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Functional Analysis of *Tadhn1312* by Virus-Induced Gene Silencing (VIGS) in Common Wheat (*Triticum aestivum* L.)

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Abstract Virus-induced gene silencing (VIGS) is an important tool for gene function analysis in plants. In the present study, the function identification of a candidate gene named *Tadhn1312* encoding Dehydrin protein obtained from the high-throughput transcriptome was carried out by VIGS. Leaves appeared the chlorotic phenotype and the transcript level of *TaPDS* decreased rapidly at 7 days after the inoculation by BSMV: TaPDS, which indicated that the virus had successfully infected wheat leaves and the BSMV system was efficient. The chlorophyll content of leaves with BSMV: TaPDS and BSMV: Tadhn1312 inoculation decreased at 7 days, and reached significant level compared with the control. After the inoculation by BSMV: Tadhn1312, the transcript level of *Tadhn1312* was rapidly decreased at 7 days, and reached the minimum value at 21 days, indicated that *Tadhn1312* had been silenced. The spike differentiation procession of wheat plants inoculated by BSMV: Tadhn1312 was late than that inoculated by BSMV: 00. The result showed that the silencing of *Tadhn1312* prolonged the spike differentiation process, illustrating that *Tadhn1312* gene was involved in the spike differentiation process of wheat directly or indirectly.
Keywords Wheat; Gene silencing; BSMV; Spike differentiation

Wheat (*Triticum aestivum L.*) is one of the most widely grown crop and essential for global food security, and its planting area is determined by the developmental characteristics of wheat cultivars (Reynolds et al., 2011). Vernalization is a crucial phase and also a complicated process regulated by many genes in wheat development process. Until now, four genes (*VRN1*, *VRN2*, *VRN3* and *VRN-D4*) have been cloned (Distelfeld et al., 2009; Kippes et al., 2014; Jin and Wei, 2016; Muterko et al., 2016). *VRN1* encodes a MADS-box transcriptional factor and the dominant *VRN-A1* allele exhibits spring growth habit (Tanaka et al., 2018). *VRN2*, including two repeated genes *ZCCT1* and *ZCCT2* that encode proteins carrying putative a zine finger and a CCT domain, acts as long day repressors of flowering and is down-regulated by vernalization (Kippes et al., 2016). *VRN3* is highly similar to *Arabidopsis* protein flowering locus t (FT) and also a flowering promoting gene (Jin and Wei, 2016). According to the current research results of *VRN* genes, the genotype between spring and winter wheat variety can be distinguished. Yuan reported that the genotypes among semi-winter, winter and strong winter were not distinguished by the allelic composition of *VRN1*. For example, the genotype of YM49-198 (semi-winter), J841 (winter), FM (strong winter) was *van-A1*, *vrn-B1*, *vrn-D1*, respectively (Yuan et al., 2008), so it is necessary to further explore new genes or molecular markers related to vernalization (Huang et al., 2018).

Plants have evolved multiple physiological and biochemical strategies at gene or protein level to adapt unfavorable environmental state during developmental process. Under low-temperature, multiple genes are induced and their expression products include both regulatory and functional proteins (Kosova et al., 2014). These proteins directly participate in the resistance to low-temperature, such as dehydrin (DHN) (Hossain et al., 2013). Dehydrin, also was known as LEA II protein, which was composed of approximately 82-575 amino acids. The dehydrin protein typically has a conserved lysine-rich K fragment composed of 15 amino acids (EKKGIMDKIKEKLPG), S fragment composed of a series of serine residues and Y fragment composed of conservative motifs T/VDEYGNP (Banerjee and Roychoudhury, 2016; Hill et al., 2016). According to the

difference in amino acid sequence of Y, S and K fragments, dehydrin proteins also can be divided into 5 categories: Kn, SKn, KnS, YsKn and YnSK2 (Allagulova et al., 2003). Among them, SKn, KnS and YsKn are acidic proteins, YnSK2 is a neutral and basic protein, Kn is an acidic and neutral protein (Shekhawat et al., 2011; Wang et al., 2014). YnSK2 can be induced by drought and Abscisic acid (ABA) (Suprunova et al., 2004; Binott et al., 2017); SKn can be induced by low temperature, but it is less expressed under drought and ABA (Fowler et al., 2001), Kn, KnS and YsKn can be induced by low temperature (Ismail et al., 1999; Kosova et al., 2010; Archambault and Stromvik, 2012). Therefore, the different conserved sequences of dehydrin determine its acidity and alkalinity, and further determine its function.

