Journal of Experimental Botany, Vol. 64, No. 8, pp. 2243–2253, 2013
doi:10.1093/jxb/ert084 Advance Access publication 1 April, 2013
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Transgenic wheat expressing *Thinopyrum intermedium* MYB transcription factor *TiMYB2R-1* shows enhanced resistance to the take-all disease

Xin Liu[1,*], Lihua Yang[1,2,*], Xianyao Zhou[1,*], Miaoping Zhou[3,*], Yan Lu[1], Lingjian Ma[2], Hongxiang Ma[3] and Zengyan Zhang[1,†]

[1] The National Key Facility for Crop Gene Resources and Genetic Improvement/Key Laboratory of Biology and Genetic Improvement of Triticeae Crops of the Agriculture Ministry, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[2] College of Agronomy, Northwest A&F University, Yangling 712100, China
[3] Biotechnology Institute, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: zhangzengyan@caas.cn

Received 7 November 2012; Revised 25 February 2013; Accepted 28 February 2013

Abstract

The disease take-all, caused by the fungus *Gaeumannomyces graminis*, is one of the most destructive root diseases of wheat worldwide. Breeding resistant cultivars is an effective way to protect wheat from take-all. However, little progress has been made in improving the disease resistance level in commercial wheat cultivars. MYB transcription factors play important roles in plant responses to environmental stresses. In this study, an R2R3-MYB gene in *Thinopyrum intermedium*, *TiMYB2R-1*, was cloned and characterized. The gene sequence includes two exons and an intron. The expression of *TiMYB2R-1* was significantly induced following *G. graminis* infection. An *in vitro* DNA binding assay proved that TiMYB2R-1 protein could bind to the MYB-binding site *cis*-element ACI. Subcellular localization assays revealed that TiMYB2R-1 was localized in the nucleus. *TiMYB2R-1* transgenic wheat plants were generated, characterized molecularly, and evaluated for take-all resistance. PCR and Southern blot analyses confirmed that *TiMYB2R-1* was integrated into the genomes of three independent transgenic wheat lines by distinct patterns and the transgene was heritable. Reverse transcription–PCR and western blot analyses revealed that *TiMYB2R-1* was highly expressed in the transgenic wheat lines. Based on disease response assessments for three successive generations, the significantly enhanced resistance to take-all was observed in the three *TiMYB2R-1*-overexpressing transgenic wheat lines. Furthermore, the transcript levels of at least six wheat defence-related genes were significantly elevated in the *TiMYB2R-1* transgenic wheat lines. These results suggest that engineering and overexpression of *TiMYB2R-1* may be used for improving take-all resistance of wheat and other cereal crops.

Key words: *Gaeumannomyces graminis* var. *tritici*, MYB transcription factor, take-all resistance, *Thinopyrum intermedium*, transformation, *Triticum aestivum.*

Introduction

Wheat (*Triticum aestivum*) is one of the most important food crops in the world. Root diseases have considerable economic impacts on wheat production. The disease known as take-all, caused by the necrotrophic fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is one of the most destructive root diseases of wheat worldwide (Gutteridge *et al*., 2003; Daval *et al*., 2011). The disease begins by *Ggt* hyphae penetrating the cortical cells of the root and progresses upwards into the base of the stem, and even leads to premature death of the infected plant. The symptoms of the disease are manifested as black lesions on

the roots. Symptoms on above-ground parts of the infected plant include stunting, premature ripening, and white heads (bleached white and empty spikes) (Cook, 2003; Guilleroux and Osbourn, 2004). Take-all can affect the quality and yield of wheat [i.e. yield losses of 40–60% (Gutteridge *et al*., 2003)]. Take-all also impacts the production of barley (*Hordeum vulgare*) and triticale (*Secale*×*Triticum*) (Gutteridge *et al*., 2003; Bithell *et al*., 2011).

Breeding wheat varieties with resistance is the most promising and reliable way to protect wheat from take-all. Since no effective resistance has been identified in wheat accessions (Gutteridge *et al*., 2003; Yang *et al*., 2011), to breed take-all-resistant wheat varieties using traditional methods does not appear to be feasible. The advent of genetic engineering and its application to the production of crops make it possible to generate wheat materials with resistance to this disease.

Plants have evolved sophisticated defence mechanisms to cope with pathogens. Many transcription factor (TF) families have been shown to play important roles in defence responses through regulating the expression of defence-related genes. MYB TFs are characterized by a MYB domain conferring an ability to bind the *cis*-acting elements of targeted genes (Dubos *et al*., 2010). Based on the numbers of adjacent repeats in the MYB domain, MYB proteins are classified into four subfamilies, namely R1MYB, R2R3-MYB, 3R-MYB, and 4R-MYB (Dubos *et al*., 2010). Since the first plant MYB gene required for synthesis of anthocyanins, *C1*, was isolated from maize (*Zea mays*) (Paz-Ares *et al*., 1987), a large number of MYB proteins have been identified in various plant species, namely *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), cotton (*Gossypium hirsutum*), grapevine (*Vitis vinifera* L.), poplar (*Populus tremuloides*), apple (*Malus domestica*), wheat, and *Avicennia marina* (Rabinowicz *et al*., 1999; Cedroni *et al*., 2003; Dias *et al*., 2003; Chen *et al*., 2006; Dubos *et al*., 2010; Zhang *et al*., 2011; Ganesan *et al*., 2012). MYB proteins perform diverse biological functions in the cell cycle and in development, regulation of primary and secondary metabolism, and abiotic stress response (Ma *et al*., 2009; Seo *et al*., 2009; Dubos *et al*., 2010; Seo and Park, 2010; He *et al*., 2011; Xue *et al*., 2011; Zhang *et al*., 2011, 2012; Qin *et al*., 2012; Romano *et al*., 2012; Shen *et al*., 2012). Some plant MYB proteins are involved in defence responses to pathogens. For instance, *Arabidopsis* R2R3-MYB proteins, including AtMYB108 and AtMYB96, participate in disease resistance (Mengiste *et al*., 2003; Seo and Park, 2010). Our previous study showed that overexpression of a wheat MYB gene *TaPIMP1* enhanced resistance to biotic and abiotic stresses in transgenic tobacco and wheat (Liu *et al*., 2011; Zhang *et al*., 2012).

Thinopyrum intermedium (*Agropyron intermedium*, intermedium wheatgrass; 2*n*=42), a wild relative of wheat, is naturally resistant to wheat diseases, such as leaf rust, yellow rust, and stem rust (Cauderon *et al*., 1973), *Wheat streak mosaic virus* (WSMV; Sharma *et al*., 1984), *Barley yellow dwarf virus* (BYDV; Sharma *et al*., 1984), Fusarium head blight (FHB; Fedak and Han, 2005), and eyespot (Li *et al*., 2005). The resistance to wheat rusts, BYDV, WSMV, FHB, and eyespot has been introgressed into the wheat background and characterized by different groups (Cauderon *et al*., 1973; Xin *et al*., 1991; Banks *et al*., 1995; Sharma *et al*., 1995; Fedak and Han, 2005; Li *et al*., 2005). However, it is not clear whether R2R3 MYB TFs in *T. intermedium* are involved in defence responses. Therefore, a study of the species-specific MYB genes may provide insights into *T. intermedium* defence mechanisms.

