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Promoter DNA hypermethylation of *TaGli-γ-2.1* positively regulates gluten strength in bread wheat

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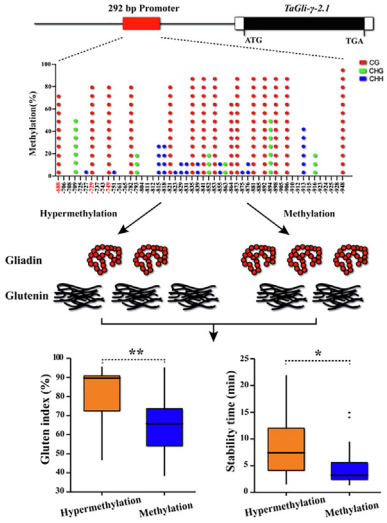
HIGHLIGHTS

- *TaGli-γ-2.1* belonged to a subgroup of -gliadin multigene family.
- *TaGli-γ-2.1* was a negative regulatory factor in gluten strength.
- Methylation of pTaGli-γ-2.1 played a key role in regulating *TaGli-γ-2.1* expression.
- Lower γ-gliadin content followed with hypermethylation of pTaGli-γ-2.1.
- Decreasing *TaGli-γ-2.1* expression could be used to improve gluten strength in wheat breeding.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Gliadins are the major components of gluten proteins with vital roles on properties of end-use wheat product and health-relate quality of wheat. However, the function and regulation mechanisms of γ-gliadin genes remain unclear.
Objectives: Dissect the effect of DNA methylation in the promoter of γ-gliadin gene on its expression level and gluten strength of wheat.
Methods: The prokaryotic expression and reduction–oxidation reactions were performed to identify the effect of *TaGli-γ-2.1* on dough strength. Bisulfite analysis and 5-Aza-2′-deoxycytidine treatment were used to verify the regulation of *TaGli-γ-2.1* expression by pTaGli-γ-2.1 methylation. The content of gluten proteins composition was measured by RP-HPLC, and the gluten strength was measured by Gluten Index and Farinograph.
Results: *TaGli-γ-2.1* was classified into a subgroup of *γ-gliadin* multigene family and was preferentially expressed in the later period of grain filling. Addition of TaGli-γ-2.1 protein fragment into strong gluten

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wheat flour significantly decreased the stability time. Hypermethylation of three CG loci of pTaGli-γ-2.1 conferred to lower *TaGli-γ-2.1* expression. Treatment with 5-Aza-2′-deoxycytidine in seeds of strong gluten wheat varieties increased the expression levels of *TaGli-γ-2.1*. Furthermore, the accumulations of gliadin and γ-gliadin were significantly decreased in hypermethylated wheat varieties, corresponding with the increasing of gluten index and dough stability time.

Conclusion: Epigenetic modification of pTaGli-γ-2.1 affected gluten strength by modulating the proportion of gluten proteins. Hypermethylation of pTaGli-γ-2.1 is a novel genetic resource for enhancing gluten strength in wheat quality breeding.

Introduction

Wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) is a major staple food crop cultivated in over 100 countries, supplying a total of 763.90 million tons grains in 2020 (http://www.worldagriculturalproduction.com/crops/wheat.aspx) and providing approximating 25% of calories and nutrients for human being in the world. Wheat flour can be processed into a wide range of food products, including baked bread, steamed bread, noodle, pasta, dumpling, cookies, and so on [1,2]. The quality of various wheat end products is mainly determined by gluten proteins, which are composed of monomeric gliadins and polymeric glutenins. Gliadins can interact with glutenin by intermolecular disulphide bonding to form a gluten complex, conferring dough viscoelasticity during wheat flour processing [3]. Gliadins can be classified into α/β-, γ- and ω-gliadins according to the mobility on A-PAGE (Acidic Polyacrylamide Gel Electrophoresis). Gamma-gliadins accounted for approximately 30% of gliadins, and played an important role on end-use properties and health-relate quality [4].

Gamma-gliadins are encoded by the *Gli-1* loci located on the short arm of the homologous group 1 chromosome [4]. *Gamma-gliadin* genes are a multigene family with 10 to 40 members in wheat [5–7]. Eleven *γ-gliadins* were identified in Xiaoyan 81 through RNA-sequencing [7]. The protein sequences of γ-gliadins generally start with a signal peptide (SP), followed by N-terminal non repetitive domain, a highly variable repetitive domain, a non-repetitive domain (containing six conserved cysteine residues), a rich glutamine domain and the C-terminal nonrepetitive domain (containing two conserved cysteine residues) [8]. The variable repetitive domain usually contains 7–16 Celiac disease (CD) epitopes which lead to an aberrant inflammatory response to gliadins in genetically susceptible individuals [7,9].

It has been reported by many studies that γ-gliadins were negative regulators of wheat quality [10,11]. In durum wheat, γ-42 gliadin was reported to be associated with decrease of SDS-sedimentation volume, a key parameter used to evaluate bread-making quality [10]. Addition of γ-gliadin into wheat flour decreases the mixing time and resistance to extension, and weakens the gluten strength of dough [11]. Silencing *γ-gliadins* in wheat by RNAi led to 33%–43% reduction of the γ-gliadin content accompanied by an increase in SDS-sedimentation volume [12,13].

The accumulation of γ-gliadins is an important contributor to the dough rheology and end-use properties [2,3,11]. A few transcription factors have been demonstrated to regulate the accumulation of γ-gliadins, such as wheat prolamin-box binding factor (WPBF) and storage protein activator (SPA) [14–16]. Except for transcription factors, DNA methylation also contributed to the expression regulation of gliadins gene [17–19]. DNA methylation is a well-studied epigenetic modification adding of a methyl groups to DNA, extensively detected in cytosine bases of CG, CHG, and CHH (H = A, T, or C) genome-wide. DNA methylation has been proved to play crucial roles in regulating gene expression through altering chromosome structure, DNA conformation and transcript factors binding ability, etc [20]. In wheat genome, the average methylation level of CG, CHG, and CHH were 53.3%, 3.48% and 1.41%, respectively [21]. Distinct methylation level between different wheat varieties were detected at huge amount of cytosines, resulting in the varying expression of the host genes. The expression of a serial of genes located in *Glu-1*, one of the most important loci for glutenin, were reported to be regulated by the DNA methylation level of this locus [22]. Wen *et al.* [17] reported that the accumulation of gliadins in wheat grains was significantly reduced by suppressing *DEMETER* gene which is an activator for DNA demethylation. The soft white wheat transformants with silenced *DEMETER* homolog genes can enhance the gluten strength, so that the dough quality of soft white wheat are comparable to hard red wheat varieties [18].

However, the regulatory mechanism of a single *γ-gliadin* gene and its precise role in dough strength are yet to be elucidated. In the present study, a multigene family of 28 *γ-gliadins* was identified from a weak gluten wheat variety, Zhengmai 004. One gene from this family, *TaGli-γ-2.1*, was rarely expressed in the seeds of strong gluten wheat varieties. Bisulfite analysis suggested that methylation in the promoter region of *TaGli-γ-2.1* (pTaGli-γ-2.1) inhibited its expression. Based on the methylation level of three cytosines in pTaGli-γ-2.1, 62 wheat varieties were divided into two types as hypermethylated varieties and methylated varieties. The accumulations of total gliadin and γ-gliadin in hypermethylated wheat varieties were significantly decreased. Accordingly, the gluten index and dough stability time were increased in hypermethylated wheat varieties. Epigenetic regulation of pTaGli-γ-2.1 played an important role in quality parameters of wheat grains.

