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RESEARCH ARTICLE

Functional analysis of a wheat pleiotropic drug resistance gene involved in Fusarium head blight resistance

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Abstract

The pleiotropic drug resistance (PDR) sub-family of adenosine triphosphate (ATP)-binding cassette (ABC) transporter had been reported to participate in diverse biological processes of plant. In this study, we cloned three novel *PDR* genes in Fusarium head blight (FHB) resistant wheat cultivar Ning 7840, which were located on wheat chromosomes 6A, 6B and 6D. In phylogeny, these genes were members of cluster I together with *AePDR7* and *BdPDR7*. Subcellular localization analysis showed that *TaPDR7* was expressed on the plasmalemma. The quantitative real time PCR (RT-PCR) analysis showed that this gene and its probable orthologues in chromosomes 6B and 6D were both up-regulated sharply at 48 h after infected by *Fusarium graminearum* and trichothecene deoxynivalenol (DON) in spike. When knocking down the transcripts of all *TaPDR7* members by barely stripe mosaic virus-induced gene silencing (BSMV-VIGS) system, it could promote the *F. graminearum* hyphae growth and made larger pathogen inoculation points in Ning 7840, which suggested that *TaPDR7* might play an important role in response to *F. graminearum*. Although salicylic acid (SA), methyl jasmonate (MeJA) and abscisic acid (ABA) had been reported to possibly regulate wheat FHB resistance, here, we found that the three members of *TaPDR7* were negatively regulated by these three hormones but positively regulated by indoleacetic acid (IAA).

Keywords: PDR, FHB, VIGS

1. Introduction

Pleiotropic drug resistance (PDR) family is one of the three sub-families of adenosine triphosphate (ATP)-binding cassette (ABC)-transporters found only in fungi and plants (Higgins 2001). In general, it was formed by two repetitive unites of nucleotide binding domain (NBD) and trans-membrane motif domain (TMD), in which NBD was a cytosolic domain involved in ATP-binding and hydrolysis and TMD was a hydrophobic trans-membrane domain involved in translocation and probably binding the substrate (Holland and Blight M A 1999; Higgins 2001). The first plant *PDR* gene *SpTUR2*

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was found to associate with abiotic stress resistance in *Spirodella polyrhiza*. In the past decades, many *PDR* genes had been identified with divergent functional roles. Till now, a total of 15 gene members in *Arabidopsis* and 23 in *Oryza sativa* had been identified, which were grouped into five clusters (Sánchez *et al*. 2001; Martinoia *et al*. 2002; Van Den Brûle *et al*. 2002; Jasinski *et al*. 2003; Garcia *et al*. 2004). Most of the reported *PDR* genes, especially in cluster I, were found to be involved in abiotic and biotic stresses tolerance such as *PgPDR1*, *OsPDR9*, *SpTUR2*, *AtPDR12*, etc. (Van Den Brûle *et al*. 2002; Campbell *et al*. 2003; Moons 2003; Lee *et al*. 2005; Zhang *et al*. 2013). Under the abiotic stresses, *PgPDR1* was up-regulated by salicylic acid (SA) or chilling, down-regulated by abscisic acid (ABA) and exhibited different expression patterns at transcript and protein levels induced by methyl jasmonate (MeJA). However, the function of *PgPDR1* is still not well established now (Zhang *et al*. 2013). Multiple responses were found for *OsPDR9*, the expression of which could be induced by polyethylene glycol, salt, heavy metals of Cd and Zn, MeJA, and ABA. Besides, its expression could be induced both by the antioxidant dithiothreitol (DTT) and oxidant hydrogen peroxide suggesting that *OsPDR9* might response to redox perturbations and oxidative conditions as well (Moons 2003). The gene of SpTUR2 was found to possibly detoxify fungicide under the regulation of ABA, over-expressing which in *Arabidopsis* resulted in improved tolerance against sclareol and no visible accumulation of anthocyanins. This is consistent with the symptom when pretreating with ABA before the addition of sclareol that the stress response was almost totally abrogated (Smart *et al*. 1996; Van Den Brûle *et al*. 2002). Furthermore, the expression of *AtPDR12* was induced by SA, JA and ethylene (ET), the knockout plants (*atpdr12*) of which grew less well and had higher Pb (II) contents than those of wild-type plants when cultivated in Pb (II)-containing medium. In contrast, over-expressions of *AtPDR12* plants were more resistant to Pb (II) and had lower Pb (II) contents than wild types, which was proposed that *AtPDR12* contributed to Pb (II) resistance in *Arabidopsis* (Lee *et al*. 2005). Moreover, *AtPDR12* showed strong response to fungi, such as *Alternaria brassicicola*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, and *Pseudomonas syringae* (Campbell *et al*. 2003). However, there were rare reports on wheat *PDR* gene and *F. graminearum* interactions.

Fusarium head blight (FHB) was mainly caused by a hemi-biotropic pathogen *Fusarium graminearum*. It is generally thought that anther is the most susceptible part of wheat for FHB infection. At early stage, the conidium sprouted in the 6–12 h after inoculation and hyphae was generated in the germ tube (Pritsch *et al*. 2000). At 24–36 h, after penetrating the cuticle, glume, lemma, palea, and ovary were all filled with hyphae. 48 h after inoculation, the subcuticular growth of hyphae could be seen in the inoculated spikelet. From the histological study on the growth of *F. graminearum* hyphae, along with inoculation pollen grain, lemma, palea, xylem, and phloem were invaded gradually (Ribichich *et al*. 2000; Xu and Nicholson 2009). In the process of *F. graminearum* infection, wheat could accumulate deoxynivalenol (DON), which was a serious threat to the health of human and animals (Bai and Shaner 2004). Currently, the resistance mechanism of wheat is not clear. Several transcriptomic studies using near-isogenic lines (NIL) have revealed the importance of JA in FHB defense (Xiao *et al*. 2013). A combination of transcriptomic and proteomic study demonstrated that SA, ET and JA might play important roles in the defense of *F. graminearum* infection (Li and Yen 2008; Ding *et al*. 2011; Xiao *et al*. 2013). However, only limited genes had been reported to be involved in resistance to FHB so far. After silencing a JA-dependent signaling gene *TaJRLL1*, the infection of *F. graminearum* was more serious than negative control in detached leaves (Xiang *et al*. 2011). Furthermore, *AtPGIP* which translated polygalacturonase-inhibiting proteins to restrict pectin degradation during fungal infection could restrict the development of FHB symptoms when overexpressing in wheat (Ferrari *et al*. 2011). *TaLTP5*, a lipid transfer protein gene, could evaluate the ability to suppress disease development in transgenic wheat (Zhu *et al*. 2012). Recently, *AtNPR1*, a key plant defence regulator in *Arabidopsis* SA pathway, was reported to effectively suppress FHB symptom expanding to flanking spikelet after over-expressing it in wheat (Makandar *et al*. 2006). This is relatively amazing as more JA response was found at late stage of *F. graminearum* infection while rare SA responsive genes were observed at the very early stage of infection (Ding *et al*. 2011).

