Effect of high molecular weight glutenin subunit Dy10 on wheat dough properties and end-use quality

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Abstract High-molecular-weight glutenin subunits (HMW-GSs) are the most critical grain storage proteins that determine the unique processing quality of wheat. Although it is part of the superior HMW-GS pair (Dx5+Dy10), the contribution of the Dy10 subunit to wheat processing quality remains unclear. In this study, we elucidated the effect of Dy10 on wheat processing quality by generating and analyzing a deletion mutant (with the *Dy10-null* allele) and by elucidating the changes to wheat flour following the incorporation of purified Dy10. The *Dy10-null* allele was transcribed normally, but there was a lack of the Dy10 subunit. These findings implied that the *Dy10-null* allele decreased the glutenin:gliadin ratio and negatively affected dough strength (i.e., Zeleny sedimentation value, gluten index, and dough development and stability times) and the bread-making quality; however, it positively affected the biscuit-making quality. The incorporation of various amounts of purified Dy10 into wheat flour had a detrimental effect on biscuit-making quality. The results of this study demonstrate that the Dy10 subunit is essential for maintaining wheat dough strength. Furthermore, the *Dy10-null* allele may be exploited by soft wheat breeding programs.

Keywords: HMW-GS, nonsense mutation, *Dy10-null* allele, end-use quality[1]

1. Introduction

Among cereal crops, wheat is unique because the biomechanical properties of gluten proteins when hydrated enable its flour to be used to make various food products, including bread, noodles, and biscuits. Gluten proteins are classically divided into gliadins and glutenins. Gliadins are usually monomeric and mainly influence the extensibility of wheat dough (Qi *et al*. 2011; Barak *et al*. 2015). Glutenins consist of high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs), which can form glutenin macropolymers (GMPs) through intermolecular disulfide bonds, thereby enhancing dough strength and elasticity (Shewry *et al*. 2003).

The HMW-GSs account for only 7–15% of the glutenins in common wheat (*Triticum aestivum*), but their composition and concentration have critical effects on gluten structure and determine the viscoelastic properties of dough (Shewry *et al*. 2003; Rustgi *et al*. 2019). Additionally, HMW-GS genes are included in the *Glu-A1*, *Glu-B1*, and *Glu-D1* loci on the long arms of chromosomes 1A, 1B, and 1D, respectively (Payne *et al*. 1982). Each locus contains two closely linked genes encoding a small y-type subunit and a large x-type subunit. However, because of allelic variation and gene silencing, only 3–5 HMW-GS genes are typically expressed in common wheat cultivars (Payne *et al*. 1982). Moreover, HMW-GSs possess a long central repetitive domain consisting of repeating units surrounded by highly conserved non-repetitive N- and C-terminal domains (Shewry and Halford 2002). The N-terminal domain of HMW-GSs usually has three or five cysteine (Cys) residues, whereas the C-terminal domain

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has only one Cys and the central repetitive domain lacks Cys or contains only one (Shewry and Halford 2002). The length of the HMW-GS repetitive domain has crucial effects on wheat processing quality (Shewry and Halford 2002; Shewry *et al*. 2003; Rustgi *et al*. 2019). Because the Cys residues are important for generating intermolecular disulfide bonds, the number and distribution of Cys residues affect the GMP structure and dough strength (Shewry *et al*. 2003). Consequently, HMW-GS alleles vary in terms of their contribution to processing quality.

Previous research indicated that Dx5+Dy10 is the superior HMW-GS subunit pair for wheat processing quality (Anderson and Bekes 2011). The lack of Dx5+Dy10 adversely affects gluten strength and bread quality (Jiang *et al*. 2019). The absence of only Dx5 decreases the sodium dodecyl sulfate sedimentation value (Wu *et al*. 2010). The overexpression of *Dx5* increases the mixing time and lowers the peak resistance, which may result in overstrong dough unsuitable for bread-making (Blechl *et al*. 2007; León *et al*. 2009). The expression of *Dy10* in transgenic wheat increases dough development times and mixing tolerance (Blechl *et al*. 2007; León *et al*. 2009). However, the critical effects of Dy10 on wheat processing quality remain uncharacterized.

