A Novel Homeobox-Like Gene Associated with Reaction to Stripe Rust and Powdery Mildew in Common Wheat

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ABSTRACT

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Stripe rust and powdery mildew, caused by *Puccinia striiformis* f. sp. *tritici* and *Blumeria graminis* f. sp. *tritici*, respectively, are severe diseases in wheat (*Triticum aestivum*) worldwide. In our study, differential amplification of a 201-bp cDNA fragment was obtained in a cDNA-amplified fragment length polymorphism (AFLP) analysis between near-isogenic lines *Yr10NIL* and Avocet S, inoculated with *P. striiformis* f. sp. *tritici* race CYR29. A full-length cDNA (1,357 bp) of a homeobox-like gene, *TaHLRG* (GenBank accession no. EU385606), was obtained in common wheat based on the sequence of GenBank accession AW448633 with high similarity to the above fragment. The genomic

DNA sequence (2,396 bp) of *TaHLRG* contains three exons and two introns. *TaHLRG* appeared to be a novel homeobox-like gene, encoding a protein with a predicted 66-amino-acid homeobox domain. It was involved in race-specific responses to stripe rust in real-time quantitative polymerase chain reaction (PCR) analyses with *Yr9NIL*, *Yr10NIL*, and Avocet S. It was also associated with adult-plant resistance to stripe rust and powdery mildew based on the field trials of doubled haploid lines derived from the cross Bainong 64/Jingshuang 16 and two $F_{2:3}$ populations from the crosses Lumai 21/Jingshuang 16 and Strampelli/Huixianhong. A functional marker, *THR1* was developed based on the sequence of *TaHLRG* and located on chromosome 6A using a set of Chinese Spring nulli-tetrasomic lines.

Additional keywords: durable resistance.

Stripe rust and powdery mildew, caused by *Puccinia striiformis* f. sp. *tritici* and *Blumeria graminis* f. sp. *tritici*, respectively, are serious wheat diseases worldwide, resulting in both yield losses and downgrading in quality (37). Use of resistant cultivars is the most economical and environmentally sound method to control these diseases. Disease resistance in crop plants has been generally characterized as race-specific resistance and non-race-specific or adult-plant resistance (APR) (14). Race-specific resistance is often overcome by new pathogen races. In contrast, APR is often long-lived under field conditions and is therefore preferred by breeders aiming to achieve durable resistance, and has been emphasized to increase crop resistance in breeding (37). For example, the wheat cultivar Knox and its derivative Massey showed stable and effective resistance against powdery mildew for 20 years (9). Forty-four stripe rust resistance and 49 powdery mildew resistance genes in wheat have been formally catalogued (17,26-28,30,33). Some of them were transferred from wild relatives. A considerable number of them (*Yr11*, *Yr12*, *Yr13*, *Yr14*, *Yr16*, *Yr18*, *Yr29*, *Yr30*, *Yr31*, *Yr36*, and *Yr39*) confer APR to stripe rust and two (*Pm38* and *Pm39*) provide APR to powdery mildew (18,19,21,28).

Previous studies indicated that APR genes involved in resistance to different diseases are clustered together on wheat chromosomes (35,39,41). For example, *Yr18*, *Lr34*, and *Pm38*

were located on the same position of chromosome 7DS conferring resistance to stripe rust, leaf rust, and powdery mildew, respectively. They were also associated with leaf tip necrosis (*Ltn*) (28,37,41). Similarly, *Yr29*, *Lr46*, and *Pm39*, which are associated with leaf tip necrosis, were mapped to the same location of chromosome 1BL and co-localize with genes encoding β 1-proteasome subunits (35). The association of APR genes is likely due to close linkage or the pleiotropic effects of a single locus for resistance to different diseases. However, few resistance genes have been cloned in wheat and none is of the APR type.

Many resistance genes contain conserved nucleotide-binding domains (31). Homeobox genes encode DNA-binding domains with a helix-turn-helix structure, many of which are involved in gene regulation (8). Typical homeodomains contain 60 amino acids and atypical domains have more or fewer amino acids (4,8). In plants, homeobox proteins are divided into several families, such as Knox, WOX, Hd-Zip, PHD-finger, GLABRA-, and BEL1-like proteins, according to sequence conservation within the homeodomain and adjacent regions (5,11,12,20,23,34). Most homeobox genes participate in plant development processes (4,5,7,8). Some plant HD-Zip genes regulate a biochemical pathway for drought stress (1), and *H52* from tomato encoding a distinct HD-Zip protein was involved in cellular protection from programmed cell death (25). *OCP3* in *Arabidopsis* mediates resistance to infection by necrotrophic pathogens (6). Here we report a novel homeobox-like gene in common wheat, designated *TaHLRG*, which is associated with race-specific reaction to stripe rust and APR to both stripe rust and powdery mildew. The role of *TaHLRG* for resistance to wheat disease will be discussed.

