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ORIGINAL PAPER

Efficient isolation of ion beam-induced mutants for homoeologous loci in common wheat and comparison of the contributions of *Glu-1* loci to gluten functionality

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Abstract

Key message **Ion beam mutations can be efficiently isolated and deployed for functional comparison of homoeologous loci in polyploid plants, and *Glu-1* loci differ substantially in their contribution to wheat gluten functionality.**

Abstract To efficiently conduct genetic analysis, it is beneficial to have multiple types of mutants for the genes under investigation. Here, we demonstrate that ion beam-induced deletion mutants can be efficiently isolated for comparing the function of homoeologous loci of common wheat (*Triticum aestivum*). Through fragment analysis of PCR products from M_2 plants, ion beam mutants lacking homoeologous *Glu-A1*, *Glu-B1* or *Glu-D1* loci, which encode high molecular weight glutenin subunits (HMW-GSs) and affect gluten functionality and end-use quality of common wheat, could be isolated simultaneously. Three deletion lines missing *Glu-A1*, *Glu-B1* or *Glu-D1* were developed from the original mutants, with the *Glu-1* genomic regions deleted in these lines estimated using newly developed DNA markers. Apart from lacking the target HMW-GSs, the three lines all showed decreased accumulation of low molecular weight glutenin subunits (LMW-GSs) and increased amounts of gliadins. Based on the test data of five gluten and glutenin macropolymer (GMP) parameters obtained with grain samples harvested from two environments, we conclude that the genetic effects of *Glu-1* loci on gluten functionality can be ranked as *Glu-D1* > *Glu-B1* > *Glu-A1*. Furthermore, it is suggested that *Glu-1* loci contribute to gluten functionality both directly (by promoting the formation of GMP) and indirectly (through keeping the balance among HMW-GSs, LMW-GSs and gliadins). Finally, the efficient isolation of ion beam mutations for functional comparison of homoeologous loci in polyploid plants and the usefulness of *Glu-1* deletion lines for further studying the contribution of *Glu-1* loci to gluten functionality are discussed.

Abbreviations

EST	Expressed sequence tag
FPC	Flour protein content
G′	Elastic modulus
G″	Viscous modulus
Gli/Glu	Gliadins/glutenins
GMP	Glutenin macropolymer
HMW-GS	High molecular weight glutenin subunit
LMW-GS	Low molecular weight glutenin subunit
NILs	Near isogenic lines
RP-HPLC	Reverse-phase high-performance liquid chromatography
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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SDS-SV	SDS-sedimentation volume
SIG	Swelling index of glutenin
STS	Sequence-tagged site
TGW	Thousand grain weight

Introduction

Common wheat (*Triticum aestivum* L., AABBDD) is the most widely cultivated food crop in the world, and provides valuable dietary energy and protein sources for over 60 % of the world population (Dixon et al. 2009; Braun et al. 2010). However, the large (about 17 Gb) hexaploid genome has made gene function analysis and molecular breeding studies difficult in common wheat (Brenchley et al. 2012). With the advent of next generation sequencing technologies, draft genome sequence information has become available for common wheat and its relatives *T. urartu* and *Aegilops tauschii* (Brenchley et al. 2012; Jia et al. 2013; Ling et al. 2013). To efficiently exploit the published genome information for functional studies of important chromosomal loci (genes) of common wheat, it is desirable to have multiple types of mutants for the genomic loci under investigation.

To date, T-DNA insertion-related mutants and those created by transposon tagging, whose development generally requires highly efficient genetic transformation, have been widely used for gene function analysis in model plants (*Arabidopsis thaliana* and rice, Alonso and Ecker 2006; Boutros and Ahringer 2008; Hirochika 2010; Bolle et al. 2011). These mutants, however, are not yet available for common wheat, which cannot be genetically transformed in high efficiency at present (Blechl and Jones 2009). Chemical mutagenesis by ethylmethane sulfonate is effective in introducing point mutations into well-defined gene sequences and has been extensively used in the molecular genetic studies of important genes in many plant species including common wheat (Slade et al. 2005; Kurowska et al. 2011). In addition, deletion mutants caused by radiation treatment with cobalt-60 γ rays or fast neutrons have also been found useful for investigating the function of important chromosomal loci or genes in higher plants (e.g., Roberts et al. 1999; Al-Kaff et al. 2008).

