Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2021) **19**, pp. 1863–1877 doi: 10.1111/pbi.13604

The MYB family transcription factor *TuODORANT1* from *Triticum urartu* and the homolog *TaODORANT1* from *Triticum aestivum* inhibit seed storage protein synthesis in wheat

Guangbin Luo[1], Lisha Shen[1,2], Yanhong Song[1,4], Kang Yu[1,5], Jingjing Ji[5], Chi Zhang[5], Wenlong Yang[6], Xin Li[1], Jiazhu Sun[1], Kehui Zhan[4], Dangqun Cui[4], Yanpeng Wang[1], Caixia Gao[1], Dongcheng Liu[3,*] and Aimin Zhang[1,*]

[1]*State Key Laboratory of Plant Cell and Chromosome Engineering, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology/ Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing, China*

[2]*University of Chinese Academy of Sciences, Beijing, China*

[3]*State Key Laboratory of North China Crop Improvement and Regulation, College of Agronomy, Hebei Agricultural University, Baoding, Hebei, China*

[4]*College of Agronomy/Collaborative Innovation Center of Henan Grain Crops, Henan Agricultural University, Zhengzhou, China*

[5]*BGI Genomics, BGI-Shenzhen, Shenzhen, China*

[6]*Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China*

*Correspondence (email liudongcheng@hebau.edu.cn (DL); Tel 86 10 6480 6618; email amzhang@genetics.ac.cn (A.Z.); Tel 86 10 6480 6618)
Guangbin Luo, Lisha Shen and Yanhong Song have contributed equally to this work.

Keywords: seed storage protein, transcriptional regulator, repressor, *Triticum urartu*, *Triticum aestivum*.

Summary

Seed storage proteins (SSPs) are determinants of wheat end-product quality. SSP synthesis is mainly regulated at the transcriptional level. Few transcriptional regulators of SSP synthesis have been identified in wheat and this study aims to identify novel SSP gene regulators. Here, the R2R3 MYB transcription factor *TuODORANT1* from *Triticum urartu* was found to be preferentially expressed in the developing endosperm during grain filling. In common wheat (*Triticum aestivum*) overexpressing *TuODORANT1*, the transcription levels of all the SSP genes tested by RNA-Seq analysis were reduced by 49.71% throughout grain filling, which contributed to 13.38%–35.60% declines in the total SSP levels of mature grains. In *in vitro* assays, *TuODORANT1* inhibited both the promoter activities and the transcription of SSP genes by 1- to 13-fold. The electrophoretic mobility shift assay (EMSA) and ChIP-qPCR analysis demonstrated that TuODORANT1 bound to the *cis*-elements 5′-T/CAACCA-3′ and 5′-T/CAACT/AG-3′ in SSP gene promoters both *in vitro* and *in vivo*. Similarly, the homolog *TaODORANT1* in common wheat hindered both the promoter activities and the transcription of SSP genes by 1- to 112-fold *in vitro*. Knockdown of *TaODORANT1* in common wheat led to 14.73%–232.78% increases in the transcription of the tested SSP genes, which contributed to 11.43%–19.35% elevation in the total SSP levels. Our data show that both *TuODORANT1* and *TaODORANT1* are repressors of SSP synthesis.

Introduction

Wheat is used as a staple food for humans and livestock and is the most important grain crop for human society (Shewry, 2009). Considering its undisputed economic importance, wheat is counted as one of the 'big three' cereal crops worldwide. For example, in 2018, global wheat production was ~725.1 million tons, ~510 and ~141 million tons of which were used as food and feed, respectively (http://faostat.fao.org/). In addition to the adaptability and high yield, the unique elasticity and extensibility of doughs that enable flour to be processed into a wide range of foods, such as bread, pasta and noodles, have contributed to wheat success. The elasticity and extensibility are conferred by two kinds of seed storage proteins (SSPs), namely glutenins and gliadins, respectively. Glutenins can be subdivided into high-molecular-weight subunits (HMW-GSs, 70–90 kDa) and low-molecular-weight subunits (LMW-GSs, 20–45 kDa) based on their molecular weight (D'Ovidio and Masci, 2004).

In addition to the composition, the content or the ratio of a single SSP might play a role in gluten functionality and dough processing properties (Barro *et al*., 1997; Ragupathy *et al*., 2008; Ravel *et al*., 2014). For example, the increase in the HMW-GS *Glu-1Bx7* in wheat cultivars containing the *Glu-1Bx7*^{OE} allele results in increased dough strength (Ragupathy *et al*., 2008). The synthesis of SSP is spatiotemporally regulated at the transcriptional level by synergetic interactions between transcriptional regulators and the *cis*-elements distributed in SSP gene promoters (Rubio-Somoza *et al*., 2006a,b; Verdier and Thompson, 2008; Xi and Zheng, 2011; Yamamoto *et al*., 2006). Intriguingly, an increase or decrease in either glutenin or gliadin would not result in a variation in the total SSPs attributed to the compensation

between glutenin and gliadin genes (Gil-Humanes *et al*., 2010; Sanchez-Leon *et al*., 2018; Yang *et al*., 2014; Zorb *et al*., 2013). The compensation indicates that glutenin and gliadin genes are regulated coordinately. In cereal crops, a few transcriptional regulators of SSP synthesis have been identified, such as *O2*, *ZmbZIP22* and *PBF* in maize (Lohmer *et al*., 1991; Marzabal *et al*., 2008; Schmidt *et al*., 1992; Vicente-Carbajosa *et al*., 1997); *RISBZ1*, *RPBF* and *OsMYB5* in rice (Onodera *et al*., 2001; Suzuki *et al*., 1998; Yamamoto *et al*., 2006); *BLZ1*, *BLZ2*, *BPBF* and *HvGAMYB* in barley (Diaz *et al*., 2002; Mena *et al*., 2002; Oñate *et al*., 1999; Vicente-Carbajosa *et al*., 1998); and *SPA*, *WPBF*, *GAMYB-D* and *SHP* in wheat (Albani *et al*., 1997; Boudet *et al*., 2019; Dong *et al*., 2007; Guo *et al*., 2015; Holdsworth *et al*., 1995; Plessis *et al*., 2013). All the above transcriptional regulators are transcription factors (TFs), and all of them are SSP synthesis activators except for *SHP*, which is a repressor of SSP synthesis. Due to the complexity of the genome, all the transcriptional regulators of SSP genes in wheat were identified by homology cloning. For example, *SPA* and *WPBF* are homologs of *O2* and *PBF* in maize, respectively, and *SHP* is the homolog of the activator *BLZ1* in barley.

As the A genome donor of common wheat, *Triticum urartu* is diploid and has a high-quality genome sequence (Ling *et al*., 2018; Ling *et al*., 2013). The homologous genes between *T. urartu* and common wheat always share an identical function. For instance, both *TuGASR7* in *T. urartu* and the homolog *TaGASR7* in common wheat determine grain length (Ling *et al*., 2013). Moreover, TFs (1,238) in *T. urartu* have been predicted (http://planttfdb.cbi.pku.edu.cn) at the genome scale, and SSP genes in *T. urartu*, including HMW-GS genes, LMW-GS genes and gliadin genes, have also been identified through the combination of gene prediction and PCR-based cloning in our previous work (Luo *et al*., 2015; Zhang *et al*., 2015). Therefore, *T. urartu* could be a good model to identify novel transcriptional regulators of SSP synthesis in wheat. Considering that SSPs are synthesized in immature endosperm of wheat (Shewry, 2009), TFs preferentially expressed in developing endosperm are more likely to be regulators of SSP synthesis.

In common wheat, the MYB family TF *TaODORANT1* was found to be expressed in roots, stems and leaves of both young seedlings and mature plants, and *TaODORANT1* was involved in drought and salt stress responses when overexpressed in transgenic tobacco (Wei *et al*., 2017). However, the expression pattern of *TaODORANT1* in endosperm during grain filling and its function in the biosynthesis of reserve materials in grains remain elusive. In this study, *TaODORANT1*'s homolog in *T. urartu*, *TuODORANT1*, was found to be preferentially expressed in the developing endosperm during grain filling. The SSP content decreased in *TuODORANT1*-overexpressing common wheat but increased in wheat with knockdown of *TaODORANT1*, which is the homolog of *TuODORANT1* in common wheat. The mechanism by which *TuODORANT1* suppressed SSP synthesis was elucidated. Both *TuODORANT1* and *TaODORANT1* identified in this work will undoubtedly expand our knowledge of the transcriptional regulation of SSP synthesis.

Results

TuODORANT1 is preferentially expressed in the developing endosperm of *Triticum urartu*

Reserve materials, such as SSPs and starch, are synthesized in the immature endosperm of wheat (Shewry, 2009); TFs preferentially expressed in developing endosperm are more likely involved in the regulation of reserve material synthesis. In the quantitative RT-PCR (qRT-PCR) analysis of *T. urartu* plants during grain-filling stages, the transcription levels of an R2R3-type MYB TF *TRIUR3_07775* in developing endosperm from 10 days post-anthesis (DPA) to 25 DPA were ~160 times the levels in the leaves, roots and stems of plants at 15 DPA (Figure 1a,b). The preferential expression in the endosperm during grain-filling stages indicated that *TRIUR3_07775* played a role in the grain-filling process. Indeed, the SSP content in common wheat overexpressing *TRIUR3_07775* was reduced in the following analyses. Therefore, *TRIUR3_07775* was selected for further functional verification of SSP gene regulation.

A BLASTP search showed that the 14–61 aa and 67–111 aa protein fragments of TRIUR3_07775 were Myb-like DNA-binding domains, which are signatures of MYB family TFs (Figure 1c). In the BLASTP search, TRIUR3_07775 shared identical Myb-like DNA-binding domains and 91% protein sequence identity with *TaODORANT1* (GenBank accession No. ASW31170.1) (Wei *et al*., 2017), which was previously identified in common wheat. Therefore, *TRIUR3_07775* was designated as *TuODORANT1*. IWGSC BLAST using the V1 version of the genome sequence of *T. urartu* showed that *TuODORANT1* was located on the short arm of the group 7 chromosome (7AS). To investigate the subcellular localization, TuODORANT1 was fused with green fluorescent protein (GFP). The nuclear GFP fluorescence detected in the wheat protoplast cells indicated that TuODORANT1 was located in the nucleus (Figure 1d).

SSP accumulation is diminished in *TuODORANT1*-overexpressing common wheat

To investigate the function in wheat, the overexpression construct of *TuODORANT1* (*Glu-1Bx14*$_{pro}$*:TuODORANT1*) was delivered into the common wheat cultivar Kenong 199 because no robust genetic transformation system has been established in *T. urartu*. Three overexpression lines, namely OE34, OE38 and OE59, were obtained (Figure S1a), and the T_3 generation was planted in both Dishang and Zhaoxian during the 2017–2018 standard growing season and in Zhaoxian during the 2018–2019 standard growing season. Agronomically important traits, such as the SSP content, thousand kernel weight (TKW) and grain weight per plant, of all three overexpression lines in Dishang and Zhaoxian during the two growing seasons were determined, and wild-type Kenong 199 (WT) was used as a control.

According to the reverse-phase high-performance liquid chromatography (RP-HPLC) analyses, the total HMW-GSs of all three overexpression lines decreased by 8.55%–48.18% compared with the 14.66 μg/mg flour in WT (Figure 2a). Compared with the 9.58 μg/mg flour in WT, the total LMW-GSs of all overexpression lines showed a reduction of 12.37%–70.02% (Figure 2b). As a result of the reduction in both HMW-GSs and LMW-GSs, the total glutenins of all overexpression lines decreased by 23.00%–44.89% compared with the 24.12 μg/mg flour in WT (Figure 2c). Similarly, the total gliadin content of all the overexpression lines decreased by 4.91%–34.91% compared with the 40.63 μg/mg flour in WT (Figure 2d). Attributed to the declines in both glutenins and gliadins, the total SSPs of all overexpression lines decreased by 13.38%–35.60% compared with the 64.87 μg/mg flour in WT (Figure 2e).

