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Cloning and Characterization of Disease Resistance Protein *RPM1* Genes against Powdery Mildew in Wheat Line N9134

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Powdery mildew (*Blumeria graminis* f. sp. *tritici*) is one of the most devastating wheat diseases. The wheat line N9134 contains *PmAS846* that was transferred to N9134 from wild emmer wheat, and is still one of the most effective resistance genes in China. A full-length wheat *RPM1* gene was obtained by rapid amplification of cDNA ends (RACE) based on the up-regulated probe sequence from differentially expressed transcripts during the N9134 and powdery mildew interaction. The gene was named *TaRPM1*, and the open reading frame (ORF) is 2721 nucleotides and encodes a polypeptide of 907 amino acids with a predicted isoelectric point of 4.86. Phylogenetic analysis revealed that *TaRPM1* was highly homologous on both *Aegilops tauschii* and *Triticum urartu* at both the nucleotide and protein level. Using real-time quantitative PCR, the *TaRPM1* gene expression level in wheat leaves was found to be sharply up-regulated, while the transcript level was lowly induced in the root and stem. Under the powdery mildew treatment, the transcription profile of *TaRPM1* was very strongly expressed at 48 hour post inoculation (hpi), which increased again to 96 hpi and reaching a high level at 120 hpi. Based on sequence similarities and positions, we inferred that the *TaRPM1* gene was on wheat chromosome 3D. These results suggested that *TaRPM1* plays an important role in the mechanism of innate immunity to infection by the powdery mildew pathogen.

Keywords: gene clone, sequence characterization, expression analysis, resistance protein

Introduction

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is one of the most destructive diseases of wheat worldwide. Genetic resistance is the most economical and effective method to control this disease. To date, 69 powdery mildew resistance alleles have been identified and designated at 49 loci. Wheat is a hexaploid crop with a tremendous genome of 16,000 Mb (Appels et al. 2018), which contains three sub-genomes (A, B and C) with a large number of repeat sequences. Thus far, few wheat powdery mildew resistance genes (*Pm21*, *Pm3b*, *Pm3c*, *Pm3f*, and *Pm3g*) have been cloned (Cao et al. 2011; Yahiaoui et al. 2004; Srichumpa et al. 2005; Tommasini et al. 2006).

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RPM1 was one of the first plant resistance genes to be identified from natural variation in *Arabidopsis thaliana* (Grant et al. 1995). Through analysis of conservative sequences, research has shown that resistance (R) genes in plants share conserved regions, including leucine-rich repeat (LRR), nucleotide-binding site (NBS), transmembrane domain, protein kinases and serine/threonine kinase regions (Collins et al. 2001; Swiderski et al. 2009).

Among the wheat R genes, the *Sr35* and *Sr33* genes have an NBS-LRR resistance domain (Periyannan et al. 2013; Saintenac et al. 2013). However, the nucleotide-binding sites and leucine-rich repeat domains, as well as variable amino domains (Gong et al. 2013; McHale et al. 2006), of NBS-LRR genes may have evolved rapidly in bread wheat (Gu et al. 2015). The role of the *RPM1* gene in wheat with respect to resistance to powdery mildew is not fully understood.

The wheat line N9134 contains a powdery mildew resistance gene, *PmAS846*, that was introgressed into common wheat from the wild emmer accession As846 (Xue et al. 2012). *PmAS846* provides a potent resistance that is effective against 21 Chinese *Bgt* isolates with different virulence patterns (Wang et al. 2007), and should be a valuable resource in wheat breeding and research programs. Previously, the gene was physically located on wheat chromosome 5BL bin 0.75–0.76 (Xue et al. 2012). The wheat line N9134 inoculated powdery mildew at 12 h, 24 h, and 48 h was analyzed by gene chip (Illumina). The results showed that thirty probes were differentially expressed, with sequences showing a NBS, LRR, and serine/threonine protein kinases domain.

