Knock-down the expression of TaH2B-7D using virus-induced gene silencing reduces wheat drought tolerance

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Abstract

Background: Drought is a major abiotic stress affecting global wheat (Triticum aestivum L.) production. Exploration of drought-tolerant genes is essential for the genetic improvement of drought tolerance in wheat. Previous studies have shown that some histone encoding genes are involved in plant drought tolerance. However, whether the H2B family genes are involved in drought stress response remains unclear.

Methods: Here, we identified a wheat histone H2B family gene, TaH2B-7D, which was significantly up-regulated under drought stress conditions. Virus-induced gene silencing (VIGS) technology was used to further verify the function of TaH2B-7D in wheat drought tolerance. The phenotypic and physiological changes were examined in the TaH2B-7D knock-down plants.

Results: In the TaH2B-7D knock-down plants, relative electrolyte leakage rate and malonaldehyde (MDA) content significantly increased, while relative water content (RWC) and proline content significantly decreased compared with those in the non-knocked-down plants under drought stress conditions. TaH2B-7D knock-down plants exhibited severe sagging, wilting and dwarf phenotypes under drought stress conditions, but not in the non-knocked-down plants, suggesting that the former were more sensitive to drought stress.

Conclusion: These results indicate that TaH2B-7D potentially plays a vital role in conferring drought tolerance in wheat.

Keywords: Drought, Triticum aestivum L., TaH2B-7D, Knock-down

Background

Drought stress is the principal abiotic factor limiting wheat (Triticum aestivum L.) productivity in arid and semi-arid areas [1]. More than 50% of the wheat growing areas in the world are impacted by drought stress [2]. A large number of studies have been carried out on the physiological changes of wheat plants under drought stress and their molecular mechanisms in response to drought stress [3–10]. However, although significant progress has been made [11, 12], the mechanisms of drought tolerance in hexaploid wheat have not been fully explored. Further exploration of drought-tolerant genes is of vital importance for the genetic improvement of wheat drought tolerance.

Studies have shown that the histones are involved in multiple stress responses in plants. Histone proteins contain large amounts of basic amino acids such as arginine and lysine, which are up to about 1/4 of all amino acid residues. The histones proteins bind to the negatively charged double helix DNA to form a chromatin complex [13, 14]. According to the composition of amino acid and molecular weight, histones can be divided into five major families: H1, H2A, H2B, H3, H4 [15, 16]. Altering the activity or level of histone variants has been demonstrated to be associated with abiotic stress responses [17]. Epigenetic modifications of histone proteins such as...
deacetylation [18], methylation [19, 20] and ubiquitination [21] are involved in plant drought response. Moreover, knock-down the drought-inducible H1-S variant of tomato by antisense technology promotes stomatal closure and enhances drought tolerance [17]. The H2A.Z variant of Arabidopsis is involved in the response to phosphate deficiency [22] as well as in the perception of ambient temperature [23]. Overexpression one of the TaH2A variant TaH2A.7 in Arabidopsis significantly lowered water loss rate, and promoted ABA-induced stomatal closure and enhanced drought tolerance in Arabidopsis [24]. Histone H2B is one of the four main histone proteins involved in the structure formation of nucleosomes of chromatin in eukaryotic cells [25]. However, whether H2B proteins are involve in the drought stress response is unclear.

Virus-induced gene silencing (VIGS) is an efficient post-transcriptional gene silencing (PTGS)-based technique for gene functional study [26]. It employs the natural defense mechanisms used by plants to protect against invading viruses [27]. Viruses that do not have or have only weak gene silencing suppressors are modified to VIGS systems to induce PTGS-mediated degradation of target plant mRNAs [28–30]. So far, several VIGS systems have been established for monocots [30, 31]. Barley stripe mosaic virus (BSMV) is a tripartite RNA virus that can infect many agronomically important crops like barley, wheat, rice, maize and oat, and the BSMV-derived VIGS system has been widely used among monocots [32]. Similarly, Brome mosaic virus (BMV) is another RNA virus that has been adopted for VIGS in barley, rice, and maize [33]. The VIGS system developed from the Rice tungro bacilliform virus (RTBV) is a convenient and efficient method using agroinoculation, which can reduce the expression levels of target genes by more than 90%. In important horticultural specie orchids, a VIGS vector system has also been successful established employing the symptom free Cymbidium mosaic virus (CymMV) [30, 33]. In this study, we identified a drought-responsive histone H2B family gene on chromosome 7D, TaH2B-7D, which was significantly up-regulated under drought stress conditions. As the BSMV-derived VIGS system has been widely used for identification of stress responsive genes in hexaploid wheat [34–38], it was used here to further investigate the function of the drought responsive gene TaH2B-7D. The phenotypic and physiological changes were examined in the VIGS-based TaH2B-7D gene knock-down plants. Our results demonstrate that relative electrolyte leakage rate and malonaldehyde (MDA) content significantly increased, while the relative water content (RWC) and proline content significantly decreased in the TaH2B-7D knock-down plants under drought stress conditions. Moreover, the TaH2B-7D knock-down plants were more sensitive to drought stress. This work shows that TaH2B-7D potentially plays a vital role in conferring drought tolerance in common wheat.