Together with the reduction in SSPs, the TKW of all overexpression lines decreased by 4.95%–8.04% compared with 43.59 g in WT (Figure S1b). As the main dimensions of TKW (Campbell *et al*., 1999; Dholakia *et al*., 2003), the grain width of all overexpression lines decreased by 1.39%–3.33%, while the

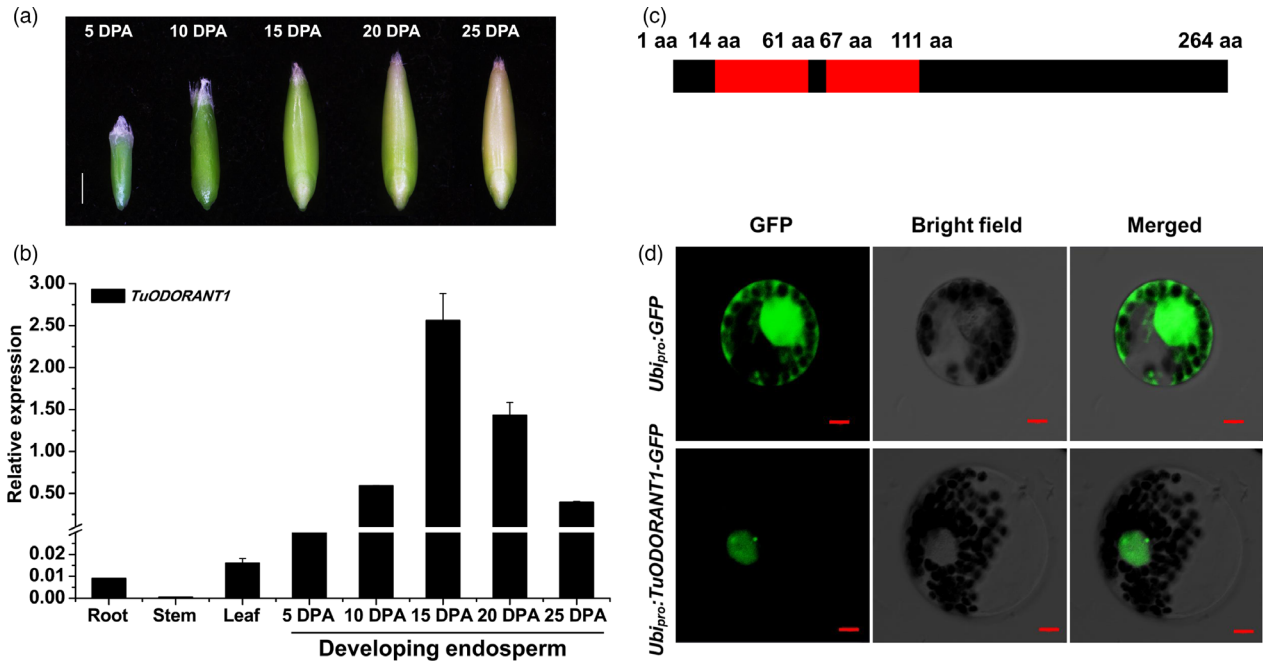

Figure 1 *TuODORANT1* was preferentially expressed in the developing endosperm of *Triticum urartu*. (a) Morphological characters of developing seeds of *T. urartu* at 5, 10, 15, 20 and 25 days post-anthesis (DPA). Bar, 2 mm. (b) *TuODORANT1* was preferentially expressed in developing endosperm of *T. urartu*. Endosperm at 5, 10, 15, 20 and 25 DPA as used. Root, stem and leaf samples were from the plant at 15 DPA. The data are the means ± SEs (n = 3). (c) Graphical summary of TuODORANT1. Red indicates the Myb-like DNA-binding domains (14–61 aa and 67–111 aa) predicted by NCBI Protein BLAST (BLASTP). aa, amino acid. (d) TuODORANT1 localizes in the nucleus of wheat protoplast cells. Ubi_{pro} is the maize ubiquitin (Ubi-1) promoter. Bars, 5 μm.

grain length showed no obvious changes compared with that of WT (Figure S1c). The grain weight per plant of all overexpression lines also declined by 8.83%–13.32% compared with the 26.61 g of WT (Figure S1d). Regarding other agronomically important traits of overexpression lines, plant height, spike number per plant, spikelet number per spike, spike length and grain number per spike showed no clear variations compared with those of WT.

The transcription of SSP genes is hampered in *TuODORANT1*-overexpressing common wheat

To explore the mechanisms of SSP reduction in *TuODORANT1*-overexpressing common wheat, the transcriptome in developing endosperm at 10, 15 and 20 DPA of all three overexpression lines (OE34, OE38 and OE59) was analysed by RNA-Seq. Considering the similar phenotypic traits, only the endosperm of overexpression lines planted in Zhaoxian during the 2017–2018 growing season was used in the RNA-Seq analysis. Developing endosperm of WT at the same stages was used as a control. In the RNA-Seq analysis, ~36 324 947 clean reads were obtained in individual replicons of each sample. Approximately 63.27% of the clean reads were mapped to annotated gene-coding regions, and ~18.57% of the clean reads were uniquely mapped (Table S1). The expression levels of transcripts were described as fragments per kilobase of exon model per million reads mapped (FPKM) values. According to the FPKMs, transcripts of *TuODORANT1* were detected in the developing endosperm at 10, 15 and 20 DPA of the three overexpression lines but were not observed in developing endosperm of WT (Figure S2a). Our qRT-PCR analysis also showed high expression of *TuODORANT1* in developing endosperm at 10, 15 and 20 DPA in the three overexpression lines, while trace amounts of expression were observed in developing WT endosperm (Figure S2b). Moreover, the expression of the three subgenome copies of endogenous *TaODORANT1*, namely *TaODORANT-A1*, *TaODORANT-B1* and *TaODORANT-D1*, in the developing endosperm at 10, 15 and 20 DPA of the three overexpression lines showed no variations compared with that in WT according to the FPKM values in RNA-Seq analysis (Figure S2c). Therefore, *TuODORANT1* was successfully overexpressed, and the overexpression had no influence on the expression of endogenous *TaODORANT1* in the overexpression lines. Intriguingly, the fluorescence in situ hybridization (FISH) analyses showed that OE38 (two) had more *TuODORANT1* expression cassette insertions than OE34 (one) and OE59 (one), which was consistent with the higher transcription level of *TuODORANT1* in OE38 (Figure S3).

At 10 DPA, the average transcription of individual HMW-GS genes (*Glu-1Ax1*, *Glu-1Bx7*, *Glu-1By9*, *Glu-1Dx2* and *Glu-1Dy12*), LMW-GS genes (*A3-620*, *D3-394*, *D3-441* and *D3-578*) and gliadin genes (*TaGli-α-11*, *TaGli-α-14*, *TaGli-γ-6*, *TaGli-γ-7* and *TaGli-ω-1*) in all three overexpression lines was reduced by 73.67%–81.83% compared with that in WT (Figure 3). At 15 DPA, the transcription of SSP genes in all overexpression lines continued to decline compared with that in WT, and the reduction amplitudes were much lower than those at 10 DPA, with values of ~44.59% in HMW-GS genes, ~32.93% in LMW-GS genes and ~41.36% in gliadin genes (Figure 3). At 20 DPA, transcription declines were detected in most HMW-GS and LMW-GS genes, with lower amplitudes than those at 15 DPA. Intriguingly, transcription increases were observed in most gliadin genes at this stage, such as the 31.52%–84.18% increase in *TaGli-α-11*, *TaGli-α-14* and *TaGli-ω-1* in both OE34 and OE59 and the 45.78%–103.92% increase in *TaGli-γ-7* in all three overexpression lines (Figure 3). The transcriptional increases in specific

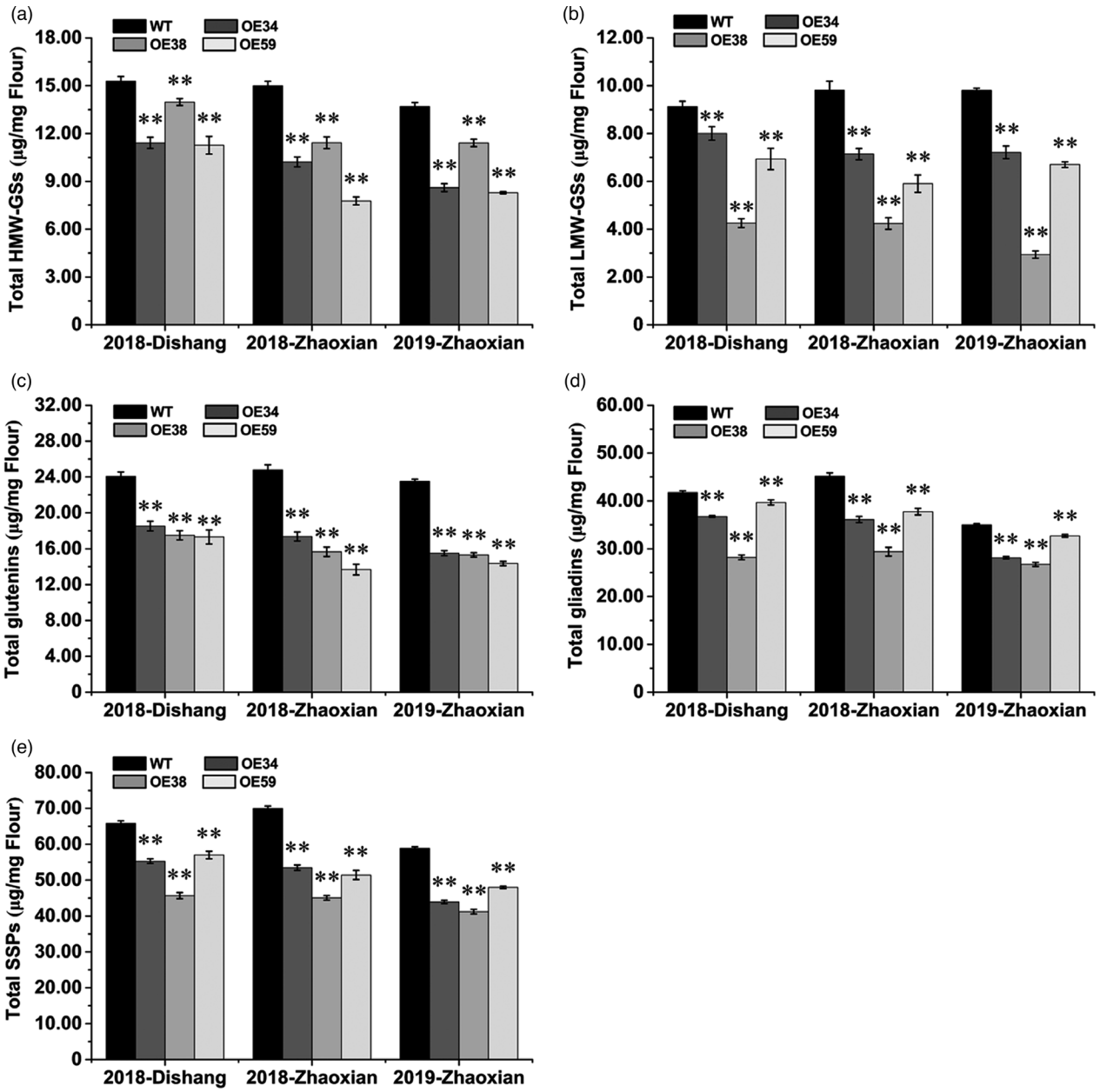

Figure 2 The SSP content decreased in *TuODORANT1*-overexpressing common wheat. (a–e) The levels of total HMW-GSs (a), total LMW-GSs (b), total glutenins (c), total gliadins (d) and total SSPs (e) in all three *TuODORANT1* overexpression lines planted in both Dishang and Zhaoxian during the 2017–2018 and 2018–2019 growing seasons are reduced compared with those of WT, respectively. The data are the means ± SEs (n = 6). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between overexpression lines and WT, as determined by one-way ANOVA in SPSS.

SSP genes at 20 DPA were correlated with the decreases in the number of specific SSP groups. For example, the transcription levels of the gliadin genes *TaGli-α-11*, *TaGli-α-14*, *TaGli-γ-6*, *TaGli-γ-7* and *TaGli-ω-1* between lines OE34 and OE59 had similar reductions from 10 to 15 DPA (Figure 3). However, the transcription increase (85.57%) of *TaGli-γ-7* at approximately 20 DPA in OE59 was higher than that (45.78%) in OE34, which contributed to the higher total gliadin level in OE59 (37.75 μg/mg) than in OE34 (36.11 μg/mg) (Figure 2). The transcription increases in gliadin genes at 20 DPA might compensate for the transcript loss in glutenin genes, and this phenomenon was reported in previous studies (Dumur *et al*., 2004; Galili *et al*., 1986; Yang *et al*., 2014). Our qRT-PCR analyses validated the transcription variations in SSP genes at 10, 15 and 20 DPA in the RNA-Seq analysis (Figure S4). The transcriptional decline in SSP genes throughout endosperm development could be the reason for the reduction in SSP accumulation in *TuODORANT1*-overexpressing common wheat.