In this study, the first R2R3-MYB gene isolated from *T. intermedium*, *TiMYB2R-1*, was cloned. Its R2R3-MYB activity was confirmed by subcellular localization and *cis*-element binding activity assays. The functional characteristics of *TiMYB2R-1* in defense responses to take-all pathogen *Ggt* were also explored through its expression in generated transgenic wheat lines. The results showed that the ectopic expression of *TiMYB2R-1* significantly increased resistance to take-all in transgenic wheat.

Materials and methods

Plant and fungal materials and treatments

Thinopyrum intermedium cultivar (cv.) Z1146 was provided by Dr Lihui Li, Institute of Crop Science, CAAS. The wheat cv. Yangmai 12, provided by Lixiahe Agricultural Institute of Jiangsu, China, was used as the recipient of *TiMYB2R-1* transformation. Yangmai 12 is a Chinese commercial wheat variety with susceptibilty to *Ggt* and is a good material for this study.

The fungal pathogen *Ggt* XNQS-2 was isolated, identified, and provided by Dr Yang Wang, College of Plant Protection, Northwest A&F University, China.

For inoculation, the *Ggt* fungus was cultured on potato dextrose agar (PDA) at 25 °C for ~10 d, then 1 cm^2 plugs from the edge of *Ggt* colonies were placed onto the surface of sand in pots. One seed germinated for 2 d was put on the top of each *Ggt* plug, and covered with 2 cm of sand. The plants were cultured in a growth chamber at a 23 °C, 14 h light/15 °C, 10 h dark regime at 70% relative humidity. The roots were collected at 0, 4, 7, 14, and 21 days post-inoculation (dpi) for RNA extraction.

DNA and RNA extraction and first-strand cDNA synthesis

Genomic DNA was extracted from leaf tissues of *T. intermedium* Z1146 or wheat as described by Sharp *et al*. (1988). Total RNA was extracted from roots of *T. intermedium* or wheat using TRIZOL reagent (Invitrogen), and then subjected to RNase-free DNase I (TaKaRa) treatment and purification.

A 5 µg aliquot of RNA per sample was used to synthesize the first-strand cDNA using a Superscript II First-Strand Synthesis Kit for RT-PCR (Invitrogen).

Cloning and sequence analysis of the TiMYB2R-1 gene

Based on the sequence of the wheat MYB gene *TaPIMP1* (accession no. EF587267), a pair of primers (MYB-OF, 5'-ACTCGC GTACGTCTTCCTGA-3'; and MYB-OR, 5'-GCGCTCTAGTTA AGTTCATCGTC-3') was designed and used to amplify the full-length cDNA sequence of the MYB gene *TiMYB2R-1* from cDNA of *T. intermedium* Z1146 roots at 4 d post-challenge with *Ggt*. The PCR fragment corresponding to *TiMYB2R-1* was excised, cloned, and its sequence was analysed. The cDNA sequence of *TiMYB2R-1* of 1038 bp in length was deposited in the National Center for Biotechnology Information (NCBI) with accession number JX683795. *TiMYB2R-1* contains an open reading frame (ORF) of 972 bp (NCBI accession no. JQ663861). The genomic sequence of *TiMYB2R-1* was amplified from genomic DNA of Z1146 using the primers MYB-OF and MYB-OR, then cloned and sequenced.

The genomic sequence was deposited in the NCBI with accession no. JX683794.

DNA and protein sequences were analysed using DNAMAN software, DNASTAR software, and BLAST online (http://www.ncbi.nlm.gov/blast).

Subcellular localization of TiMYB2R-1

The coding region of *TiMYB2R-1* without the stop codon was amplified using gene-specific primers with *Hin*dIII and *Xba*I restriction sites (TIM-NLF, 5'-G*aagctt*ATGGACATGGACAAGGAGTA-3'; and TIM-NLR, 5'-C*tctaga*TCAGCAGTAAATGTCCTCTAG-3'). *TiMYB2R-1* was fused in-frame to the 5' terminus of the green fluorescent protein (*GFP*) gene in the *ph16318* vector (Dr Daowen Wang, CAS), and controlled by the *Cauliflower mosaic virus* (CaMV) 35S promoter. The resulting *TiMYB2R-1-GFP* fusion construct or *GFP* alone were transformed separately into white onion epidermal cells using a PDS-1000/He gene gun (Bio-Rad, USA) at 1100 psi. After incubation for 40 h at 25 °C, GFP fluorescence in the transformed onion cells was observed under 488 nm excitation using a confocal laser scaning microscope (Zeiss LSM 700, Germany) with a Fluar 10×/0.50 M27 objective lens and a SP640 filter.

The cis-element binding assay of TiMYB2R-1

Previously R2R3-MYB proteins were shown to bind to MYB-binding site (MBS) AC *cis*-elements including ACI (core sequence: ACCTACC, Patzlaff *et al*., 2003). To investigate if TiMYB2R-1 protein binds to the MBS ACI *cis*-element, the ORF sequence of TiMYB2R-1 was subcloned in-frame to the 3' terminus of a glutathione *S*-transferase (*GST*) gene in the *pGEX-4T-1* vector (GE Amersham), resulting in the recombinant expression vector *pGST-TiMYB2R-1*. The *pGST-TiMYB2R-1* construct was transformed into competent cells of *Escherichia coli* BL21. The recombinant protein GST–TiMYB2R-1 was expressed after induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 16°C, and purified using a MicroSpin module (GE Amersham).

The forward and reverse oligonucleotides containing the MBS ACI *cis*-element (Zhang *et al*., 2012) were synthesized and used to prepare the probe. Electrophoretic mobility shift assays (EMSAs) were conducted following the protocol of Zhang *et al*. (2012). A 1 µg aliquot of the ACI probe plus 3 µg of purified GST–TiMYB2R-1 or GST protein alone were mixed with the binding buffer.

TiMYB2R-1 transformation vector and wheat transformation

The full ORF sequence of the *TiMYB2R-1* gene was subcloned into the *Sma*I and *Sac*I sites of a plant expression vector pAHC25 (Christensen and Quail, 1996), resulting in the transformation vector *pA25-TiMYB2R-1*. It contained a *Ubi::TiMYB2R-1-Tnos* chimera, where *TiMYB2R-1* was driven by the maize *ubiquitin* (*Ubi*) promoter and terminated by the terminator of the *Agrobacterium tumefaciens* nopaline synthase gene (*Tnos*). In all, 1200 immature embryos of wheat Yangmai 12 were transformed by biolistic bombardment using *pA25-TiMYB2R-1*. All aspects of transformation were conducted according to the protocol described by Chen *et al*. (2008).

