CRISPR/Cas9-Mediated Disruption of Xylanase inhibitor protein (XIP) Gene Improved the Dough Quality of Common Wheat

Zhengjuan Sun, Mingxia Zhang, Yanrong An, Xu Han, Baojin Guo, Guangde Lv, Yan Zhao, Ying Guo* and Sishen Li*

State Key Laboratory of Crop Biology, College of Agronomy, Shandong Agricultural University, Tai’an, China

The wheat dough quality is of great significance for the end-use of flour. Some genes have been cloned for controlling the protein fractions, grain protein content, starch synthase, grain hardness, etc. Using a unigene map of the recombinant inbred lines (RILs) for “TN 18 × LM 6,” we mapped a quantitative trait locus (QTL) for dough stability time (ST) and SDS-sedimentation values (SV) on chromosome 6A (QSt/Sv-6A-2851). The peak position of the QTL covered two candidate unigenes, and we speculated that TraesCS6A02G077000 (a xylanase inhibitor protein) was the primary candidate gene (named the TaXip gene). The target loci containing the three homologous genes TaXip-6A, TaXip-6B, and TaXip-6D were edited in the variety “Fielder” by clustered regularly interspaced short palindromic repeats–associated protein 9 (CRISPR/Cas9). Two mutant types in the T_{2:3} generation were obtained (aaBBDD and AAbbdd) with about 120 plants per type. The SVs of aaBBDD, AAbbdd, and WT were 31.77, 27.30, and 20.08 ml, respectively. The SVs of the aaBBDD and AAbbdd were all significantly higher than those of the wild type (WT), and the aaBBDD was significantly higher than the AAbbdd. The STs of aaBBDD, AAbbdd, and WT were 2.60, 2.24, and 2.25 min, respectively. The ST for the aaBBDD was significantly higher than that for WT and was not significantly different between WT and AAbbdd. The above results indicated that XIP in vivo can significantly affect wheat dough quality. The selection of TaXip gene should be a new strategy for developing high-quality varieties in wheat breeding programs.

Keywords: wheat, dough quality, xylanase inhibitor protein (XIP), CRISPR/Cas9, SDS-sedimentation values (SV), stability time (ST)

INTRODUCTION

Wheat (Triticum aestivum. L) is the main source of nutrition and feeds more than 30% of the world’s population (Rasheed et al., 2018; Wang et al., 2019). The demand for wheat with high-quality attributes has increased globally due to the growing population and the rising living standards in countries worldwide (Peña et al., 2008; Kumar et al., 2019). The presence of wheat gluten gives the dough viscoelasticity and ductility, and it can be processed into a variety of foods to meet people’s needs (Veraverbeke and Delcour, 2002).
The stability time (ST) and SDS-sedimentation value (SV) are the key quality traits for wheat and play a critical role in determining end-use products. ST is the main parameter of the farinograph, which determines the final quality of bread, steamed bread, noodles, and other wheat foods (Tsilo et al., 2011). The length of the ST reflects the resistance of the dough to kneading, and doughs with high ST values have good flexibility and high gluten strength (Veraverbeke and Delcour, 2002; Dangi et al., 2019). The SV can be used as an essential indicator for detecting the quality of gluten, which positively correlates with dough rheological properties (Kaur et al., 2013). In general, the strong flour demands that the ST and SV are greater than 8 min and 40 ml, respectively (Ma et al., 2021). Moreover, because the mensuration of SV is simple and convenient and uses less flour, it is usually used as an indicator of early generation selection in wheat quality breeding programs (Peña et al., 2008).

The ST and SV are parameters highly influenced by the protein compositions, i.e., glutenins and gliadins, their fractions, the ratio between them as well as the total protein content (Payne et al., 1988; Deng et al., 2006; Rogers et al., 2006; Caballero et al., 2008; Dhaka and Khakkar, 2015). Some genes have been cloned in controlling the protein compositions, high molecular weight glutenin subunits (HMW-GS; Thompson et al., 1985; Anderson and Greenee, 1989), low molecular weight glutenin subunits (LMW-GS) (D’Ovidio et al., 1992; Ikeda et al., 2002), and gliadin (Rafalski et al., 1984; Sugiyama et al., 1986). The genes regulated other wheat quality traits, such as grain protein content (Uauy et al., 2006; Hagenblad et al., 2012) and grain hardness (Gautier et al., 1994; Chen et al., 2006), were also cloned. Some transcription factors, such as TaNAC019, directly activated the expression of HMW-GS genes by binding to a specific motif in their promoters and interacting with the TaGlI-1 regulator (Gao et al., 2021).

