

# A RADISH (*RAPHANUS SATIVUS* L.) E3 UBIQUITIN LIGASE GENE *RHA2b* ENHANCES SEED DORMANCY AND TOLERANCE TO PREHARVEST SPROUTING IN TRANSGENIC WHEAT (*TRITICUM AESTIVUM* L.)

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**Abstract.** The *RHA2b* gene from radish encodes a transcription factor that is involved in abscisic acid (ABA) signal transduction and is associated with seed dormancy and preharvest sprouting. *RsRHA2b* gene may have the function to improve dormancy and tolerance to PHS in transgenic wheat. The *RsRHA2b* gene was cloned and transferred into Zhengmai 9023 via *Agrobacterium*-mediated stem apex transformation. The results of RT-PCR and southern blot indicated that the *RsRHA2b* gene was integrated into the genomes of the wheat transformants. Seed dormancy and PHS tolerance was significantly enhanced in transgenic plants that stably expressed *RsRHA2b*. In addition, in the presence of 0.5  $\mu\text{mol/L}$  ABA and 5  $\mu\text{mol/L}$  ABA, a significant reduction in weight was found between the transgenic wheat plants and the non-transgenic Zhengmai 9023 plants. Furthermore, the expression of genes involved in *RHA2b*-mediated ABA signal transduction, such as *ABI5*, *FUS3* and *MAL*, significantly increased at the second leaf stage in ABA-treated transgenic wheat plants. These results show that *RsRHA2b* improves dormancy and preharvest sprouting tolerance in transgenic wheat.

**Keywords:** *agrobacterium-mediated stem apex transformation, southern blot, abscisic acid, signal transduction, reverse transcription-polymerase chain reaction*

## Introduction

Preharvest sprouting (PHS) in wheat (*Triticum aestivum* L.) is the germination of grains in the ears when long-range rainfall or damp conditions occur prior to harvest (Li et al., 2004). PHS is an important issue, which is not conducive to the stability of wheat production worldwide (Xiao et al., 2002). Seed viability and the hydrolysis of starch and protein in the endosperm are always reduced after PHS (Mohan, 2008). Moreover, PHS creates a favorable environment for serious infections by saprophyte fungi and thus causes heavy losses to grain yield (Castor and Frederiken, 1977). Therefore, there is a need to breed for increased resistance to PHS. Molecular breeding may be an effective method to reduce PHS susceptibility. Wheat is an important cereal crop worldwide, and it is a staple food for many populations. Genetically modified (GM) crops can act as powerful complement to the crops produced by laborious and time consuming conventional breeding methods to meet the worldwide demand for quality foods and help fight malnutrition by enhanced yield, nutritional quality and increased resistance to various biotic and abiotic stresses (Kamthan et al., 2016). PHS and dormancy in wheat have been focuses of numerous researches. *TaRHA2b* and *RsRHA2b* gene may play an important role in seed

dormancy during germination, which could be used to improve the PHS resistance of wheat. Because of homologous co-suppression in transgenic plants, exogenous genes are usually used for genetic manipulation.

To date, most researchers have agreed that PHS is the result of interactions between genotypes and the environment. Seed dormancy is an adaptive trait that inhibits seed germination and is positively correlated with resistance to PHS (Derera et al., 1977; Gerjets et al., 2010; Gubler et al., 2005; Chen et al., 2008b). The resistance of a seed to PHS is partially influenced by the external environment, but seed dormancy is the most critical determinant (Kucera et al., 2005; Holdsworth et al., 2008). As has been confirmed in many species, seed dormancy is influenced by the internal abscisic acid (ABA) content and the sensitivity to ABA (Groot and Karssen, 1992; Tan et al., 1997). ABA plays a critical role in plant development. Therefore, ABA, including its metabolism and signaling, has long been a focus of research (Grill and Himmelbach, 1998; Raghavendra et al., 2010; Zhu, 2002). ABA is important for plant growth and developmental processes, such as seed maturation, germination and seedling growth. ABA also regulates plant adaptation to various environmental challenges, including drought, salt, cold and other abiotic stresses (Nambara and Marion-Poll, 2003; Yamaguchi Shinozaki and Shinozaki, 2006; Lopez-Molina et al., 2001). During seed maturation, the ABA content in the seed increases significantly, resulting in strong dormancy. After the mature seed absorbs water, it quickly changes from a state of dormancy to a state of germination.

Many regulatory factors and response regulators in ABA signaling act in the signal transduction cascade during seed maturation and in young seedlings. These factors include ABA receptors, regulators and really interesting new gene (RING) finger transcription factors, which regulate the regulatory factors via ubiquitination and via the expression of ABA downstream response regulators (Koornneef et al., 1984). The genes related to ABA include ABI5, FUS3, MAL, and so on (Chiu et al., 2012; Huang et al., 2012; Li et al., 2011). The ubiquitin-proteasome pathway comprises two major components, the ubiquitination machinery, which attaches a ubiquitin or a poly-ubiquitin chain to a substrate, and the 26S proteasome, which degrades poly-ubiquitin-tagged protein substrates (Dreher and Callis, 2007; Moon et al., 2004; Schwechheimer and Schwager, 2004; Smalle and Vierstra, 2004). The ubiquitination of substrate proteins requires three types of enzymes, including E1 enzymes, which are ubiquitin-activating enzymes; E2 enzymes, which are ubiquitin-conjugating enzymes; and E3 enzymes, which are ubiquitin ligases. These three enzyme types act sequentially to catalyze the covalent attachment of the 76-amino-acid ubiquitin to the target protein. The E3 ligases recruit specific substrates and are the key determinants of the specificity of ubiquitination (Smalle and Vierstra, 2004).

