




# A novel NAC family transcription factor *SPR* suppresses seed storage protein synthesis in wheat

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## Summary

The synthesis of seed storage protein (SSP) is mainly regulated at the transcriptional level. However, few transcriptional regulators of SSP synthesis have been characterized in common wheat (*Triticum aestivum*) owing to the complex genome. As the A genome donor of common wheat, *Triticum urartu* could be an elite model in wheat research considering its simple genome. Here, a novel NAC family transcription factor *TuSPR* from *T. urartu* was found preferentially expressed in developing endosperm during grain-filling stages. In common wheat transgenically overexpressing *TuSPR*, the content of total SSPs was reduced by c. 15.97% attributed to the transcription declines of SSP genes. Both *in vitro* and *in vivo* assays showed that *TuSPR* bound to the *cis*-element 5'-CANNTG-3' distributed in SSP gene promoters and suppressed the transcription. The homolog in common wheat *TaSPR* shared a conserved function with *TuSPR* on SSP synthesis suppression. The knock-down of *TaSPR* in common wheat resulted in 7.07%–20.34% increases in the total SSPs. Both *TuSPR* and *TaSPR* could be superior targets in genetic engineering to manipulate SSP content in wheat, and this work undoubtedly expands our knowledge of SSP gene regulation.

## Introduction

Common wheat (*Triticum aestivum* L.,  $2n = 6x = 42$ , AABBDD) is a staple food in the diet of the world's population and contributes 20% energy and 22% proteins in the daily intake of people (Faris, 2014; Shewry, 2009). The ability of common wheat dough to be processed into kinds of foods, such as bread and noodles, is mainly attributed to its visco-elasticity, which is determined by seed storage proteins (SSPs; Weegels *et al.*, 1996). Generally, SSPs consist of two groups of polypeptides, glutenins and gliadins. Glutenins are divided into high-molecular-weight subunits (HMW-GSs, 70–90 kDa) and low-molecular-weight subunits (LMW-GSs, 20–45 kDa) according to their molecular weight (D'Ovidio and Masci, 2004), and gliadins are classified into  $\alpha$ -( $\alpha$ - $\beta$ )-,  $\gamma$ - and  $\omega$ -types on the basis of the mobility in acid polyacrylamide gel electrophoresis (A-PAGE; Payne, 1987). SSPs are encoded by multi-gene families. In each of A, B and D sub-genomes of common wheat, there are one x-type and one y-type HMW-GS genes at the *Glu-1* loci, 3–8 LMW-GS genes at the *Glu-3* loci, and dozens of gliadin genes at the *Gli-1* and *Gli-2* loci

(Nieto-Taladriz and Carrillo, 1996; Payne *et al.*, 1981; Zhang *et al.*, 2013).

The syntheses of SSPs are spatial-temporally regulated at the transcriptional level by synergetic interactions between transcription factors (TFs) and *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). However, only a few TFs, most of which belong to bZIP, Dof and MYB families, have been proved to regulate SSP gene transcription in cereal crops. For example, *SPA* (bZIP), *WPBF* (Dof) and *TaGAMYB* (MYB) are SSP gene regulators in common wheat (Albani *et al.*, 1997; Dong *et al.*, 2007; Guo *et al.*, 2015; Ravel *et al.*, 2009; Ravel *et al.*, 2006); *O2* (bZIP), *OHP1* (bZIP), *OHP2* (bZIP), *ZmbZIP22* (bZIP) and *PBF* (Dof) are regulators in maize (Li *et al.*, 2018; Lohmer *et al.*, 1991; Marzabal *et al.*, 2008; Pysh *et al.*, 1993; Schmidt *et al.*, 1992; Vicente-Carbajosa *et al.*, 1997); *RISBZ1* (bZIP), *RPBF* (Dof), *OsMYB5* (MYB) are regulators in rice (Onodera *et al.*, 2001; Suzuki *et al.*, 1998; Yamamoto *et al.*, 2006) and *BLZ1* (bZIP), *BLZ2* (bZIP), *BPBF* (Dof), *HvGAMYB* (MYB), *HvMCB1* (MYB) and *HvMYB53* (MYB) are regulators in barley

(Churin *et al.*, 2003; Diaz *et al.*, 2002; Mena *et al.*, 2002; Mena *et al.*, 1998; Oñate *et al.*, 1999; Rubio-Somoza *et al.*, 2006a,b; Vicente-Carbajosa *et al.*, 1998). All the above TFs act as activators in SSP synthesis. Intriguingly, the homolog of the activator *BLZ1* in barley, *SHP* was recently found to repress SSP synthesis in common wheat (Boudet *et al.*, 2019). In addition to the bZIP, Dof and MYB TFs, barley *Fusca3* and wheat *TaFusca3* are also regulators of SSP genes (Moreno-Risueno *et al.*, 2008; Sun *et al.*, 2017). Due to the complexity of the polyploid genome, all the regulators of SSP synthesis in common wheat were identified through homology-based cloning and none of them was first characterized in common wheat.

NAC is one of the largest TF families in higher plants and comprises more than 100 members in both monocot and dicot species (Murozuka *et al.*, 2018). In common wheat, NAC family TFs are involved in responses to abiotic and biotic stresses such as drought, salt tolerance and stripe rust (Feng *et al.*, 2014; Huang *et al.*, 2015; Mao *et al.*, 2014; Mao *et al.*, 2012; Tang *et al.*, 2012; Wang *et al.*, 2015; Xia *et al.*, 2010a,b; Xue *et al.*, 2006), as well as senescence, nutrient remobilization and grain-filling duration (Alhabbar *et al.*, 2018b; Uauy *et al.*, 2006; Zhao *et al.*, 2015). Recently, a NAC TF *TaNAC2-5A* was found promoting nitrogen use efficiency and seed vigour in common wheat (He *et al.*, 2015; Li *et al.*, 2020), and two NAC TFs *ZmNAC128* and *ZmNAC130* were identified as activators of zeins in maize (Zhang *et al.*, 2019).

As the A genome donor of common wheat, *T. urartu* is diploid and has a high-quality genome sequence (Ling *et al.*, 2018; Ling *et al.*, 2013). Gene homologs between *T. urartu* and common wheat always share a conserved function. For example, both *TuGASR7* in *T. urartu* and the homolog *TaGASR7* in common wheat control grain length (Ling *et al.*, 2013). Moreover, TFs (1238) in *T. urartu* have been predicted (<http://plantfdb.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 characterized using both gene prediction and traditionally PCR-based cloning methods in our previous work (Luo *et al.*, 2015; Zhang *et al.*, 2015). Therefore, *T. urartu* could be a superior model to identify new transcriptional regulators of SSP synthesis in wheat.

In this study, a novel NAC family TF *TuSPR* from *T. urartu* was preferentially expressed in developing endosperm during grain-filling stages. The SSP content in mature grains of *TuSPR* overexpression common wheat was reduced. The molecular mechanisms of *TuSPR*'s suppression of SSP synthesis were elucidated. Moreover, the function of the homolog in common wheat *TaSPR* was also investigated. Both *TuSPR* and *TaSPR* identified in this work undoubtedly expands our knowledge of the transcriptional regulation of SSP synthesis.

## Results

### The accumulation of SSPs decreases in common wheat transgenically overexpressing *TuSPR*

Using the *T. urartu* genome sequence (Ling *et al.*, 2018; Ling *et al.*, 2013), 1238 TFs were predicted at the genome scale (<http://plantfdb.cbi.pku.edu.cn>). We assumed that TFs preferentially expressed in endosperms during grain filling were prone to be involved in the transcriptional regulation of major reserves, such as SSP and starch. Thus, we analysed the tissue-specific expression of all the 1238 TFs in *T. urartu* using quantitative reverse transcription PCR (qRT-PCR) analysis. Among these TFs, a NAC family TF *TRIUR3\_21467* had the

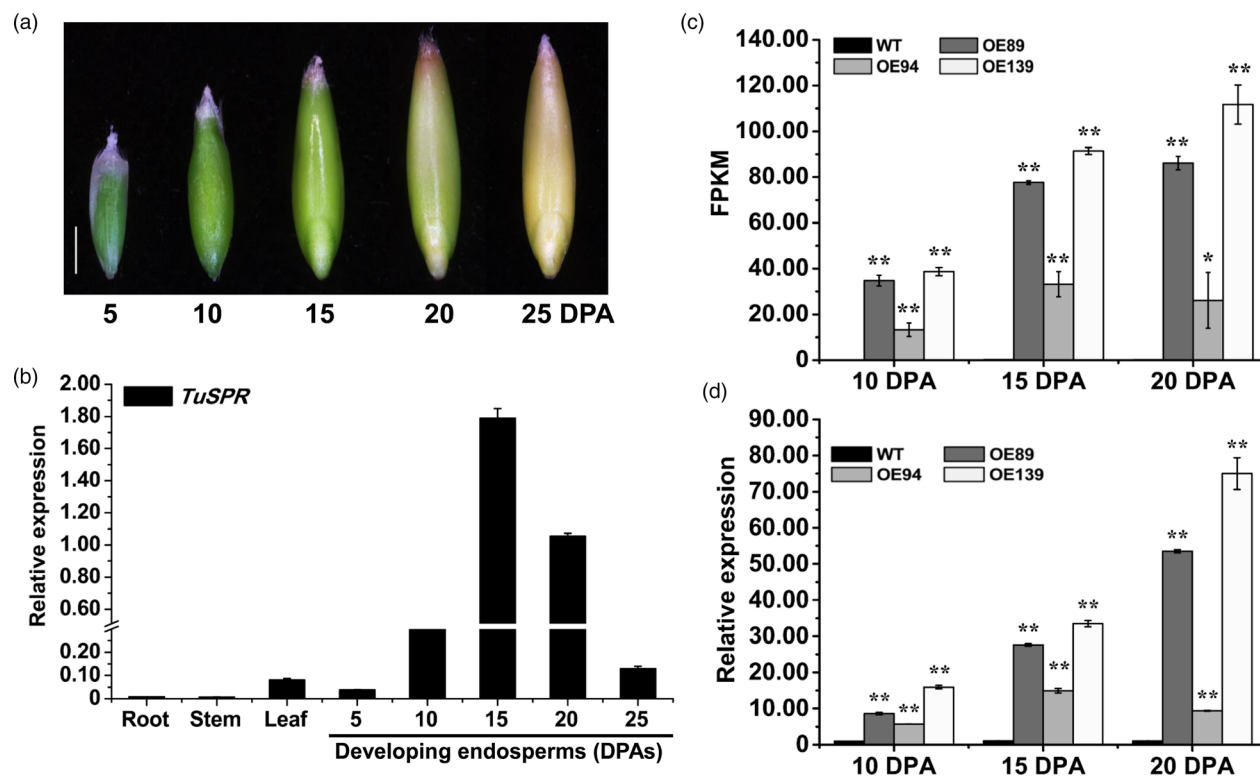
highest expression level in developing endosperms (with the embryo, aleurone, seed coat and pericarp removed) while trace levels in other tissues including roots, stems and leaves in qRT-PCR analysis (Figure 1a,b). The endosperm preferential expression indicated that *TRIUR3\_21467* might be involved in the regulation of reserve biosynthesis in endosperms. Considering its inhibition on SSP biosynthesis in subsequent research, *TRIUR3\_21467* was nominated as a 'storage protein repressor (*TuSPR*)'.

