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Isolation and Functional Characterization of a B3 Transcription Factor Gene *FUSCA3* Involved in Pre-Harvest Sprouting Resistance in Wheat

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Abstract

Pre-harvest sprouting (PHS) reduces yields and grain quality, resulting in seriously economic losses in wheat. It has been showed that PHS is significantly correlated to seed dormancy levels. *FUSCA3* (*FUS3*) gene is considered to be the key regulator of seed dormancy. However, little information is available about the function of *FUS3* gene (*TaFUS3*) in wheat. In this study, three homologous genes were identified in wheat grain, and their functions were investigated by gene silencing. Three full-length DNA (3477, 3534 and 3501 bp) and cDNA (1015, 1012 and 1015 bp) sequences encoding a B3 transcription factor, designated *TaFUS3-3A*, *TaFUS3-3B* and *TaFUS3-3D*, were first isolated from common wheat. The transcription of three *TaFUS3* genes in seed development and germination process was detected. *TaFUS3-3B* and *TaFUS3-3D* had similar expression profiles, and high levels of gene transcripts were detected in seeds at 25 DAP (days after pollination) and after 24 h of imbibition. However, the transcription of *TaFUS3-3A* was not detected. Silencing of *TaFUS3* in common wheat spikes resulted in increased seed germination and PHS. Compared with wild-type, the *TaFUS3*-silenced plants showed increased expression of genes related to GA biosynthesis and ABA metabolism, and decreased expression of genes associated with ABA biosynthesis. Moreover, silencing of *TaFUS3* in wheat plants led to a decrease in embryo sensitivity to ABA and changed the expression of genes involved in ABA signal transduction. The results of gene silencing indicated that *TaFUS3* plays a positive role in wheat seed dormancy and PHS-resistance, which might be associated with ABA, GA level and signal transduction.

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Keywords

Wheat (*Triticum aestivum* L.), *FUSCA3*, Molecular Cloning, Virus-Induced Gene Silencing (VIGS), Pre-Harvest Sprouting

1. Introduction

Pre-harvest sprouting (PHS) denotes grains germinating on the mother plants when they are subjected to the rainy and humid environment prior to harvest [1]. PHS of wheat grains not only causes the decline in grain yield, but also affects the end-use quality of the grain, resulting in severe economic losses [2] [3]. PHS is a worldwide problem. In some wheat-growing countries, such as the United States, Canada, Australia, and Japan, the problems associated with PHS occur frequently [4] [5] [6] [7]. In China, heavy PHS sometimes occurs on >83% of the wheat acreage [8]. Therefore, increasing resistance to PHS is an important current objective in improving wheat varieties.

Wheat PHS is a complex quantitative trait regulated by multiple genes [9]. Many studies have shown that seed dormancy level is one of the main factors determining the resistance to PHS in wheat [2] [10]. Seed dormancy is finely controlled by the seed maturation program, and ABA plays a key role in regulating seed dormancy [11] [12]. Genetic and molecular studies have shown that many of the genes participating in seed dormancy are known to be involved in ABA synthesis and signal transduction [10]. Studies in *Arabidopsis* showed that seed dormancy and germination were controlled by at least four major regulators, namely *ABA INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), *LEAFY COTYLEDON2* (*LEC2*), and *LEAFY COTYLEDON 1* (*LEC1*) [13] [14] [15]. *ABI3*, *FUS3* and *LEC2* are plant-specific genes, encoding transcription factor of the B3 domain family, whereas *LEC1* encodes a HAP3 subunit of the CCAAT binding factor [16] [17] [18] [19]. The loss-of-function mutants of these genes showed reduced accumulation of seed storage proteins and enhanced precocious germination of immature embryos [20] [21] [22]. Although these genes have similar functions, their expression patterns differ during seed development [23] [24]. *LEC1* and *LEC2* are expressed during the first period of embryonic development, followed by *FUS3* and finally by *ABI3*; all genes are active in the embryo, and *LEC2* and *FUS3* are active also in the endosperm [17] [19]. Moreover, the spatio-temporal expressions of *LEC2/ABI3/FUS3/LEC1* are transcriptionally cross-regulated and act together to control seed maturation and dormancy [25].

Recently, it has been demonstrated that *FUS3* is a crucial molecular switch in regulating a transition from seed dormancy to germination [26] [27]. *FUS3* usually acts as both activator and repressor of seed germination and dormancy by interacting with hormone signaling and synthesis pathways [20] [28]. It has been reported that *FUS3* accumulates preferentially in the epidermis, vasculature and radicle tips of embryos. *FUS3* expression is regulated by auxin [26]. The

C-terminal domain (CTD) of FUS3 is responsible for instability of the FUS3 protein and is involved in hormone sensitivity [29]. During late seed maturation, *FUS3* can promote dormancy and inhibit precocious germination of immature seeds by increasing ABA concentration and repressing GA biosynthesis genes. In addition, FUS3 protein is regulated by ABA and GA. The stability and concentration of the FUS3 are regulated negatively by ABA and positively by GA [26] [29] [30]. Similarly, during seed imbibition, FUS3 protein acts as a seed germination repressor through regulating ABA/GA ratio [31]. Furthermore, over-expression of *Arabidopsis FUS3* increases the sensitivity to high temperature and delays seed germination due to a high ABA level [32] [33]. These results indicated that *FUS3* plays a critical role in inducing dormancy and inhibiting germination of seed by regulating ABA and GA levels.