Materials and methods

Plant materials

A total of 62 wheat varieties were planted in Yuanyang, Henan Province. Zhengmai 004 is a weak gluten variety, whereas Zhengmai 366 is a strong gluten variety. The seeds at five different development stages (7, 14, 21, 28 and 35 day after pollination, DAP) and four other tissues (root, stem, anther and leaves) of Zhengmai 004 were collected for transcript levels quantification by qRT-PCR. The mature seeds of 62 wheat varieties were also collected for qRT-PCR analysis.

RNA and DNA extraction

Total RNA was extracted using TransZol^{TM} Plant Kit (K21229, TransGen Biotech, Beijing, China). NanoDrop2000 (Thermo Scientific, Massachusetts, USA) was used to determine the ratio of A260/A280. RNA integrity was assessed by 1.2% (w/v) agarose gel electrophoresis. Genomic DNAs from the mature seeds of 62 wheat varieties were extracted using DNeasy Plant Mini Kit (163043067, Qiagen, Hilden, Germany) following the protocol.

Transcriptome analysis

Two wheat varieties, Zhengmai 366 and Zhengmai 004, with different gluten strength were chosen for RNA sequencing. Total

RNA was isolated from the seeds at 7, 14, 21, 28 and 35 DAP (day after pollination). Following concentration measurement and integrity control, the total RNA was reverse-transcribed to complementary DNA (cDNA) for library construction. Illumina HiSeq™ 2000 system (Illumina, San Diego, USA) was used for RNA sequencing. The raw data was filtered to get clean reads, which were assembled into contigs and scaffolds using Trinity [23]. A threshold of fold change 2 and a *P* value 0.01 were used for differentially expressed genes analysis.

Quantitative Real Time-PCR

Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (00310953, Thermo Scientific, Massachusetts, USA). PCR was conducted with 1 μg of total RNA and anchored oligo-$(dT)_{18}$ primers in a 20 μL reaction buffer for 1 h at 42 °C and 5 min at 72 °C.

Primers for differential expression analysis were designed based on the partial sequences obtained by RNA sequencing (Table S1), and were used to detect the expression levels of gliadin genes in the mature seeds of Zhengmai 366 and Zhengmai 004 by qRT-PCR. *TaGli-γ-2.1* transcripts in different tissues of Zhengmai 004 and the mature seeds of different wheat varieties were also analyzed. qRT-PCR was conducted in a 20 μL reaction buffer with 2 × SYBR qPCR Mix following the manufacturer's instruction of THUNDERBIRD SYBR qPCR Mix Kit (453300, Toyobo, Tokyo, Japan) and was performed using the Real Time PCR Bio-Rad CFX96 (1855200, Bio-Rad, California, USA). *Actin1* (GenBank accession AB181991.1) was used as an internal control. Three biological replicates were conducted for each sample.

Cloning the full length cDNA of TaGli-γ-2.1

TaGli-γ-2.1 partial sequences were used for BLASTN search in NCBI database. Three sequences (GenBank accession *JX081265*, *JX081266* and *JX081267*) were identified in wheat and related species [24]. According to the conserved open reading frame (ORF) region, degenerate primers were designed using Primer3 software [25] to clone *TaGli-γ-2.1* coding regions (Table S1). Genomic DNA and cDNA from Zhengmai 004 were used as templates for PCR amplification.

According to *TaGli-γ-2.1* sequences, primers were designed to clone 3′ downstream and 5′ upstream sequences, respectively. Total RNA was extracted to synthesize cDNA following the protocol of 3′- and 5′-Full RACE Kit (AK1501, TaKaRa, Shiga, Japan), respectively. The 3′ and 5′ Untranslated Region (UTR) were obtained by Nested-PCR. The promoter sequence of *TaGli-γ-2.1* was cloned with the promoter specific primers designed based on the wheat reference genome sequence (Table S1) using Phusion® High-Fidelity DNA Polymerase (M0530, New England Biolabs, Massachusetts, USA). The PCR products were cloned into the pEASY-Blunt3 vector (TransGen Biotech, Beijing, China), and were transformed into DH5α competent cells (TaKaRa, Shiga, Japan).

Bioinformatic analysis

The γ-gliadin sequences (*TaGli-γ-2.1* to *TaGli-γ-2.28*) were assembled and aligned by Lasergene 7.0, ClustalW, MEGA 6.0 and Genedoc software [26,27]. The Open Reading Frame was identified and aligned by Lasergene 7.0 and ClustalW, respectively. The phylogenetic tree was constructed by the neighbor-joining (NJ) method in MEGA 6.0 with 1000 bootstrap replicates. The multiple sequence alignment result was visualized and edited by Genedoc. ProtParam (https://web.expasy.org/protparam/) was used to analyze the properties of amino acid. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the cis-acting elements in the promoter.

Prokaryotic expression and reduction–oxidation reaction

According to the coding sequence of *TaGli-γ-2.1* with no signal peptide, prokaryotic expression primers were designed with restriction enzyme sites (Table S1). The recombinant pMD-18T-γ-gliadin plasmid and pET32a plasmid were digested with *HindIII* and *BamHI* at 37 °C for 30 min for ligating using T4 DNA ligase. The product was then transformed into *E. coli* Rosetta (DE3) competent cells (Novagen, Wisconsin, USA). The target protein was induced by 1 mM isopropyl β-△-thiogalactopyranoside (IPTG, M21008, Beijing, China) at 37 °C for 6 h. The target protein was further extracted from DE3 cells by sonicate treatment followed by purification with B-PER 6 × His Fusion Protein Purification Kits (Thermo Scientific, Massachusetts, USA) [28]. The purified target protein was detected by SDS-PAGE and was identified by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOFF-MS) [29].

The effects of *TaGli-γ-2.1* on dough quality were investigated using reduction–oxidation method [28]. The fusion protein extract was mixed with the flour of Xinong 979 and Zhengmai 366, respectively, at the ration of 1:400 using micro farinograph (Perten, Stockholm, Sweden). Distilled water and 0.25 mL of reducing agent DTT (50 $\mu g \cdot mL^{-1}$) were mixed for 0.5 min and then 0.25 mL of oxidating agent KIO_3 (200 $\mu g\ mL^{-1}$) was added. Mixing parameters were recorded and every test was done in triplicate.

Bisulfite sequencing

Genomic DNAs were extracted from mature seeds of 62 varieties. Bisulfite treatment was conducted using the EpiTect Bisulfite Kit (160022793, Qiagen, Hilden, Germany). Based on *TaGli-γ-2.1* promoter sequences (1,585 bp), the bisulfite primers were designed through Meth Primer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) to test the methylation status of target region (-660 to −952 bp). The bisulfite-treated DNA was used for PCR amplification with Maxima Hot Start - Taq DNA Polymerase (00332877, Thermo Scientific, Massachusetts, USA). The PCR products were cloned into the pMD18-T vector (AJF1708A, TaKaRa, Shiga, Japan) and 10 independent clones were sequenced for each variety. The number and position of methylated residues in *TaGli-γ-2.1* promoter regions in wheat varieties were analyzed by DNAStar Lasergene software and Bisulfite Analysis (http://katahdin.mssm.edu/kismeth/revpage.pl). After sodium bisulfite treatment, the methylated cytosines (5-methylcytosine and 5-hydroxymethycytosine) would not change, while the unmethylated cytosines (C) would be converted into uracil (T). The thresholds of methylation in common wheat were set for CG sites at over 75%, CHG sites at over 25% and CHH sites at over 10% [21]. In this study, hypermethylation of *TaGli-γ-2.1* was assigned that methylation level of the three CG sites (−688 bp, −729 bp and −749 bp) on its promoter region demonstrated over 75%.

