transcriptional activation domain (AD) in the N-terminus (from amino acids 51 to 75) of TiERF1 (Liang et al., 2008). The yeast one-hybrid assay was used to investigate the function of the putative AD domain of TiERF1. As shown in Fig. 2B, significant activity of β-galactosidase (encoded by LacZ) could be detected only in the yeast cells containing the wild-type TiERF1 effector plus the pGCC-LacZ reporter, Y-pYTIERF1/pGCC-LacZ, but no activity of β-galactosidase was present in the other combinations, Y-pYTIERF1ΔAD/pGCC-LacZ, Y-pYTIERF1/pmGCC-LacZ, and Y-pYTIERF1/pLacZi. The results demonstrated that the putative transcription AD functions as a transcriptional domain, which is essential for TiERF1 activating the transcription of the reporter gene LacZ.

**Transgenic wheat plants expressing TiERF1**

To elucidate if TiERF1 confers enhanced resistance of the transgenic wheat to *R. cerealis*, the wheat cv. Yangmai12, which is susceptible to *R. cerealis*, was used to generate transgenic wheat plants. Based on detection of the positive TiERF1 transgenic wheat plants from the T0-T4 generations by PCR analysis, stable inheritance of the TiERF1 gene was detected in these progeny derived from 13 lines, i.e. E111, E117, E199, E231, E259, E314, E319, E334, E348, E512, E765, E767, and E787 (Fig. 3B). The result of Southern blotting analysis showed that one hybridization band with a different pattern was present in each transgenic line E199, E767, and E787 digested with EcoRV and HindIII (Fig. 3C), indicating that the transgenic TiERF1 gene was integrated into the wheat genome with a single copy, and these three lines were independently derived. When the genomic DNAs were digested by BglII, one strong and one weak hybridization bands were present, respectively, in each transgenic line; two weak bands were present in the genomic DNA of wild-type wheat cv. Yangmai12, indicating further that the wheat genome contained at least one homologous sequence of the TiERF1 gene.

RT-PCR analysis indicated that the TiERF1 transcript was overexpressed in the positive transgenic plants (Fig. 3D), such as T3 progeny plants of lines E111, E117, E199, E231, E259, E319, E334, E348, E512, E765, E767, and E787 showing resistance to sharp eyespot, but there was no detectable TiERF1 expression in the T3 plants of E314-3-3 with susceptibility to sharp eyespot, presumably expressing a different copy number due to genetic variation.

Fig. 1. Southern blotting analysis of genomic DNAs from *T. intermedium* (1) and wheat cv. Yangmai12 (2) digested with *BglII, EcoRV, HindIII*, and *BamHI*. Fragments were hybridized with an [α-32P]dCTP-labelled probe from the TiERF1-specific fragment (nucleotides 407–747).

Fig. 2. The transcriptional activation activity of TiERF1. (A) Scheme of the reporter and effector constructs. GAP, the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene. (B) The yeast cells selected in SD-Ura-Trp medium (plate) and quality assay of the β-galactosidase activity (lifted-filter) for four kinds of reporter and effector combinations. 1, Y-pYTIERF1/pGCC-LacZ; 2, Y-pYTIERF1/pLacZi; 3, Y-pYTIERF1/pmGCC-LacZ; 4, Y-pYTIERF1ΔAD/pGCC-LacZ.
resulting from silencing of the TiERF1 gene. Q-RT-PCR analysis indicated that TiERF1 was still overexpressed in the T4 progeny plants of the transgenic lines E111, E117, E199, E231, E259, E319, E334, E348, E512, E765, E767, and E787, all of which contained the TiERF1 gene and showed resistance to sharp eyespot (Fig. 3E).

**Overexpression of TiERF1 enhances resistance to R. cerealis**

The results of the sharp eyespot test demonstrated that the transgenic wheat lines overexpressing TiERF1 showed enhanced resistance (ITs: 0–1), whereas the non-transgenic wheat Yangmai 12 showed susceptibility to R. cerealis (ITs: 3–4) (Fig. 4, Table 2). From T1–T4 plants, high resistance to sharp eyespot was present in the TiERF1-expressing plants, but not in the segregated plants lacking the TiERF1 gene or in the silenced transgenic plants, such as E314-3-3. The results suggested that the enhanced resistance in the transgenic lines overexpressing TiERF1 could be stably inherited, and was associated with accumulation of TiERF1 transcript.

**Overexpression of TiERF1 activates the transcript of some PR genes**

Q-RT-PCR was used to investigate the expression of wheat PR genes in the transgenic wheat lines expressing TiERF1 and the wild-type Yangmai12. The results showed that overexpression of TiERF1 in wheat dramatically increased the transcript levels of ChiI 1200- to 1800-fold and those of D-GLU 800- to 1200-fold over those in the wild-type wheat parent Yangmai12, and increased the transcript abundance of PR10, 12- to 20-fold over that in the wild type (Fig. 5), but did not noticeably change the transcript level of PR1 (data not shown) in the transgenic plants compared with the wild-type plants. However, the transcript levels of TiERF1, ChiI, D-GLU, and PR10 in the silenced transgenic plant E314-3-3 were similar to those in the wild-type Yangmai12.