**Methods**

**Plant material and growth conditions**

An elite drought-tolerant wheat variety in China, XN979, was used for in vitro transcribed RNA inoculation in the VIGS trial [39]. Pot culture was employed in the trial. Firstly, seeds were germinated for 16 h at 22 °C; then, twelve germinated seeds were sown in each pot with a soil water content of 90% field capacity (FC). The incubator temperature was set at 21 ± 1 °C in the daytime and 19 ± 1 °C at night (15 h light/9 h dark). Wheat plants were thinned to nine plants per pot after emergence. Sixteen days after sowing, wheat seedling plants (Zadoks growth scale 12) were used for in vitro transcribed RNA inoculation in the VIGS trial. The procedure for vector construction and in vitro transcribed RNA inoculation will be described in detail later. After the inoculation, the pots were divided into two groups and the following two treatments were performed separately: (1) non-stress conditions (NS, maintained the soil water content at 80–90% FC), and (2) drought stress conditions (DS, no watering after sowing). Sixteen days after the inoculation (about 44% FC under DS conditions), the leaves of each pot were collected for measurement of proline and MDA content, RWC and rate of relative electrolyte leakage. In the meanwhile, another trial comprising low nitrogen treatment (LN), salt stress treatment (SS) and non-stressed control were carried out according to previous literatures [40, 41].

**Vector construction and in vitro transcribed RNA inoculation**

Vectors for VIGS trial were constructed as previously described [42]. Firstly, a 135 bp-fragment of TaH2B-7D cDNA coding region was cloned and then inserted into the γ vector (forward primer containing the Pac I restriction site and two protective bases (CC) at 5 prime end: 5′CCCTAATTAAAGACAAAGAAGAAGAAGGCC3′; reverse primer containing the Not I restriction site and three protective bases (TAT) at 5 prime end: 5′TATTGGGGCCCGCGCTCGTTGATGAGAGGATCC′). The BSMV _0_ derived from the original empty pSL038-1 vector and acted as a negative control. BSMV _POS_ was used as a positive control to monitor the time course of VIGS [35, 39]. Then, the constructs were linearized and used to synthesize α, β, γ RNAs of the BSMV genome using Ribo MAX TM Large Scale RNA Production System-T7 (Promega, Madison) [43]. The α, β, γ RNAs were mixed in equal amounts and diluted with an equal volume of RNAase-free water and added to FES buffer [34]. Each
of the constructs consisted of BSMV α, β, and γ with the TaH2B-7D gene fragment (BSMV_TaH2B-7D) or phytoene desaturase (GenBank: FJ517553.1, BSMV_ppds) or null insertion (BSMV0). The inoculation of each viral construct was performed according to previously described procedures [35]. The incubator temperature was set at 23 ± 1 °C, with darkness for 24 h, followed by a 15 h light/9 h dark photoperiod [39].

Real-time PCR analysis
Leaf total RNA was extracted using Trizol reagent according to the product instructions (Trizol; Invitrogen). Two-Step Prime-Script™ RT reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa) was used for the cDNA synthesis. The temperature procedure was set as follows: 2 min at 42 °C, 15 min at 37 °C, 5 s at 85 °C, and then 4 °C. The primers used for real-time PCR were designed using Primer 5.0 software (forward primer: 5′GACAAGAGAAGAAAGAAGC3′; reverse primer: 5′GTCGTGGATGGAAGAGGTC3′). Real-time PCR was performed on a Bio-Rad IQ5 Real-Time PCR Detection System. Each reaction contained 0.4 μmol of forward and reverse primers respectively, 12.5 μl of SYBR Premix Ex Taq (Tli RNaseH Plus), 4 μl diluted cDNA templates. The reaction volume was added to 25 μl with nuclease-free water. The temperature procedure was set as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The internal reference gene TaActin (forward primer: 5′ACCTTCAGTTGCCAGCAAT 3′; reverse primer: 5′CAGAGTCGAGCA CAATACCGTGT 3′) and TaGAPDH (forward primer: 5′ TGTCGTGGTGCATGGAAGAGG 3′; reverse primer: 5′GCAAGAGAAGAAGCAGTTAGT 3′) were used to normalize the expression level of TaH2B-7D. Three biological replicates were performed. Relative gene expression levels of TaH2B-7D were calculated using 2−ΔΔCT method.