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MATERIALS AND METHODS

Plant materials. The near-isogenic lines *Yr9/6*Avocet S* (*Yr9NIL*), *Yr10/6*Avocet S* (*Yr10NIL*), and *Avocet S*, kindly provided by C. R. Wellings, Plant Breeding Institute Cobbitty, University of Sydney, Australia, were used for stripe rust tests in the greenhouse, cDNA-amplified fragment length polymorphism (AFLP) assay, and cloning of the homeobox-like gene. Chinese Spring nulli-tetrasomic (NT) lines used for determining the location of the homeobox-like gene were provided by R. A. McIntosh, University of Sydney. A doubled haploid (DH) population (128 lines) derived from the cross Bainong 64/Jingshuang 16 and two $F_{2.3}$ populations from the crosses Lumai 21/Jingshuang 16 (186 lines) and Strampelli/Huixianhong (226 lines) were used for the assessment of APR to stripe rust and powdery mildew in the field. Strampelli was introduced from Italy and showed good APR to stripe rust in China for the last 35 years, whereas Huixianhong is highly susceptible to all Chinese *P. striiformis* f. sp. *tritici* races. Bainong 64 and Lumai 21 were previous leading Chinese wheat cultivars with good APR to powdery mildew, whereas Jingshuang 16 is highly susceptible to current *B. graminis* f. sp. *tritici* races in China. The $F_{2.3}$ lines generated from F_2 plants were planted and harvested as bulks with over 50 plants of each line to produce $F_{2.3}$ populations that can be maintained as bulk populations, each deriving from a single F_2 plant.

Inoculation of *P. striiformis* f. sp. *tritici*. The *Yr9NIL*, *Yr10NIL*, and *Avocet S* were inoculated with two *P. striiformis* f. sp. *tritici* races, CYR29, and/or culture 78028. *Yr9NIL* was susceptible to CYR29 and resistant to 78028, whereas *Yr10* was resistant, and *Avocet S* was susceptible to both isolates. Wheat seedlings were grown in a growth chamber at 17°C with 50% relative humidity (RH), and inoculated with *P. striiformis* f. sp. *tritici* urediospores on the first fully expanded leaves as described by Stubbs (40) and Sun et al. (42). After the inoculation, the seedlings were maintained in the dark at 9 to 11°C with 100% RH. As a control, wheat seedlings were mock-inoculated with water and taken through the same process. Inoculated and corresponding mock-inoculated leaves for each treatment (*Yr9*-CYR29, *Yr9*-78028, *Yr10*-CYR29, and *Avocet*-CYR29) were collected at 12, 15, 18, 21, 24, 28, 32, and 36 h post-inoculation (hpi) for subsequent cDNA-AFLP and quantitative polymerase chain reaction (Q-PCR) analysis. Meanwhile, the seedlings of susceptible check cultivar Mingxian169 in each treatment were transferred into a growth chamber with identical conditions, i.e., the day/night regime of 14 h light (28,000 lx) at 17°C and 10 h of darkness at

12°C, with 70% RH. All checks were fully susceptible (IT = 4) 2 weeks after inoculation, indicating a successful inoculation in all treatments.

cDNA-AFLP assay. Total RNA was prepared from the first leaves of CYR29-inoculated and corresponding mock-inoculated *Yr10NIL* and *Avocet S*, collected at five time points (15, 18, 21, 24, and 36 hpi) using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Dnase I-treated (Qiagen, Hilden, Germany) total RNA was then used as a template for double-stranded cDNA amplification by a double-stranded cDNA amplification kit (*TaKaRa*, Dalian, China) following the manufacturer's recommendations. The double-stranded cDNA was digested with restriction enzymes *Pst*I and *Mse*I (New England Biolabs, Ipswich, CT), and the 3' regions were captured on streptavidin Dynabeads (Invitrogen, Carlsbad, CA). The pre-amplifications were performed with the *Pst*I and *Mse*I primers, with either T or C as the 3' nucleotide. From a 40-fold dilution of the pre-amplified samples, 5 µl was used for the final selective amplifications using *Pst*I and *Mse*I primers with three selective nucleotides (CGA or TAC). Amplification products were separated on 5% polyacrylamide gels and visualized by silver staining (3). The differential PCR fragments amplified from the cDNA-AFLP assay were cloned following the method described by Xu et al. (44).

Gene cloning by 3' RACE, 5' RACE, and PCR amplification of genomic DNA. Oligonucleotides (Table 1) used for 3' and 5' RACE were designed based on the sequence of a wheat EST (GenBank accession no. AW448633). This EST was detected in a BLAST search, and was highly similar to a 201-bp differential cDNA-AFLP fragment. Dnase I-treated (Qiagen, Hilden, Germany) total RNA from seedlings of *Yr10NIL* inoculated with CYR29 was used as a template for 3' and 5' rapid amplification of cDNA ends (RACE). For 3' RACE, an oligo-dT adapter primer (AP), 3'AP1, was used in RT-PCR for rapid amplification of the first-strand cDNA using an RT kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. A gene-specific primer (GSP), 3'GSP1, and an adapter primer, 3'AP2, permitted capture of 3'-mRNA sequence for the 563- to 1357-bp region. PCR was performed using *LA Taq* DNA polymerase (*TaKaRa*, Dalian, China) with an annealing temperature of 60°C according to the manufacturer's recommendation.