In this study, we identified a *Dy10* deletion mutant (SM482-Dy10null) by screening an ethyl methanesulfonate (EMS)-induced mutant population of common wheat *cv*. 'Shumai 482'. The effect of Dy10 on wheat dough properties and processing quality was thoroughly investigated.

2. Materials and methods

2.1. Plant materials and growth conditions

Common wheat (*T. aestivum*) *cv*. 'Shumai 482' produces five HMW-GSs (i.e., Ax1, Bx7+By9, and Dx5+Dy10). The *Dy10* deletion mutant (SM482-Dy10null) was isolated from 'Shumai 482' seeds treated with 0.8% EMS (Sigma-Aldrich, St. Louis, MO) on the basis of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Qi *et al*. 2011). The glutenins analyzed by SDS-PAGE were extracted from 10 mg whole-seed powder using 100 μL extraction buffer (62.5 mmol L^{-1} Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 1.5% (w/v) dithiothreitol).

To examine quality-related properties, the *Dy10* deletion mutant was backcrossed two or three times with 'Shumai 482' (Appendix A). The wild-type (WT) 'Shumai 482' and *Dy10* deletion mutant homozygous plants were grown at the experimental farm of Sichuan Agricultural University (30°43′16″N, 103°52′15″E) for two wheat growing seasons (2019–2020 and 2020–2021). The field trials were performed using a randomized block design, with seven replicates for the WT and mutant lines. Each replicate was grown in a 2 m×2 m area, with 20 cm between rows and 60 plants per row. A compound fertilizer (N:P:K=15:15:15) was applied before sowing at a rate of 450 kg ha^{-1}. The WT and mutant lines were compared in terms of their agronomic performance at maturity. After harvesting, the grains were dried under the sun at about 35°C and stored for 2 months at room temperature for the subsequent

analysis. The harvested seeds for each replicate were analyzed by SDS-PAGE and acid polyacrylamide gel electrophoresis (A-PAGE) as described by Lafiandra and Kasarda (1985).

2.2. Nucleic acid extraction and gene cloning

Genomic DNA was extracted from fresh leaves using the Plant Genomic DNA Kit (Biofit, Chengdu, China). Immature seeds (25 days post-anthesis) were collected and ground to a fine powder in liquid nitrogen. Total RNA was extracted from the ground material using the MiniBEST Universal RNA Extraction Kit with DNase (TaKaRa, Dalian, China). The RNA concentration was determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA samples were reverse transcribed using the Prime Script™ First Strand cDNA Synthesis Kit (TaKaRa). A primer pair (F1: 5′-ATGGCTAAGCGGCTGGTCCTCTTTG-3′ and R1: 5′-CTATCACTGGCTAGCCGACAATGCG-3′) was designed and used to amplify the full *Dy10* coding sequence (CDS) (Wang *et al*. 2021a). The PCR mixtures consisted of 100 ng genomic DNA or cDNA template, 100 μM each dNTP, 1.5 mM Mg^{2+}, 4 pmol each primer, 0.75 U high-fidelity LA Taq polymerase (Takara), and 2.5 μL 10 × buffer. The PCR amplification was conducted in an Applied Biosystems™ Veriti™ PCR instrument (Thermo Fisher Scientific), with the following program: 94 °C for 5 min; 35 cycles of 94°C for 45 s, 61°C for 30 s, and 72°C for 2 min; 72°C for 12 min. The PCR products were separated on 1.0% agarose gels. The expected PCR fragments were purified and inserted into the pMD19-T vector (Takara) according to the manufacturer's instructions. The recombinant plasmids in the positive colonies were sequenced by Tsingke Biotechnology (Chengdu, China). All experiments were independently repeated at least three times.

2.3. Determination of the glutenin and gliadin contents

The glutenin and gliadin contents in white flour samples were analyzed by reversed phase high-performance liquid chromatography. The total glutenin (including HMW-GSs and LMW-GSs) and gliadin contents were estimated by combining the relevant chromatogram peak areas as described by Zheng *et al*. (2018). The glutenin:gliadin ratio was then calculated.