With the rapid application of high-throughput sequencing technologies, many genes have been isolated and cloned from wheat. But how to validate gene functions has become a bottleneck because of the lower genetic transformation efficiency comparing with Arabidopsis and rice, due to its complicated and huge genome and polyploidy characteristics. Nowadays, Virus-induced gene silencing (VIGS) has been developed as an effective transgenic technology for determining gene functions in dicot and monocot cereals (Ma et al., 2012), which only partial gene sequence is sufficient for silencing gene (Senthil-Kumar and Mysore, 2011). The function of hundreds of plant genes involved in defense response pathways, plant development, and metabolism have been identified by VIGS (Guo et al., 2010; Rivas et al., 2014).

We had conducted a high-throughput transcriptome sequencing analysis using winter wheat cultivar Jing841 (J841) and spring wheat cultivar Liaochun10 (LC10) as experimental materials under vernalization and non-vernalization treatments (Feng et al., 2016), and obtained many differentially expressed genes; among them, a differentially expressed candidate Unigene1312 encoding Dehydrin protein was selected and temporarily named *Tadhn1312*. Expression analysis showed that *Tadhn1312* was expressed in LC10 and J841, but the expression level in J841 was higher than that in LC10 under vernalization treatment, indicating that the gene was responsive to vernalization treatment. In the present study, we employed the Barley Stripe Mosaic Virus (BSMV) based VIGS system for the primary function verification of *Tadhn1312* gene

1 Results and Analysis

1.1 Phenotypes of wheat leaves after the inoculation with BSMV Virus

Virus-Induced Gene Silencing is a powerful reverse genetics approach for knocking down target genes expression to study their functions and is widely used in the regulation of plant metabolism, growth, and development (Guo et al., 2010). A 242 bp coding sequence of *Tadhn1312* gene was amplified and used to construct the BSMV vector, and designated as BSMV: Tadhn1312 (Figure 1). When wheat plants were at two-leaf stage, BSMV: Tadhn1312 was used to inoculate wheat leaves, BSMV: TaPDS as the positive control and BSMV: 00 as the negative control.

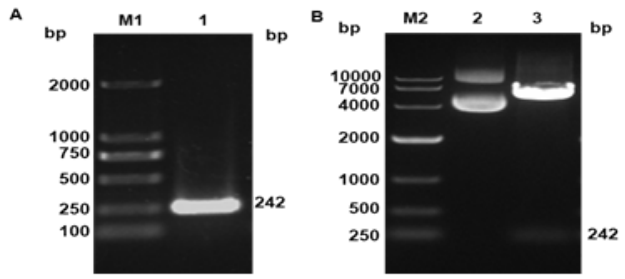

Figure 1 PCR amplification of *Tadhn1312* (A) and double enzyme digestion of BSMV: Tadhn1312 (B)

Note: M1:DL2000 DNA marker; 1: PCR product of *Tadhn1312*; M2: DL10000 DNA marker; 2: Recombinant plasmid BSMV: Tadhn1312; 3: Double digestion of BSMV: Tadhn1312

The second leaves of wheat plants appeared virus symptom and chlorotic phenotype at 7 days after inoculated with BSMV: TaPDS and BSMV: Tadhn1312; and the obvious photo bleaching phenotype were shown at 14 days, indicating that the virus had successfully infected wheat leaves. At 21 days, the third leaves showed radial banded white spots or the entire leaves showed photo bleaching (Figure 2A). As time went by, the phenotype gradually subsided, but the young leaves showed mottled chlorotic phenotype at 28 days. Obvious bleaching lasted for 35

days after inoculation. The leaves of negative control BSMV: 00 had no bleaching phenotype. Among 50 wheat leaves infected by BSMV: TaPDS and BSMV: Tadhn1312, respectively, 32 and 29 leaves showed chlorosis with silencing efficiencies of 64% and 58% and reached the significant level (Figure 2B), indicating that the VIGS system is effective.