5-Aza-2′-deoxycytidine treatment

Five strong gluten wheat varieties, including Zhengmai 366, Xinmai 26, Gaocheng 8901, Xinong 979 and Zhengmai 9023, were selected for demethylation treatment by 5-Aza-2′-deoxycytidine (A3656, Sigma, Missouri, USA). One hundred seeds of each variety were soaked in water for 16 h at 37 °C [30], which were then treated with 1 mM 5-Aza-2′-deoxycytidine. The solutions with and without 50 mL 5-Aza-2′-deoxycytidine (1 mM/L) containing 1 mL Tris-HCL (1 mol/L, pH 7.5) were prepared as the experimental

group and control, respectively. The seeds were collected in a Petri dish and were immersed with 4 mL solution for 3 days in the dark at 25 °C. The 5-Aza-2′-deoxycytidine-treated seeds were washed with water for five times, and were then cultivated in the field. Total RNA of mature seeds of treated and untreated wheat varieties were extracted, and the expression levels of *TaGli-γ-2*.1 were analyzed by qRT-PCR.

Reversed-phase high performance liquid chromatography (RP-HPLC) analysis

The glutenin and gliadin proteins were extracted from the grains of 62 wheat varieties for RP-HPLC analysis using the method in a previous study with some modifications [31,32]. Forty-five mg of whole wheat flour from each sample was used in extracting the glutenin or gliadin proteins. The glutenin and gliadin extracts were filtered by 0.45 μm nylon filter and 10 μL of filtered extracts were analyzed for RP-HPLC (Waters e2695 equipped with PDA 2998 detector, Waters Corporation, Massachusetts, USA) with the chromatographic column of Vydac 218TP C18 (E140314-1–2, Massachusetts, USA). The elution condition was performed according to Gao *et al.* [33] and Zhang *et al.* [34]. The content of glutenin, gliadin and γ-gliadin were calculated from chromatograms based on the peak areas with the retention times.

Quality test and data analysis

Flour moisture content, gluten index, and farinograph parameters were determined according to AACC methods 44-15A, 38-12A and 54–21, respectively. SPSS v22.0 software (IBM crop, Chicago, USA) was used for statistical analysis (*t*-test).

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Transcriptome data can be found in National Center for Biotechnology Information with SRA number SRP322490 (https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP322490).

Results

TaWG04 is generally suppressed in strong gluten wheat varieties

Two wheat varieties, Zhengmai 366 (strong gluten wheat) and Zhengmai 004 (weak gluten wheat), were selected for the identification of gluten strength related genes. The glutenin content, glutenin-to-gliadin ratio, gluten index and dough stability time of Zhengmai 366 were significantly higher than that of Zhengmai 004, with an increment of 2.36-, 1.63-, 10.90- and 2.63-fold, respectively (Fig. S1).

The seeds of Zhengmai 366 and Zhengmai 004 at 7, 14, 21, 28 and 35 DAP, were used for RNA sequencing. A total of 63,258,020 and 64,827,022 clean reads were obtained from Zhengmai 366 and Zhengmai 004, respectively. Finally, 114,418 unigenes were assembled and 375 differentially expressed genes (DEGs) including eight gliadin genes were identified. All the eight gliadin genes were expressed in higher levels in Zhengmai 004 than that in Zhengmai 366, which were further confirmed by qRT-PCR (Fig. 1a, Table S2). The expression level of one DEG, *TaWG04*, in Zhengmai 004 was 35.7-fold of that in Zhengmai 366 (Fig. 1a). Therefore, it was selected for further analysis.

Extensive expression variations of *TaWG04* were detected among different wheat varieties. *TaWG04* was expressed in low level in strong gluten wheat varieties, such as Gaocheng 8901, Xinong 979, Zhengmai 9023, Zhengmai 366 and Xinmai 26 (Fig. 1b). In contrast, high expression levels of *TaWG04* were detected in non-strong gluten wheat varieties including Yumai 13, Aikang 58, Zhengmai 004, Jingdong 1 and Zhoumai 13.

TaGli-γ-2.1 was preferentially expressed in the later period of grain filling

We performed a BLASTN search against the NCBI database with the partial sequence of *TaWG04*. As a result, three sequences (*JX081265*, *JX081266* and *JX081267*) were found to be aligned to *TaWG04* with high similarity [24]. Primers were developed based on the conserved regions of the three sequences to clone the entire sequence of *TaWG04* (Fig. S2, Table S1). The full-length of *TaWG04* was 1,114-bp, with a 945-bp Open Reading Frame (ORF) flanked by 68-bp 3′ and 101-bp 5′ flanking sequences. The protein encoded by the ORF was predicted with 314 amino acids. *TaWG04* had a typical structure of γ-gliadin and eight conserved cysteine residues linked by four intra-chain disulfide bonds. Subsequently, *TaWG04* was renamed as *TaGli-γ-2.1*.

The expression pattern of *TaGli-γ-2.1* in Zhengmai 004 was investigated by qRT-PCR, which showed that the expression level of *TaGli-γ-2.1* increased continuously from 14 to 21 DAP, peaked at 28 DAP and decreased rapidly at 35 DAP (Fig. 1c). No expression was detected in root, stem, leaf, anther and seeds at 7 DAP. The expression level of *TaGli-γ-2.1* in seeds at 28 DAP was approximately ten-time higher than that in seeds at 14 DAP. In addition, *TaGli-γ-2.1* was highly expressed in endosperm rather than in embryo at 28 DAP (Fig. 1d).

Phylogenetic analysis of TaGli-γ-2.1

Twenty-eight *γ-gliadin* ORF sequences ranging from 912 to 975 bp were isolated from Zhengmai 004 by degenerate PCR (Table S1) and these sequences were highly similar with each other (Table S3). The number of predicted amino acids ranged from 303 to 324, and the predicted pI varied from 6.85 to 7.72 (Table S4). All the 28 γ-gliadin genes contained the typical γ-gliadin structures, a signal peptide of 21 residues and five polypeptide domains (Fig. 2). Domain II and IV were variable in length and amino acids composition caused by repeat insertions/deletions. Except for TaGli-γ-2.24 missing the second cysteine residue in domain V, the other 27 γ-gliadins contained six cysteine residues in domain III and two cysteine residues in domain V, which formed the intra-chain disulfide bonds. TaGli-γ-2.1 and TaGli-γ-2.2 contained additional amino acids (PLFPQKEPQQ/PLFPQKEPQQPFPLQQ) at position 57 in domain II. Most γ-gliadins contained a conserved short glutamine with 3 to 20 amino acids residues in domain IV. In addition, the γ-gliadins have been analyzed to determine the position of CD toxic epitopes. DQ2.5-glia-γ1 (PQQSFPEQQ) or DQ8.5-glia-γ1 (PQQSFPEQE) was presented in 27 gliadins, whereas *TaGli-γ-2.2* was none of epitopes (Fig. 2, Table S5). Sequence analysis revealed a mutation from proline (P) to leucine (L) at the sixth amino acid in domain II of TaGli-γ-2.2.

To gain insight into the evolution of gliadin genes in wheat, 31 *α-gliadins*, 14 *ω-gliadins* and 70 *γ-gliadins* were obtained from Chinese Spring and related species (Table S6). All the 143 *gliadins* (including 28 sequences cloned in the present study) were divided into three major clusters, designated as *α-gliadins* cluster, *ω-gliadins* cluster and *γ-gliadins* cluster (Fig. S3). Obviously, *α-gliadins* and *γ-gliadins* were clustered together, showing closer evolutionary relationship. The 70 *γ-gliadins* were categorized into subgroup I, and the 28 sequences from Zhengmai 004 were assigned to subgroup II. Subgroup II presented closer phylogenetic relationship with *γ-gliadins* from Chinese Spring (*JX679680*, *JX679683* and *FJ006599*) and synthetic hexaploid wheat (*FJ006607*, *FJ006617* and *FJ006619*).

