CLONING AND CHARACTERIZATION OF A WHEAT RING FINGER GENE *TaRHA2b* WHOSE EXPRESSION IS UP-REGULATED BY ABA TREATMENT

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(Received 27th Feb 2019; accepted 3rd May 2019)

Abstract. Preharvest sprouting in wheat (*Triticum aestivum L.*) is the germination of grains in the ears when long range rainfall or damp conditions prior to harvest occur. E3 ubiquitination (type RING-H2) RING finger protein plays a key role in dealing with abiotic stresses in plants, which could be used to improve the PHS resistance of wheat. The full-length cDNA of a wheat RING finger gene named *TaRHA2b* was firstly cloned from wheat. Bioinformatic analysis and expression profile analysis of the *TaRHA2b* gene were carried out. The results indicated that the full-length cDNA of *TaRHA2b* was 845bp containing 465bp open reading frame which encoded 154 amino acid residues. The genomic *TaRHA2b* gene had no introns. The protein encoded by *TaRHA2b* gene was consisted of Zinc finger RING-type profile. Blast and phylogenetic analysis showed that the protein encoded by *TaRHA2b* shared the identity with *RHA2b* from the *Arabidopsis*. The result of semi RT-PCR showed that expression of *TaRHA2b* gene was significantly tissue-specific. The result of qRT-PCR showed that the expression of *TaRHA2b* in the seeds was significantly higher than the expression of gene expression after soaking germination. The sensitivity of this gene to abscisic acid was significantly increased. *TaRHA2b* gene may play an important role in seed dormancy during germination, which could be used to improve the PHS resistance of wheat.
Keywords: *wheat, bioinformatics analysis, expression profile, seed dormancy, preharvest sprouting*

Abbreviations: ABA, abscisic acid; PHS, preharvest sprouting; RING, Really Interesting New Gene; E1, ubiquitin-activating enzymes; E2, ubiquitin-conjugating enzymes; E3, ubiquitin protein ligase; ORF, open reading frame; Rel.Exp, relative expression of gene

Introduction

Pre-harvest sprouting (PHS) is one of the major adverse effects of high yield and stable yield of wheat (Xiao et al., 2002a; Li et al., 2004). In China, the phenomenon of wheat spike germination is particularly serious. Spike germination occurred frequently and seriously in the world's major wheat producers, including Canada, the United States, Britain, Australia, Brazil, Germany, Sweden and so on. Direct annual losses caused by PHS approach $1 billion dollars worldwide (Liu et al., 2013). The yield reduction caused by the problem of wheat spike germination accounts for nearly 83% of the total wheat planting area. Seed viability and hydrolysis of starch and protein in the endosperm are always reduced after PHS (Xiao et al., 2002b). At present, most of the wheat varieties used in the production have certain ear sprouting characteristics. The way to solve the PHS was as follows: firstly, the application of chemical control technology; secondly, breeding varieties with resistance to PHS. Breeding resistant cultivars is the best way to solve the problem of sprouting, but the selection and application was restricted by limited resistance source. It is very important to excavate

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1704_74957510
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good genes of PHS resistance, which is an ideal way to solve the PHS problem. In order to make full use of the RING finger gene to improve the resistance to PHS in wheat, a RING finger transcription factor gene *TaRHA2b* was cloned from wheat by RT-PCR and RACE amplification. Bioinformatic analysis and expression profile analysis of the *TaRHA2b* gene with the treatment of different abscisic acid (ABA) concentration were carried out. The research could provide some reference for studying the mechanism of *TaRHA2b* gene in wheat dormancy, which may be used as an excellent gene resource to improve PHS resistance.

Review of literature

The ubiquitination process is accomplished by the continuous action of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin protein ligases (E3), and the specificity of the ubiquitin-conjugating enzyme E3 binding substrate to a great extent (Smalle and Vierstra, 2004). In the *Arabidopsis* genome, more than 1300 genes are predicted to encode different types of E3 ligases, which means that ubiquitination is involved in a wide range of cellular metabolic processes and fine adjustment of life activities (Li et al., 2017). It was found that ubiquitination linked many components of plant biology through molecular genetic analysis, including cell cycle, embryonic development, light morphogenesis, regulation of day and night patterns, hormone signaling, homologous transformation, resistance to disease and aging (Liu et al., 2017).

Ubiquitin E3 ligases are mainly classified into three groups: RING (Really Interesting New Gene) finger protein family, HECT protein family and U-box family. At present, Ring finger protein family was found to be the largest among them. There were 469, 488, 399, 725, 330 and 688 possible RING finger E3 ligase family members in *Arabidopsis thaliana*, rice, poplar, soybean, grape and apple, respectively (Kraft et al., 2005; Du et al., 2009; Lim et al., 2010; Li et al., 2011).

RING finger protein consists of a large family of proteins, which is ubiquitous in eukaryotic organisms. The RING finger domain is a typical structural feature, and it has been shown that RING finger domain plays an important role in abiotic stress of plants (Zeba et al., 2009). The RING finger domain contains a conserved amino acid sequence: Cys-X2-Cys-Xn (9-39)-Cys-X (1-3) -His-X (2-3) –Cys/His-X2-Cys-X (4-48) -Cys-X2-Cys (Cys is a cysteine residue, His is a histidine residue, X is an arbitrary amino acid residue) (Kraft et al., 2005). According to the number and location of Zn^{2+} binding residues Cys and His, the RING finger family can be divided into nine subgroups: RING-HC, RING-H2, RING-v, RING-C2, RING-D, RING-S/T, RING-G, RING-mH2 and RING-mHC (Li et al., 2011). At present, many RING finger proteins have been isolated from many plants.