TuODORANT1 suppresses both the promoter activities and the transcription of SSP genes *in vitro*

To verify the *TuODORANT1*-mediated down-regulation of SSP genes, a dual-luciferase reporter assay was conducted. In the dual-luciferase reporter assay, the LUC/REN of all tested HMW-GS gene promoters (*TuGlu-1Ax*$_{pro}$ and *TuGlu-1Ay*$_{pro}$) decreased by ~13 times in protoplast cells transformed with *35S*$_{pro}$*:BD-VP16-*

TuODORANT1 compared with that of the negative control in which an empty overexpression vector (*35S$_{pro}$:BD-VP16*) was transformed (Figure S5a). Similarly, the LUC/REN of all tested LMW-GS gene promoters (*TuA3-520$_{pro}$* and *TuA3-538a$_{pro}$*) and gliadin promoters (*Gli-α-8$_{pro}$*, *Gli-γ-1$_{pro}$* and *Gli-ω-1$_{pro}$*) was reduced by ~10-fold compared with that of the negative control (Figure 4a). To investigate the suppression of SSP gene transcription, the overexpression plasmid *Ubi$_{pro}$:TuODORANT1* was transformed into the developing endosperm of Chinese Spring (Figure S5b) for transient overexpression of *TuODORANT1*. qRT-PCR analysis showed that the transcription levels of all examined SSP genes in the endosperm transiently overexpressing *TuODORANT1* were reduced by 5- to 13-fold for HMW-GS genes (*Glu-1Bx7*, *Glu-1By8* and *Glu-1Dx2*) and 1- to 3-fold for LMW-GS (*A3-620* and *D3-578*) and gliadin (*TaGli-α-8*, *TaGli-γ-1* and *TaGli-ω-10*) genes, compared with those in the endosperm transformed with an empty vector (Figure 4b). These data indicated that *TuODORANT1* hampered both the promoter activities and the transcription of SSP genes *in vitro*.

TuODORANT1 binds to SSP gene promoters

To investigate *TuODORANT1*'s interaction with SSP genes, electrophoretic mobility shift assays (EMSAs) and ChIP-qPCR analyses were conducted. In all SSP gene promoters of both *T. urartu* and Chinese Spring, 1–7 of the *cis*-elements 5′-T/CAACCA-3′ and 5′-T/CAACT/AG-3′, which were predicted as binding motifs of MYB family TFs in PlantCARE, were detected (Figure S6). EMSA was performed to investigate the binding of

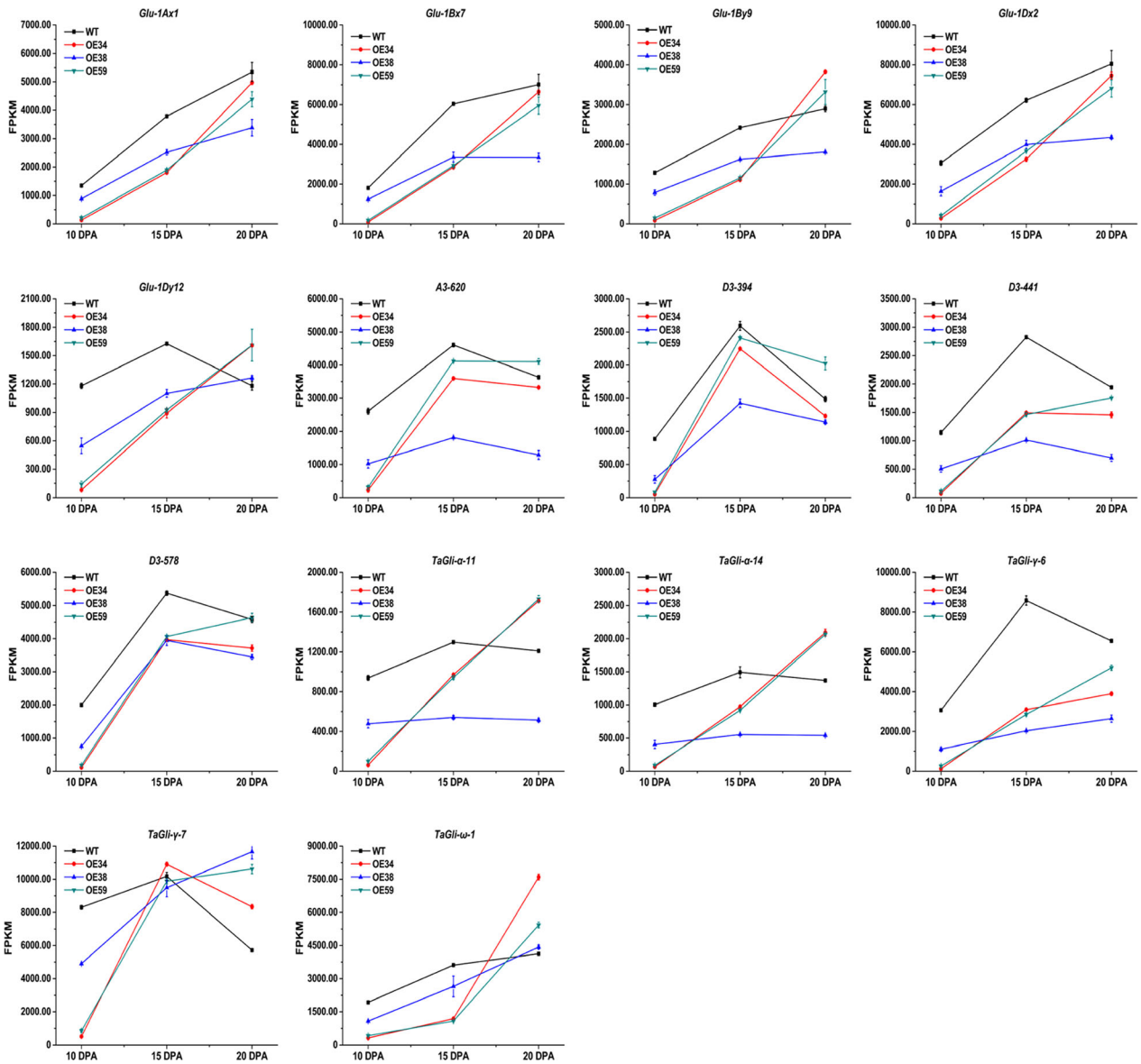

Figure 3 The transcription of SSP genes was inhibited during endosperm development in the overexpression lines of *TuODORANT1*. The transcription of all the tested SSP genes in the *TuODORANT1* overexpression lines was inhibited at 10 and 15 DPA, whereas the transcription of SSP genes was less suppressed, even increased, at 20 DPA. The data are the means ± SEs (n = 3). Only the endosperm transcriptome of the *TuODORANT1* overexpression lines planted in Zhaoxian during the 2017–2018 growing season was analysed. Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between overexpression lines and WT, as determined by one-way ANOVA in SPSS.

TuODORANT1 to these two *cis*-elements. Considering the distribution in all SSP gene promoters, only 5′-CAACCA-3′ and 5′-CAACTG-3′ in the promoter of the HMW-GS gene *TuGlu-1Ay* were used (Figure 5a). In the EMSA analysis, a shifted band was observed when TuODORANT1-GST was mixed with the biotin-labelled probe I, which was the promoter oligo containing 5′-CAACCA-3′ in the middle (Figure 5a, b). Upon addition of the nonlabelled competitive probe, the shift became less clear. No shifted band was detected when TuODORANT1-GST was mixed with the biotin-labelled mutant probe in which 5′-CAACCA-3′ was exchanged with 5′-AAAAAA-3′ (Figure 5b). Similarly, a shifted band was observed when TuODORANT1-GST was mixed with biotin-labelled probe II containing 5′-CAACTG-3′ in the middle, and the band became less clear with the addition of the competitive probe (Figure 5a, c). Upon addition of the mutant probe, in which 5′-CAACTG-3′ was exchanged with 5′-AAAAAA-3′, no shifted band was detected (Figure 5c). When either probe I or probe II was mixed with the glutathione S-transferase (GST) tag, no shifted band was observed. The EMSA results indicated that TuODORANT1 bound to both the *cis*-elements 5′-CAACCA-3′ and 5′-CAACTG-3′ *in vitro*.

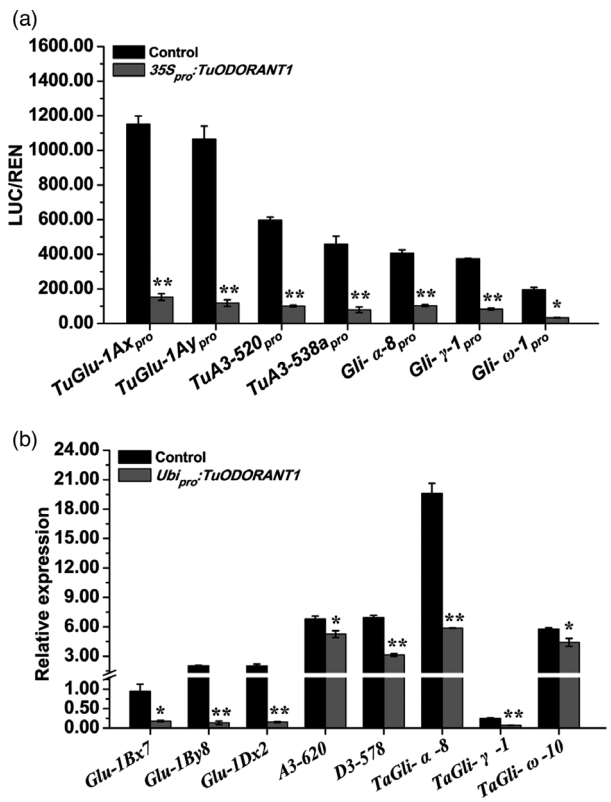

Figure 4 *TuODORANT1* inhibited both the promoter activities and transcription of SSP genes *in vitro*. (a) The promoter activity of each tested SSP gene was suppressed by *TuODORANT1* in the dual-luciferase reporter assay. The ratio of firefly LUC activity to Renilla LUC activity (LUC/REN) was used to reveal the transactivation ability of *TuODORANT1*. The empty construct was used as a negative control. $35S_{pro}$, the 35S promoter. The data are the means ± SEs (n = 3). (b) The transcription of each examined SSP gene was hindered when *TuODORANT1* was transiently overexpressed in the immature endosperm of common wheat. The empty vector was used as a negative control. Ubi_{pro} is the maize ubiquitin promoter (Ubi-1). The data are the means ± SEs (n = 3). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**), as determined by one-way ANOVA in SPSS.

To verify the binding to the *cis*-elements 5′-T/CAACCA-3′ and 5′-T/CAACT/AG-3′ *in vivo*, ChIP-qPCR analysis was conducted using endosperms at 10 DPA of *TuODORANT1* overexpression lines. Primer pairs amplifying a fragment containing the *cis*-elements 5′-T/CAACCA-3′ and/or 5′-T/CAACT/AG-3′ distributed in each SSP gene promoter were used in the qRT-PCR analysis (Figure S6; Table S2). Considering the multiple distributions of these two *cis*-elements, only the fragments within the key functional domain (0 ~ −500 bp) (Korkuc *et al*., 2014; Ravel *et al*., 2014) of HMW-GS and gliadin gene promoters were amplified, and the fragments containing the *cis*-elements at −1,260 bp, −1,106 bp and −478 bp in promoters of the LMW-GS genes *A3-620*, *D3-575* and *D3-578*, respectively, were amplified due to the lack of distribution within the functional domain. In the ChIP-qPCR analysis, the enrichment of the promoter regions containing the *cis*-elements 5′-T/CAACCA-3′ and/or 5′-T/CAACT/AG-3′ of all the tested HMW-GS genes (*Glu-1Ax1*, *Glu-1By9*, *Glu-1Dx2* and *Glu-1Dy12*), LMW-GS genes (*A3-620*, *D3-575* and D*3-578*) and gliadin genes (*TaGli-α-6*, *TaGli-γ-10* and *TaGli-ω-10*) in the experimental sample (treated with the monoclonal antibody of TuODORANT1) was 2–14 times higher than that in the negative control (not treated with the antibody, No-AB) (Figure 5d). Altogether, these data indicated that TuODORANT1 bound to SSP gene promoters both *in vitro* and *in vivo*.

TaODORANT1 inhibits both the promoter activities and transcription of SSP genes *in vitro*

The application of this gene in wheat breeding would be broadened if the homolog of *TuODORANT1* in common wheat, *TaODORANT1*, also shared an identical function in SSP synthesis suppression. To resolve this issue, the three subgenome copies of *TaODORANT1*, namely *TaODORANT-A1*, *TaODORANT-B1* and *TaODORANT-D1*, from Chinese Spring, which is widely used as the reference for genetic studies in wheat research (Shen *et al*., 2005), were cloned. In addition to the identical Myb_DNA-binding domains (14–61 aa and 67–111 aa), *TaODORANT-A1*, *TaODORANT-B1* and *TaODORANT-D1* shared 100%, 93.2% and 95.1% identity with *TuODORANT1*, respectively, in the protein sequence alignment (Figure S7). Both IWGSC BLAST and WheatExp BLAST showed that *TaODORANT-A1*, *TaODORANT-B1* and *TaODORANT-D1* were located on the short arms of the group 7 chromosomes 7AS, 7BS and 7DS, respectively. qRT-PCR analyses demonstrated that all three *TaODORANT1* subgenome copies were preferentially expressed in the developing endosperm despite the trace amounts of expression in roots, stems and leaves (Figure S8). The high sequence identity and the same expression patterns in developing endosperm implied that *TaODORANT1* had a function similar to that of *TuODORANT1*.