Wheat is a globally important crop, accounting for 20% of the calories consumed by humans. Research that focuses on mechanisms of seed dormancy regulation at the molecular level is very important for accelerating the process of improving wheat varieties. Although the functions of *FUS3* gene have been studied extensively in *Arabidopsis*, and putative orthologs have been described in several monocot species [34] [35], *FUS3* gene has not experimentally been proven to have a role in PHS tolerance in wheat, and wheat homologues for *FUS3* have not been identified. Therefore, in this study, we isolated the sequences of *Ta-FUS3* homoeologs in hexaploid wheat, analyzed the effect of silencing the *FUS3* gene using the barley stripe mosaic virus (BSMV)-mediated virus-induced gene silencing (VIGS) technique. These research data will provide fundamental insight for future studies regarding the functions of *FUS3* gene in seed dormancy and PHS.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Wheat cultivar (*Triticum aestivum* L.) Huaimai 0360 (highly PHS-resistant cultivar), was used for this study. For gene cloning and expression experiments, 100 surface-sterilized seeds were germinated on moist filter paper in growth chambers at 25˚C under 12-h light/12-h dark conditions and transplanted into pots in a naturally lit glasshouse with standard irrigation and fertilization until mature. During this period, developing seeds were collected at 15, 20, 25, and 30 days after pollination (DAP). For germination, mature seeds were surface-sterilized and then imbibed on water moistened filter paper in Petri dishes in a temperature-controlled cultivation chamber (darkness at 25˚C). 3 - 5 seeds were collected at 0, 6, 12, 24, 36, and 48 h after imbibition commenced. All the collected plant samples were snap-frozen in liquid nitrogen immediately after harvesting and stored at −80˚C until use. Each treatment was repeated thrice.

For VIGS experiments, wheat cultivar Huaimai 0360 was grown in the agricultural and experimental field of Henan Agricultural University (Zhengzhou, China) during the wheat-growing season. The plot area was 15 m^2 (length 5.0 m

× width 3.0 m) and was watered and fertilized as needed throughout the growth period.

At anthesis, an arch shed (length 5.0 m × width 3.0 m × height 1.5 m) was built directly above the wheat plants according to the method described by Liu *et al.* [36]. One hundred wheat spikes in the same heading stage and of similar size were labeled. During 1 day before and after BSMV inoculation, the PVC plastic film was covered with soil to prevent water loss from affecting the spread of the virus. Developing grains in the middle of BSMV-infected spikes were collected at 21, 28, 35, and 45 days post inoculation (dpi) at 10:00 a.m., snap-frozen in liquid nitrogen and then stored at −80˚C for transcription analysis. At maturity, spikes of BSMV-infected wheat plants were harvested and stored at room temperature until use. Uniform spikes at the anthesis stage were used for BSMV-VIGS inoculation.

2.2. Isolation of the Wheat FUS3 Homologues

Genomic DNA was isolated from seed at 15~30 DAP using the CTAB method. Gene specific primers TaFUS3-F (5'-TCC TCC GCC TTG ACC TCC T-3') and TaFUS3-R (5'-CAA GGC TGG TGA CTC TGA ACT-3') were designed based on the sequence of *TaFUS3* [37] using Primer 5.0 software. The PCR was performed in an Applied Biosystems-2720 thermal cycler in total volumes of 50 μL, including 5 μL of 10 × PCR buffer, 100 mM dNTP, 0.4 μM of each primer, 2.5 unit of Taq DNA polymerase (TIANGEN, Beijing), and 100 ng of template DNA. The PCR cycling conditions comprised an initial cycle at 95˚C for 3 min followed by 35 cycles of 94˚C for 30 s, annealing at 58˚C for 1 min and 72˚C for 2 min, with a final extension at 72˚C for 10 min. PCR products were separated by electrophoresis in 1.0% agarose gels. Targeted fragments of expected size were recovered and cloned into the pMD18-T vector. Selected 20 positive clones were sequenced by Henan Shangya Technology Co., Ltd. Sequence alignment was performed by using DNAMAN software. Chromosome locations of cloned sequences were identified at urgi (https://urgi.versailles.inra.fr/blast/blast.php).

2.3. Functional Analysis by BSMV-VIGS

For VIGS experiments, a *γ*RNA-based BSMV vector was utilized to silence *FUS3* gene in wheat as described previously [38]. Since wheat is hexaploid, there are usually three copies of genes. To make *TaFUS3* gene silenced completely, a 247 bp cDNA fragment from the wheat *FUS3* gene that contains *Nhe* I sites was amplified with specific primers designed in the conserved regions of the three copies by Primer 5.0 software. The forward and reverse primers were 5'-GCG GGA GAT GAT CTA GTT GC-3' and 5'-AAT CAC TGA ATG GGT CGA AGA-3', respectively. Then, the amplified PCR products and the BSMV-*γ* empty vector were individually digested with restriction enzyme *Nhe* I. The digestion results were verified by agarose gel electrophoresis. The correct target fragments were ligated into the BSMV-*γ* empty vector and sequenced. The inserted clone in re-

verse was selected as the recombinant vector BSMV: TaFUS3 and subsequently used for gene silencing. For identifying wheat plants with the VIGS response, the BSMV: GFP was used as an infection control.

In vitro transcription of viral RNAs was carried out as described by Feng *et al.* [38]. The plasmids of BSMV-α, BSMV-β, BSMV-γ-derivative clones (BSMV-TaFUS3 and BSMV-GFP) were digested with restriction enzymes *Mlu* I and *Spe* I. Then, the linearized products were examined by electrophoresis. The correct linearized products were purified, and transcription was performed using a RiboMAX^{TM} Large Scale RNA Production Systems-T7 kit (Promega, USA) following the manufacturer's protocol. Transcripts of each of the BSMV plasmids were mixed in a 1:1:1 ratio and 22.5 volumes of FES buffer [39] were added to the transcript mixture. This mixture was then applied to the young wheat spikes by rub inoculation. 15 μL of BSMV: TaFUS3 or BSMV: GFP transcript mixture was used for each spike. The details of the BSMV inoculation and wheat plant growth conditions were described by Liu *et al.* [36].