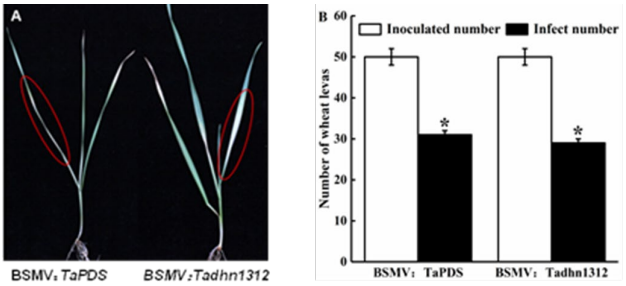

Figure 2 Magnified image of leaves at 21d after BSMV: Tadhn1312 inoculation showing chlorotic phenotype and Infection rate statistics

Note: A: chlorotic phenotype; B: Infection rate statistics. Each value is the mean±standard deviation of three independent biological replicates; * indicate the significant difference at 0.05 level (p<0.05)

1.2 Chlorophyll content

Chlorophyll content was measured every 7 days after the inoculation. The result showed that the chlorophyll content of leaves with BSMV: TaPDS inoculation decreased at 7 days, and reached the minimum level at 21 days, accounted for 43.8% of BSMV: 00, the difference was extremely significant. At 28 d, the chlorophyll content gradually increased, but was still lower than that of negative controls BSMV: 00 (Figure 3 A).

1.3 Transcription level of target gene after BSMV recombinant vector inoculation

qRT-PCR was used to measure the *Tadhn1312 and TaPDS* transcript abundances at different time after inoculation. The relative expression level of *TaPDS* rapidly decreased at 7 days, and continued to decrease and reached the lowest level at 21 days. At 28 days, the transcript level *TaPDS* gradually increased, and recovered to about half of the initial level at 35 days, indicating that the expression of *TaPDS* gene had been significantly inhibited. The expression of target gene *Tadhn1312* exhibited similar expression trend as *TaPDS* after BSMV virus inoculation. The transcript level of *Tadhn1312* was rapidly decreased at 7 days, and reached the minimum value at 21 days, accounting for 27% of that of negative control and reached the significant level, suggesting that the target gene *Tadhn1312* was inhibited successfully (Figure 3B).

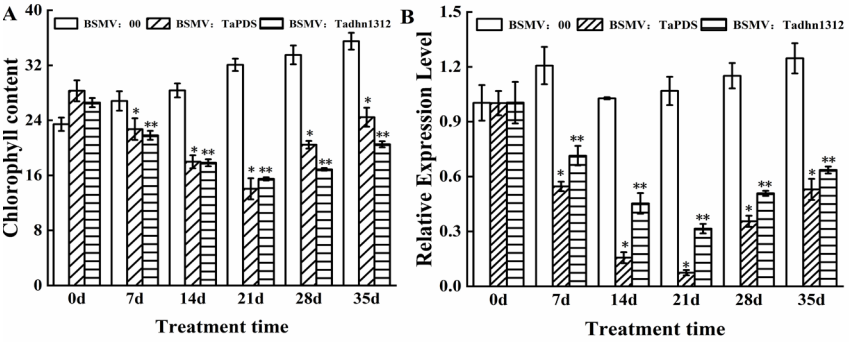

Figure 3 Chlorophyll content and relative expression analysis inoculated by BSMV recombinant vector from 0 to 35d in wheat with BSMV: TaPDS and BSMV: Tadhn1312.