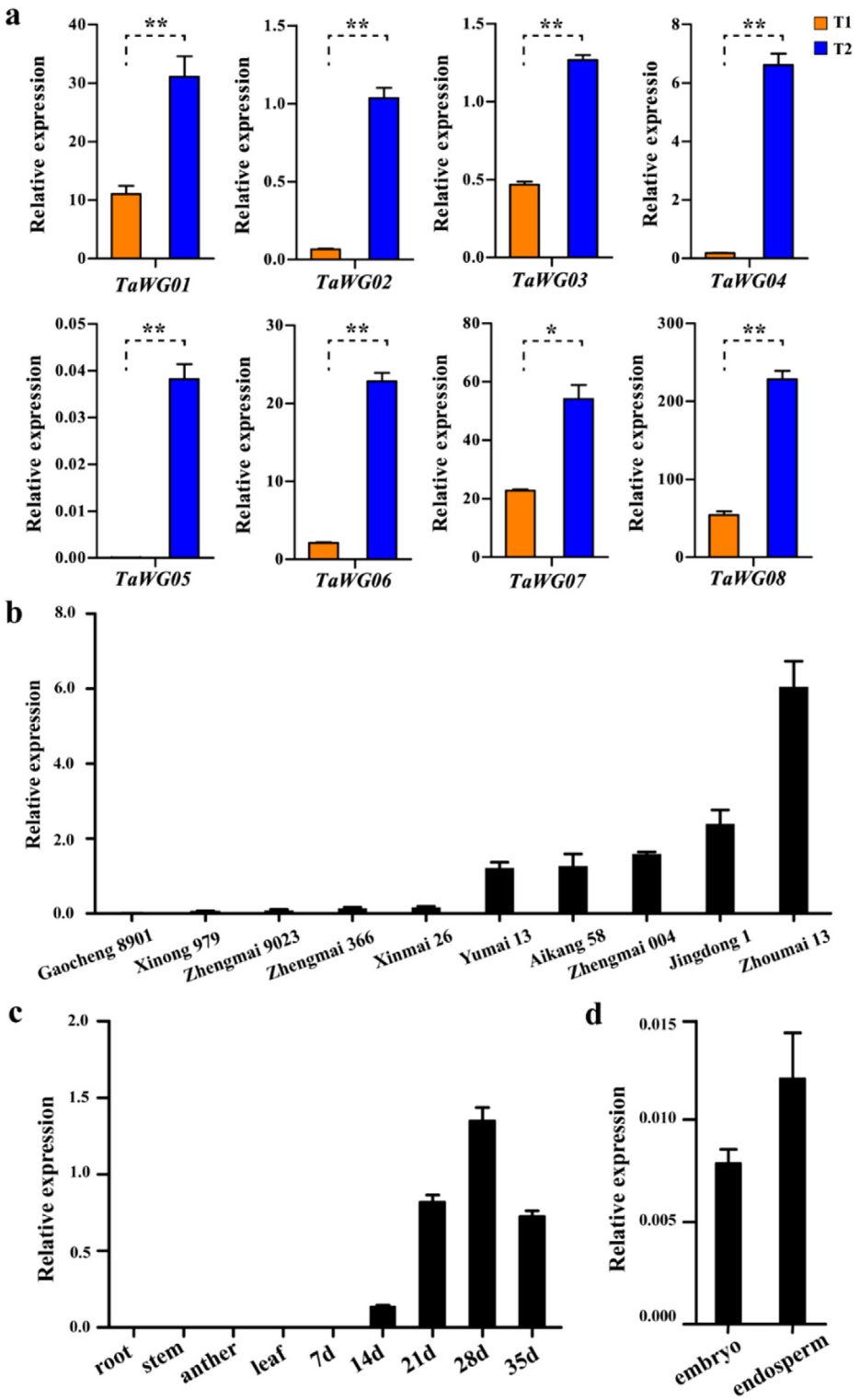

Fig. 1. The expression levels of *γ-gliadins* in different gluten strength wheat varieties. a: qRT-PCR analysis of the differentially expressed genes (*γ-gliadins*) in two wheat varieties. T1 and T2 represented Zhengmai 366 (a strong gluten wheat) and Zhengmai 004 (a weak gluten wheat), respectively. *P* values were calculated using *t*-test. * and ** indicated significant difference of $p < 0.05$ and $p < 0.01$, respectively. b: The expression levels of *TaGli-γ-2.1* in different gluten wheat varieties by qRT-PCR. Gaocheng 8901, Xinong 979, Zhengmai 9023, Zhengmai 366 and Xinmai 26 were strong gluten wheat varieties; Yumai 13, Aikang 58, Zhengmai 004, Jingdong 1 and Zhoumai 13 were non-strong gluten wheat varieties. c: *TaGli-γ-2.1* transcripts in different tissues of Zhengmai 004. Root, stem, anther and leaf were collected at the anthesis period. Denotations of 7d, 14d, 21d, 28d and 35d separately represented the seeds were collected at 7, 14, 21, 28 and 35 DAP. d: *TaGli-γ-2.1* transcripts in embryo and endosperm of Zhengmai 004 at 28 DAP. *Actin1* was an internal control.