In the dual-luciferase reporter assay, all three copies of *TaODORANT1* reduced the LUC/REN values of all the tested SSP gene promoters by 6- to 112-fold (Figures 6a and S4c). Moreover, when transiently overexpressed in the developing endosperm of Chinese Spring, all three copies of *TaODORANT1* suppressed the transcription of almost all the examined SSP genes, that is, the HMW-GS genes (*Glu-1Bx7*, *Glu-1By8* and *Glu-1Dx2*; by 5- to 9-fold), LMW-GS genes (*A3-620* and *D3-578*; 1- to 3-fold) and gliadin genes (*TaGli-α-8*, *TaGli-γ-1* and *TaGli-ω-10*) (Figures 6b and S4d). Similar to *TuODORANT1*, *TaODORANT1*

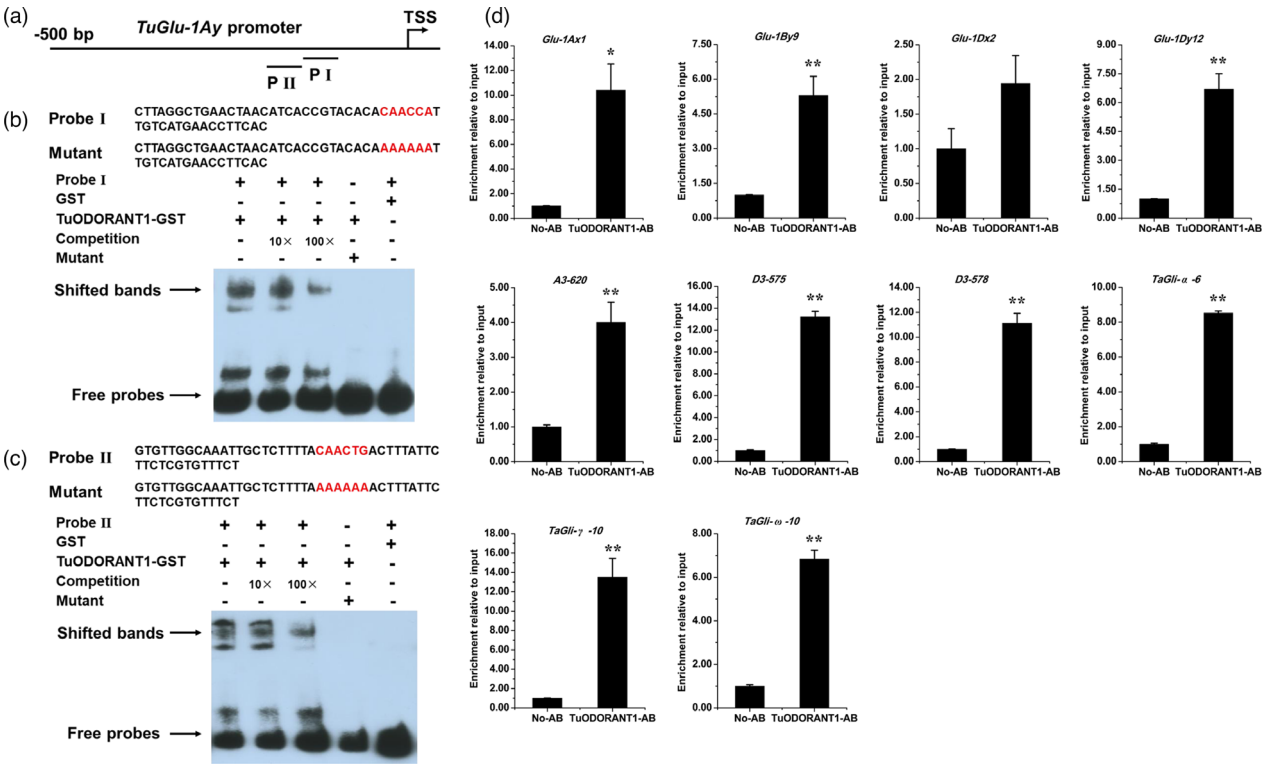

Figure 5 TuODORANT1 binds to the promoter regions of SSP genes both *in vitro* and *in vivo*. (a) Positions of probes I and II in the promoter region of the HMW-GS gene TuGlu-1Ay. P I, probe I; P II, probe II. (b–c) TuODORANT1 binds to the probes containing the *cis*-element 5′-CAACCA-3′ (b) and the *cis*-element 5′-CAACTG-3′ (c) distributed in the promoter region of HMW-GS gene *TuGlu-1Ay* (−100 ~ −300 bp), as determined by EMSA. Lane 1 is the reaction containing both the wild-type probe with biotin and the TuODORANT1-GST fusion protein. Lanes 2 and 3 are competition reactions containing 10X and 100X wild-type probe without biotin, respectively. Lane 4 is the reaction containing the mutant probe with biotin and the TuODORANT1-GST fusion protein. Lane 5 is the reaction containing the wild-type probe with biotin and the GST protein. (d) The promoter fragments of SSP genes containing the *cis*-elements 5′-T/CAACCA-3′ and/or 5′-T/CAACT/AG-3′ were enriched in the ChIP-qPCR analysis. AB is the antibody. No-AB is the negative control in which the antibody of TuODORANT1 was not added. The data are the means ± SEs (n = 3). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**), as determined by one-way ANOVA in SPSS.

hindered both the promoter activities and the transcription of SSP genes *in vitro*.

The SSP content increases in *TaODORANT1*-knockdown wheat

To investigate the suppression of SSP synthesis *in vivo*, *TaODORANT1* of Kenong 199, which is one of the most widely used genotypes in common wheat transformation, was knocked down using RNA interference (RNAi). Three lines, namely RNAi#1, RNAi#2 and RNAi#3, were obtained, and the T_3 generation was planted in both Dishang and Zhaoxian during the 2017–2018 growing season and in Zhaoxian during the 2018–2019 growing season. qRT-PCR analysis was performed to examine the transcription variations in both *TaODORANT1* and SSP genes; wild-type Kenong 199 (WT) was used as a control. Considering the similar phenotype between the two planting sites during the two growing seasons, only the RNAi lines planted in Zhaoxian during the 2017–2018 growing season were used in the qRT-PCR analysis. Only the endosperm at 15 DPA was used because *TaODORANT1* had the highest expression level in this stage during grain filling. In the qRT-PCR analysis, the transcription levels of *TaODORANT1* in all three RNAi lines were ~10 times lower than those in WT, indicating successful knockdown (Figure S9a). Compared with those in WT, the transcription levels of all the tested HMW-GS genes, namely *Glu-1Ax1*, *Glu-1Bx7*, *Glu-1Dx2* and *Glu-1Dy12*, increased by 39.72%–232.78%. Similarly, the transcription levels of all the examined LMW-GS genes (*A3-620* and *D3-575*) and gliadin genes (*TaGli-α-8* and *TaGli-ω-10*) were elevated by 42.98%–119.20% and 14.73%–84.96%, respectively (Figure S9b).

To investigate whether the transcriptional elevation led to increased protein accumulation, the SSP content in mature grains of the RNAi lines was determined by RP-HPLC analysis. In Dishang during the 2017–2018 growing season, the total HMW-GSs of all three RNAi lines increased by 8.65%–16.84% compared with the 13.10 μg/mg flour in WT (Figure 7a). The total LMW-GSs of all the RNAi lines increased by 15.64%–22.88% compared with the 9.69 μg/mg flour in WT (Figure 7b). Due to the increase in both HMW-GSs and LMW-GSs, the total glutenin content of all the RNAi lines increased by 11.62%–19.41% compared with the 22.79 μg/mg flour in WT (Figure 7c). Compared with the 36.78 μg/mg flour in WT, the total gliadin content of all the RNAi lines increased by 10.52%–19.32% (Figure 7d). Attributed to the increase in both glutenins and gliadins, the total SSP content of all the RNAi lines was elevated by 11.43%–19.35% compared with the 59.56 μg/mg flour in WT (Figure 7e). Similarly, the total HMW-GS, total LMW-GS and total gliadin levels of almost all the RNAi lines in Zhaoxian during the two seasons also increased; this contributed to the 13.55%–19.81% elevation in the total SSPs. Taken together, the results show that the accumulation of SSPs in

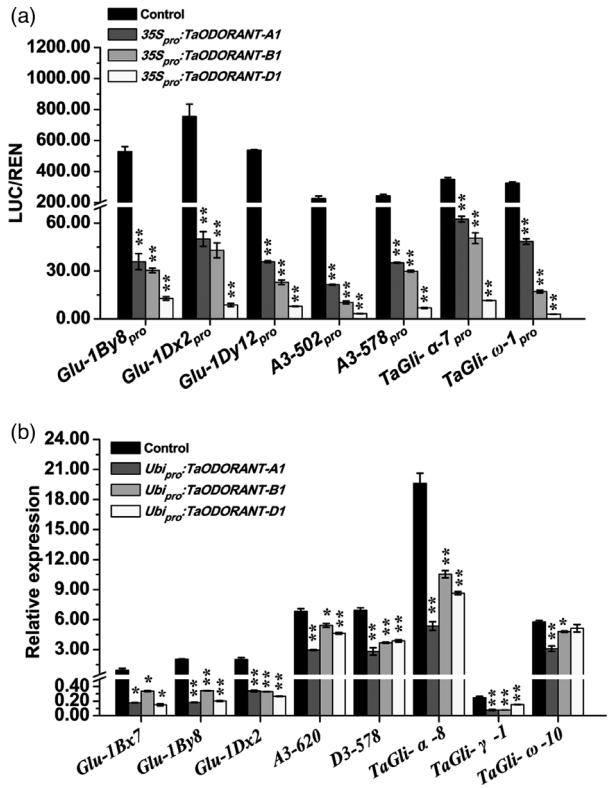

Figure 6 *TaODORANT1* suppressed both the promoter activities and transcription of SSP genes *in vitro*. (a) The promoter activity of each tested SSP gene was suppressed by all three subgenome copies of *TaODORANT1* in the dual-luciferase reporter assay. The ratio of firefly LUC activity to Renilla LUC activity (LUC/REN) was used to reveal the transactivation ability of *TaODORANT1*. The empty construct was used as a negative control. Ubi_{pro} is the maize ubiquitin promoter (Ubi-1). The data are the means ± SEs (n = 3). (b) The transcription of individual SSP genes was hindered when each subgenome copy of *TaODORANT1* was transiently overexpressed in the immature endosperm (at 15 DPA) of common wheat. The empty vector as used as a negative control. Ubi_{pro} is the maize ubiquitin promoter (Ubi-1). The data are the means ± SEs (n = 3). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**), as determined by one-way ANOVA in SPSS.

TaODORANT1-knockdown wheat increased due to the transcriptional elevation of SSP genes.

Together with the elevation in SSPs, the TKW of all the RNAi lines increased by 3.67%–7.77% compared with the 41.75 g in WT (Figure S10a). As two dimensions of TKW, the grain width of all the RNAi lines increased by 1.18%–3.54% compared with that of WT, while the grain length showed no obvious changes (Figure S10b). The grain weight per plant of all the RNAi lines also increased by 9.86%–16.39% compared with the 22.74 g in WT (Figure S10c). Regarding the other agronomically important traits of the RNAi lines, plant height, spike number per plant, spikelet number per spike, spike length and grain number per spike showed no clear changes compared with those of WT.

Discussion

Triticum urartu is a tool for gene identification in wheat

In this work, TFs that were preferentially expressed in the endosperm were first identified in *T. urartu* since our laboratory was one of the participants in the *T. urartu* genome sequencing project (Ling *et al*., 2018; Ling *et al*., 2013). The genome sequences of tetraploid and hexaploid wheat are also currently available. However, our identification of TFs preferentially expressed in the developing endosperm of *T. urartu* occurred much earlier than the release of genome sequences of tetraploid and hexaploid wheat (IWGSC, 2014, 2018; Maccaferri *et al*., 2019). Moreover, we also tried to identify TFs that were preferentially expressed in the developing endosperm of tetraploid and hexaploid wheat. Unfortunately, serious expression biases were detected between or among subgenome copies of numerous TFs in tetraploid and hexaploid wheat, which hindered the accurate characterization of TFs that were preferentially expressed in the endosperm. The expression bias among copies also complicated the functional prediction of a single TF. Therefore, we selected *T. urartu* as a subject to identify TFs that were preferentially expressed in the endosperm.