2.4. Evaluation of the processing quality parameters

Wheat seeds were milled to produce white flour using the CD1 Laboratory Mill (CHOPIN Technologies, Villeneuve-la-Garenne Cedex, France) according to AACC Approved Method 26-70 (AACC International 2010). The processing quality parameters, including grain protein content, Zeleny sedimentation value, wet gluten content, gluten index, and GMP content, were assessed as described by Wang *et al*. (2021a). Rheological properties were evaluated using a standard farinograph (Brabender GmbH & Co., KG, Germany) as described by AACC Approved Method 54-21 (AACC International 2010).

Bread-making and biscuit-making qualities were examined as described by Wang *et al*. (2021a). The loaf volume was determined using the BVM6630 volume meter (Perten, Stockholm, Sweden) following the manufacturer's instructions. The biscuit surface area (SA), which is an accurate indicator of the biscuit diameter ($SA=\pi d^2/4$; d=diameter and $\pi=3.14$), was measured using the C-Cell imaging

system (Calibre Control International Ltd., Warrington, UK). Biscuit hardness and thickness were determined using the TA.XTC texture analyzer (BisinTech, Shanghai, China) (Wang *et al*. 2021a). The spread ratio was calculated as the biscuit diameter:biscuit thickness ratio.

2.5. *In vitro* expression of *Dy10* and western blot analysis

The 'Shumai 482' *Dy10* allele CDS (without the signal peptide-encoding fragment) was amplified by PCR using the F2/R2 primer pair (F2 with the *Nde*I restriction site: 5′-ACCCATATGGAAGGTGAGGCCTCTAGGC-3′ and R2 with the *Xho*I restriction site: 5′-TTCCTCGAGCTATCACTGGCTAGCCGAC-3′). The amplified sequence was inserted into the bacterial expression vector pET-30a (Novagen, Merck, Darmstadt, Germany). *Escherichia coli* strain BL21 (DE3) chemically competent cells (Weidi, Shanghai, China) were transformed with the recombinant plasmid and grown at 37°C until the culture optical density at 600 nm reached 0.6. Bacterial expression was induced by adding 0.8 mmol L^{-1} isopropyl *β*-D-thiogalactopyranoside (IPTG; Solarbio, Beijing, China) to the culture, which was then incubated for 6 h at 37°C. The Dy10 subunit was purified from *E. coli* cells as described by Uthayakumaran *et al*. (2000) and confirmed by SDS-PAGE and western blot analyses. Immunoblotting was performed using the mouse anti-HMW-GS polyclonal antibody (1:4000) and the HRP-conjugated anti-mouse secondary antibody (1:5000; Sangon Biotech, Shanghai, China) as described by Wang *et al*. (2021a). The mouse anti-HMW-GS polyclonal antibody, which was obtained from Zoonbio Biotechnology (Nanjing, China), was generated using the peptide "GYYPTSPQQPGC" as described by Denery-Papini *et al*. (1996).

To express a truncated Dy10 peptide (196 residues) fused to the glutathione S-transferase (GST) tag, the *Dy10-null* allele CDS (without the signal peptide-encoding fragment) was amplified by PCR using the mutant and the F3/R3 primer pair (F3 with the *BamH*I restriction site: 5′-TTCCAGGGGCCCCTGGGATCCGAAGGTGAGGCCTCTAGG-3′ and R3 with the *EcoR*I restriction site: 5′-CTCGAGTCGACCCGGGAATTCTTATTGCCTTTGTCCTGTGTGCTGCA-3′). The amplified sequence was inserted into the pEGX-6p-1 vector (Novagen), after which *E. coli* cells were transformed with the recombinant plasmid. The bacterial cells were cultured and protein expression was induced as described above. Additionally, an immunoblotting assay using the mouse anti-HMW-GS antibody (1:4000), the HRP-conjugated anti-mouse secondary antibody (1:5000), the rabbit anti-GST polyclonal antibody (1:5000; Sangon Biotech), and the HRP-conjugated anti-rabbit secondary antibody (1:5000; Sangon Biotech) was completed as described above.