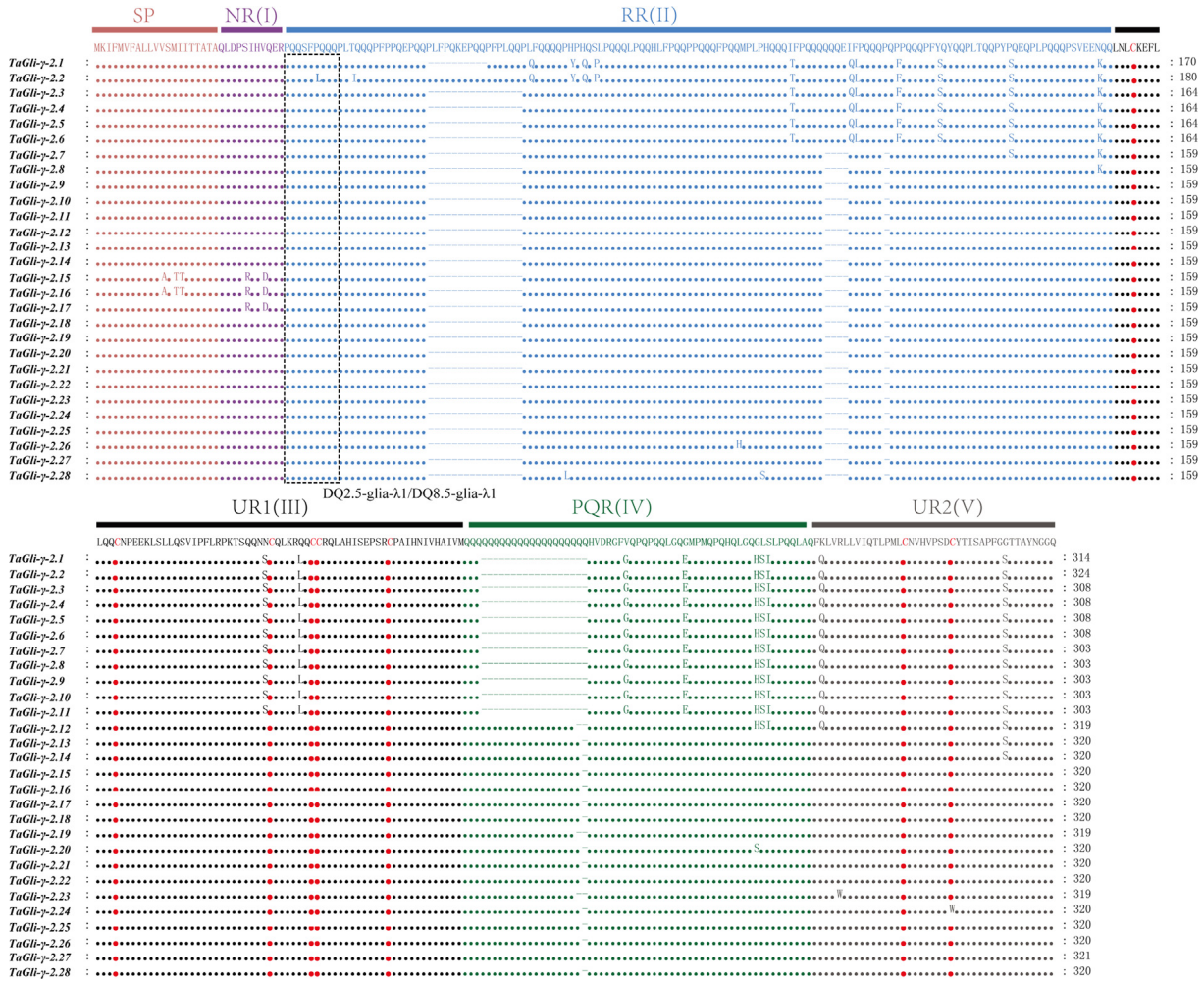

Fig. 2. Alignment analysis of 28 deduced amino acid sequences of γ-gliadins. SP (red) represented signal peptide. NR (I) in purple, RR (II) in blue, UR1 (III) in black, PQR (IV) in green and UR2 (V) in gray represented N-terminal non Repetitive I domain, Repetitive II domain, Non Repetitive III domain, Rich Glutamine IV domain and C-terminal non Repetitive V domain, respectively. Eight conserved cysteine residues (C) were colored in red. Box with dotted black line indicated the toxic epitopes of DQ2.5-glia-γ1 or DQ8.5-glia-γ1.

TaGli-γ-2.1 was a negative regulatory factor of gluten strength

To further investigate the function of TaGli-γ-2.1, the prokaryotic expression vector of pET-32a-TaGli-γ-2.1 was successfully constructed and 1 mM IPTG was used to induce the expression of the target protein fragment. The fusion protein stripe size was about 55 kD and was located in inclusion bodies by SDS-PAGE (Fig. 3a). Five peptides were mapped into TaGli-γ-2.1 by MALDI-TOFF-MS (Table S7), suggested that the fusion protein was the target protein, which was used for further analysis.

Two strong gluten wheat varieties, Xinong 979 and Zhengmai 366, were chosen to evaluate the effect of the target protein on the rheological properties of dough by Farinograph. The flour with the target protein extract was set as the experimental group (B), while the flour without the target protein extract was set as the control (A). The result of reduction–oxidation reactions showed that the stability time was significantly decreased while the development time was increased after adding the protein fragment of TaGli-γ-2.1 in the flour of Xinong 979 and Zhengmai 366 (Fig. 3b, Table S8). In detail, the mean stability time in Xinong 979 and Zhengmai 366 with the target protein were 5.2 min and 4.6 min, with a decrease of 9.36% and 24.04% than that of the control, respectively. However, the mean development time of the two varieties with the target protein were 6.7 min and 7.6 min, with an increment of 7.53% and 6.51% than that of the control, respectively. These results indicated that *TaGli-γ-2.1* probably play a negative role in the regulation of gluten strength.

Low TaGli-γ-2.1 expression was associated with high pTaGli-γ-2.1 methylation

The promoter sequence of *TaGli-γ-2.1* was cloned from Zhengmai 004 and Zhengmai 366. Only one single nucleotide polymorphism was detected between Zhengmai 366 and Zhengmai 004 in the 1585-bp promoter region. The motifs including TATA-box, MBS, 4 cl-CMA2b, AT1-motif, Box4, GA-motif, I/L-box and TCT-motif were identified in pTaGli-γ-2.1 (Fig. 4a, Table S1 and Fig. S4). Particularly, two endosperm specific motifs (GCN4 and Skn-1) were identified in pTaGli-γ-2.1 (Fig. 4b, Table S9), which may explain the endosperm-specific expression characteristic of *TaGli-γ-2.1*.

The significant differential expression of *TaGli-γ-2.1* and the high sequence identity in the promoter of *TaGli-γ-2.1* between Zhengmai 366 and Zhengmai 004 led us to detect the epigenetic regulation of *TaGli-γ-2.1*. DNA (cytosine) methylation status of pTaGli-γ-2.1 in mature seeds of Zhengmai 366 and Zhengmai 004 were evaluated by bisulfite sequencing. Significant differentially methylated regions were identified in a continuous 292-bp region (from −660 bp to −952 bp) of pTaGli-γ-2.1 (Fig. 4c). Comparing

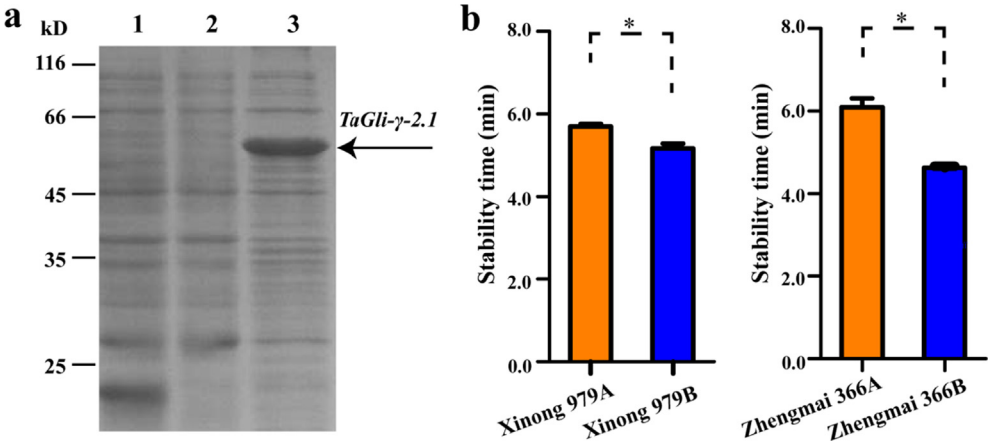

Fig. 3. Prokaryotic expression of *TaGli-γ-2.1* and the effects on dough stability time in different wheat varieties. a: SDS-PAGE analysis of recombinant plasmid pET-32a-TaGli-γ-2.1 after ultrasonic disruption. 1 represented the Marker; 2 represented the supernatant solution after ultrasonic disruption; 3 represented the precipitation solution after ultrasonic disruption. b: The ranges of stability time after reduction–oxidation reaction in Xinong 979 and Zhengmai 366. *P* values were calculated using *t*-test. * indicated significant difference of $p < 0.05$.