RING finger proteins have motifs containing conserved cysteines and histidines. The RING motifs can be classified into three subgroups, including C3HC4 (RING-HC), C3H2C3 (RING-H2) and modified RING domain proteins. RING finger proteins always mediate protein-protein interactions and the formation of multi-protein complexes (Borden, 2000; Stone et al., 2005). At least 477 RING motif-containing proteins that play a critical role in plant developmental processes have been found in *Arabidopsis* (Vierstra, 2009). Some evidence has indicated that RING class E3 enzymes are critical for regulating ABA signaling and are associated with abiotic stress responses in plants (Ko et al., 2006; Zhang et al., 2005, 2007). *RHA2a* and *RHA2b*, which are RING finger E3 ligases, can positively regulate the ABA-mediated

inhibition of germination and act in the early seedling stage. RHA2a can activate positive regulators by mono-ubiquitinating and stabilizing certain key regulators of the ABA signaling pathway (Bu et al., 2009). ANAC019 and ANAC055, two closely related NAC family proteins, were identified as RHA2a-interacting proteins (Bu et al., 2008; Jiang et al., 2009). RHA2b acts additively with RHA2a in regulating ABA signaling. Seeds over-expressing *RHA2a* and *RHA2b* showed increased sensitivity to exogenous ABA, increased dormancy and a significant reduction in germination capacity. RHA2b targets MYB30 degradation to regulate ABA signal transduction (Zheng et al., 2018). Although some progress has been made in understanding the molecular and biological functions of *RHA2b* in *Arabidopsis*, the molecular mechanism of *RHA2b*-mediated ABA signaling is largely unknown. The *AtRHA2b* gene encodes an active E3 ubiquitin ligase that plays a positive regulatory role in ABA signaling and stress responses. The over-expression of *AtRHA2b* leads to ABA-associated phenotypes, such as ABA hyper-sensitivity in seed germination and seedling growth (Li et al., 2011).

In our previous studies, *TaRHA2b* and *RsRHA2b* genes were cloned from wheat and radish (*Raphanus sativus* L.), respectively. The results showed that the proteins encoded by *TaRHA2b* and *RsRHA2b* were consisted of Zinc finger RING-type profiles. Blast and phylogenetic analysis showed that the protein encoded by *TaRHA2b* and *RsRHA2b* shared the identity with *AtRHA2b* from the *Arabidopsis*. The expression of *TaRHA2b* and *RsRHA2b* genes was significantly tissue-specific. The sensitivity of the *TaRHA2b* and *RsRHA2b* genes to ABA was significantly increased (Li et al., 2019a). The differences in nucleotide polymorphism sites of *RsRHA2b* gene resulted in differences in the three cleavage sites of *BstD102I*, *Cfr10I*, and *HpaII/MspI* (Li et al., 2019b). These results facilitate the exploration of the correlation between the *TaRHA2b* gene and wheat's enhanced resistance to PHS and the development of molecular markers for resistance to PHS.

In the present study, the *RsRHA2b* gene from radish was cloned and then transferred into wheat using *Agrobacterium tumefaciens*. Seed dormancy and ABA sensitivity were significantly enhanced in transgenic wheat lines. These results displayed that *RsRHA2b* gene has the function to improve dormancy and tolerance to PHS in transgenic wheat.

## Materials and methods

To clone the *RsRHA2b* gene, mRNA was isolated from the embryos of a local radish variety, "New generation 791". The elite Chinese bread wheat cultivar Zhengmai 9023, a hexaploid wheat cultivar with weak resistance to PHS, which is widely cultivated in Henan Province, was used for transformation.

### Construction of a binary vector

The coding sequence and 3'-untranslated region (3'-UTR) of the *RsRHA2b* gene were amplified from radish cDNA via PCR with primers *RsRHA2b*-F2/*RsRHA2b*-R2 (Table 1). The PCR-amplified products were cloned into TA vectors (Takara, Dalian, China) and sequenced. A binary vector was constructed based on pCAMBIA3301. The TA vectors including the *RsRHA2b* gene and the pCAMBIA3301 vector were digested with *Bgl*III and *Bst*EII. The two digested fragments were ligated together using T4 ligase. The resulting binary vector carried the *phosphinothricin* gene (*bar*) for selection.

### ***Agrobacterium-mediated stem apex transformation of wheat***

Wheat seeds were sterilized with 0.1% HgCl<sub>2</sub> for 10 min, then washed five times with sterilized distilled water. The sterilized seeds were germinated at 25 °C in the dark for 2–3 days on filter-containing plates. When the coleoptiles of the seeds reached a certain height (2–4 cm), the shoots were cut using a clean knife. Then, the stem apices were exposed. Finally, 2–4 µL of resuspended *Agrobacterium* harboring the *RsRHA2b* gene and that had been induced with 100 µmol/L acetosyringone was dropped onto the stem apex for inoculation.

The inoculated seedlings were maintained on medium-containing plates until new leaves grew out, and then the seedlings were transferred into pots. Selection for resistance was performed by spraying the seedlings with a solution containing 160 mg/L basta when the seedlings had 3–4 leaves. The resistant plants were planted in the field.

### ***Propagation of the transgenic lines***

The seeds were harvested from the field in 2011. Then, they were grown in a digital biochemical incubator after rigorous screening. When the seedlings had 3–4 leaves, selection for resistance was performed by spraying the seedlings with a solution containing 160 mg/L basta. Genomic DNA and RNA were extracted from young wheat leaves. Then, PCR, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were conducted on the resistant plants. The seeds of *RsRHA2b* transgenic plants were harvested while the plants were in the digital biochemical incubator. Then, they were grown in the field. They were then grown and screened as in prior years.