In the previous study, RHA2b played an important positive role in ABA-mediated seed maturity and early germination in *Arabidopsis* (Li et al., 2011). In order to solve the problem of wheat PHS, scholars at home and abroad have conducted a series of studies on the mechanism of wheat ear germination and made many remarkable achievements. The apparent physical and physiological characteristics of wheat seeds, including ear morphology (Zanetti et al., 2000), the color of the seed coat (Torada and Amano, 2002; Bassoi and Flintham, 2005), seed structure and water absorption (King and von Wettstein-Knowles, 2000), seed dormancy (Andreoli et al., 2006; Hughes et al., 2010), ABA content (Gerjets et al., 2010) and α-amylase activity (Major et al., 2001), was studied. The characteristics of genes related to PHS or seed dormancy and the

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positional cloning of QTLs (Zanetti et al., 2000; Major et al., 2001; Bassoi and Flintham, 2005; Somers et al., 2007; Chen et al., 2008) was studied. PHS resistance in wheat is a quantitative trait, and relevant quantitative trait locuses linked to PHS have been reported on almost all chromosomes (Ogbonnaya et al., 2008; Munkvold et al., 2009). From the existing research results, most researchers agree that the germination of wheat spike is the result of the interaction between genotype and environment. The dormancy characteristics of plants are closely related to their spike germination characteristics. PHS-resistant wheat germplasm resources are scarce. The *VP1* and *Trx* genes have been used to increase the PHS resistance of wheat (Li et al., 2009; Huang et al., 2012). Although these attempts have yielded some progress in controlling seed dormancy and PHS, more work is required.

Materials and methods

The wheat variety "Zhengmai 9023" was tested. Zheng mai 9023 is an excellent wheat variety, but it is prone to sprouting. Further research will be carried out based on the transgenic operation of this variety to improve the resistance of PHS. Plant expression vector PGM-T, Escherichia coli DH5a and Taq DNA polymerase were purchased from Tiangen (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). MiniBEST Plant Genomic DNA Extraction Kit, Plant total RNA extraction reagent Trizol, PrimeScript^{TM} RT reagent Kit (Perfect Real Time) kit, T4 DNA ligase, TranZol^{TM} Plant kit and SYBR Premix Ex Taq^{TM} II (Tli RNaseH plus) were purchased from TaKaRa (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Japan).

Extraction of total DNA and RNA and synthesis of cDNA first strand

DNA was extracted from wheat germ with MiniBEST Plant Genomic DNA Extraction Kit. Trizol reagent was used to extract the total RNA from wheat germ. The extracted RNA was tested for quality and purity and then stored in a refrigerator at -80°C for reserve. PrimeScript^{TM} RT reagent Kit (Perfect Real Time) was used to synthesize cDNA from total RNA. All operations are performed according to the instructions of the kits. The cDNA was stored at -80°C for later use.

Cloning of TaRHA2b gene

According to the amino acid sequence of RING finger gene *AtRHA2b* of *Arabidopsis thaliana*, GenBank EST data was searched and many expression sequences of its highly homologous origin were obtained. By software splicing, analysis and in vitro splicing, a complete cDNA sequence with RING finger structure domain was obtained from wheat database (National Center for Biotechnology information). According to splicing products, specific Primer5.0 software was used to design the primers for the experiment (*Table 1*), and target gene prediction and in vitro splicing were conducted.

The wheat cDNA was used as template for PCR amplification. PCR reaction system 20 μL: 10x PCR Buffer 2.0 μL, 2.5 mM dNTPs 1.6 μL, 10 mM upstream and downstream primers each 0.8 μL, Taq enzyme 0.3 μL, cDNA template 1.0 μL, ddH_2O complement 20 μL. Response procedures: 94°C pre degeneration 3 min; 94°C modified 30 s, 50°C annealing 40 s, 72°C for 1 min, 30 cycle; 72°C extension time for 10 min.

The primer pairs P1-F and P1-R were designed according to the sequence. By using bioinformatics mosaic and RACE amplification, the unknown segment of the gene was

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cloned. The full-length cDNA sequence of wheat *TaRHA2b* gene was obtained by RT-PCR amplification with specific primers P2-F and P2-R designed according to the above product sequences. The PCR products were recovered and connected with the pGEMT vector, and the recombinant plasmid transformed into E. coli DH5α competent cells, and the positive clones were screened and sent to BGI (HuaDa Biotechnology co., ltd., China) for sequencing.

Table 1. *Primers sequences of the experiment*

Primers name	Primer sequence (from 5'sequence-3'sequence)
P1	F: TGCCCGAGGAGGTCAAGGAG
	R: CGTGATTGGATGGCTACTATACAAAGTG
P2-1	F: CAGGTGGTCCGGCCGAGGTCGAT
	R: CCAGGCACACGATGCACGTCGCCG
P2-2	F: GCGGTGGCGGCCGACACAGAGA
	R: CAAAATTGGGATTTTATTTAGCTTATTATTAG
P2	F: CGCAAACGATAGACAGGCCTG
	R: GCTCGACAACTGTAAAGTCTAGGT
P2-3	F: GTCTCCGGCCATGGGGTTCCC
	R: CGAGCTGTTGACATTTCAGATCCACTAGC
P3	F: GTTCCAATCTATGAGGGATACACGC
	R: GAACCTCCACTGAGAACAACATTACC
P4	F: GGTGGATCGACCTCGGC
	R: GCTGGGAAAACGAAAGACG

Bioinformatics analysis of TaRHA2b gene

Based on cDNA sequence, using NCBI site ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) speculation TaRHA2b open reading frame, SingaIP TaRHA2b protein 4.1 (http://www.cbs.dtu.dk/services/SignalP/) analysis of signal peptide, TMHMM Server2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict transmembrane regions (Krogh et al., 2001). Using SMART online server (http://smart.embl-heidelberg.de/) domain analysis of protein structure. The consistency of *TaRHA2b* homologous genes with other species was analyzed using DNAMAN, and the evolution tree was constructed with the software MEGA 7.0. The RHA2b interacting proteins network can be predicted by string software.

