

EXPRESSION AND BIOINFORMATIC ANALYSIS OF THE ROP FAMILY GENE *TaRAC* IN WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract. The Ras related C3 botulinum toxin substrate (RAC) play a key role in dealing with abiotic stresses in plants. The full-length open reading frame of a *TaRAC* was firstly cloned from wheat. Bioinformatic analysis and expression profile analysis of the *TaRAC* gene were carried out. The results indicated that the full-length open reading frame of *TaRAC* was 594bp which encoded 197 amino acid residues. The genomic *TaRAC* gene had no introns. The protein encoded by the *TaRAC* gene consisted of a small GTPase Rho family domain profile. Blast and phylogenetic analysis showed that the protein encoded by *TaRAC* shared the identity with *RAC* from corn (*Zea mays* L.). The result of qRT-PCR showed that expression of *TaRAC* gene was significantly tissue-specific. The result of qRT-PCR showed that *TaRAC* in the seeds was significantly higher than the expression of gene expression after soaking germination. The sensitivity of this gene to abscisic acid (ABA) significantly increased. *TaRAC* gene may play an important role in seed dormancy during germination, which could be used to improve the preharvest sprouting (PHS) resistance of wheat.

Keywords: wheat, preharvest sprouting, seed dormancy, abscisic acid, tissue-specific

Introduction

Preharvest sprouting (PHS) in wheat (*Triticum aestivum* L.) is the germination of grains in the ears when long range rainfall or damp conditions prior to harvest occur (Li et al., 2019c). Small GTP binding proteins have a class of GTP binding proteins with molecular weight of 20-40 KD, including five families of Ras, Ran, Rab, Rho and Arf, which are involved in the regulation of eukaryotic cell proliferation, signal transduction and material transport (Takai et al., 2001; Kahn et al., 1992; Christensen et al., 2004). At present, more than 100 small GTP binding proteins have been found in eukaryotes, forming a huge superfamily.

There are many members in Rho subfamily. A group of small GTP binding proteins related to Rho subfamily were isolated by sequencing *Arabidopsis* genome. These proteins are evolutionarily independent and unique to plants. Because of their highest similarity with Ras related C3 botulinum toxin substrate (RAC) protein in animals, they are called RAC (Rho related GTPase from plant) or Rop protein family. The small GTP binding proteins of the Rop family have many functions, including the regulation of plant cell polarity, cell growth, morphological development, cytokinesis, hormone signaling, plant pathogen interaction, stress response and so on (Berken and Wittinghofer, 2008).

Several reports have suggested a function of RAC/ROPs as direct or indirect modulators of plant gene expression (Poraty-Gavra et al., 2013; Scheler et al., 2016). RHO GTPases were considered as transcriptional regulators because their downstream factors strongly affect gene expression and chromatin structure (Rajakyla and

Vartiainen, 2014; Yu and Brown, 2015). It was found that a subset of plant Rac/Rop GTPases functions in mediating the auxin signal to downstream responsive genes basing on the tobacco overexpressing Rac/Rop GTPase, NtRac1 (Tao et al., 2002). Barley susceptibility factor RACB modulates transcript levels of signaling protein genes in compatible interaction with *Blumeria graminis* F. sp. *hordei* (Schnepf et al., 2018).

Transgenic crops can be used as powerful supplements to traditional breeding methods to meet the world's demand for high-quality food, and help combat malnutrition by improving yield, nutritional quality and resistance to various biological and abiotic stresses (Kamthan et al., 2016). *RsRHA2b* improves dormancy and preharvest sprouting tolerance in transgenic wheat (Bornman et al., 2019). Exogenous *RsRHA2b* gene promoted the expression of *YTH2456* (Li et al., 2019a). *TaRHA2b* interacts with *TaRAC* and other proteins (Li et al., 2019b). Blast analysis of the *YTH2456* show the sequence (AK251632.1) in barley and the *RAC* gene sequence (GU452718.1) in Wheat.

