A 4-bp deletion in the 5’UTR of TaAFP-B is associated with seed dormancy in common wheat (Triticum aestivum L.)

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Abstract

Background: AFP is a negative regulator of ABA signaling that promotes ABI5 protein degradation and weakens regulation of ABA signaling by targeting upstream genes of ABI5, and TaABI5 gene was seed-specific, and accumulated during wheat grain maturation and dormancy acquisition, which played an important role in seed dormancy; TaAFP has a conserved domain with AFP, so TaAFP may also play an important role in seed dormancy in wheat.

Results: Two allelic variants of TaAFP were identified on chromosome 2BS in common wheat, and designated as TaAFP-B1a and TaAFP-B1b. Sequence analysis showed a 4-bp deletion in the 5’UTR region of TaAFP-B1b compared with TaAFP-B1a. Based on the 4-bp deletion, a co-dominant functional marker of TaAFP-B was developed and designated as AFPB. The genotype generating a 203-bp fragment (TaAFP-B1b) was more resistant to pre-harvest sprouting than the genotype producing a 207-bp fragment (TaAFP-B1a) in a test of 91 white-grained Chinese wheat cultivars and advanced lines. The average germination index(GI) values of TaAFP-B1a and that of TaAFP-B1b were 45.18 and 30.72%, respectively, indicating a significant difference (P < 0.001). Moreover, the 4-bp deletion located in the 5’UTR not only affected the transcription level of TaAFP-B, but also affected the mRNA decay, reduced the translation level of GUS and tdTomatoER and GUS activity in wheat leaves of transient expression. The transcript expression and the mRNA half-life value of TaAFP-B1a in developing seeds and mature seeds were much higher than those of TaAFP-B1b.

Conclusion: We identified a 4-bp InDel in the 5’UTR of TaAFP-B, which affected the mRNA transcription level, mRNA decay, translation levels of GUS and tdTomatoER, GUS activity, and was significantly associated with seed dormancy in common wheat. A functional marker was developed and validated based on this InDel.

Keywords: Wheat, Seeds dormancy, TaAFPs, Marker, Transcription, Translation

Background

A high level of seed dormancy plays a pivotal role in resistance to pre-harvest sprouting (PHS), providing a mechanism for plants to delay germination until conditions are optimal for survival of the next generation [1, 2]. The balance of abscisic acid (ABA) and gibberellin (GA) levels and sensitivity is a major regulator of dormancy status. The mechanism of ABA sensitivity in seeds has been extensively studied in Arabidopsis. Some genes associated with seed dormancy have been identified as factors in the ABA signaling and ABA synthesis pathway [3–6]. abi5 is insensitive to an ABA-induced post-germination growth arrest [7], and alters activity of an ABA-inducible late embryo genesis abundant (LEA) gene promoter [8]. ABI5 is mainly expressed in dry seeds, and its expression significantly decreases after germination [9, 10]. Expression of ABI5 is induced strongly by exogenous ABA [4], and is regulated by ABI3, HYL1 and HYS [11, 12], but is repressed by WRKY2, WRKY40, WRKY18 and WRKY60 [13, 14]. Both abi3 and abi5 mutants were initially recovered by virtue of their ability to germinate in the presence of ABA [7, 15, 16]. The Arabidopsis ABI3 gene is an ortholog of Vp-1 and is required for appropriate ABI5 expression [11, 10, 17]. ABI3 encodes a transcription factor and acts together with ABI5 to govern embryonic gene expression and seed sensitivity to ABA [7, 15, 16].
ABI5 binding protein (AFP), a novel negative regulator of ABA signaling that works by facilitating the degradation of ABI5 [18], was isolated using yeast two-hybrid assays; AFP functions in developing seeds and young seedlings [18]. AFP gene transcription and translation increased during seed development and desiccation, ultimately reaching plateau values in mature seeds [18]. ABI5 acts as a critical factor in maturation, dormancy development of seeds, or the dehydration tolerance of young seedlings of Arabidopsis [19, 20].