Tissue specific expression analysis of TaRHA2b gene

The Root, stem, leaf, cob, lemma and endosperm from 25d flowering wheat plants were taken. RNA was extracted, and reverse transcription was conducted to synthesize the first strand of cDNA.

And PCR amplification was conducted with the first strand of cDNA as template. The reaction system was the same as above. Reaction conditions: 94°C for 3 min, 94°C for 30 s, 54°C for 30 s, 72°C for 40 s, a total of 28 cycle, 72°C extension time for 10 min, electrophoresis detection of PCR products.

The *Actin* gene products amplified by wheat *Actin* gene specific primers were taken as internal reference. The primers of *Actin* gene used in PCR reaction were P3-F and P3-R (*Table 1*). And the primers of *TaRHA2b* gene were P4-F and P4-R (*Table 1*). According to the PCR reaction mixture system, PCR amplification conditions as follows: 94°C 3 min, 94°C for 30 s, 54°C for 30 s, 72°C for 35 s, a total of 28 cycle. 72°C

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DOI: http://dx.doi.org/10.15666/aeer/1704_74957510
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for 10 min, RT-PCR product after 1.0% agarose electrophoresis detecting camera, preservation. The experiment was repeated for three times.

Analysis of TaRHA2b gene expression under ABA treatment

To further study the ABA response of *TaRHA2b* gene during seed germination, qRT-PCR was used to analyze the expression of *TaRHA2b* gene by ABA treatment at different concentrations and at different times at the same concentration. ABA solutions of different concentrations (*Table 2*) were used to treat wheat seeds with full and equal grain size three days after germination after disinfection. RNA was extracted with TranZol^{TM} Plant kit after 12 hours of sampling. PrimeScript^{TM} RT reagent Kit (Perfect Real Time) was used to synthesize cDNA from total RNA. All operations are performed according to the instructions of the kits. The cDNA was stored at -80°C for later use.

Table 2. *Different content of the ABA in the present study*

Group number	ABA content (μM)
1	0
2	0.25
3	0.5
4	1.0
5	2.0
6	3.0
7	4.0
8	5.0

Wheat seeds with full and equal size after disinfection were soaked with ABA solution of 0.5 μM, treatment without ABA was set for control, and samples were taken at different time (*Table 3*). RNA of dry seeds without soaking was extracted at the same time. PrimeScript^{TM} RT reagent Kit (Perfect Real Time) was used to synthesize cDNA from total RNA. The cDNA was numbered and stored at -80°C for later use.

Table 3. *Sampling time of the experiments with the treatment of 0.5 μM ABA*

Group number	Sampling time (h)
1	0
2	12
3	24
4	36
5	48

Fluorescence quantitative PCR was used to analyze the *TaRHA2b* gene expression under ABA treatment with the primers P4-F and P4-R and internal reference *Actin* gene primers P3-F and P3-R (*Table 1*). According to the relative quantitative method to calculate: The relative expression of gene (Rel.Exp) = $2^{-\Delta\Delta Ct}$, among them $-\Delta\Delta Ct$ = Calibrator ΔCt - ΔCt (the unknown sample), ΔCt (unknown sample) = (Ct) internal gene - (Ct) target gene, Calibrator ΔCt = (Ct) reference sample internal gene - (Ct) reference sample target gene. The reaction system of fluorescence quantitative PCR was 20 μL: SYBR Premix Ex Taq^{TM} II, 10 μL, PCR Forward Premer (10 μM) 0.8 μL, PCR Reverse Premer (10 μM) 0.8 μL, ROX Reference Dye II (50x), cDNA template 2 μL, ddH_2O up to 20 μL.

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Statistical analysis

The software package SPSS 13.0 was used for statistical analysis. For comparing results of different treatments, the Tukey ANOVA test was performed. Differences were considered significant for $P<0.05$.

Results

Analysis of TaRHA2b gene sequence and its coding protein structure

cDNA cloning of wheat *TaRHA2b* gene was synthesized using bioinformatics splicing combined with RACE amplification. First, the amino acid sequence of RING finger gene *AtRHA2b* was used to search for GenBank EST data, and the wheat EST (CA741783) with high homology was obtained (*Fig. 1A*). Secondly, specific primers P2-1-F and P2-1-R, P2-2-F and P2-2-R were designed according to the wheat EST sequence, and 5 'RACE (*Fig. 1B*) and 3' RACE (*Fig. 1C*) were respectively used to clone the unknown regions of the TaRHA2b gene. Finally, according to the above product sequence design, the specific primers P2-F and P2-R were used to amplify the full-length *TaRHA2b* fragment by RT-PCR (*Fig. 1D*), and the target sequence was obtained. The sequencing results confirmed that the length of *TaRHA2b* gene was 845 bp, including the 5 '-end non-coding sequence of 79 bp, the 3' -end non-coding sequence of 301 bp and the open reading frame (ORF) of 465 bp, encoding 155 amino acids (*Fig. 2*), the molecular weight was about 16.94 kD, and the isoelectric point was 7.77, belonging to weakly alkaline protein. This gene was named *TaRHA2b*. The GenBank accession number of the *TaRHA2b* gene is JN661690.1.