In order to make full use of the Rop family gene to improve the resistance to PHS in wheat, a Rop gene *TaRAC* was cloned from wheat by RT-PCR amplification. Bioinformatic analysis and expression profile analysis of the *TaRAC* gene with the treatment of different abscisic acid (ABA) concentration were carried out. The research could provide some reference for studying the mechanism of *TaRAC* gene in wheat dormancy, which may be used as an excellent gene resource to improve PHS resistance.

Materials and methods

Materials and ABA treatment

The wheat variety “Zhengmai 9023” was tested. The elite Chinese bread wheat cultivar Zhengmai 9023, a hexaploid wheat cultivar with weak resistance to PHS, which is widely cultivated in Henan Province.

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™ 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 *TaRAC* gene

According to the amino acid sequence of the *RAC* gene sequence (GU452718.1) in Wheat, 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 pM upstream and downstream primers each 0.8 µL, Taq enzyme (5 U/µL) 0.3 µL, cDNA template 1.0 µL, ddH₂O complement 20 µL. Reaction conditions: 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 and P2 were designed according to the sequence of wheat *TaRAC* gene. The target sequence was obtained by RT-PCR amplification with primers

P1 and P2. The PCR products were recovered and connected with the pGEMT vector, and the recombinant plasmid transformed into *E. coli* DH5 α 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	ATGAGCGCTTCTCGGTTTCAT
P2	TTACAAAAAGGTGGATCCTT
P3	GTTCCAATCTATGAGGGATACACGC
P4	GTTCCAATCTATGAGGGATACACGC
P5	CAGGTGGTCCGGCCGAGGTCGAT
P6	CCAGGCACACGATGCACGTCGCCG

Biological information analysis of *TaRAC* gene

A comprehensive bioinformatics analysis was conducted using tools including the NCBI (<https://www.ncbi.nlm.nih.gov/>), Swiss-Prot databases (<https://prosite.expasy.org/>), DNAMAN and MEGA 7.0. Based on cDNA sequence, using NCBI site ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) speculation *TaRAC* open reading frame. The consistency of *TaRAC* homologous genes with other species was analyzed using DNAMAN, and the evolution tree was constructed with the software MEGA 7.0.

***TaRAC* gene expression analysis**

The root, stem, leaf, cob, lemma and endosperm from 25 d 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. PCR reaction system 20 μ L: 10x PCR Buffer 2.0 μ L, 2.5 mM dNTPs 1.6 μ L, 10 pM upstream and downstream primers each 0.8 μ L, Taq enzyme (5 U/ μ L) 0.3 μ L, cDNA template 1.0 μ L, ddH₂O complement 20 μ L. Reaction conditions: 94 $^{\circ}$ C for 3 min, 94 $^{\circ}$ C for 30 s, 54 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 40 s, a total of 28 cycle, 72 $^{\circ}$ 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 and P4 (Table 1). And the primers of *TaRAC* gene were P5 and P6 (Table 1). According to the PCR reaction mixture system, PCR amplification conditions as follows: 94 $^{\circ}$ C 3 min, 94 $^{\circ}$ C for 30 s, 54 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 35 s, a total of 28 cycle. 72 $^{\circ}$ C for 10 min, RT-PCR product after 1.0% agarose electrophoresis detecting camera, preservation. The experiment was repeated for three times.

Wheat seeds (n = 150) with full and equal size after disinfection were soaked with ABA solution of 0.5 μ M (Li et al., 2019c), treatment without ABA was set for control, and samples were taken at different time (Table 2). RNA of dry seeds without soaking was extracted at the same time. PrimeScriptTM RT reagent Kit (Perfect Real Time) was used to synthesize cDNA from total RNA. The cDNA was numbered and stored at -80 $^{\circ}$ C for later use.