### ***DNA and RNA extraction***

Genomic DNA was extracted from young wheat leaves. Total RNA was isolated from seeds and leaves using TRIZON reagent (CoWin Biotech Co., China) according to the manufacturer's instructions and treated with RNase-free DNase I (Tiangen, China) before final precipitation with ethanol.

### ***PCR, RT-PCR analysis and southern blot***

For the PCR analysis of transgenic plants, a 565 bp *RsRHA2b* product was amplified using PCR with the primers *RsRHA2b*-F1/*RsRHA2b*-R1 (Table 1), which were designed based on pCAMBIA3301-*RsRHA2b*. The primers were specific for radish.

For RT-PCR analyses of transgenic plants, approximately 5 µg of total RNA from the shoots of transgenic plants was reverse-transcribed into cDNA using reverse transcriptase (Tiangen, China) and oligo-dT<sub>18</sub> primers. PCR amplifications were performed using the primers *RsRHA2b*-F2/*RsRHA2b*-R2.

Southern blot analysis was performed using standard procedures. Genomic DNA samples (30 µg) from PCR-positive transgenic wheat plants were digested with *Hind*III overnight at 37 °C. The DNA fragments were separated on 1.0% (w/v) agarose gels and transferred to Hybond N<sup>+</sup> nylon membranes. The PCR-amplified fragment to be used as a probe was further purified and labeled with digoxigenin (DIG)-dUTP using a DIG-High Prime Kit (Roche, Mannheim, Germany). Hybridization was conducted at 68 °C. Hybridization and detection were performed using a DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions.

### **qRT-PCR analysis of transgenic wheat plants**

For qRT-PCR, the RNA from wheat leaves at 12 h, 24 h, 48 h after treatment with 0  $\mu\text{mol/L}$ , 0.5  $\mu\text{mol/L}$ , or 5  $\mu\text{mol/L}$  ABA was used for reverse transcription (Toyobo, Osaka, Japan). qRT-PCR was performed in a volume of 20  $\mu\text{L}$  containing SYBR Green I PCR Master Mix (Promega, Madison, USA) 10 pmol each of forward and reverse gene-specific primers (Table 1), and cDNA (0.2  $\mu\text{mol/L}$ ). Gene-specific primers were designed using Primer Premier 5 software. The wheat *ACTIN* gene was co-amplified as an internal control to normalize the total amount of cDNA present in each reaction and to eliminate the differences among the samples. PCR amplification was performed in an iQ5 Cycler (Bio-Rad) under the following conditions: 95 °C for 3 min, followed by 39 cycles of 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. The plate was read at 72 °C for 30 s. To determine the specificity of each PCR primer pair, a melt curve was generated by incubating the reaction at 95 °C for 10 s, cooling to 65 °C for 10 s and increasing to 95 °C at a rate of 0.5 °C/10 s.

**Table 1.** Primer sequences used for PCR, RT-PCR, qRT-PCR and southern blot analysis

Name	Sequence (5'-3')
<i>RsRHA2b</i> -F1	CCATGGTCCAGCAACAGCAGCAAACA
<i>RsRHA2b</i> -R1	GGTGACCGGAGAAAGCCGCGAGATTA
qRT- <i>RsRHA2b</i> -F	TGCCCCGAGGAGGTCAAGGAG
qRT- <i>RsRHA2b</i> -R	CGTGATTGGATGGCTACTATACAAAGTG
<i>RsRHA2b</i> -F2	AGATCTATGGGGTTACAAGGTCAACTCT
<i>RsRHA2b</i> -R2	GGTGACCGGAGAAAGCCGCGAGATTA
Trans(gDNA)-F	AGATCTATGGGGTTACAAGGTCAACTCT
Trans(gDNA)-R	GGTGACCGGAGAAAGCCGCGAGATTA
Trans(cDNA)-F	AGATCTATGGGGTTACAAGGTCAACTCT
Trans(cDNA)-R	GGTGACCGGAGAAAGCCGCGAGATTA
Southernblot(probe)-F	AGATCTATGGGGTTACAAGGTCAACTCT
Southernblot(probe)-R	GGTGACCGGAGAAAGCCGCGAGATTA
Ta <i>ACTIN</i> -F	GCTGTTCCAGCCATCTCATGT
Ta <i>ACTIN</i> -R	CGATCAGCAATTCAGGAAAC
<i>ABI5</i> -F	GACGGAAGAAGCGGCAGTG
<i>ABI5</i> -R	TCTGAAGCAGCACGAGAAAGG
<i>FUS3</i> -F	GTGGCTGGGTTGCGAGTTAT
<i>FUS3</i> -R	ATCCTGCTCTTGTGTTTGGC
<i>MAL</i> -F	CTTCCTGTGCTGCCTATTCTG
<i>MAL</i> -R	CCACATGCACTGGACGATAAC

### **Seed germination test**

Intact spikes were harvested at physiological maturity. Grains from the wheat spikes were hand-threshed. The dormant seeds were used for further analysis. Thirty sound grains of each plant were placed crease down on moist filter paper in Petri dishes at 20 °C in the dark for 7 d. The germinated grains were counted every day and

calculated in terms of the germination index (GI).  $GI = (7n_1 + 6n_2 + \dots + 1n_7) / 7N$ , where  $n_1, n_2, n_3, \dots, n_7$  are the number of grains that had germinated on the 1st day, 2nd day, ... 7th day, and  $N$  is the total number of grains in the assay. The grains from each plant composed a replication, and the experiments were performed in three replicates. Additionally, 30 sound grains of each plant were placed crease down on moist filter paper in Petri dishes at 20 °C in normal conditions (16 h, light:8 h, dark; 20 °C). The germination capacity on the 39<sup>th</sup> day after sowing was recorded.