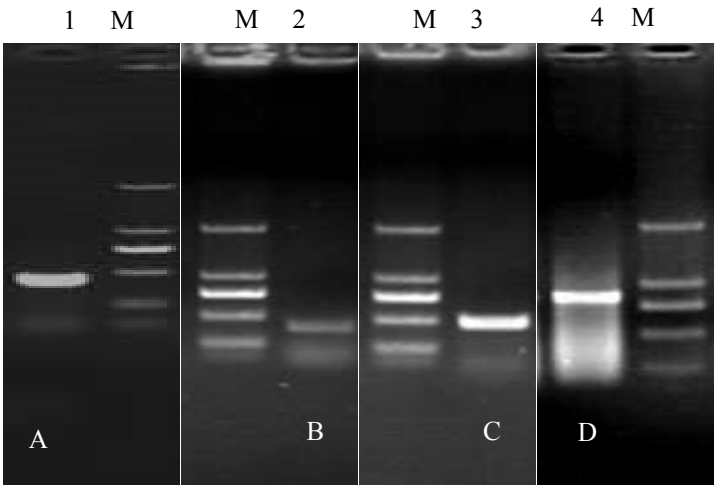

Figure 1. *Amplification of cDNA fragments of TaRHA2b gene in wheat immature endosperm. A, EST of TaRHA2b fragment; B, 3' RACE of TaRHA2b fragment; C, 5' RACE of TaRHA2b fragment; D, full-length TaRHA2b fragment; M, marker*

To further obtain the detailed information of the *TaRHA2b* gene, PCR amplification was performed using primers P2-3-F and P2-3-R (*Table 1*) with wheat genomic DNA as the template. The amplification products were sequenced. The analysis of sequencing showed that *TaRHA2b* gene had no introns.

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The results of SingaIP 4.1 analysis of the signal peptide software showed that TaRHA2b contains a signal peptide sequence with a length of 32 (S^1-F^{32}) and 39 (M^1-M^{40}) amino acid residues, indicating that this kind of protein needs transmembrane transfer (localization) during the synthesis process, and then the signal peptide can be removed under the specific protease to form the mature protein sequence. Further structural analysis revealed that all RHA transcription factors, including TaRHA2b, contained motif with a length of 49 amino acid residues (RING-H2 motif), $CX_2CX_{14-15}CXHX_2HX_2CX_3WX_{6-8}CPXC$.

```
1          CGCAAACGATAGACAGGCCTGGCCGGCGCAGCCTGTGACGTTCGAGCTCCCGCCGGGCGT
61         CCACGCGTCGTCTCCGGCCATGGGGTTCCCCCTGGTGTGCTACTGCGTCGCCATCCCCAA
1                              M  G  F  P  L  V  C  Y  C  V  A  I  P  K

121        GCCGGTCATCGCCTTCTGCAAGCTCGTCGCCGCCGTCAGGGACGCCCTCCTCCTGCTGCT
15           P  V  I  A  F  C  K  L  V  A  A  V  R  D  A  L  L  L  L  L

181        CTCCCTCGTCGGCCTCTGCCGCTCCCCGCGCCGCTCTGTGGACGACGCCCCGCTGCCCGA
35           S  L  V  G  L  C  R  S  P  R  R  S  V  D  D  A  P  L  P  E

241        GGAGGTCAAGGAGCGCCTCCCGGCCGTCGAGTTCGGCTGCCTGGCGCGTCCGGCGCAGCA
55           E  V  K  E  R  L  P  A  V  E  F  G  C  L  A  R  P  A  Q  Q

301        GCAGCAGCACGACGGGGACGACGACGAGGTCGCCGCCGCGGCGACGTGCATCGTGTGCCT
75           Q  Q  H  D  G  D  D  D  E  V  A  A  A  A  T  C  I  V  C  L

361        GGAGAGGCTGCGGGCGACGGACGAGGTGCGGCGGCTGGGCAACTGCGCGCACGCCTTCCA
95           E  R  L  R  A  T  D  E  V  R  R  L  G  N  C  A  H  A  F  H

421        CCGGGGCTGCATCGACGGGTGGATCGACCTCGGCCGGACCACCTGCCCGCTGTGTCGCTC
115          R  G  C  I  D  G  W  I  D  L  G  R  T  T  C  P  L  C  R  S

481        CCACCTACTGCCTCGCGCGCGGAGGGACGGCCCGCTCGCCAGCCTCCTCACGCGCGTTTG
135          H  L  L  P  R  A  R  R  D  G  P  L  A  S  L  L  T  R  V  W

541        GTGACGACCAACCGCGCCAGGTTAGCTCGAAGGCCACGTCTTTCGTTTTCCCAGCGCGGT
155          *

601        GGCGGCCGACACAGAGATTCGCGATCTCGACCGGCTGCATGTGTATTTAATGTGGGTTTA
661        CATCATCTCAAAATTGGGATTTTATTTAGCTTATTATTAGGGCGCCACTTTGTATAGTAG
721        CCATCCAATCACGAGGTCACCAATAGGTGCATACGCATAGTTTTTTCCCCCCGTAGAGAA
781        GGATTCTTTGGTCGAACATATGTGTACTAGCGTTTGGCTAGTGGATCTGAAATGTCAACA
841        GCTCG
```