The 5' end of the cDNA was amplified using the 5'-RACE technique following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The primer 5'GSP1 was used to obtain the first-strand cDNA and a poly-C tail was added to the 3' end of the cDNA. In the second PCR, a cDNA fragment (1 to 286 bp) was amplified with the primer set 5'GSP2/5'AP1 and sequenced. Then, 5'GSP3/5'AP2 were used in PCR amplification with the above 286-bp PCR product as template, and a 157-bp cDNA fragment (1 to 157 bp) was amplified. The two fragments overlapped by 157-bp with 100% sequence identity. The sequence of the 5'RACE fragment confirmed that the 286-bp cDNA fragment was the 5'-end sequence (1 to 286 bp) of *TaHLRG*. The primer set OP4/OP5 was designed from the AW448633 sequence to obtain a 380-bp (8 to 387 bp) cDNA fragment that overlapped with the 5'-end sequence, with 100% sequence identity in 279 bp. The primer set OP1/OP2 was used to amplify the region 266 to 665 bp, overlapping with the OP4/OP5 amplification region and 3' RACE cDNA sequence in 122 and 103 bp, respectively, with 100% sequence identity. PCR was performed using *Pfu* DNA Polymerase (TIANGEN, Beijing, China) with an annealing temperature 55 to 65°C following the manufacturer's recommendation.

Genomic DNA was extracted from *Yr10NIL* seedlings (36). The genomic DNA sequence of the *TaHLRG* gene was assembled with PCR amplification of four primer sets (OP4/OP5, OP1/OP2, 3'GSP1/OP6, and OP7/OP8; Table 1), amplifying the regions covering 1 to 477, 259 to 755, 653 to 1985, and 1,916 to 2,396 bp,

TABLE 1. Oligonucleotides used for 3' rapid amplification of cDNA ends (RACE), 5' RACE, reverse transcription-polymerase chain reaction (PCR), and PCR amplification of genomic DNA

Primer	Primer sequence (5'-3')
3'AP1	CTGATCTAGAGGTACCGATCCTTTTCTTTTCTTTT
3'AP2	CTGATCTAGAGGTACCGGATCC
3'GSP1	TGGACCCGAGGAGGATGAATGG
5'AP1	GGCCACGCGTCTGACTAGTACGGGIIIGGGIIIGGGIIG
5'AP2	GGCCACGCGTCTGACTAGTAC
5'GSP1	TGGGGGACCATCATCT
5'GSP2	GAGCGGTCCGGGGAGGCGTTGAA
5'GSP3	CTTGGCGCTGCCCGTCATACTTGTC
OP1	CAACGCCTCCCCGACCGCTCC
OP2	GAGATCCCCAGGAACACAGC
OP3	GTAAGCTTCTCTCCACCCACCCCTCTCC
OP4	TCTCCTCCACCCACCCCTCTCCA
OP5	CGGCCACTGGGGGACCATCATCT
OP6	CAGCGGACCCCTTACATC
OP7	AGGCCCTCTATTTGACGCTCGTGTCT
OP8	AAGCGCACAGGGAAGCGAAACAAC
Tub2U	TGTGCCCCGTGCTGTTCTTATG
Tub2L	CCCTTGGCCAGTTGTTACCC

respectively, with 100% identity in the overlapping sequences. PCR was performed using *Pfu* DNA Polymerase (TIANGEN, Beijing, China) with an annealing temperature of 55 to 65°C according to the manufacturer's recommendation.

Real-time quantitative PCR. Total RNA samples were extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) from the inoculated and corresponding mock-inoculated leaves of each treatment (*Yr9*-CYR29, *Yr9*-78028, *Yr10*-CYR29, and Avocet-CYR29) at eight time points (12, 15, 18, 21, 24, 28, 32, and 36 hpi), following protocols recommended by the manufacturer. One-step RT-PCR (RT kit, Qiagen, Hilden, Germany) was performed using DNase I-treated (Qiagen, Hilden, Germany) total RNA as template.

In the Q-PCR assay, sequence-specific primer pairs, 3'GSP1/OP6 and Tub2U/Tub2L designed by DNAStar, were used to quantify accumulation of *TaHLRG* transcripts and to normalize the amount of cDNA samples, respectively. Tub2U/Tub2L was an endogenous control from the sequence of the *Triticum aestivum* *beta-tubulin* 2 gene (GenBank accession U76745), amplifying a 135-bp fragment (176 to 310 bp). The cDNA region amplified with the primer set 3'GSP1/OP6 was 201 bp (563 to 763 bp), whereas the corresponding genomic region included an intron (771 to 1,902 bp), facilitating a check of genomic DNA contamination in cDNA samples. All Q-PCR products from the two primer sets were sequenced to confirm the sequence identity with the template cDNA. Q-PCR experiments were performed in an ICycler iQ (Bio-Rad) with high amplification efficiency using a SYBR GREEN kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

The $2^{-\Delta\Delta C_T}$ method (22) was used to normalize and calibrate the *TaHLRG* C_T values relative to the *beta-tubulin* 2 endogenous control and the corresponding mock-inoculated controls. C_T values included in the analyses were based on three replicates for each treatment at each time point. All statistical analyses were performed using Optical System Software version 3.1.

Field trials for stripe rust and powdery mildew reactions. The 226 F_{2.3} lines from cross Strampelli/Huixianhong were tested for stripe rust reaction in Qingshui following inoculation with *P. striiformis* f. sp. *tritici* race CYR32 in the 2004 to 2005, 2005 to 2006, and 2006 to 2007 cropping seasons. Qingshui, located in the Gansu province, is considered a stripe rust 'hot spot' in China, having ideal conditions for rust infection and spread. Field trials were conducted in a randomized complete block design with three replicates; each plot consisted of two 1.5-m rows spaced 25 cm apart. Approximately 150 seeds were sown in each plot. The highly susceptible line Tiaogan 601 was used as a susceptible check and was planted after every 10 plots. Spreader rows of Tiaogan 601 were planted perpendicular and adjacent to the rows of tested lines. Stripe rust severities were assessed around 25 June for the first time and then at weekly intervals for two further weeks using the modified Cobb scale (29,32).