2.4. Seed Germination and Spike Sprouting Test

180 wheat seeds from both the BSMV-TaFUS3 and BSMV-GFP plants (40 dpi) with uniform size and plumpness were surface sterilized in 70% alcohol for 1 min, and then in 0.1% $HgCl_2$ for 5 - 10 min and washed four times with sterile distilled water. Six groups (30 grains/group) were distributed on two layers of filter paper in a 9-cm Petri dish containing 10 mL of sterilized water, and germinated at 25˚C under continuous dark for 7 days. Three of them were used to investigate the germination rate and the other three groups were used for the expression analysis of ABA/GA synthetic and metabolic genes. The number of germinated seeds (radicle protruding through the seed coat) was recorded daily. Germination ratio refers to the number of germinated seed as a proportion of the total number of seeds. For the gene expression analysis, germinated seeds were harvested at 24, 48, 72, and 96 h snap-frozen in liquid nitrogen and stored at −80˚C for further analysis.

Spike sprouting tests were performed with intact spikes (40 and 45 dpi). Twelve spikes each from the BSMV-TaFUS3 and BSMV-GFP plant groups were surface sterilized in 0.1% $HgCl_2$ for 10 min, and washed four times with sterilized water, vertically inserted into a foam board (length 20 cm × width 20 cm × height 3 cm), and covered by a plastic film to prevent water loss. Water was sprayed once every 6 h. After 7 days, the test spikes were taken out and immediately threshed manually to record seed germination. The calculation method of germination rate was the same as for the seed germination test. In addition, the plumule and radicle length was measured on germinated seeds. All tests were performed in a climate chamber at 25˚C (24 h darkness).

2.5. ABA Treatment

Seeds from BSMV-TaFUS3 and BSMV-GFP plants (40 dpi) were soaked in steri-

lized water for 4 h, and then the embryos were isolated with dissecting needles under aseptic conditions and were placed on two layers of filter paper in a 9 cm Petri dish containing 10 mL of sterilized water with 50 μM ABA at 25˚C in darkness for 48 h. RNA was extracted from embryos after 0 and 48 h of treatment. The experiments were performed in triplicate.

2.6. Quantitative Real-Time PCR (qRT-PCR)

The RNA extraction and the first strand cDNA synthesis were described in our previous study [40]. Based on the sequences of the genes, including *TaFUS3-3A*, *TaFUS3-3B*, *TaFUS3-3D*, *TaNCED1*, *TaNCED2*, *TaCYP707A2*, *TaGA20ox-D4*, *TaGA20ox1d*, *TaABI3*, *TaABI4*, *TaABI5*, *TaPKABA1*, and *β-actin*, specific primers were designed using Primer 5.0 software. The primer sequences and amplified product are provided in Table 1. The qRT-PCR analysis was performed with a SYBR Green quantitative RT-PCR kit (TaKaRa, Dalian, China) on a CFX connect real-time system (Bio-RAD, USA) following the manufacturer's instructions. The expression level of each gene was determined using the comparative Ct method. Relative quantification for each gene was calculated by the $2^{-\Delta\Delta Ct}$ method using wheat *β-actin* gene as an internal control [41].

3. Results

3.1. Isolation and Characterization of TaFUS3 Homologues in Wheat

Based on the known cDNA sequence of *TaFUS3* gene, a pair of specific primers

Table 1. Primer sequences for gene expression assay.

Gene	GenBank No.	Primer sequence (5' - 3')		Amplicon length (bp)
		Forward	Reverse	
TaFUS3-3A	MZ408248	GTGGCTGGGTTGCGAGTT	TGTTCGGCCAGTATCTGTTCC	196
TaFUS3-3B	MZ408249	GCCGAACAACAAGAGCAGG	GTGGTGGGATTAGAGACACATACTT	239
TaFUS3-3D	MZ408250	GTGGCTGGGTTGCGAGTTA	GTGCATTTAGCAAATCATGCGTA	160
TaFUS3-VIGS	-	CGAGATGTTTGATGGGATTTT	GGTTGGGAAACAAAGAAAGC	188
TaNCED1	JQ772528	GTCGGAGATGATGTGGGTG	CCGTGTCGTTGAAGATGGA	137
TaNCED2	LC077862	GTCGGAGATGGTGTGGGT	TCGTTGAAGATGGAGTCGG	132
TaCYP707A2	AB849504	CTGCCCCCTGGCTCCAT	GCCGTACCGCTTCTGCTT	102
TaGA20ox-D4	LN828669	GCGGCAGCAAAAACAAAT	GTCGACCACAGGCACGTC	94
TaGA20ox1d	FR716527	TCGCTGGAGATCATGGAG	GCACGGCGGGTAGTAGTT	114
TaABI3	DQ517494	CTGGTGACTTTGTTCGGTCC	TGGCATTCTTGTGCTTGG	138
TaABI4	AY781355	GGATGCTGCCCGTGCTTAT	TGAGTGGTTGGCTGATGTTGTAG	178
TaABI5	KX002276	TCCTGTGGTGGGTGCTGG	GCTGCTGTGAGGGTTGTGC	188
TaPKABA1	DQ343302	CCCTGATGAGCCAAGGAACT	CGGGACAGTAGATGTACGCAGT	111
β-actin	AB181991	TTTGAAGAGTCGGTGAAGGG	TTTCATACAGCAGGCAAGCA	196