Fluorescence quantitative PCR was used to analyze the *TaRAC* gene expression under ABA treatment with the primers P5 and P6 and internal reference *Actin* gene primers P3 and P4 (Table 1). According to the relative quantitative method to calculate: The relative expression of gene (Rel.Exp) = $2^{-\Delta\Delta Ct}$, among them $-\Delta\Delta Ct = \text{Calibrator } \Delta Ct - \Delta Ct$ (the unknown sample), ΔCt (unknown sample) = (Ct) internal gene - (Ct) target gene, Calibrator $\Delta Ct =$ (Ct) reference sample internal gene - (Ct) reference sample target gene. The reaction system of fluorescence quantitative PCR was 20 μL : SYBR Premix Ex TaqTM II, 10 μL , PCR Forward Primer (10 μM) 0.8 μL , PCR Reverse Primer (10 μM) 0.8 μL , ROX Reference Dye II (50x), cDNA template 2 μL , ddH₂O up to 20 μL .

Table 2. Sampling time of the experiments with the treatment of 0.5 μM ABA

Group number	Sampling time (h)
1	0
2	1
3	2
4	4
5	8
6	12

Statistical analysis

The GraphPad Prism 8 was used for statistical analysis and drawing. For comparing results of different treatments, Variance analysis is followed by a post-hoc test in order to determine pairwise differences. Differences were considered significant for $P < 0.05$.

Results

Sequence analysis of gDNA and cDNA of *TaRAC* gene

It shows that the ORF region of *TaRAC* is 594 bp in length (Fig. 1). The obtained plasmid pGEM-T-*TaRAC* was sequenced. The results of sequences are listed in Figure 2. The sequencing results were matched with the accession number GU452718.1 after blast on NCBI, encoding 197 amino acid residues in an open reading frame with molecular weight of 21.662 kD and isoelectric point of 9.26. The obtained pGEM-T-*TaRAC* plasmids from cDNA and gDNA were sequenced and the results showed that there was no intron in the *TaRAC* gene in Figure 3.

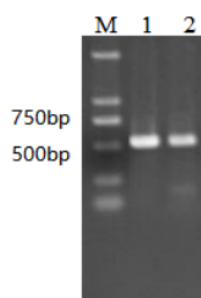


Figure 1. Sequence of the *TaRAC* gene. M, marker. 1 and 2, the target *TaRAC* fragment

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1      ATGAGCGCTT CTCGGTTCAT CAAGTGCCTG ACGGTGGGGG ACGGTGCCGT CGGCAAGACT
61     TGCCCTCTCA TCTCCTACAC ATCCAACACC TTCCCACCG ACTATGTCCC AACAGTCTTC
121    GACAACTTCA GCGCTAACGT TGTGGTTGAC GGGAGCACCG TCAACCTCGG ATTATGGGAT
181    ACTGCAGGAC AAGAGGACTA TAATAGACTA CGCCACTGA GCTACCAGAG TGCCGATGTC
241    TTCCTGCTCG CCTTTTCTCT TATCAGCAA GCAAGCTACG AGAATGTAC TAAGAAGTGG
301    ATCCCTGAGC TACGGCACTA TGCTCCTGGT GTGCCATAA TTCTTGTGCG GACAAAAGCTT
361    GATCTGCGGG ATGACCAGCA GTTTTTCTGT GATCACCTG GGGCTGTTCC TATTTCCACC
421    GCTCAGGGTG AAGAGCTGAA GAAGGTAATT GCGCGACCG CCTACATCGA GTGCAGCTCA
481    AAAACACAGC AGAACATCAA GGGGGGGTTT GATGGGGGAA TCAAGGGGTT TCTCCACCTT
541    CCAAACCAGA AGCGGAAGAA GAGGAAGTCG CAAAAGGAT CCACCTTTT GTAA

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Figure 2. The *TaRAC* fragment amplified by PCR