Note: A: chlorophyll content. B: The relative expression pattern of *TaPDS* and *Tadhn1312* by using qRT-PCR; *β-actin* was used as internal control; Each value is the mean±standard deviation of three independent biological replicates; * indicate the significant difference between BSMV: TaPDSP and BSMV: 00 at 0.05 level (p<0.05). ** indicate the significant difference between BSMV: Tadhn1312 and BSMV: 00 at 0.05 level (p<0.05)

1.4 Spike differentiation Observation after Inoculation with BSMV: Tadhn1312

Wheat spike differentiation is affected by various factors, including cultivar, illumination and temperature. The double ridge stage is an important indicator for passing through vernalization process (Baloch et al., 2003). The spike differentiation of negative control plants (BSMV: 00) and BSMV: Tadhn1312 remain single ridge stage at 14 d after BSMV virus inoculation. At 21 days, the spike differentiation of negative control plants (BSMV: 00) entered the early double ridge stage, while the spike differentiation of plants inoculated with BSMV: Tadhn1312 still remained in single ridge stage. When the spike differentiation of plants inoculated with BSMV: Tadhn1312 entered into the early double ridge stage at 28 days, the spike differentiation of control plants inoculated with BSMV: 00 has been in the middle of the double edges (Figure 4). The result showed that the silencing of *Tadhn1312* prolonged the spike differentiation process, illustrating *Tadhn1312* gene was involved in the spike differentiation process of wheat directly or indirectly.

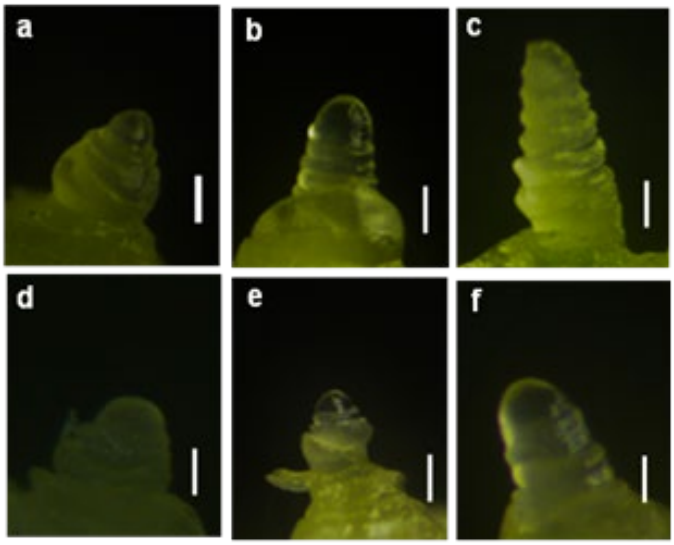

Figure 4 Spike differentiation process of wheat plants after BSMV virus inoculation with BSMV: 00 and BSMV: Tadhn1312

Note: a, b, c: BSMV virus inoculation with BSMV:00; d, e, f: BSMV virus inoculation with BSMV: Tadhn1312; a, d: BSMV virus inoculation at 14 days; b, e: BSMV virus inoculation at 21 days; c, f: BSMV virus inoculation at 28 days. The proposed scale is 0.15mm

2 Discussion

As a hydrophilic protein with high thermal stability, dehydrin widely exists in plants and can protect intra-cellular proteins and membrane structure from damage (Abedini et al., 2017). In recent years, dehydrin has attracted much attention due to its protective effect on plants under abiotic stress (Ramakrishna and Gokare, 2011; Yu and Yang, 2016). The expression of *CaDHN1* from pepper was markedly upregulated in response to cold, salt, osmotic stresses and salicylic acid (SA) treatment, while the silencing of *CaDHN1* using the virus-induced gene silencing (VIGS) technique led to decreased tolerance to cold, salt and osmotic induced stresses (Chen et al., 2015). In another work, seven dehydrin genes were up-regulated under low-temperature stress in loquat, and the expression of dehydrin gene in the cultivars with strong cold-resistance was greater compared to those with weak cold resistance (Xu et al., 2014). The transgenic Arabidopsis plants with over-expressed *IpDHN* gene showed a significant enhancement in tolerance to salt/drought stresses, less accumulation of hydrogen peroxide (H_2O_2) and the superoxide radical (O-2(-)), accompanied by increasing activity of the antioxidant enzyme system in vivo (Zhang et al., 2018). There are many reports about dehydrin related to abiotic or biotic stress (Guo et al., 2000; Sun et al., 2000; Kumar et al., 2014), while there are fewer literatures about dehydrin related to wheat development. Sequencing analysis showed that *Tadhn1312* was highly similar with T.durum Dehydrin mRNA pTd38 and TaCOR80, with 99% and 96% identity, respectively. Kobayashi et al. reported that COR genes were

up-regulated and the COR proteins were accumulated more in winter cultivar than spring one in the cold acclimation process (Kobayashi et al., 2005).