TaFUS3-F and TaFUS3-R were designed in the 5' and 3' non-coding regions, respectively, to amplify the complete gene DNA sequence from wheat cultivar "Huaimai 0360". An amplicon of about 3500 bp was obtained. After sequencing, three genes, containing seven exons and six introns, and with sequence lengths of 3477, 3534 and, 3501 bp was obtained (Figure 1). The URGI BLAST analysis showed that the three sequences were highly similar to the, respectively, 3A, 3B and 3D sequences from the long arm of chromosome 3 in Chinese spring. So, the three *TaFUS3* homologues were named *TaFUS3-3A* (GenBank accession MZ408248), *TaFUS3-3B* (GenBank accession MZ408249) and *TaFUS3-3D* (GenBank accession MZ408250), respectively. The corresponding cDNA sequences of *TaFUS3-3A*, *TaFUS3-3B* and *TaFUS3-3D* were 1015, 1012 and 1015 bp in length, and contained 906, 909 and 906 bp open reading frames (ORF), respectively. *TaFUS3-3A*, *TaFUS3-3B* and *TaFUS3-3D* were predicted to encode proteins containing 301, 302 and 301 amino acid, respectively, and shared 98.57% similarity.

3.2. Transcription Profiling of the TaFUS3 Homoeologs during Seed Development and Germination in Wheat

The expression profiles of three *TaFUS3* genes in seed development and germination stages were investigated by qRT-PCR using gene-specific primer pairs TaFUS3-3A-F/R, TaFUS3-3B-F/R and TaFUS3-3D-F/R (Table 1). Expression analysis indicated that the significant differences in transcription were observed among the three homoeologous genes. *TaFUS3-3B* and *TaFUS3-3D* had similar expression patterns (Figure 2), whereas the expression of *TaFUS3-3A* was almost undetectable in all samples (data not shown). During grain filling stage, the transcription of *TaFUS3-3B* and *TaFUS3-3D* showed a gradual upward trend at 15 - 25 DAP, and then declined quickly at 30 DAP (Figure 2(a)). During seed imbibition, the expression of *TaFUS3-3B* and *TaFUS3-3D* showed an up-regulation trend in the 0 - 24 h period. Afterwards (36 - 48 h), the transcripts of the two genes showed gradual declined (Figure 2(b)). Additionally, the two *TaFUS3* genes also shared different transcript abundances during seed development and germination process. For instance, *TaFUS3-3D* had higher expression level than

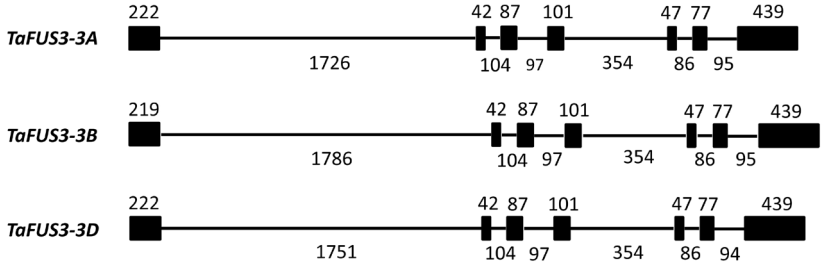
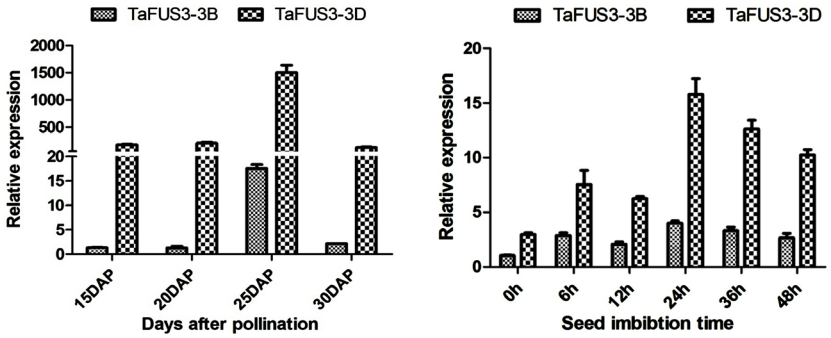


Figure 1. Schematic comparison of the *TaFUS3* homologs genes. Black boxes indicate exons and solid lines indicate introns. The numbers above the lines and below the boxes indicate the size of exons and introns in bp, respectively.



(a) (b)

Figure 2. Expression profiles of the *TaFUS3* genes determined by quantitative real-time PCR Analysis. ((a) and (b)) The expression levels of *TaFUS3-3B* and *TaFUS3-3D* in seed development (a) and germination (b) in wheat cultivar Huaimai 0360.

that of *TaFUS3-3B*. These results indicated that the two *TaFUS3* homoeologs might play an important role in wheat seed development and germination.