In wheat (*Triticum aestivum*), many genes or QTL are associated with tolerance to PHS, and molecular markers have been developed based on these genes and QTL [21–26]. Several major QTL [27–29] associated with PHS tolerance were found and some genes associated with PHS tolerance were cloned [21, 30–37]. *TaABI5* genes one of which is the same as *TaABF*, and *TaABF* mRNA was seed-specific and accumulated during wheat grain maturation and dormancy acquisition, which played an important role in seed dormancy were isolated in wheat [32]. *TaABI5s* were expressed in developing grain, roots, and leaves [38]. Three wheat *AFP* genes (*TaAFPs*) were isolated, located on the short arms of chromosomes 2A, 2B, and 2D, and designated *TaAFP-A*, *TaAFP-B*, and *TaAFP-D*, respectively [38]. The structure of *TaAFP* consisted of one intron and two exons, including a nuclear localization domain (119–133 aa of *AtAFP*) in the middle of the deduced amino acid sequence and an ABI5-binding domain (284–335 aa of *AtAFP*) in the C-terminal region.

ABI5 has an important function in maturation and dormancy development of seeds in Arabidopsis, while AFP promotes ABI5 protein degradation [18]. Moreover, *TaAFP* has a conserved domain (60 and 69%) with *AtAFP* in the region of nuclear localization and the ABI5 binding domain, respectively [38]. Therefore, *TaAFP* may play an important role in seed dormancy in wheat. The objectives of the present study were to: (1) identify allelic variations at the *TaAFP* locus in Chinese wheat varieties with different levels of seed dormancy; (2) develop a functional marker for use in marker-assisted selection for PHS tolerance; and (3) characterize the transcription regulation mechanism of *TaAFP*. The identification of new alleles of *TaAFP* associated with different seed dormancy could also contribute to our understanding of the mechanisms underlying seed dormancy or PHS tolerance in common wheat.

### Results

#### Sequence analysis of three *TaAFP* homologs in varieties with different levels of seed dormancy

Full sequences of *TaAFP-A*, *TaAFP-B*, and *TaAFP-D* were cloned using genome-specific primers (Table 1). Six new alleles of *TaAFPs* were found and named according to the 2005 Supplement of the Wheat Gene Catalogue [39]. In these germplasm, *TaAFP-A1a* and *TaAFP-A1b* were on chromosome 2AS; *TaAFP-B1a* and *TaAFP-B1b* were on chromosome 2BS; and *TaAFP-D1a* and *TaAFP-D1b* were on chromosome 2DS. *TaAFP-A* was amplified and sequenced with three primer sets *TaAFP-AF1/R1*, *TaAFP-AF2/R2*, and *TaAFP-AF3/R3* from 10 varieties mentioned above. Two new alleles of *TaAFP-A* were found and designated *TaAFP-A1a* and *TaAFP-A1b*.