In the present study, we identified a fragment of the *RPM1* gene (GenBank accession number: AJ606014.1) that was up-regulated by powdery mildew infection. We obtained the full-length sequence of the corresponding wheat gene named *TaRPM1* using the rapid amplification of cDNA ends (RACE), delineating its expression profile in the presence of powdery mildew infection and various stress treatments. The results of these studies indicate that the activation of *TaRPM1* may be a general response to powdery mildew infection.

Materials and Methods

Plant materials and treatments

Winter wheat (*Triticum aestivum* L.) genotype N9134 and powdery mildew race E09 were used in this study for the full-length cloning of the wheat *TaRPM1* gene and its expression analysis. The *Bgt* isolate E09 was maintained on susceptible wheat ‘Shaanyou 225’. The germinated seedlings were grown in sterilized vermiculite and cultured with a cycle of 12 h-artificial light at 22 °C and 12 h dark at 14 °C with 70% relative humidity. At the three-leaf development stage, twenty-four plants of each genotype were inoculated with E09. Talc was used to aid in the spread and adhesion of spores over leaf surfaces. Eight strains of each control flats were treated the same way except for the absence of spores in the talc.

In order to perform tissue-specific expression analyses of *TaRPM1*, intact root, stem, and leaf tissues of 3-week-old wheat seedlings were collected from inoculated plants. All samples in these experiments were collected, quickly frozen in liquid nitrogen and stored at –80 °C prior to RNA extraction. Three independent biological replications were performed for each treatment.

RNA isolation and purification

Total RNA was extracted independently for each individual leaf sample using Trizol reagent according to the manufacturer's instructions (Invitrogen Corporation). The RNA was purified using the RNeasy Kit (Takara) according to the manufacturer's instructions. RNA quality and quantity were assessed using a spectrophotometer and agarose gel electrophoresis. The RNA acceptable purity was indicated by A260:280 ratios of 1.9–2.1 in 10 mM Tris-HCl (pH 7.5).

Rapid amplification of cDNA ends (RACE)

The up-regulated probe Ta.30657.1.S1_at (GenBank accession No. AJ606014.1) was a candidate gene of 574 base pairs (bp) in length, encoding the *Triticum aestivum* partial mRNA for putative NB-ARC protein. To obtain a full-length cDNA of the wheat *TaRPM1* gene, a pair of gene-specific primers (forward primer: 5′-ATGGATGCTCGTCGTCT-GTC-3′, reverse primer: 5′-GGGAGTGACCTGACCCTAGT-3′) was designed based on the AJ606014.1 assembly sequence and synthesized (Shanghai GeneCore BioTechnologies Co., Ltd.).

Sequence and phylogenetic analysis

The cDNA sequence data was analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the CAP3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3), ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/), and PROSITE (https://prosite.expasy.org/) network services. The signal peptide was predicted using TargetIP (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane analysis was performed using TMPRED (https://embnet.vital-it.ch/software/TMPRED_form.html). The alignments of the deduced protein sequences and phylogenetic trees were computed using the MEGA software version 7.0 programs, respectively, employing standard parameters.

Expression analysis of RPM1 gene in different tissues

Use qRT-PCR to detect the specific expression of *TaRPM1* in wheat different tissues, including roots, stems and leaves of wheat new germplasm N9134 after inoculation with powdery mildew E09 at 48 h.

Use the Expression Atlas website (https://www.ebi.ac.uk/gxa/genes/TraesCS3B02G483600?bs=%7B%22triticum%20aestivum%22%3A%5B%22ORGANISM_PART%22%2C%22DEVELOPMENTAL_STAGE%22%2C%22SAMPLING_TIME_POINT%22%5D%7D&ds=%7B%22kingdom%22%3A%5B%22plants%22%5D%7D#baseline) further verified the tissue-specific expression analysis of *RPM1* gene.