The 128 DH lines from cross Bainong 64/Jingshuang 16 and 186 F_{2.3} lines from cross Lumai 21/Jingshuang 16 were tested in the field for powdery mildew reaction in Beijing and Anyang located in Henan province, during the 2004 to 2005 and 2005 to 2006 cropping seasons. In Beijing, they were artificially inoculated with the prevalent *B. graminis* f. sp. *tritici* isolate E20, whereas in Anyang a mixture of pathotypes was used. Field trials were planted in randomized complete block designs with three replicates; each plot was comprised of two 1.5-m rows spaced 25 cm apart. Approximately 150 seeds were sown in each plot. Spreader rows of the susceptible cultivar Jingshuang 16 surrounded the tested cultivars and were also planted after every 10 plots. In Beijing, the infection severity on F1 leaves (the leaf below the flag leaf) of 10 arbitrarily selected plants from each plot were scored, based on the actual percentage (0 to 100%) leaf area covered by powdery mildew (10). Disease scoring was carried out for the first time 3 weeks after inoculation and then at

weekly intervals until leaves were physiologically mature. Disease severities of 10 selected plants were averaged to obtain a mean severity for each line. In Anyang, powdery mildew severities on the F-1 leaf of each line were visually rated as percentage leaf area covered by powdery mildew, when the disease severities of the susceptible check cv. Jingshuang 16 reached a maximum level around 18 May 2005 and 2006. The maximum stripe rust and powdery mildew severity were used to analyze their association with *TaHLRG* gene.

A functional marker, *THR1* was developed from the sequence of *TaHLRG* using the software SSRHunter1.3. One-way analysis of variance (ANOVA), using the software SAS 6.12, was conducted to determine the association between the functional marker *THR1* and APR reaction to powdery mildew and stripe rust.

RESULTS

Cloning a homeobox-like gene. In the cDNA-AFLP assay, 192 transcript derived fragments (TDF) were obtained and sequenced, ranging from 80 to 750 bp in size. A 201-bp cDNA fragment was expressed at five time points (15, 18, 21, 24, and 36 hpi) in *Yr10NIL* inoculated with *P. striiformis* f. sp. *tritici* race CYR29 and matched known genes involved in pathogen response. A wheat EST (GenBank accession no. AW448633) was detected through a BLAST search using the 201-bp cDNA sequence in a search against the wheat EST database in GenBank. Oligonucleotide primers were designed based on the AW448633 sequence, and a new *Triticum aestivum* homeobox-like resistance gene, designated *TaHLRG*, was cloned in *Yr10NIL* by 3' RACE, 5' RACE, and PCR amplification of genomic DNA, encoding a protein with a predicted HOMEBOX_2 domain. In the genomic clone the sequence was 2,396 bp (GenBank accession no. EU385606), with three exons from nucleotides 131 to 313, 411 to 770, and 1903 to 2373. The assembled full-length cDNA sequence (GenBank accession no. EU364815) was 1,357 bp containing an open reading frame (ORF) of 1,014 bp. It encoded a protein of 337 amino acid residues with a predicted 66-amino-acid HOMEBOX_2 domain (PS500071, at positions 11 to 76) close to the N terminus that formed a conserved helix-turn-helix structure including a first helix (amino acids 11 to 43), a turn (amino acids 44 to 55) and a second helix (amino acids 56 to 76) as predicted by ScanProsite. *TaHLRG* was compared with 20 known genes with HOMEBOX_2 domain sequences (Fig. 1) in maize, *Arabidopsis thaliana*, barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.), which contained the conserved residues in alignment of Swiss-Prot true positive hits by PS500071 and were chosen from their representative sequence homology in each gene family. The predicted homeobox of *TaHLRG* contained six conserved residues (Leu-39, Phe-43, Trp-65, Phe-66, Asn-68, and Arg-70) and six frequently occurring residues (Arg-25, 69, 75; Gln-61, 67; and Val-62). The other residues of *TaHLRG* were not homologous to any known homeodomain proteins, indicating that *TaHLRG* is a new homeobox-like gene.

Expression analysis of *TaHLRG*. In a Q-PCR assay, the transcript level of *TaHLRG* was up-regulated in the compatible (*Yr9*-CYR29) interaction at 15 hpi and no significant changes were found at seven other time points (12, 18, 21, 24, 28, 32, and 36 hpi) (Fig. 2). In the incompatible interaction (*Yr10*-CYR29), the transcript level of *TaHLRG* was firstly down-regulated at 24 hpi and then up-regulated at 28 hpi, respectively, and no significant changes were detected at six other time points (Fig. 2). These results indicated that *TaHLRG* was constitutively expressed in both inoculated and mock-inoculated samples, and might be involved in both compatible and incompatible race-specific responses.