Compensation between glutenins and gliadins in *TuODORANT1*-overexpressing common wheat

In RNAi and deletion lines of glutenin genes, the reduction in glutenins is compensated to some extent by the increase in gliadins (Dumur *et al*., 2004; Galili *et al*., 1986; Yang *et al*., 2014). Conversely, when the accumulation of gliadins decreases, the glutenin content also increases (Becker *et al*., 2012; Gil-Humanes *et al*., 2012; Gil-Humanes *et al*., 2010; Piston *et al*., 2011). Although the compensation between glutenins and gliadins in wheat has been known for a long time, the mechanisms underlying the coordinated regulation of these two groups of genes are far from being elucidated. At 10 and 15 DPA, the transcription of all the tested glutenin and gliadin genes in the three overexpression lines was suppressed by *TuODORANT1* (Figure 3). However, although *TuODORANT1* was still highly overexpressed at 20 DPA (Figure S2), the transcription of *TaGli-α-11*, *TaGli-α-14* and *TaGli-ω-1* in both OE34 and OE59 and *TaGli-γ-7* in all three overexpression lines increased by 31.52%–103.92% (Figure 3), which might compensate for the transcriptional declines in glutenin genes at 10 and 15 DPA. Similarly, the transcription of *Glu-1By9* in OE34 and *Glu-1Dy12* in both OE34 and OE59 increased by 32.16%–36.41% at 20 DPA, and the expression of *A3-620* and *D3-394* in OE59 increased by 13.04% and 36.23%, respectively, at this stage (Figure 3). The increases in the transcription of glutenin genes at 20 DPA might compensate for the reduction in gliadin transcription at 10 and 15 DPA. As the transcription levels of all well-characterized activators of SSP genes, such as *SPA* (bZIP), *WPBF* (Dof), *GAMYB-D* (MYB) and *TaGAMyb* (MYB) (Albani *et al*., 1997; Dong *et al*., 2007; Guo *et al*., 2015; Holdsworth *et al*., 1995; Plessis *et al*., 2013), showed no obvious changes at 20 DPA compared with those in WT (Table S3), the force driving the transcriptional elevation of SSP genes at this stage remained unknown.

Additional functions of *TuODORANT1* and *TaODORANT1*

Despite the suppression of SSP synthesis, the additional functions determine the potential value of *TuODORANT1* and *TaODORANT1* in wheat breeding. In addition to SSP, starch is a main reserve material in wheat grain and is related to yield. The total starch content in all three *TuODORANT1* overexpression lines decreased by 3.10%–7.66% in both Dishang and Zhaoxian during the 2017–2018 growing season compared with the 60.78% (percentage of mg per 100 mg flour) in WT

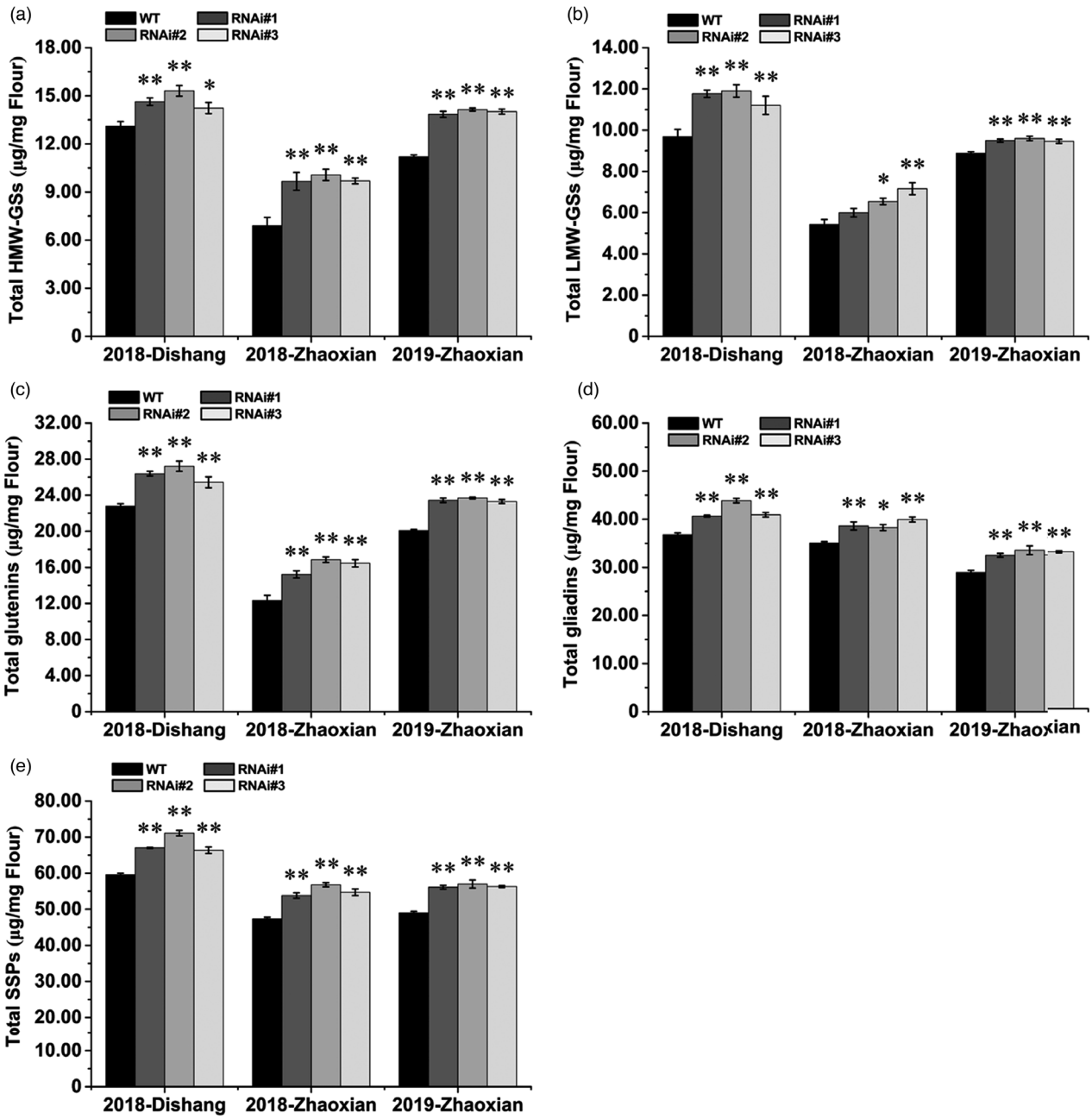

Figure 7 The SSP content increased in *TaODORANT1*-knockdown wheat. (a–e) The levels of total HMW-GSs (a), total LMW-GSs (b), total glutenins (c), total gliadins (d) and total SSPs (e) in all three *TaODORANT1* RNAi lines planted in both Dishang and Zhaoxian during the 2017–2018 and 2018–2019 growing seasons were elevated compared with those of WT. The data are the means ± SEs ($n = 6$). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between RNAi lines and WT, are determined by one-way ANOVA in SPSS.

(Figure S11a). In contrast, the total starch content in all three *TaODORANT1* RNAi lines increased by 3.57%–6.51% in both Dishang and Zhaoxian during the 2017–2018 growing season compared with the 60.66% (percentage of mg per 100 mg flour) in the WT (Figure S11b). The reduction in overexpression lines and the elevation in RNAi lines indicated that both *TuODORANT1* and *TaODORANT1* suppressed starch synthesis. In the process of starch synthesis, *AGPase* is the first key regulatory and rate-limiting enzyme (Smidansky *et al*., 2002; Tuncel and Okita, 2013), and cytosolic *AGPase* is expressed specifically in the endosperm of cereal crops and play a major role in starch synthesis during grain-filling stages (Burton *et al*., 2002; Denyer *et al*., 1996; Sikka *et al*., 2001; Tetlow *et al*., 2003; Thorbjørnsen *et al*., 1996). Sucrose synthase (SuSy) and UDP-glucose pyrophosphorylase (UGPase) are two other enzymes related to starch accumulation in cereal endosperm (Comparot-Moss and Denyer, 2009). In the RNA-Seq analysis, the transcription of the small subunit of cytosolic *AGPase* (AGPS-cyto) in all three *TuODORANT1* overexpression lines was reduced at 10 DPA but was elevated at 15 DPA compared with that of WT (Table S3). The transcription of the large subunit of cytosolic *AGPase* (AGPL-cyto) decreased in the OE34 and OE59 overexpression lines but increased in OE38 at 20 DPA (Table S3).

The transcription of *SuSy1* and *SuSy2* increased in OE38 at 15 and 20 DPA but showed no obvious changes in OE34 and OE59 at 10, 15 and 20 DPA (Table S3). The transcription of *UGPase* increased in OE38 at 15 and 20 DPA but was reduced in OE59 at 20 DPA and showed no obvious variations in OE34 at 10, 15 and 20 DPA (Table S3). Similarly, the transcription of the remaining starch synthesis-related genes, such as *GBSSI* in amylose synthesis and *SSI*, *SSII*, *SSIII*, *SSIV*, *SBEI*, *SBEIIa*, *SBEIIb*, *ISAI*, *ISAII*, *ISAIII* and *PUL* in amylopectin synthesis, also showed no consistent changes in any of the three overexpression lines (Table S3). Therefore, the regulatory effect of *TuODORANT1* on starch synthesis remains elusive, and further research is needed.

Regarding the other agronomically important traits, the plant height, spike number per plant, spikelet number per spike and grain number per spike of both the *TuODORANT1* overexpression lines and *TaODORANT1* RNAi lines showed no clear variations compared with those of WT, which indicated that *TuODORANT1* and *TaODORANT1* had no influence on these traits.

In this work, the MYB family TF *TuODORANT1* was preferentially expressed in the developing endosperm of *T. urartu*. Further *in vitro* assays demonstrated that *TuODORANT1* suppressed both the promoter activities and the transcription of all the tested SSP genes. In the overexpression lines, *TuODORANT1* inhibited the transcription of SSP genes during endosperm development by binding to the promoter regions of the latter. Consequently, the total SSP content of the overexpression lines was reduced significantly. Similar to *TuODORANT1*, the homolog *TaODORANT1* in common wheat also suppressed both the promoter activities and the transcription of SSP genes. The total SSP content increased when *TaODORANT1* was knocked down in RNAi lines. Both *TuODORANT1* and *TaODORANT1* could act as elite genetic engineering targets in wheat quality improvement.

Methods

Plant materials and growth conditions

The common wheat (*T. aestivum*) varieties Chinese Spring and Kenong 199 and the genome-sequenced *T. urartu* accession PI 428198 were planted at the experimental station (40°11′N, 116°42′E) of the Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences (IGDB, CAS), Beijing, China, during the 2015–2016 standard growing season. The endosperm at 5, 10, 15, 20 and 25 DPA and roots, stems and flag leaves at 15 DPA of *T. urartu* and Chinese Spring were used for qRT-PCR analysis to explore the expression patterns of *TuODORANT1* and *TaODORANT1*, respectively. Kenong 199 was used to generate overexpression lines of *TuODORANT1* and knockdown (RNAi) lines of *TaODORANT1*. Three overexpression lines (OE34, OE38 and OE59) of *TuODORANT1* and three RNAi lines (RNAi#1, RNAi#2 and RNAi#3) of *TaODORANT1* were obtained. The T_3 generations of the overexpression lines and RNAi lines were planted at two experimental stations located at Dishang (37°95′N, 114°73′E; Hebei, China) and Zhaoxian (37°45′N, 114°46′E; Hebei, China), respectively, during the 2017–2018 standard growing season. The T_3 generations of both the overexpression lines and RNAi lines were grown again in Zhaoxian during the 2018–2019 standard growing season. For individual overexpression and RNAi lines, 15 seeds were sown in a 1.5-m-long row, and the row spacing was 20 cm. Each line had three lines. The growth of these transgenic wheat plants was performed under normal conditions to avoid the influence of flooding or drought on the accumulation of SSPs and starch. Traditional cultivation practices for common wheat on the North China Plain, where these two planting sites were located, were used. For this purpose, irrigation with 60.00 mm of water was conducted once at each of the jointing, heading and filling stages; 140.00 kg and 60.00 kg of nitrogen fertilizer were applied at sowing and at the jointing stage, respectively, in each hectare. At each planting site, *TuODORANT1*-overexpressing and *TaODORANT1*-knockdown common wheat lines were planted in different plots. In every plot, the wild type (WT) was also planted for more accurate comparison with the transgenic lines in the measurement of SSP levels and other agronomically important traits.

Considering the similar phenotypes, only the endosperm of the overexpression lines and RNAi lines planted in Zhaoxian were used for further molecular analyses. Among the overexpression lines of common wheat, the endosperms of all three lines at 10, 15 and 20 DPA were used in the RNA-Seq and qRT-PCR analyses to explore the transcriptional variations in SSP genes throughout endosperm development, and the endosperm of all three lines at 10 DPA was used for the ChIP-qPCR analysis to reveal the interaction between TuODORANT1 and SSP gene promoters. In *TaODORANT1* RNAi wheat, the endosperm of all three lines at 15 DPA was used in qRT-PCR analysis to reveal the transcriptional variations in SSP genes during endosperm development.