PCR detection of TiMYB2R-1 transgenic wheat plants

The presence of the introduced *TiMYB2R-1* gene in transgenic wheat plants was monitored by PCR using *Ubi::TiMYB2R-1*-specific primers (Fig. 4A): UbiP-F, 5'-GCTCTGCCTTCATACGCTAT-3' (located in the *Ubi* promoter of the transformation vector); and TiMYB1-R, 5'-TCCGCCAGTAGTTCTTGACC-3' (located in the *TiMYB2R-1* coding sequence). PCR was performed in a 25 µl volume containing 50 ng of genomic DNA, 1× GC PCR buffer I (TaKaRa), 0.4 µM of each primer, 200 µM of each dNTP, and 1 U of *Taq* polymerase (TaKaRa). The amplified product (533 bp, Fig. 4A, B) specific to the *Ubi::TiMYB2R-1* transgene was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis

Southern blotting was conducted following a modified protocol of Sharp *et al*. (1988). Genomic DNAs (~20 µg each) of the transgenic wheat plants and non-transformed Yangmai 12 were digested separately by the restriction enzyme *Eco*RV, and then blotted onto a Hybond N+ nylon membrane (GE Amersham). The amplified fragment (533 bp, Fig. 4A) specific to the chimeric *Ubi::TiMYB2R-1* was labelled by [α-^{32}P]dCTP and then used as the probe. Hybridization was performed at 65 °C for 20 h. The hybridized membrane was washed twice in 1× SSC, 0.1% SDS, twice in 0.5× SSC, 0.1% SDS, and once in 0.2× SSC, 0.1% SDS at 65 °C for 10 min each time.

Transcription analyses of TiMYB2R-1 and wheat defence-related genes in transgenic wheat

In a previous microarray analysis, wheat *TaPIMP1* overexpression activated transcription of defence-related genes (Zhang *et al*., 2012), including *PR1a* (TC384382), *PR17c* (TC398605), *Chitinase 2* (*Chit2*; TC426538), class III *chitinase* (*Chit3*; TC386649), *nsLTP1* (TC411506) encoding a non-specific lipid transfer protein1, and *GST22* (TC372250). These TC numbers and sequences can be found at the website: http://compbio.dfci.harvard.edu/cgi-bin/tgi/tc_ann.pl?gudb=wheat.

Reverse transcription–PCR (RT–PCR) and real-time quantitative RT–PCR (Q-RT–PCR) were used to analyse transcription levels of *TiMYB2R-1* and the above-mentioned wheat defence-related genes in *TiMYB2R-1* transgenic and control wheat lines. The RT–PCRs for *TiMYB2R-1* transcription were set up with 31 cycles (95 °C for 1 min, 56 °C for 40 s, 72 °C for 35 s) to illustrate high expression of *TiMYB2R-1* in the transgenics with primers (MRT-F and MRT-R) specific to the *TiMYB2R-1* sequence (Table 1); the third from last nucleotide of the reverse primer MRT-R is different from that of TaPIMP1 (Supplementary Fig. S1 available at *JXB* online). Q-RT–PCR was performed using SYBR Green I Master Mix (TaKaRa, Japan) in a volume of 25 µl on an ABI 7300 RT–PCR system (Applied Biosystems). Reactions were set up with the following thermal profile: 95 °C for 5 min, followed by 41 cycles of 95 °C for 15 s and 60°C for 35 s. The wheat *actin* gene was used to normalize amounts of cDNAs among the samples. The relative transcript level of a target gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative transcript levels of the tested genes in the transgenics were relative to those in the wild-type (WT) recipient since the genetic backgrounds of the transgenics and the WT recipient are the same except for the introduced *TiMYB2R-1* and the reactions (including defence genes) caused by *TiMYB2R-1* overexpression. In Q-RT–PCR analysis, transcription of *TaPIMP1*, a wheat endogenous homologue of *TiMYB2R-1*, may be detected in *TiMYB2R-1* transgenics and WT recipient wheat. The relative transcript levels of *TiMYB2R-1* were the transcript levels of *TiMYB2R-1* and *TaPIMP1* in transgenics minus those of *TaPIMP1* in WT plants. Three replications for each treatment were performed.

The primer sequences for *TiMYB2R-1*, and wheat defence-related and *actin* genes, and related information on (Q)-RT–PCR analyses are listed in Table 1.

Western blotting assay

The expression of the *TiMYB2R-1*-encoded protein in the transgenic wheat lines was tested by western blotting analysis. Total proteins were extracted from 0.3 g of ground root powder. About 12 µg of total soluble proteins for each line were separated on 12% SDS–polyacrylamide gels and transferred to a polyvinyl difluoride

Table 1. Primers used in (Q-)RT–PCR.

Gene name	Accession no.	MBS position and sequence	Sequence of gene-specific primer	Species
TiMYB2R-1	JX683795		F: 5'-ACGGACAACGAGGTCAAGAAC-3' R: 5'-GAAATGGGCTCCGTACG-3'	*Thinopyrum intermedium*
PR1a	TC384382	–43 to –39, GGATA	F: 5'-CGTGGGTGTCGGAGAAGC-3' R: 5'-AAGTTGCCTGGCGGGTTG-3'	*Triticum aestivum*
PR17c	TA65181	–58 to –54 GGATA	F: 5'-ACGACATCACGGCGAGGT-3' R: 5'-CACGGGGAAAGAGAGGATGA-3'	*Triticum aestivum*
nsLTP1	TC411506	–86 to –81(–), CTGTTA	F: 5'-ATGCGGGTTGGCGTGAAG-3' R: 5'-TGTTGCGGTGGTAGGTTGTTG-3'	*Triticum aestivum*
GST22	TC372250	–211 to –206(–), CCGTTG	F: 5'-GGGATTGGGGCAGGAG-3' R: 5'-TCGGGAGGGAGGAAGC-3'	*Triticum aestivum*
Chit2	TC426538	No promoter sequence	F: 5'-TTCTGGATGACGGCACAAG-3' R: 5'-CCTTAGTGTGACCAGTCGTTTT-3'	*Triticum aestivum*
Chit3	TC386649	No promoter sequence	F: 5'-TAAGATGAGCCCCTACCTGTTC-3' R: 5'-GTACACGGCATTTATTTAGTCCC-3'	*Triticum aestivum*
Actin	BE425627		F: 5'-CACTGGAATGGTCAAGGCTG-3' R: 5'-CTCCATGTCATCCCAGTTG-3'	*Triticum aestivum*
18SrRNA	AY049040		F: 5'-GTGACGGGTGACGGAGAATT-3' R: 5'-GACACTAATGCGCCCGGTAT-3'	*Triticum aestivum*
Ggt 18SrRNA	FJ771002		F: 5'-CGAACTCGGTCGTTTAGAGG-3' R: 5'-GGTATGTTCACAGGGGTTGG-3'	*Gaeumannomyces graminis* var. *tritici*

membrane (Amersham). The western blots were incubated with the polyclonal GST–TiMYB2R-1 antibody (1:100 dilution), which was developed in mice from GST–TiMYB2R-1 protein. TiMYB2R-1 protein in these lines was visualized with the ECL Western Blot Detection and Analysis System (GE Healthcare).