### ***Spike sprouting test***

Ten spikes of each T5-generation transgenic line harvested from the experimental field were inserted into floral foam (floral foam can retain water to prevent the detached wheat spikes from wilting) at approximately 20-25 °C in a greenhouse. Misting was controlled by a humidifier and checked automatically for 4 d. Under these conditions, a high relative humidity (> 98%) was maintained.

### ***ABA sensitivity assay***

Wheat seeds from the transgenic lines and from the non-transgenic Zhengmai 9023 whose dormancy had been broken were tested for ABA sensitivity. Seeds were germinated at 20 °C. At the second leaf stage, they were treated with different concentrations of ABA, including 0 µmol/L, 0.5 µmol/L and 5 µmol/L ABA. Fourteen seedlings from each transgenic line and from non-transgenic Zhengmai 9023 were assayed after being treated for 13 d (Saad et al., 2013). The initial fresh weight and final fresh weight of the plants were measured. Then, the difference in weight was calculated. The experiments were performed on three replicates.

### ***Statistical analysis***

The GIs, germination capacity and average weight difference of each line were analyzed using Excel software, and Student's *t* test was calculated for relevant data using SPSS software. The figures were created using Origin8 software and Adobe Photoshop CS5 software.

## **Results**

### ***Cloning and analysis of the *RsRHA2b* gene***

The EST database was BLASTed based on the amino acid sequence encoded by the *Arabidopsis* RING finger gene *AtRHA2b* (Gene ID: 814644). Many highly homologous ESTs were found. A cDNA sequence including the entire ORF of the radish RING finger gene *RHA2b* was found using artificial splicing and software analysis based on the radish database. The length of the entire sequence was 697 bp (*Fig. 1*). The ID of the *RsRHA2b* gene was KR349970. The entire coding sequence, plus a 93 bp sequence in the 3'-UTR, was amplified from radish cDNA and was 565 bp in length. The sequence was then used to construct the binary vector pCAMBIA3301-*RsRHA2b* (*Fig. 2*). The constructed vector was further confirmed by sequence analysis (*Fig. 1*) and was then transferred into *Agrobacterium tumefaciens* EHA105. Transgenic wheat plants were obtained to investigate the function of *RsRHA2b*.

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1      CCATGGTCCAGCAACAGCAGCAAACACTCTCTCTCTAAAAGAATCTCTCTCTCTCAGGAA
61     GAAGAAGAAGATGGGGTTACAAGGTCAACTATCTGACGTTTCATCAGATTCAATCCCTCT
1      M G L Q G Q L S D V S S D S I P L

121    CATGCTCGTTGCTCTCCTCGCTACTTTGTTCAAACACGTCGCTCTTTCTTCCCTCCGCTT
18     M L V A L L A T L F K H V R S F F L R F

181    CTCCTCCTCCTCCTCTGTCGTCGAAGATGCTTCTCTCTCCATTTCTCAGGGTTTGGCAA
38     S S S S S V V E D A S L S I S S G F A N

241    CATCGCCGTACTIONCGCCGACCAGCTCAAACCTCAACCGTCTTCTCATAACCCCTACGACCA
58     I A V L A D Q L K L N R L F S Y P Y D H

301    TAAGGCCGCCGAGCCGCATCCGACTGCATCGTGTGCTTGTCTACACTCAAGACCGGAGA
78     K A A A A S D C I V C L S T L K T G E

361    AGAAGTGAGGAAGCTGGGATGCAGACACGTGTTCCACAAACAGTGTGGAAAGGTTGGCT
98     E V R K L G C R H V F H K Q C L E G W L

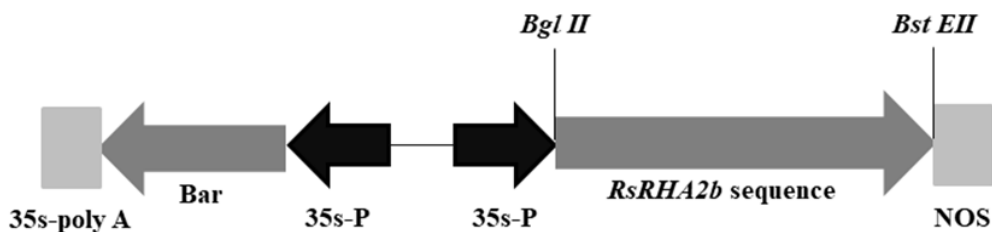
421    TCAACATCTCAATTTTAATTGTCCGCTTTGTAGATCTCCGTTGGTTGGTCGTGGAGGAGG
118    Q H L N F N C P L C R S P L V G R G G G