3.3. Molecular Identification of the Barley Stripe Mosaic Virus-Wheat FUSCA3 (BSMV-TaFUS3) Infected Wheat Plants

To functionally test the role of *TaFUS3* gene, we used a virus-induced gene silencing (VIGS) approach in the cultivar "Huaimai 0360". This cultivar has a relatively high seed dormancy level. A 247-bp cDNA fragment of the *TaFUS3* gene was isolated. Then, a recombinant viral vector BSMV: TaFUS3 was constructed and used to inoculate the wheat spikes during the heading stage under field conditions. We collected developing seeds at 21, 28, 35, and 45 dpi and then mature seeds (45 dpi) were imbibed for 24, 48, 72, and 96 h. The qRT-PCR analysis showed that the transcript level of *TaFUS3* in the BSMV: TaFUS3-silenced plants was significantly decreased (by 61%, 41%, 55%, and 25% at 21, 28, 35, and 45 dpi, respectively) compared with the control plants (**Figure 3(a)**). During seed germination, the transcript abundance of *TaFUS3* declined by 18% - 24% in mature seeds (45 dpi) imbibed for 48 - 96 h (**Figure 3(b)**). These findings indicated the target gene was successfully repressed in BSMV: TaFUS3-silenced plants.

3.4. Silencing of TaFUS3 Increased Seed Germination and Spike Sprouting Capacities

The effect of *TaFUS3* knockdown on the seed germination was characterized at 40 dpi. The seed germination capacity of BSMV-TaFUS3-silenced plants was always higher than that of control plants (BSMV: GFP infected) for 7 days of imbibition (**Figure 4(a)**). Moreover, the germination speed was faster in BSMV:TaFUS3 than BSMV:GFP infected plants following the addition of water. As shown in **Figure 4(b)**, the seed germination rate in TaFUS3-silenced plants reached 16.7%, 24.5%, and 27.8% on the first, second and third day of germination, which were 5 times, 3.7 times and 2.8 times of the control, respectively.

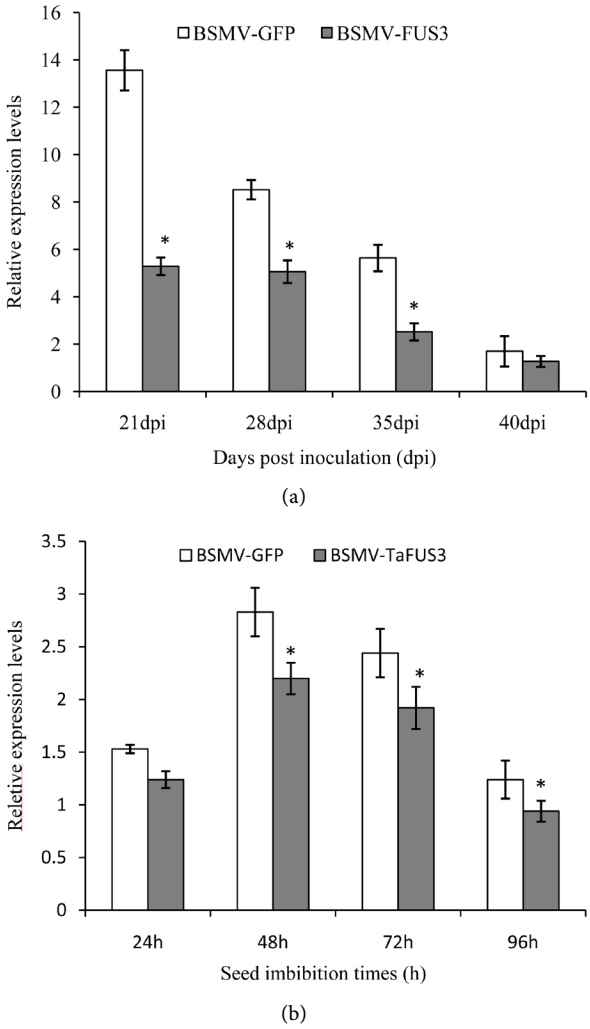


Figure 3. Identification of the BSMV-VIGS wheat plants and expression profiles of the *TaFUS3* genes determined by quantitative real-time PCR Analysis. ((a) and (b)) Relative expression level of the *TaFUS3* gene during seed development (a) and germination (b) in the BSMV-TaFUS3-inoculated and the BSMV-GFP-inoculated plants. Asterisks indicate significant differences ($p < 0.05$).

Similar results in PHS experiment were observed at 40 and 45 dpi. The data showed that the sprouting capacity was obviously higher in spikes of BSMV:TaFUS3 than BSMV:GFP plants (**Figure 4(c)**). Compared with the control, the germination rates in seed of BSMV-TaFUS3 wheat spike increased on average 50% (40 dpi) and 24.1% (45 dpi) (**Figure 4(d)**). In addition, the plumule and radicle lengths in grains of BSMV: TaFUS3 wheat spike were increased by, respectively, 60.4% and 55.8% at 40 dpi (**Figure 4(e)**) and 14.3% and 32.5% at 45 dpi (**Figure 4(f)**). These findings indicated that the sprouting speed was significantly faster in spikes of BSMV: TaFUS3 than BSMV-GFP wheat. Above results suggested that *TaFUS3* silencing resulted in enhanced seed germination and PHS in wheat grains; thus, *TaFUS3* may act as a negative regulator of wheat seed germination.