TaRac1-cDNA.txt	ATGAGCGCATCTCGGTTTCATCAAGTGCCTGACGGTGGGGGACGGCGCCGT	50
TaRac1-gDNA.txt	ATGAGCGCATCTCGGTTTCATCAAGTGCCTGACGGTGGGGGACGGCGCCGT	50
Consensus	atgagcgcatctcggtttcatacaagtgctgacggtgggggacggcgccgt	
TaRac1-cDNA.txt	GGGAAAGACATGCCTCCTCATCTCATACACATCCAACACCTTCCCCACAG	100
TaRac1-gDNA.txt	GGGAAAGACATGCCTCCTCATCTCATACACATCCAACACCTTCCCCACAG	100
Consensus	gggaaagacatgcctcctcatctcatacacatccaacaccttccccacag	
TaRac1-cDNA.txt	ACTATGTCCCAACAGTTTTTCGACAACCTCAGCGCTAACGTCGTGGTTGAC	150
TaRac1-gDNA.txt	ACTATGTCCCAACAGTTTTTCGACAACCTCAGCGCTAACGTCGTGGTTGAC	150
Consensus	actatgtcccaacagtttttcgacaacctcagcgctaaagtcgtggttgac	
TaRac1-cDNA.txt	GGCAGCACCGTCAACCTCGGATTATGGGATACTGCAGGACAAGAAGACTA	200
TaRac1-gDNA.txt	GGCAGCACCGTCAACCTCGGATTATGGGATACTGCAGGACAAGAAGACTA	200
Consensus	ggcagcacctgcaacctcggattatgggatactgcaggacaagaagacta	
TaRac1-cDNA.txt	TAATCGACTACGCCACTAAGCTACCGTGGTGCCTGATGTCCTTCTGCTCG	250
TaRac1-gDNA.txt	TAATCGACTACGCCACTAAGCTACCGTGGTGCCTGATGTCCTTCTGCTCG	250
Consensus	taatcgactacgccactaagctaccgtggtgctgctgcttctgctcgcg	
TaRac1-cDNA.txt	CCTTTTCTCTCATCAGCAAAGCAAGCTACGAGAATGTACTAAGAAGTGG	300
TaRac1-gDNA.txt	CCTTTTCTCTCATCAGCAAAGCAAGCTACGAGAATGTACTAAGAAGTGG	300
Consensus	ccttttctctcatcagcaaagcaagctacgagaatgtactaagaagtgg	
TaRac1-cDNA.txt	ATTCCAGAGTTACGGCACTATGCTCCTGGCGTGCCCATAAITCTTGTGG	350
TaRac1-gDNA.txt	ATTCCAGAGTTACGGCACTATGCTCCTGGCGTGCCCATAAITCTTGTGG	350
Consensus	attccagagttacggcactatgctcctggcgtgccccataaitcttgtgg	
TaRac1-cDNA.txt	AACAAAGCTTGATCTGCGGGATGACAAGCAGTTTTTTGTGGATCACCCCTG	400
TaRac1-gDNA.txt	AACAAAGCTTGATCTGCGGGATGACAAGCAGTTTTTTGTGGATCACCCCTG	400
Consensus	aacaaagcttgatctgcgggatgacaagcagttttttgtggatcacccctg	
TaRac1-cDNA.txt	GCGCGGTTCTTATTTCCACTGCTCAGGGTGAAGAGCTGAAGAAGGTGATT	450
TaRac1-gDNA.txt	GCGCGGTTCTTATTTCCACTGCTCAGGGTGAAGAGCTGAAGAAGGTGATT	450
Consensus	gcgcggttcttattttccactgctcagggtgaagagctgaagaaggtgatt	
TaRac1-cDNA.txt	GGCGCGACTGCCTACATCGAGTGCAGCTCAAAAACACAGCAGAACATCAA	500
TaRac1-gDNA.txt	GGCGCGACTGCCTACATCGAGTGCAGCTCAAAAACACAGCAGAACATCAA	500
Consensus	ggcgcgactgcctacatcgagtgcagctcaaaaacacagcagaacatcaa	
TaRac1-cDNA.txt	GGCGGTGTTTGATGCGGCGATCAAGGTGGTCTCCAGCCTCCGAAGCAGA	550
TaRac1-gDNA.txt	GGCGGTGTTTGATGCGGCGATCAAGGTGGTCTCCAGCCTCCGAAGCAGA	550
Consensus	ggcggtgtttgatgcgcgatcaaggtggtctccagcctccgaagcaga	
TaRac1-cDNA.txt	AGCGGAAGAAGGAAGTACAGAAAGGATGCAGCATCTTGTAA	594
TaRac1-gDNA.txt	AGCGGAAGAAGGAAGTACAGAAAGGATGCAGCATCTTGTAA	594
Consensus	agcggaagaaggaagtacagaaaggatgcagcatcttgtaa	