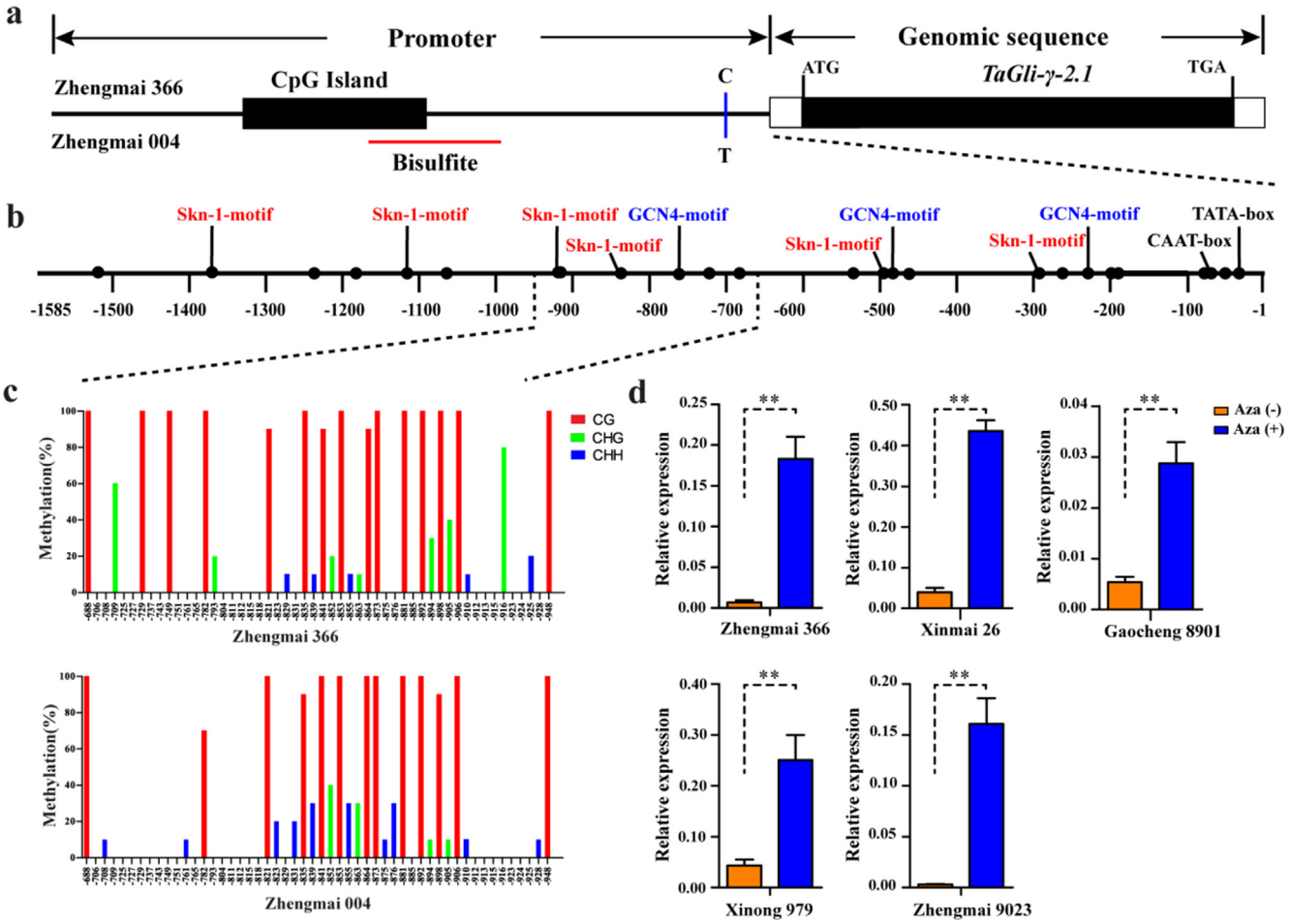

Fig. 4. Methylation analysis of pTaGli-γ-2.1 in several wheat varieties. a: Gene and promoter structure of *TaGli-γ-2.1* in Zhengmai 366 and Zhengmai 004. Boxes represented exon (black), untranslated regions (white) and CpG Island (black). Vertical line (blue) showed the SNP site in promoter region between Zhengmai 366 and Zhengmai 004. The red line represented the 292-bp sequence for DNA methylation analysis by bisulfite sequencing. b: The prediction of *cis*-acting elements in pTaGli-γ-2.1. Three GCN4 and six Skn-1 motifs which involved in endosperm expression existed in pTaGli-γ-2.1. c: DNA methylation characteristics in 292-bp region of *TaGli-γ-2.1* promoter by bisulfite sequenced analysis in Zhengmai 366 and Zhengmai 004. Histograms indicated the percentages of CG (red), CHG (green) and CHH (blue), respectively. d: The expression levels of *TaGli-γ-2.1* in five strong gluten wheat varieties with (+) or without (-) the 5-Aza-2′-deoxycytidine treatment. Aza (-) and Aza (+) were abbreviated by 5-Aza-2′-deoxycytidine (-) and 5-Aza-2′-deoxycytidine (+), respectively. *P* values were calculated using *t*-test. ** indicated significant difference of $p < 0.01$.

with Zhengmai 004, higher CG and CHG methylation and lower CHH methylation level were detected in Zhengmai 366. Most significant difference of DNA methylation were observed at −709 (CHG), −729 (CG), −749 (CG) and −916 (CHG) in the promoter (Fig. 4c). In detail, approximately 60%, 100%, 100% and 80% of DNA methylation in the four sites were detected in Zhengmai

366, respectively. In contrast, no methylation was detected at any of the four sites in Zhengmai 004.

Another four strong gluten wheat varieties (Xinmai 26, Gaocheng 8901, Xinong 979 and Zhengmai 9023) were further analyzed for DNA methylation. Compared with Zhengmai 004, higher DNA methylations were observed (Fig. S5). Next, the seeds from all the five strong gluten wheat varieties were treated with 5-Aza-2′-deoxycytidine. As expected, the expression levels of *TaGli-γ-2.1* were increased in the seeds of all the five strong gluten wheat varieties after demethylation treatment, with increments ranging from 4.84% to 56.86% (Fig. 4d). These results proved that the low expression level of *TaGli-γ-2.1* was caused by the high DNA methylation level in its promoter.

Hypermethylation and methylation analysis of pTaGli-γ-2.1 in different wheat varieties

DNA methylation level of pTaGli-γ-2.1 in 62 wheat varieties were further evaluated. As a result, a total of 52 methylation sites was identified, including 15 CG sites, 7 CHG sites and 30 CHH sites (Fig. 5). Among the 15 CG sites, the largest methylation variation among the 62 wheat varieties was detected at −688 bp site. Based on the variations in CG methylation level between Zhengmai 366 and Zhengmai 004, three sites (-688, −729 and −749 bp) were used to distinguish the hypermethylation and methylation of pTaGli-γ-2.1 of the 62 wheat varieties. Thirteen hypermethylated varieties were screened out and their methylation level varied significantly among different varieties (Table S10). The variation ranges of high-level methylation at CG sites and low-level methylation at CHG or CHH sites in hypermethylated varieties were 83%-100%, 29%-40% and 10%-14%, respectively. The methylation proportions of 49 methylated varieties were 34%-98%, 6%-38% and 2%-9%, respectively.

Gluten quality was significantly associated with methylation level of pTaGli-γ-2.1

The values of *TaGli-γ-2.1* expression, the contents of gluten proteins and the parameters of dough quality in 62 wheat varieties were obtained (Table S10). The expression levels of *TaGli-γ-2.1* varied significantly among the 62 wheat varieties, ranging from 0.001 to 7.11. The contents of gliadin ranged from 29.29 to 69.19 AU, and the mean contents were approximately two-fold than that of glutenin in wheat varieties. The glutenin-to-gliadin ratio, an