Take-all assessments in transgenic wheat

Take-all responses of *TiMYB2R-1* transgenics in the T_3–T_5 generations and untransformed wheat controls were evaluated following inoculation with *Ggt*. At 3 weeks post-inoculation, the disease severity in each plant was assessed as the percentage area of take-all lesions covering the root system (Daval *et al*., 2011). The infection types (ITs) were categorized from 0 to 4 according to Bithell *et al*. (2011) (i.e. IT 0, no take-all; IT 1, >0% and ≤10%; IT 2, >10% and ≤30%; IT 3, >30% and ≤60%; IT 4, >60%). The take-all index (TAI) was $(10\times N_1+30\times N_2+60\times N_3+100\times N_4)/(N_1+N_2+N_3+N_4)$, where N was the number of plants with each infection type (Bithell *et al*., 2011). At least 70 plants per line were tested.

To investigate the take-all resistance of transgenic wheat lines further, Q-RT–PCR was used to assess the relative abundance of *Ggt* in transgenic wheat plants based on *Ggt* 18S rRNA (FJ771002; Daval *et al*., 2011) in reference to wheat 18S rRNA (AY049040). The primer sequences for *Ggt* 18S rRNA and wheat 18S rRNA in Q-RT–PCR analyses are listed in Table 1.

Results

TiMYB2R-1 is an R2R3-MYB transcription factor in T. intermedium

Both the full-length cDNA and genomic sequences of the *TiMYB2R-1* gene were isolated from *T. intermedium*. The gene sequence includes two exons and an intron of 240 bp (Fig. 1A). The ORF of *TiMYB2R-1* encodes a deduced protein, TiMYB2R-1, with 323 amino acids and a mol. wt of 35.4 kDa. The TiMYB2R-1 protein possesses two conserved SANT domains [one (R2) located at amino acids 46–94 and the other (R3) at amino acids 100–145], two acidic transcriptional activation domains (at amino acids 15–60 and 288–307), and two basic nuclear localization signal (NLS) regions (at amino acids 80–89 and 142–150) (Supplementary Fig. S2 at *JXB* online).

The overall sequence of the TiMYB2R-1 protein displayed various identities with known R2R3-MYB proteins from diverse species, namely wheat MYB proteins, including TaPIMP1 (ABU93236.1, 95.99%), TaMYB73 (AEW23186.1, 30.03%), TaMYB32 (AEV91155.1, 28.4%), TaMYB13-1 (AER38255.1, 27.24%), and TaMYB2 (AAT37168.1, 26.99%), and *Arabidopsis* MYB TFs, including AtMYB108 (AEE74402.1, 41.26%), AtMYB2 (BAA03534.1, 38.58%), AtMYB72 (AEE33352.1, 22.87%), and AtMYB96 (AED97611.1, 22.6%). Phylogenetic analysis indicated that TiMYB2R-1 clustered with R2R3-MYB proteins (Fig. 1B), among which AtMYB108, AtMYB96, and TaPIMP1 are implicated in responses to biotic and abiotic stresses in *Arabidopsis* and wheat (Mengiste *et al*., 2003; Seo and Park, 2010; Zhang *et al*., 2012). The above results suggest that TiMYB2R-1 is probably an R2R3-MYB protein.

TiMYB2R-1 transcript level increases in T. intermedium after Ggt infection

Following *Ggt* inoculation, the transcription of *TiMYB2R-1* in *T. intermedium* was significantly induced. The transcription level at 7 dpi reached a peak (8-fold above that of non-treated plants, 0 h), declined afterwards, but remained higher than the non-treatment control (Fig. 2). The results suggested that *TiMYB2R-1* is potentially involved in the host defence response to *Ggt*.

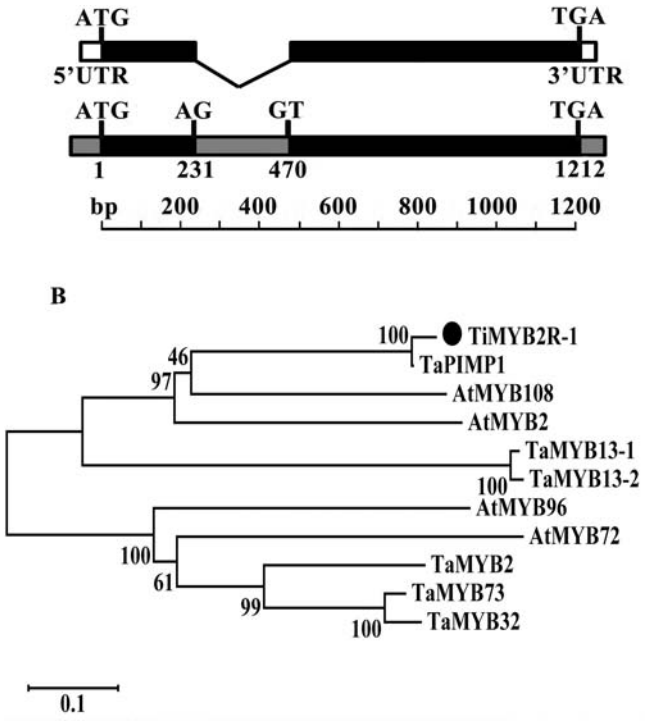

Fig. 1. The gene structure and phylogenetic tree of *TiMYB2R-1*. (A) The *TiMYB2R-1* predicted mRNA structure is shown above the genomic sequence with introns (grey), exons (black), and untranslated regions (white). ATG represents the start methionine codon and TGA represents the stop codon. (B) Phylogenetic tree constructed by Neighbor–Joining algorithms of MEGA 5.05 software after the multiple MYB protein sequence alignment using the CLUSTAL W program. Accession numbers for the other MYB proteins are: TaPIMP1 (ABU93236.1), TaMYB73 (AEW23186.1), TaMYB32 (AEV91155.1), TaMYB13-2 (AER38258.1), TaMYB13-1 (AER38255.1), TaMYB2 (AAT37168.1), AtMYB108 (AEE74402.1), AtMYB2 (BAA03534.1), AtMYB72 (AEE33352.1), and AtMYB96 (AED97611.1).