In this study, we had cloned a *PDR* gene from wheat, the transcripts of which was up-regulated by *F. graminearum*. For further functional characterization, the FHB resistance of wheat was investigated when transiently knocking down the transcripts of this gene by barely stripe mosaic virus induced gene silencing (BMSV-VIGS).

2. Materials and methods

2.1. Plant materials

Wheat cultivar Ning 7840 was used in this study for gene cloning and FHB infection. The seedlings of the wheat were grown in chamber under 20–24°C, 16 h light/8 h dark. Spikes of wheat were treated with *F. graminearum* on anthesis as described previously (Kong *et al*. 2005, Makandar *et al*. 2006). *Nicotiana benthamiana* plants were grown in

chamber at 20–25°C, 14 h light/10 h dark. Genomic DNA was extracted from seedling leaves using Genomic DNA Extraction Kit (Tiangen, Beijing, China) as described by Zhang *et al*. (2011). Total RNA from spikes with different treatments and seedling leaves were extracted using the Trizol regeant (TransGene, Beijing). The first strand-cDNA was synthesized using TransScipt First-Strand cDNA Synthesis Supermix (TransGen, Beijing).

2.2. Full-length cDNA amplification of *TaPDR7*

Primers were designed according to the wheat unigene Ta.53363 (JV864962.1) which was searched from National Center for Biotechnology Information (NCBI), and was 2628 bp in length. Partial fragment of *TaPDR7* was amplified using Phusion *Taq* (Themofisher, USA). The fragment was cloned to pEASY-blunt vector (TransGen, Beijing) for sequencing. According to the partial sequence of *TaPDR7,* primers 5´GSP-1 and 5´GSP-2 (Table 1) were designed and synthesized in Sangon Biotech (Shanghai). 5´-RACE (rapid amplification of cDNA ends) was performed using SMARTer RACE cDNA Amplification Kit (Clontech, Japan). As for the nest PCR, both inner and outer 5´-RACE reactions were performed as follows: predenaturation at 98°C for 2 min; 38 cycles of amplification (98°C for 10 s, 65°C for 20 s and 72°C for 2 min) and then 10 min at 72°C. The inner 5´-RACE PCR products were separated on 1% agarose gel and the band was excised, purified and cloned into pEASY-blunt vector for sequencing.

3´-RACE was performed using the SMARTer RACE cDNA Amplification Kit. 3´GSP-1 and 3´GSP-2 (Table 1) were also designed and synthesized according to the partial fragment of *TaPDR7*. The first round of PCR was performed with primer 3´GSP-1 and the universal primer (Table 1). PCR products were diluted 100-fold and used as the template of the nest PCR with primer 3´GSP-2. Both outer and inner PCRs were performed as follows: predenaturation at 98°C for 2 min; 38 cycles of amplification (98°C for 10 s, 65°C for 20 s and 72°C for 2 min) and then 10 min at 72°C. The inner 3´-RACE PCR products were separated on a 1% agarose gel and the band was excised, purified and cloned into pEASY-blunt vector for sequencing.

For amplification of the full-length cDNA, primers of *TaPDR7* ORF were designed following the reverse transcript polymerase chain reaction (RT-PCR) procedure: predenaturation at 98°C for 2 min; 38 cycles of amplification (98°C for 10 s, 58°C for 20 s and 72°C for 4 min) and then 10 min at 72°C. The purified PCR product was cloned into pEASY-

Table 1 Primers used in this study

Primer name	Description	Sequence[1)]
Unigene53363	Sense	5´-TGATCAGCCAGATGGCATCG-3´
Unigene53363	Antisense	5´-TAGCTATGGCGATCTGAAGCTG-3´
5´GSP-1	Antisense	5´-GTCTGCAACAACCATATCCCTTCC-3´
5´GSP-2	Antisense	5´-CATACATCAGAGGGGAGCACCAGTAG-3´
3´GSP-1	Sense	5´-ATGGCTGTCGGTGTTGTAGGATTC-3´
3´GSP-2	Sense	5´-AAACAGCTCAGATCGCCAAGCTC-3´
TaPDR7 ORF	Sense	5´-ATGGGGAGCTCGGCGGGGG-3´
TaPDR7 ORF	Antisense	5´-AGCCTCTCTATCTTCTTTGG-3´
TaPDR7 RT A	Sense	5´-CTTCCCAAAACAGTATGCA-3´
TaPDR7 RT A	Antisense	5´-GTCCTGCCTCTTGCCGAT-3´
TaPDR7 RT B	Sense	5´-CAACTTTGCCGACGTATACAC-3´
TaPDR7 RT B	Antisense	5´-CTTCCACAGGCAAGCAACA-3´
TaPDR7 RT D	Sense	5´-CAGGAAGATGCCTTGGGTC-3´
TaPDR7 RT D	Antisense	5´-CTTCCACAGGCAAGCAACA-3´
TaPDR7 VIGS	Sense	5´-AAGGAAGTTTAACTATTGTCGACGTCGAG-3´
TaPDR7 VIGS	Antisense	5´-AACCACCACCACCGTCTAAGGAAACCGGCAAAG-3´
TaPDR7 semi RT	Sense	5´-TCCATCATACACTGCAACTAG-3´
TaPDR7 semi RT	Antisense	5´-GTGGGATCTCAATGAAAACC-3´
BS-α	Sense	5´-AATATGCCGTTTCATCCTCA-3´
BS-α	Antisense	5´-AGTTGTCGCGATGTATTTGC-3´
BS-β	Sense	5´-GAGGACACTACAACGAGGAT-3´
BS-β	Antisense	5´-GAACCAACTGTGGTCTTCAT-3´
BS-10	Sense	5´-GGTGCTTGATGCTTTGGATAAGG-3´
BS-32	Antisense	5´-TGGTCTTCCCTTGGGGGAC-3´
18srRNA	Sense	5´-GTGACGGGTGACGGAGAATT-3´
18srRNA	Antisense	5´-GACACTAATGCGCCCGGTAT-3´

[1)] Sequences underlined represented the adaptor of VIGS vector.

blunt vector for sequencing.

2.3. *In silico* analysis

Homologue genes of *TaPDR7* were searched in the *Brachypodium distachyon* genome sequences in ensemble (http://plants.ensembl.org/Brachypodium_distachyon/Info/Index), *Triticum aestivum* genome project (http://mips.helmholtz-muenchen.de/plant/wheat/uk454survey/search-jsp/blast.jsp) and Triticeae full-length coding sequence (CDS) database (http://trifldb.psc.riken.jp/index.pl) using BLASTX. Weblab (http://weblab.cbi.pku.edu.cn/) was used to calculate molecular weight and isoelectric point of the deduced protein. Multiple sequence alignments and phylogenetic analysis were carried out using ClustalX. Conserved domains were analyzed by MEME (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). The phylogenetic tree was constructed using neighbour-joining method by MEGA5 (Tamura *et al*. 2011).