Figure 3. Comparative analysis between gDNA and cDNA sequences of *TaRAC*

TaRAC encoding amino acid sequence alignment and phylogenetic tree

After functional domain analysis of the encoded amino acids, it was found that there was a small GTPase Rho family profile, as shown in *Figures 4* and *5*. It contains a small

GTPase Rho family domain. It is a polypeptide formed by 1-177 amino acids and belongs to an alkaline protein. *TaRAC* shared the identity with *RAC* from the corn.

Expression analysis of the *TaRAC* gene

The relative expression of *TaRAC* gene in different tissues (*Fig. 6*) showed that the specific expression bands of *TaRAC* gene could also be detected in the root, stem, leaf, cob, lemma and endosperm samples of wheat. The highest expression bands were found in leaves, cob, lemma and endosperm, and the signal intensity was significantly higher than that in roots and stems, which indicated that the transcriptional expression of *TaRAC* gene had strong tissue specificity.

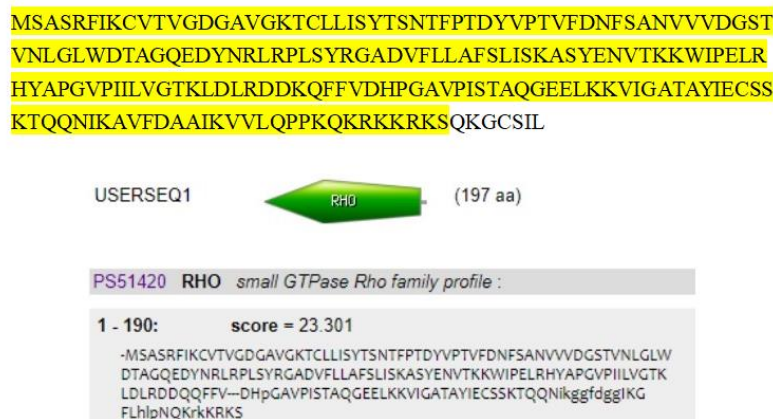


Figure 4. Domain analysis of amino acids encoded by *TaRAC* gene. The yellow part of amino acid corresponds to the green part of the functional domain