2.6. Micro-biscuit processing test

The Dy10 subunit purified from *E. coli* cells was incorporated into the WT and mutant flour according to a slightly modified reduction–oxidation protocol (Bekes *et al*. 1994). Briefly, 10 g wheat flour (14% moisture content) and the purified Dy10 (0, 20, 40, and 60 mg) were mixed with 1.5 mL dithiothreitol (3 mg mL^{-1} in distilled water) for 2 min and then rested for 5 min. After adding 0.75 mL KIO_3 oxidant (25 mg mL^{-1} in distilled water), the sample was mixed for 2 min and then rested for 10 min. To prepare micro-biscuits, the following ingredients were added to the dough: 6 g sucrose, 300 mg nonfat milk

powder, 100 mg $NaHCO_3$, 50 mg NH_4Cl, 45 mg NaCl, 3 g shortening, and 0.5 mL H_2O. The micro-biscuit processing test was performed in triplicate.

2.7. Statistical analysis

Student's *t*-test was performed using the Data Procession System software (version 17.10) (Zhejiang University, Hangzhou, China) to determine the significance of any differences in the mean values for the processing parameters and agronomic characteristics.

3. Results

3.1. Identification of the *Dy10* deletion mutant

A *Dy10* deletion mutant line (SM482-Dy10null), which produces four HMW-GSs (Ax1, Bx7+By9, and Dx5), was identified in an EMS-mutagenized 'Shumai 482' population that was analyzed by SDS-PAGE (Appendix A). There were no significant differences in the agronomic characteristics between the WT and mutant lines (Appendices B and C). The alignment of the mutant *Dy10* (*Dy10-null* allele; GenBank No. OK482716) and the WT *Dy10* (GenBank No. X12929) sequences revealed a nonsense mutation (C to T) in the *Dy10-null* allele (Appendix D), which resulted in a premature termination of translation at the 217th amino acid residue (Fig. 1-A). Reverse transcription (RT)-PCR results indicated that the *Dy10-null* allele was normally expressed at the transcript level (Appendix E). The anti-HMW-GS antibody cross-reacted with the truncated Dy10 peptide (with a predicted molecular mass of 21.6 kDa) produced by the heterologous expression system (Fig. 1-C). In contrast, the anti-HMW-GS antibody did not detect the Dy10 peptide in the mutant seeds (Fig. 1-B), thereby confirming the lack of Dy10 in the mutant.

3.2. Effects of Dy10 on processing quality

The HMW-GS and gliadin contents were respectively significantly lower and higher in the mutant line than in the WT line (Fig. 2). Additionally, the LMW-GS content was slightly higher in the mutant line than in the WT line, but this difference was not significant (Fig. 2). The glutenin:gliadin ratio was significantly lower in the mutant than in the WT control.

The grain protein and wet gluten contents were similar between the mutant and WT lines (Table 1). In contrast, the gluten index, Zeleny sedimentation value, and GMP content were significantly lower in the mutant than in the WT control. With regard to the dough rheological characteristics, the development and stability times of the mutant were significantly shorter than those of the WT control. As expected, the mutant dough was softer than the WT dough (degree of softening in Table 1). Therefore, the lack of Dy10 resulted in decreased dough strength, but it had no effect on the grain protein content.

The loaf volume was significantly smaller for the mutant than for the WT (Fig. 3-A and B; Table 1). Compared with the WT biscuits, the mutant biscuits were not as thick and had a larger diameter, a higher spread ratio, and a similar hardness (Fig. 3-C and D; Table 1). There were no obvious differences in the loaf and biscuit sensory properties between the mutant and WT samples.

To confirm its effect on the biscuit-making quality, the Dy10 subunit was purified from *E. coli* cells (Fig. 4), after which various amounts (0, 20, 40, and 60 mg) were incorporated into the WT and mutant flour as described by Bekes and Gras (1999). In all cases, the addition of the purified Dy10 adversely affected the biscuit-making quality. More specifically, the incorporation of purified Dy10 into the wheat flour resulted in a decrease in the biscuit area, an increase in biscuit thickness, and a decrease in the spread ratio (Fig. 5). Moreover, the biscuit quality was negatively correlated with the Dy10 content.