To further investigate its function, the overexpression construct of *TuSPR* (*Glu-1Bx14<sub>pro</sub>:TuSPR*) was transformed into the common wheat variety Kenong 199, considering that no robust genetic transformation system had been established in *T. urartu*. To drive the overexpression of *TuSPR* specifically in developing endosperms, the promoter of the HMW-GS gene *Glu-1Bx14* (Geng *et al.*, 2014) was used in *Glu-1Bx14<sub>pro</sub>:TuSPR*. Three independent *TuSPR* overexpression lines OE89, OE94 and OE139 were obtained and the T<sub>3</sub> generation of these lines were planted in Dishang and Zhaoxian. The following RNA-Seq analysis showed the overexpression of *TuSPR* in all three lines, which was verified by qRT-PCR analysis (Figure 1c,d). SSPs in mature grains of all three *TuSPR* overexpression lines were separated and quantified using the reverse-phase high-performance liquid chromatography (RP-HPLC) analysis. Compared with the c. 14.40 µg/mg flour in WT, the total HMW-GSs of all the three overexpression lines planted in both Dishang and Zhaoxian was decreased by 45.59%–67.73% (Figure 2a). Similarly, the total LMW-GSs of all three overexpression lines in both Dishang and Zhaoxian had 26.71%–45.17% reductions compared with the c. 9.33 µg/mg flour in WT (Figure 2b). The total glutenins of the three overexpression lines in the two planting sites were decreased by 37.56%–58.48% compared with the c. 23.56 µg/mg flour in WT (Figure 2c), which was consistent with reductions in HMW-GSs and LMW-GSs. Compared with the c. 42.25 µg/mg flour in WT, the total gliadins of all three overexpression lines in the two planting sites had no obvious changes (Figure 2d). The total SSPs of all three overexpression lines in the two planting sites was decreased by 11.70%–20.92% compared with the c. 65.94 µg/mg flour in WT (Figure 2e). SDS-PAGE analysis also showed that the bands of HMW-GSs and LMW-GSs from all three overexpression lines were less clear than those of WT (Figure S1a), and the bands of gliadins from both overexpression lines and WT were of the similar staining intensity (Figure S1b).

Consistent with the reduction of total SSPs, the thousand kernel weight (TKW) of all three overexpression lines was decreased by 3.75%–8.18% compared with the c. 44.91 g in WT (Figure S2a). In both Dishang and Zhaoxian, the grain weight per plant of all three overexpression lines was reduced by 11.15%–13.92% compared with the 24.52 g in WT (Figure S2b). These data collectively demonstrated that in common wheat transgenically overexpressing *TuSPR*, the accumulation of total SSPs was reduced with a concomitant reduction in total grain weight per plant. Intriguingly, the other agronomically important traits of *TuSPR* overexpression lines, such as plant height and spike number, had no obvious variations compared with WT (Figure S2c).

### The transcription of SSP genes is inhibited in common wheat transgenically overexpressing *TuSPR*

To explore the mechanisms of SSP decline in *TuSPR* overexpression common wheat, the transcriptomes of developing

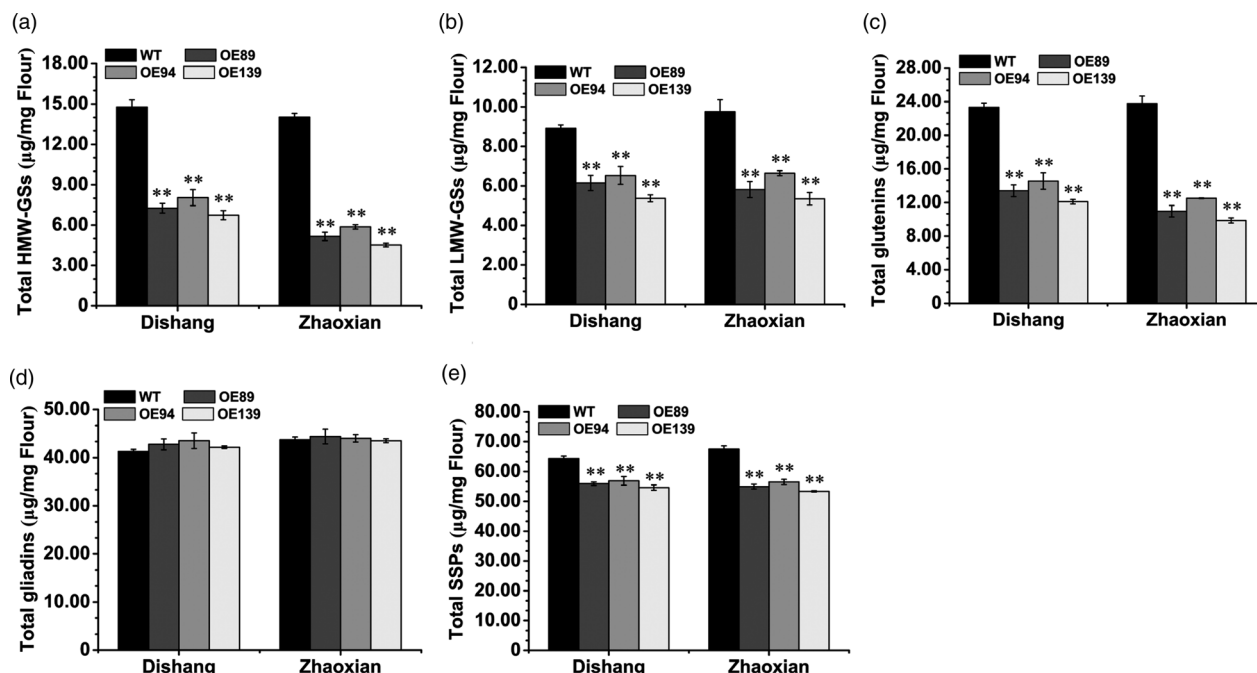


**Figure 1** The tissue-specific expression of *TuSPR* in *Triticum urartu* and the overexpression of *TuSPR* in overexpression common wheat lines. (a) Morphological variations of developing seeds of *T. urartu* at 5, 10, 15, 20 and 25 days post-anthesis (DPAs). Bar, 2 mm. (b) Tissue-specific expression assay of *TuSPR* in *T. urartu* using qRT-PCR analysis. Endosperms at 5, 10, 15, 20 and 25 DPAs are used. Root, stem and leaf are from plant at 15 DPA. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. (c) The overexpression of *TuSPR* calculated in RNA-Seq analysis. (d) The overexpression of *TuSPR* in RNA-Seq analysis is verified by qRT-PCR analysis. Considering the similar phenotype, only the endosperms of overexpression lines planted in Zhaoxian were used in RNA-Seq and qRT-PCR analyses. Primers characterizing SNPs are used to distinguish *TuSPR* and *TaSPR* in the qRT-PCR analysis. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) between overexpression lines and WT by one-way ANOVA in the SPSS program.

endosperms from all three overexpression lines OE89, OE94 and OE139 planted in Zhaoxian at 10, 15 and 20 DPAs were analysed by RNA-Seq. The developing endosperms of WT at the same stages were used as negative controls. Approximately 39 804 868 clean reads were obtained in individual replicons of each sample, 63.63% of which were mapped to annotated gene-coding regions, and 18.72% were uniquely mapped (Table S1). The expression level of transcripts was described by the fragments per kilobase of exon model per million reads mapped (FPKM).

Although dozens even hundreds of SSP genes exist in the genome of common wheat, only the ones predicted in the genome sequence and had an intact open reading frame (ORF) were considered in the FPKM analysis. At 10 DPA, the transcription levels of all the analysed glutenin and gliadin genes in all three overexpression lines were decreased with varying magnitudes compared with those of genes in WT according to the FPKMs (Figure 3a). In more detail, the transcription of HMW-GS genes, including *Glu-1Ax1*, *Glu-1Bx7*, *Glu-1By9*, *Glu-1Dx2* and *Glu-1Dy12*, decreased by 50.55%–62.35%; the transcription of LMW-GS genes including *A3-620*, *D3-394*, *D3-441* and *D3-578* had reductions of 46.88%–56.74% and the transcription of gliadin genes including *TaGli- $\alpha$ -7*, *TaGli- $\gamma$ -1* and *TaGli- $\omega$ -2* declined by 16.79%–47.40%. At 15 DPA, the transcription of all the analysed

HMW-GS and LMW-GS genes was still reduced, but the reduction amplitudes were lower than those at 10 DPA (Figure 3b). However, the transcription of almost all the analysed gliadin genes had no obvious variations even 24.18%–62.55% increase in all overexpression lines at this stage. At 20 DPA, the transcription of analysed HMW-GS and LMW-GS genes continued to decline except for those of *Glu-1Dy12* in all three lines, *Glu-1By9* in OE89 and *A3-620* and *D3-394* in OE94, all of which had 20.36%–46.75% increases (Figure 3c). Nevertheless, the transcription of all analysed gliadin genes in all three overexpression lines was increased by 17.69%–92.50%. The transcription decreases of gliadin genes at 10 DPA might be compensated by the transcription increases at 15 and 20 DPAs, which resulted in the no reduction in the total gliadins in mature grains of overexpression common wheat (Figure 2d). qRT-PCR was conducted to validate the results in the RNA-Seq analysis and overall correlation was found between the two datasets (Figure S3). These dynamic transcriptomic data demonstrated that all the active SSP genes predicted in common wheat genome were reduced throughout the endosperm development in *TuSPR* overexpression common wheat, and the reduction strength was the highest at the early stage (10 DPA), then decreased with endosperm maturation, even the transcription of some SSP genes, especially gliadin genes, increased at later stages (15 and 20 DPAs).