481    ATGTGAATCGATCACTTCTTCTTCTCTCTCTCAGACGCTCAGTGAGTTCTCTTCAT
138    C E S I T S S F S L L S D A Q *

541    TGTCTGATGAACAAAAAAAAAAAAAGAAAAGGAAAGAGAGAAGAAGAGTTTGGGCGTTTT
601    TTTAATCTCGCGGCTTTCTCCGGTCAAC

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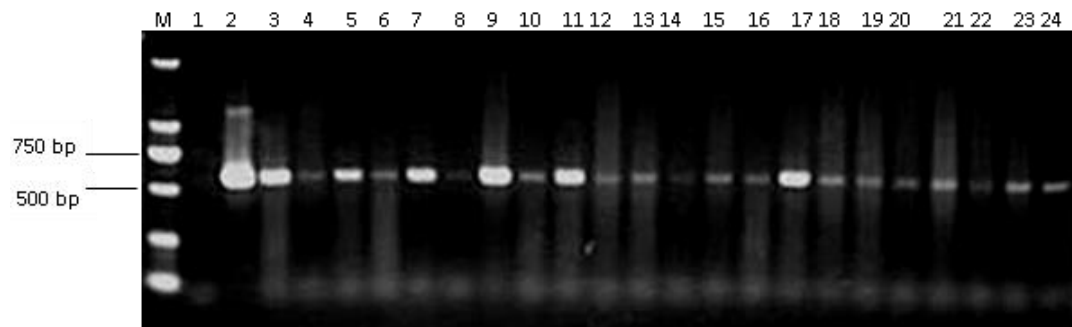
**Figure 1.** Nucleotide and deduced amino acid sequences of the *RsRHA2b* gene



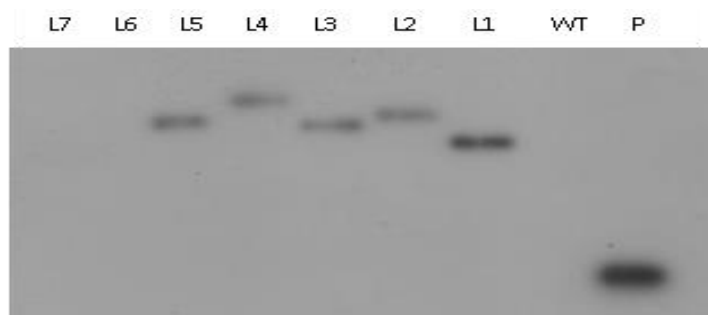
**Figure 2.** Structure of the binary vector *pCambia3301-RsRHA2b*, which was used for transformation. *RsRHA2b* sequence, *RsRHA2b* coding sequence plus 93 bp sequence at 3'-UTR; 35S-P, 35S-promoter; *Bar*, phosphinothricin gene

### Identification of transgenic wheat plants

The transgenic plants were identified using PCR and RT-PCR. As shown in *Figure 3*, the *RsRHA2b* gene fragment was found in the genome of transgenic wheat, and *RsRHA2b* transcripts were also detected. These results showed that the *RsRHA2b* gene was stable in the wheat genome and was stably expressed. Each of the five lines (L1, L2, L3, L4 and L5) showed a single band, indicating independent transformation events (*Fig. 4*). As expected, there was no significant hybridization signal from WT DNA. These results indicated that the *RsRHA2b* gene was integrated into the genomes of the wheat transformants in the selected lines and was inherited by the progeny as a single copy. However, the segregation was not consistent with heredity of a single-copy gene. Ultimately, four transgenic lines, 1471 (100 wheat heads), 1477 (102 wheat heads), 1492 (105 wheat heads) and 1569 (103 wheat heads), were selected for further characterization. Their morphology was similar to that of the non-transgenic Zhengmai 9023.



**Figure 3.** Identification of transgenic plants by PCR and RT-PCR analyses. DNA was extracted from the leaves of each T5 transgenic line and from non-transgenic Zheng9023. RNA was extracted from the shoots of each T5 transgenic line and non-transgenic Zheng9023. (3-13) DNA fragments between the *RsRHA2b* coding sequence and the NOS sequence amplified by primers *RsRHA2b-F/RsRHA2b-R*. (14-24) RT-PCR products of the *RsRHA2b* sequence amplified from cDNA using primers *RsRHA2b-F/RsRHA2b-R*. M, marker 2000; 1, non-transgenic Zheng9023; 2, plasmid pCAMBIA3301-*RsRHA2b*; 3-13, transgenic lines (DNA); 14-24, transgenic lines (cDNA)



**Figure 4.** Southern blot analysis. L1-7, seven randomly selected T2 PCR-positive lines (L1-7); WT, non-transgenic Zheng9023; P: purified probe product (positive control). Thirty micrograms of *HindIII*-digested total wheat leaf DNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a probe labeled with DIG-11-dUTP

### ***RsRHA2b* has an important role in the regulation of *RsRHA2b*-mediated seed dormancy**

To assay the seed dormancy of transgenic wheat, GIs were investigated using the seeds from the T5 generation. The GIs were significantly lower in the transgenic wheat lines than in the non-transgenic Zhengmai 9023 (Table 2). The GIs of the seeds of the transgenic lines ranged from 0.098 to 0.387, a significant reduction, up to 89.9%. In particular, the GI of transgenic line 1477 was 0.098. The germination capacity of the transgenic lines ranged from 0.367 to 0.724, a significant reduction of up to 63.3%. In particular, the germination capacity of the transgenic line 1477 was 0.367. GI value (less than 0.4) was defined as resistance to spike germination. GI value (0.4-0.8) is medium resistance. GI value (more than 0.8) is prone to spike germination (Ma et al., 2014). The GI values of the seeds of the four transgenic lines in this study were all less than 0.4, while the average GI values of the control group were 0.973. Therefore, the retarded seed germination in the transgenic wheat with *RsRHA2b* gene is stably inherited, and *RsRHA2b* can increase seed dormancy.