Cereals contain proteinaceous inhibitors of endo-xylanases (McLauchlan et al., 1999). The inhibitors of xylanase in wheat are grouped into three classes: TAXI (Triticum aestivum xylanase inhibitor; Debyser et al., 1997, 1999), XIP (xylanase inhibitor protein; McLauchlan et al., 1999; Elliott et al., 2003), and TLXI (thraumatin-like xylanase inhibitor; McLauchlan et al., 1999). The XIP-type xylanase inhibitor genes are responsible for plant defense (Takahashi-Ando et al., 2007). The effect of the XIP in vivo on the grain quality of wheat has not been reported.

Gene editing is an important tool to study gene function. In the past few years, clustered regularly interspersed short palindromic repeats (CRISPR) have achieved the ability to control the specific introduction of directed sequence variation (Gilbert et al., 2013; Shan et al., 2013; Soda et al., 2018). This technology has been applied to the genetic studies of wheat quality. Zhang S. J. et al. (2018) used CRISPR/Cas9 to silence HWM-GS in wheat, which significantly reduced dough strength and bread-baking quality. Sánchez-León et al. (2018) found that CRISPR/Cas9 could be used to produce low-gluten foodstuff and serve as source material to introgress this trait into elite wheat varieties. Li et al. (2020) edited TaSBEIIa in both winter and spring wheat varieties by CRISPR/Cas9 modification of starch composition, structure, and properties.

In this study, we found a quantitative trait locus (QTL) under multi-environments for the ST and SV, QSt/Sv-6A-2851, on chromosome 6A using TL-recombinant inbred lines (RIL) population. This locus contains the xylanase inhibitor protein (Xip) gene. We performed functional validation of the TaXip gene using the CRISPR/Cas9 mutagenesis system.

**MATERIALS AND METHODS**

**RIL Population, Field Trials, and Quality Trait Measurements**

The RIL population of 184 lines used in the study was derived by single-seed descent (SSD) methods from a cross of “TN18 × LM6” (TL-RIL, F11 in 2015; Zhang et al., 2019). TN18 is a cultivated variety developed by our research group and LM6 is an excellent line developed by the Linyi Academy of Agricultural Sciences. The field trials were conducted by Guo et al. (2020) at the experimental farm of Shandong Agricultural University in Tai’an for three growing seasons with two replications: 2011–2012 (E1), 2012–2013 (E2), and 2013–2014 (E3).

The seed samples for the TL-RIL population obtained from the harvested grains were stored at room temperature for approximately three months and then milled using a Bühler experimental mill (Bühler mill, Bühler-Miag Company, Braunschweig, Germany; Guo et al., 2020). The flour was used to test the SV and farinograph parameters including ST. The SV was determined with a sedimentation volume instrument (BAU-A type) and the farinograph parameters were determined by a farinograph (Brabender GmbH and CoKG).

**The Genetic Map of Unigenes and Quantitative Trait Locus Analysis for the TL-RILs**

By RNA sequencing each line of the TL-RIL population, a genetic map of unigenes based on the physical position in reference genome RefSeq v1.1 (IWGSC, 2018) was previously constructed by our group (Zhang, 2019). The map included 27,452 sites; 28,811 unigenes; 31,445 sub-unigenes; and 117,758 SNP/InDels. Using the unigene map and phenotypic data (Guo et al., 2020), we mapped QTLs by IciMapping4.1 and MapQTL5.0 software. For IciMapping4.1, inclusive composite interval mapping (ICIM) was carried out with a step size of 0.5 cm. The parameter for handling missing phenotypic data was “Deletion.” For MapQTL 5.0, the multiple-QTL model (MQM) package with a mapping step size of 0.5 cm was used to map QTLs. The LOD threshold for declaring a significant QTL in both the software was a LOD > 2.5.

**DNA and RNA Extraction and Primer Design**

For the variety “Fielder” and the progenies of gene editing, total DNA was extracted using a DNA extraction kit (Tiangen, Beijing, China). The quality and concentration of the total DNA were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). Total RNA was extracted using an AG21019 RNA extraction kit.
Amplification of the TaXip Genes