Vernalization is a periodic characteristic, and the vernalization treatment duration and optimum temperature of wheat varieties with different developmental patterns are different. Yin et al. reported that spring wheat cultivars could accomplish the transition from the vegetative to the reproductive phase under non-vernalization condition and finish the spike differentiation process; the spike differentiation was still at the single ridge stage in winter wheat cultivar BJ No.10 and semi-winter cultivar ZM 9405 under non-vernalization after sowing 62-d, 48 d, respectively, indicating that winter wheat and semi-winter cultivars could flower and head normally only after vernalization treatment (Yin et al., 2017). The VIGS technique is conducted to silence target genes by using the plant's RNAi-mediated antiviral defense mechanism (Lee et al., 2017). After constructing recombinant BSMV infected wheat leaves in this study, the transcript level of the target gene decreased sharply, imaging that BSMV had successfully silenced the endogenous gene. The spike differentiation process caused by silencing of the *Tadhn1312* gene was later than that of the negative control, indicating that *Tadhn1312* has a promoting effect on wheat developmental process. While the silencing duration caused by VIGS can only be maintained for approximately 30 days, which may bring difficulties for long-term observation of plant phenotype, but VIGS technique still has incomparable advantages than other gene function research methods.

3 Materials and Methods

3.1 Plant materials and treatment

The spring wheat cultivar Liaochun10 (LC10) was used in this study, provided by the National Engineering Research Center for Wheat. Plump seeds were placed on moist filter paper in petri dishes and placed in the greenhouse at (25±2)°C with 16 h/8 h day/night photoperiod cycles and photon flux density of approximately 300μmol/ ($m^{-2}·s^{-1}$) and 60% relative humidity. After germination, seeds were placed in the vernalization box for 30 days and then put into pots containing sterilized vermiculite, the growth conditions were the same as above. The seedlings with consistent growth state were selected at the two-leaf stage for inoculation; leaf samples were picked every 7 days after inoculation and immediately frozen in the liquid nitrogen.

3.2 BSMV Vector construction, in-vitro transcription and inoculation

A conserved cDNA fragment 242 bp of *Tadhn1312* was amplified and used to construct the BSMV recombinant vector. The BSMV vectors, including α, β and γ tripartite genome, were transcribed into RNA *in vitro* by T7 RNA polymerase. Equal amount of the three RNA was inoculated into wheat leaves. Target genes were cloned into BSMV-γ through the *NheI* restriction site. *TaPDS* is essential in the carotenoid pigment biosynthetic pathway and the suppression of its activity results in photolysis of chlorophyll, also referred to as photobleaching in the affected tissues.

The recombinant plasmids of BSMV:00, BSMV:TaPDS, BSMV:Tadhn1312, and BSMV-α were linearized with *Mlu I* digestion, the BSMV- β backbone was linearized via *Spe I*, at 37°C for 4 h. The linearizing products were purified and transcribed *in vitro* using the RiboMAX™ Large Scale RNA Production System-T7 kit (Promega, USA) as the manufacturer's instructions. The RNA-α, RNA-β, and RNA-γ (or its derivative) transcripts were mixed in a 1:1:1 ratio, and diluted with nine volumes of DEPC water. In addition, 12 volumes of 2 × GKP buffer were added to the diluted transcript mixture for subsequent inoculation. Plants with consistent growth were chosen for virus inoculation at the two-leaf stage using 10 μL transcript mixtures per leaf. After inoculation, BSMV-inoculated leaves were fog-sprayed with RNase-free water and covered with plastic film for 3 days and

cultured in an illumination incubator (16 h light/8 h dark photoperiod at 25°C). The control plants BSMV: 00 and BSMV: TaPDS were used as negative control and positive control, respectively (Wang et al., 2010).