**Figure 2** Content variations in SSPs of *TuSPR* overexpression lines in RP-HPLC analysis. (a–e) The variations in the total HMW-GSs, the total LMW-GSs, the total glutenins, the total gliadins and the total SSPs of mature grains of *TuSPR* overexpression lines in the RP-HPLC analysis, respectively, compared with those of wild type (WT), in both Dishang and Zhaoxian. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) between overexpression lines and WT by one-way ANOVA in the SPSS program.

### *TuSPR* suppresses both the promoter activities and the transcription of SSP genes

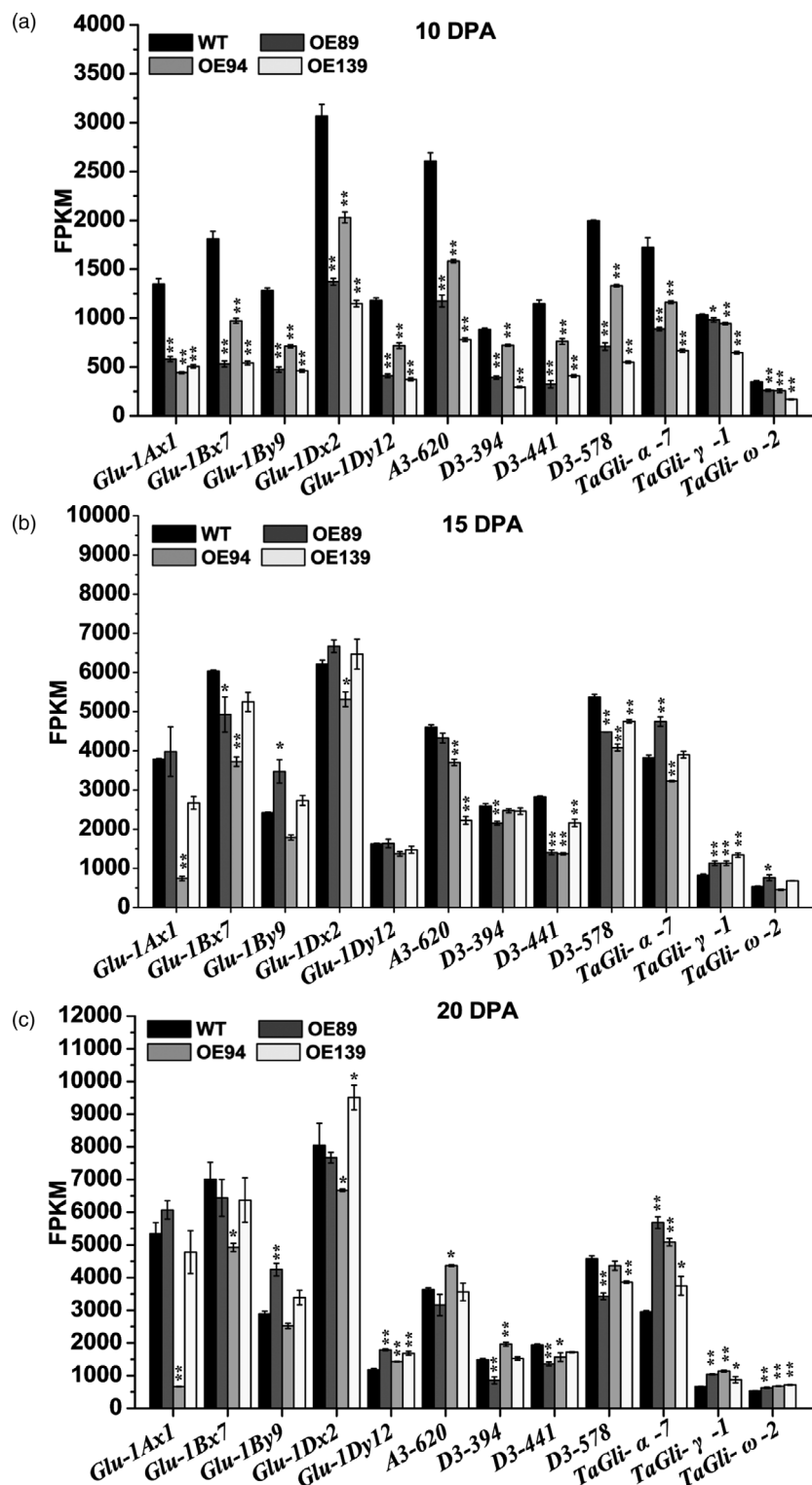
As a candidate regulator of SSP genes, molecular characteristics of *TuSPR* were analysed. Based on the IWGSC BLAST search ([https://urgi.versailles.inra.fr/blast\\_iwgsc/?dbgroup=wheat\\_iwgsc\\_refseq\\_v1\\_chromosomes&program=blastn](https://urgi.versailles.inra.fr/blast_iwgsc/?dbgroup=wheat_iwgsc_refseq_v1_chromosomes&program=blastn)), *TuSPR* was located on the long arm of group 2 chromosome (2AL). The BLASTP ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)) search showed that the fragment of 7–133 amino acids (AAs) in *TuSPR* was the no apical meristem (NAM) domain, which is the signature motif of NAC family TFs (Figure 4a). In the transrepression activity analysis, the relative LUC activity was significantly reduced when the reporter was co-transformed with the *TuSPR* overexpression plasmid into Arabidopsis leaf protoplast cells, indicating that *TuSPR* was a transcriptional repressor (Figure 4b,c). The NAM domain (1–133 AA) and the region of 134–392 AA of *TuSPR* were further truncated in the transrepression activity analysis. Only the region of 134–392 AA had a decreased relative LUC activity, demonstrating that it was the domain of transrepression activity (Figure 4b,c). Fused with the green fluorescent protein (GFP), the GFP signal was detected in the nucleus of common wheat leaf protoplast cells (Figure 4d), demonstrating the nuclear localization of *TuSPR*.

To investigate the molecular mechanisms of the SSP gene transcription reduction in overexpression common wheat, the regulation of *TuSPR* on SSP gene promoters was analysed using the dual-luciferase reporter assay. Compared with those of promoters co-transformed with the empty plasmid, the relative LUC activity of each tested SSP gene (including HMW-GS genes *TuGlu-1Ax* and *TuGlu-1Ay*, LMW-GS genes *TuA3-520* and

*TuA3-538a* and gliadin genes *Gli- $\alpha$ -8*, *Gli- $\gamma$ -1* and *Gli- $\omega$ -1*) promoter co-transformed with *35S<sub>pro</sub>:BD-VP16-TuSPR* was decreased by c. 374 times (Figures 4e and S4a). To investigate its suppression on SSP gene transcription, the overexpression plasmid (*Ubi<sub>pro</sub>:TuSPR*) of *TuSPR* was transformed into endosperms of common wheat at 15 DPA (Figure S4c). Compared with those in endosperms transformed with the empty plasmid, the transcription levels of each examined SSP gene in the endosperms, where *TuSPR* was transiently overexpressed, were repressed by 2- to 3-fold in the qRT-PCR analysis (Figure 4f). These data indicated that *TuSPR* hampered the promoter activities *in vitro* and inhibited the transcription of SSP genes *in vivo*, which was consistent with the transcription reduction of SSP genes in developing endosperms of overexpression common wheat.

### *TuSPR* binds to SSP gene promoters both *in vitro* and *in vivo*

To explore *TuSPR*'s interactions with SSP genes, the candidate binding motifs of NAC family TFs in the SSP gene promoters were analysed. In the New PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>), four to fifteen occurrences of the *cis*-element 5'-CANNTG-3', which was supposed to be bound by the NAC family TFs, were found distributed in the promoters (0–2000 bp) of all the SSP genes predicted in the genomes of both *T. urartu* and common wheat (Figure S5). And one to four occurrences of the *cis*-element 5'-CANNTG-3' were located within the functional promoter, which was from 0 to –500 bp (Korkuc *et al.*, 2014; Ravel *et al.*, 2014). Considering the identical *cis*-element in all SSP gene promoters, only the binding of *TuSPR* to the *cis*-element 5'-CATGTG-3' distributed at –933 bp in the promoter of the HMW-GS gene *TuGlu-1Ay* was tested using