**Table 2.** Seed germination indexes and germination capacity (on the 39<sup>th</sup> day after germination) in different T5 transgenic wheat compared with those of non-transgenic Zhengmai 9023

Transgenic line	Germination index <sup>a</sup> (GI)	Germination capacity <sup>b</sup>
1471	0.217 ± 0.010 b	0.650 ± 0.024 b
1477	0.098 ± 0.003 b	0.367 ± 0.047 b
1492	0.244 ± 0.017 b	0.600 ± 0.024 b
1569	0.387 ± 0.007 b	0.724 ± 0.024 b
CK <sup>c</sup>	0.973 ± 0.012 a	1.000 ± 0.000 a

<sup>a</sup>Germination indexes are the average ± standard error from 10 plants per line

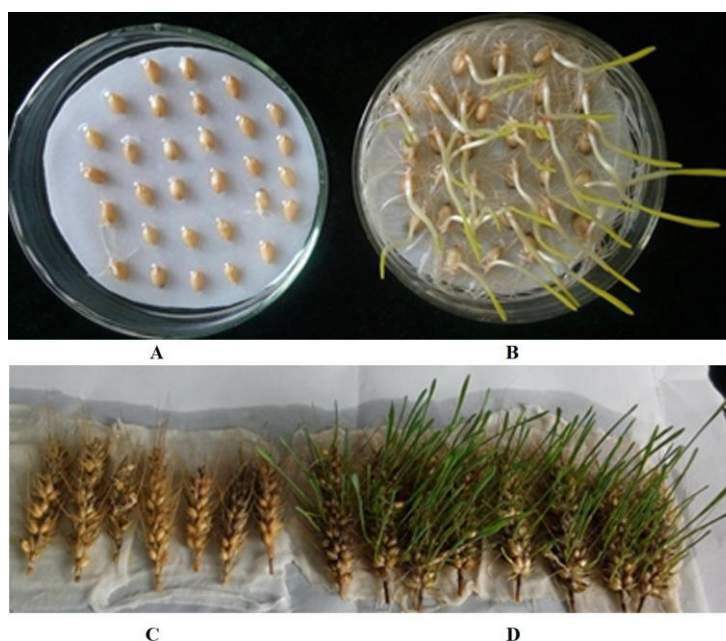
<sup>b</sup>Germination capacity is the average ± standard error from 10 plants per line on the 39<sup>th</sup> day after germination

<sup>c</sup>CK: non-transgenic Zhengmai 9023

The letters 'a' and 'b': Significant at P < 0.05 with Student's *t* test

### *RsRHA2b* effectively regulates *RsRHA2b*-mediated tolerance to PHS in spikes

To ascertain whether the *RsRHA2b* gene mediates the PHS response, 10 T5-generation plants from each of four transgenic lines, as well as non-transgenic Zhengmai 9023, were assayed for their PHS tolerance. The GI of the transgenic wheat line 1477 was significantly lower than that of the non-transgenic Zhengmai 9023 (Table 2; Fig. 5). Seeds from 1477 had just started to germinate while Zhengmai 9023 had a long first leaf. Spikes of 1477 maintained dormancy while Zhengmai 9023 displayed substantial leaves after 4 d of misting. The results showed that the *RsRHA2b* gene can improve PHS tolerance in transgenic wheat plants.



**Figure 5.** Seed germination phenotypes of representative seeds and spikes from the T5 transgenic line 1477 and the non-transgenic Zheng9023. (A) Seeds of 1477 and (B) Zheng9023 incubated on a moist filter for 7 d. (C) Spikes of 1477 and (D) Zheng9023 incubated for 4 d under misting conditions

### Higher sensitivity to exogenous ABA in transgenic plants

The ABA sensitivity of the *RsRHA2b* transgenic wheat plants was assayed. At the second leaf stage, the plants were transferred to Hoagland's liquid medium containing different concentrations of ABA (0  $\mu\text{mol/L}$ , 0.5  $\mu\text{mol/L}$  or 5  $\mu\text{mol/L}$ ). In the absence of ABA, the weights of transgenic wheat plants and non-transgenic Zhengmai 9023 were 0.701 g and 0.743 g, respectively (Table 3); there was no significant difference between them. However, in the presence of 0.5  $\mu\text{mol/L}$  ABA, the weights of the transgenic wheat plants and non-transgenic Zhengmai 9023 were 0.417 g and 0.597 g, respectively (Table 3), and the difference was significant. The inhibitory effect was more apparent when 5  $\mu\text{mol/L}$  ABA was applied. As shown in Table 3, with 5  $\mu\text{mol/L}$  ABA, the weights of transgenic wheat plants and of non-transgenic Zhengmai 9023 were 0.274 g and 0.448 g, respectively. Taken together, the ABA sensitivity was significantly improved in transgenic wheat lines containing the *RsRHA2b* gene.

**Table 3.** Weight differences in wheat plants with ABA treatment

Transgenic line	ABA		
	0 $\mu\text{mol/L}$	0.5 $\mu\text{mol/L}$	5 $\mu\text{mol/L}$
CK <sup>a</sup>	0.743 $\pm$ 0.015 (g)	0.597 $\pm$ 0.037 (g) a	0.448 $\pm$ 0.006 (g) a
1477	0.701 $\pm$ 0.008 (g)	0.417 $\pm$ 0.009 (g) b	0.274 $\pm$ 0.015 (g) b