Vector construction and plant transformation

Full-length cDNAs of *TuODORANT1* and the three copies of *TaODORANT1*, namely *TaODORANT-A1*, *TaODORANT-B1* and *TaODORANT-D1*, were cloned from PI 428198 and Chinese Spring, respectively. To drive the expression of *TuODORANT1* specifically and highly in the developing endosperm of transgenic wheat, the promoter of the HMW-GS gene *Glu-1Bx14* (GenBank accession No. AY367771.1) (Geng *et al*., 2014) was used in the overexpression construct (*Glu-1Bx14*$_{pro}$*:TuODORANT1*). In the RNAi constructs, the third exon-distributed fragment (653–990 bp), which was conserved among the three *TaODORANT1* copies in Kenong 199, was used as the target sequence in the RNAi hairpin. The fourth intron of the wheat *Wx* (GenBank accession No. LC373577) gene was used as a spacer in the RNAi hairpin. Each overexpression and RNAi plasmid was transformed into immature embryos of Kenong 199 by following the biolistic particle delivery methods of Rasco-Gaunt *et al*. (2001) and Wang *et al*. (2014). In brief, embryos of Kenong 199 at the early-medium milk stage were isolated and bombarded using the biolistic particle delivery system (Bio-Rad). Before bombardment, the *TuODORANT1* overexpression plasmid and the plasmid pAHC20 (a plasmid encoding the bar gene that confers resistance to the herbicide glufosinate) were mixed in a 1 : 1 molar ratio, and 1.5 μg/kb of the *TuODORANT1* overexpression plasmid was used in each shot. Bombarded embryos were transferred to callus induction medium. Three to four weeks later, induced calli were transferred to regeneration media with selection (containing 5 mg/L phosphinothricin). After four weeks, regenerated calli were transferred to elongation media with selection (containing 5 mg/L phosphinothricin). During regeneration and elongation, subculture was conducted every 2 weeks. Each T_0 putative line was tested by normal PCR with a primer pair covering the *TuODORANT1* coding sequence and the 35S terminator in the overexpression plasmid. Lines that tested positive by PCR were transferred into soil and grown in a greenhouse. In addition, the T_1 to T_3 generations were grown in the field. PCR testing was performed for each generation. Lines in the T_3 generation were

homozygotes, because no segregation was detected by PCR testing of the T_4 generation. The primers used for vector construction are listed in Table S2.

Subcellular localization assays

The full-length cDNA of *TuODORANT1* was inserted downstream of the maize ubiquitin promoter (Ubi-1) in the pJIT163-hGFP vector. The GFP fusion construct (*Ubi$_{pro}$:TuODORANT1-GFP*) was subsequently transformed into wheat protoplasts according to the methods of Shan *et al*. (2014). After 18 h of incubation in the dark at 25 °C, GFP fluorescence of wheat protoplasts was observed using a laser confocal microscope (Zeiss LSM 710 NLO, Oberkochen, Germany).

Measurement of the SSP content

To measure the SSP content in mature seeds of *TuODORANT1* overexpression lines and *TaODORANT1* RNAi lines, RP-HPLC was used according to the manufacturer's instructions. For each line, seeds of six randomly selected plants were ground into whole-wheat flour, and 45 mg of flour from each plant was used for glutenin and gliadin extraction following the methods of Tilley *et al*. (1993) and Yang *et al*. (2014). After filtration through a 0.45-μm organic nylon filter, 10 μL of the glutenin and gliadin fractions was analysed by RP-HPLC using an Agilent 1260 Infinity Quaternary LC system with an XBndge TMBEH300-C_{18} column (5 μm, 4.6 × 250 mm i.d., Agilent Technologies, Santa Clara, CA) following the method of González-Torralba *et al*. (2011). To quantify the SSP extracts, a 1.00 mg/mL BSA standard solution was used. The amounts of SSPs were calculated by integrating the relevant peak areas in the chromatograms. The SSP content is represented as μg/mg flour. All samples used in the RP-HPLC analysis had three biological replicates.

Measurement of the total starch content

The Total Starch Assay Kit (K-TSTA, Megazyme, Bray, Ireland) was used to determine the total starch content of *TuODORANT1* overexpression lines and *TaODORANT1* RNAi lines according to the manufacturer's instructions. The flour from plants that were subjected to SSP measurement were used for measurement of the total starch content.

Scanning electron microscopy

Crosscut with a razor blade and sputter-coated with gold, the inner structure of the mature seeds of *TuODORANT1* overexpression lines was observed using scanning electron microscopy (SEM; Hitachi S-3000N and Quorum PP3000T, Tokyo, Japan). The scanning was performed three times.

Measurements of agronomically important traits

For each *TuODORANT1* overexpression and *TaODORANT1* RNAi line planted in both Dishang and Zhaoxian, 20 independent plants were randomly selected to measure the agronomically important traits. The plant height, spike number per plant, spike length, spikelet numbers per spike, grain number per spike and grain weight per plant were measured or counted manually. The TKW, grain length and grain width were determined using an automatic analyser (Model SC-G, Wseen Ltd, Zhejiang, China). The data for each trait had three replicates.

RNA extraction and qRT-PCR analysis

TRIzol Universal Reagent (TIANGEN, Beijing, China) was used to extract the total RNA from the developing endosperm (without pericarp), roots, stems and leaves of *TuODORANT1* overexpression lines and *TaODORANT1* RNAi lines following the manufacturer's instructions. The Fast Quant RT Kit (Tiangen, Beijing, China) was used to synthesize first-strand cDNA. qRT-PCR analysis was performed using SYBR Green I Master Mix (Roche, Mannheim, Germany) in a LightCycler 480 (Roche, Mannheim, Germany). The relative expression levels were normalized to the expression of the *Ta4045* (ubiquinol-cytochrome C reductase iron-sulphur subunit) gene (Paolacci *et al*., 2009). Experiments were performed with three biological replicates. The primers used for the qRT-PCR analysis are listed in Table S2.

RNA-Seq analysis

Total RNA extracted from endosperm (without pericarp) at 10, 15, and 20 DPA of all three *TuODORANT1* overexpression lines planted in Zhaoxian was used for RNA-Seq analysis. Each sample had three biological replicates. A library with an insert length of ~350 bp for each sample was sequenced using an Illumina HiSeq 2000 (San Diego, CA). All clean reads were mapped to the Chinese Spring reference genome (TGACv1) (Clavijo *et al*., 2017). SSP genes were predicted from this genome. Using gene-specific primers (Table S2), qRT-PCR analyses of both *TuODORANT1* and SSP genes were performed to verify the transcript abundance calculated in the RNA-Seq analysis.

Fluorescence in situ hybridization

The fluorescence in situ hybridization (FISH) was conducted according to Han *et al*. (2004) and Tang *et al*. (2014) using the root tips of *TuODORANT1* overexpression lines and WT. The *TuODORANT1* overexpression plasmid was labelled with Alexa Fluor-488-dUTP (green) and used as a probe. Fluorescence signals were observed using the epifluorescence ZEISS AXIO Imager 2.0.

Dual-luciferase reporter assay

To investigate the influence of *TuODORANT1* on SSP gene promoter activity, a dual-luciferase reporter assay was conducted following the methods of Wei *et al*. (2015) with some modifications. To increase the expression of the LUC gene in reporters, the GAL4-VP16 fusion protein (Chasman *et al*., 1989; Croston *et al*., 1992; Sadowski *et al*., 1988) was used. In this fusion protein, GAL4 binds to the five GAL4 binding domains (5 × GAL4) upstream of the LUC gene, and the activator VP16 boosts the expression of the LUC gene. Therefore, we placed *TuODORANT1* downstream of the GAL4-VP16 fusion protein to generate the effector (*35Spro:BD-VP16- TuODORANT1*) and placed a single SSP gene promoter upstream of 5 × GAL4 to create reporters (*SSPpro + 5 × GAL4:LUC*) (Figure S5a). In the dual-luciferase reporter assay, the reporter contained the LUC gene, and the *Renilla reniformis* luciferase (REN) gene driven by the 35S promoter (*35Spro:REN*) was used as an internal control. In experimental samples, each reporter (*SSPpro + 5 × GAL4:LUC*) was cotransformed in Arabidopsis leaf protoplasts with the internal control (*35Spro:REN*) and the effector (*35Spro:BD-VP16-TuODORANT1*). In the control experiment, each reporter (*SSPpro + 5 × GAL4:LUC*) was cotransformed in Arabidopsis leaf protoplasts with the internal control (*35Spro:REN*) and the empty effector (*35Spro:BD-VP16*). Each sample had three replicates. The ratio of firefly luciferase activity to *R. reniformis* luciferase activity (LUC/REN) was used to estimate the inhibitory effect of *TuODORANT1* on the SSP gene promoter. The dual-luciferase reporter assay of *TaODORANT1* was performed with the same method as that used for *TuODORANT1*.

Transient expression of *TuODORANT1* and *TaODORANT1* in immature endosperm

To investigate the influence on SSP gene transcription during endosperm development, *TuODORANT1* and the three subgenome copies of *TaODORANT1* were transiently overexpressed in the developing endosperm of Chinese Spring at 15 DPA. As a robust transient expression system using immature endosperm of *T. urartu* has not yet been established and almost all SSP genes have been predicted in the genome of Chinese Spring (IWGSC, 2014, 2018), immature endosperm of Chinese Spring was used in this transient expression analysis. The early-medium milk stage grains of Chinese Spring were harvested, surface-sterilized in 0.5% sodium hypochlorite for 25 min and rinsed in sterile water six times. The seed coat of each sterilized grain was removed, and the isolated endosperm was placed on basic medium (MS + 30 g/L sucrose + 3 g/L phytagel, pH = 5.8). In the overexpression constructs, the maize ubiquitin promoter (Ubi-1) was used to drive the high expression of *TuODORANT1* and *TaODORANT1* subgenome copies (Figure S5b,d). The empty overexpression vector was used as a negative control. Each plasmid was transformed into endosperm using the biolistic particle delivery method of Wang *et al*. (2014). After 48 h of incubation in the dark at 25 °C, the total RNA of these bombarded endosperm was extracted and used for qRT-PCR analysis. Each sample had three replicates.

Electrophoretic mobility shift assay

The full-length cDNA of *TuODORANT1* was cloned into pGEX-4T-1 and transferred to *Escherichia coli* strain BL21 (*Transseta*) to obtain the TuODORANT1-GST fusion protein. The TuODORANT1-GST fusion protein was induced and purified following the methods of Qiao *et al*. (2016). In the promoter regions of all SSP genes, two *cis*-elements, namely 5′-T/CAACCA-3′ and 5′-C/TAACTA/G-3′, which were supposed to be bound by MYB family TFs, were predicted using PlantCARE (Figure S6). Two fragments (~50 bp) in the *TuGlu-1Ay* promoter containing 5′-CAACCA-3′ (at −121 bp) and 5′-CAACTG-3′ (at −179 bp) in the middle were labelled with biotin and used as probes I and II, respectively (Figure 5a). In these two probes, the *cis*-elements 5′-CAACCA-3′ and 5′-CAACTG-3′ were mutated to 5′-AAAAAA-3′ and 5′-AAAAAA-3′, respectively, to generate mutant probes. Stand reaction mixture and electrophoresis procedures for EMSA were conducted following the protocol of Qiao *et al*. (2016). The shifted bands were detected using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham) according to the manufacturer's instructions. The EMSA was performed three times.

ChIP-qPCR analysis

To investigate the binding of TuODORANT1 to 5′-T/CAACCA-3′ and 5′-C/TAACTA/G-3′ *in vivo*, ChIP-qPCR analysis was conducted using the developing endosperm at 10 DPA. Endosperms of OE34, OE38 and OE59 were mixed to generate an experimental sample. Using the peptide PPTATSWQQLDGAE in TuODORANT1 (143–156 aa), a monoclonal antibody (C3, TuODORANT1-AB) was produced by Abmart Antibody Company (Shanghai, China). The ChIP assay was performed following the method of Bowler *et al*. (2004). After reverse cross-linking, the precipitated DNA was used for qRT-PCR analysis. Primer pairs (Table S2) amplifying an ~100 bp fragment that contained the *cis*-elements 5′-T/CAACCA-3′ and/or 5′-C/TAACTA/G-3′ in the promoter region of each SSP gene were used in the qRT-PCR analysis. Each sample had three replicates.

Statistical analysis

Statistical analysis in this work was performed using one-way ANOVA with the SPSS 19.0 package for Windows (SPSS, Inc., Chicago, IL). All data are expressed as the mean ± SE. Differences with $P < 0.05$ were considered statistically significant.