Total DNA and cDNA were used to amplify TaXip-6A, TaXip-6B, and TaXip-6D. Sequence amplification was performed with FastPfu high-fidelity enzyme (TransGen Biotech, Beijing, China). The primers were TAXIP6A-F: ccttaggtatcacttgcc; TAXIP6A-R: gttccgagtcgagtgca; TAXIP6B-R: ggacctggagcatgcct; TAXIP6B-F: gtc ggatacgaattggcg; and TAXIP6D-R: aactgtgcgaccaatctgttc). PCR was run at 95°C for 5 min, followed by 33 cycles of denaturing at 94°C, annealing for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min on an ABI PCR system 2400. The PCR products were separated by electrophoresis, recovered, and ligated to a pEASY-Blunt Cloning Kit (TransGen Biotech, Beijing, China). They were transformed into E. coli competent cells and selected on LB plates containing 50 µg/ml ampicillin. Positive clones were sequenced. Complete multiple alignments of sequences (Supplementary Figure 1) and translations of nucleotides into amino acid sequences were performed by DNAMAN Version 5.0 (Gong et al., 2013).

Acquisition and Culture of Gene Editing Plants

The sequences of the TaXip genes were obtained from the cloned sequence from “Fielder” and used to design sgRNA target sequences in CRISPR-direct1 and CRISPOR. The sgRNA2 was 5′-GTGCAACCCGCTCCGGGCTG-3′ from 374 to 393 bp (start at ATG) with protoscaler adjacent motif (PAM) site CCC, and sgRNA1 was 5′-ACCAATCCGCCGCCGCAGCC-3′ from 550 to 569 bp with PAM site GGG.

Using the designed primer (MT1T2-XF: gacaacatcggccgc gccggcgttttagctagaataatt; MT1T2-XR: gtcacaacgccgctgcgtcgc gttttggcc; MT1T2-XF: aataatggtcacgccaaactccggcggcgcggc; and MT1T2-XR: attattggctctaaactgctccgctgcgtc) and MT1T2 vector as templates, PCR amplification was conducted to obtain the intermediate vector fragment containing two sgRNAs (964 bp). The sgRNAs were connected to the pBUE11 vector by an enzymatic ligation reaction. The schematic map of the binary vector is shown in Supplementary Figure 2. The RB/LB represent the left and right borders of the vector; TaU3P was the wheat U3 gene promoter and used to drive sgRNAs; sgRNA site refers to the guide RNA clone site; sgRNA SC was the sgRNA wheat U3 gene promoter and used to drive sgRNAs; sgRNA site zCas9 (Xing et al., 2014). After sequencing the target sites, the binary vector was transformed into the wheat variety “Fielder” by Agrobacterium tumefaciens-mediated transformation (Zhang S. J. et al., 2018). A total of 29 T0 plants were yielded in August 2019. In the greenhouse of Shandong Agricultural University, T0 generation seeds of different genotypes were selected and T1 plants were planted. After identifying the mutant types of T2 plants, the seeds of T2 mutant plants were sown in flowerpots with 4 plants in each flowerpot and 30 flowerpots for each edited type (T2:3 lines). The seeds harvested in the T2 generation were the T2:3 lines that were used for ST and SV measurements. All plants were grown in the greenhouse with 16 h of light and 8 h of darkness at 25°C. The grains from WT plants were similarly harvested and served as controls in the experiments.

Genomic DNA was extracted from the leaves of the genome-edited plants. To identify mutation types of T0, T1, and T2 generations, specific primers (g6aF: gagaatgattc gtgtaTGCCCGGCTACGGGACC; g6aR: gagaatgattcgtgccgc gCACGGGACCCGCTCCGG; g6bF: gagaatgattccgtgccgcc gCACGGGACCCGCTCCGG; g6bR: gagaatgattccgtgccgcc gCACGGGACCCGCTCCGG; g6dF: gagaatgattccgtgccgcc gCACGGGACCCGCTCCGG; g6dR: gagaatgattccgtgccgcc gCACGGGACCCGCTCCGG) were designed to sequence the target regions of the A, B, and D genomes. Then, the PCR products were amplified and sequenced by Hi-Tom (Liu et al., 2019). Next-generation sequencing technology was used to detect the mutation sites. Li et al. (2021) reported that the lowest average ratio for plants with albino phenotype was over 80% after the TaPDS gene was edited. This indicated an editing threshold for displaying loss of function phenotype. We used this strategy to classify different genotypes.

Sedimentation Values and Stability Time Determination and Statistical Analysis for Mutant Lines and “Fielder”

For the variety “Fielder” (wild type, WT) and the T2:3 mutant lines, about 500 g grains were obtained from mixed samples of 120 individual plants, respectively. The flour was milled by a small experimental mill of Quadrumat Junior (Brabender GmbH and CoKG, Germany) with no xylanases added and then passed through a 80-mesh sieve. The separate flour samples were used to test the SV and ST. The SV was tested based on Peña et al. (1990) and corrected to 14% flour moisture content. The ST is a farinograph parameter which was determined using Automatic Farinograph-AT (Brabender GmbH and CoKG, Germany) referred to Tian (2006).