***TaHLRG* was associated with adult-plant resistance to stripe rust and powdery mildew.** A functional marker, *THR1*

ANL2_ARATH/132-192 PPRKKRYHRHTPQQIQEESMFKE..... CPHPDEKQRLLELSKRL..... CLETRQVKFTEONRRITQMTQ
ATML1_ARATH/60-120 PNKKRYHRHTPQQIQEESMFKE..... CPHPDDKQRLKELSRRL..... SLEPLQVKFTEONRRITQMTQAAQ
PDF2_ARATH/60-120 PNKKRYHRHTPQQIQEESMFKE..... CPHPDDKQRLKELSRDL..... NLEPLQVKFTEONRRITQMTQAAQ
ROC8_ORYSJ/13-73 QRRKKRYHRHTPQQIQEESMFKE..... CPHPDENQRAQLSRRL..... GLEPRQVKFTEONRRITQMTQAAQ
HDG10_ARATH/14-74 GIDSNNRRHSHNHQVQRPEAFHE..... CPHPDDSQRRQLGNEL..... NLKHKQVKFTEONRRITQMTQAAQ
ATB20_ARATH/82-142 TMLGEKKKRLQLEQVKADEKSEEL..... GNKLEPERKIQALAKAL..... GMQPRQVKFTEONRRITQMTQAAQ
HAT7_ARATH/114-170 ---EKKKRLNLEQVRADEKSEEL..... GNKLEPERKIQALAKAL..... GLQPRQVKFTEONRRITQMTQAAQ
ATHBX_ARATH/64-124 GGRRRKKLRLTKEQSHLDEESFIQ..... NHTLTPKQKDLATFL..... KLSQRQVKFTEONRRITQMTQAAQ
HOX25_ORYSI/17-78 SSAAARKRRLTAEQVRADEKSEEL..... KRKLEPERKSELARRL..... GIAPRQVKFTEONRRITQMTQAAQ
TF1_ORYSJ/64-124 EKRKKRLQRLTGKQSEVDEGFSI..... CGHPDDGQKRLSETT..... GLGLDQVKFTEONRRITQMTQAAQ
WOX8_ORYSJ/86-151 KITARRRWTPTPMQLQIPENIDQg..... NGTPSKQKIKDITAElsq... hgQISETNVYNTEONRRITQMTQAAQ
WOX3B_MAIZE/2-67 PQTPSTRWCPTPEQLMIDHEMYSRg..... VRTPNAAEIQQITAHlay... ygrIEGKNVYFTEONRRITQMTQAAQ
HOX1A_MAIZE/557-617 SNSTARKGHFGPVINQKHEHEKT..... QPYPSRSVKESLAEL..... GLTFRQVKFTEONRRITQMTQAAQ
PRH_ARATH/451-511 KGGRRRMFRLPRNAVEKQRQVFAE..... TELPSKAVRDRILAKEL..... SLDPKQVKFTEONRRITQMTQAAQ
KNOX8_MAIZE/21-84 FSKKKKKGLPKPEARQQLHWELhy... kWPYPSETEKMALETT..... GLDPKQVKFTEONRRITQMTQAAQ
KNOX3_HORVU/266-329 LSKKKKKGLPKPEARQQLSWEMhy... kWPYPSESQKVALAEST..... GLDLKQVKFTEONRRITQMTQAAQ
KNX10_MAIZE/24-87 LSRKKKKGLPRDARQQLHWELhy... rWPYPSELEKAALEST..... GLEAKQVKFTEONRRITQMTQAAQ
KN1_MAIZE/262-325 LSKKKKKGLPKPEARQQLSWEDhy... kWPYPSETQKVALAEST..... GLDLKQVKFTEONRRITQMTQAAQ
RSH1_MAIZE/252-315 FSKKKKKGLPKPEARQQLHWELhy... kWPYPSETEKIALAEST..... GLDQKQVKFTEONRRITQMTQAAQ
HLG3_MAIZE/21-84 FLKKKKGLPKDARTVLEWNTThy... rWPYPTEEDKVRILAAMT..... GLDPKQVKFTEONRRITQMTQAAQ
TAHLRG/11-76 RFTDTEVAKMEEVLRLDNAMPKHPVIQGTDDF..... NASPDRSGDGKV..... PVQYNQVKFTEONRRITQMTQAAQ

Fig. 1. HOMEBOX_2 domain sequence comparison among *TaHLRG* and 20 known homeobox-like genes. The shaded amino acids are conserved residues. The numbers following the gene names indicate the position of helix-turn-helix domain in the deduced peptides.

DISCUSSION

TaHLRG is a novel homeobox-like gene identified through a BLAST search against the nr database in GenBank. The specific motif, HOMEBOX_2, contains 12 conserved residues, without a consensus pattern with other homeobox-like genes. Apart from the 12 conserved residues in the homeobox domain of *TaHLRG* (Fig. 1), other residues were not homologous to any known homeodomain proteins. Therefore, *TaHLRG* may encode a new homeobox protein.