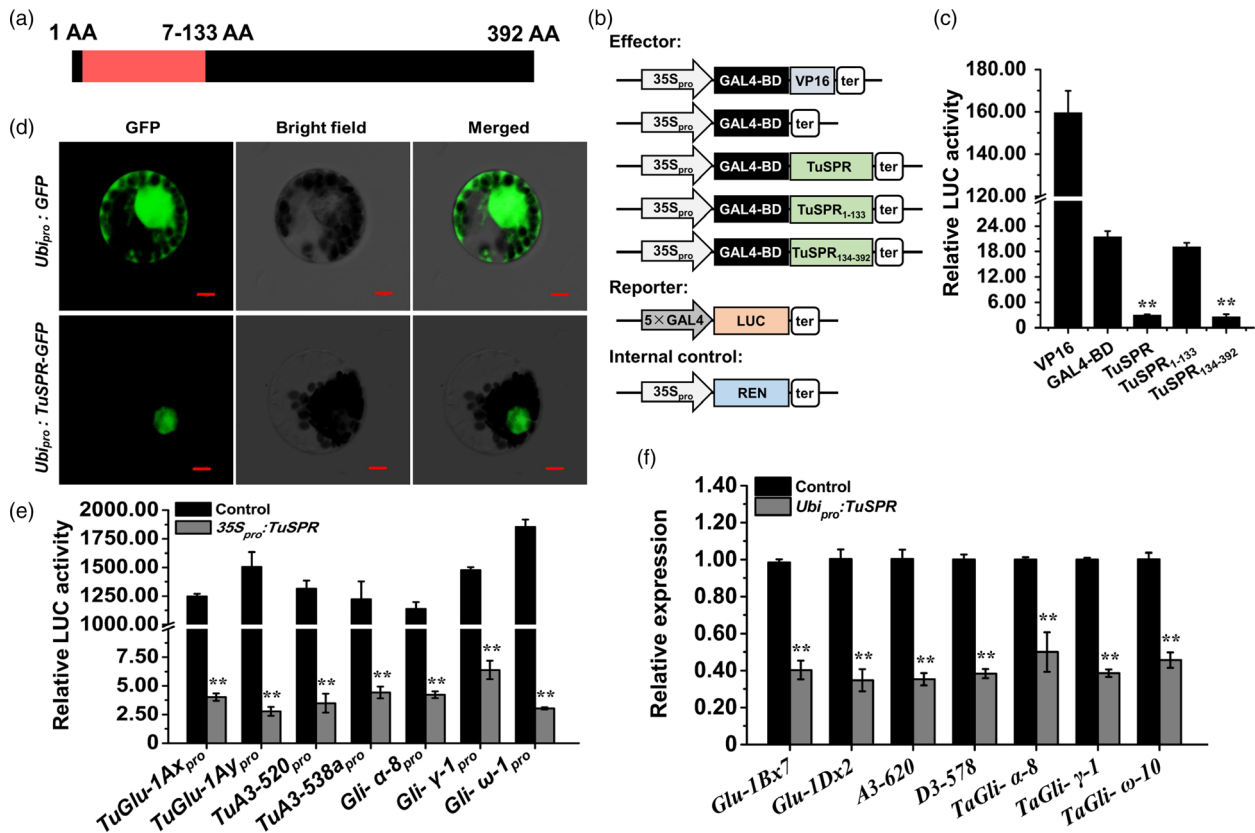


**Figure 3** The transcription changes of SSP genes in *TuSPR* overexpression common wheat endosperms during grain filling in RNA-Seq analysis. (a–c) The transcription changes of SSP genes in *TuSPR* overexpression common wheat endosperms at 10, 15 and 20 DPAs, respectively, compared with those of WT. Considering the similar variations in the contents of SSPs between Dishang and Zhaoxian, only the endosperms of lines planted in Zhaoxian were used. Since dozens even hundreds of SSP genes exist in the genome of common wheat, only the ones predicted in the genome sequence and had an intact open reading frame (ORF) were considered in the FPKM analysis. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) between overexpression lines and WT by one-way ANOVA in the SPSS program.

electrophoretic mobility shift assay (EMSA). In the EMSA analysis, a shifted band was observed when TuSPR-GST was mixed with the biotin-labelled probe, which was the promoter oligo containing 5'-CATGTG-3' in the middle (Figure 5a,b). Added with the non-labelled competitive probe, the shift was turning less clear. No shifted band was detected when TuSPR-GST was mixed with the biotin-labelled mutant probe in which the 5'-CATGTG-3' was exchanged with 5'-AAAAAA-3' (Figure 5b). When the

biotin-labelled probe was mixed with the GST tag, no shifted band was observed. The EMSA results indicated that TuSPR bound to the *cis*-element 5'-CATGTG-3' *in vitro*.

To verify the binding to the *cis*-element 5'-CANNTG-3' *in vivo*, chromatin immunoprecipitation (ChIP) assay was conducted using endosperms at 15 DPA from *TuSPR* overexpression lines. The promoter regions containing the *cis*-element 5'-CANNTG-3' distributed within 0–500 bp of HMW-GS (*Glu-1Ax1*, *Glu-1Bx7*,



**Figure 4** The influences of *TuSPR* on the promoter activities and the transcription of SSP genes in both the *in vitro* and *in vivo* assays. (a) Graphical summary of *TuSPR*. Red indicates the no apical meristem (NAM) domain (7–133 amino acids) predicted in NCBI Protein BLAST (BLASTP). AA, amino acid. (b) Schematic diagrams of the effector and reporter constructs used in the transrepression activity analysis. 35S<sub>pro</sub>, the 35S promoter; ter, terminator; 5 × GAL4, five GAL4 binding domain; LUC, firefly luciferase; REN, Renilla luciferase. (c) The transrepression activity analysis of *TuSPR*. GAL4-VP16 fusion protein is a positive control in which GAL4 would bind to the GAL4 binding domain and VP16 would burst the expression of LUC. GAL4-BD is a negative control. In addition to the full-length cDNA, the fragment of 1–133 AA (*TuSPR*<sub>1-133</sub>) containing the NAM domain and the fragment of 134–392 AA (*TuSPR*<sub>134-392</sub>) of *TuSPR* were used. (d) The subcellular localization of *TuSPR* in common wheat leaf protoplast cells. Ubi<sub>pro</sub> is the maize ubiquitin (Ubi-1) promoter. Bars, 5 μm. (e) The influence of *TuSPR* on the promoter activities of SSP genes in the dual-luciferase reporter assay. Empty construct is used as control. The ratio of firefly LUC activity to Renilla LUC activity (LUC/REN) is used to reveal the *trans*-activation ability of *TuSPR*. 35S<sub>pro</sub> is the 35S promoter. (f) The influence of *TuSPR* on the transcription of SSP genes in the endosperm transient overexpression analysis. The immature endosperms of common wheat at 15 DPA are used. Empty vector is used as the negative control. Ubi<sub>pro</sub> is the maize ubiquitin promoter (Ubi-1). Data are means ± SE (*n* = 3) of three independent biological replicates. Asterisks indicate statistically significant differences at *P* < 0.05 (\*) and *P* < 0.01 (\*\*) by one-way ANOVA in the SPSS program.

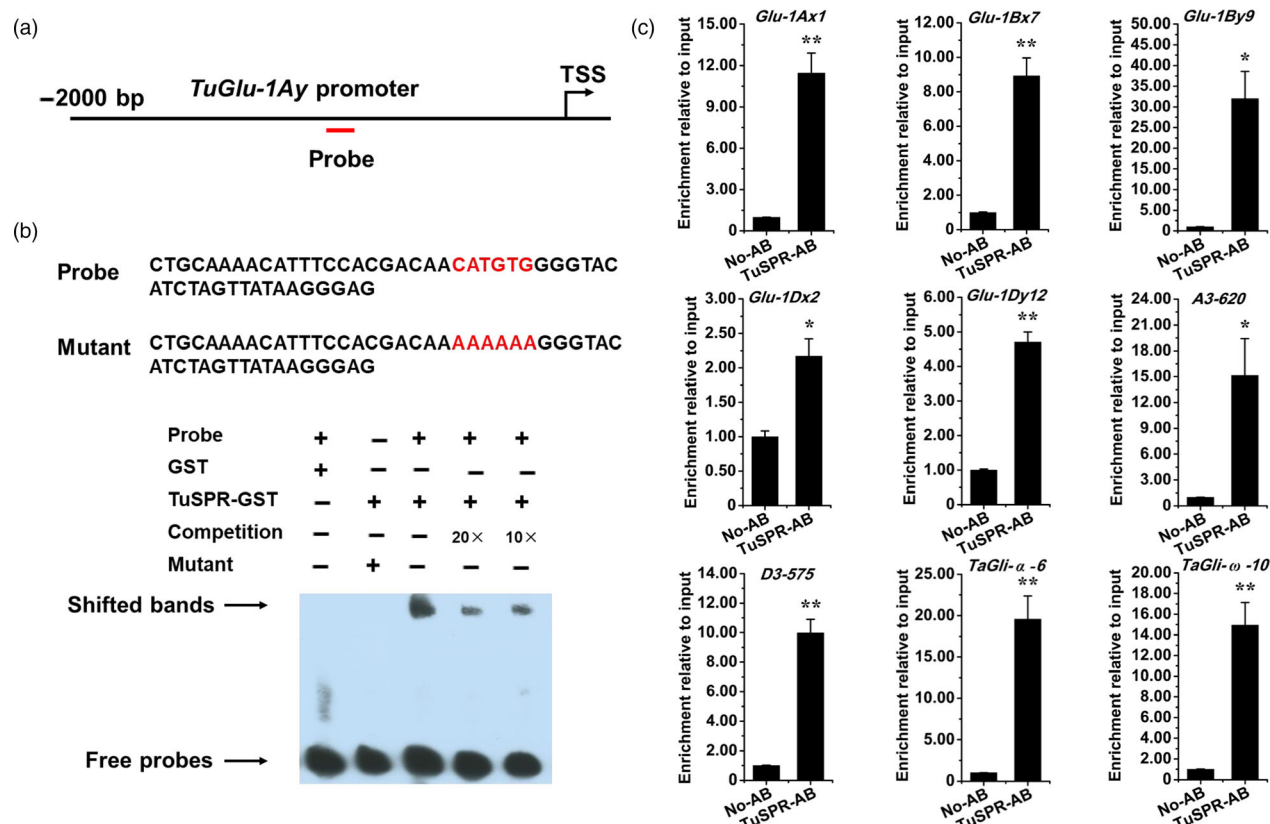
*Glu-1By9*, *Glu-1Dx2* and *Glu-1Dy12*), LMW-GS (A3-620 and D3-575) and gliadin genes (*TaGli-α-6* and *TaGli-ω-10*), which were active and could represent most of the sequence diversity in each of the HMW-GS, LMW-GS and gliadin gene sub-families in the phylogenetic analysis, respectively, were determined (Figure S6b). Primer pairs, whose last three nucleotides were based on single nucleotide polymorphisms (SNPs), were used to distinguish the promoter regions of SSP gene members sharing high sequence identities. In the ChIP-PCR analysis, the enrichment of the promoter regions of all the above-listed SSP genes in the experimental sample (*TuSPR*-AB) was 2–32 times higher than that in the negative control (No-AB; Figure 5c). Taken together, these data indicated that *TuSPR* bound to SSP gene promoters both *in vitro* and *in vivo*. Previously identified regulatory elements of SSP genes were always distributed within a region <500 bp upstream of the start codon (Forde *et al.*, 1985; Halford *et al.*, 1989; Hammond-kosack *et al.*, 1993; Muller and Knudsen, 1993). However, regulatory elements were also predicted recently

in the regions much far from 500 bp upstream of the start codon of HMW-GS and LMW-GS genes (Juhász *et al.*, 2011; Makai *et al.*, 2015; Ravel *et al.*, 2014). Whether the *cis*-element 5'-CANNTG-3' predicted here in the regions 500–2000 bp upstream of the start codon was involved in the regulation of SSP genes still needs more evidence.