Real time-PCR analysis

Expression profile of *TaRPM1* after inoculation with *Bgt* was detected by qRT-PCR analysis with a pair of specific primers Q-*RPM1*-FP and Q-*RPM1*-RP (*RPM1*-FP: GGGAGGACACGAAAAGAGCAA; *RPM1*-RP: GGCTTCAAGGATGACTGGGAT). For qRT-PCR, SYBR® Premix DimerEraser™ (TaKaRa) was used according to the manufacturer's protocols and analyzed using an ABI 7300 RT-PCR system (Applied Biosystems). The qRT-PCR amplification mixture (25 μL) contained 2 μL 20 × first-strands cDNA, 12.5 μL of SYBR Green PCR Master Mix, and 2 μL of forward and reverse primers (10 μM). The wheat tubulin gene (GenBank accession No. Y049040) was used as a control for normalization. Three independent experiments were performed, including three non-template controls. Each 25 μl reaction was denatured at 95 °C with an initial denaturation step (95 °C/2 min), followed by 40 cycles of 95 °C/10 s, 60 °C/20 s, and 72 °C/20 s. A melting curve analysis was performed over the range 80–95 °C at 0.5 °C intervals. Dissociation curves were generated for each reaction to ensure specific amplification. The transcript concentration was calculated as $2^{-\Delta\Delta Ct}$. To avoid errors caused by operational factors, and each data point was expressed as the average ± SD of three independent biological replicates.

Chromosome localization prediction of TaRPM1

The gene sequence of *TaRPM1* was compared with the genome of Chinese spring wheat (http://plants.ensembl.org/Triticum_aestivum/Info/Index?tdsourcetag=s_pcqq_aiomsg). The structure of *TaRPM1* analyzed using GSDS 2.0 (Gene Structure Display Server) version for intron and exon prediction. A highly consistent sequence was screened and the target gene was mapped to the wheat chromosome (Qiao et al. 2016).

Results

Isolation and sequence analysis of TaRPM1 *cDNA*

The probe Ta.30657.1.S1_at (AJ606014.1) was an infection-induced up-regulated gene fragment identified in a previous experiment using gene chips (Xue 2012). Following 3′ and 5′ RACE analyses, two cDNA fragments of approximately 1246 (Fig. 2) and 944 bp (Fig. 1), respectively, were detected on 1% agarose gel. The results showed the full-length sequence of wheat *RPM1*, which consisted of a 3034 nucleotide and cDNA containing an

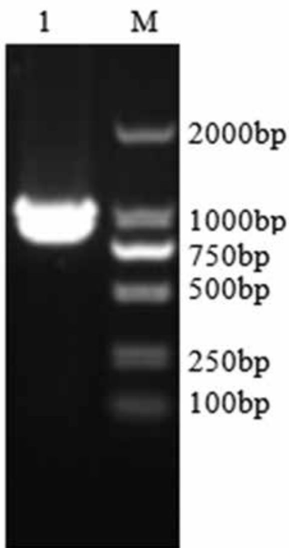

Figure 1. 5'RACE amplification results 1: Amplified product of 5′RACE PCR M: DL2000 Marker

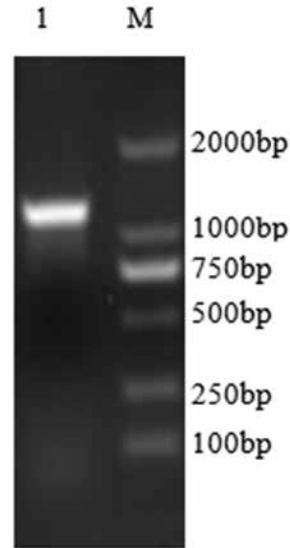

Figure 2. 3'RACE amplification results 1: Amplified product of 3′RACE PCR M: DL2000 Marker

ORF of 2721 nucleotides (Fig. S1*). The predicted protein encoded by the *TaRPM1* gene consisted of 907 amino acids (Fig. S2) with a predicted isoelectric point of 4.86.

Characterization of the primary structure of the TaRPM1 *homolog*

PROSITEScan analysis suggested that the *TaRPM1* protein consists of five conserved domains: RX-CC_like, AAA_22, LRR_4, NB-ARC, and LRR_8. The NB-ARC domain was located at positions 175–454 aa, and the LRR subdomain of LRR_4 and LRR_8 was located at 594–633 aa and 594–645 aa, respectively.