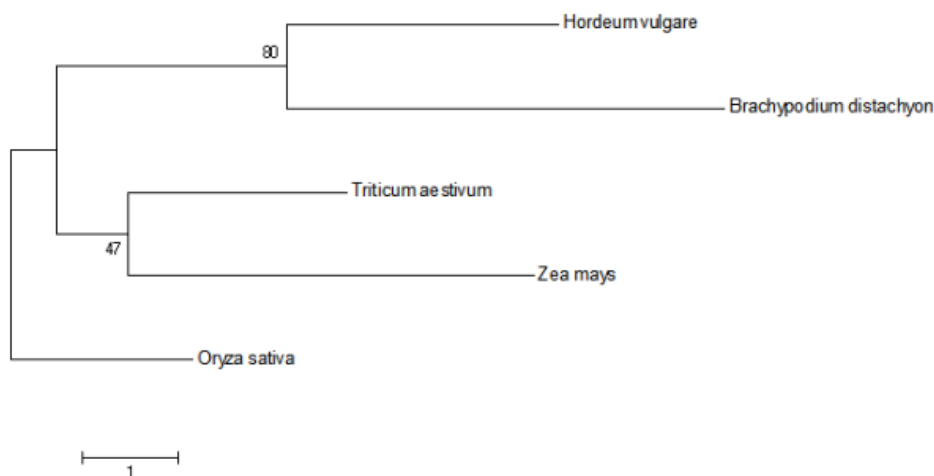


Figure 5. A phylogenetic tree of homology sequence with the *TaRAC* gene (with the software MEGA7.0). GU452718.1, *Triticum aestivum*, *rac*-type small GTP-binding protein mRNA; AK251632.1, *Hordeum vulgare*; XM_003570698.4, *Brachypodium distachyon*, *rac*-like GTP-binding protein 5 mRNA; EU968843.1, *Zea mays* clone 324497, *rac*-like GTP-binding protein 5 mRNA; AF218381.1, *Oryza sativa*, subsp. *japonica* small GTP binding protein *RACP* (*RAC*) mRNA

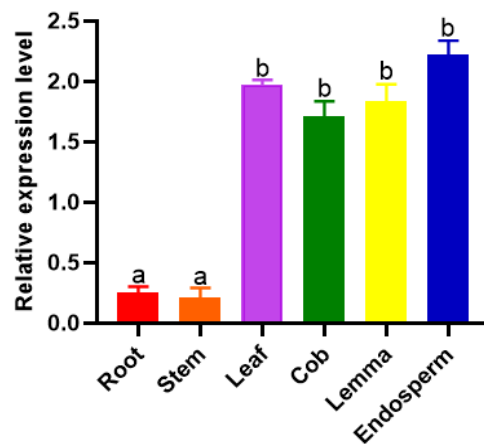


Figure 6. Expression analysis of *TaRAC* gene in different tissues of wheat. The letters 'a' to 'b' indicate statistically significant differences $P < 0.05$ (Tukey's least significantly difference test). Data are expressed as mean values standard errors from three replicates and error bars represent standard errors

***TaRAC* gene expression is sensitive to exogenous ABA treatment**

Different concentrations of ABA were used to treat wheat seedlings at two-leaf stage. Real-time fluorescence quantitative PCR (RT-PCR) amplification was performed with primers to analyze the response of *TaRAC* gene to ABA in early germination seedlings. Quantitative fluorescence assay (Fig. 7) showed that under 5 mol/L ABA treatment, the expression of *TaRAC* gene peaked at 8 h, and the response was obvious. The results showed that *TaRAC* gene was sensitive to the change of ABA concentration. Within a certain ABA concentration range, the response time of *TaRAC* gene would be shortened, but when beyond a specific ABA concentration range, the response of *TaRAC* gene was passivated.

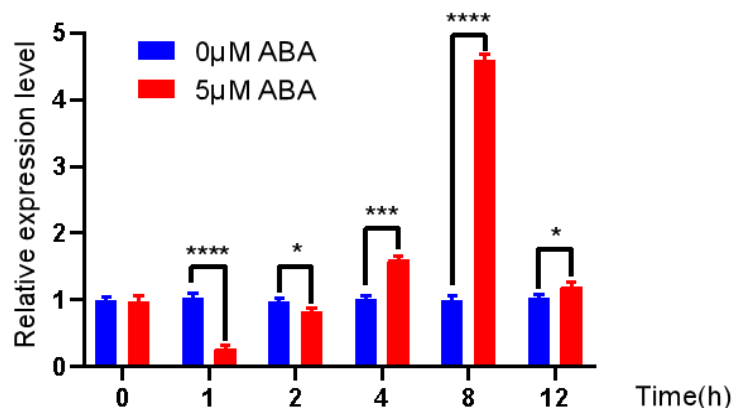


Figure 7. Relative expression levels of *TaRAC* gene at different times with 0 μM and 5 μM ABA treatment. The symbol '*' indicates statistically differences $P < 0.05$, the symbol '**' indicates statistically differences $P < 0.01$; the symbol '***' indicates statistically differences $P < 0.001$; the symbol '****' indicates statistically differences $P < 0.0001$ (Turkey's least significantly difference test). Data are expressed as mean values standard errors from three replicates and error bars represent standard errors