#### *TaSPR* inhibits both the promoter activities and the transcription of SSP genes

The application of this gene in breeding would be broadened if the homolog of *TuSPR* in common wheat *TaSPR* also shared a conserved function with *TuSPR* on SSP synthesis suppression. To resolve this question, *TaSPR* in Chinese Spring was identified through homology-based cloning. In protein sequence alignment, the three sub-genome copies of *TaSPR*, *TaSPR*-A (GenBank accession No. MN022886), *TaSPR*-B (GenBank accession No. MN022887) and *TaSPR*-D (GenBank accession No. MN022888), shared 90.30%–94.70% identity with *TuSPR*, and they had the





**Figure 5** The interaction between TuSPR and SSP gene promoters in EMSA assay and ChIP-PCR analysis. (a) Location of the probe containing the *cis*-element 5'-CATGTG-3' (−933 bp) in the promoter region of HMW-GS gene *TuGlu-1Ay*. (b) The binding of TuSPR to the probe containing the *cis*-element 5'-CATGTG-3' and the mutant in the EMSA analysis. (c) The binding of TuSPR to the promoter regions (0–500 bp) of SSP genes containing the *cis*-element 5'-CANNTG-3' in the ChIP-PCR assay. AB, the antibody. Only the promoters of genes which are active and could represent most of the sequence diversity in each the HMW-GS, LMW-GS and gliadin gene sub-family are selected in the ChIP-PCR. Data are means ± SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) by one-way ANOVA in the SPSS program.

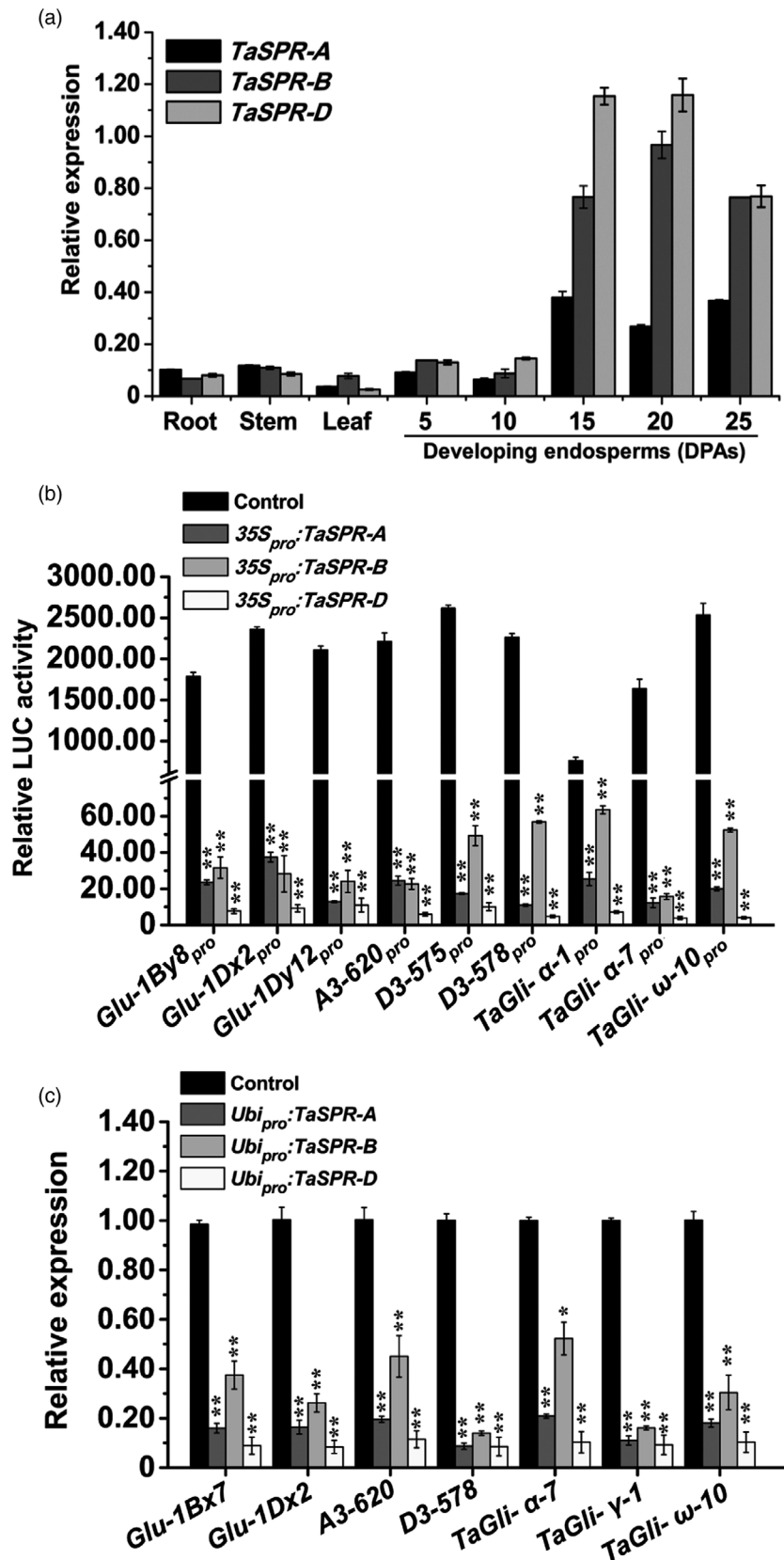
identical NAM domain (Figure S7). Like *TuSPR*, all *TaSPR-A*, *TaSPR-B* and *TaSPR-D* were preferentially expressed in developing endosperms and had trace expressions in roots, stems and leaves during grain-filling stages (Figure 6a). To investigate the trans-repression activity of *TaSPR* on SSP genes, the dual-luciferase reporter assay was performed. In the dual-luciferase reporter assay, the relative LUC activity of each representative SSP gene promoter co-transformed with each *35S<sub>pro</sub>:BD-VP16-TuSPR-A*, *35S<sub>pro</sub>:BD-VP16-TuSPR-B* and *35S<sub>pro</sub>:BD-VP16-TuSPR-D* was decreased by c. 169 times, compared with those of promoters co-transformed with the empty plasmid (Figure 6b). To explore their regulation on SSP gene transcription, each *TaSPR-A*, *TaSPR-B* and *TaSPR-D* was transiently overexpressed in endosperms of common wheat at 15 DPA. qRT-PCR analyses showed that the transcription levels of all representative SSP genes were repressed by 2- to 12-fold compared with those in endosperms transferred with the empty vector (Figure 6c). These data indicated that all three *TaSPR* copies suppressed the SSP gene promoter activities *in vitro* and inhibited the transcription of SSP genes *in vivo*.

### The SSP accumulation and bread-making quality are enhanced in *TaSPR* knock-down common wheat

To investigate its suppression on SSP synthesis in common wheat, *TaSPR* in Kenong 199 was knocked down by RNA interference (RNAi). To determine the transcription changes of *TaSPR* copies

and SSP genes during grain filling, the endosperms of all three obtained independent RNAi lines (RNAi#1, RNAi#2 and RNAi#3) at 15 DPA, which was at the middle of grain-filling stage (6–24 DPA; Simmonds and O'Brien, 1981), were used for qRT-PCR analysis. Considering the similar variations in SSP content, only the endosperms of the three *TaSPR* knock-down common wheat lines (RNAi#1, RNAi#2 and RNAi#3) planted in Zhaoxian was used. The transcription levels of *TaSPR* in all three RNAi lines were c. 7 times lower than that in WT, indicating the successful knock-down (Figure S8b). Compared with WT, the transcription levels of almost all representative SSP genes including HMW-GS genes (*Glu-1Ax1*, *Glu-1Bx7*, *Glu-1Dx2* and *Glu-1Dy12*), LMW-GS genes (*A3-620* and *D3-575*) and gliadin genes (*TaGli-α-7* and *TaGli-ω-10*) were elevated by 22.92%–126.19% in RNAi lines (Figure S8c). Intriguingly, the transcription levels of glutenin genes (c. 69.40%) had higher increases than those of gliadin genes (c. 48.73%). The accumulation of SSPs in mature grains of RNAi lines was determined by RP-HPLC analysis. In the RP-HPLC analysis, the total HMW-GSs of all three RNAi lines was increased significantly with amplitudes of 9.38%–20.64% in Dishang and of 35.41%–50.96% in Zhaoxian compared with that of WT (Figure 7a). Compared with that in WT, the total LMW-GSs of all three RNAi lines was elevated by 11.28%–16.65% in Dishang and by 16.57%–30.13% in Zhaoxian (Figure 7b). Owing to the increases in both HMW-GSs and LMW-GSs, the total glutenins of

**Figure 6** The influences of *TaSPR* on the promoter activities and the transcription of SSP genes in both the *in vitro* and *in vivo* assays. (a) Tissue-specific expression analysis of *TaSPR* in common wheat (Chinese Spring). Endosperms at 5, 10, 15, 20 and 25 DPAs are used. Root, stem and leaf are from plant at 15 DPA. (b) The influence of *TaSPR* on the promoter activities of SSP genes in the dual-luciferase reporter assay. Empty construct is used as a negative control. The ratio of firefly LUC activity to Renilla LUC activity (LUC/REN) is used to reveal the trans-activation ability of *TaSPR*. (c) The influence of *TaSPR* on the transcription of SSP genes in the endosperm transient overexpression analysis. The immature endosperms of common wheat at 15 DPA are used. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) by one-way ANOVA in the SPSS program.



all three RNAi lines was elevated with amplitudes of 7.07%–15.02% in Dishang and of 27.53%–41.79% in Zhaoxian, compared with that of WT (Figure 7c). Although increased in

both the two planting sites, the total gliadins of all RNAi lines rose more (by 15.77%–29.01%) in Dishang than in Zhaoxian (by 2.80%–9.56%), which was in contrast with the elevation in the



total glutenins (Figure 7d). In Zhaoxian, the higher increase in glutenin accumulation than that of gliadins was consistent with the higher elevations in the transcription of glutenin genes during grain filling. Attributed to the increases of both glutenins and gliadins, the total SSPs of all three RNAi lines was elevated by 10.89%–20.34% in Dishang and by 7.07%–17.72% in Zhaoxian compared with that of WT (Figure 7e). Altogether, transcription of SSP genes during endosperm development and accumulations of SSPs in mature grains of *TaSPR* knock-down common wheat were elevated. Attractively, the other agronomically important traits of *TaSPR* knock-down lines had no obvious variations compared with WT (Figure S9a).