Measurement of physiological indices
The RWC was measured according to Hodges et al with minor modifications [47]. In brief, 0.5 g fresh leaves were sampled and fast-frozen in liquid nitrogen. Then the samples were fully grinded using a tissue grinder. 5 ml of 5% (w/v) trichloroacetic acid (TCA) was added to each sample and mixed thoroughly. The mixture was centrifuged at 4 °C, 4000×g for 20 min, and 1 ml of supernatant was transferred to equal volume of 0.5% (v/v) TBA in 20% TCA. The mixture was boiled at 100 °C for 30 min, and then placed on ice for 30 min. After centrifuged at 4000×g for 10 min at 4 °C, the absorbance of 2 ml supernatant was recorded at 450 nm, 532 nm and 600 nm, respectively. MDA content was calculated by using the following formula: MDA content (μmol g FW−1) = (6.45 OD532 − OD600) − 0.56 OD450 × V/W. In the formula, V represents the volume of extracts (5 ml) and W represents the fresh weight of sample (0.5 g).

Results
The expression of TaH2B-7D under NS and DS conditions
Firstly, we examined the expression of TaH2B-7D under non-stress (NS) and drought stress (DS) conditions. Result shows that the expression of TaH2B-7D in XN979 was significantly up-regulated by drought stress (Fig. 1a, b). Since previous studies have shown that histones are involved in multiple stress responses, we examined the expression of TaH2B-7D under low nitrogen and salt stress conditions. Results show that TaH2B-7D was also significantly up-regulated by low nitrogen stress and salt stress (Fig. 1c–f). To check the effect of VIGS in our study, the expression level of TaH2B-7D was investigated in four independent BSMV_TaH2B-7D-infected plants (BSMV_TaH2B-7D-1, BSMV_TaH2B-7D-2, BSMV_TaH2B-7D-3 and BSMV_TaH2B-7D-4) and controls. Results show that the
expression of \( TaH2B-7D \) were significantly down-regulated in the BSMV\( _{TaH2B-7D} \)-infected plants compared with that in the non-infected and BSMV\( _{0} \)-infected plants (negative control) under DS conditions (Fig. 2), indicating that the expression levels of \( TaH2B-7D \) have been successfully knocked-down in the BSMV\( _{TaH2B-7D} \)-infected individuals.

**Phenotypic changes in the TaH2B-7D knock-down plants**

In the VIGS trial, 10 days after inoculation, all the BSMV constructs-infected plants exhibited slight chlorosis owing to the plant immunity to virus. The BSMV\( _{PDS} \)-infected plants emerged visible bleached leaves (Fig. 3a), indicating the success of the viral inoculation [39]. Twenty days after inoculation, there were no obvious phenotypic changes of BSMV\( _{TaH2B-7D} \)-infected plants under NS conditions compared with the non-infected and BSMV\( _{0} \)-infected plants (Fig. 3b). However, severe leaf sagging, wilting and slow growth (dwarf) were presented in the BSMV\( _{TaH2B-7D} \)-infected plants under DS conditions (Fig. 3c).

**Physiological changes of the TaH2B-7D knock-down plants**

We also checked physiological changes in the TaH2B-7D knock-down plants. Under DS conditions, leaf RWC in the non-infected plants and BSMV\( _{0} \)-infected plants only decreased by 16.4% and 14.5%, respectively, compared with that in the NS non-infected plants. However, leaf RWC in the BSMV\( _{TaH2B-7D} \)-infected plants under DS conditions reduced by 67.0% compared with that in the NS non-infected plants (Fig. 4a). At the same time, relative electrolyte leakage rate in the BSMV\( _{TaH2B-7D} \)-infected plants increased by 446.2% under DS conditions compared with that in the NS non-infected plants, which was significantly higher than that in the non-infected (173.5%) and BSMV\( _{0} \)-infected (159.6%) plants under DS conditions (Fig. 4b). Moreover, MDA content in the BSMV\( _{TaH2B-7D} \)-infected plants increased by 410.4% under DS conditions compared with that in the NS non-infected plants, which was also significantly higher than that in the non-infected and BSMV\( _{0} \)-infected individuals (negative controls) under DS conditions (Fig. 4c). In addition, proline content of the BSMV\( _{TaH2B-7D} \)-infected plants under DS conditions increased by 93% compared with that in the NS non-infected plants, which is obvious lower than was the case in both non-infected (211.8%) and BSMV\( _{0} \)-infected (196.9%) individuals (Fig. 4d).