### Table 1

The primer sets used in this study

| Primer Set | Upstream (5′-3′) | Downstream (5′-3′) | Primer Anneal Temperature (°C) | Fragment Size (bp) |
|------------|-----------------|-------------------|-------------------------------|-------------------|
| TaAFP-AF1/R1 | GATTCTACTGCTGCTGCTTT | GAGCAAGGAAGCGCAATAG | 57 | 808 |
| TaAFP-AF2/R2 | CCGTCCTGACCCACAGGGGAAG | CAGACTCTGAGCCTACGTAACCTC | 63 | 568 |
| TaAFP-AF3/R3 | GAGATGGCTCTTACGGCATG | GAGGAAGTGCCGCTGGCA | 60 | 613 |
| TaAFP-BF1/R1 | GATTCTGCTGGCTCTGCTTT | CAAGGGAACGCAATAAGAC | 57 | 828 |
| TaAFP-BF2/R2 | CCGTCCTGACCCACAGGGGAAG | ACCCTGAGCCTACGTAACCTC | 62 | 587 |
| TaAFP-BF3/R3 | GAGATGGCTCTTACGGCATG | GCCCTAGTACATCCTCGGA | 59 | 733 |
| TaAFP-D1/R1 | GATTCTGCTGGCTCTGCTTT | TTGAGTAGCAGGGGAAGG | 57 | 770 |
| TaAFP-D1/R2 | CCGTCCTGACCCACAGGGGAAG | TCCCTCCTTCAATAAATAGTGAAC | 62 | 618 |
| TaAFP-D1/R3 | GAGATGGCTCTTACGGCATG | AGCATGTTGGCAGGCGGA | 60 | 706 |
| AFBF/R | CTTCTTGAGAATTGGCCGGTG | TGAAGTGCAGGACCCACACTCACAC | 61 | 207,203 |
| ACTINF/R | GATCTTTCAATGCTACGTTCGATG | CATATATATACACGAGCAG | 64 | 410 |
| Q-TaAFP-BF/R | ACCTTGCTCAGGCAACG | ACGTGTCATGCTGCTACCG | 66 | 102 |
| Q-TaAFP-AF/R | CCGTCTCAGAAGCATGCCGG | GCTGGAACGGTATTCATAG | 59 | 100 |
| Q-TaAFP-D1/R | CTTCTCTCAACGGCATGCCGGA | CGCCACCTTGGAGGAGACT | 56 | 289 |
| Q-ABI5F/R | GGAAGAAGAGCATCCACC | GAGGCAAGAGGAGAAGGACT | 62 | 310 |

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Compared with the TaAFP-A (AB360911) [38], 6 SNPs were found in the full sequence of TaAFP-A1a: an A to G transversion was observed at 197 bp in the 5’UTR; 2 SNPs were located in exons (i.e. C to T at position 94 bp in the first exon, and G to A at position 1523 bp in the second exon) that cause changes of amino acids Gla to Val and Glu to Lys, respectively; and the other 3 SNPs (i.e. G to T, T to A, and G to A) were present in the introns at positions of 529, 717, and 980 bp, respectively. For TaAFP-A1b, 4 SNPs were found: a C to T transversion at 94 bp in the first exon causing a change of amino acid from Gla to Val; and the other 3 SNPs (i.e. T to A, C to A, and C to G) were located in the introns at positions of 717, 764, and 976 bp, respectively (Additional file 1: Figure S1).

TaAFP-B was amplified with the primer sets TaAFP-BF1/R1, TaAFP-BF2/R2, and TaAFP-BF3/R3. Two alleles of TaAFP-B were found, designated TaAFP-B1a and TaAFP-B1b. Compared with the TaAFP-B gene (GenBank accession AB360912) [38], TaAFP-B1a had 8 SNPs and 2 insertions. Four SNPs (i.e. G to A, G to A, T to C, and G to T at positions 199 bp, 152 bp, 27 bp, and 26 bp, respectively) were found in the 5’UTR; 2 SNPs (i.e. A to G at position 203 bp that causes change of amino acid from Tyr to Cys, and T to C at 1238 bp that is a synonymous mutation) were found in the first and second exon, respectively; 2 SNPs (i.e. T to C and G to A at position 574 bp and 1102 bp) were observed in introns; and the 2 insertions (G insertion and CT insertion at positions 45 bp and 25 bp) were present in the 5’UTR.

TaAFP-B1b had 3 SNPs, 1 insertion, and 1 deletion. One SNP was a C to T at position 199 bp was found in the 5’UTR; another SNP was A to G at position 1252 bp that causes a change of amino acid from Glu to Gly; and the final SNP was T to C at position 574 bp was identified in an intron; there was a G insertion at position 45 bp and CT deletion at position 25 bp were found in the 5’UTR. In addition, a polymorphic fragment was detected in the 10 varieties with different levels of seed dormancy amplified with primer set TaAFP-BF1/R1. An 830-bp fragment was amplified in genotypes of TaAFP-B1a, whereas an 826-bp fragment was generated in genotype of TaAFP-B1b. Compared with the TaAFP-B1a genotype, a 4-bp deletion (CTCT) in the 5’UTR was present in TaAFP-B1b (Additional file 2: Figure S2).