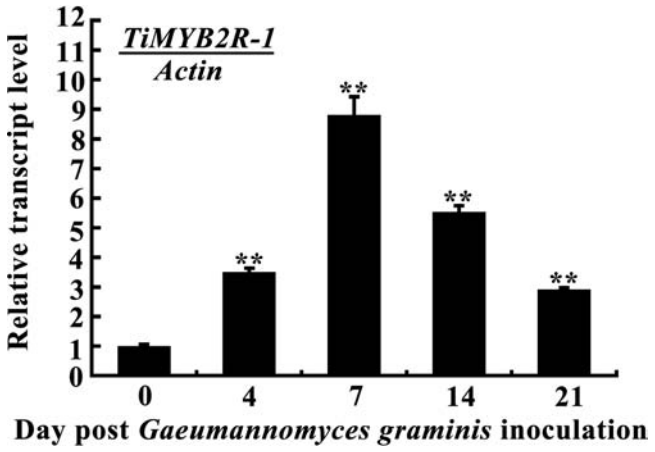

Fig. 2. Transcription of *TiMYB2R-1* in *T. intermedium* following *Ggt* challenge. The transcription level of *TiMYB2R-1* in *T. intermedium* was measured by Q-RT–PCR relative to an untreated control (0 h). Three biological replicates for each time point were averaged, with the standard error of the mean (SE) indicated. Asterisks indicate statistically significant variation calculated using the Student's *t*-test (**$P < 0.01$).

TiMYB2R-1 localizes to nuclei and binds to the MBS ACI cis-element

To study the subcellular localization of TiMYB2R-1, a chimeric *p35S::TiMYB2R-1-GFP* expression vector or control vector *p35S::GFP* was introduced separately into onion epidermal cells. Confocal imaging of transient expression in the epidermal cells showed that TiMYB2R-1–GFP accumulated only in the nucleus, whereas GFP alone was present throughout the whole cell, indicating that TiMYB2R-1 is a nuclear-localized protein (Fig. 3A). These results were consistent with those of TFs that typically function in the nuclei.

To test whether this TiMYB2R-1 protein bind to MBS ACI *cis*-elements, purified GST–TiMYB2R-1 recombinant protein was used in EMSAs. The results proved that the GST–TiMYB2R-1 could bind to the ACI *cis*-element, whereas GST protein alone did not (Fig. 3B). Thus TiMYB2R-1 is indeed an R2R3-MYB TF.

Generation and molecular characterization of TiMYB2R-1 transgenic wheat

To evaluate the role of *TiMYB2R-1* in wheat, transgenic wheat lines expressing *TiMYB2R-1* were generated via bombarding the *TiMYB2R-1* expression vector *pA25-TiMYB2R-1* (Fig. 4A) into 1200 immature Yangmai 12 embryos. Four independent transgenic lines containing the *Ubi::TiMYB2R-1* transgene were obtained with a transformation efficiency of 0.33%. Using the primers specific for the chimeric *Ubi::TiMYB2R-1* (Fig.4A), genomic PCR assays of the T_0–T_5 generation plants showed that the specific band of the introduced *TiMYB2R-1* was detected in progeny derived from three transgenic wheat lines O1, O3, and O5 with enhanced resistance to take-all, but not in non-transformed (WT) Yangmai 12 (recipient) and null-segregants lacking the transgene (Fig. 4B). Southern blot analysis of T_4 transgenic wheat lines indicated that the three transgenic lines O1, O3, and O5 had one, one, and two copies, respectively, with different hybridization patterns (Fig. 4C), suggesting that the threee lines resulted from independent transformation events. Additionally, a common band was present in these transgenic lines and WT Yangmai 12, suggesting that the transgenic lines and Yangmai 12 contain a sequence (*TaPIMP1*) homologous to the probe derived from the *Ubi::TiMYB2R-1* chimera (Fig.4A, C). Collectively these results proved that the transgene was integrated into the genomes of the three transgenic wheat lines and was stably transmitted to subsequent generations.

The expression of *TiMYB2R-1* was examined in roots of three transgenic lines (O1, O3, and O5) with enhanced resistance at 21 d after *Ggt* inoculation. The RT–PCRs for *TiMYB2R-1* transcription were set up with 31 amplified cycles to illustrate the higher expression of *TiMYB2R-1* in the three transgenic lines, while no corresponding products were observed in WT Yangmai 12 and null-transgenic lines (Fig. 4Di). In Q-RT–PCR analysis, as *TaPIMP1* transcription should be amplified at the same level in the transgenic and WT recipient plants, the relative transcript levels of

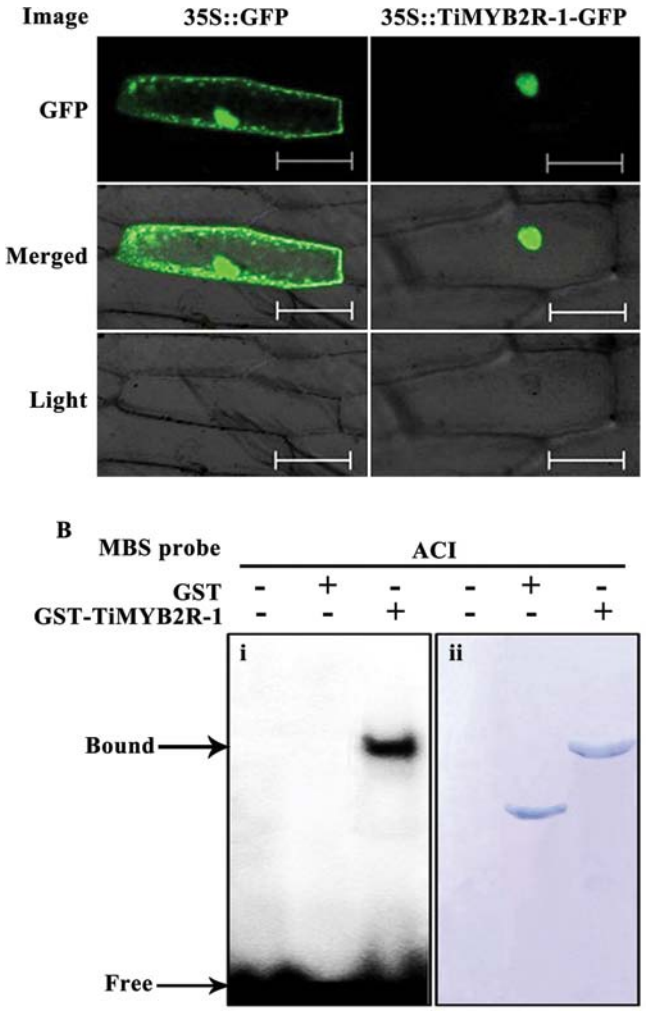

Fig. 3. The subcellular localization and electrophoretic mobility shift assay (EMSA) of TiMYB2R-1 protein. (A) Subcellular localization of 35S::TiMYB2R-1–GFP fused protein in onion epidermal cells. *p35S::TiMYB2R-1-GFP* and *p35S::GFP* constructs were introduced separately into onion cells by bombardment, expressed, and observed under a confocal microscope. Bars=100 μM. (B) EMSA of TiMYB2R-1. Lane 1, only the ACI probe; lane 2, GST alone with the ACI probe; lane 3, GST–TiMYB2R-1 fusion protein with the ACI probe. (i) EMSA of DNAs in the gel stained by ethidium bromide. The free ACI probe and the retarded band of GST–TiMYB2R-1 fusion protein binding to the ACI probe are indicated. (ii) The proteins in the gel stained by Coomassie colloidal blue after EMSA. (This figure is available in colour at *JXB* online.)