Accession numbers

Considering the large gene number and high nucleotide sequence identities (>90%) among members of each SSP type, only the expressed genes that could represent most of the sequence variations in the phylogenetic analyses according to Shen *et al*. (2020) were selected in both *in vivo* and *in vitro* assays in this work. The DNA sequences of all genes in this work can be found in GenBank and in the genome sequence data of *T. urartu* and *T. aestivum* under the following accession numbers: *TuODORANT1* (GenBank accession No. *TRIUR3_07775*), *TaODORANT-A1* (GenBank accession No. MN022889), *TaODORANT-B1* (GenBank accession No. MN022890), *TaODORANT-D1* (GenBank accession No. MN022891), *TuGlu-1Ax* (TRIUR3_25207), *TuGlu-1Ay* (GenBank accession No. FJ404595.1), *TuA3-520* (GenBank accession No. KM085275.1), *TuA3-538a* (GenBank accession No. KM085182.1), *Gli-α-8* (GenBank accession No. KP280202.1), *Gli-γ-1* (GenBank accession No. KP280192.1), *Gli-ω-1* (GenBank accession No. KP280195.1), *Glu-1Ax* (TRIAE_CS42_1AL_TGACv1_002470_A A0042200), *Glu-1Bx7* (TRIAE_CS42_1BL_TGACv1_031273_AA0110720), *Glu-1By8* (TRIAE_CS42_1BL_TGACv1_030818_A A0101450), *Glu-1By9* (GenBank accession No. X61026.1), *Glu-1Dx2* (TRIAE_CS42_1DL_TGACv1_063795_AA0230870), *Glu-1Dy12* (TRIAE_CS42_1DL_TGACv1_062418_AA0214200), *A3-502* (TRIAE_CS42_1AS_TGACv1_0 19801_AA0071580), *A3-620* (GenBank accession No. JF339169.1), *D3-394* (GenBank accession No. JF339158.1), *D3-441* (GenBank accession No. JF339160.1), *D3-575* (GenBank accession No. JF339165.1), *D3-578* (GenBank accession No. JF339166.1), *TaGli-α-6* (GenBank accession No. BAS02405.1), *TaGli-α-7* (TRIAE_CS42_6AS_TGACv1_486025_AA1555680), *TaGli-α-8* (TRIAE_CS42_U_TGACv1_665230_AA2153870), *TaGli-α-11* (TRIAE_CS42_6AS_TGAC v1_486025_AA1555680), *TaGli-α-14* (TRIAE_CS42_6AS_TGACv1_487904_AA1573520), *TaGli-γ-1* (TRIAE_CS42_1DS_TGACv1_080625_AA0251270), *TaGli-γ-6* (TRIAE_CS42_1D S_TGACv1_080991_AA0256470), *TaGli-γ-7* (TRIAE_CS42_1AS_TGACv1_019696_AA0070 097), *TaGli-γ-10* (TRIAE_CS42_1AS_TGACv1_019078_AA0059980), *TaGli-ω-1* (TRIAE_CS4 2_U_TGACv1_640817_AA2075610) and *TaGli-ω-10* (TRIAE_CS42_1AS_TGACv1_019 512_AA0067500).

Acknowledgments

This work was supported by the National Key R&D Program of China (2016YFD0102002, 2017YFD0101004), the Transgenic Research Projects (2016ZX08009-003-004) and the National Natural Science Foundation of China (31871617, 31571643). We thank Mr. Brendan Crumrine from the University of Florida for his help in language editing.

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

AMZ, DCL, KHZ, DQC, YPW and CXG conceived and designed the study. GBL, LSS and YHS performed the experiments. GBL, LSS, JYX, JJJ, CZ, WLY, XL and JZS analysed the data. GBL prepared the manuscript, and AMZ, DCL and WLY improved the manuscript. YWL, KHZ and DQC contributed reagents and materials. AMZ and DCL provided guidance on the whole study. All the authors read and approved the final manuscript.

References

Albani, D., Hammond-Kosack, M.C.U., Smith, C., Conlan, S., Colot, V., Holdsworth, M. and Bevan, M.W. (1997) The wheat transcriptional activator SPA: a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. *Plant Cell* **9**, 171–184.

Barro, F., Rooke, L., Békés, F., Gras, P., Tatham, A.S., Fido, R., Lazzeri, P.A. *et al*. (1997) Transformation of wheat with high molecular weight subunit genes results in improved functional properties. *Nat. Biotechnol.* **15**, 1295–1299.

Becker, D., Wieser, H., Koehler, P., Folck, A., Mühling, K.H. and Zörb, C. (2012) Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference. *J. Appl. Bot. Food Qual.* **85**, 23–33.

Boudet, J., Merlino, M., Plessis, A., Gaudin, J.C., Dardevet, M., Perrochon, S., Alvarez, D. *et al*. (2019) The bZIP transcription factor SPA heterodimerizing protein represses glutenin synthesis in *Triticum aestivum*. *Plant J.* **97**, 858–871.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M. and Paszkowski, J. (2004) Chromatin techniques for plant cells. *Plant J.* **39**, 776–789.

Burton, R.A., Johnson, P.E., Beckles, D.M., Fincher, G.B., Jenner, H.L., Naldrett, M.J. and Denyer, K. (2002) Characterization of the genes encoding the cytosolic and plastidial forms of ADP-glucose pyrophosphorylase in wheat endosperm. *Plant Physiol.* **130**, 1464–1475.

Campbell, K.G., Bergman, C.J., Gualberto, D.G., Anderson, J.A., Giroux, M.J., Hareland, G., Fulcher, R.G. *et al*. (1999) Quantitative trait loci associated with kernel traits in a soft x hard wheat cross. *Crop Sci.* **39**, 1184–1195.

Chasman, D.I., Leatherwood, J., Carey, M., Ptashne, M. and Kornberg, R.D. (1989) Activation of yeast polymerase-II transcription by herpesvirus VP16 and GAL4 derivatives *in-vitro*. *Mol. Cell. Biol.* **9**, 4746–4749.

Clavijo, B.J., Venturini, L., Schudoma, C., Accinelli, G.G., Kaithakottil, G., Wright, J., Borrill, P. *et al*. (2017) An improved assembly and annotation of the allohexaploid wheat genome identifies complete families of agronomic genes and provides genomic evidence for chromosomal translocations. *Genome Res.* **27**, 885–896.

Comparot-Moss, S. and Denyer, K. (2009) The evolution of the starch biosynthetic pathway in cereals and other grasses. *J. Exp. Bot.* **60**, 2481–2492.

Croston, G.E., Laybourn, P.J., Paranjape, S.M. and Kadonaga, J.T. (1992) Mechanism of transcriptional antirepression by GAL4-VP16. *Genes Dev.* **6**, 2270–2281.

Denyer, K., Dunlap, F., Thorbjornsen, T., Keeling, P. and Smith, A.M. (1996) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol.* **112**, 779–785.

Dholakia, B.B., Ammiraju, J.S.S., Singh, H., Lagu, M.D., Röder, M.S., Rao, V.S., Dhaliwal, H.S. *et al*. (2003) Molecular marker analysis of kernel size and shape in bread wheat. *Plant Breed.* **122**, 392–395.

Diaz, I., Vicente-Carbajosa, J., Abraham, Z., Martinez, M., Isabel-La Moneda, I. and Carbonero, P. (2002) The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. *Plant J.* **29**, 453–464.

Dong, G.Q., Ni, Z.F., Yao, Y.Y., Nie, X.L. and Sun, Q.X. (2007) Wheat Dof transcription factor WPBF interacts with TaQM and activates transcription of an alpha-gliadin gene during wheat seed development. *Plant Mol. Biol.* **63**, 73–84.

D'Ovidio, R. and Masci, S. (2004) The low-molecular-weight glutenin subunits of wheat gluten. *J. Cereal Sci.* **39**, 321–339.

Dumur, J., Jahier, J., Bancel, E., Lauriere, M., Bernard, M. and Branlard, G. (2004) Proteomic analysis of aneuploid lines in the homeologous group 1 of the hexaploid wheat cultivar Courtot. *Proteomics* **4**, 2685–2695.

Galili, G., Levy, A.A. and Feldman, M. (1986) Gene-dosage compensation of endosperm proteins in hexaploid wheat *Triticum aestivum*. *Proc. Natl Acad. Sci. USA* **83**, 6524–6528.

Geng, Y.K., Pang, B.S., Hao, C.Y., Tang, S.J., Zhang, X.Y. and Li, T. (2014) Expression of wheat high molecular weight glutenin subunit 1Bx is affected by large insertions and deletions located in the upstream flanking sequences. *PLoS ONE* **9**, e105363.

Gil-Humanes, J., Piston, F., Tollefsen, S., Sollid, L.M. and Barro, F. (2010) Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. *Proc. Natl Acad. Sci. USA* **107**, 17023–17028.

Gil-Humanes, J., Piston, F., Gimenez, M.J., Martin, A. and Barro, F. (2012) The introgression of RNAi silencing of γ-gliadins into commercial lines of bread wheat changes the mixing and technological properties of the dough. *PLoS ONE* **7**, e45937.

González-Torralba, J., Arazuri, S., Jaren, C. and Arregui, L.M. (2011) Stable quality traits of soft winter wheat under nonlimiting nitrogen conditions. *Crop Sci.* **51**, 2820–2828.

Guo, W.W., Yang, H., Liu, Y.Q., Gao, Y.J., Ni, Z.F., Peng, H.R., Xin, M.M. *et al*. (2015) The wheat transcription factor TaGAMyb recruits histone acetyltransferase and activates the expression of a high-molecular-weight glutenin subunit gene. *Plant J.* **84**, 347–359.

Han, F.P., Liu, B., Fedak, G. and Liu, Z.H. (2004) Genomic constitution and variation in five partial amphiploids of wheat–*Thinopyrum intermedium* as revealed by GISH, multicolor GISH and seed storage protein analysis. *Theor. Appl. Genet.* **109**, 1070–1076.

Holdsworth, M.J., MunozBlanco, J., HammondKosack, M., Colot, V., Schuch, W. and Bevan, M.W. (1995) The maize transcription factor Opaque-2 activates a wheat glutenin promoter in plant and yeast cells. *Plant Mol. Biol.* **29**, 711–720.

International Wheat Genome Sequencing Consortium (IWGSC). (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science* **345**, 1251788.

International Wheat Genome Sequencing Consortium (IWGSC). (2018) Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* **361**, eaar7191.

Korkuc, P., Schippers, J.H.M. and Walther, D. (2014) Characterization and identification of *cis*-regulatory elements in *Arabidopsis* based on single-nucleotide polymorphism information. *Plant Physiol.* **164**, 181–200.

Ling, H.Q., Ma, B., Shi, X.L., Liu, H., Dong, L.L., Sun, H., Cao, Y.H. *et al*. (2018) Genome sequence of the progenitor of wheat A subgenome *Triticum urartu*. *Nature* **557**, 424–428.

Ling, H.Q., Zhao, S.C., Liu, D.C., Wang, J.Y., Sun, H., Zhang, C., Fan, H.J. *et al*. (2013) Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* **496**, 87–90.

Lohmer, S., Maddaloni, M., Motto, M., Difonzo, N., Hartings, H., Salamini, F. and Thompson, R.D. (1991) The maize regulatory locus *Opaque-2* encodes a DNA-binding protein which activates the transcription of the B-32 gene. *EMBO J.* **10**, 617–624.

Luo, G.B., Zhang, X.F., Zhang, Y.L., Yang, W.L., Li, Y.W., Sun, J.Z., Zhan, K.H. *et al*. (2015) Composition, variation, expression and evolution of low-molecular-weight glutenin subunit genes in *Triticum urartu*. *BMC Plant Biol.* **15**, 68.

Maccaferri, M., Harris, N.S., Twardziok, S.O., Pasam, R.K., Gundlach, H., Spannagl, M., Ormanbekova, D. *et al*. (2019) Durum wheat genome highlights past domestication signatures and future improvement targets. *Nat. Genet.* **51**, 885–895.

Marzabal, P., Gas, E., Fontanet, P., Vicente-Carbajosa, J., Torrent, M. and Ludevid, M.D. (2008) The maize Dof protein PBF activates transcription of γ-zein during maize seed development. *Plant Mol. Biol.* **67**, 441–454.

Mena, M., Cejudo, F.J., Isabel-Lamoneda, I. and Carbonero, P. (2002) A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone. *Plant Physiol.* **130**, 111–119.