The full sequence of TaAFP-D was amplified with the genome-specific primer sets TaAFP-DF1/R1, TaAFP-DF2/R2, and TaAFP-DF3/R3. Two new alleles of TaAFP-D were found and designated TaAFP-D1a and TaAFP-D1b. Compared with the TaAFP-D gene (GenBank accession AB360913) [38], 3 SNPs were found in TaAFP-D1a: 2 SNPs were located in the second exon (i.e. A to G transversion at positions 1502 and 1530 bp, resulting in changes of amino acids Ile to Met and Ser to Gly, respectively), and another SNP was located in the intron at 1273 bp. Five SNPs were found in the full sequence of TaAFP-D1b: 2 SNPs (i.e. A to G transition at positions 1502 and 1530 bp) were located in the second exon leading to change of amino acids Ile to Met and Ser to Gly, respectively; and the other 3 SNPs (i.e. G to A, T to C, and G to C) were detected in the intron at positions of 742, 995, and 1071 bp (Additional file 3: Figure S3).

Development and validation of the STS marker AFPB for PHS tolerance

Based on the sequence analysis, an STS marker of TaAFP-B, designated AFPB and located in the 5’UTR, was developed and used for association analysis with 91 Chinese varieties and advanced lines. Among the 91 varieties and lines tested, 23 possessed the allele TaAFP-B1a with a 207-bp fragment, whereas 68 had TaAFP-B1b with a 203-bp fragment (Additional file 4: Table S1 and Fig. 1). The GI values of the 91 varieties were consistent over the 2 years (r = 0.631, P < 0.01), with mean values and standard deviations of 0.345 ± 0.17 in 2006 and 0.343 ± 0.16 in 2007. Analysis of variance indicated significant differences (P < 0.001) between the two genotypes. The genotype TaAFP-B1a with a 207 bp fragment was more susceptible to PHS with an average GI value of 0.452, compared with TaAFP-B1b with an average GI value of 0.307, exhibiting a significant association of TaAFP-B with PHS tolerance.

Expression of the TaAFP-B gene in Zhou 8425B and Wanxianbaimaizi at different developmental stages

To evaluate the potential influence of different alleles and characterize the expression patterns of TaAFP-B1a and TaAFP-B1b in different cultivars, the expression patterns of Zhou 8425B (TaAFP-B1a) and

![Fig. 1](image-url) PCR fragments amplified with the primer set TaAFP-BF1/R1 from 10 wheat cultivars and lines. 1: Jimai 19 (TaAFP-B1a); 2: Jinan 16 (TaAFP-B1b); 3: Zhou 8425B (TaAFP-B1a); 4: Yangxiaomai (TaAFP-B1b); 5: Zhoumai 16 (TaAFP-B1a); 6: Yanfu 188 (TaAFP-B1a); 7: Langzhongbaimaizi (TaAFP-B1b); 8: Wanxianbaimaizi (TaAFP-B1b); 9: Yongchuanbaimaizi (TaAFP-B1a); 10: Xiaobaiyuhua (TaAFP-B1b).
Wanxianbaimaizi (TaAFP-B1b) were determined using real-time Quantitative PCR (RT-qPCR) analysis. The transcript expression levels of TaAFP-B1a were higher than that of TaAFP-B1b in seeds at 10 days after pollination (DAP), 20 DAP, 30 DAP, 40 DAP, and dry mature seeds immersed in water for 24 h (Fig. 2). In addition, the transcript expression levels of TaAFP-B1a and TaAFP-B1b had a trend of first increasing and then decreasing in seeds of different developmental stages, reaching the highest level at 20 DAP and the lowest at 40 DAP (Fig. 2). No transcript expression of TaAFP-B1a and TaAFP-B1b was detected in dry seeds. The results illustrated that allelic variation in the 5‘UTR of TaAFP-B affected the transcript expression level in seeds at 10 DAP, 20 DAP, 30 DAP, and 40 DAP and in dry mature seeds immersed in water for 24 h.