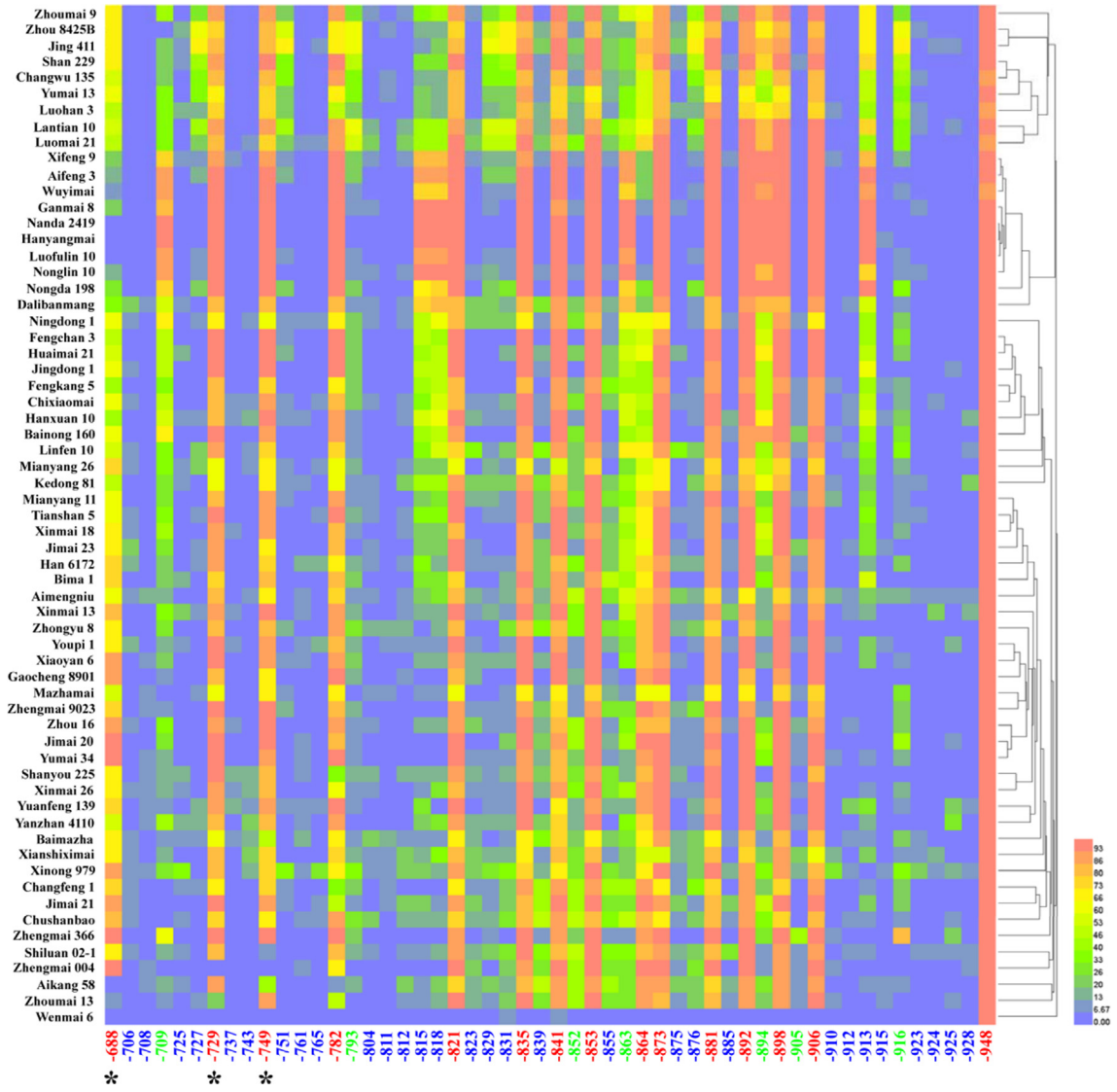

Fig. 5. The methylation status in 292-bp promoter regions of *TaGli-γ-2.1* in 62 wheat varieties. The numbers with different colors at bottom represented the different methylation types. Red, green and blue separately represented the types of CG, CHG and CHH. * indicated the three sites of CG type with significant difference to divide wheat varieties into two groups (hypermethylation and methylation).

important factor affecting dough properties and end-use quality, ranged from 0.29 to 0.94. Gluten index, the most important parameter to determine the quality of gluten proteins, ranged from 38.09% to 95.90%. The stability time which closely linked with dough strength, ranged from 1.2 to 22.0 min. These results implied the existence of extensive variation in gluten proteins content among the 62 wheat varieties.

The expression levels of *TaGli-γ-2.1* in 13 wheat varieties with hypermethylation were significantly lower than that in methylated varieties (Fig. 6a). The mean values of relative expression of *TaGli-γ-2.1* in the hypermethylated and methylated varieties were 0.05 and 1.49, respectively. The decreased expression of *TaGli-γ-2.1* in hypermethylated varieties resulted in the reduction of gliadin and γ-gliadin accumulation, corresponding with the increasing of glutenin accumulation and glutenin-to-gliadin ratio (Fig. 6b–e). The mean values of gliadin and γ-gliadin accumulation in hypermethylated varieties were 41.6AU and 14.6AU, much lower than that of methylated varieties (46.8AU and 16.9AU). On the contrary, the glutenin accumulation and glutenin-to-gliadin ratio in hypermethylated varieties were 20.92% and 28.84% higher than that of methylated varieties, respectively.

Furthermore, the hypermethylated wheat varieties had significantly higher gluten index and dough stability time than those in methylated varieties (Fig. 6f–g). The mean values of gluten index in hypermethylated wheat varieties and methylated wheat varieties were 80.7% and 64.0%, respectively. The mean values of dough stability time were 9.5 min and 4.2 min for hypermethylated and methylated wheat varieties. These results suggested a positive correlation between hypermethylation of pTaGli-γ-2.1 and wheat gluten quality.

Discussion

TaGli-γ-2.1 belongs to a subgroup of γ-gliadin multigene family

About 10–40 copies of *γ-gliadin* genes were identified in different wheat varieties in previous studies, while 29 γ-gliadin genes were annotated in the Chinese Spring reference genome [6]. However, the identification of γ-gliadin genes from different varieties is still challenging because of the copy number variation and allelic divergence [2]. In the present study, 28 γ-gliadins were identified from a weak gluten wheat variety, Zhengmai 004. They were assigned to a subgroup of γ-gliadins by phylogenetic analysis and were closely related with γ-gliadins annotated in the Chinese Spring and synthetic hexaploid wheat. All of those genes contained two highly variable domains of γ-gliadins: domain II and IV (Fig. 2), agreed with the results of Qi [6] and Wang [35]. One γ-gliadin, designated as *TaGli-γ-2.1*, was identified to be differentially expressed between wheat varieties of different gluten strength, encoding a protein sharing eight conserved cysteines with other 26 sequences (excluded TaGli-γ-2.24) in this subgroup.

Gliadins can cause serious health-related issues, such as celiac disease (CD). The issue of CD is caused by an aberrant inflammatory response to gliadins in genetically susceptible individuals, which was stimulated by the epitopes commonly found in gliadin repetitive domain [36]. Due to containing several sets of CD epitopes, γ-gliadins cause CD [2,7,12]. The subgroup gliadin genes reported in the present study contain less CD epitopes than other gliadins that would benefit to human health. Among the 28 genes, 27 contained only one CD epitope, DQ2.5-glia-γ1 or DQ8.5-glia-γ1, in the repeat domain II. Whereas TaGli-γ-2.2 was found free of the