The conserved region of the *TaRPM1* homolog was compared with *RPM1* regions from other plant species. The nucleotide sequences were highly conserved in *RPM1* genes, with sequence identities ranging from 75 to 99%, including *Aegilops tauschii* at 99%, *Hordeum vulgare* at 86%, and *Oryza sativa* at 75%. In addition, the predicted protein identities were compared with seven plant species: *Aegilops tauschii* at 98%, *Triticum urartu* at 95%, *Hordeum vulgare* at 79%, *Sorghum bicolor* at 70%, *Oryza sativa Indica Group* at 70%, *Brachypodium distachyon* at 68% and *Zea mays* at 67%.

The overall sequence alignment indicated that *RPM1* is relatively conserved among the seven plant species. Seven available *RPM1* sequences of various plant species were downloaded from GenBank and then employed to establish a neighbor-joining phylogenetic tree using MEGA software version 7.0 with 1000 bootstraps (Fig. 3). Results showed that the plant *RPM1s* can be separated into four main divisions. No transmembrane or

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

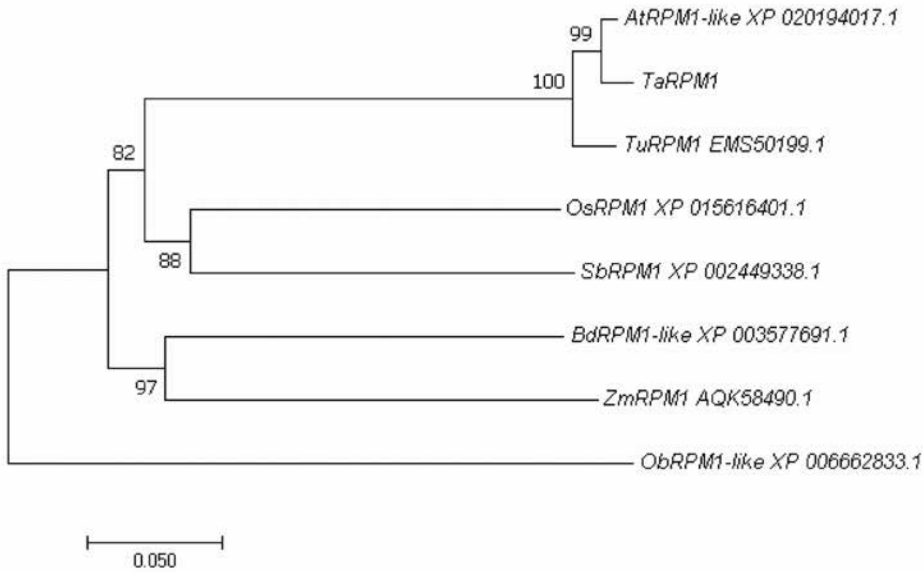

Figure 3. Neighbor-joining phylogenetic tree of whole RPM1 protein sequences from different species using MEGA 7.0 software. Branches are labeled with plant species names (At – *Aegilops tauschii*; Sb – *Sorghum bicolor*; Tu – *Triticum urartu*; Bd – *Brachypodium distachyon*; Os – *Oryza sativa*; Zm – *Zea mays*; Ob – *Oryza brachyantha*), followed by the gene name and GenBank accession number

signal peptide were confirmed by TMpred and Signal IP 3.0 analysis. Through location prediction using PSORT, TaRPM1 was found to be outside of cells with a probability of 67.1%.

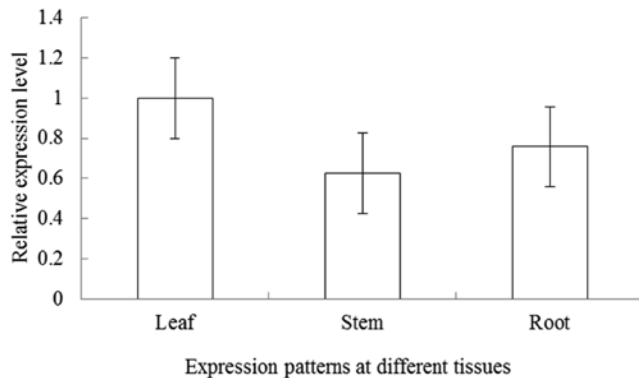

Figure 4. qRT-PCR analyses of the *TaRPM1* expression in wheat leaf, stem, and root tissues, and expression patterns of *TaRPM1* in wheat leaves after inoculation with E09. Data were normalized to wheat tubulin expression level. The mean expression value was calculated with three independent replicates. Vertical bars represent the standard deviation