Effect of the 4-bp deletion in the 5′UTR on the mRNA decay of TaAFP-B
The results showed that TaAFP-B mRNA half-life (t_{1/2}) of Zhou 8425B with the allele TaAFP-B1a was 144.38 min, 154.00 min, and 115.50 min, in flag leaves, dry mature seeds, and seeds at 20 DAP, respectively, whereas those of Wanxianbaimaizi were 39.60 min, 46.20 min, and 33.32 min, respectively (Fig. 3). This indicated that TaAFP-B mRNA of Zhou 8425B with TaAFP-B1a was more stable than that of Wanxianbaimaizi with TaAFP-B1b.

The mRNA patterns of TaAFPs and TaABI5 at different seed developmental stages
RT-qPCR showed that TaAFP-B had the highest transcription level of the four genes: TaAFP-A, TaAFP-B, TaAFP-D, and TaABI5 at 10 DAP, 20 DAP, 30 DAP, and 40 DAP, respectively, in two genotypes of Zhou 8425B and Wanxianbaimaizi. Transcription levels of these four genes also had the general trend of gradually increasing and then decreasing during seed development. At the same time, TaAFP-B had a higher transcriptional level in Zhou 8425B than in Wanxianbaimaizi at each observation time point (Fig. 4). The most abundant TaAFP-B transcript levels in Zhou 8425B and Wanxianbaimaizi were detected at 20 DAP, while the highest transcriptional level of TaABI5 occurred at 20 DAP in Zhou 8425B and at 30 DAP in Wanxianbaimaizi (Fig. 4). These results indicated that the time of the highest transcription level of TaABI5 was later than that of TaAFP-B in Wanxianbaimaizi; this might be another reason why Wanxianbaimaizi has a higher capacity of seed dormancy than Zhou 8425B.

Transient expression of tdTomatoER and GUS-tagged pITV1-TaAFP-Ba/bF in wheat leaves
The plant transient expression vectors pITV1-TaAFP-BaF, pITV1-TaAFP-BbF, and pITV1#1534 (control) were separately transformed into wheat leaves of Zhou 8425B using biolistic bombardment. The fluorescence intensity of tdTomatoER gene expression in leaves of wheat were observed with a confocal laser scanning microscope after the transformation for 12–18 h in a dark environment, then the GUS expression was observed with an optical microscope after treatment with dye and decoloring solution of GUS. The results showed that the fluorescence intensity of tdTomatoER gene expression and GUS gene expression showed the same trend with the fluorescence intensity of tdTomatoER gene expression (Fig. 5: a, b, and c). In addition, GUS gene expression showed the same trend with the fluorescence intensity of tdTomatoER gene expression (Fig. 5: d, e, and f). These results indicated that the 4-bp deletion in the 5′UTR of TaAFP-B reduced the translation level of tdTomatoER and GUS.
Quantitative analysis of TaAFP-Ba/bf activity
To investigate the quantitative of TaAFP- Ba/bf sequence of promoter activity, total proteins were extracted from wheat leaves of transient expression. The GUS activity was determined by fluorometric assays. The GUS expression level of TaAFP-BaF::GUS and TaAFP-BbF::GUS transient expression leaves were significantly higher than Col ($P < 0.01$)(Fig. 6). Otherwise, the GUS expression level and activity in TaAFP-BaF::GUS were higher than TaAFP-BbF::GUS ($P < 0.01$). The results indicated that the 4 bp deletion in the 5'UTR of TaAFP-B decreased the GUS activity.

Discussion
The 800-bp region in the 5'UTR upstream from the start codon ATG of TaAFPs is relatively well conserved [38]. Nevertheless, in the present study, five allelic variants in the 5'UTR of TaAFP-A and TaAFP-B were found in 10 varieties (Additional file 1: Figure S1 and Additional file 2: Figure S2), indicating rich allelic variations in the 5'UTR of TaAFP-A and TaAFP-B in these germplasms. In addition, no alleles with the same to sequences as AB360911, AB360912, or AB360913 [38] were found in these 10 varieties, which may be attributed to the limited varieties sequenced. Furthermore, among the allelic variations present in TaAFP-A, TaAFP-B and TaAFP-D loci, only a CTCT deletion at the TaAFP-B1b locus was associated with seed dormancy in 91 varieties.