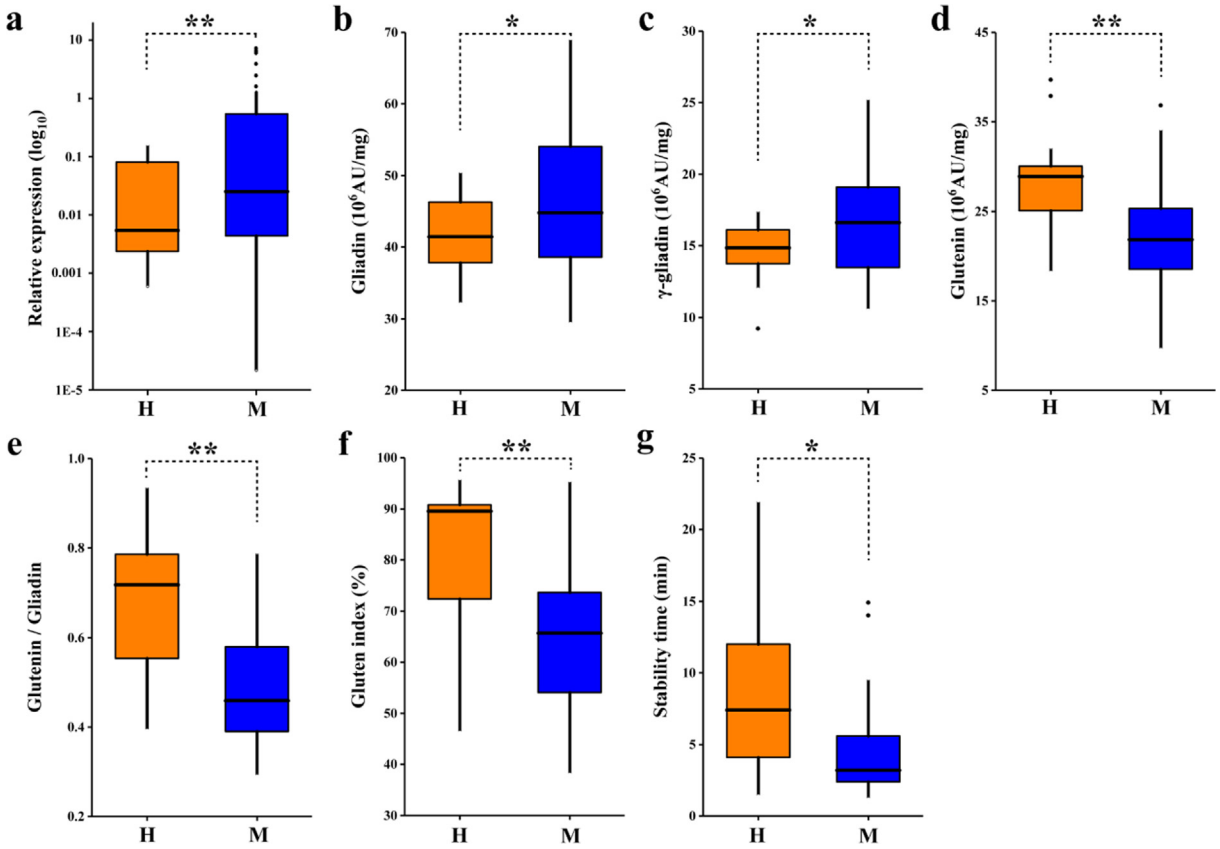

Fig. 6. Characterization of gluten proteins and dough properties in hypermethylated and methylated varieties. a: The expression levels of *TaGli-γ-2.1* in hypermethylated and methylated varieties. b-g: The contents of gliadin, γ-gliadin and glutenin, glutenin-to-gliadin ratio, gluten index and dough stability time in hypermethylated and methylated varieties. H and M separately represented hypermethylation and methylation. *P* values were calculated using *t*-test. * and ** indicated significant difference of $p < 0.05$ and $p < 0.01$, respectively.

epitopes (Fig. 2). Jouanin *et al.* [37] reported that small in-frame mutations in the epitope region would produce non-immunogenic gliadins which retained the desired rheological properties. The *γ-gliadin* genes identified in the present study, especially *TaGli-γ-2.2*, would be useful to develop healthier wheat food products.

The expression of TaGli-γ-2.1 is regulated by the DNA methylation level in its promoter

It has been widely accepted that alteration of DNA hypermethylation in promoter region is associated with gene activity [38–40]. The precise pattern of DNA methylation in promoter region is associated with temporal and spatial expression of gene [41]. Generally, DNA hypermethylation in promoter region gave rise to silencing the host genes in *Arabidopsis* and *rice* [42,43]. In this study, 62 wheat varieties were collected for evaluation of the DNA methylation status of pTaGli-γ-2.1 and the expression level of *TaGli-γ-2.1*. The results clearly demonstrated that the expression level of *TaGli-γ-2.1* was suppressed by the high methylation level of pTaGli-γ-2.1 in strong gluten wheat. *TaGli-γ-2.1* transcript was down-regulated by DNA hypermethylation in its promoter region. In plants, DNA methylation is categorized into CG, CHG, and CHH (H = A, T, or C), according to cytosines in three sequence contexts [38]. In the present study, 52 methylation sites, including 15 CG, 7 CHG and 30 CHH sites were identified in 62 wheat varieties. Interestingly, in a continuous 292-bp sequence of pTaGli-γ-2.1, only three CG sites (-688, −729 and −749 bp) were found with important roles in regulating the expression of *TaGli-γ-2.1*.

Furthermore, DNA demethylation treatment confirmed the function of pTaGli-γ-2.1 hypermethylation. Zhu *et al.* [22] reported that 5-Aza-2′-deoxycytidine treatment could increase demethylase expression in wheat grains, which resulted in a hypomethylation in promoter region and increased *Glu-1* expression. Therefore, seeds from five strong gluten wheat varieties were treated with 5-Aza-2′-deoxycytidine. As expected, significant increase in the expression levels of *TaGli-γ-2.1* were detected in the five strong gluten wheat varieties (Fig. 4d). It was also confirmed that DNA methylation was a major factor affecting *TaGli-γ-2.1* transcription.

TaGli-γ-2.1 is a candidate gene for improvement of gluten strength in wheat

Gluten strength is a crucial characterization for determining dough property. Gamma-gliadin accumulation is a major negative regulator of gluten strength [11]. Therefore, decreasing γ-gliadin accumulation is an effective approach for increasing gluten strength. *TaGli-γ-2.1* was confirmed as a negative factor for gluten strength in wheat for three reasons. First, the expression levels of *TaGli-γ-2.1* were suppressed in strong gluten wheat varieties. The addition of TaGli-γ-2.1 protein fragment significantly reduced the stability time which is closely related with dough strength (Fig. 3b). These results implied that expression of *TaGli-γ-2.1* can affect dough strength in wheat. Second, the methylation levels of pTaGli-γ-2.1 in strong gluten wheat varieties were significantly higher than that in other wheat varieties (Fig. 4, Fig. S5), suggesting that dough strength in wheat could be regulated by pTaGli-γ-2.1 methylation. Third, the low content of γ-gliadin was associated with high glutenin content, glutenin-to-gliadin ratio, gluten index and stability time in hypermethylated wheat varieties (Fig. 6), which advocated that *TaGli-γ-2.1* could be one of the main factors to regulate dough strength. Conclusion, silencing of *TaGli-γ-2.1* in wheat might be an effective way to decrease gliadin content and improve gluten strength for bread making.

Conclusions

The present study revealed *TaGli-γ-2.1* which belonged to a subgroup of γ-gliadin multigene family negatively regulated dough strength in wheat. Expression variation of *TaGli-γ-2.1* depended on the divergent methylation level of three CG sites in *TaGli-γ-2.1* promoter region. Hypermethylation of these CG sites played a key role in reducing *TaGli-γ-2.1* expression. Decreasing the accumulation of γ-gliadin significantly increased the contents of glutenin and glutenin-to-gliadin ratio. Furthermore, the quality parameters (gluten index and dough stability time) in hypermethylated wheat varieties performed better than that in methylated varieties. Taken together, our results indicate that the methylation level of pTaGli-γ-2.1 is a vital factor for regulating gliadin content in the grains of wheat, which could be applied to improve dough strength of wheat.

CRediT authorship contribution statement

Zhengfu Zhou: Conceptualization, Methodology, Writing - original draft. **Congcong Liu:** Investigation, Writing - original draft. **Maomao Qin:** Investigation. **Wenxu Li:** Data curation. **Jinna Hou:** Writing - review & editing. **Xia Shi:** . **Ziju Dai:** Validation. **Wen Yao Data analysis:** . **Baoming Tian:** Methodology. **Zhensheng Lei:** Supervision, Funding acquisition. **Yang Li:** Methodology, Writing - review & editing. **Zhengqing Wu:** Supervision, Project administration.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.06.021.

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