SPSS 17.0 software (SPSS, Chicago, IL, United States) was used for the comparison of statistical analysis of the ST and SV. Multiple comparisons using LSD to identify where the differences lay. P-value less than 0.05 or 0.01 was considered as significant or extremely significant.

RESULTS

Acquisition of the Candidate Gene of the Stability Time and Sedimentation Values

Using the TL-RIL population and their genetic map of unigenes based on RNA-Seq technology (Zhang, 2019), we mapped

1http://crispr.dbcls.jp/
2http://crispor.tefor.net/
the QTLs for quality traits. Of these, a QTL for ST and SV, QSt/Sv-6A-2851, was detected under multi-environments with peak positions ranging from 2850.3 to 2860.4 cM by IciMapping 4.1 and MapQTL 5.0 software (Figure 1A and Supplementary Table 1). The peak position of QSt/Sv-6A-2851 covered STRG.003769 and two annotated unigenes in RefSeq v1.1 (IWGSC, 2018), TraesCS6A02G076900 (46974751–46979670 bp) and TraesCS6A02G077000 (47104969–47106326 bp). The STRG.003769 is a transcript with no complete gene structure. The TraesCS6A02G076900 was annotated as an ABC-2-type transporter family protein. The TraesCS6A02G077000 was annotated as a xylanase inhibitor protein (named the TaXip gene). Sequence analysis revealed that TraesCS6A02G076900 had three SNPs in the 5′UTR, two SNPs in the intron region, and one SNP in the 3′UTR without amino acid changes. But the TraesCS6A02G077000 had one SNP at the 390 bp (start at ATG) in the exon region that led to amino acid changes (cysteine to tryptophan). Additionally, our group found a QTL cluster that contained SV and ST on chromosome 6A between TraesCS6A02G070900 and TraesCS6A02G125200 using the same TL-RIL population and its molecular marker map (Guo et al., 2020). Xylanases are widely used as additives in the baking industry to improve processing and product quality (Goesaert et al., 2005). The xylanase inhibitor protein is likely to inhibit the decomposition of xylanase in vivo (Simpson et al., 2003). Therefore, we speculated that TraesCS6A02G077000 was the candidate gene of the QTL QSt/Sv-6A-2851.

Amplification of the TaXip Homologous Genes

Three homologs of TaXip were identified by BLAST analysis in the IWGSC database: TaXip-6A (TraesCS6A02G077000), TaXip-6B (TraesCS6B02G103900), and TaXip-6D (TraesCSU02G026500). These homologous genes were amplified separately in the parents TN18 and LM6 of the TL-RILs and the variety “Fielder” (used in gene editing). For TN18 and LM6, the predicted amplicons were from 161 bp to 313 bp. For “Fielder”, the predicted amplicons were from 229 bp to 305 bp. The amplicons were sequenced for each of the 186 parents and “Fielder” and the sequences were aligned using the BLAST program for confirmation. The sequencing results showed the similarity between the amplicons and the BLAST hits for each of the homologs. The homologs were named according to the chromosome where they were located: TaXip-6A, TaXip-6B, and TaXip-6D.

The functional validation of TaXip homologous genes was performed by CRISPR/Cas9-mediated genome editing in wheat. The CRISPR protospacer-adjacent motif (PAM) was designed as NGG. All the homologous genes were amplified from the parents and edited by CRISPR/Cas9. The deletion mutants were obtained for each of the homologs. The knockouts of TaXip homologous genes were confirmed by sequencing the amplified fragments. The knockouts were then subjected to a series of quality trait evaluations to determine the effects of TaXip on wheat dough quality.
TaXip-6A and TaXip-6D RNA-Seq result. The exon sequences of TaXip-6B and tryptophan, respectively, which is in accordance with the gene. It was base C in TN18 and G in LM6, encoding cysteine and tryptophan, respectively. In “Fielder,” TaXip-6A and TaXip-6D have one exon with 915 bp open reading frames and encode 305 amino acids. In contrast, TaXip-6B has one intron and two exons, 939 bp open reading frames, and encodes 313 amino acids (Figure 1B). Based on the amino acid sequences and domains, paired comparison results showed that the identities between TaXip-6A and TaXip-6D, between TaXip-6A and TaXip-6B, and between TaXip-6B and TaXip-6D were 94.75, 91.69, and 91.37%, respectively.