2.4. Subcellular localization of *TaPDR7*

The coding region of *TaPDR7* without a stop codon was amplified and cloned into the pBIN35S:EGFP vector, which was then transformed into the *Agrobacterium EAH105* using a freezethaw procedure (Höfgen and Willmitzer 1988). The resultant *Agrobacterium* culture was then resuspended in an infiltration medium (10 mmol L^{-1} 4-morpholineethanesulfonic acid hydrate (MES), pH 5.6, 10 mmol L^{-1} $MgCl_2$, and 200 mmol L^{-1} acetosyringone) to an optical density of 0.6 OD at 600 nm, and injected into 4-wk-old *N*. *benthamiana* leaves. The pBIN35S:EGFP vector was also transformed into *Agrobacterium*, which was used as a negative control. Plants grown in an incubator for 3 days post inoculation (DPI) and then were used for microscopic observation. Furthermore, pBIN35S:TaPDR7:EGFP and the negative control pBIN35S:EGFP were transformed into the onion (*Allium cepa*) epidermis using a Bio-Rad PDS-1000/He Biolistic Particle Delivery System (Das *et al*. 2009). The onion pieces grown on MS agar (4.3 g Murashige & Skoog salts, 10 g sucrose, 8 g agar per litre; pH 5.7) plates were incubated at 28°C for 8–12 h. The results were obtained by the Zeiss LSM 510 confocal laser microscope (Carl Zeiss Vision, Oberkochen, Germany) using excitation with a 488-nm laser, coupled with a 530-nm band pass filter.

2.5. Quantitative real time PCR analysis of *TaPDR7*

Quantitative RT-PCR analysis was performed using a Roche LC480 (Roche Diagnostics, Penzberg, Germany). Each reaction was performed in a total volume of 20 μL containing 10 μL 2.5× Real Master Mix (SYBR Green)(Tiangen, Germany) solution, 2 μL 40 ng μL^{-1} cDNA, 0.4 μL of each primer (10 μmol L^{-1}), 7.2 μL sterilized H_2O. Gene specific primers used for quantitative RT-PCR was shown in Table 1. The quantitative RT-PCR conditions were as follows: predenaturation at 95°C for 2 min; 40 cycles of amplification (95°C for 2 min, 59°C for 18 s and 72°C for 12 s) and then a final round (95°C for 5 s, 65°C for 1 min, 97°C for 5 s, and 50°C for cooling) to generate melting curve. All of the amplified DNA fragments were sent for sequencing. Quantitative RT-PCR primers were used to amplify nullitetrasomics lines of Chinese Spring to make sure their specificities. The expression level was calculated by the comparative cycle threshold (C_T) method with the wheat 18S rRNA gene as the endogenous reference for normalization. The relative expression of *TaPDR7* was calculated with its transcript level in comparison to that in the mock controls across time point. Error bars represent the variations among three replications (Kong *et al*. 2007).

2.6. Virus induced gene silencing of *TaPDR7*

BSMV-VIGS was performed as described previously (Cheng *et al*. 2011; Hou *et al*. 2015). The fragment of *TaPDR7* was amplified and cloned in vector of pCass-γ. Then, each of the BSMV α, β and γ plasmid was independently transformed into *A*. *tumefaciens* strain EHA105, which was used for inoculation medium preparation by culturing at 28°C, 200 r min^{-1} with proper antibiotics. The obtained bacterial cells were pelleted at 2 200 r min^{-1} for 10 min, resuspended in infiltration buffer (10 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} 2-N-morpholinoethanesulfonic acid (MES), pH 5.2, and 0.1 mmol L^{-1} acetosyringone) to 0.7 OD_{600} and incubated at room temperature for 3–5 h with equal amounts of cells each harboring α, β and γ. Then the mix was infiltrated into leaves of *N*. *benthamiana* with a 1-mL needleless syringe. After maintenance in a growth chamber for 5–12 d, the infiltrated leaves were harvested, grounded in phosphate buffer saline (PBS) (pH 7.2) containing 1% celite, and the sap was mechanically inoculated onto the 2-leaf stage of wheat. The success of virus inoculation was verified by semi quantitative RT-PCR. Phytoene desaturase (*PDS*) which was a key gene involved in the carotenoids synthetic was used to verify the feasibility of BSMV mediated gene silencing in wheat, a mottled photo-bleaching phenotype could be detached after silencing of it.

2.7. *F. graminearum* culture and pathogen inoculation assays

The *in vitro F*. *graminearum* infection assay on wheat was modified by the method as described previously (Chen *et al*. 2009; Xiang *et al*. 2011; Hou *et al*. 2015). Briefly, 5-cm leaf

sections were cut from the central part of the third wheat leaf and then placed on the 1% water-ager medium. A conidial suspension of *F. graminearum* (5 μL of 1×10^6 conidia mL^{-1}) was applied to the fresh wound on the adaxial surface. After inoculation, the plates were sealed and placed in a growth chamber at 18–22°C, 14 h light/10 h dark. Conidial spores were counted 4 DPI. To investigate cell death, trypan blue staining was carried out by vacuum pumping leaf tissues for 3 min in solution containing phenol, lactic acid (85%), glycerol, distilled water, and 1.5 mg mL^{-1} trypan blue (1:1:1:1). All the above reagents were prepared immediately before use. The stained leaves were boiled for 5 min, and then sit for 6–8 h. After that, the tissues were de-colored overnight in a solution of 2.5 mg mL^{-1} chloral hydrate. All samples were examined with an Olympus BX50 (Olympus, Japan) light microscope.

3. Results and discussion

3.1. Isolation and sequence analysis of *TaPDR7*

Initially, a 2 771-bp cDNA was amplified by RACE in *F. graminearum* treated wheat samples using primers of 5´GSP-1 and 3´GSP-1 (Table 1). To obtain the full length sequence, we designed new primers 5´GSP-2 and 3´GSP -2 (Table 1) for a second RACE. Finally, the full-length cDNA sequence of *TaPDR7* was identified, with the mRNA of which as 4 708 bp in length, the ORF as 4 329 bp, 3´UTR as 221 bp, and 5´UTR as 158 bp. Then we blasted this sequence against the Ensembl Plants database (http://plants.ensembl.org/index.html) and confirmed it as a sequence on chromosome 6A. Upon the sequence information of gene alleles on 6B and 6D, we finally cloned the full-length cDNA sequence of *TaPDR7*B (ORF as 4 347 bp) and *TaPDR7*D (ORF as 4 350 bp) respectively. Within the *TaPDR7*, ABC signature could be identified at position 996 (LSTEQRKRLTIA), Walker A motif was identified at position 889 (GVSGAGK) and Walker B motif was identified at position 1 016 (IIFMD) (Fig. 1).