**Discussion**

Plants inevitably come across complicated environmental changes during their life cycle. Drought is one of the major limiting factors for plant growth and productivity [48, 49]. Identification of drought tolerance-related genes is very important for the genetic improvement of plant drought tolerance. Currently, many drought-responsive genes/proteins have been identified in different species such as wheat, maize, rice, peanut and soybean in previous studies [50–55]. These results are of great significance for exploring the molecular mechanisms and genetic improvement of wheat drought tolerance [56, 57]. However, most of these genes/proteins have not been functionally verified, especially in hexaploid wheat. Gene functional verification by genetic transformation in wheat is time-consuming and high-cost. Verification the functions of the large number of drought responsive
Fig. 2 Detection of the expression levels of TaH2B-7D in the gene knock-down and control plants. NS, non-stress; DS, drought stress. BSMV₀, negative control of the VIGS system; BSMV₀TaH2B-7D, TaH2B-7D knock down plants; BSMV₀TaH2B-7D-1, BSMV₀TaH2B-7D-2, BSMV₀TaH2B-7D-3 and BSMV₀TaH2B-7D-4 are four independent TaH2B-7D knock down plants. The expression level of TaH2B-7D in the TaH2B-7D knock down plants was detected under DS conditions. Different letters above the columns indicate significant differences at $P \leq 0.01$ levels.

Fig. 3 The phenotypes of TaH2B-7D knock down plants. a Leaf; b, c whole plants; b non-stress treatment (NS); c drought stress treatment (DS). BSMV₀ represents the negative control of VIGS system; BSMVₚDS represents the positive control monitoring time course of VIGS; BSMVₜₐₜH₂B-7D represents TaH2B-7D knock down plants.
genes/proteins is a big challenge in hexaploid wheat. VIGS technology is an alternative approach for preliminary functional analysis of these genes/proteins because of its rapidity and high efficiency [34–38]. In this study, VIGS was used to further analyse the function of a drought stress up-regulated histone H2B family gene, TaH2B-7D.

Histone proteins have been proved to be involved in multiple stress response [17]. For example, some histone protein variants are involved in the response to low phosphate and drought stress response, and temperature perception [17, 22, 23]. Moreover, deacetylation, methylation and ubiquitination of histone proteins are also involved in plant drought response [18–21]. A recent study showed that overexpression Arabidopsis AtHUB2 gene in cotton increases the global H2B monoubiquitination (H2Bub1) level through a direct interaction with GhH2B1 and up-regulates the expression of drought-related genes in transgenic cotton plants [21]. Coincidentally, the expression level of TaH2B-7D was also up-regulated by drought stress in wheat (Fig. 1a, b). The evidences indicate that H2B proteins may play a role in plant drought stress response. Since the expression of TaH2B-7D was significantly up-regulated by DS (Figs. 1, 2), VIGS as a post-transcriptional gene silencing technology, is suitable for the functional study of this gene. In the VIGS trial, we observed a significant decrease of TaH2B-7D expression level in all the four independent BSMVTaH2B-7D-infected lines, indicating that the expression level of TaH2B-7D was efficiently knocked-down (Fig. 2). Conventionally, the degree of leaf drooping is less, and leaves can maintain a relatively higher RWC in drought-tolerance plants than drought-sensitive individuals under DS conditions [58, 59]. Thus, the RWC of plant leaves can be used to at least partially assess the drought tolerance of a plant. In the TaH2B-7D knocked-down plants, leaf RWC...
We designed and the sequence of the inserted cDNA fragment was confirmed by Sanger sequence when constructing the VIGS vectors, the possibility of knocking down some homologous genes of TaH2B-7D could not be completely ruled out. Therefore, further studies are needed to generate transgenic lines that overexpress and/or underexpress TaH2B-7D to better understand the function of this gene.

**Conclusion**

Knock-down the expression level of TaH2B-7D in wheat plants significantly increased leaf relative electrolyte leakage rate and MDA content, decreased leaf RWC and proline accumulation, and reduced wheat drought tolerance. Therefore, TaH2B-7D potentially plays a vital role in conferring drought tolerance in hexaploid wheat.

**Abbreviations**

VIGS: virus-induced gene silencing; MDA: malonaldehyde; RWC: relative water content; DS: drought stress; NS: no stress; FC: field capacity; PTGS: post-transcriptional gene silencing; BSMV: barley stripe mosaic virus; BMV: brome mosaic virus; RTBV: rice tungro bacilliform virus; CymMV: cymbidium mosaic virus; LN: low nitrogen; SS: salt stress.

**Authors’ contributions**

XW and YR performed most of the experiments; JL took part in partial work of this research; ZX and ZW gave many advices during the research; YR and TL designed the experiments; YR wrote the paper. TL gave many helpful suggestions on the writing. All authors read and approved the final manuscript.

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**Competition of interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

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