The HMW-GS/LMW-GS ratio, Gliadin/Glutenin (Gli/Glu) ratio and SDS sedimentation volume are pivotal indicators of bread-making quality (Blackman and Gill, 1980; Blumenthal *et al.*, 1994; Doekes and Wennekes, 1982; Gupta *et al.*, 1992; MacRitchie, 1987; MacRitchie and Gupta, 1993; Pechanek *et al.*, 1997). The HMW-GS/LMW-GS ratios, the Gli/Glu ratios and SDS sedimentation volumes of *TaSPR* knock-down common wheat were calculated. In all the three *TaSPR* knock-down lines planted in Zhaoxian, the HMW-GS/LMW-GS ratios were increased by 23.49% while the Gli/Glu ratios were decreased by 24.27%, compared with those in WT (Figure 7f,g). The HMW-GS/LMW-GS ratios and the Gli/Glu ratios of *TaSPR* knock-down lines planted in Dishang had no obvious variations (Figure 7f,g). Attractively, the SDS sedimentation volumes of all *TaSPR* knock-down lines planted in both the two sites were increased by 14.16% (Figure 7h). Considering the HMW-GS/LMW-GS ratio and SDS sedimentation volume were positively correlated with bread-making quality while the Gli/Glu ratio was negatively correlated, *TaSPR* knock-down wheat should have improved bread-making quality. In concert with the increase in SSPs, the TKW and the grain weight per plant of *TaSPR* knock-down common wheat were increased by 4.16% and 12.38%, respectively, in both the two planting sites (Figure S9b,c). Taken together, the suppression of *TaSPR* could improve both the bread-making quality and the yield in common wheat.

## Discussion

### *TuSPR* regulates SSP genes directly

Considering the interactions with SSP gene promoters in the dual-luciferase reporter assay (Figures 4e and 6b) and the binding to SSP gene promoters in both EMSA and ChIP-PCR assays (Figure 5), *TuSPR* and *TaSPR* regulated SSP genes directly. A bZIP family TF *SHP* was recently found to repress SSP synthesis in common wheat (Boudet *et al.*, 2019). However, *SHP* is not first identified in common wheat, but the homolog of *BLZ1*, which is an activator of SSP synthesis in barley. Similarly, all the well-characterized *SPA*, *WPBF* and *TaGAMYB* in common wheat (Albani *et al.*, 1997; Dong *et al.*, 2007; Guo *et al.*, 2015; Ravel *et al.*, 2006) were identified through homology-based cloning. To the best of our knowledge, *TuSPR* and *TaSPR* are transcriptional repressors of SSP synthesis first characterized in wheat.

In our qRT-PCR analysis, *TaSPR-A*, *TaSPR-B* and *TaSPR-D* had the lowest, medium and highest expression levels, respectively, in endosperms of common wheat throughout grain filling. However, *TaSPR-A*, *TaSPR-B* and *TaSPR-D* had medium, lowest and highest the activities (regulation strength on SSP genes), respectively, in both the dual-luciferase reporter assay and the endosperm transient overexpression assay. Therefore, the activity of a single copy was not consistent with its expression level.

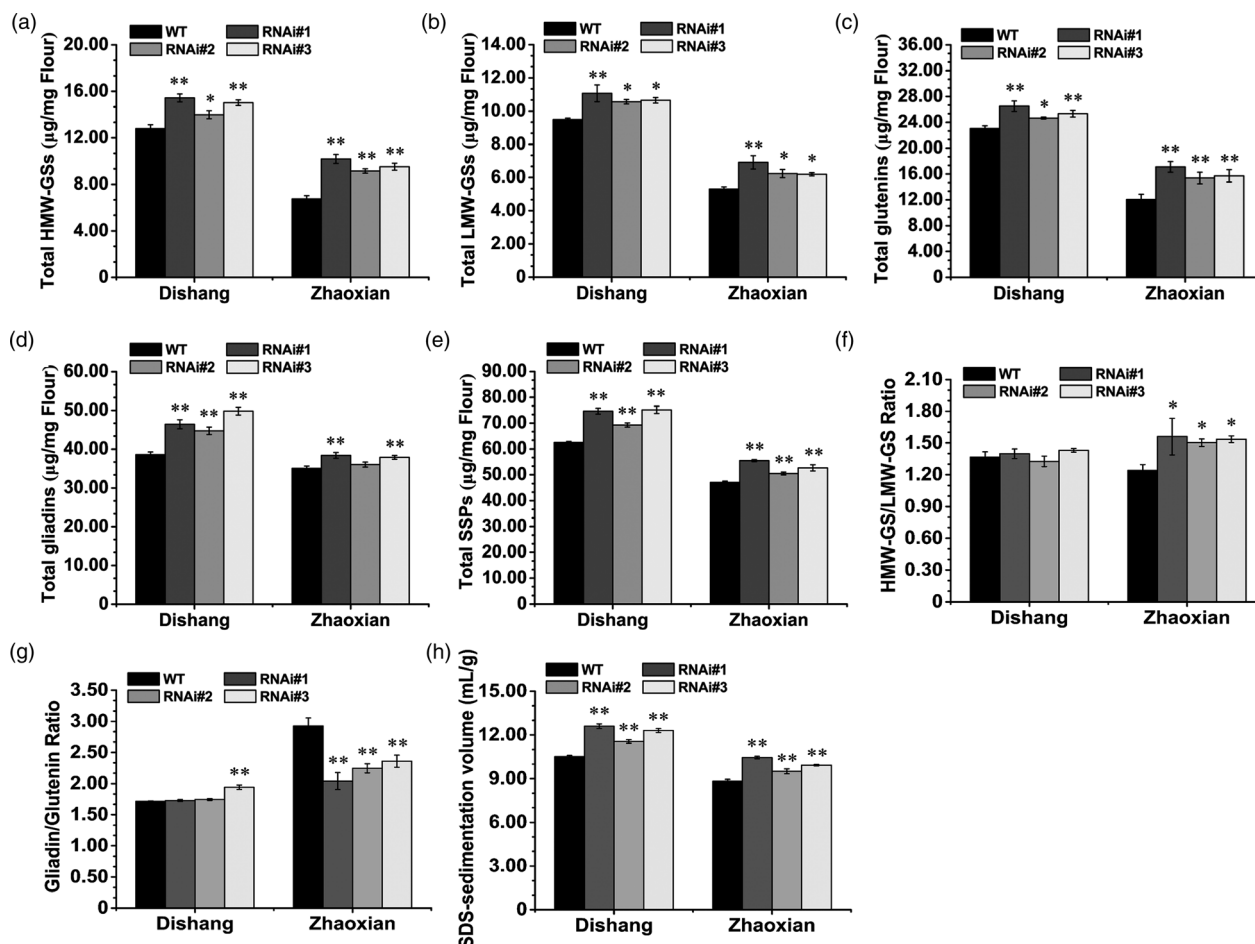
Transcriptional regulators always form a complex in the regulation of SSP genes. For example, the well-characterized regulators *GAMYB*, *WPBF* and *SPA* in wheat form a *GAMYB*-*WPBF*-*SPA* complex in the activation of SSP genes (Kawakatsu and Takaiwa, 2010). Yeast two-hybrid assay was conducted to investigate the interactions between *TuSPR* and each of the three well-characterized TFs in wheat. The yeast two-hybrid assay showed that *TuSPR* interacted with none of the above three TFs, this might indicate that *TuSPR* and the *GAMYB*-*WPBF*-*SPA* complex worked independently in the regulation of SSP synthesis in wheat (Figure S10a,b).

### *TuSPR* and *TaSPR* might not be involved in N regulation

Nitrate (N) affects SSP accumulation in common wheat grain (Barneix, 2007; Zheng *et al.*, 2018). Several NAC family TFs are involved in the regulation of N use in major cereal crops, such as *NAM-A1*, *NAM-B1* and *TaNAC2-5A* in common wheat and *OsNAP* in rice (Alhabbar *et al.*, 2018b; He *et al.*, 2015; Liang *et al.*, 2014; Uauy *et al.*, 2006). This raises the question of whether *TuSPR* and *TaSPR* regulate the N uptake, transportation or reduction, which in turn affects SSP accumulation. Distributed in different clades in the phylogenetic analysis, *TuSPR* and *TaSPR* had rather distant relationships with the previously identified N regulating NAC family TFs (Figure S11). N acquisition happens in root and N transport occurs mainly in leaf and stem (Tegeeder and Masclaux-Daubresse, 2018). However, during the grain-filling stages, both *TuSPR* and *TaSPR* were preferentially expressed in developing endosperms and had trace expression in leaves, roots and stems (Figures 1b and 6a). Although all *NAM-A1*, *NAM-B1* and *OsNAP* increase the grain protein accumulation, they accelerate leaf senescence by enhancing N allocation to grains (Alhabbar *et al.*, 2018a; Liang *et al.*, 2014; Uauy *et al.*, 2006). Throughout both vegetative and reproductive growth, delayed leaf senescence was not observed in *TuSPR* overexpression common wheat with decreased SSP content (Figure S2c); and early leaf senescence was not detected in *TaSPR* knock-down common wheat with increased SSP content (Figure S9a). In the RNA-Seq analysis of *TuSPR* overexpression common wheat, the expression levels of the N transport and assimilation related genes, for example *TaNRT2.1* (He *et al.*, 2015), had no obvious changes in endosperms throughout grain-filling stages compared with WT (Table S2). Altogether, these data indicated that *TuSPR* and *TaSPR* are unlikely to be involved in wheat N regulation. As transcription factors regulated SSP synthesis specifically in developing endosperms, the trace expression of *TuSPR* and *TaSPR* in leaves was intriguing. Considering the fact that no growth retardation was detected during leaf development and no phenotypic changes were observed in mature leaves when *TaSPR* was knocked down in common wheat (Figure S9a) and the exclusion of involvement in N regulation, the functions of *TuSPR* and *TaSPR* in wheat leaf development remain to be investigated.