Changes for the SV and ST Between Wild and Mutant Genotypes

The SVs of the two mutant genotypes, aaBBDD and AAbbdd, and WT were 31.77, 27.30, and 20.08 ml, respectively (Supplementary Table 2). The SVs of aaBBDD and AAbbdd were significantly higher than that of the WT (Figure 1D); the SV of aaBBDD was significantly larger than that of AAbbdd and WT; and the SV of AAbbdd was significantly larger than that of WT. These results indicated that TaXip-6A, TaXip-6B, and TaXip-6D significantly influenced the SV, but the effect of TaXip-6A was greater than those of TaXip-6B and TaXip-6D. The STs of aaBBDD, AAbbdd, and WT were 2.60, 2.24, and 2.25 min, respectively (Supplementary Table 3). The ST of the mutant genotype aaBBDD was significantly higher than that of WT and AAbbdd, but was not significantly different between WT and AAbbdd (Figure 1D). This indicates that TaXip-6A significantly affected the ST, while TaXip-6B and TaXip-6D have little effect on the ST.

DISCUSSION

For the genes of XIP class, Xip-I, Xip-III, Xip-R1, Xip-R2, Xip-II, and xip-9023, xip-366 (Elliott et al., 2002, 2009; Igawa et al., 2005; Takahashi-Ando et al., 2007; Liu et al., 2017) have been cloned in wheat with the functions of plant defense by binding glycoside hydrolase families 10 (GH10) and 11 (GH11; Payan et al., 2004). TaXIP-6A was most similar to wheat XIP-III, XIP-I, and rice acidic class III chitinase (Park et al., 2002) with a protein sequence identity of 98.69, 87.25, and 36.59%, respectively. The sequence alignment of TaXIP-6A, TaXIP-6B, and TaXIP-6D conservation of the two Arg residues (Figure 2; red boxed) proved to be engaged in salt bridges with the catalytic Glu residue (Figure 2; Supplementary Figure 3).

Obtaining of Gene Editing Plants by CRISPR/Cas9

The sgRNA targets for TaXip-6A, TaXip-6B, and TaXip-6D were designed based on the conserved domains in all the three subgenomes. Two sgRNAs (sgRNA1 and sgRNA2) were designed to target the first exon on TaXip-6A and TaXip-6D, the second exon on TaXip-6B (Figure 1C). The pbU411 vector was designed to create In/Del in the fourth base after PAM. In the T0 generation, a total of 29 mutated plants were identified with 7, 7, 5, and 10 mutant plants that were edited for A, D, AB, and ABD subgenomes, respectively. In the T2-3 generation, two genotypes of mutants were obtained (Figure 1C): the aaBBDD genotype with an editing ratio greater than 80% for subgenome A and less than 20% for B and D, and the AAbbdd genotype with an editing ratio greater than 80% for subgenomes B and D and less than 20% for A. In the aaBBDD, 1 bp was deleted or 1 bp was inserted for 6A and in the AAbbdd 22 bp was deleted for 6B and 1 bp was inserted for 6D (Figure 1C). After protein prediction,4 we found that all these mutations would lead to frameshift mutations and make the termination codon appear in advance, leading to protein functional inactivation (Supplementary Figure 3).

4http://www.detaibo.com/sms2/translate.html
blue bold and red boxed) in XIP-I. This feature suggests that TaXIP-6A, TaXIP-6B, and TaXIP-6D lack chitinolytic activity, as demonstrated in the case of XIP-I (Payan et al., 2003). It indicated that TaXIP functions as an inhibitor of xylanase. The TaXIP-6A gene was expressed notably in the grain at the later stage of grain development. In this study, we indicated that the knock-out XIP in vitro significantly affected the SV and ST. That is to say, the endogenous XIP affected dough quality. The addition of fungal xylanase significantly affected the SV and ST. That is to say, the mechanism of XIP and xylanase interaction, and how the xylanase improves the dough quality needs to be further studied.

The factors affecting wheat quality have mainly been focused on protein (Sharma et al., 2020), starch (Rakszegi et al., 2006), and fat (Bonnand-Ducasse et al., 2010). HMW-GS is one of the most important factors affecting dough rheological properties and end-product quality of bread (Wang et al., 2005; Ghoshal et al., 2013, 2017; Ahmad et al., 2014; Leys et al., 2019). So we speculated that the SV and ST, as important indicators for dough quality, may be affected by XIP through xylanase. But what is the mechanism of XIP and xylanase interaction, and how the xylanase improves the dough quality needs to be further studied.

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