TiMYB2R-1 were the transcript levels of *TiMYB2R-1* and *TaPIMP1* in transgenics relative to that of *TaPIMP1* in the WT (Fig. 4Dii). As shown in Fig. 4Dii, *TiMYB2R-1* in the transgenic lines was transcribed at a significantly higher level. Western blot results indicated that these lines (O1, O3, and O5) expressed high levels of TiMYB2R-1 protein, although the WT and transgenic lines contained a tiny amount of TaPIMP1 (Fig. 4E). Thus, the introduced *TiMYB2R-1* gene can be highly expressed in the three transgenic lines with enhanced resistance.

Expression of TiMYB2R-1 improves resistance to take-all in transgenic wheat

Take-all reactions of transgenic wheat lines overexpressing *TiMYB2R-1* along with the null-segregants and WT Yangmai 12 were evaluated 21 d after *Ggt* inoculation. The results showed that most plants of *TiMYB2R-1*-overexpressing transgenic wheat lines O1, O3, and O5 displayed significantly increased resistance relative to the WT Yangmai 12 and null-segregant plants (Table 2, Fig. 5A). The average disease severities of the three transgenic wheat lines were 9.32–25.84%, whereas those of the null-segregants and WT Yangmai 12 were 43.13–49.25% and 44.74–50.14%, respectively. The average TAIs of these transgenic wheat lines were 18.75–35.47, whereas those of the null-segregant and WT Yangmai 12 were 63.33–66.75 and 61.67–68.89, respectively (Table 2). Moreover, using *Ggt* 18S rRNA levels as an indicator of the fungal biomass and infection, the relative *Ggt* abundance was significantly lower in the three *TiMYB2R-1*-overexpressing transgenic lines than in WT Yangmai 12 and null-segregants (Fig. 5B), further supporting that *TiMYB2R-1* transgenic wheat plants were more resistant to *Ggt* infection. Thus, *TiMYB2R-1*-overexpressing transgenic wheat exhibited significantly enhanced resistance to take-all.

Expression of TiMYB2R-1 activates defence-related genes in transgenic wheat

To investigate putative molecular mechanisms of TiMYB2R-1 overexpression in enhanced disease resistance, RT–PCR and Q-RT–PCR were used to analyse the transcription levels of six defence-related genes, namely *PR1a*, *PR17c*, *Chit2*, *Chit3*, *nsLTP1*, and *GST22*, in roots of *TiMYB2R-1*-overexpressing transgenics and of WT Yangmai 12 and null-segregant plants at 21 dpi with *Ggt*. As shown in Fig. 6, the transcription levels of all six genes were markedly elevated in *TiMYB2R-1*-overexpressing lines relative to those in the null-segregant and WT plants. The results suggested that overexpression of *TiMYB2R-1* activates transcription of these defence-related genes in the transgenic wheat lines.

Discussion

In plant species, some MYB proteins have been implicated in defence responses (Mengiste *et al.*, 2003; Seo and Park, 2010; Zhang *et al.*, 2012). Although *T. intermedium* possesses resistance to diverse pathogens, it is not known if and how MYB TFs in *T. intermedium* regulate defence responses to the pathogens. In this study, the first MYB gene of *T. intermedium*, namely *TiMYB2R-1*, was successfully isolated. Sequence analysis showed that the gene sequence of *TiMYB2R-1* includes two exons and an intron, and the deduced TiMYB2R-1 protein possesses the structural characteristics of R2R3-MYB TFs. Sequence alignment and phylogenetic analysis indicated that TiMYB2R-1 protein was most closely related to a wheat R2R3 MYB TaPIMP1, followed by *Arabidopsis* AtMYB108. The results of subcellular localization and MBS *cis*-element binding analyses proved

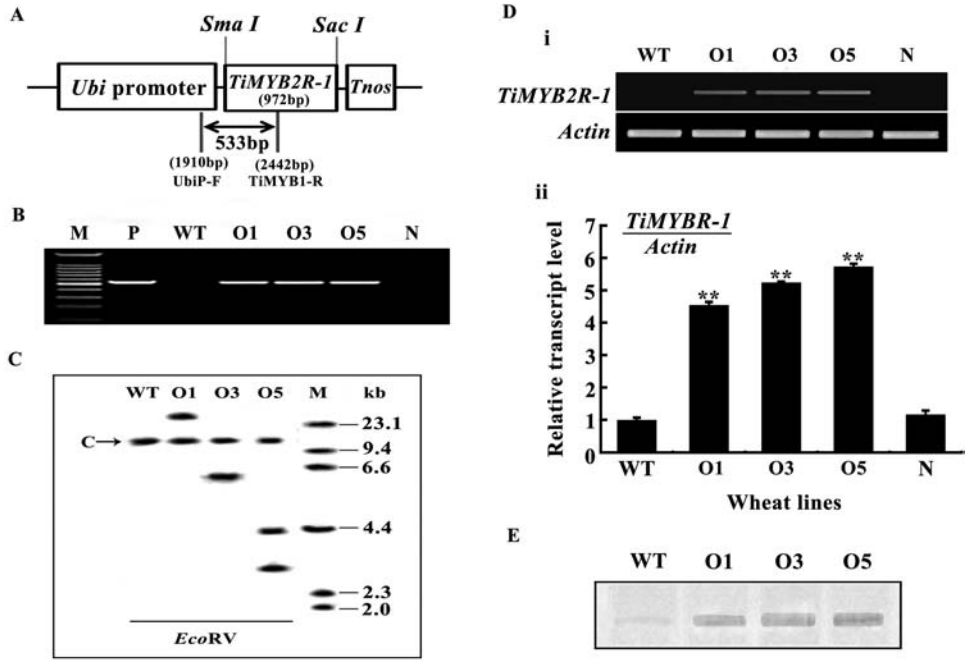

Fig. 4. *TiMYB2R-1* transformation vector and molecular characterization of transgenic wheat. (A) In transformation vector *pA25-TiMYB2R-1*, the *TiMYB2R-1* gene was driven by the maize *ubiquitin* (*Ubi*) promoter and terminated by *Tnos*. The arrow indicates the region amplified in the PCR assays using UbiP-F and TiMYB1-R primers and used for the Southern blot probe. (B) PCR pattern of *TiMYB2R-1* transgenic and wild-type wheat plants using the transgene-specific primers UbiP-F and TiMYB1-R. M, 100 bp DNA ladder; P, transformed vector plasmid pA25-TiMYB2R-1. (C) A Southern blot analysis of *Eco*RV-digested genomic DNAs from non-transformed and *TiMYB2R-1* transgenic plants hybridized with an amplified fragment specific for the transgene. M, λDNA/*Hin*dIII markers. C band indicates the common bands in all wheat plants. (D) RT–PCR (i) and Q-RT–PCR (ii) analyses of *TiMYB2R-1* transcript levels in the roots of T_5 transgenics and control plants. In Q-RT–PCR analysis, three replicates for each sample were averaged, with the standard error of the mean (SE) indicated. Asterisks indicate statistically significant variation (**$P < 0.01$). (E) Western blot pattern of TiMYB2R-1 protein expression in roots of *Ggt*-resistant T_5 transgenics and susceptible wild-type wheat lines. WT, non-transformed wheat Yangmai 12; O1, O3, and O5, three *TiMYB2R-1* transgenic lines; N, segregant plants lacking *TiMYB2R-1*.

that TiMYB2R-1 indeed is an R2R3-MYB TF in *T. intermedium*, consistent with its sequence traits.