In polyploid plants (such as common wheat), chromosomal loci usually have homoeologous counterparts that frequently differ in functional potency (Hegarty and Hiscock 2008; Akhunov et al. 2013). Thus, it is often necessary to compare functional difference among homoeologous copies to facilitate the selection of important one(s) for further investigations. For this type of investigations, it is often beneficial to have deletion mutants for a complete set of homoeologs. Toward this end, we have been testing if ion beam mutagenesis, mediated by charged particles, may be deployed for developing deletion lines for functional comparison of homoeologous chromosomal loci. Previously, ion beam mutagenesis has been found highly potent in inducing DNA mutations in both diploid and polyploid plants (Shikazono et al. 2005; Magori et al. 2010), some of which have been successfully exploited for developing elite crop varieties (Ishikawa et al. 2012). In several studies, ion beam mutants have been used for investigating gene function (Hase et al. 2000, 2006; Tanaka et al. 2002; Rahman et al. 2006; Shitsukawa et al. 2007). From the data available, the deletion caused by ion beam radiation may range from several base pairs to several hundred kilobases (Shikazono et al. 2005; Shitsukawa et al. 2007), and the fragment deleted may carry the target gene as well as the non-target genes adjacent to the gene of interest (Rahman et al. 2006). Despite many studies published so far, there is still no report on the efficient isolation and mapping of ion beam mutants and their application in comparing the function of homoeologous loci in common wheat and other polyploid species.

In common wheat, the end-use quality is strongly influenced by three families of gluten proteins, high molecular weight glutenin subunits (HMW-GSs), low molecular weight glutenin subunits (LMW-GSs) and gliadins (reviewed in Wrigley et al. 2009). The three families of gluten proteins are the main components of the gluten complex, and are thus important determinants of gluten functionality and dough processing properties (reviewed in Delcour et al. 2012). The x and y types of HMW-GSs are largely responsible for the elasticity of gluten by forming glutenin macropolymer (GMP) particles with LMW-GSs, whereas gliadins influence gluten viscosity by acting as monomeric proteins (Weegles et al. 1996a, b; Shewry et al. 2002; Don et al. 2003a, b, 2006; Delcour et al. 2012). The genes encoding HMW-GSs are carried in three homoeologous loci (*Glu-A1*, *Glu-B1* and *Glu-D1*) located on group one chromosomes (Lawrence and Shepherd 1981; Payne 1987). Furthermore, the three *Glu-1* loci, each with multiple alleles (Payne and Lawrence 1983; Payne 1987), have been found to differ in the effects on gluten functionality and dough processing performance by many genetic and breeding studies (Wrigley et al. 2009). A number of studies have also compared the effects of three *Glu-1* loci on gluten, dough and end-use quality-related parameters (e.g., Lawrrence et al. 1988; Uthayakumaran et al. 2002; Beasley et al. 2002; Liu et al. 2005; Zhang et al. 2009; Jin et al. 2013). In general, *Glu-D1* was found to have the strongest effects, followed by *Glu-B1* and *Glu-A1*. The plant materials used in these studies included recombinant lines (Lawrence et al. 1988; Uthayakumaran et al. 2002; Beasley et al. 2002; Zhang et al. 2009), variety population (Liu et al. 2005), or near isogenic lines (NILs, Jin et al. 2013). To date, there is still no report on the development of a complete set of locus deletion lines for *Glu-1* loci from a single

bread wheat variety and their use in comparing the function of the three loci.