3.2. Phylogenetic analysis of *TaPDR7*

To further analyze the phylogeny of *TaPDR7*, this gene was blasted against the current available databases (materials and methods). Previously, *PDR* gene family was grouped into five clusters and *TaPDR7* fell in cluster I (Fig. 2-A) (Sánchez *et al*. 2001). The three copies of *TaPDR7* together with their homologue genes of *AePDR7*, *BdPDR7* and *OsPDR7* in *Poaceae* fell in the same branch in clade I (Fig. 2-B). In order to identify the chromosome distributions of these gene copies, specific primers were designed to amplify the corresponding gene fragment using Chinese Spring origin nullitetrasomic lines. The Fig. 3 showed that *TaPDR7* was placed on chromosome 6A, while the other two copies were identified in 6B and 6D. There is a major QTL associated with the resistance to Fusarium head blight on chromosome 6B in some resistant wheats (Cuthbert *et al*. 2007). Two flanking simple-sequence repeats (SSR) markers (GWM133 and WMC397) of *Fhb2* were blasted against the database of International Wheat Genome Sequencing Consortium

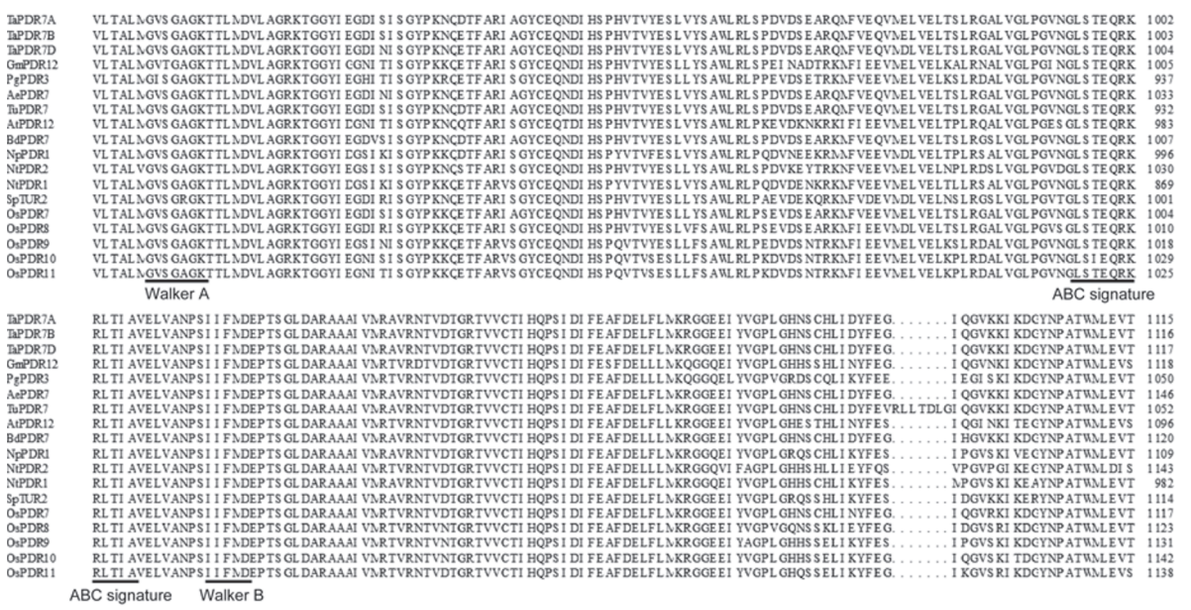

Fig. 1 Alignment of amino acid sequence of *TaPDR7*. The pleiotropic drug resistance (PDR) domain of putative genes in clade I was aligned with Clustal X program. ABC, adenosine triphosphate (ATP)-binding cassette.

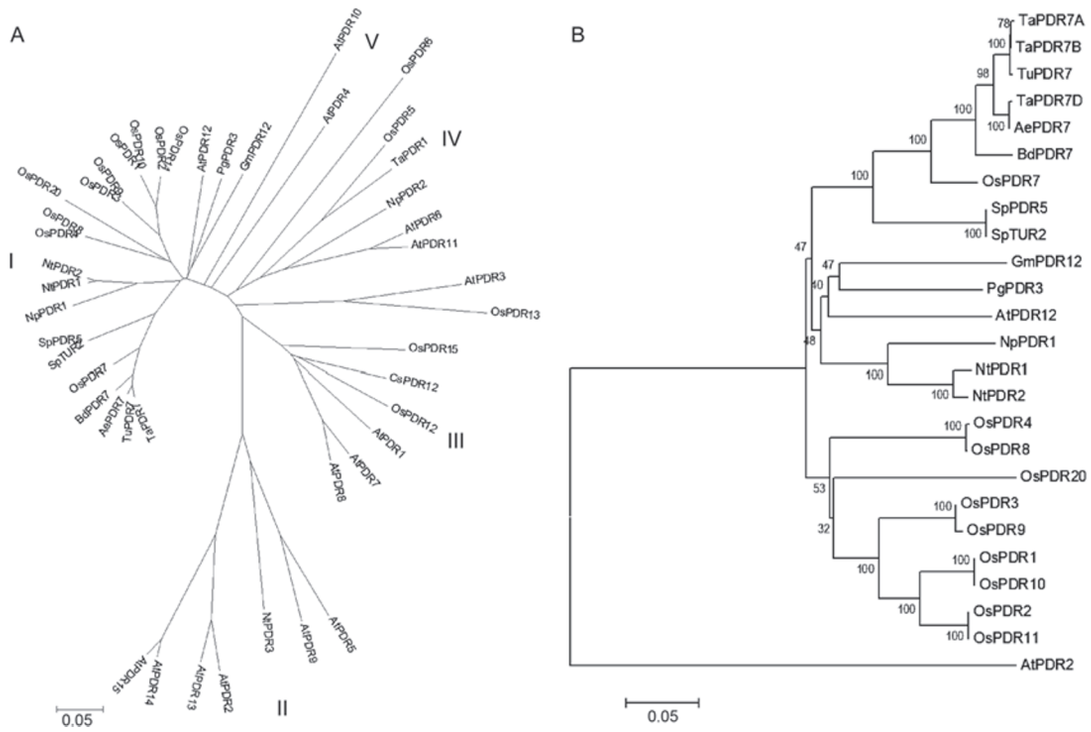

Fig. 2 Phylogenetic tree of *TaPDR7*. A, phylogenetic tree of *TaPDR7* in the PDR sub-family. B, phylogenetic tree of three *TaPDR7* copies in the clade I. Evolutional relationships were inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 1 000 replicates was taken into represent the evolutionary history of the taxa. In the scale, the bar 0.05 was equal to 5% sequence divergence.