**Expression characteristics of PR genes in wheat and T. intermedium**

To establish the expression characteristics of ChiI, D-GLU, PR10, and PR1 genes, Q-RT-PCR analysis was used to investigate the effects of SA, JA, ET, JA+ET, and the ET synthesis inhibitor (CoCl2) on the transcript levels of the above PR genes in wild-type Yangmai12 and T. intermedium. The results of these experiments are shown in Figs 6 and 7. The transcript abundances of ChiI and D-GLU were dramatically increased by ET treatment, and increased by JA treatment, but could not be detected after CoCl2 treatment, suggesting that ChiI and D-GLU are downstream PR genes primarily in the ET-dependent pathway. The transcript abundance of PR10 was significantly increased by JA treatment, increased by ET
treatment, and not affected by CoCl2 treatment, suggesting that wheat **PR10** is a downstream PR gene primarily in the JA-dependent pathway. The transcript abundance of **PR1** was significantly increased by SA treatment, but not obviously affected by ET, JA, and CoCl2 treatments, suggesting that wheat **PRI** is a downstream defence-related gene primarily in the SA-dependent pathway. These results indicated that these PR genes in *T. intermedium* were regulated through similar signal pathways of wheat.

**Discussion**

Wheat sharp eyespot has been becoming a devastating disease of wheat in China. It is urgent to elucidate the host
defence mechanism against the pathogen, a typical soil-borne fungus R. cerealis. Arabidopsis ERF1 and AtERF2 have been shown to play an important modulated role in mediating resistance to several necrotrophic fungi and a soil-borne fungus Fusarium oxysporum (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005). Therefore, ERFs in wheat and its relative T. intermedium were used as the starting point to study host defence against R. cerealis.

In a previous study, an ERF gene in T. intermedium, TiERF1, was isolated (Liang et al., 2008), which could be induced by R. cerealis challenge, and played a positively modulating role in certain defence responses of transgenic tobacco plants (Liang et al., 2008). TiERF1 belongs to the B3 subgroup of the ERF family. Several ERF proteins in the B3 subgroup were shown to regulate the plant defence response positively through activating PR genes with a GCC-box cis-element, such as Arabidopsis ERF1, AtERF2, and AtERF14, tomato PtI4 and TSRF1, tobacco OPBP1, and rice OsBIERF3 (Berrocal-Lobo et al., 2002; Gu et al., 2002, 2004; Berrocal-Lobo and Molina, 2004; Zhang et al., 2004; McGrath et al., 2005; Cao et al., 2006; Oñate-Sánchez and Singh, 2007). In this study, further yeast one-hybrid analysis confirmed that the putative transcriptional AD of TiERF1 is a transactivation domain essential for TiERF1 as a transcriptional activator following binding to the GCC-box, suggesting that TiERF1 should play modulating roles in the defence response through activating transcripts of PR genes with a GCC-box. These findings gave rise to the hypothesis that overexpression of TiERF1 may positively regulate the host defence against R. cerealis. This report confirmed the hypothesis.

In this study, since the genome of T. intermedium has not yet been sequenced, Southern blotting was used to analyse the copy number of the TiERF1 gene in the T. intermedium genome. The Southern blotting results suggested that at least two copies of TiERF1 exist in the T. intermedium genome. One clear hybridization band was present in the genomic DNA of wheat Yangmai12 digested by Bgl II, and a weak hybridization band was present in the wheat genomic DNA digested by BamHI and EcoRV (Fig. 1), suggesting that the wheat genome contained at least one homologous sequence of the TiERF1 gene. Sequence alignment analysis indicated that the probe of the TiERF1 sequence shares 87% identity with the corresponding sequence of a wheat ERF gene TaERF3 (Zhang et al., 2007), supporting the Southern blotting results with Bgl II. The highly specific TiERF1 hybridization in BamHI and EcoRV digestions (Figs 1, 3C) may result from the high stringency for hybridization, blot washing, or the restriction sites in wheat genome.