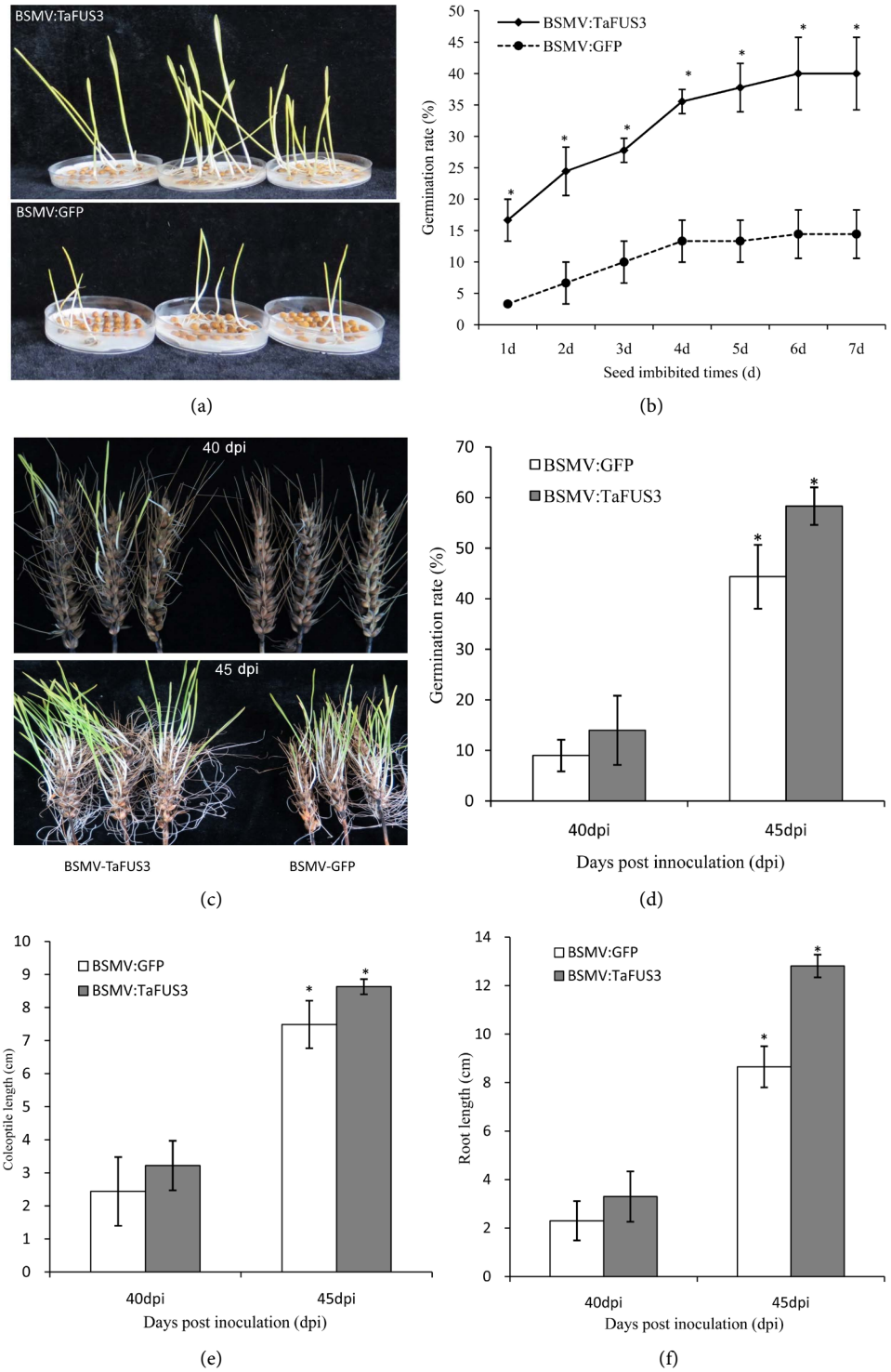


Figure 4. Characterization of seed germination and PHS from the BSMV-TaFUS3-infected and BSMV-GFP-infected wheat plant. ((a) and (b)) Seed germination phenotypes and germination rate of BSMV-TaFUS3-infected and BSMV-GFP-infected wheat plants at 40 dpi incubated on moist filter paper for 7 d. ((c) and (d)) Spike sprouting phenotypes and germination rate of BSMV-TaFUS3-infected and BSMV-GFP-infected plants incubated for 7 d under misting conditions at 40 and 45 dpi. ((e) and (f)) Coleoptile and radicle lengths of the BSMV-TaFUS3-infected and BSMV-GFP-infected wheat spikes after 7 d under misting conditions at 40 and 45 dpi.

3.5. Silencing of TaFUS3 Changed the Transcript Levels of Genes Related to GA/ABA Biosynthesis and ABA Metabolism during Seed Germination

To define the role of *TaFUS3* in regulating seed germination and PHS, qRT-PCR analyses were carried out to detect the expression levels of ABA biosynthesis genes *TaNCED1*, *TaNCED2*, GA biosynthesis genes *TaGA20ox-D4*, *TaGA20ox1d* and ABA metabolism gene *TaCYP707A2* (Table 1). The result revealed that the transcription of one GA synthesis genes (*TaGA20ox1d*) and one ABA metabolism gene (*TaCYP707A2*) was evidently induced and their transcript levels in TaFUS3-silenced plants were up-regulated on average by 1.9 and 2.0 times, respectively, in the seeds imbibed for 48 hours (Figure 5). However, the expression of ABA biosynthesis genes *TaNCED1* and *TaNCED2* was obviously inhibited and the transcription levels of *TaNCED2* in TaFUS3-silenced plants decreased by 32.86% (Figure 5), indicating that *TaFUS3* might control seed dormancy and PHS -resistance by positively regulating ABA synthesis genes and negatively regulating ABA metabolism and GA synthesis genes.