Oñate, L., Vicente-Carbajosa, J., Lara, P., Diaz, I. and Carbonero, P. (1999) Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 *in vivo* and

activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. *J. Biol. Chem.* **274**, 9175–9182.
Onodera, Y., Suzuki, A., Wu, C.Y., Washida, H. and Takaiwa, F. (2001) A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *J. Biol. Chem.* **276**, 14139–14152.
Paolacci, A.R., Tanzarella, O.A., Porceddu, E. and Ciaffi, M. (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol. Biol.* **10**, 11.
Piston, F., Gil-Humanes, J., Rodriguez-Quijano, M. and Barro, F. (2011) Down-regulating γ-Gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength. *PLoS ONE* **6**, e24754.
Plessis, A., Ravel, C., Bordes, J., Balfourier, F. and Martre, P. (2013) Association study of wheat grain protein composition reveals that gliadin and glutenin composition are *trans*-regulated by different chromosome regions. *J. Exp. Bot.* **64**, 3627–3644.
Qiao, Z.Y., Qi, W.W., Wang, Q., Feng, Y.N., Yang, Q., Zhang, N., Wang, S.S. *et al*. (2016) ZmMADS47 regulates zein gene transcription through interaction with Opaque2. *PLoS Genet.* **12**, e1005991.
Ragupathy, R., Naeem, H.A., Reimer, E., Lukow, O.M., Sapirstein, H.D. and Cloutier, S. (2008) Evolutionary origin of the segmental duplication encompassing the wheat *GLU-B1* locus encoding the overexpressed Bx7 ($Bx7^{OE}$) high molecular weight glutenin subunit. *Theor. Appl. Genet.* **116**, 283–296.
Rasco-Gaunt, S., Riley, A., Cannell, M., Barcelo, P. and Lazzeri, P.A. (2001) Procedures allowing the transformation of a range of european elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. *J. Exp. Bot.* **52**, 865–874.
Ravel, C., Fiquet, S., Boudet, J., Dardevet, M., Vincent, J., Merlino, M., Michard, R. *et al*. (2014) Conserved *cis*-regulatory modules in promoters of genes encoding wheat high-molecular-weight glutenin subunits. *Front. Plant Sci.* **5**, 621.
Rubio-Somoza, I., Martinez, M., Diaz, I. and Carbonero, P. (2006a) HvMCB1, a R1MYB transcription factor from barley with antagonistic regulatory functions during seed development and germination. *Plant J.* **45**, 17–30.
Rubio-Somoza, I., Martinez, M., Abraham, Z., Diaz, I. and Carbonero, P. (2006b) Ternary complex formation between HvMYBS3 and other factors involved in transcriptional control in barley seeds. *Plant J.* **47**, 269–281.
Sadowski, I., Ma, J., Triezenberg, S. and Ptashne, M. (1988) GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563–564.
Sanchez-Leon, S., Gil-Humanes, J., Ozuna, C.V., Gimenez, M.J., Sousa, C., Voytas, D.F. and Barro, F. (2018) Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J.* **16**, 902–910.
Schmidt, R.J., Ketudat, M., Aukerman, M.J. and Hoschek, G. (1992) Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD-zein genes. *Plant Cell* **4**, 689–700.
Shan, Q.W., Wang, Y.P., Li, J. and Gao, C.X. (2014) Genome editing in rice and wheat using the CRISPR/Cas system. *Nat. Protoc.* **9**, 2395–2410.
Shen, B., Wang, D.M., McIntyre, C.L. and Liu, C.J. (2005) A 'Chinese Spring' wheat (*Triticum aestivum* L.) bacterial artificial chromosome library and its use in the isolation of SSR markers for targeted genome regions. *Theor. Appl. Genet.* **111**, 1489–1494.
Shen, L., Luo, G., Song, Y., Xu, J., Ji, J., Zhang, C., Gregová, E. *et al*. (2020) A novel NAC family transcription factor SPR suppresses seed storage protein synthesis in wheat. *Plant Biotechnol. J.* https://doi.org/10.1111/pbi.13524. [Epub ahead of print].
Shewry, P.R. (2009) Wheat. *J. Exp. Bot.* **60**, 1537–1553.
Sikka, V.K., Choi, S.B., Kavakli, I.H., Sakulsingharoj, C., Gupta, S., Ito, H. and Okita, T.W. (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADP glucose pyrophosphorylase. *Plant Sci.* **161**, 461–468.
Smidansky, E.D., Clancy, M., Meyer, F.D., Lanning, S.P., Blake, N.K., Talbert, L.E. and Giroux, M.J. (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. *Proc. Natl Acad. Sci. USA* **99**, 1724–1729.
Suzuki, A., Wu, C.Y., Washida, H. and Takaiwa, F. (1998) Rice MYB protein OSMYB5 specifically binds to the AACA motif conserved among promoters of genes for storage protein glutelin. *Plant Cell Physiol.* **39**, 555–559.
Tang, Z.X., Yang, Z.J. and Fu, S.L. (2014) Oligonucleotides replacing the roles of repetitive sequences pAs1, pSc119.2, pTa-535, pTa71, CCS1, and pAWRC.1 for FISH analysis. *J. Appl. Genet.* **55**, 313–318.
Tetlow, I.J., Davies, E.J., Vardy, K.A., Bowsher, C.G., Burrell, M.M. and Emes, M.J. (2003) Subcellular localization of ADPglucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. *J. Exp. Bot.* **54**, 715–725.
Thorbjørnsen, T., Villand, P., Denyer, K., Olsen, O.A. and Smith, A.M. (1996) Distinct isoforms of ADP glucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J.* **10**, 243–250.
Tilley, K.A., Lookhart, G.L., Hoseney, R.C. and Mawhinney, T.P. (1993) Evidence for glycosylation of the high-molecular-weight glutenin subunit 2, 7, 8, and 12 from Chinese Spring and TAM 105 Wheats. *Cereal Chem.* **70**, 602–606.
Tuncel, A. and Okita, T.W. (2013) Improving starch yield in cereals by over-expression of ADPglucose pyrophosphorylase: expectations and unanticipated outcomes. *Plant Sci.* **211**, 52–60.
Verdier, J. and Thompson, R.D. (2008) Transcriptional regulation of storage protein synthesis during dicotyledon seed filling. *Plant Cell Physiol.* **49**, 1263–1271.
Vicente-Carbajosa, J., Moose, S.P., Parsons, R.L. and Schmidt, R.J. (1997) A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proc. Natl Acad. Sci. USA* **94**, 7685–7690.
Vicente-Carbajosa, J., Oñate, L., Lara, P., Diaz, I. and Carbonero, P. (1998) Barley BLZ1: a bZIP transcriptional activator that interacts with endosperm-specific gene promoters. *Plant J.* **13**, 629–640.
Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Qiu, J.L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* **32**, 947–951.
Wei, Q.H., Luo, Q.C., Wang, R.B., Zhang, F., He, Y., Zhang, Y., Qiu, D. *et al*. (2017) A wheat R2R3-type MYB transcription factor TaODORANT1 positively regulates drought and salt stress responses in transgenic tobacco plants. *Front. Plant Sci.* **8**, 1374.
Wei, W., Zhang, Y.Q., Tao, J.J., Chen, H.W., Li, Q.T., Zhang, W.K., Ma, B. *et al*. (2015) The Alfin-like homeodomain finger protein AL5 suppresses multiple negative factors to confer abiotic stress tolerance in Arabidopsis. *Plant J.* **81**, 871–883.
Xi, D.M. and Zheng, C.C. (2011) Transcriptional regulation of seed storage protein genes in Arabidopsis and cereals. *Seed Sci. Res.* **21**, 247–254.
Yamamoto, M.P., Onodera, Y., Touno, S.M. and Takaiwa, F. (2006) Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. *Plant Physiol.* **141**, 1694–1707.
Yang, Y.S., Li, S.M., Zhang, K.P., Dong, Z.Y., Li, Y.W., An, X.L., Chen, J. *et al*. (2014) Efficient isolation of ion beam-induced mutants for homoeologous loci in common wheat and comparison of the contributions of *Glu-1* loci to gluten functionality. *Theor. Appl. Genet.* **127**, 359–372.
Zhang, Y.L., Luo, G.B., Liu, D.C., Wang, D.Z., Yang, W.L., Sun, J.Z., Zhang, A.M. *et al*. (2015) Genome-, transcriptome- and proteome-wide analyses of the gliadin gene families in *Triticum urartu*. *PLoS ONE* **10**, e0131559.
Zorb, C., Becker, D., Hasler, M., Muhling, K., Godde, V., Niehaus, K. and Geilfus, C.M. (2013) Silencing of the sulfur rich α-gliadin storage protein family in wheat grains (*Triticum aestivum* L.) causes no unintended side-effects on other metabolites. *Front. Plant Sci.* **4**, 369.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The yield of *TuODORANT1*-overexpressing wheat was reduced compared with that of WT. (a) PCR testing of transgenic lines. Lane M is the marker, and lanes 1 to 5 are the WT, *TuODORANT1* overexpression plasmid, OE34, OE38 and OE59. (b) to (d) The thousand kernel weight (TKW), grain width and grain weight per plant of all *TuODORANT1* overexpression lines, planted in both Dishang and Zhaoxian during 2017–2018 and

2018–2019 growing seasons, were reduced compared with those of WT. The data are the means ± SEs (n = 20). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between the overexpression lines and WT by one-way ANOVA in the SPSS program.

Figure S2 *TuODORANT1* was overexpressed throughout endosperm development in the overexpression line of common wheat. (a) and (b) The expression levels of *TuODORANT1* in developing endosperms revealed by the FPKM in RNA-Seq analysis and the relative expression level in qRT-PCR analysis, respectively. (c) The expression levels of the three endogenous *TaODORANT1* copies have no obvious variations compared with those of WT. The data are the means ± SEs (n = 3). DPA, day post anthesis. Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between the overexpression lines and WT by one-way ANOVA in the SPSS program.

Figure S3 The overexpression line OE38 had more *TuODORANT1* expression cassette insertions than OE34 and OE59 in the fluorescence in situ hybridization (FISH) analyses. (a), (b), (c) and (d) Zero, one, two and one *TuODORANT1* expression cassette insertions were detected in WT, OE34, OE38 and OE59, respectively. Arrows indicated the probe signal. Blue indicated chromosomes counterstained with DAPI. The two chromosomes with probe signals in each of (b) and (d) were homologous chromosomes, and the two chromosomes with the same probe signal location were also homologous chromosomes. Bar = 10 μm.

Figure S4 Verification of the transcription variations in SSP genes in *TuODORANT1*-overexpressing common wheat during endosperm development using qRT-PCR analysis. (a) to (c) The transcriptional suppression of SSP genes in developing endosperms at 10, 15 and 20 DPA of *TuODORANT1* overexpression lines were confirmed by qRT-PCR analyses. The data are the means ± SEs (n = 3). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between the overexpression lines and WT, as determined by one-way ANOVA in SPSS.

Figure S5 Schematics of the constructs used in *in vitro* assays of *TuODORANT1* and *TaODORANT1*. (a) Schematic diagrams of the effector and reporter constructs used in the dual-luciferase reporter assay of *TuODORANT1*. The empty vector was used as the negative control. $35S_{pro}$, the 35S promoter; ter, terminator; SSP_{pro}, SSP gene promoter (0~-2,000 bp); LUC, firefly luciferase; REN, Renilla luciferase. (b) Schematics of the *TuODORANT1* overexpression constructs used in the endosperm transient expression assay. (c) Schematic diagrams of the effector and reporter constructs used in the dual-luciferase reporter assay of *TaODORANT1*. The empty vector was used as a negative control. (d) Schematics of the *TaODORANT1* overexpression constructs used in the endosperm transient expression assay.

Figure S6 Distributions of the *cis*-elements 5′-T/CAACCA-3′ and 5′-T/CAACT/AG-3′ in promoters of SSP genes from *T. urartu* and common wheat. *TuGlu-1Ax*, *TuGlu-1Ay*, *TuA3-520* and *TuA3-538a* were from the *T. urartu* accession PI 428198, and the remaining genes were from the common wheat variety Chinese Spring. These two MYB TF binding *cis*-elements are predicted in New PLACE and PlantCARE.

Figure S7 The three subgenome copies of TaODORANT1 share the identical Myb like DNA-binding domains and high protein sequence identities with TuODORANT1. The MYB like DNA-binding domains I and II are distributed in the 14–61 aa and 67–111 aa regions, respectively.

Figure S8 All the three subgenome copies of *TaODORANT1* were preferentially expressed in the developing endosperm of common wheat, as shown by qRT-PCR analysis. The three *TaODORANT1* copies were expressed at different rates. The transcription levels of *TaODORANT-B1* were much higher than the similar levels of *TaODORANT-A1* and *TaODORANT-D1*. Endosperms at 5, 10, 15, 20 and 25 DPA of the common wheat cultivar Chinese Spring were used. Root, stem and leaf samples were from the plants at 15 DPA. The qRT-PCR analysis was performed with three biological replicates.

Figure S9 The transcription of SSP genes increased in the developing endosperm at 15 DPA of the *TaODORANT1* RNAi line of common wheat. (a) The expression of *TaODORANT1* in the developing endosperm at 15 DPA of all the three RNAi lines planted in Zhaoxian during the 2017–2018 growing season was reduced compared with that in WT. Universal primers for all the three subgenome copies of *TaODORANT1* were used. (b) The transcription of all the tested SSP genes in the developing endosperm at 15 DPA of all three RNAi lines planted in Zhaoxian was enhanced compared with that in WT. The data are the means ± SEs (n = 3). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between the RNAi lines and WT, as determined by one-way ANOVA in SPSS.

Figure S10 The yield of *TaODORANT1*-knockdown common wheat was elevated compared with that of WT. (a) to (c) The thousand kernel weight (TKW), grain width and grain weight per plant of all the three *TuODORANT1* RNAi lines planted in both Dishang and Zhaoxian during the 2017–2018 and 2018–2019 growing seasons increased, respectively, compared with those of WT. The data are the means ± SEs (n = 20). Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between the RNAi lines and WT, as determined by one-way ANOVA in SPSS.

Figure S11 Variations in the starch content of *TuODORANT1* overexpression and knockdown lines of common wheat. (a) The total starch content was reduced in *TuODORANT1* overexpression lines in both Dishang and Zhaoxian during the 2017–2018 growing season compared with that in WT. (b) The total starch content was reduced in *TaODORANT1* RNAi lines in both Dishang and Zhaoxian during the 2017–2018 growing season compared with that in WT. The data are the means ± SEs (n = 3). % represents percent per 100 mg wheat flour. Asterisks indicate statistically significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) between overexpression lines and WT, as determined by one-way ANOVA in SPSS.

Table S1 Mapped reads of the RNA-Seq data

Table S2 Primers used in this study

Table S3 The FPKM variations in SSP genes and well-characterized transcriptional activators in the overexpression lines of *TuODORANT1*