Analysis of TaAFP sequence showed some transcription factor binding elements in the region of 800 bp located upstream from the start code ATG, including ABA response elements ABRE, G-box, CACA, AACAA, Dof, RAVI, Myb-type transcription factor elements P, GAmyb, DRE/CRT and elements for cold and dehydration response [38]. Although the 4-bp deletion in the 5'UTR of TaAFP-B1b did not reside in any of the elements mentioned above, it changed the number of adjacent CT repeats from 9 to 7. Previous reports indicated that the number of repeat sequences in the promoter region of a gene could affect the expression level [40, 41].
Further study also showed that the 4-bp (CTCT) deletion changed the mRNA half-life and further transcription level (Figs. 2 and 3). The sequence with 9 CT repeats might increase the stability of mRNA and the expression level of TaAFP-B1a, which could enhance the binding of related transcription factors through changing the secondary structure of the upstream region of TaAFP-B1a during transcription.

Altering mRNA stability under some conditions plays an important role in the dynamic control of gene expression [42]; the rate of mRNA decay is an essential element of post-transcriptional regulation in all organisms, because the stability of mRNA determines how fast the equilibrium level of a new protein will be reached [43]. Thus, the half-life of mRNA will influence the stochastic fluctuation in the production rate of the corresponding protein [44]. mRNA transcripts are protected from degradation by exoribonuclease by 5′ capping and 3′ poly A structures [45]. In this study, compared with the sequence of TaAFP-B1a, a 4-bp deletion (CTCT) located at −25 bp of the 5′UTR was found in TaAFP-B1b, and the mRNA half-life values of TaAFP-B1b and TaAFP-B1a were significantly different. More stable mRNA and a higher transcriptional level existed in TaAFP-B1a genotype.

The mRNA degradation data (Fig. 4) in this study suggest that the 4-bp deletion (CTCT) in the 5′UTR of TaAFP-B1b forms a “hot spot” for degradation by endogenous ribonucleases, whereas the region of TaAFP-B1a with “CTCT” sequence might be engineered to be more stable, leading to increased mRNA half-life. Consequently, more protein production and higher expression
levels of tdTomatoER and GUS were observed in pITV1-TaAFP-BaF compared to pITV1-TaAFP-Bbf, and higher GUS activity were detected in TaAFP-BaF::GUS transient expression wheat leaves than TaAFP-Bbf::GUS, which indicated that the difference of the 4-bp deletion (CTCT) affected not only mRNA decay and transcription expression level, but also the translation expression level of its downstream gene.

Seed dormancy is a complex quantitative trait. In wheat, ABA signaling is the main factor for seed dormancy [46, 47]. TaAFP is a negative regulator in seed dormancy in wheat. In the present study, an STS marker AFPB associated with seed dormancy in Chinese wheat cultivars with different GI values was developed. In this set of germplasms, there are significant differences in GI between the TaAFP-B1a and TaAFP-B1b genotypes ($P = 0.0002$). Several STS markers like Vp1A3, Vp1B3, TaSdr and Tamyb10D were associated with seed dormancy in wheat [21, 22, 25]. The TaVp-1, TaAFP, and TaSdr genes were involved in the mechanism of embryo-imposed dormancy, while Tamyb10 was involved in the mechanism of coat-imposed dormancy. It is better to obtain highly efficient marker-assisted selection for PHS-resistant varieties by combining both the embryo-imposed and coat-imposed dormancy.

Conclusions
In this study, two allelic variations of TaAFP-B were identified, including TaAFP-B1a and TaAFP-B1b. TaAFP-B had a 4-bp InDel in the 5'UTR, which affected the mRNA stability, mRNA transcription expression level, translation expression level of tdTomatoER and GUS, and GUS activity and was significantly associated with PHS tolerance in common wheat. Based on the 4-bp InDel, a functional marker was developed and validated.