Figure 2. *Nucleotide and deduced amino acid sequences of the TaRHA2b gene*

TaRHA2b encoding amino acid sequence alignment and phylogenetic tree

The bioinformatics software DNAMAN was used to sequence and predict the amino acid sequence of *TaRHA2b* gene (*Fig. 2*). The protein encoded by *TaRHA2b* gene was consisted of Zinc finger RING-type profile (*Fig. 3*).

```
MGFPLVCYCVAIPKPVIAFCKLVAAVRDALLLLLSLVGLCRSPRRSVDDAPLPEEVKERLPAVEFG
CLARPAQQQQHDGDDDEVAAAATCIVCLERLRATDEVRRLGNCAHAFHRGCIDGWIDLGRTTCPLC
RSHLLPRARRDGPLASLLTRVW
```

Figure 3. *Domain analysis of TaRHA2b protein. The yellow part of the amino acid corresponds to the blue part of the functional domain*

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DOI: http://dx.doi.org/10.15666/aeer/1704_74957510
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The sequence combination and similarity analysis of RING-H2 motif with 4 types of RING finger proteins (BOXSHADE 1.80) were performed (*Fig. 4*). The results showed that the amino acid sequence from wheat was similar with RHA2a and RHA2b from the *Arabidopsis*, indicating that this RING finger transcription factor and AtRHA2b belonged to the same family.

Figure 4. *Comparison of the RING finger motif sequences of four type RHA proteins. The missed base is marked with "-"; conserver residues are marked with "*"; TaRHA2b (AEQ67396), AtRHA1a (NP_192876), AtRHA1b (NP_192875), AtRHA2a (NP_172962), AtRHA2b (OAP11108), AtRHA3a (NP_179337), AtRHA3b (NP_195273) and NaRHA4a (XP_019266728) are the RING finger motif sequences of four type RHA proteins from Triticum aestivum, Arabidopsis thaliana and Nicotiana attenuate*

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By applying MAGE software, the protein sequence of RING finger transcription factor was compared multiple times to map the phylogenetic tree (*Fig. 5*). The results showed that *TaRHA2b* gene was very similar to *AtRHA2b* gene.

The interacting protein network of RHA2b includes KIN1(AT5G15960.1), ATDI8(AT5G66400.1), AT5G12110(AT5G12110.1), KUOX1(AT5G07480.1), KUF1(AT1G31350.1), AIRP1(AT4G23450.2), SDIR1(AT3G55530.1), KEG(AT5G13530.1), AT5G58410(AT5G58410.1) and NAC019(AT1G52890.1) (*Fig. 6*). RHA2b is involved in the ubiquitination of proteins, cell protein metabolism, and response to acidic compounds. The function of proteins interacting with RHA2b is listed in *Table 4*. The results showed that *RHA2b* gene is essential for plant growth and development and stress response.

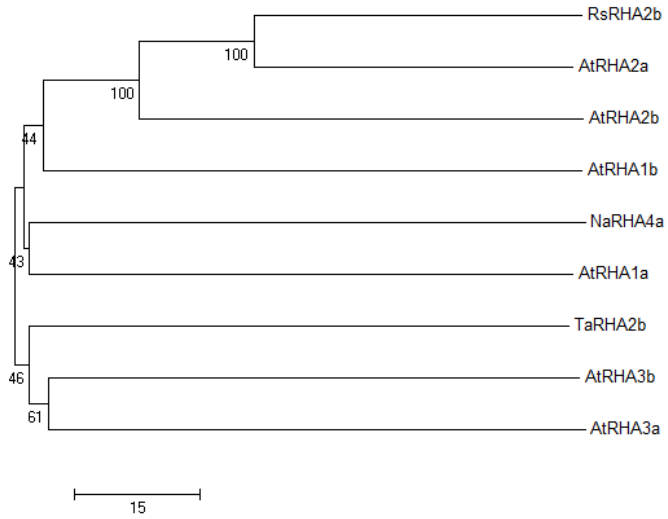

Figure 5. *A phylogenetic tree of the RING finger motifs (with the software MEGA 7.0)*

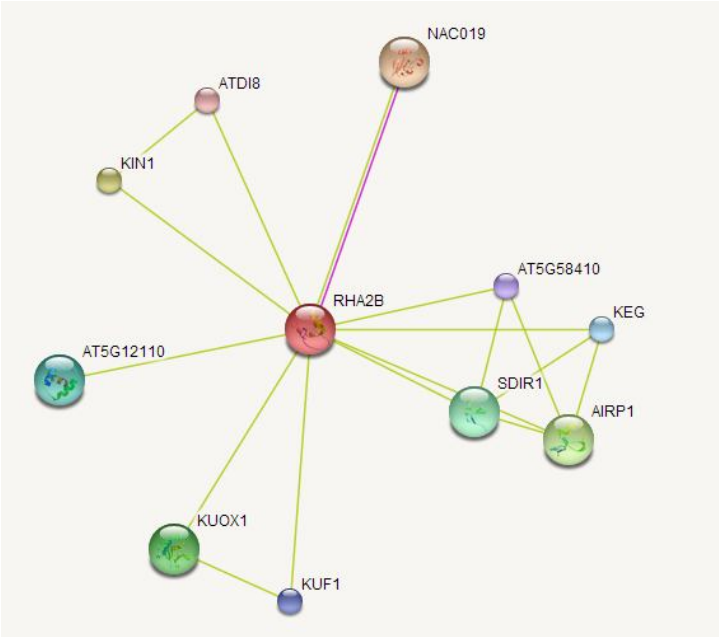

Figure 6. *Interaction network of RHA2b protein (prediction by string software)*

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Table 4. *The function of proteins interacting with RHA2b (prediction by string software)*