A hypersensitive response (HR) is an efficient plant defense against biotrophic pathogens, resulting in localized cell death and formation of necrotic lesions. In incompatible interactions between resistant wheat cultivars and stripe rust races, the host cells start to become necrotic at 24 hpi (16). At the same time, the structural defense reactions such as formation of cell wall apposition, collar or papillae are more pronounced in the infected leaves of the resistant cultivars than in those of the susceptible ones (15). After 24 hpi, the infected cells become necrotic and collapse inhibiting the proliferation of the stripe rust fungus (16). In the incompatible interaction (*Yr10*-CYR29), the expression of *TaHLRG* was detected in the above two phases. At the beginning of the structural defense reaction, 24 hpi, *TaHLRG* was down-regulated (Fig. 2). *TaHLRG* was strongly up-regulated at 28 hpi, corresponding to a period of cell death, which may stop further development of pathogen. In contrast, no significant change of *TaHLRG* transcripts was found in the compatible interaction of Avocet-CYR29. This indicated that *TaHLRG* might play an important role in wheat line *Yr10*NIL against stripe rust race CYR29. In the compatible interaction of *Yr9*-CYR29, the transcript level of *TaHLRG* was strongly up-regulated as early as 15 hpi, before host cells became necrotic. In contrast, the expression of *TaHLRG* showed no significant changes among different time points in the incompatible interaction of *Yr9*-78028. These reactions may reflect the specific incompatible and compatible interactions between resistance gene and stripe rust races. A number of DNA-binding proteins are important in the transmission of defense signals for the plant-pathogen interaction to either activate or suppress downstream gene expression, and in the regulation of cross-talk between different signaling pathways (2,24). *TaHLRG* has the characteristics of genes with a DNA-binding

Fig. 2. *TaHLRG* transcript levels relative to corresponding mock-inoculated samples at eight sampling points. Interactions were *Yr9*NIL-CYR29 (compatible), *Yr9*NIL-78028 (incompatible), *Yr10*NIL-CYR29 (incompatible), and Avocet-CYR29 (compatible).

(primer set OP3/5'GSP3), was developed from the sequence of *TaHLRG*, amplifying two PCR fragments of 145 and 157 bp in two parents of the populations, respectively. The sequence of the 157-bp PCR fragment is 100% identical to that of *TaHLRG*, whereas two simple sequence repeats were absent in the 145-bp PCR fragment.

THR1 was used to genotype three populations. One-way ANOVA indicated that the gene explained 9.3 to 9.7% and 11.9 to 20.5% of the phenotypic variance for powdery mildew reaction in populations Bainong 64/Jingshuang 16 and Lumai 21/Jingshuang 16, respectively, and accounted for 11.8 to 22.5% of the phenotypic variance for stripe rust resistance in the population from Strampelli/Huixianhong across different environments (Table 2). The results indicated that *TaHLRG* was strongly associated with APR for both stripe rust and powdery mildew resistance in wheat.

Chromosomal location of *TaHLRG*. In PCR amplification with the marker *THR1*, no PCR product was amplified from the Chinese Spring (CS) nulli-tetrasomic (NT) lines N6A/T6B and N6A/T6D (Fig. 3). However, a 145-bp fragment was amplified from CS and all other NT lines (data not shown), indicating that *TaHLRG* was located on wheat chromosome 6A.

TABLE 2. One-way analysis of variance for the association between *TaHLRG* and adult-plant reaction to stripe rust and powdery mildew in three populations

Population	Disease	Location	Cropping season	R^{2a}	<i>P</i>
Bainong 64/Jingshuang 16	Powdery mildew	Beijing	2004-05	9.3	0.0005
		Anyang	2004-05	9.7	0.0004
Lumai 21/Jingshuang 16	Powdery mildew	Beijing	2004-05	20.5	<0.0001
		Beijing	2005-06	11.9	<0.0001
		Anyang	2005-06	17.5	<0.0001
		Anyang	2005-06	17.5	<0.0001
Strampelli/Huixianhong	Stripe rust	Qingshui	2004-05	11.8	<0.0001
		Qingshui	2005-06	22.5	<0.0001
		Qingshui	2006-07	12.8	<0.0001

^a Coefficient of determination. The maximum disease severities scored in the field trials were used to investigate the association between *TaHLRG* and adult-plant reaction to powdery mildew or stripe rust.

Fig. 3. Chromosomal location of *TaHLRG* by amplifying Chinese Spring (CS), N6A-T6B, and N6A-T6D with the marker *THR1* and separating products on a 5% denaturing polyacrylamide gel. M, DNA ladder DL2000 (TaKaRa, Dalian, China); CK, check with no template DNA in the PCR reaction; CS, Chinese Spring; N6A-T6B, nullisomic 6A-tetrasomic 6B; N6A-T6D, nullisomic 6A-tetrasomic 6D.

region. Possibly, *TaHLRG* is involved in cross-talk between different signaling pathways.

To date, three powdery mildew resistance genes (13) and five stripe rust resistance genes (26,27,28) are located on chromosome 6 in wheat. Among them, *Yr36-6BS* was characterized as a high-temperature APR gene that was susceptible to 15 different stripe rust races at the seedling stage and resistant to PST100 and PST101 at the adult plant stage under high temperature. *Pm11*, *Pm14*, *Pm31*, *Yr4a*, *Yr4b*, *Yr20*, and *Yr23* are major genes with specific resistance to different pathotypes. *Yr35-6BS* and *Yr38-6A* are dominant genes with resistance to a range of *P. striiformis* f. sp. *tritici* pathotypes. Based on its location, *TaHLRG* may be a new quantitative trait locus (QTL) associated with resistance to both powdery mildew and stripe rust. In addition, the EST AW448633 showed high homology (93%) to a wheat EST BG262981 on chromosomes 6A and 6B from a blast search of mapped wheat EST in the GrainGenes website, confirming the location of *TaHLRG*.