Many TFs are induced under environmental stress conditions. For example, transcriptional levels of wheat TaMYB73 and *Arabidopsis* AtMYB44 were up-regulated under salinity stress conditions (He *et al.*, 2011). The expression of the wheat MYB gene *TaPIMP1* was increased after *Bipolaris sorokiniana* infection and dehydration treatment (Zhang *et al.*, 2012). They can offer potential genes for improving biotic and abiotic stress *in planta.* The transcription of *TiMYB2R-1* in *T. intermedium* was significantly induced after inoculation with *Ggt* or *B. sorokiniana* (Supplementary Fig. S3 at *JXB* online), suggesting that *TiMYB2R-1* is probably involved in host response to infection by *Ggt* or *B. sorokiniana*.

To investigate defence roles of *TiMYB2R-1 in planta*, here *TiMYB2R-1*-overexpressing transgenic wheat lines were generated through transformation and characterized in detail. Based on PCR detection for T_0–T_5 transgenic wheat lines and

Table 2. Take-all responses of TiMYB2R-1 transgenic and control wheat lines.[a]

Lines	T_4 generation		T_5 generation	
	Disease severity (%)	Take-all index	Disease severity (%)	Take-all index
O1	20.495**	28.5**	23.99**	35.47**
O3	22.26**	32.33**	25.84**	34.75**
O5	9.32**	18.75**	19.24**	28.12**
Null	43.13	63.33	49.25	66.75
Yangmai12 (recipient)	44.74	61.67	50.14	68.89

Significant difference between *TiMYB2R-1* transgenic lines and untransformated Yangmai 12 (recipient) at **P <0.01.
Null indicates the segregants lacking *TiMYB2R-1*.
[a] The values derived from the average of 70 plants of each line tested in T_4 and T_5 transgenics and control wheat lines.

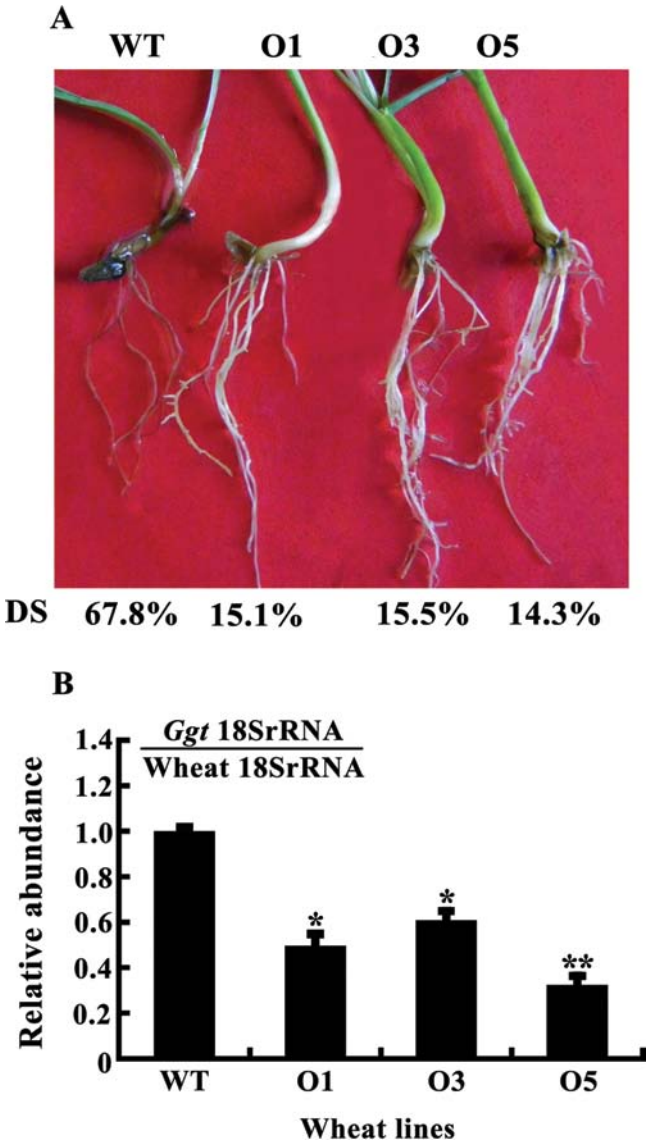

Fig. 5. Typical phenotypes of take-all and relative abundance of *Ggt* in *TiMYB2R-1* transgenics and non-transformed wheat Yangmai 12 plants. (A) Responses of resistant *TiMYB2R-1* transgenic wheat lines, and a susceptible plant of non-transformed Yangmai 12 to take-all. DS indicates the disease severity of take-all in wheat roots. (B) The relative abundance of *Ggt* in resistant transgenic wheat lines (O1, O3, and O5) relative to that in non-transformed Yangmai 12 (WT) based on *Ggt* 18S rRNA in reference to wheat 18S rRNA. Three replicates of each line were averaged, with the standard error of the mean (SE) indicated. Asterisks indicate statistically significant variation (**$P < 0.01$, *$P < 0.05$). (This figure is available in colour at *JXB* online.)