Materials and methods
Plant materials
Ten wheat varieties were used for cloning of TaAFP-A, TaAFP-B, and TaAFP-D, including five PHS-resistant varieties (Xiaoyan6, Langzhoubaimaizi, Wanxianbaimaizi, Yangxiaomai, and Xiaobaiyuhua, with GI values of 0.137, 0.084, 0.076, 0.075, and 0.04, respectively) and five PHS-susceptible varieties (Yongchuanbaimazi, Xinong 979, Zhoumai 16, Zhou 8425B, and Jinan 16, with GI values of 0.232, 0.327, 0.494, 0.560, and 0.616, respectively). Ninety-one Chinese wheat varieties and advanced lines with different PHS tolerances, from the China Autumn-sown Wheat Region (CAWR) representing more than 85% of wheat production areas in China, were used for association analysis of TaAFP-B allelic variations and PHS tolerance. The GI was determined based on the average date across two cropping seasons at two locations in Beijing and Anyang (Henan) in 2005–2006 and 2006–2007 (Additional file 4: Table S1). Grains of Zhou 8425B and Wanxianbaimaizi planted in Hohhot in Inner Mongolia after seeds vernalization in 2016, and collected the spikes at 10, 20, 30, and 40 DAP, and at maturity were frozen in liquid nitrogen, and stored at −80 °C for analysis of mRNA transcription. Gene gun transformation was conducted during the period when there was one leaf and one terminal bud of wheat.

DNA and RNA extraction
Genomic DNA was extracted from 3 g of seedlings grown in the dark at 25°C for 7 days by the CTAB method [48]. Total RNA was extracted from five whole grains at different developmental stages with the Trans-Zol Plant Kit (TransGenBiotech).

PCR amplification and RT-qPCR analysis
PCR reactions for gene cloning and molecular marker tests were performed in an MJ Research PTC-200 thermal cycler in a total volume of 15 μl, including 1.5 μl of 10 x PCR buffer, 1.2 μl of 2.5 μM dNTP each, 4 pmol of each primer, 0.75 U of TaLaaq polymerase (TaKaRa), and 500 ng of template DNA, then up to 15 μl with ddH2O. PCR amplification were 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 57°C–65°C for 45 s, and 72°C for 45 s, with a final extension of 72°C for 10 min. Amplified PCR fragments were separated on 1.5% agarose gel with the nucleic acid dye Gelview (TaKaRa).

The cDNA was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase (TaKaRa) with random hexamer primer Oligo (dT)18 according to the manufacturer’s instructions. RT-qPCR reactions were performed in a LightCycler®480 Real Time PCR System following the introduction book of SYBR® Premix Ex Taq™ II (TliRnaseH Plus). PCR cycling was performed
at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C – 66 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Then, the expression level of the TaAFP-B gene was estimated based on the size of the 102-bp fragment amplified with Q-TaAFP-BF/R primers designed in the exon region. The gene transcriptional expression analysis of TaAFP and TaABI5 were also estimated also with RT-qPCR; which primers showed in Table 1.

mRNA half-life assay
The TaAFP-B mRNA half-life (t_{1/2}) was determined in flag leaves at the heading stage, dry mature seeds immersed in water for 24 h and seeds at 20 DAP of cultivars Zhou 8425B (TaAFP-B1a) and Wanxianbaimaizi (TaAFP-B1b), treated with 200 ng/mL Cordycepin (Sigma-Genosys, USA) for 0 min and 60 mins, respectively. The mRNA half-life (t_{1/2}) was calculated based on the formula: T\(1/2\) = 0.693/kd [49], where the denominator kd is a gradient constant determined by the value of Ct in the real-time RT-PCR reaction.

DNA sequencing
The PCR products were sequenced from both strands by the Beijing Genomics Institute (http://www.genomics.cn). Sequence analysis and characterization were performed using the software DNAMAN (http://www.lynnon.com).