From the information presented above, the main objectives of this work were to efficiently isolate and map ion beam mutants lacking *Glu-A1*, *Glu-B1* or *Glu-D1* and to use the resultant deletion lines to compare the role of *Glu-1* loci in gluten functionality. To facilitate the comparison, we determined the values of SDS-sedimentation volume (SDS-SV), swelling index of glutenin (SIG), GMP content, and the elastic (G′) and viscous (G″) moduli of GMP gel for the grain samples of Xiaoyan 81 and the three deletion lines harvested from both field and glasshouse environments. SDS-SV (Peña et al. 1990; Ross and Bettge 2009) and SIG (Wang and Kovacs 2002a, b) have been widely considered as important indicators of gluten strength. GMP content is highly correlated with the amounts of HMW-GSs and LMW-GSs (Don et al. 2006), while G′ and G″ reflect the elastic and viscous properties of gluten, respectively (Don et al. 2006; Kieffer 2006). Together, SDS-SV, SIG and the three GMP parameters should provide a more comprehensive assessment of gluten strength and quality, and thus, gluten functionality. Concomitantly, potential influences of *Glu-1* deletions on flour protein content (FPC) and balance among the accumulation levels of HMW-GSs, LMW-GSs and gliadins were evaluated.

Materials and methods

Development of ion beam mutant population and screening for *Glu-1* deletions

The common wheat variety used in this work is Xiaoyan 81, which is winter hardy and carries *Glu-A1a* (encoding 1Ax1 subunit), *Glu-B1h* (specifying 1Bx14 and 1By15 subunits) and *Glu-D1a* (coding for 1Dx2 and 1Dy12 subunits) alleles at *Glu-A1*, *Glu-B1* and *Glu-D1* loci, respectively. The *1Ay* gene in the *Glu-A1a* allele of Xiaoyan 81 is silenced. Five HMW-GS genes (*1Ax1, 1Bx14, 1By15, 1Dx2* and *1Dy12*) are consistently expressed in Xiaoyan 81 grains. After radiating the dry seeds of Xiaoyan 81 by nitrogen ions (with an energy level of 30 keV and a dose of 8×10^{17} ions/cm^2), an M_2 population composed of 2,400 families was developed. For screening the deletion mutants of *Glu-1* loci, genomic DNA samples were prepared from 5,600 seedlings grown from randomly selected M_2 seeds and were used as templates for genomic PCR.

By comparing the nucleotide sequences of HMW-GS genes contained in *Glu-A1* (GenBank accession DQ537335), *Glu-B1* (GenBank accession DQ537336) and *Glu-D1* (GenBank accession DQ537337) loci, a pair of oligonucleotide primers Glu1p-F (5′-TTGAACTCATTTGGGAAGT-3′) and Glu1p-R (5′-GTCTGCTAAAGCCACGTAAT-3′) was designed from the conserved portions of the promoter region. This primer set allowed the amplification of six fragments (with different sizes) in genomic PCR, which were specific for the *1Ax1* (528 bp), *1Ay1*^{null} (463 bp), *1Bx14* (595 bp), *1By15* (554 bp), *1Dx2* (526 bp) and *1Dy12* (543 bp) genes of Xiaoyan 81, respectively. To facilitate the screening of *Glu-1* deletions by fragment analysis, Glu1p-F was labeled with 6-FAM and the resultant PCR products were separated by capillary electrophoresis as described by Zhang et al. (2011). The deletion mutants were identified based on simultaneous absence of the fragments for *1Ax1* and *1Ay*^{null}, *1Bx14* and *1By15*, or *1Dx2* and *1Dy12*. The mutants in three M_2 families (M7, J84 and R41) were recovered, followed by backcrossing three times with Xiaoyan 81 as recurrent parent. Homozygous *Glu-1* deletion lines, DLGluA1, DLGluB1 and DLGluD1, were selected from BC_3F_2, and their seeds were multiplied after a further round of selfing. The lack of the HMW-GSs encoded by *Glu-A1*, *Glu-B1* or *Glu-D1* in the three *Glu-1* deletion lines was verified by SDS-PAGE analysis (see below).