To unravel the roles of TiERF1 in the host defence mechanism against R. cerealis, overexpressing and silencing lines of the gene should be developed and investigated. Because the transformation system of T. intermedium has not been established, transgenic wheat lines expressing TiERF1 were developed in this study. Based on PCR detection results on TiERF1 present in
T₀–T₄ individuals, stable inheritance of the TiERF1 gene was detected in these progeny derived from 13 lines. Southern analysis results indicated that a different pattern of TiERF1 gene integration was detected in stable inheritance lines, such as E199, E765, and E767. The transgenic wheat lines with different integration patterns show the genetic independence of transgenic lines, like the transgenic wheat plants expressing AtNPR1 (Ragiba et al., 2006), demonstrating further the transformation randomness in micro-projectile bombardment. Stable integration and inheritance of the TiERF1 gene were confirmed in the five examined generations (from T₀ to T₄). Results of RT-PCR, Q-RT-PCR, and the disease resistance assays indicated the stable overexpression of the TiERF1 gene in most of the transgenic lines which showed enhanced resistance to sharp eyespot. However, the progeny of a few lines had no detectable TiERF1 expression, leading to susceptibility to sharp eyespot. The reason behind the non-expression of the transgenic plants is not clear, but presumably results from silencing at the transcript level of the introduced genes. Similar silencing transgenic events occurred in other transgenic wheat plants (Anand et al., 2003; Ragiba et al., 2006). Furthermore, the transgenic wheat lines overexpressing TiERF1 showed enhanced resistance to sharp eyespot compared with non-transgenic wheat Yangmai12 and the silenced transgenic wheat E314 (Table 2), indicating that the enhanced resistance of the transgenic plants was associated with accumulation of TiERF1 transcript, and overexpression of TiERF1 conferred the enhanced resistance to sharp eyespot in transgenic wheat plants.

To elucidate if TiERF1 plays positive regulatory roles in defence against R. cerealis through up-regulating the expression of some downstream PR genes, the expression of some PR genes with a GCC-box cis-element in transgenic wheat plants overexpressing TiERF1 should be analysed. However, there is a lack of information regarding the promoter sequence of known wheat PR
genes. Based on our analysis of a PR gene (D-GLU) involved in wheat defence against *R. cerealis* (unpublished data) and other reports about some PR genes in wheat and rice (*Kong et al.*, 2005; *Qiu et al.*, 2007), expression of some wheat defence-related genes (*ChiI*, *D-GLU*, *PR10*, and *PR1*), which may be regulated by different signalings, was investigated in the transgenic wheat lines. The Q-RT-PCR analysis of expression of the targeting genes showed that overexpression of *TiERF1* in the transgenic wheat lines dramatically increased the transcript levels of wheat *ChiI* and *D-GLU*, and also activated the expression of *PR10* with the silenced transgenic wheat and wild-type Yangmai12 plants, but did not noticeably change the transcript level of the wheat *PR1* gene. As expected, the disease resistance of the *TiERF1*-expressing wheat plants was associated with higher transcript levels of the wheat PR genes. Although the expression characteristics of *Arabidopsis* PR genes are well documented, this information is not available for the wheat PR genes assayed in the analyses of the transgenic wheat plants described above. To understand further in which signal pathway the PR genes regulated by *TiERF1* are involved, in this study the effects of ET, CoCl2, JA, ET+JA, and SA on the expression of the above wheat PR genes in wheat and *T. intermedium* were investigated by Q-RT-PCR analysis. Here, the results indicated that *ChiI* and *D-GLU* are downstream defence-related genes primarily in the ET-dependent pathway; wheat *PR10* is a downstream defence-related gene primarily in the JA-dependent pathway; and wheat *PR1* is a downstream defence-related gene primarily in the SA-dependent pathway. The expression characteristics of the defence-related genes in *T. intermedium* are the same as those in wheat. These results indicated that *TiERF1* overexpression may activate primarily the PR genes (*ChiI* and *D-GLU*) in the ET-dependent pathway, and may up-regulate the PR gene (*PR10*) in the JA-dependent pathway, leading to enhanced resistance to sharp eyespot. It was also found that the transgenic wheat overexpressing *D-GLU* improved resistance to sharp eyespot (unpublished result). The above results suggested that *TiERF1*, and *ChiI* and *D-GLU* may participate positively in wheat resistance against *R. cerealis*. These results support the view on ERFs as important components of the ET signal pathway for regulating PR genes with or without a GCC-box (*Solano et al.*, 1998; *Chakravarthy et al.*, 2003; *Guo and Ecker*, 2004; *Mazarei et al.*, 2007; *Oñate-Sánchez* and *Singh*, 2007), and may provide evidence to bridge the gap between the transgenic *TiERF1*, *ChiI*, and *D-GLU* genes and transgenic resistance, and these three genes may be allocated to the ET-dependent pathway.

In conclusion, all the results supported the hypothesis that overexpression of *TiERF1* could confer enhanced resistance to *R. cerealis* infection in transgenic wheat plants without a major resistance gene background. *TiERF1* was a positive regulator of wheat resistance against *R. cerealis* through activating the transcript of PR genes mainly in the ET-dependent pathway. Genetic engineering with *TiERF1* provides an effective strategy for developing wheat germplasm with resistance to sharp eyespot. To our knowledge, this is the first report about the roles of an ERF and wheat PR genes in the ET-dependent pathway in wheat defence against *R. cerealis* infection. This study provides new insights into wheat defence mechanisms against *R. cerealis*.

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