4. Discussion

Deletion mutants are suitable for clarifying the effects of HMW-GSs on wheat processing quality. In this study, a *Dy10* deletion mutant line (SM482-Dy10null) was isolated from an EMS-mutagenized population (Appendix A). Previous research verified the utility of EMS for modifying single nucleotides to introduce a stop codon or a missense mutation (a C-to-T mutation is most common) (Kim *et al*. 2006). The *Dy10-null* allele had a single point mutation (C-to-T; Appendix D) that introduced a premature stop codon (TAA). Two major gene-silencing mechanisms in plants have been described, namely transcriptional gene silencing (TGS) and post-TGS (PTGS); the silenced gene is transcribed normally during PTGS (Sijen *et al*. 2001). In the current study, the *Dy10-null* allele in the mutant line and the *Dy10* allele in the WT line were similarly transcribed (Appendix E), implying the silencing of the *Dy10-null* allele was via PTGS (Fig. 1). Previous research suggested that a premature stop codon in HMW-GS genes leads to the production of truncated proteins (De Bustos *et al*. 2000; Chen *et al*. 2021), which is detrimental for investigations regarding the effect of HMW-GSs on processing quality. The western blot analysis performed in this study revealed the lack of Dy10 subunit in the mutant with the *Dy10-null* allele (Fig. 1-B and C). Therefore, our analysis of the *Dy10-null* allele has clearly shown that wheat processing quality is influenced by the Dy10 subunit.

The absence of Dy10 in the mutant likely results in the restructuring of the inherent gluten network, ultimately leading to decreased dough strength. Wheat HMW-GSs, especially the Dx5+Dy10 subunit pair, are major determinants of bread-making quality (Shewry *et al*. 2003). Additionally, *Dx5*, which encodes a protein that contains one more Cys residue in the repetitive domain than the other HMW-GSs, is always expressed as part of an allelic pair with *Dy10*. Compared with Dx5, the Dy10 subunit contains more Cys residues that form intermolecular disulfide bonds during dough development, enabling the extensive cross-linking of glutenin polymers (Shewry and Halford 2002). There is evidence that dimers comprising these two subunits are present as “building blocks” in glutenin polymers (Wieser 2007; Wang *et al*. 2021b). In the present study, the lack of Dy10 decreased the gluten index, Zeleny sedimentation value, dough development and stability times, and GMP content, which helps to explain the observed decrease in bread-making quality (Table 1).

The balance between different gluten protein fractions is an important factor governing the end-use quality of wheat. For different types of food products, diverse dough strength and extensibility values may reflect the optimum balance between gluten fractions (Oliver and Allen 1992). Our results indicated that the *Dy10-null* allele in the mutant decreased the glutenin:gliadin ratio, but did not alter the grain protein content. The *Dy10-null* allele significantly decreased the HMW-GS contents, while

significantly increasing the gliadin contents. These findings are in accordance with the reported compensatory interaction between HMW-GSs and gliadins (Dia *et al*. 2022; Liu *et al*. 2022; Scossa *et al*. 2008; Zhang *et al*. 2018). Several studies revealed that HMW-GSs and gliadins mainly contribute to the viscoelastic properties and extensibility of wheat dough, respectively (Shewry *et al*. 2003; Barak *et al*. 2015). Therefore, it is not surprising that in response to the deletion of Dy10, the biscuit-making quality (Fig. 3-C and D; Table 1) and bread-making quality (Fig. 3-A and B; Table 1) increased and decreased, respectively. Furthermore, the incorporation of the purified Dy10 subunit had adverse effects on the biscuit-making quality of the wheat dough (Fig. 5). In contrast, supplementing wheat flour with purified gliadins reportedly leads to enhanced biscuit-making quality (Kuragano *et al*. 1991). Unexpectedly, both positive and negative effects of the silencing of HMW-GS genes on biscuit-making quality have been reported. For example, the deletion of a single HMW-GS gene in *T*. *aestivum cv*. 'Ningmai 9' (which normally contains Ax1, Bx7+By8, and Dx2+Dy12) results in improved biscuit-making quality (Zhang *et al*. 2016), whereas the absence of the Dy12 subunit in *T*. *aestivum cv*. 'Kenong 199' (which normally contains Ax1, Bx7+By9, and Dx2+Dy12) leads to decreased biscuit-making quality (Chen *et al*. 2021). We speculate that the glutenin:gliadin ratio and the HMW-GS composition are essential determinants of biscuit-making quality.