Proteins	Function
KIN1, ATDI8, SDIR1 and KEG	ABA stress response
KIN1, ATDI8, SDIR1 and NAC019	water loss stress response
RHA2b, SDIR1, KEG, AT5G58410 and AIRP1	the process of protein ubiquitination
KIN1, ATDI8, SDIR1, NAC019, RHA2b and KUF1	non-biological stress response
KIN1, ATDI8, SDIR1, NAC019 and KEG	acidic and oxygen-containing stress response
KIN1 and ATDI8	low temperature stress response
SDIR1, KEG, RHA2b, AT5G58410, AT5G12110 and KUF1	the process of cell protein metabolism

Tissue specific expression analysis of TaRHA2b gene

The root, stem, leaf, cob, lemma and endosperm of wheat were used to detect expression arrays of *TaRHA2b* gene (*Fig. 7*). There was strong expression of *TaRHA2b* gene in the cob, lemma and endosperm tissue, while weak expression of *TaRHA2b* gene in root, stem and leaf tissue. The result showed that the expression of *TaRHA2b* gene in wheat is tissue-specific.

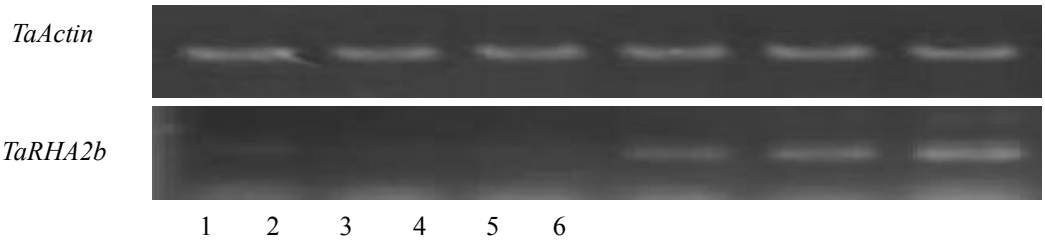

Figure 7. *Expression analysis of TaRHA2b gene in different organs of wheat. Expression was analyzed by semi-quantitative RT-PCR.1, roots; 2, stems; 3, leaves; 4, cob; 5, lemma; 6, endosperm*

Expression profile of TaRHA2b gene under ABA treatment

The results of tissue specific expression analysis of *TaRHA2b* gene showed that *TaRHA2b* gene was highly expressed in endosperm, and it can be inferred that *TaRHA2b* gene may be involved in seed germination. ABA is an important plant hormone that regulates seed maturation, development and germination. Therefore, in order to study whether *TaRHA2b* gene is involved in ABA signaling pathway, we analyzed the response of *TaRHA2b* gene to exogenous ABA during wheat seed germination.

The results showed that the transcription expression of *TaRHA2b* gene was induced by ABA (*Fig. 8*). The different ABA concentration (0 μM, 0.25 μM, 0.5 μM, 1.0 μM, 2.0 μM, 3.0 μM, 4 μM and 5 μM) was used to explore whether the *TaRHA2b* gene was sensitive to ABA or not. With the increase of ABA concentration, the gene expression level gradually increased. The gene expression level was at the expression peak at 0.5 μM, which was extremely significant compared with the control ($p < 0.01$). After that, the *TaRHA2b* gene expression level gradually decreased. And The expression level of the *TaRHA2b* gene was similar between the experimental group and the control group. So the 0.5 μM ABA concentration was used for follow-up research.

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ABA response analysis of TaRHA2b gene in early stage after germination

It showed that *TaRHA2b* gene expression is highest in dry seeds (*Fig. 9*). During seed germination, *TaRHA2b* gene expression decreased as a whole compared with dry seeds. It decreased most significantly at the stage of 12 h with 0.5 μM ABA treatment and increased at the stage of 24 h with 0.5 μM ABA treatment. The expression of *TaRHA2b* gene in the samples with the treatment of 0.5 μM ABA was higher than the samples without the treatment of ABA, and the second half of the S curve of *TaRHA2b* gene expression in the treatment group showed a tendency to be straightened.

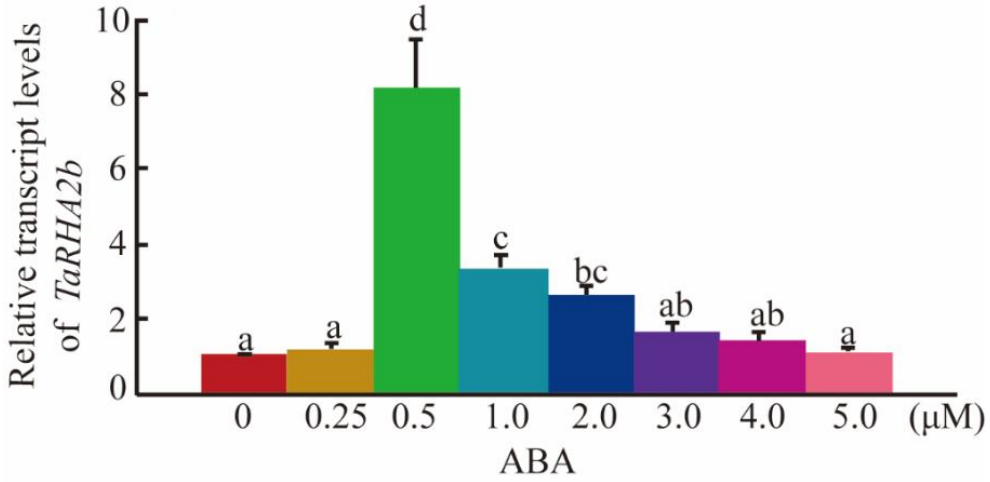

Figure 8. *TaRHA2b expression in 3-d-old seedlings with the treatment of different ABA content for 12h. The letters 'a' to 'd' indicate statistically significant differences by Turkey's least significantly difference test ($p<0.05$)*