Spatial expression patterns of TaRPM1

We next examined the transcription patterns of *TaRPM1* in tissues, including root, stem and seedling leaves, in response to infection with powdery mildew E09 using qRT-PCR analyses (Fig. 4). The transcription of *TaRPM1* in wheat leaves was higher than in both stem and root samples. Followed by the root, the stem samples had the lowest expression, which was approximately half of the expression level than that of the leaf samples. The expression of *TaRPM1* in wheat roots, stems, and leaves was not significantly statistically different. We also analyzed the spatial tissues expression of *TaRPM1* using Expression Atlas website. The result showed the expression level of *RPM1* in Arabidopsis leaves, roots were expression. There was also no significant difference in expression levels.

Expression changes of TaRPM1 *in response to Bgt infection*

Real-time PCR results showed that *TaRPM1* expression increased between 0 to 48 hpi in an incompatible interaction (Fig. 5). The expression of *TaRPM1* was up-regulated as early as 6 hpi. The highest level was reached at 48 hpi, showing a transcript level was approximately twenty-five-fold higher than the control. The level then slightly decreased to 72 hpi and increased again to 96 hpi and stayed at a high level until 120 hpi.

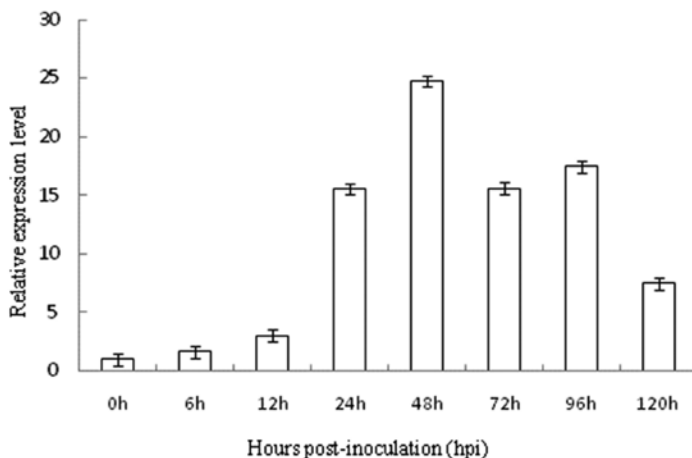

Figure 5. Expression patterns of the *RPM1* assessed by real-time PCR. Total RNA was isolated from young leaves of N9134 induced by powdery mildew infection at 0, 6, 12, 24, 48, 72, 96, and 120 hpi. The a-tubulin genes were used as internal controls. Y-axis indicats the fold changes of miRNA expression in the stress samples compared with the mock samples. Data were averages of three independent biological experiments. Standard deviation (SD) is shown as an error bar

Figure 6. Structural prediction of *TaRPM1* using GSDS 2.0. The blue portion is the upstream or downstream sequence and the yellow is the coding sequence

In silico *mapping of* TaRPM1

The chromosome location of *TaRPM1* was determined using in silico mapping analysis. By comparing the *TaRPM1* gene with the latest sequencing data from Chinese Spring, the sequence similarity with 3A, 3B and 3D was 93.60%, 98.16%, and 98.73%, respectively. We also used the GSDS 2.0 for structural prediction of *TaRPM1* that has a CDS domain (Fig. 6). Based on an analysis of sequence similarities, we inferred that the *TaRPM1* gene is likely to be located on the wheat 3D chromosome.

Discussion

In this study, we described the isolation and characterization of the *TaRPM1* gene from the wheat germplasm N9134 after inoculation with powdery mildew E09. The *TaRPM1* gene was located on chromosome 3D. In addition, we also investigated the expression pattern of the *TaRPM1* in response to treatments with powdery mildew.