### Proteome rebalancing between glutenins and gliadins

Proteome rebalancing has long been aware in endosperms of cereal crops. In maize, the loss of zeins could be compensated by the synthesis of non-zein proteins, which contributed the stable seed protein content (Holding and Larkins, 2009; Schmidt *et al.*, 2011; Wu and Messing, 2012b, 2014; Wu *et al.*, 2012; Zhang *et al.*, 2015). In addition to the rebalancing between SSPs and non-SSPs, compensation between glutenins and gliadins is also observed in common wheat endosperms. When the accumulation of glutenins decreases in RNAi or deletion lines, the content



**Figure 7** The variations in SSP content and bread-making quality of *TaSPR* knock-down common wheat. (a–e) The variations in the total HMW-GSs, the total LMW-GSs, the total glutenins, the total gliadins and the total SSPs of mature grains of *TaSPR* knock-down common wheat lines in the RP-HPLC analysis, respectively, compared with those of wild type (WT), in both Dishang and Zhaoxian. (f–h) The variations in the HMW-GS/LMW-GS ratio, the Gliadin/Glutelin ratio and the SDS sedimentation volume of *TaSPR* knock-down common wheat lines, respectively. Data are means  $\pm$  SE ( $n = 3$ ) of three independent biological replicates. Asterisks indicate statistically significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) by one-way ANOVA in the SPSS program.

of gliadins increases (Dumur *et al.*, 2004; Galili *et al.*, 1986; Yang *et al.*, 2014). Conversely, the reduction of gliadins results in elevation of glutenins (Becker *et al.*, 2012; Gil-Humanes *et al.*, 2012; Gil-Humanes *et al.*, 2010; Piston *et al.*, 2011). In *TaSPR* knock-down common wheat, the total glutenins was increased more in Zhaoxian (27.53%–41.79%) than in Dishang (7.07%–15.02%). However, the total gliadins was elevated more in Dishang (15.77%–29.01%) than in Zhaoxian (2.80%–9.56%; Figure 7c,d). These data indicated the rebalancing between glutenins and gliadins. Although gliadin genes were suppressed by *TuSPR*, the total gliadins in *TuSPR* overexpression common wheat had no obvious reductions, and this might be a compensation for the loss in the total glutenins. At 15 and 20 DPAs, the transcription of a portion of gliadin genes in *TuSPR* overexpression common wheat was enhanced by 24.18%–92.50%, which might compensate for the transcription reduction of gliadin genes at 10 DPA and resulted in the stable total gliadins in mature grains. Our RNA-Seq data showed that *TuSPR* was still overexpressed at 15 and 20 DPAs and the transcription levels were even 2–3 times higher than that at 10 DPA (Figure 1c). Moreover, the expression of previously identified transcriptional activators of SSP genes in common wheat, such as *SPA*, *WPBF* and *TaGAMYB*

(Albani *et al.*, 1997; Dong *et al.*, 2007; Guo *et al.*, 2015; Ravel *et al.*, 2006) did not rise at 15 and 20 DPAs (Table S2). Therefore, the driving force of the gliadin gene transcription increases at 15 and 20 DPAs was unclear. Both the total glutenins and the total gliadins of all three *TaSPR* knock-down common wheat lines planted in Zhaoxian were lower than those in Dishang. However, both the total glutenins and the total gliadins of WT planted in the same plot with *TaSPR* knock-down lines in Zhaoxian were also lower than those of WT planted in the same plot with *TaSPR* knock-down lines in Dishang. Considering the similar variations in WT planted in the same plot, the different glutenin and gliadin amounts of *TaSPR* knock-down lines between the two planting sites were less likely due to the plasmids or constructs used in the transformation and might be attributed to the environmental condition variations. Despite the compensation between glutenins and gliadins, the total SSPs was decreased in *TuSPR* overexpression common wheat and was increased in *TaSPR* knock-down common wheat, indicating that *TuSPR* and *TaSPR* could be elite targets in genetic engineering to manipulate SSP content in wheat.

In conclusion, a novel NAC family TF *TuSPR* in *T. urartu* was preferentially expressed in developing endosperm. *TuSPR* bound

to the promoters of SSP genes and suppressed the transcription of the latter in both *in vivo* and *in vitro* assays. In *TuSPR* overexpression common wheat, both the transcription of SSP genes and the accumulation of SSPs were hampered. The homolog *TaSPR* in common wheat also suppressed SSP synthesis. In *TaSPR* knock-down common wheat, the content of total SSPs was elevated. Both *TuSPR* and *TaSPR* could be optimal targets for genetic engineering to manipulate SSP content in wheat.

## Experimental procedures

### Plant materials and growth conditions

The genome-sequenced *T. urartu* accession PI 428198 and common wheat (*T. aestivum*) variety Chinese Spring were planted in 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 2016–2017 standard growing season. The common wheat variety Kenong 199 was used to generate overexpression and RNA interference (knock-down) lines. All the T<sub>3</sub> generation of *TuSPR* overexpression lines (OE89, OE94 and OE139), T<sub>3</sub> generation of *TaSPR* RNAi lines (RNAi#1, RNAi#2 and RNAi#3) and the wild type (WT) Kenong 199 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. Located in the North China Plain, both Dishang and Zhaoxian are two of the main cultivation areas of Chinese winter wheat with a different climate. The cultivation practices in both Dishang and Zhaoxian were identical and were described in the work of Song et al. (2020). For each line, 45 seeds were sown in three 1.50-m-long rows, which were spaced 23.00 cm apart. The growth of these transgenic common wheats was under normal conditions to avoid the influence of flood and drought on the accumulation of SSPs.

### Vector construction and plant transformation

In the overexpression construct (*Glu-1Bx14<sub>pro</sub>:TuSPR*), the promoter of the HMW-GS gene *Glu-1Bx14* (GenBank accession No. AY367771; Geng et al., 2014) was used to drive the overexpression of *TuSPR* specifically in developing endosperms of transgenic common wheat. In the RNAi vector construction, the fragment (901–1091 bp) distributed in the fourth exon of *TaSPR* which was amplified from the common wheat variety Kenong 199 was used as the target sequence in the RNAi hairpin to build the *Ubi<sub>pro</sub>:TaSPR* RNAi vector (Figure S8a). *Ubi<sub>pro</sub>* was the maize ubiquitin (Ubi-1) promoter. The fourth intron of common wheat *Wx* (GenBank accession No. LC373577) gene was used as a spacer in the hairpin. Each of the above constructs was transformed into the immature embryos of Kenong 199 using the biolistic particle delivery system following the method of Wang et al. (2014). All the transgenic lines were obtained from independent transformation events. The primers used for vector constructions and transgenic common wheat screening are listed in Table S3.

### RNA extraction and quantitative reverse transcription PCR analysis

Total RNA was extracted from wheat developing endosperms (with the embryo, aleurone, seed coat and pericarp removed), roots, stems and leaves using the TRNzol Universal Reagent (TIANGEN, Beijing, China). The first-strand cDNA was synthesized from 2.00 µg of total RNA by the Fast Quant RT Kit (TIANGEN).

qRT-PCR was performed with the Lightcycler 480 (Roche, Mannheim, Germany) using the SYBR Green I Master (Roche) kit. For the tissue-specific analyses of both *TuSPR* and *TaSPR*, the relative expression of *TuSPR* or *TaSPR* was calculated using  $2^{-\{C_t(\text{Reference gene}) - C_t(\text{TuSPR or TaSPR})\}}$ . As to the transcription changes of *TuSPR* in overexpression lines and *TaSPR* copies in knock-down lines, the transcription variations of SSP genes in both overexpression and knock-down lines and the transcription changes of SSP genes in the endosperm transient overexpression analyses, the normalized relative quantities (NRQs) relative to the reference gene were calculated using the formula  $2^{-\Delta\Delta C_t}$  (Schmittgen and Livak, 2008). Three reference genes, *Ta4045* (Ubiquinol-cytochrome C reductase iron-sulphur subunit) (Paolacci et al., 2009), *TaActin* and *TaTubulin*, were used in parallel for the best results. And only the data calculated using *Ta4045* were shown considering the similar results obtained from all the three reference genes. Primers used for the qRT-PCR analysis are listed in Table S3.

### Transrepression activity analysis of *TuSPR*

The transrepression activity analysis assay was performed according to the method of Ohta et al. (2001). In the transrepression activity analysis, the full-length cDNA of *TuSPR*, the fragment of 1–133 AA (*TuSPR<sub>1-133</sub>*) and the fragment of 134–392 AA (*TuSPR<sub>134-392</sub>*) were inserted into the *35S<sub>pro</sub>:GAL4-BD* vector to generate effector plasmids (Figure 4b). The vector *35S<sub>pro</sub>:GAL4-BD* was used as a negative control and the vector (*35S<sub>pro</sub>:GAL4-BD-VP16*) containing the GAL4-VP16 fusion protein expression cassette was used as the positive control (Chasman et al., 1989; Croston et al., 1992; Sadowski et al., 1988). The firefly luciferase (LUC) gene driven by the minimal TATA box of the 35S promoter plus five GAL4 binding elements in series was used as a reporter. The *Renilla reniformis* luciferase (REN) gene driven by the 35S promoter (*35S<sub>pro</sub>:REN*) was used as an internal control. Each effector was co-transformed in Arabidopsis leaf protoplasts with the reporter and the internal control, and the fluorescence was measured according to the protocol of Liao et al. (2008). The ratio of firefly LUC activity to *R. reniformis* luciferase activity was used to reveal the transrepression ability.

### Subcellular localization assay

The full-length cDNA of *TuSPR* was inserted into the pJIT163-hGFP vector and was driven by the maize ubiquitin (Ubi-1) promoter. The construct was subsequently transformed into common wheat protoplasts following the methods of Shan et al. (2014). After 18 h of dark incubation in a chamber at 25 °C, GFP fluorescence of common wheat protoplasts was observed using a laser confocal microscope (Zeiss LSM 710 NLO, Oberkochen, Germany). The primers used for vector construction are listed in Table S3.

### Measurement of the SSP content

Reverse-phase high-performance liquid chromatography (RP-HPLC) was used to measure the content of SSPs including glutenins (HMW-GSs and LMW-GSs) and gliadins in mature grains of *TuSPR* overexpression lines and *TaSPR* knock-down lines following the methods described elsewhere (Tilley et al., 1993; Yang et al., 2014). For each transgenic line, c. 20.00 g mature grains of four randomly selected plants were ground into whole-wheat flour, and 45.00 mg whole-wheat flour of each plant was used for glutenin and gliadin extraction. Both the glutenin and gliadin fractions were filtered through a 0.45 µm organic nylon

filter and analysed by RP-HPLC according to the methods described by González-Torralba *et al.* (2011). The amounts of glutenins and gliadins were estimated by integrating the relevant peak areas in the chromatograms and 1.00 mg/mL BSA standard solution was used to quantify the gliadin and glutenin extracts. The content of these SSP components was represented by µg/mg flour. To verify the quantification in RP-HPLC analysis, the same SSP extracts of transgenic lines were separated in SDS-PAGE analysis following the methods of Luo *et al.* (2015).

### Measurements of the agronomically important traits

For each *TuSPR* overexpression and *TaSPR* RNAi line in both Dishang and Zhaoxian, more than 15 independent plants were randomly selected to measure the agronomically important traits. The grain weight per plant and the thousand kernel weight (TKW) were measured manually.