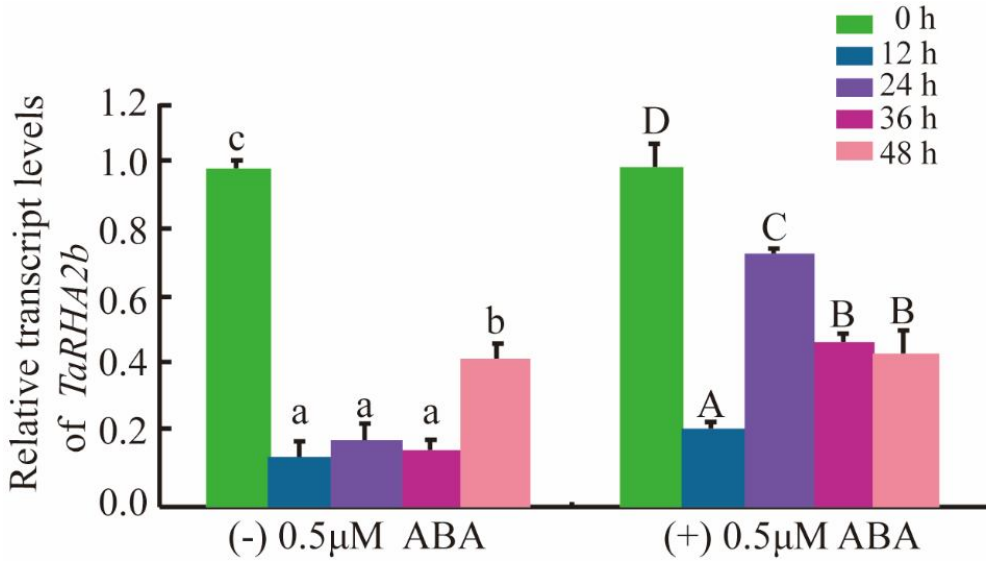

Figure 9. *TaRHA2b expression in seeds during germination with the treatment of 0.5μM ABA. (-) 0.5μM ABA, the letters 'a' to 'd' indicate statistically significant differences by Turkey's least significantly difference test ($p<0.05$); (+) 0.5μM ABA, the letters 'A' to 'D' indicate statistically significant differences by Turkey's least significantly difference test ($p<0.05$)*

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Discussion

Many studies have shown that over-expression of RING-H2 protein in model plant can affect abiotic stress (Lyzenga and Stone, 2012; Stone, 2014). Some RING finger type E3 ubiquitin ligase is involved in some specific plant signaling pathways, mainly ABA signaling pathways (Devoto and Turner, 2003; Moon et al., 2004; Schwechheimer and Schwager, 2004; Hoecker, 2005; Dreher and Callis, 2007). Several RING finger genes have been reported. AIP2 and KEG, two proteins with RING finger structure in ABA signaling pathway, are two important positive regulators of ABI3 and ABI5, respectively, which are negative regulators of ABA signaling pathway (Stone et al., 2006). It was found that CIPK26 interacted with KEG through yeast two-hybridization, and proved that CIPK26 participated in ABI5-mediated and KEG-mediated ABA stress response (Lyzenga et al., 2013).Whether there is an interaction among the three protein (CIPK26, ABI5 and KEG) is unclear. SDIRIP1, the substrate of SDIR1, was degraded by ubiquitin-proteasome pathway. SDIR1 controlled ABA related germination and stress response. SDIRIP1 participates in ABA and salt stress pathways by negatively regulating ABI5 expression (Zhang et al., 2015). Overexpression of *AtXerico* in *Arabidopsis thaliana* and rice significantly increased plant sensitivity to ABA and salt, and significantly increased plant drought resistance (Ko et al., 2006; Zeng et al., 2015). Overexpression of *AtXerico* homologous gene *OsRHP1* in rice showed increased drought and salt tolerance, ABA content, and the expression of ABA biosynthetic genes and related genes (Zeng et al., 2014). The overexpression of *ZmXerico* in maize can improve drought resistance and ABA content. It is also proved that *ZmXerico1* plays a role in ABA dynamic equilibrium by regulating the stability of ABA 8 '-hydroxylase protein and is a new control point in the ABA regulation pathway (Brugiere et al., 2017).

A RING finger transcription factor gene *TaRHA2b* was cloned by RT-PCR and RACE in this study. It was found that the gene belonged to the typical RING-H2 finger family. The bioinformatics analysis results showed that *TaRHA2b* gene was very similar to *AtRHA2b*. Therefore, it is speculated that *TaRHA2b* is similar in function to *AtRHA2b*.

It can be seen that spike germination caused certain physiological damage to wheat (*Fig. 10*). The dormancy and germination of seeds are regulated by hormones and metabolic pathways inside the seeds. PHS resistance is closely related to seed dormancy. Varieties with strong dormancy have strong PHS resistance, while those with weak dormancy are easy to germinate. The external environment can influence the PHS resistance of wheat to some extent, but the most critical determinant is seed dormancy, especially the endogenous ABA content and the sensitivity of seeds to ABA. Genetic studies of several species have also confirmed that ABA can effectively induce seed dormancy.