To investigate the changes in gluten and GMP parameters caused by the deletion of *Glu-1* loci, DLGluA1, DLGluB1, DLGluD1 and Xiaoyan 81 were grown in two different environments during 2010 and 2011. The first environment was in the field with three replications planted for each of the four genotypes. The replications were arranged following a randomized block design, and each replication included three rows of plants. The length of each row was 2 m, and the distance between adjacent rows was 20 cm. The second environment was in the glasshouse, with the temperature and photoperiod settings adjusted for optimal wheat plant growth (Wang et al. 2011). The grains harvested from the field and glasshouse environments were conditioned to a moisture content of 14 % and milled in the Brabender Quadrumat Junior mill (Brabender, Duisburg, Germany). The flour yield was approximately 70 % for all four genotypes. While cultivating the four genotypes in two different environments, efforts were also made to compare the growth performance of DLGluA1, DLGluB1 and DLGluD1 with that of Xiaoyan 81 in terms of plant height, flowering time, spike morphology and thousand grain weight (TGW).

Analyzing *Glu-1* deletions with newly developed DNA markers

For mapping *Glu-1* deletions in DLGluA1, DLGluB1 and DLGluD1, we developed sequence-tagged site (STS) markers from the genes syntenically conserved between wheat and *Brachypodium distachyon*. Previous studies revealed that the *Glu-1* orthologous region in *B. distachyon* was on chromosome 2 (Bd2) (Vogel et al. 2010). The

Table 1 Summary of the STS markers developed for *Glu-1* region in this work

Marker	Wheat EST	Ortholog in *B. distachyon*	Primer sequence (5′–3′)	T_m (°C)	Marker specificity[a]
STS$^{Glu\text{-}1}$*-1*	BJ256501	*Bradi2g20020*	F: GGTGCTGGTTACTCCTCTGG R: GCCATGTGCCATATCTCCTT	60	Dominant for *Glu-B1*
STS$^{Glu\text{-}1}$*-2*	CJ682658	*Bradi2g20141*	F: TGCTGGAGTGTGGAGAATCA R: TGCTGCTGCTTTCTGTATGC	60	Co-dominant for *Glu-A1* and *Glu-B1*
STS$^{Glu\text{-}1}$*-3*	CA692060	*Bradi2g20250*	F: TCGAGTTGTTTGCAATGTGTC R: ACAATTCTTGCTGGCAGCTT	60	Co-dominant for *Glu-A1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-4*	CJ630263	*Bradi2g20347*	F: GATGCGTGCCATGTTTCAACAAGTC R: TCCAGTCCCATCCTGCTCATAAGAA	60	Dominant for *Glu-A1*
STS$^{Glu\text{-}1}$*-5*	HX195951	*Bradi2g20400*	F: CAAGGTTACTGAAGACGGGACAATC R: TCACCCTCACTGCTCTCCCTGT	60	Co-dominant for *Glu-A1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-6*	CJ923101	*Bradi2g20460*	F: TTGACACGGAGGATGATTAC R: CACAGATACCTGAGGACGAG	60	Dominant for *Glu-A1*
STS$^{Glu\text{-}1}$*-7*	CD916315	*Bradi2g20570*	F: GGCACACATCCTGTCTCCTT R: TGCCTTGAATCACTGCTGAG	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1* region (RFLP-*Hae*III)
STS$^{Glu\text{-}1}$*-8*	HX174154	*Bradi2g20767*	F: GGGTCCTTGTTCAGCCCTTAC R: CTCCACGTCGCACTTCCTTC	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1* (RFLP-*Hae*III or *Taq*I)
STS$^{Glu\text{-}1}$*-9*	CD909229	*Bradi2g20920*	F: GGCAGGAAGAGTCTGACCAC R: CACCCTGAAGCTGAATGACC	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-10*	CJ822930	*Bradi2g21040*	F: GGCAGCAAAGAATCGTCTTC R: CGGAGATGATCCACCAACTT	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-11*	CK162420	*Bradi2g21050*	F: ACAGAGAGGAAATGGAAAGGCACAA R: GGTAGTCAGCAACTTCCTTAGCAGC	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1* region (RFLP-*Taq*I)
STS$^{Glu\text{-}1}$*-12*	BQ240584	*Bradi2g21110*	F: GGGACTCCCTCTTCTTCCAC R: TGCACGAGCTCTGTGAAATC	60	Dominant for *Glu-A1*
STS$^{Glu\text{-}1}$*-13*	CJ571436	*Bradi2g21210*	F: GGGTTTGCTGTTCGCTTTAG R: TCTCAAGCGGCGAATAATCT	60	Dominant for *Glu-A1*
STS$^{Glu\text{-}1}$*-14*	BJ284894	*Bradi2g21790*	F: GAACATCTCGTCCAGCTTCC R: CAGGTGCTGAAAGCAATGAA	60	Co-dominant for *Glu-A1, Glu-B1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-15*	CB307285	*Bradi2g22150*	F: TCTGGGACGTCCTTGTTGAT R: TCCCTCTGGAGCAGAGTACG	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1*
STS$^{Glu\text{-}1}$*-16*	CA597665	*Bradi2g22160*	F: ATGTTGGGAAGCAGATCCAG R: CTCTTCTCCGACTCCACCAG	60	Co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1*