3.3 Determination of chlorophyll content

Chlorophyll meter SPAD-502 (Konica Minolta sensing Inc., Japan) was used to determine the chlorophyll content of wheat leaves. Two leaves were measured per plant. Each leaf was measured 5 to 6 times from the leaf base to the tip and 10 samples were randomly selected for each treatment.

3.4 RNA extraction and reverse transcription

Total RNA was extracted from wheat leaves using RNAiso Plus kit (TaKaRa, Dalian, China), solubilized with DEPC-treated ddH_2O and stored at -80°C. The integrity of the RNA was assessed by 1.0% agarose gel electrophoresis. The first strand of cDNA was synthesized using the Prime Script™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) as the manufacturer's instructions.

3.5 Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was performed using the first-strand cDNA as template using 2×SYBR® *Premix Ex Taq™* (TaKaRa, Dalian China). The reaction contained 10 μL of 2 × SYBR, 10 μL of Premix Ex Taq TMII, 1 μL of cDNA template, 2 μL of real-time PCR primers (10 μM) (Table 1), and ddH_2O supplemented to 20 μL. Reaction conditions were as follows: 95°C for 30 s; 95°C for 5 s and 60°C for 30 s, circulating 40 times. Fluorescence signals were collected during the reaction steps at 60°C; 3 repeat samples were set for each sample to correct the copy number of PCR templates. *β*-actin (GenBank accession no. AB181991) was used as internal control (Liu et al., 2016). The $2^{-\Delta\Delta Ct}$ method used to calculate relative changes in gene expression determined from qRT-PCR experiments (Livak and Schmittgen, 2016). Each value is the mean±standard deviation of three independent biological replicates. Asterisks indicate significant differences (p<0.05). BSMV: 00 and BSMV: TaPDS were used as a negative control and a positive control, respectively.

3.6 Observing the process of spike differentiation

Spike differentiation of inoculated and control wheat plants was observed at 14 days, 21 days and 28 days after virus inoculation and three samples were observed for each time. The spike differentiation stage for each main shoot was determined using a binocular stereoscopic microscope (OLYMPUS SZX12) with magnification of 40×. All primers used in the study are listed in Table 1.

Table 1 Primers for gene cloning and vector construction

Primer name	Primer sequence(5'→3')	Usage	Product size
β-actin-F	TTTGAAGAGTCGGTGAAGGG	internal control	176 bp
β-actin-R	TTTCATACAGCAGGCAAGCA		
TaPDS-F1	AGCGTCCAGGCACTAAA		251 bp
TaPDS-R1	TAACTTTCCGCCCAACA	qRT-PCR	
Tadhn1312-F2	TCCCGAGTGACAGGTTGAGC		242 bp
Tadhn1312-R2	TCCCAGTGCCAGTCGTTCC		
TaPDS-F2	GCTAGCAGCGTCCAGGCACTAAA	Vector construction	251 bp
TaPDS-R2	GCTAGCTAACTTTCCGCCCAACA		
Tadhn1312-F3	GCTAGCTCCCGAGTGACAGGTTGAGC		242 bp
Tadhn1312-R3	GCTAGCTCCCAGTGCCAGTCGTTCC		

Note: the underline part in primer sequence is enzyme loci.

3.7 Statistical analysis

All data were subjected to analysis of variance using the SAS 9.3 program. And the significant difference among group means tested by using Duncan's multiple range tests at 0.05 ($p < 0.05$) level.

Authors' contributions

The work presented here was carried out in collaboration between all authors. ZPY , WTC and WL defined the research theme and co-designed experiments, and discussed analyses. ZPY designed methods, experiments, and wrote this paper. ZPY and QX carried out the laboratory experiments and analyzed the data. WGR worked on experiments design and obtaining test data. WL provide financial support. All authors read and approved the final manuscript.

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