5. Conclusion

A common wheat mutant carrying the *Dy10-null* allele was identified by screening an EMS-mutagenized 'Shumai 482' population. The *Dy10-null* allele resulted in the absence of Dy10. On the basis of an analysis of this mutant, we demonstrated that the Dy10 subunit is essential for maintaining dough strength. Furthermore, the *Dy10-null* allele positively affects the biscuit-making quality of wheat dough, making it potentially useful for soft wheat breeding programs.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (31971939, 32072054, and 31901961), the Science and Technology Department of Sichuan Province, China (2019YFH0066 and 2020YFH0150), and the Designing Future Wheat strategic program (BB/P016855/1) of the UK.

Conflicts of interest

The authors declare that they have no conflict of interest.

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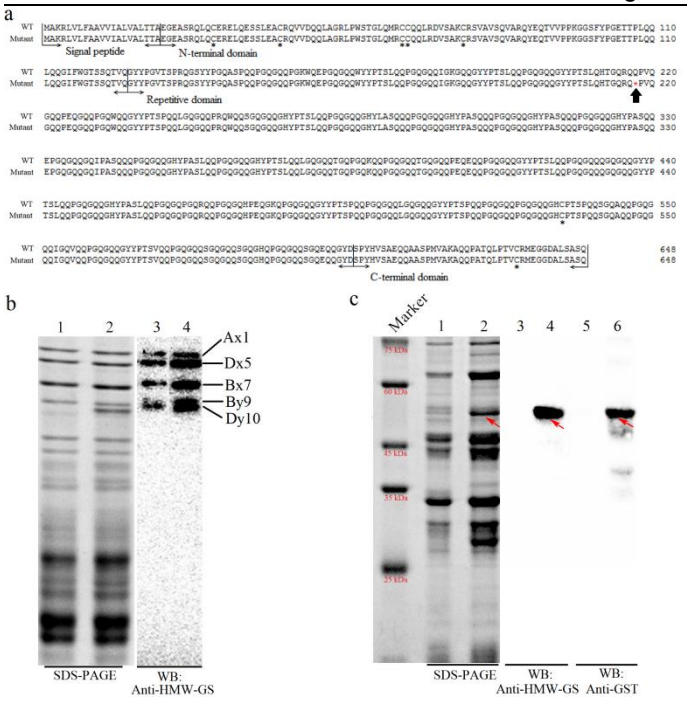

Fig. 1 The *Dy10-null* allele results in a lack of Dy10 in seeds. A, alignment of the deduced amino acid sequences of *Dy10* in the WT and mutant lines. Cysteine residues are indicated by black asterisks. The thick black arrow indicates the premature stop codon site (red asterisk). The signal peptide, N-terminal domain, repetitive domain, and C-terminal domain are indicated. B, Western blot analysis of the gluten proteins in the mutant (lanes 1 and 3) and WT (lanes 2 and 4) lines using the anti-HMW-GS antibody. C, Western blot analysis of the GST-tagged truncated peptide (196 residues) using the anti-HMW-GS antibody or anti-GST antibody. The proteins extracted from *E. coli* cells expressing the GST-tagged truncated peptide (196 residues) with (lanes 2, 4, and 6) and without (lanes 1, 3, and 5) the addition of IPTG are presented. Red arrowheads indicate the target protein band.