### RNA-Seq analysis

Total RNAs extracted from endosperms (with the embryo, aleurone, seed coat and pericarp removed) of *TuSPR* overexpression common wheat lines OE89, OE94, OE139 and WT at 10, 15, and 20 DPAs were used for RNA-Seq analysis. Considering the similar variations in the contents of SSPs between Dishang and Zhaoxian, only the endosperms of lines planted in Zhaoxian were used. Each line and WT had three independent plants for RNA extraction. For individual samples, a library with an insert length of c. 350 bp was sequenced using the Illumina HiSeq 2000 (San Diego, CA). All clean reads were mapped to the Chinese Spring reference genome (TGACv1; Clavijo *et al.*, 2017). To verify the transcript abundance calculated in RNA-Seq, qRT-PCR analyses of both *TuSPR* and SSP genes were performed using gene-specific primers (Table S3).

### Electrophoretic mobility shift assay

The full-length cDNA of *TuSPR* was cloned into pGEX-4T-1 and transferred to *Escherichia coli* BL21 (*Transseta*) to obtain the fusion protein. The induction and purification of the GST (Glutathione S-transferase) recombinant protein of *TuSPR* were following the methods of Qiao *et al.* (2016). EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The primers used for vector construction are listed in Table S3.

### Chromatin immunoprecipitation assay

To investigate the binding of *TuSPR* to SSP gene promoters *in vivo*, ChIP assay was conducted using the developing endosperm at 15 DPA of *TuSPR* overexpression lines following the method of Bowler *et al.* (2004) with minor modifications. Considering the similar phenotype among overexpression lines, endosperms of OE89, OE94 and OE139 were mixed to generate experimental samples. A monoclonal antibody (*TuSPR*-AB) was produced in the Abmart antibody company (Shanghai, China) using the peptide NPEGQHGPSSDLTT (263–276 AA of *TuSPR*). In the ChIP assay, the experimental sample was added with the antibody (*TuSPR*-AB) to immunoprecipitate the protein-DNA complex, and the sample without the antibody was used as a negative control (No-AB). After reverse cross-linking, the precipitated DNA was used for ChIP-PCR analysis. Primer pairs (Table S3) amplifying a c. 100 bp fragment containing the *cis*-element 5'-CANNTG-3' in the promoter region (0–500 bp) of each SSP gene were used in the ChIP-PCR analysis.

### The dual-luciferase reporter assay

To investigate the interaction between *TuSPR*/*TaSPR* and SSP gene promoters, the dual-luciferase reporter assay was performed following the methods of Ohta *et al.* (2001). Each full-length cDNA of *TuSPR* and *TaSPR* (*TaSPR*-A, *TaSPR*-B and *TaSPR*-D) was inserted downstream of the GAL4-VP16 fusion protein expression cassette in the backbone to generate individual effector plasmids (Figure S4a,b). For a single reporter plasmid, each SSP gene promoter was inserted at the upstream of five GAL4 binding elements (5 × GAL4) in series, which formed a unit to drive the expression of the firefly luciferase (LUC) reporter gene. For individual promoters, the 2000 bp region upstream of the translation initiation codon (ATG), which was supposed to contain most of the key *cis*-elements in SSP gene expression, were used (Juhász *et al.*, 2011; Lamacchia *et al.*, 2001; Ravel *et al.*, 2014; Wang *et al.*, 2020). Considering the large number of SSP gene in the *T. urartu* genome ([https://plants.ensembl.org/Triticum\\_urartu/Info/Index](https://plants.ensembl.org/Triticum_urartu/Info/Index)) and the common wheat genome ([http://plants.ensembl.org/Triticum\\_aestivum/Info/Index](http://plants.ensembl.org/Triticum_aestivum/Info/Index)), only the promoters of genes that were active and could represent the sequence diversity in each of the HMW-GS, LMW-GS and gliadin gene sub-families in the phylogenetic analysis were selected in the dual-luciferase reporter assay (Figure S6). In *T. urartu*, the promoters of HMW-GS genes *TuGlu-1Ax* and *TuGlu-1Ay*, LMW-GS genes *TuA3-520* and *TuA3-538a* and gliadin genes *Gli-α-8*, *Gli-γ-1* and *Gli-ω-1* were used. In common wheat, promoters of HMW-GS genes *Glu-1By8*, *Glu-1Dx2* and *Glu-1Dy 12*, LMW-GS genes *A3-620*, *D3-575* and *D3-578* and gliadin genes *TaGli-α-1*, *TaGli-α-7* and *TaGli-ω-10* were selected.

The GAL4-VP16 fusion protein would bind to the GAL4 binding elements and burst the expression of LUC, which could compensate for the weak activities of SSP gene promoters in Arabidopsis leaf protoplast cells. The *R. reniformis* luciferase (REN) gene driven by the 35S promoter (*35S<sub>pro</sub>:REN*) was used as an internal control. The GAL4-VP16 fusion protein without *TuSPR* or *TaSPR* insertion was used as control. In experimental samples, each reporter (*SSP<sub>pro</sub> + 5 × GAL4:LUC*) was co-transformed in Arabidopsis leaf protoplasts with the internal control (*35S<sub>pro</sub>:REN*) and the effector (*35S<sub>pro</sub>:BD-VP16-TuSPR* or *35S<sub>pro</sub>:BD-VP16-TaSPR*). In the control experiment, each reporter (*SSP<sub>pro</sub> + 5 × GAL4:LUC*) was co-transformed in Arabidopsis leaf protoplasts with the internal control (*35S<sub>pro</sub>:REN*) and the empty effector (*35S<sub>pro</sub>:BD-VP16*). The fluorescence was measured according to the protocol of Liao *et al.* (2008). The ratio of firefly LUC activity to *Renilla* LUC activity (LUC/REN) was used to reveal the trans-activation abilities of *TuSPR* and *TaSPR*. Experiments were done with three biological replicates.

### Transient overexpression of *TuSPR* and *TaSPR* in developing common wheat endosperms

*TuSPR* and the three sub-genome copies of *TaSPR* (*TaSPR*-A, *TaSPR*-B and *TaSPR*-D) were driven by the maize ubiquitin promoter (Ubi-1) to generate overexpression constructs (*Ubi<sub>pro</sub>:TuSPR* or *Ubi<sub>pro</sub>:TaSPR*; Figure S4c,d). Each overexpression construct was transferred into the developing endosperms of Chinese Spring at 15 DPA following the method of Wang *et al.* (2014). The empty overexpression vector (*Ubi<sub>pro</sub>:*) was used as a negative control. After 48 h of dark incubation at 25 °C, the total RNA of these bombarded endosperms was extracted and the expression levels of SSP genes were quantified by qRT-PCR analysis. Considering the numerous SSP gene members in the common

wheat genome ([https://plants.ensembl.org/Triticum\\_aestivum/Info/Index](https://plants.ensembl.org/Triticum_aestivum/Info/Index)), only the genes (HMW-GS genes *Glu-1Bx7* and *Glu-1Dx2*, LMW-GS genes *A3-620* and *D3-578* and gliadin genes *TaGli- $\alpha$ -7*, *TaGli- $\gamma$ -1* and *TaGli- $\omega$ -10*) that were active and could represent most of the sequence diversity in each of the HMW-GS, LMW-GS and gliadin gene sub-families in the phylogenetic analysis were selected for qRT-PCR analysis (Figure S6b).

### Phylogenetic analysis

Protein sequences of the 488 NAC family TFs predicted in the common wheat genome (Borrill *et al.*, 2017; Guerin *et al.*, 2019) were used in the phylogenetic analysis following the method of Luo *et al.* (2018).

### Evaluation of the SDS sedimentation volume

The SDS sedimentation volume (SDS-SV) of *TaSPR* RNAi lines and WT was measured following the method described earlier (Zeleny, 1947).

### Statistical analysis

The one-way ANOVA with the SPSS 17.0 package for Windows (SPSS, Inc., Chicago, IL) was used for statistical analysis in this work. The data were expressed as mean  $\pm$  SE. Differences of  $P < 0.05$  were considered statistically significant.

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### Conflict of interest

The authors declare that they have no conflict of interests.

### Author contributions

A.Z., D.L., K.Z. and D.C. conceived and designed the study. L.S., G.L. and Y.S. performed the experiments. G.L., L.S., J.X., J.J., C.Z., E.G., W.Y., X.L. and J.S. analysed the data. G.L. prepared the manuscript, A.Z., D.L. and W.Y. improved the manuscript. K.Z. and D.C. contributed reagents and materials. A.Z. and D.L. provided guidance on the whole study. All authors read and approved the final manuscript.

### Accession number

Accession numbers of genes used in this study are listed in Table S4.

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## Supporting information

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

**Figure S1** Comparison of stained bands of HMW-GSs, LMW-GSs and gliadins in SDS-PAGE analysis between *TuSPR* overexpression common wheat and WT.

**Figure S2** Yield variations in *TuSPR* overexpression common wheat.

**Figure S3** The verification of the transcription declines of SSP genes in developing endosperms of *TuSPR* overexpression common wheat in the RNA-Seq analysis using qRT-PCR analysis.

**Figure S4** Schematics of the constructs used in both the *in vitro* and *in vivo* assays of *TuSPR* and *TaSPR*.

**Figure S5** Distributions of the *cis*-element 5'-CANNTG-3' in SSP gene promoters of *Triticum urartu* and common wheat.

**Figure S6** The phylogenetic analysis of SSP genes in *Triticum urartu* and common wheat.

**Figure S7** Protein sequence alignment between *TuSPR* and the three copies of *TaSPR*.

**Figure S8** The transcription changes of SSP genes in developing endosperms of *TaSPR* knock-down common wheat in the qRT-PCR analysis.

**Figure S9** Yield variations in *TaSPR* knock-down common wheat.

**Figure S10** The interactions between *TuSPR* and other well-characterized SSP gene regulators in yeast two-hybrid analysis.

**Figure S11** Phylogenetic analysis of *TuSPR*, *TaSPR* and all the NAC family TFs predicted in the common wheat genome.

**Table S1** Mapped reads of the RNA-Seq data

**Table S2** The FPKM variations of SSP genes and well-characterized transcriptional activators of SSP synthesis in *TuSPR* overexpression lines

**Table S3** Primers used in this study

**Table S4** Accession numbers of genes used in this study