Most of the resistance proteins encoded by NBS-LRR disease-resistant genes are associated with plant disease resistance processes (Belkhadir et al. 2004). A resistance gene of *TaRPM1* was cloned which, contained a complete ORF, consisting of 2721 nucleotides. Compared with the Chinese Spring sequence at 3D chromosome published by Appels et al. (2018), the sequence identity was 98.73%. The differences in the sequence were mainly due to mutations at individual sites. The main cause of this phenomenon may be the difference in research materials. It may also be caused by the evolution of R genes, which is driven by selection on allelic variation created by mutation and reassortment (Hulbert et al. 2001). Sequence structure analysis showed that the *TaRPM1* gene had a conserved region typical of a serine/threonine kinase structure. Studies have shown that NBS-LRR genes have evolved rapidly in bread wheat (Gu et al. 2015). However, our phylogenetic analysis showed that the *TaRPM1* gene was highly homologous in the plant at deduced amino acid levels. The results further suggest that the *TaRPM1* gene evolved slowly in wheat.

The domain architecture of NBS-LRR was consistent with a role in pathogen recognition and defense response signaling (Amelinetorregrosa et al. 2008). In this study, the *TaRPM1* gene did not contain a transmembrane structure and a signal peptide, suggesting that *TaRPM1* is made of soluble cytoplasmic proteins. This finding is consistent with previous research results (Boyes et al. 1998).

Most R genes are constitutively expressed in the plant (Thurau et al. 2003). *TaRPM1* was found to show no tissue-specific expression and to be regulated by powdery mildew, *TaRPM1* gene expression was observed in roots, stems and leaves, respectively. Mean-

while, the expression level of *RPM1* was also not significantly different in the leaves and roots of Arabidopsis using the Expression Atlas website. Some R genes were required for maintaining or enhancing the induction of signal transduction pathways, leading to resistance (Li et al. 2011). The *psoRPM1* gene is capable of helping set up a resistance mechanism against *M. incognita* infection in tobacco (Li et al. 2013). The expression of the *psoRPM1* gene was highest 12 hours after inoculation with root-knot nematodes (Li et al. 2011). In this study, the RT-PCR results demonstrated that *TaRPM1* expression was increased in N9134 after powdery mildew infection. The expression level of *TaRPM1* was the highest at 48 hpi. Compared with N9134 results at 6 hpi, the expression level of *TaRPM1* increased 8 times. The results of the study were inconsistent with those of Jiang et al. (2015). However, the results indicated that the expression level of the *RPM1* gene has a high expression level in disease resistant materials.

With the continuous improvement of wheat genome sequencing data, the silico mapping method has the advantages of simplicity and quickness compared with the traditional Chinese Spring deletion lines positioning method. In the wheat genome, 2406 sequences of NBS domain were isolated (Qiao et al. 2016), which is much higher than other monocotyledonous crops.

The sequences having the NBS sequence structure on the 3A, 3B, and 3D chromosomes of wheat were 71, 133, and 71, respectively (Qiao et al. 2016). In our past research, the *PmAS846* gene from wheat line N9134 was located on chromosome 5BL (Xue et al. 2012). Line N9134 is highly resistant to immunity against powdery mildew in Guanzhong, Shaanxi Province (Ji et al. 1999). In this study, however, *TaRPM1* was located at chromosome 3D, and is apparently different than *PmAS846*. Due to *TaRPM1* was highly expressed in wheat line N9134 after the powdery mildew inoculated. Therefore, we hypothesize that *TaRPM1* is involved in *PmAS846*-mediated resistance to powdery mildew.

In summary, the *TaRPM1* gene was isolated from the wheat germplasm N9134 and infected by powdery mildew. *TaRPM1* was expressed in root, stem and leave of wheat, and the difference in expression was not statistically significant. *TaRPM1* had a strong response to powdery mildew at 48 hpi. In addition, *TaRPM1* is located on the 3D arm of the wheat chromosome when using silico mapping. We found that *TaRPM1* plays a significant role in response to powdery mildew infection.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at https://akademiai.com/loi/0806

Electronic Supplementary *Figure S1*. *TaRPM1* gene sequence

Electronic Supplementary *Figure S2*. Protein sequence