3.6. Decreased Expression of TaFUS3 Gene in Wheat Granted Reduced ABA Sensitivity through Negatively or Positively Regulating Different Genes Involved in the ABA Signaling Pathway

The expression levels of *TaFUS3* and endogenous *ABI3*, *ABI4*, *ABI5*, and *PKABA1* (Table 1) were analyzed in embryos of the BSMV-infected plants upon ABA treatment. As shown in Figure 6, after ABA treatment, the expression of these genes was induced. Compared with the control, the relative transcript level of *TaFUS3* in BSMV:TaFUS3 plants was significantly lower. In addition, the expression of *TaFUS3* in TaFUS3-silenced plants significantly up-regulated the expression of *PKABA1* and markedly down-regulated the expression of *ABI5*

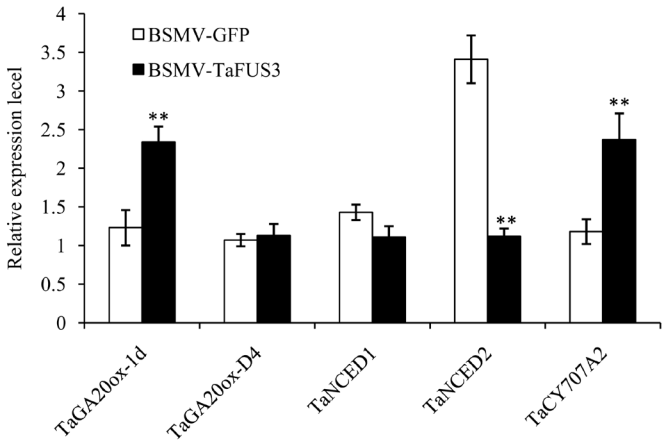


Figure 5. Expression profiles of *TaGA20ox1d*, *TaGA20ox-D4*, *TaNCED1*, *TaNCED2*, and *TaCYP707A2* genes determined by qRT-PCR analysis in the process of seed germination. Each value is the mean ± standard deviation of three independent biological replicates. "**" indicate significant differences at $p < 0.01$ level.

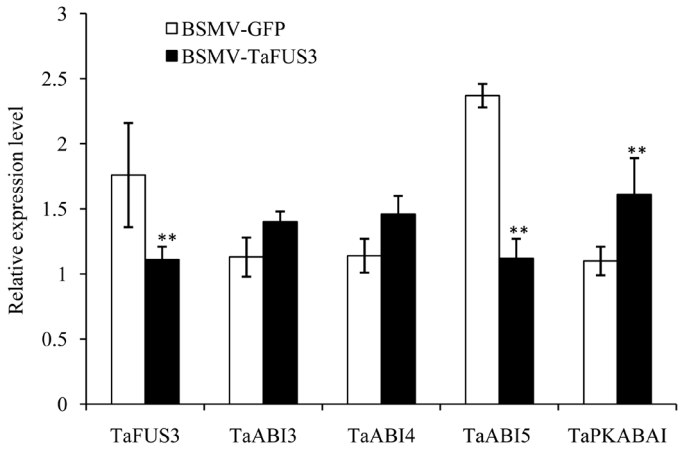


Figure 6. Transcription levels of the *TaFUS3*, *TaABI3*, *TaABI4*, *TaPKABA1*, and *TaABI5* genes in embryos of the BSMV-infected plants after 50 μmol/L ABA treatment. Each value is the mean ± standard deviation of three independent biological replicates. "**" indicate significant differences at $p < 0.01$ level.

(**Figure 6**). Although the expression of *ABI3* was also up-regulated, the difference was not significant. These findings suggested that repressed expression of *TaFUS3* gene reduced ABA sensitivity through negatively regulating *ABI4* and *PKABA1* genes and positively regulating *ABI5* gene involved in the ABA signaling pathway.

4. Discussion

FUS3 is a key regulator in inducing dormancy and inhibiting seed germination in *Arabidopsis thaliana*. In the present study, in order to clarify the functions of *FUS3* orthologs in wheat seed germination and PHS resistance, the cDNA and genomic DNA sequences of *TaFUS3* homoeologs from wheat were isolated and characterized for the first time. Sequence analysis revealed that the *TaFUS3* homoeologs had the similar structures and numbers of amino acids, and all encode a protein with a conserved B3 domain (**Figure 1**). Although the three *TaFUS3* genes had high homology with barley *HvFUS3* (GenBank No.CAL91173) at amino acid level, there were great differences in gene structure among them. Wheat *TaFUS3* homoeologs had seven exons and six introns, whereas barley *HvFUS3* gene had same six exons and five introns as *AtFUS3*, implying *TaFUS3* gene might have different function from *HvFUS3* and *AtFUS3*.

The qRT-PCR analysis demonstrated that only two copies of the *TaFUS3* genes were constitutively transcribed at different developmental and germinating stages. Moreover, transcript levels of both *TaFUS3-3B* and *TaFUS3-3D* also varied with the developmental and germinating process. During grain filling stage, the relatively high expression of *TaFUS3-3B* and *TaFUS3-3D* was recorded at 25 DAP (**Figure 2(a)**). Similar results were obtained by Luerßen *et al.* [17], who showed that the expression level of *AtFUS3* was higher in the prophase and near metaphase of seed development, but decreased before seed maturity. The

two *TaFUS3* genes were induced after seed imbibition, and relatively high expression was noted 24 h after imbibition commenced (**Figure 2(b)**); these findings were in agreement with the results observed by Chiu *et al.* at high temperatures [31]. Hence, the expression of the three *TaFUS3* genes in wheat seed was differentially regulated, which might reflect a difference in functions of the three homoeologs in the development and germination.