The 16 gene-based STS markers were tentatively designated in a sequential manner according to the order of their syntenic genes on chromosome 2 of *B. distachyon* (Online Resource 3). They were labeled with the superscript *Glu-1* to indicate that they had been developed from the genes in and around the *Glu-1* region

[a] For three markers (*STS*$^{Glu\text{-}1}$*-7*, *STS*$^{Glu\text{-}1}$*-8* and *STS*$^{Glu\text{-}1}$*-11*), their PCR products had to be digested with the indicated restriction enzyme to reveal restriction fragment length polymorphism (RFLP) for identifying the bands specific for *Glu-A1*, *B1* or *D1*

genes annotated in the *Glu-1* orthologous region of *B. distachyon* were downloaded from Phytozome (v9.0, http://www.phytozome.org/) and used as queries to search wheat expressed sequence tags (ESTs) using BLASTN (http://blast.ncbi.nlm.nih.gov/). The top-hit wheat EST sequences were used for designing STS markers with the computer program Conserved Primers (v2.0, http://probes.pw.usda.gov/cgi-bin/ConservedPrimers/ConservedPrimers.cgi). Before using the predicted primers for mapping *Glu-1* deletions, the chromosomal specificity of each primer pair and the number of homoeoallele amplified by it were investigated using the nulli-tetrasomic lines of 1A, 1B and 1D chromosomes derived from Chinese Spring (Sears 1954). Subsequently, the confirmed primer sets (Table 1) were used to analyze Xiaoyan 81 and the three *Glu-1* deletion lines by genomic PCR. In general, the amplification reaction was conducted in 20 μl volume containing 100 ng DNA, 10 μM of each primer, 2× *EcoTaq* PCR supermix (TransGen Biotech, Beijing, China). After an initial denaturation at 94 °C for

4 min, 35 amplification cycles (94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) and one final extension step (72 °C for 5 min) were performed in an Applied Biosystems Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, CA, USA). An aliquot (8 μl) of the amplification product was analyzed by electrophoresis on 6 % PAGE gel. For three STS primer sets, their amplification products had to be digested with *Hae*III or *Taq*I (1 U, at 37 or 65 °C, Table 1) to separate different homoeoalleles. The digested fragments were fractionated by electrophoresis on 4 % agarose gel as described previously (Ishikawa et al. 2009).