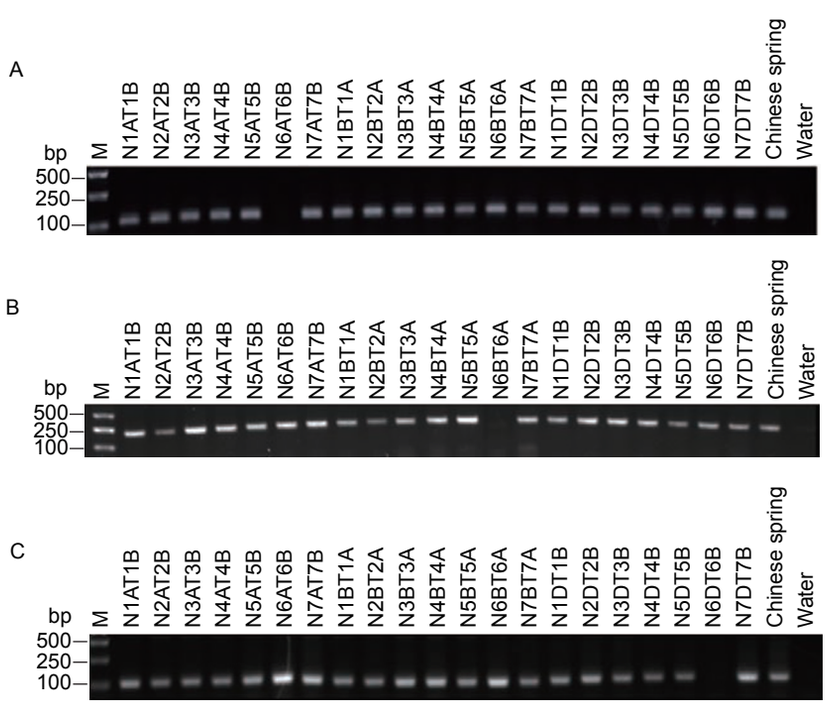

Fig. 3 Chromosome location of *TaPDR7* with Chinese Spring nullitetrasomics. A, chromosome location of *TaPDR7*A with Chinese Spring nullitetrasomic using primer *TaPDR7* RT A. B, chromosome location of *TaPDR7*B with Chinese Spring nullitetrasomic using primer *TaPDR7* RT B. C, chromosome location of *TaPDR7*D with Chinese Spring nullitetrasomic using primer *TaPDR7* RT D.

(IWGSC1.0+popseq), which located from 73 631 500 to 97 839 071. However, *TaPDR7* herein was not in the same position with QTL 6B according our results.

3.3. Subcellular localization of *TaPDR7*

To examine the subcellular location of *TaPDR7*, the construct of *pBIN35S:TaPDR7:EGFP* and the negative control *pBIN35S:EGFP* were respectively delivered to tobacco leaf cells by *A. tumefaciens* infiltration. For the negative control, the transient expression was observed in the cytosol including the nuclei and along the cell wall (Hou *et al*. 2015). The green fluorescent protein (GFP) fluorescence for TaPDR7 protein was detected only along the cell wall and plasma-lemma (Fig. 4-A1–A3). To further confirm the location of this fusion, the enhanced green fluorescent protein (EGFP) fusion protein constructed before was transformed into the onion epidermis. It was confirmed that this fusion was located on plasma-membrane actually *via* plasmolysis (Fig. 4-B1–B3).

3.4. Expression analysis of *TaPDR7*

Originally, we have attempted to isolate *TaPDR7* due to its up-regulated expression in the RNA-*seq* analysis using *F. graminearum* treated wheat samples. In order to verify the transcriptional response of *TaPDR7* to *F. graminearum* and DON, the mRNA expression profiles of three *TaPDR7* copies under those conditions were studied by quantitative RT-PCR. In Fig. 5, the expression level of *TaPDR7*A in spikes was up-regulated sharply by *F. graminearum* (Fig. 5-A1). In the first 24 h after treatment with *F. graminearum*, the expression level of *TaPDR7*A was up-regulated to 6.5-fold, and reached at a peak of 226-fold in 48 h, then the expression level dropped to 113-fold at 72 h. The expression levels of *TaPDR7*B and *TaPDR7*D in spikes were also up-regulated by *F. graminearum* but were not induced so sharply as *TaPDR7*A. In the first 24 h after treatment, the expression level of *TaPDR7*B was up-regulated to 3.8-fold, and reached at a peak of 17-fold in 48 h, then the expression level dropped to 4-fold at 72 h (Fig. 5-A2). In the first 24 h after treatment, the expression level of *TaPDR7*D was up-regulated to 5.9-fold, and reached at a peak of 50-fold in 48 h, then the expression level dropped to 18-fold at 72 h (Fig. 5-A3). Therefore, it could be revealed that these three copies of *TaPDR7* were all up-regulated, although they showed different expression patterns in response to *F. graminearum*. It was known that DON was the major toxin secreted by *F. graminearum*, which largely contributed to the fungus infection of *F. graminearum* at late stage (Bai and Shaner 2004). It was shown that the expression level of A copy increased gradually to 8-fold at 6 hours post inoculation (hpi) and maintained up-regulated in the rest of the treatment (Fig. 5-B1), while the expression levels of B and D copies increased gradually to 5- and 7-fold at 6 hpi and maintained up-regulated in the rest of the treatment (Fig. 5-B2–B3). This expression pattern of *TaPDR7* was similar with that of *TaPDR1* (Shang *et al*. 2009) in response to *F. graminearum* as well as DON. There was a channel between the two TMDs, and the change of its conformation