Previous studies indicated that APR genes against different pathogens are located on the same chromosomal regions and they may be involved in a similar biochemical pathway for APR in wheat (18,35,38,39,41). The β 1din subunit of the 20S proteasome, located in the genomic intervals carrying *Lr34/Yr18/Pm38/Ltn* and *Lr46/Yr29/Pm39/Ltn* (35), was shown to be involved in the establishment of systemic acquired resistance (43). In our study, *TaHLRG* was characterized as a gene involved in race-specific responses. Highly significant associations between *TaHLRG* and stripe rust and powdery mildew resistance were found in three populations across two or three environments, indicating a stable effect associated with *TaHLRG* for resistance to stripe rust and powdery mildew.

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LITERATURE CITED

- Agalou, A., Purwantomo, S., Övernäs, E., Johannesson, H., Zhu, X., Estiati, A., Kam, R., Engström, P., Slamet-Loedin, I., Zhu, Z., Wang, M., Xiong, L., Meijer, A., and Ouwerkerk, P. 2008. A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. *Plant Mol. Biol.* 66:87-103.
- Anderson, J. P., Badruzaufari, E., Schenk, P. M., Mannes, J. M., Desmond, O. J., Ehler, C., Maclean, D. J., Ebert, P. R., and Kazan, K. 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell* 16:3460-3479.
- Bassam, B. J., Caetano-Anolles, G., and Gresshoff, P. M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80-83.
- Bürglin, T. R. 1994. A comprehensive classification of homeobox genes. Pages 25-71 in: *Guidebook to the Homeobox Genes*. D. Duboule, ed. Oxford University Press, Oxford, UK.
- Chan, R. L., Gago, G. M., Palena, C. M., and Gonzalez, D. H. 1998. Homeoboxes in plant development. *Biochim. Biophys. Acta* 1442:1-19.
- Coego, A., Ramirez, V., Gil, M., Flors, V., Mauch-Mani, B., and Vera, P. 2005. An Arabidopsis homeodomain transcription factor, *OVEREXPRESSION OF CATIONIC PEROXIDASE 3*, mediates resistance to infection by necrotrophic pathogens. *Plant Cell* 17:2123-2137.
- Gehring, W. J., Affolter, M., and Bürglin, T. 1994. Homeodomain proteins. *Annu. Rev. Biochem.* 63:487-526.
- Gehring, W. F., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G., and Wüthrich, K. 1994. Homeodomain-DNA recognition. *Cell* 78:211-223.
- Griffey, C. A., and Das, M. K. 1994. Inheritance of adult-plant resistance to powdery mildew in Knox 62 and Massey winter wheats. *Crop Sci.* 34:641-646.
- Gustafson, G. D., and Shaner, G. 1982. The influence of plant age on the expression of slow-mildewing resistance in wheat. *Phytopathology* 72:746-749.
- Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. 2004. Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development* 131:657-668.
- Himmelbach, A., Hoffmann, T., Leube, M., Hohener, B., and Grill, E. 2002. Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. *EMBO J.* 21:3029-3038.
- Huang, X. Q., and Röder, M. S. 2004. Molecular mapping of powdery mildew resistance genes in wheat: A review. *Euphytica* 137:203-223.
- Johnson, R. 1988. Durable resistance to yellow (stripe) rust in wheat and its implications in plant breeding. Pages 63-75 in: *Breeding Strategies for Resistance to the Rusts of Wheat*. N. W. Simmonds and S. Rajaram, eds. CIMMYT Mexico.
- Kang, Z. S., Huang, L. L., and Buchenauer, H. 2002. Ultrastructural changes and localization of lignin and callose in compatible and incompatible interactions between wheat and *Puccinia striiformis*. *J. Plant Dis. Prot.* 109:25-37.
- Kang, Z. S., Wang, Y., Huang, L. L., Wei, G. R., and Zhao, J. 2003. Histology and ultrastructure of incompatible combination between *Puccinia striiformis* and wheat cultivars with low reaction type resistance. *Sci. Agric. Sin.* 2:1102-1113.
- Kuraparthi, V., Chhuneja, P., Dhaliwal, H. S., Kaur, S., Bowden, R. L., and Gill, B. S. 2007. Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. *Theor. Appl. Genet.* 114:1379-1389.
- Liang, S. S., Suenaga, K., He, Z. H., Wang, Z. L., Liu, H. Y., Wang, D. S., Singh, R. P., Sourdile, P., and Xia, X. C. 2006. Quantitative trait loci