SDS-PAGE analysis of HMW-GSs

HMW-GSs were extracted from the desired grain samples and separated in 10 % SDS-PAGE as detailed previously (Wan et al. 2000). The HMW-GSs expressed in the winter wheat variety Xiaoyan 54 (1Ax1, 1Bx14, 1By15, 1Dx2 and 1Dy12, Li et al. 2004) was used as reference.

Reverse-phase high-performance liquid chromatography

Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out to compare the accumulation profiles of major gluten protein (HMW-GS, LMW-GS and gliadin) fractions in the grains of Xiaoyan 81, DLGluA1, DLGluB1 and DLGluD1. The majority of the steps of the analysis were conducted at 25 °C except where noted. The gliadin fraction was prepared according to Lookhart et al. (1993). For each grain sample, 45 mg flour was added to 1 ml 70 % (v/v) aqueous ethanol for 1 h at room temperature with periodic agitation. After spinning at 13,000 rpm for 10 min, the supernatant was collected as the gliadin fraction. The glutenin fraction was prepared following the method 2 described by DuPont et al. (2005) with some modifications. Briefly, for each grain sample, 45 mg flour was added to 1 ml of 0.3 M NaI, 7.5 % 1-propanol. The mixture was agitated periodically for 10 min before being centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was extracted with 1 ml of 70 % ethanol for 30 min. After spinning at 13,000 rpm for 10 min, the supernatant was decanted and the pellet was resuspended in 1 ml of 55 % isopropanol. The mixture was incubated at 65 °C for 30 min with periodic agitation, followed by centrifugation at 13,000 rpm for 10 min. This step was repeated once, with the resultant pellet resuspended in 500 μl of 50 % isopropanol, 80 mM Tris–HCl (pH 8.0) and 1 % (w/v) dithiothreitol. The suspension was incubated at 65 °C for 1 h, followed by the addition of 1 % (w/v) 4-vinylpyridine. After a further incubation at 65 °C for 30 min, the mixture was centrifuged at 13,000 rpm for 10 min, with the supernatant retained as the glutenin fraction. The gliadin and glutenin fractions were both filtered through 0.45 μm nylon filter before being used for RP-HPLC analysis.

RP-HPLC was accomplished using a Waters HPLC 2695–2998 system (Waters Corporation, Milford, USA) equipped with a C_{18} column using the conditions described by González-Torralba et al. (2011). For each gliadin or glutenin extract, 10 μl were withdrawn and analyzed by RP-HPLC, with the amounts of HMW-GSs, LMW-GSs and gliadins estimated by integrating the relevant peak areas in the chromatograms. For each grain sample, the ratio of gliadins to glutenins (Gli/Glu) was also calculated by dividing the amount of gliadins with the combined total of HMW-GSs and LMW-GSs.

Evaluation of FPC, SDS-SV and SIG

Flour protein content (FPC) was measured with Kjeldahl nitrogen determination assay according to AACC Method 46-10.01 (AACCI 2010). The results were expressed as the protein percentage of the flour. SDS-SV was measured using 1 g flour (Peña et al. 1990). SIG was determined with 0.4 g flour (Wang and Kovacs 2002a).

Investigation of the content and rheology of glutenin macropolymers

The GMP content was determined as described previously (Weegles et al. 1996b) and expressed as the percentage of flour. The preparation of GMP gel from defatted flour was accomplished as described by Don et al. (2003a, b). The rheology of GMP gel was investigated essentially according to Don et al. (2006) with a Bohlin Gemini HR nano rheometer (Malvern instruments, Worcestershire, UK). Two main parameters, elastic modulus (G') and viscous modulus (G''), were recorded.

Statistical analysis

In the experiments described above, three separate tests (each with 2–3 technical repeats) were carried out for each grain sample. All data (presented as mean ± SE) were subjected to one-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparison test using IBM SPSS Statistics 19 (IBM, New York, USA). Pearson correlation coefficients between different gluten protein fractions and gluten and GMP parameters were investigated also using IBM SPSS Statistics 19. In this investigation, the data points varied from 12 to 18 for the individual gluten protein fractions and gluten and GMP parameters, and each data point was the value obtained from a technical repeat.