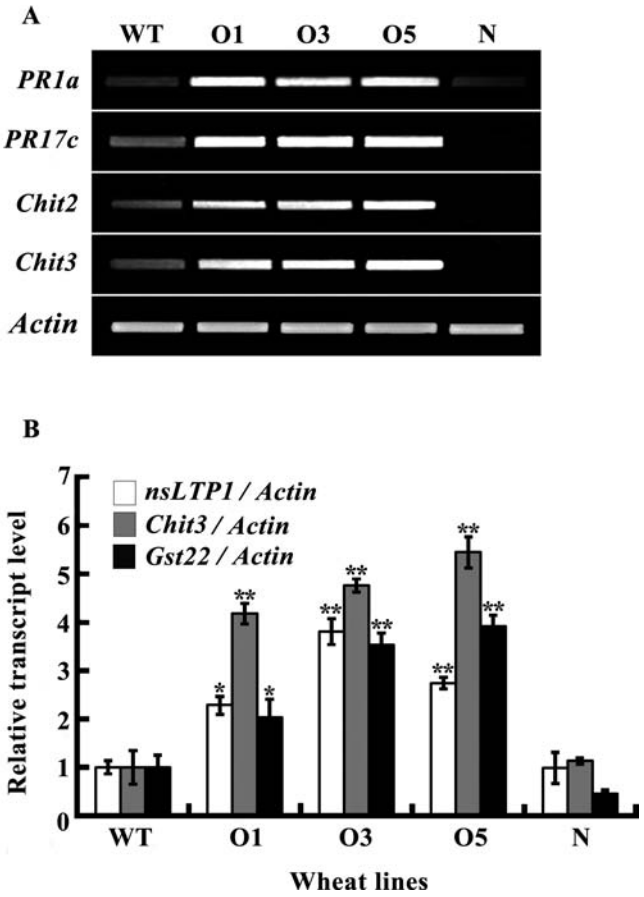

Fig. 6. Expression patterns of the defence-related genes *PR1a*, *PR17c*, *Chit2*, *Chit3*, *nsLTP1*, and *GST22* in roots of *Ggt*-resistant T_5 transgenics and WT and null-segregant plants. (A) RT–PCR analyses. (B) Q-RT–PCR analyses. O1, O3, and O5, three *TiMYB2R-1* transgenic lines; N, null-segregants lacking *TiMYB2R-1*; WT, non-transformed Yangmai 12. The transcript level of a defence-related gene in resistant transgenics (O1, O3, and O5) was relative to that in WT Yangmai 12. Three replicates for each sample were averaged, with the standard error of the mean (SE) indicated. Asterisks indicate statistically significant variation (**$P < 0.01$, *$P < 0.05$).

Southern blot analyses using a transgene-specific amplicon as a probe, the results showed that the *TiMYB2R-1* transgene was integrated into the genomes of three transgenic wheat lines and could be transmitted to subsequent generations. RT–PCR and western blot assays indicated that *TiMYB2R-1* was highly expressed in the three transgenics. Following inoculation with the take-all pathogen *Ggt*, the disease severity, TAI, and *Ggt* relative biomass assays showed that the transgenic wheat lines overexpressing *TiMYB2R-1* had more significantly enhanced resistance to take-all than WT Yangmai 12 and null-segregants, although *TaPIMP1* exists in the WT and *TiMYB2R-1* transgenic wheat lines and is induced following *Ggt* infection. The enhanced degrees of resistance in the transgenic lines were correlated with *TiMYB2R-1* expression levels. These suggested that overexpression of *TiMYB2R-1* conferred increased resistance in transgenic wheat, whereas the expression of *TaPIMP1* in WT Yangmai 12 is not enough to confer resistance after *Ggt* challenge. Take-all responses of *TaPIMP1*-overexpressing wheat will be tested in the future. Furthermore, overexpression of *TiMYB2R-1* does not affect the development and growth of the transgenic wheat under normal growth conditions. Thus, *TiMYB2R-1* can be used as an important engineering gene for improving take-all resistance of wheat. Additionally, the transgenic wheat lines expressing TiMYB2R-1 showed enhanced resistance to

common root rot caused by *B. sorokiniana* (Supplementary Table S1 at *JXB* online), similar to TaPIMP1-overexpressing wheat (Zhang *et al*., 2012). Thus, the transgenic wheat produced in this study will provide potential wheat germplasm for enhancing resistance to take-all and common root rot. This is believed to be the first report on development of take-all-resistant wheat lines.

In plants, certain activator-type TFs have been implicated in triggering disease resistance and abiotic stress tolerance through activation of defence- and stress-related genes (Ma *et al*., 2009; Zhang *et al*., 2012). Defence-related genes play vital roles in defence against pathogens in plants (Guilleroux and Osbourn, 2004; Seo and Park, 2010; Zhang *et al*., 2012). For example, transgenic wheat plants overexpressing a wheat *LTP* gene or a barley *chitinase* gene displayed increased resistance to fungal pathogens (Shin *et al*., 2008; Zhu *et al*., 2012). Although no natural highly resistant wheat cultivar has been identified, the transcription levels of some wheat defence-responsive genes were up-regulated during compatible interactions between wheat roots and *Ggt* using suppression subtractive hybridization and expressed sequence tag (EST) analysis (Guilleroux and Osbourn, 2004). Among them, two ESTs corresponded to *Chit3* and *GST2* genes (Guilleroux and Osbourn, 2004). TFs interact with specific DNA sequences (*cis*-acting elements) in target genes to modulate the transcription process. In a previous study, the microarray data showed that wheat TaPIMP1 overexpression activates the transcription of some defence-related genes in wheat, namely *PR1a*, *PR17c*, *Chit2*, *Chit3*, *nsLTP1*, and *GST22* (Zhang *et al*., 2012).

In this study, RT–PCR and Q-RT–PCR were used to investigate if TiMYB2R-1 overexpression activates the same defence-related genes in the transgenic wheat, using the null-segregant and WT wheat lines as controls. The results indicated that overexpression of *TiMYB2R-1* indeed elevated the expression of such defence-related genes in the transgenic wheat. The promoter sequences of these defence-related genes obtained from the Wheat Draft Genome Assembly database (http://www.cerealsdb.uk.net/CerealsDB/), and prediction of MBS *cis*-elements in the promoters (www.dna.affrc.go.jp/PLACE/) revealed that MBS *cis*-acting elements exist in the promoters of wheat defence genes *PR1a*, *PR17c*, *nsLTP1*, and *GST22* (Table 1). TiMYB2R-1 possibly interacts with these promoters and thereby contributes to the increased expression of these genes. The promoters of wheat *Chit2* and *Chit3* genes could not be found due to the incomplete coverage of wheat genome sequences. TiMYB2R-1 may elevate the expression of *Chit2* and *Chit3* genes through indirect means, which has yet to be verified experimentally. The results suggested that changes in expression of a subset of wheat defence-related genes regulated by *TiMYB2R-1* expression probably result in enhanced resistance to take-all in *TiMYB2R-1* transgenic wheat. The results may provide a new insight into the interaction between wheat and *Ggt*.

In summary, *TiMYB2R-1*, the first MYB gene isolated from *T. intermedium*, was characterized. It encodes an R2R3-MYB TF and has significantly higher expression levels in host plants following *Ggt* infection. *TiMYB2R*-1 overexpression in transgenic wheat lines showed significantly enhanced resistance to take-all and common root rot possibly through activation of some defence-related genes. The results contribute to further understanding of the characteristics and functions of the MYB TF family in additional plant species.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of *TiMYB2R-1* and *TaPIMP1* sequences amplified in (Q-)RT–PCR.

Figure S2. The cDNA sequence and deduced amino acid sequence of *TiMYB2R-1*.

Figure S3. Transcription analysis of *TiMYB2R-1* in *T. intermedium* following *B. sorokiniana* inoculation.

Table S1. *Bipolaris sorokiniana* responses of *TiMYB2R-1* transgenic and control wheat.

Acknowledgements

The authors are grateful to Dr Cunjin Zhang (Durham University, UK) for revision, and Professor Daowen Wang (Chinese Academy of Sciences) for providing the *ph16318* vector. This study was supported by the National '863' project (grant no. 2012AA10A309), a National 'Key Sci-Tech' projects (2011ZX08002-001 and 2013ZX08002-001), and the NSFC programme (grant no. 30871523).

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