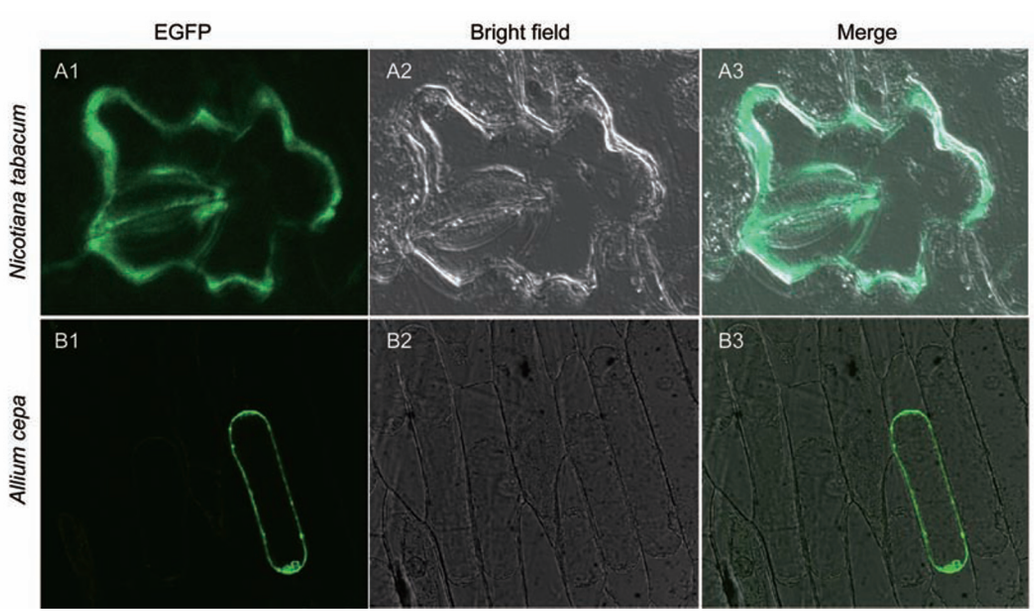

Fig. 4 Subcellular localization of *TaPDR7*. A1–A3, subcellular localization of TaPDR7:EGFP in tobacco leaf cells. B1–B3, subcellular localization of TaPDR7:EGFP in onion epidermis cells. EGFP, enhanced green fluorescent protein.

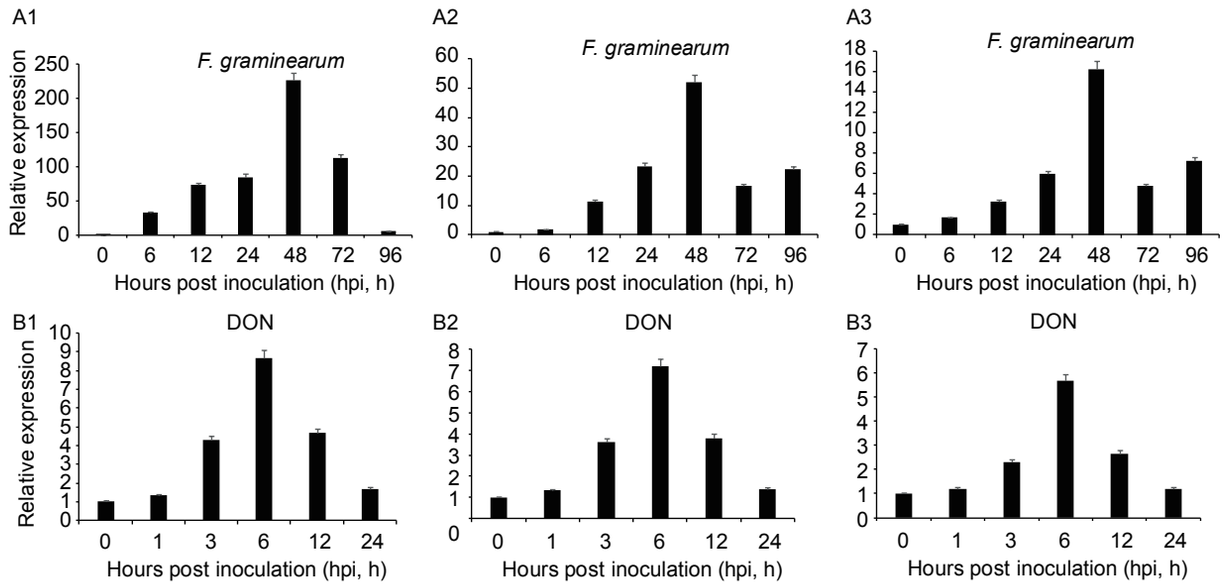

Fig. 5 Expression patterns of *TaPDR7* induced by *Fusarium graminearum* and DON. A1, A2 and A3, response of *TaPDR7* A, B and D copy against *F. graminearum* inoculation, respectively. B1, B2 and B3, response of *TaPDR7* A, B and D copies against DON treatment, respectively. The expression level was calculated by the comparative C_T method with the wheat 18S rRNA gene as the endogenous reference for normalization. The relative expression of *TaPDR7* was calculated with its transcript level in comparison to that in the mock controls across time point. Error bars represented the variations among three replications. The same as below.

could transport the substrates through cytomembrane using the energy released by adenosine triphosphate (ATP) hydrolysis (Hollenstein *et al*. 2007). It is proposed that these genes could transport DON or DON-detoxification products outside to the cell and reduce the damage of DON to plant cells, implying their contribution to FHB resistance.

In order to obtain clues of signaling pathways in which *TaPDR7* might be involved to perform functions in FHB resistance, we checked the response of *TaPDR7* to hormone treatments using wheat seedlings. As presented in Fig. 6-A1, the expression level of *TaPDR7*A was down-regulated sharply at 6 h after treated by MeJA and reached at the peak of 6-fold, then the expression gradually recovered to 2-fold. When induced with SA (Fig. 6-A2), the expression level of *TaPDR7* was down-regulated gradually from 0 to 24 h, and most suppressed at 3 h after treatment. In case of ABA (Fig. 6-A3), the expression level was down-regulated at first 12 h and recovered gradually. Interestingly, when treated with IAA (Fig. 6-A4), the expression level was up-regulated to 4-fold at the first 3 h, and then recovered to a normal expression level. The expression levels of *TaPDR7*B and *TaPDR7*D showed similar expression tendency with *TaPDR7*A when treated with these hormones (Fig. 6-B1–B4 and C1–C4). Previously, a dozen of *PDR* genes were reported to be up-regulated by MeJA or SA involved in plant defence (Moon 2003; Ducos *et al*. 2005; Eichhorn *et al*. 2006; Migocka *et al*. 2012; Zhang *et al*. 2013). In comparison with the other *PDR* genes studied before, the expression level of *TaPDR7* was not up-regulated by either MeJA or SA, which was the same to *NpPDR2* (Trombik *et al*. 2008). Furthermore, several genes such as *SpTUR2* and *AtPDR12*, which belong to the cluster I of PDR subfamily, had been reported to be involved in resistance against the fungicide sclareol regulated by ABA (Smart *et al*. 1996). ABA, however, was reported to be either a positive or a negative role in plant defense against pathogen infection (Audenaert *et al*. 2002; Anderson *et al*. 2004; Thaler and Bostock 2004; Mauch-Mani and Mauch 2005). Till now, little was known about the role of ABA in wheat defense against *F. graminearum*. Based on the expression pattern of *TaPDR7* which was down-regulated in the treatment of ABA, we indicated that the FHB resistant function of *TaPDR7* might be negatively regulated by ABA. IAA, which was recently been studied, might play roles in plant defense responses (Bari and Jones 2009). It was reported that infection of virulent necrotrophs such as *P. cucumerina* resulted in the down regulation of auxin response genes in *Arabidopsis* (Llorente *et al*. 2008). Over-expression of *GH3-8* resulted in enhanced resistance to the rice pathogen *Xanthomonas oryzae* pv. *oryzea* and which was shown to be independent of SA and JA signaling (Ding *et al*. 2008). All of the studies above indicated that auxin might act as an important component of hormone signaling net work involved in regulation of defence responses against biotrophic and necrotrophic pathogens. Up till now, we know very little about the PDR sub-family involved in IAA signaling yet. Here, the expression level of *TaPDR7* was