<sup>a</sup>CK: non-transgenic Zhengmai 9023

The letters 'a' and 'b': significant at  $P < 0.05$  with Student's *t* test

### *RsRHA2b* affects the expression of genes associated with ABA signal transduction

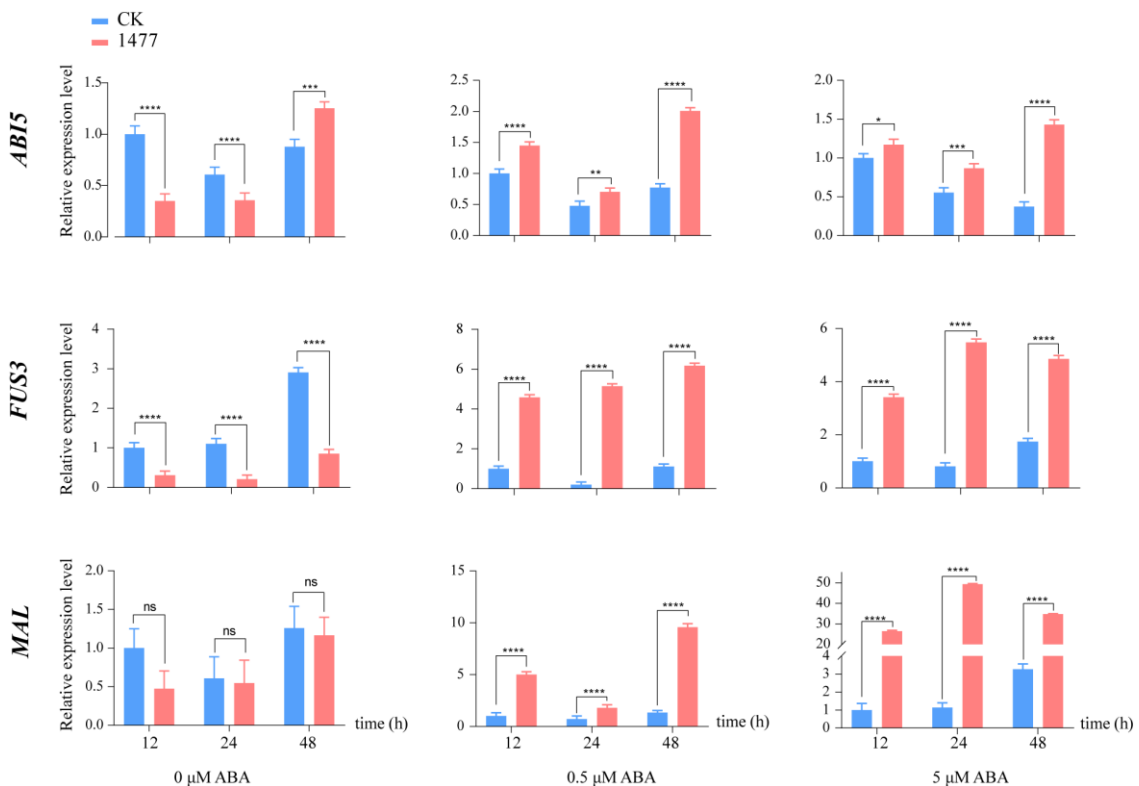
To investigate whether the expression of the *RsRHA2b* gene affected the ABA signal transduction pathways, the expression levels of several genes associated with ABA were analyzed in the T5 transgenic plants and non-transgenic Zhengmai 9023. Three genes, *ABI5* (accession no. AB362818.1), *FUS3* (accession no. AM418838.1, homologous cloning) and *MAL* (accession no. AK334873.1), were found to be closely associated with *RsRHA2b*. As shown in Figure 6A-C, the expression of three genes showed an obvious steady increase in transgenic wheat plants after ABA treatment. In the presence of 0.5  $\mu\text{mol/L}$  ABA, the expression levels of *ABI5*, *FUS3*, and *MAL* increased 2.6-fold (48 h, highest level), 25.7-fold (24 h, highest level) and 7.2-fold (48 h, highest level), respectively, in the transgenic wheat plants relative to non-transgenic Zhengmai 9023; in the presence of 5  $\mu\text{mol/L}$  ABA, the expression of the three genes increased 3.8-fold (48 h, highest level), 6.8-fold (24 h, highest level) and 43.5-fold (24 h, highest level), respectively, in the transgenic wheat plants. Taken together, these results indicate that the *RsRHA2b* gene up-regulated the expression of several genes related to seed germination in transgenic wheat.

### Discussion

To solve the problem of PHS in wheat, scholars have conducted much research to explore the mechanism of PHS resistance. The conclusions are as follows. First, studies have focused on the apparent physical and physiological characteristics of wheat seeds, such as spike 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  $\alpha$ -amylase activity (Major et al., 2001). In the breeding process, grain color, spikelet density, 1000-grain weight and grain width can be used as important reference indexes for wheat varieties resistant to spike germination (Ma et al., 2016). Second, research has focused on the characteristics of genes related to PHS or seed dormancy and the positional cloning of QTLs (Anderson et al., 1993; Chen et al., 2008a; Somers et al., 2007). Third, research has focused on the screening of molecular markers, the mapping of resistance genes and the generation of transgenic lines using genetic engineering methods. PHS resistance in wheat is a quantitative trait, and relevant quantitative trait locuses linked to PHS have been reported on almost all chromosomes (Munkvold et al., 2009; Ogonnaya et al., 2008). However, PHS-resistant wheat germplasm resources are scarce. Therefore, the use of genetic engineering provides good choices for the improvement of PHS resistance in wheat. The *VPI* and *Trx* genes have been used to improve 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.

The objective of this study was to determine whether the *RHA2b* gene from radish could improve the PHS tolerance of wheat. The *RsRHA2b* gene was used to improve seed dormancy and PHS tolerance in wheat.