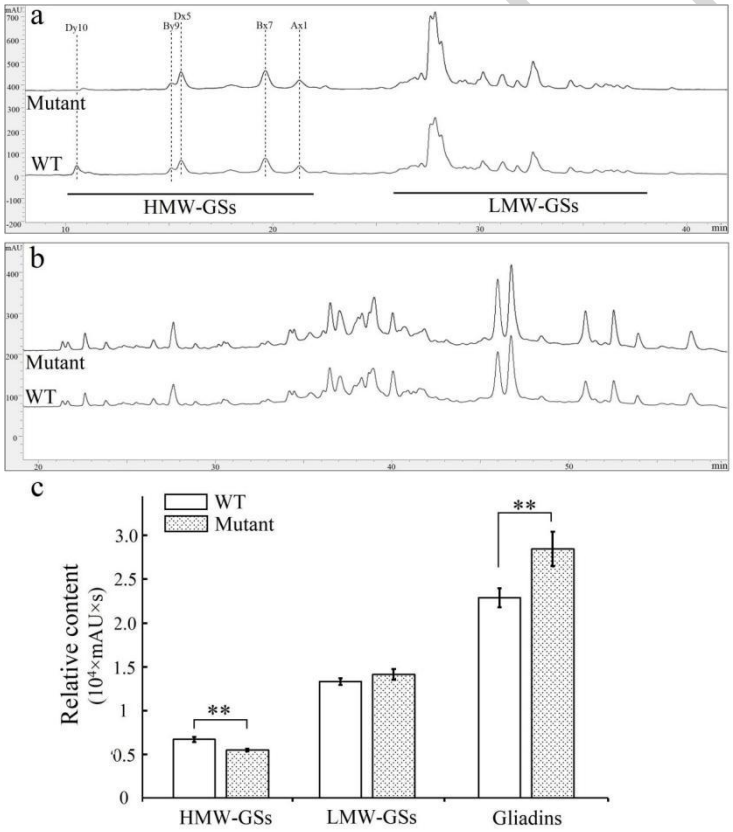

Fig. 2 Glutenin and gliadin contents in the WT and mutant lines as determined by RP-UPLC. A, RP-UPLC profile of HMW-GSs and LMW-GSs; the peaks corresponding to the different HMW-GSs are indicated. B, RP-UPLC profile of gliadins. C, relative contents of HMW-GSs, LMW-GSs, and gliadins. Data are presented as the mean±standard deviation, n=7. ** indicates significance at P<0.01.

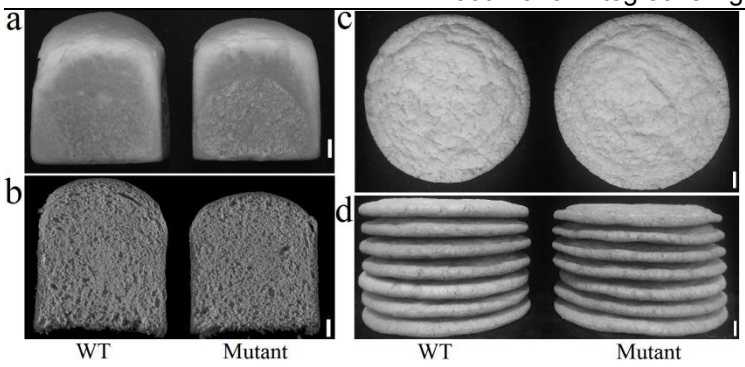

Fig. 3 Comparison of the WT and mutant loaves and biscuits. A, loaf shape. B, loaf slices. C, biscuit shape. D, biscuit thickness. Scale bar=1 cm.

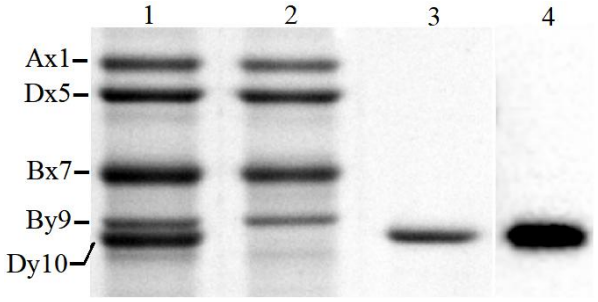

Fig. 4 Purification of the Dy10 subunit from *E. coli* cells. The glutenins extracted from the mutant and WT lines were used as the control (lanes 1 and 2). The heterologous expression of the Dy10 subunit was verified by SDS-PAGE (lane 3) and western blot (lane 4) analyses.