Statistical analysis
Analysis of variance was conducted by PROC MIXED in the Statistical Analysis System (SAS Institute, 8.0) with genotype clusters indicated by two types of fragments amplified with the STS marker AFPB, as a categorical variable to derive mean GI value from each cluster and to test significance levels. The genotype clusters were treated as fixed effects, while genotypes nested in clusters and years were treated as random effects. Pearson’s linear correlation coefficients for GI between years were obtained by SAS PROC CORR.

Plasmid constructions
The plant expression vector of pITV1#1534 was supplied by YP. Xing from Inner Mongolia Agricultural University. Then, the two different fragments (272 bp and 276 bp, the sequences were part of the 5’UTR of TaAFP-B1a and TaAFP-B1b, respectively, with an added sequence of a mini promoter in a 5’ orientation), which differ in the 4 bp deletion (CTCT), were synthesized by General Biosystems (Anhui) Co. Ltd. The confirmed sequences were cloned into NotI and NcoI sites of the binary vector pITV1#1534. Finally, two recombinant expression vectors, pITV1-TaAFP-BaF and pITV1-TaAFP-BbF, were constructed successfully (Fig. 7).

Plant transformation and observation
When the leaves of wheat grew to the period of one leaf and one terminal bud, the first leaf was cut off about 4
cm from the tip of the leaf, and soaked in 70% ethanol for 3 min. Then, the leaves were washed with distilled water 3 times, the leaf surface was cleaned, and the leaves were attached to a piece of glass. Finally, plasmid DNA (1 μg/μl) were transferred into leaves by biolistic bombardment (PDS-1000/He system of BioRad) transformation. He pressure of the rupture disc was 1100 psi and vacuum degree was 28 inHg. The bombardment distance was 9 cm [50, 51]. Every experiment had three biological replicates.

The tdTomatoER expression was viewed in whole leaves mounted on glass slides using the Olympus BX-60 of Confocal Laser Scanning Microscope (excitation light 540 nm, emitted light 580 nm). Images were processed with Adobe Photoshop (Mountain View, CA). Then, for observation of GUS expression, the samples were incubated overnight in a solution of 1 mM X-Gluc in 50 mM phosphate buffer (pH 7.0) at 37 °C. After that, tissues were cleared of chlorophyll in 70% ethanol and photographs of whole-mounted tissues were taken using an optical microscope. Finally, all transgenic plants for each construct were analyzed.

Quantitative GUS assay
All transient expression wheat leaves separately converted to pITV1-TaAFP-BaF, pITV1-TaAFP-BbF, and pITV1#1534 as a control by biolistic bombardment were frozen in liquid nitrogen, and stored at −80 °C prior to the quantitative analysis of GUS activity of the promoter of TaAFP-BaF and TaAFP-BbF, which was expressed as nanomoles 4-MU (4-methylumbelliferone) per minute per microgram protein [52]. For each construct, at least six independent transient expression wheat leaves were analyzed, and three replicates were performed.

Additional file 1: Figure S1. Sequence comparison of two new TaAFP-A alleles of TaAFP-A1a and TaAFP-A1b detected in Chinese germplasm with TaAFP-A (AB360911).SNPs are in bold letters. (DOCX 19 kb)

Additional file 2: Figure S2. Sequence comparison of three new TaAFP-B alleles of TaAFP-B1a, TaAFP-B1c and TaAFP-B1b detected in Chinese germplasm with TaAFP-B (AB360912). Insertions are underlined, deletions are shadowed, and SNPs are in bold letters. (DOCX 17 kb)

Additional file 3: Figure S3. Sequence comparison of two new TaAFP-D alleles of TaAFP-D1a and TaAFP-D1b detected in Chinese germplasm with TaAFP-D (AB360913). SNPs are in bold letters. (DOCX 19 kb)

Additional file 4: Table S1. Polymorphism of the AFB3 marker in 91 white-grained cultivars with different levelsof seed dormancy. (DOCX 20 kb)

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Not applicable.
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