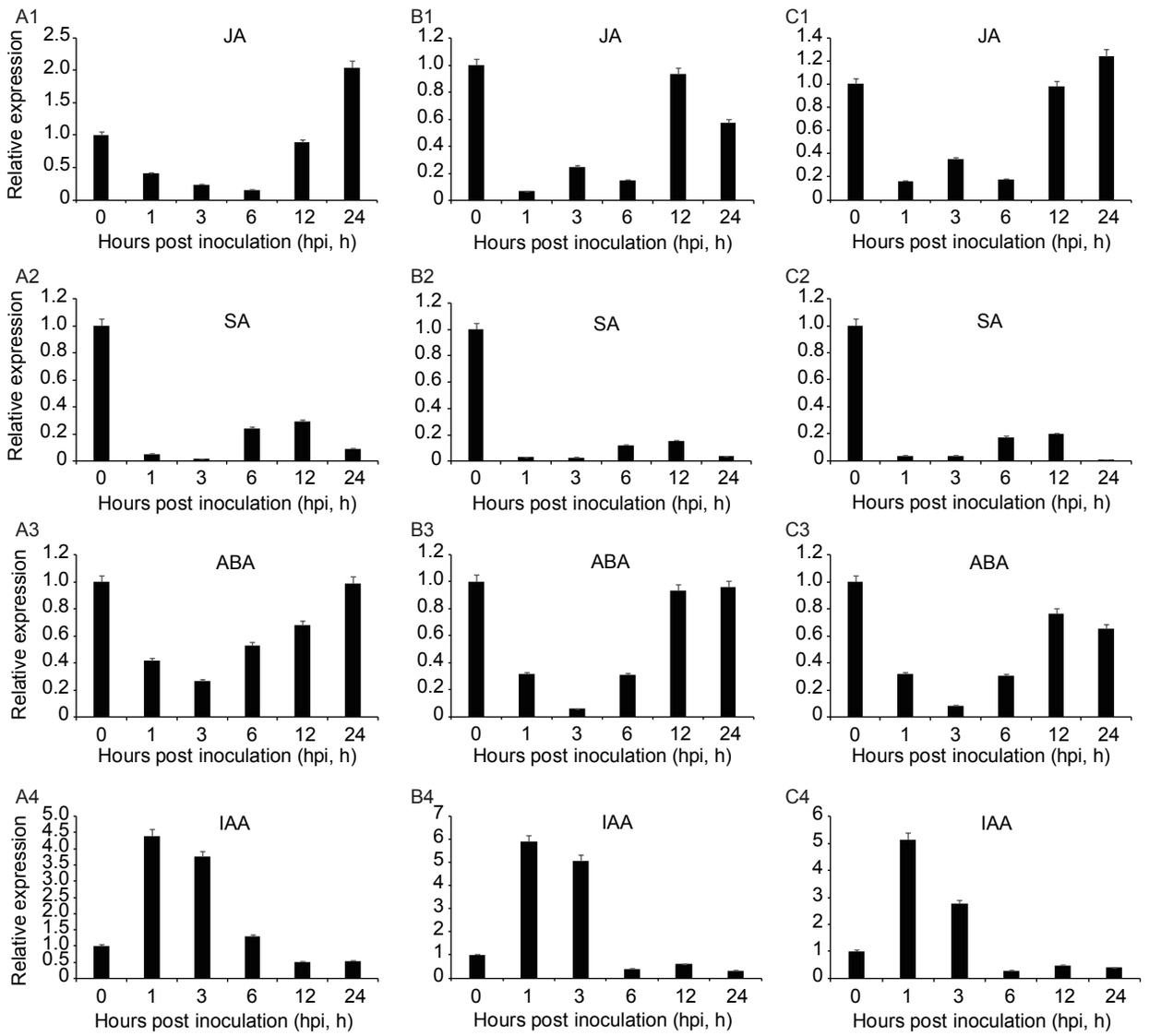

Fig. 6 Expression patterns of *TaPDR7* induced by hormone. A1–A4, the expression of *TaPDR7*A against methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), and indoleacetic acid (IAA). B1–B4, the transcriptional response of *TaPDR7* B against each hormone. C1–C4, the transcriptional response of *TaPDR7* D against each hormone. All of the hormone treatments were treated using 10-d-old seedlings of wheat.

up-regulated by IAA, which indicated that *TaPDR7* possibly participated in defense of *F. graminearum via* IAA signaling.

3.5. Virus-induced gene silencing of *TaPDR7*

To further confirm the function of *TaPDR7*, we used the BSMV-VIGS system to transitionally silence the gene and the *in vitro F. graminearum* infection assays on detached wheat leaves to determine whether *TaPDR7* was involved in resistance to *F. graminearum*. BSMV::*PDS* was used to verify the feasibility of BSMV mediated gene silencing in wheat. As expected, photo bleaching was observed in the third and forth leaves of wheat at 14 DPI with BSMV::*PDS* in the second leaf, which demonstrated that the BSMV-VIGS system used in our research was functional (Fig. 7-A).

Detached leaves from seedlings formed negative control, BSMV::00 (without target gene) and BSMV::*TaPDR7* were challenged with *F. graminearum* 12 DPI. The pathogen inoculation points were noted for all treatments on the 4 DPI. Symptoms on leaves from plants treated with BSMV::*TaPDR7* were much more severe than CK and BSMV::00 (Fig. 7-B). Significantly more and faster hyphae growth and increased conidial yield were observed in the *TaPDR7* silenced leaves than in the controls (Fig. 7-C), and the expression of *TaPDR7* was much down-regulated evaluated by the semi-RT-PCR when using a common primer set for all the three gene