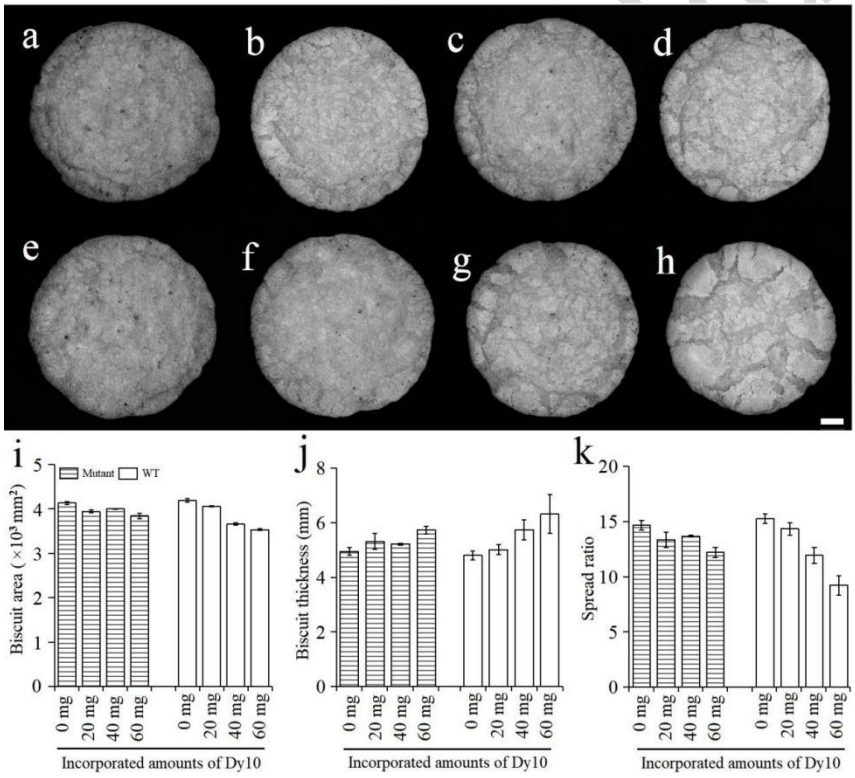

Fig. 5 Incorporation of the purified Dy10 subunit negatively affects biscuit quality. A–D, biscuits produced from the mutant flour incorporated with 0 mg (A), 20 mg (B), 40 mg (C), and 60 mg (D) Dy10 subunit. E–H, biscuits produced from the WT flour incorporated with 0 mg (E), 20 mg (F), 40 mg (G), and 60 mg (H) Dy10 subunit. Scale bar=1 cm. I–K, comparison of the biscuit area (I), biscuit thickness (J), and spread ratio (K) for the mutant and WT flour with and without the addition of Dy10.

Table 1 Comparison of the processing quality parameters of the WT and mutant lines

Parameter	2019-2020 BC_2F_4		2020-2021 BC_3F_4	
	WT	mutant	WT	mutant
Quality trait				
Grain protein content (%, dry weight)	13.62±0.35	13.71±0.23	16.40±0.71	16.32±0.50
Zeleny sedimentation value (mL)	16.97±2.94	13.49±2.37*	35.67±1.75	24.6±1.86**
Wet gluten content (%)	24.24±2.5	25.06±3.18	32.15±1.27	32.79±2.04
Gluten index (%)	64.31±6.89	48.44±8.50**	61.92±4.07	40.08±5.79**
GMP (%)	2.09±0.198	1.70±0.18**	2.84±0.28	2.43±0.14*
Rheological property				
Dough development time (min)	1.65±0.17	1.43±0.07*	3.84±0.69	2.49±0.26**
Dough stability time (min)	3.42±0.27	2.61±0.44*	8.03±2.28	4.12±0.48**
Degree of softening (FE)	100.00±5.42	115.33±6.39*	64.00±8.03	92.00±5.48**
Processing quality				
Loaf volume (mL)	203.0±1.41	184.8±8.64**	237±5.60	216.38±7.59**
Biscuit diameter (mm)	99.63±4.10	106.32±2.14**	96.44±1.10	99.56±0.94**
Biscuit thickness (mm)	9.23±1.64	8.09±0.63*	9.79±0.39	9.16±0.25*
Spread ratio (diameter/thickness)	11.17±2.6	13.22±0.60**	9.87±0.50	10.86±0.24**
Hardness (N)	138.96±18.23	129.38±12.43	133±19.56	124.42±13.28

Data are provided as the mean±standard deviation.
* and ** represent significance at $P<0.05$ and $P<0.01$, respectively.