Discussion

Plant RAC/ROP proteins are involved in a series of important biological processes, such as plant polar growth, hormone signal transduction, plant disease resistance and abiotic stress resistance, by regulating the assembly of cytoskeleton actin, the production of reactive oxygen species and the change of Ca^{2+} concentration in cells (Zheng and Yang, 2000; Li et al., 2001). *RAC1* was cloned from pea for the first time and found to be related to the growth of pea pollen tube (Yang and Watson, 1993). RAC protein was involved in the polar growth of pollen tube and root hair cells (Ye et al., 2003; Lee and Yang, 2008; Hwang et al., 2010).

The expression of *TaRAC* in different tissues of wheat was detected by qRT-PCR. The results showed that the gene had different abundances in different tissues of wheat, with certain tissue specificity, and the highest expression in leaves, cob, lemma and endosperm.

RAC/ROP protein was also involved in plant hormone signal transduction. For example, auxin related genes were also induced in tobacco and corresponding active mutants that overexpress *NTRac1* (Morel et al., 2004). Exogenous ABA can inhibit the expression of *AtRop10* in *Arabidopsis*, while the deletion mutant of this gene is more sensitive to ABA (Zheng et al., 2002). RAC/ROP protein was also involved in the response of plants to abiotic stress, and the salt resistance of *Arabidopsis* plants overexpressing tobacco *NtRop1* was improved (Cao et al., 2010). *Arabidopsis AtRop6* and *AtRop11* enhanced drought resistance of plants through stomatal closure regulated by ABA (Lemichez and E., 2001; Zheng et al., 2002).

Plant RAC/ROP protein genes are involved in many plant stress responses. *TaRAC* can promote ABA induced responses, such as seed germination, root elongation, stomatal closure, and ABA can also play a negative role in regulating *TaRAC*. In the present study, the *TaRAC* gene of wheat was cloned, and its molecular structure, tissue expression specificity and expression under ABA treatment were analyzed. It is suggested that the gene may be involved in wheat development, especially in response to abiotic stress, which provides a theoretical basis for further study of the role of *TaRAC* protein in plant resistance to spike germination.

ABA signaling pathway is closely related to plant stress resistance. In the present study, exogenous ABA promoted the expression of *TaRAC* gene. It is suggested that the increase of *TaRAC* gene expression may promote the cascade amplification of ABA signal transduction and improve plant resistance.

The expression profile of *TaRAC* gene with the treatment of different ABA concentration showed that *TaRAC* gene was highly expressed in seedlings. The results indicated that the function of *RAC* gene in plants is related with ABA signal transduction pathway. The results showed that *TaRAC* gene may be an excellent genetic resource to improve PHS resistance.

Rac GTPases play an important role in the regulation of secondary metabolism in plant cells and that overexpression of the gene(s) may be capable of enhancing the production of natural products accumulated in higher plant cells (Asano et al., 2013). The expression profile of *TaRAC* gene and the main functional domain of the protein encoded by a gene were analyzed to reveal that the gene may be involved in the regulation of wheat resistance to PHS. More work is needed for using *TaRAC* gene to improve dormancy and PHS tolerance in wheat.

Conclusion

The full-length ORF of the *TaRAC* gene was cloned from wheat. The full-length ORF of *TaRAC* encoded 197 amino acid residues, which was consisted of a small GTPase Rho family profile. The genomic *TaRAC* gene had no introns. *TaRAC* shared the identity with *RAC* from the corn. The expression of *TaRAC* gene was significantly tissue-specific. The expression of *TaRAC* in the seeds was significantly higher than the expression of gene expression after soaking germination. The sensitivity of the *TaRAC* gene to ABA was significantly increased. In the next step, we will use transgenic wheat lines to further clarify the function, subcellular localization of *TaRAC* gene and its role in ABA mediated signal transduction pathway, providing effective candidate genes for wheat resistance to PHS and stress breeding.

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Conflict of interests. The authors state no conflict of interest.

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