- mapping for adult-plant resistance to powdery mildew in bread wheat. *Phytopathology* 96:784-789.
19. Lin, F., and Chen, X. M. 2007. Genetics and molecular mapping of genes for race specific and all-stage resistance and non-specific high-temperature adult-plant resistance to stripe rust in spring wheat cultivar Alpowa. *Theor. Appl. Genet.* 114:1277-1287.
 20. Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K., and Hake, S. 1994. A *knotted 1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* 6:1859-1876.
 21. Liu, S. X., Griffey, C. A., and Saghai-Maroo, M. A. 2001. Identification of molecular markers associated with adult plant resistance to powdery mildew in common wheat cultivar Massey. *Crop Sci.* 41:1268-1275.
 22. Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402-408.
 23. Long, J. A., Moan, E. I., Medford, J. I., and Barton, M. K. 1996. A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature* 379:66-69.
 24. Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J., and Solano, R. 2003. ETHYLENE RESPONSE FACTOR 1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15:165-178.
 25. Mayda, E., Törner, P., Conejero, V., and Vera, P. 1999. A tomato homeobox gene (HD-Zip) is involved in limiting the spread of programmed cell death. *Plant J.* 20:591-600.
 26. McIntosh, R. A., Devos, K. M., Dubcovsky, J., Morris, C. F., Appels, R., and Anderson, O. A. 2005. Catalogue of gene symbols for wheat: 2005 (suppl.) <http://www.wheat.pw.usda.gov/GG2/pubs.shtml>.
 27. McIntosh, R. A., Devos, K. M., Dubcovsky, J., and Rogers, W. J. 2004. Catalogue of gene symbols for wheat: 2004 (suppl.) <http://www.wheat.pw.usda.gov/GG2/pubs.shtml>.
 28. McIntosh, R. A., Devos, K. M., Dubcovsky, J., Rogers, W. J., Morris, C. F., Appels, R., Somers, D. J., and Anderson, O. A. 2007. Catalogue of gene symbols: 2007 (suppl.) In: KOMUGI-Integrated Wheat Science Database. (<http://www.grs.nig.ac.jp/wheat/komugi>).
 29. McIntosh, R. A., Wellings, C. R., and Park, R. F. 1995. Pages 1-200 in: *Wheat Rusts: An Atlas of Resistance Genes*. CSIRO Australia.
 30. McIntosh, R. A., Yamazaki, Y., Devos, K. M., Dubcovsky, J., Rogers, W. J., and Appels, R. 2003. Catalogue of gene symbols for wheat. N. E. Pogna, M. Romano, E. A. Pogna, and G. Galterio, eds. *Proc. 10th Int. Wheat Genet. Symp.* Vol. 4:1-34.
 31. Mondragon-Palomino, M., Meyers, B. C., Michelmore, R. W., and Gaut, B. S. 2002. Patterns of positive selection in the complete NBS-LRR gene family of *Arabidopsis thaliana*. *Genome Res.* 12:1305-1315.
 32. Peterson, R. F., Campbell, A. B., and Hannah, A. E. 1948. A diagrammatic scale for estimating rust intensity of leaves and stems of cereals. *Can. J. Res. Sect. C* 26:496-500.
 33. Ramburan, V. P., Pretorius, Z. A., Louw, J. H., Boyd, L. A., Smith, P. H., Boshoff, W. H. P., and Prins, R. 2004. A genetic analysis of adult plant resistance to stripe rust in the wheat cultivar Kariega. *Theor. Appl. Genet.* 108:1426-1433.
 34. Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G. W., and Fischer, R. L. 1995. The *BEL1* gene encodes a homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium. *Cell* 83:735-742.
 35. Rosewarne, G. M., Singh, R. P., Huerta-Espino, J., William, H. M., Bouchet, S., Cloutier, S., McFadden, H., and Lagudah, E. S. 2006. Leaf tip necrosis, molecular markers and β 1-proteasome subunits associated with the slow rusting resistance genes *Lr46/Yr29*. *Theor. Appl. Genet.* 112:500-508.
 36. Saghai-Maroo, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* 81:8014-8018.
 37. Singh, R. P. 1992. Genetic association of leaf rust resistance gene *Lr34* with adult plant resistance to stripe rust in bread wheat. *Phytopathology* 82:835-838.
 38. Singh, R. P., Nelson, J. C., and Sorrells, M. E. 2000. Mapping *Yr28* and other genes for resistance to stripe rust in wheat. *Crop Sci.* 40:1148-1155.
 39. Spielmeier, W., McIntosh, R. A., Kolmer, J., and Lagudah, E. S. 2005. Powdery mildew resistance and *Lr34/Yr18* genes for durable resistance to leaf and stripe rust cosegregate at a locus on the short arm of chromosome 7D of wheat. *Theor. Appl. Genet.* 111:731-735.
 40. Stubbs, R. W. 1988. Pathogenicity analysis of yellow (stripe) rust of wheat and its significance in a global context. Pages 23-28 in: *Breeding Strategies for Resistance to the Rusts of Wheat*. N. W. Simmonds and S. Rajaram, eds. CIMMYT Mexico.
 41. Suenaga, K., Singh, R. P., Huerta-Espino, J., and William, H. M. 2003. Microsatellite markers for genes *Lr34/Yr18* and other quantitative trait loci for leaf rust and stripe rust resistance in bread wheat. *Phytopathology* 93:881-890.
 42. Sun, Q., Wei, Y., Ni, C., Xie, C., and Yang, T. 2002. Microsatellite marker for yellow rust resistance gene *Yr5* introgressed from spelt wheat. *Plant Breed.* 121:539-541.
 43. Suty, L., Lequeu, J., Lancon, A., Etienne, P., Petitot, A. S., and Blein, J. P. 2003. Preferential induction of 20S proteasome subunits during elicitation of plant defense reactions: Towards the characterization of "plant defense proteasomes". *Int. J. Biochem. Cell B.* 35:637-650.
 44. Xu, M. L., Huaracha, E., and Korban, S. S. 2001. Development of sequence-characterized amplified regions (SCARs) from amplified fragment length polymorphism (AFLP) markers tightly linked to the *Vf* gene in apple. *Genome* 44:63-70.