Overexpression of *RHA2a* in transgenic *Arabidopsis* increased the sensitivity to ABA, increased seed dormancy and decreased seed germination rate (Bu et al., 2009). The *rha2b-1* mutant with high homology between AtRHA2b and AtRHA2a also showed ABA insensitive phenotype and drought sensitivity (Li et al., 2011). The interaction between ATAF2 and RHA2a in yeast were found. RHA2a could negatively regulate Flg22-induced root growth inhibition and seedling growth inhibition in *Arabidopsis thaliana* and promote the growth of Pst DC3000. RHA2a can activate positive regulators by mono-ubiquitinating and stabilizing certain key regulators of the ABA signaling pathway. ANAC019 and ANAC055, two closely related NAC family proteins, were identified as RHA2a-interacting proteins (Bu et al., 2008; Jiang et al.,

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2009). RHA2b targets MYB30 degradation to regulate ABA signal transduction (Zheng et al., 2018).

The analysis of the expression of wheat *TaRHA2b* gene showed its tissue specificity. There was higher expression of *TaRHA2b* gene in the cob, lemma and endosperm tissues than other tissues. It showed that *TaRHA2b* gene was involved in the regulation of seed formation and dormancy.

The germination rate of *Arabidopsis thaliana* seeds over-expression *AtRHA2a* and *AtRHA2b* significantly decreased and the sensitivity to exogenous ABA of them were enhanced. It suggested that *TaRHA2b* may play an equally important role in the dormancy-germination process and could be used as an excellent genetic resource to improve the germination characteristics of spike.

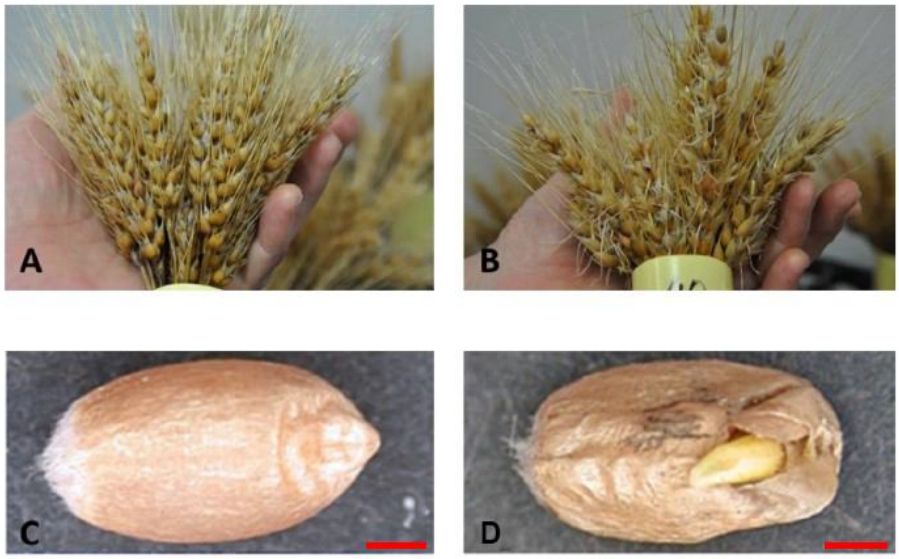

Figure 10. *Physical damage of wheat caused by PHS. A: Spike of PHS-resistant germplasm; B: Spike of PHS-susceptible germplasm; C: Grain of PHS-resistant germplasm; D: Grain of PHS-susceptible germplasm. The bar value is 1 mm*

The expression profile of *TaRHA2b* gene with the treatment of different ABA concentration showed that *TaRHA2b* gene was highly expressed in dry seeds. And its expression decreased rapidly after soaking germination, which was similar to the expression pattern of similar *AtRHA2b* gene in *Arabidopsis thaliana*. The results indicated that the function of *RHA2b* gene in plants is relatively similar, especially in ABA signal transduction pathway. The results showed that *TaRHA2b* gene may be an excellent genetic resource to improve PHS resistance. More work is needed for using *TaRHA2b* gene to improve dormancy and PHS tolerance in wheat.

Conclusion

The full-length cDNA of the RING finger gene *TaRHA2b* was cloned from wheat. The full-length cDNA of *TaRHA2b* encoded 154 amino acid residues, which was consisted of Zinc finger RING-type profile. The genomic *TaRHA2b* gene had no introns. TaRHA2b shared the identity with RHA2b from the *Arabidopsis*. The main result are as

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follows: (1) the expression of *TaRHA2b* gene was significantly tissue-specific; (1) the expression of *TaRHA2b* in the seeds was significantly higher than the expression of gene expression after soaking germination; (3) the sensitivity of the *TaRHA2b* gene to ABA was significantly increased. Some questions are as follows: (1) the distribution of *TaRHA2b* gene coding region in donor ancestors and different varieties could be studied to explore the mechanism mediated with *TaRHA2b* gene in PHS resistance;(2) the proteins interacted with TaRHA2b could be screened by yeast two hybrid system, which may be the new genes of PHS resistance genes; (3) how does the *TaRHA2b* gene regulate in ABA signal transduction. Further researches on the mechanism of wheat mediated with *TaRHA2b* gene in PHS resistance should be futher expanded in the future.

Acknowledgements. This work was supported by the "Twelfth Five-Year" National Science and Technology Projects in Rural Areas (2013BADD04B01-02) and Henan Science and Technology Project (162102110007). Hongbin Niu designed the research; Dongbing Li and Junpeng Lyu performed the experiment; Dongbing Li drafted the manuscript; Guizhen Lyu provided ideas; Xiang Wang and Jun Yin edited the manuscript; Dongbing Li and Guizhen Lyu read and approved the final manuscript. Authors state no conflict of interest.

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