Earlier experiments with the transgenic *Arabidopsis* revealed that *AtFUS3* over-expression delayed seed germination during imbibition [31]. Based on this finding, it might be expected that under-expression of *TaFUS3* would have an opposite effect, that is accelerating germination. As shown in **Figure 4**, this hypothesis was supported; germination of seeds in *TaFUS3*-silenced plants was hindered for at least 7 d following the addition of water. Spike sprouting test further revealed that grains in the *TaFUS3*-silenced plants showed copious germination in the spike 7 d after being subjected to sprouting conditions, whereas the grain in the plants with normal expression of *TaFUS3* barely germinated at 40 dpi. However, germination rate in TaFUS3-silenced plants was significantly lower than those in the control (GFP infected) plants at 45 dpi, suggesting the difference narrowed. So, we speculated that this might be related to the decrease in dormancy level of grains because the expression levels in grains of TaFUS3 silenced spikes were lower than those in the control plants. These results suggested that *TaFUS3* had an important role in regulating seed dormancy and PHS tolerance. Interestingly, the inhibition of germination occurred at the appropriate seed germination temperature (25˚C) of wheat instead of the supra-optimal temperature in case of *Arabidopsis* reported by Chiu *et al.* [31]. The reason might be related to the characteristics of different species.

It has long been known that endogenous hormones abscisic acid (ABA) and gibberellin (GA) played a key role in controlling seed dormancy and germination [12]. GAs breaks seed dormancy and promote germination. However, ABA is considered to induce and maintain seed dormancy. Seed dormancy or germination depends on the levels of ABA/GA, which are controlled by the precise balance between biosynthesis and catabolism rates of these hormones [12]. Recently, the major enzymes involved in ABA and GA metabolism pathways have been identified, such as ABA biosynthetic gene *NCED* that encodes 9-cis-epoxycarotenoid dioxygenase, ABA catabolic gene *CYP707A* that encodes ABA 8'-hydroxylase, GA biosynthetic gene GA20ox that encodes GA 20-oxidase, GA3ox that encodes GA 3-hydroxy-lase, and GA catabolic gene GA2ox that encodes GA 2-oxidase [42] [43] [45]. Gubler *et al.* [46] reported that suppressing expression of *HvCYP707A* in transgenic barley grains resulted in a higher ABA content and increased dormancy. The over-expression of *AtFUS3* delayed seed germination by regulating ABA/GA ratio at high temperatures [31] [33]. In the present study, seed germination and PHS were significantly enhanced when *TaFUS3* gene was down-regulated. Therefore, we hypothesized that the *TaFUS3* gene may be involved in the regulation of ABA/GA synthesis and metabolism

during seed germination. To test the hypothesis, we investigated the expression patterns of the ABA biosynthesis-related genes *TaNCED1* and *TaNCED2*, the GA biosynthesis-related genes *TaGA20ox-D4* and *TaGA20ox1d* and the ABA metabolism-related gene *TaCYP707A2*. The transcription levels of *TaGA20ox-D4*, *TaGA20ox1d* and *TaCYP707A2* in grains of BSMV:TaFUS3-silenced plants were significantly up-regulated (**Figure 5**), whereas the expression levels of *TaNCED1* and *TaNCED2* in BSMV:TaFUS3-silenced plants were significantly down-regulated in imbibed seed (**Figure 5**). This pattern is in accordance with the increase in seed germination and PHS capacity observed in BSMV: TaFUS3 silenced plants after 7 d of incubation (**Figure 4**). These results suggested that down-regulating expression of *TaFUS3* might lead to increased GA transcription level and decreased ABA transcription level, indicating *TaFUS3* may regulate seed dormancy and PHS-resistance by regulating the expression levels of genes in the ABA and GA metabolism pathways.

In addition to ABA levels, the ABA signal and sensitivity of embryos to ABA also play important roles in seed dormancy and PHS. Cultivars with strong dormancy have strong sensitivity to ABA [47] [48] [49]. The ABA signal regulators, such as the ABI (for ABA-insensitive) transcription factors, include *ABI3*, *ABI4* and *ABI5*. The *ABI3* belongs to the B3 type transcription factors and plays an important regulatory role in seed dormancy and ABA inhibition of seed germination [50]. The *ABI5* encodes a bZIP transcription factor whose accumulation inhibits seed germination and early seedling growth [51] [52]. Another important component of the pathway is the ABA-induced Ser/Thr protein kinase PKABA1 [53]. The PKABA1 mRNA levels in both seeds and leaves of wheat increase rapidly in response to dehydration and ABA [54]. In the present work, we examined the response of *TaFUS3* and the ABA signaling pathway-related genes *ABI3*, *ABI4*, *ABI5*, and *PKABA1* to ABA treatment. The results revealed that the expression of *TaFUS3* and *ABI5* was significantly down-regulated and the expression of *ABI3*, *ABI4* and *PKABA1* was up-regulated in the TaFUS3-silenced plants in the ABA treatment (**Figure 6**). We speculate that the mechanism underpinning reduced ABA sensitivity of *TaFUS3*-silenced plants is based on *TaFUS3* regulating the expression of genes susceptible as *PKABA1*and *ABI5*.

5. Conclusions

In this study, three *TaFUS3* homoeologous genes, encoding B3-domin proteins, were isolated and their role in seed germination and PHS were investigated using the BSMV-VIGS method under field conditions. In TaFUS3-silenced wheat plants, seed germination and PHS capacities were significantly increased, and the seed dormancy level was significantly decreased in mature grains. The transcription levels of two GA synthesis-related genes and one ABA catabolism-related gene were significantly up-regulated, and two ABA synthesis-related genes were significantly down-regulated. In addition, three signal genes involved in ABA response were markedly up-regulated, and one signal gene was markedly down-regulated

in embryos of *TaFUS3*-silenced plants under ABA treatment. These results indicated that *TaFUS3* acts as a positive regulator in inhibiting seed germination and PHS, and temporally regulates the expression of some ABA, GA metabolic-related genes and ABA signal genes in imbibed wheat seed.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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