**Figure 6.** qRT-PCR analysis of three genes in the T5 generation of transgenic line 1477 and non-transgenic Zheng9023 is presented. RNA was isolated from the leaves at the second leaf stage of the plants. *ABI5*: a positive regulator of ABA signaling; *MAL*: involved in the pathways of ABA signaling; *FUS3*: involved with the regulation of ABA biosynthesis. 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  $P < 0.0001$  (Two-way ANOVA, Sidak’s multiple comparisons test)

The success of the *Agrobacterium*-mediated transformation method used here provides a new technique for wheat genetic breeding (Zhao et al., 2006). In this study, the stem apex was used to improve *Agrobacterium*-mediated transformation. Basta was used to select the transgenic lines. The optimum half lethal concentration of basta had not been reported previously. Prior to this study, the concentration was analyzed using concentration gradient experiments. The phenotypes of plants treated with basta solutions of 80 mg/L, 100 mg/L, 120 mg/L, 140 mg/L, 160 mg/L, 180 mg/L, 200 mg/L, 220 mg/L, 240 mg/L, and 260 mg/L were recorded. The results showed that the optimum half lethal concentration of basta was 160 mg/L. This method can provide genetically stable material for further application. As shown in *Figure 3*, the transgenic lines were rigorously screened using basta, PCR and RT-PCR. The seeds from pure transgenic lines, identified based on the segregation ratio of the integrated *RsRHA2b* gene, were used for propagation.

Seed dormancy was stably inherited in transgenic wheat lines carrying the *RHA2b* gene from radish (*Figs. 3 and 4*). GIs and germination capacities were stably reduced in the T5 generation of transgenic wheat lines (*Table 2*). The transgenic lines in the present study had significantly increased tolerance to PHS, indicating that *RsRHA2b* has an important role in regulating seed dormancy and PHS tolerance.

The resistance to dormancy and PHS is regulated by ABA anabolism and ABA signal transduction (Gubler et al., 2005). Additionally, studies on wheat mutants have shown that ABA sensitivity is directly associated with seed dormancy (Kawakami et al., 1997; Noda et al., 2002). Research on the physiological characteristics of wheat has shown that ABA synthesis is important for embryos to remain dormant. However, embryo dormancy is closely associated with the internal ABA content and the sensitivity of embryos to ABA (King, 1993; Morris et al., 1989; Walker-Simmons, 1987). Cultivars with strong dormancy have strong sensitivity to ABA (Morris et al., 1989; Walker-Simmons, 1987; Corbineau et al., 2000). These results demonstrate that both ABA signal transduction and ABA cascade effectors are important for maintaining dormancy and resisting PHS. Increased ABA sensitivity in transgenic wheat may increase survival rates.

In this study, the *RsRHA2b*-regulated ABA sensitivity apparently failed to impair the growth of transgenic wheat plants without exogenous ABA, but with exogenous ABA, smaller weight increases were observed (*Table 3*). Therefore, the exogenous *RsRHA2b* gene may regulate the ABA signaling pathway in transgenic wheat.

Although the *AtRHA2b* gene plays a positive role in regulating ABA signaling and stress responses, the regulatory factors and response regulators associated with *AtRHA2b* are not known. Previous qRT-PCR assays have indicated that ABA treatment increased the expression of several ABA-inducible marker genes, including *RAB18*, *RD29A*, *KINI*, *AtADHI*, *RD22* and *RD29B* in wild-type *Arabidopsis* (Li et al., 2011). The regulators associated with ABA and ABA-inducible marker genes were chosen for analysis in transgenic wheat containing the *RsRHA2b* gene. In the present work, three genes, i.e., *ABI5*, *FUS3* and *MAL*, were found to be significantly associated with *RHA2b*. *ABI5*, a bZIP transcription factor, is a positive regulator of ABA signaling, and its expression can be activated by exogenous ABA treatment (Suzuki et al., 2003; Kobayashi et al., 2008). *FUS3* (*FUSCA3*) promotes dormancy and prevents the precocious germination of immature seeds by stimulating ABA synthesis (Chiu et al., 2012). *MAL* is involved in the metabolic pathways of ABA signaling (Huang et al., 2012). The expression of *ABI5*, *FUS3* and *MAL* significantly increased in the presence

of 0.5  $\mu\text{mol/L}$  ABA and 5  $\mu\text{mol/L}$  ABA at the second leaf stage in transgenic wheat (Fig. 6). Thus, the *RsRHA2b* gene could positively regulate ABA signaling intermediates in transgenic wheat.

The grain germination rate of the over-expressed miR9678 wheat transgenic lines was significantly lower than that of the wild type, and the content of active gibberellin was reduced, leading to significantly increased resistance to spike germination (Guo et al., 2018). The miR9678 can mediate the splicing of long-segment non-coding RNA and produce a series of phasiRNA, which eventually leads to the change of gibberellin metabolism and its regulatory pathway by affecting the expression of gibberellin synthesis gene, thus controlling the germination rate of grain and spike germination (Guo et al., 2018). Based on transgenic wheat containing *RsRHA2b*, the reason for significantly increased tolerance to PHS can be explored by further work.

## Conclusion

The *RsRHA2b* gene was integrated into the genomes of the wheat transformants. Transgenic wheat containing the *RsRHA2b* gene showed improved seed dormancy and PHS tolerance. *RsRHA2b* gene plays a significant role in regulating the genes associated with ABA signal transduction. This study provides a promising strategy for breeding wheat cultivars with improved seed dormancy and PHS tolerance using genetic engineering with the *RsRHA2b* gene. However, more work is needed to discover the mechanism of *RsRHA2b*-mediated PHS resistance.

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