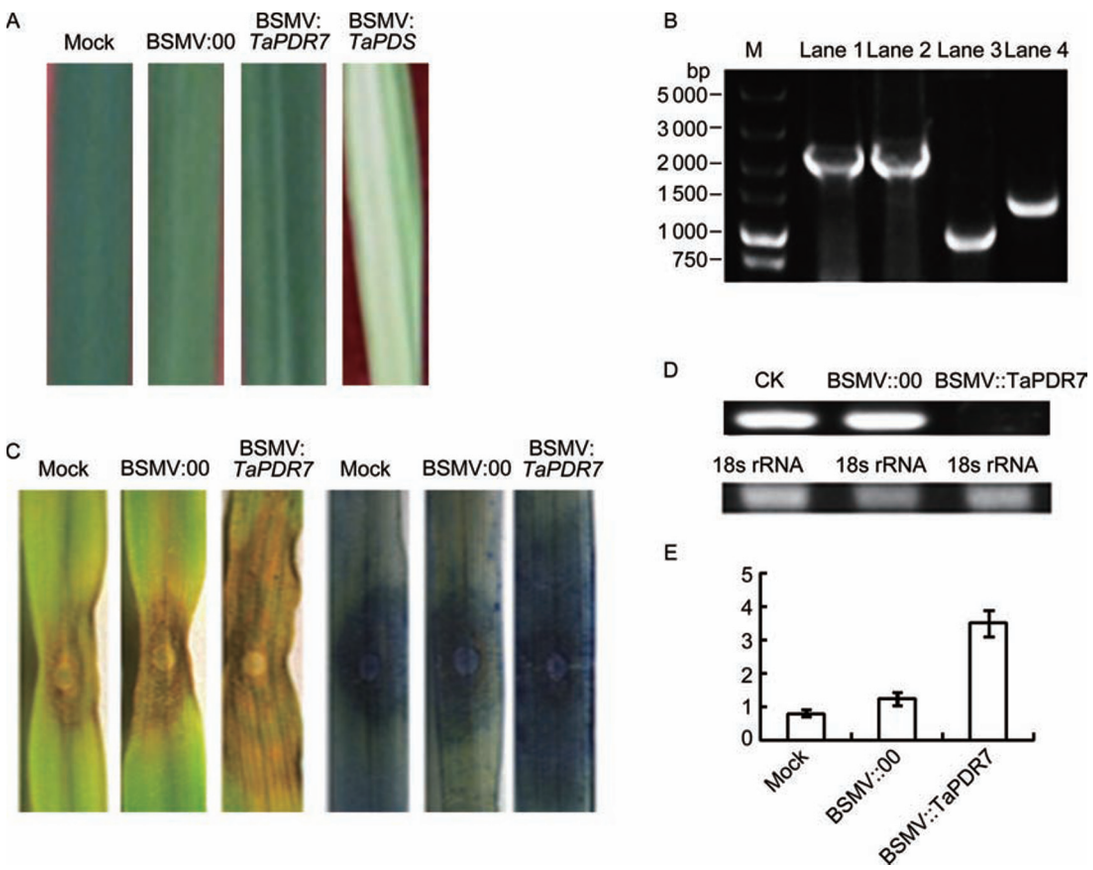

Fig. 7 Virus-induced *TaPDR7* silencing in wheat. A, symptoms of barely stripe mosaic virus-induced gene silencing (VIGS) using *PDS* gene. B, phenotypes of *TaPDR7* silenced leaves against *F. graminearum* infection. The central part of the wheat 3rd leaf was detached and cultured *in vitro* for *F. graminearum* treatment. CK, negative control of wild type wheat; BSMV::00, the VIGS treatment with only vectors; BSMV::*TaPDR7*, VIGS treatment of *TaPDR7.* C, verification of BMSV infection by semi RT-PCR. The cDNA from 6 days post inoculation (DPI) leaves was amplified by primers specific to α, β and γ plasmid. D, semi-quantitative RT-PCR of *TaPDR7* in VIGS treated seedling leaves. E, the saprophytic spot length (centimeter) of CK, BSMV::00 and BSMV::*TaPDR7*.

copies (Fig. 7-D). This result demonstrated that silencing of *TaPDR7* enhanced the susceptibility to *F. graminearum*, indicating its involvement in FHB resistance. To date, the FHB defence mechanism of wheat is still not clear and only limited genes have been functionally characterized. A series of signal pathways had been suggested to be activated after *F. graminearum* infection, such as JA, SA and ET. It was proposed that SA signaling was mainly active at early stage, followed by ET and JA signalings (Li and Yen 2008; Walter *et al*. 2010; Ding *et al*. 2011). Here, our analysis of gene expression and gene silencing indicate that *TaPDR7* might be involved in FHB resistance *via* IAA signaling pathway.

As a potent protein synthesis inhibitor, the trichothecene mycotoxin of DON has been thought to promote the disease expansion along the wheat spike. The DON accumulated in FHB-resistant wheat genotypes was generally less than that in susceptible ones (Goswami and Kistler 2005; Wilde and Miedaner 2006). Also, disruption of the *TRI5* gene of *F. graminearum*, DON-nonproducing mutants, had weakened ability of disease spread in wheat spikes (Jansen *et al*. 2005). Thus, it has been proposed that plant detoxification process of DON might play important roles in FHB resistance of wheat. One possible family is plant UDP-glycosyltransferases (UGTs) which could convert DON into inactive form D3G. Several genes such as *TaUGT3* and *HvUGT13248* have been cloned and overexpressed in *Arabidopsis*, which showed the enhanced tolerance against DON (Ma *et al*. 2010; Shin *et al*. 2012; Ma *et al*. 2015). The *HvUGT13248* and its close homologs of Bradi5g02780.1 and Bradi5g03300.1 were transformed in DON-sensitive *Saccharomyces Cerevisiae* and proved to be functional. However, till now, none of UDT genes has been transformed into wheat and functionally confirmed to show significant resistance to FHB in wheat spike. In this study, the DON and *F. graminearum* responsive ABC transporter of *TaPDR7* was cloned. As the nature of *PDR* gene, a possible mechanism of *TaPDR7* in FHB resistance might be hypothesized in DON transportation and detoxification

during *F. graminearum* infection. Previously, a similar gene of *TaPDR1* has been reported to be expressed in responsive *F. graminearum* infection, while no data has been provided to prove its resistance function to FHB (Shang *et al*. 2009). Here, our data from BMSV induced gene silencing showed that it should exert the resistance function at least in wheat leaves, which shed a light on exploring novel DON detoxifying genes except for UDP family. Actually, for *PDR* genes in cluster I, several genes were reported to be involved in response to various stresses and played important roles in other plant pathogen defense. For instance, *OsPDR8* was proved to be more prevalent in rice leaves infected with the rice blast fungus *Magnaporthe grisea* than that in non-infected leaves, which suggested the involvement in the plant pathogen defense (Jantasuriyarant *et al*. 2005). This study may help to expand the knowledge of disease resistance spectrum of this gene family.

4. Conclusion

A novel *PDR* gene *TaPDR7* was cloned from *F. graminearum* infected wheat spikes, which contains three copies distributed on A, B and D genomes, respectively. Subcellular localization analysis showed that *TaPDR7* expressed on the plasmalemma. Functional redundancy was observed for all of these three gene copies were up-regulated by *F. graminearum* and DON, possibly regulated by IAA. After silencing the gene in FHB resistant wheat cultivar Ning 7840 by BSMV-VIGS system, it could promote the *F. graminearum* hyphae growth and made larger pathogen inoculation points, which suggested that *TaPDR7* might play an important role in response to *F. graminearum*.

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