Results

Identification of ion beam mutants of *Glu-1* loci and development of *Glu-1* deletion lines

After screening 5,600 M_2 seedlings by fragment analysis of PCR products, putative homozygous ion beam mutants lacking *Glu-A1*, *B1* and *D1* were detected in five (C36, G74, L50, M7 and S40), one (J84) and one (R41) independent M_2 families, respectively (Online Resource 1). To facilitate further analysis, the homozygous *Glu-1* mutants identified in M7, J84 and R41 were backcrossed three times with their wild-type progenitor Xiaoyan 81, and three stable *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGluD1) were obtained. SDS-PAGE analysis of BC_3F_4 seed extracts verified the lack of 1Ax1 in DLGluA1, 1Bx14 and 1By15 in DLGluB1, and 1Dx2 and 1Dy12 in DLGluD1 (Fig. 1). The three deletion lines resembled Xiaoyan 81 in plant height, flowering dates, and spike and kernel morphology (Online Resource 2). Moreover, no significant difference was found in TGW among Xiaoyan 81 and the three deletion lines (data not shown). Therefore, DLGluA1, DLGluB1 and DLGluD1 were considered to share a highly similar genetic background with their WT progenitor Xiaoyan 81. This consideration was consistent with the identical compositions of LMW-GSs or gliadins observed for the grains of DLGluA1, DLGluB1, DLGluD1 and Xiaoyan 81 (see below).

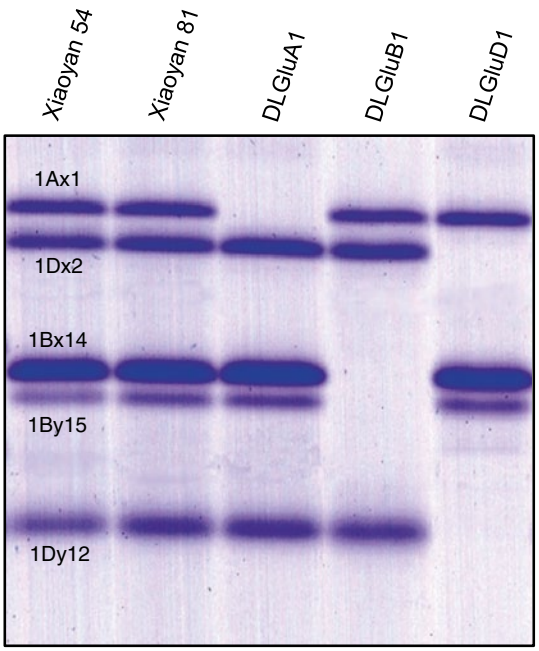

Fig. 1 SDS-PAGE analysis of HMW-GSs expressed in Xiaoyan 54, Xiaoyan 81 and three *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGlu-D1). The HMW-GSs detected in Xiaoyan 54 (namely, 1Ax1, 1Bx14, 1By15, 1Dx2 and 1Dy12) were used as reference. Xiaoyan 81 expressed an identical set of HMW-GSs as Xiaoyan 54. DLGluA1, DLGluB1 and DLGlu-D1 lacked the subunits encoded by *Glu-A1* (1Ax1), *B1* (1Bx14 and 1By15) or *D1* (1Dx2 and 1Dy12), respectively

Mapping the approximate borders of *Glu-1* deletions

As the first step to estimate the borders of *Glu-1* deletions in DLGluA1, DLGluB1 and DLGluD1, we developed a series of STS markers based on 16 genes conserved between *Glu-1* and its orthologous region in *B. distachyon* (Table 1). Among the 16 STS markers, 11 were co-dominant for *Glu-A1*, *Glu-B1* and *Glu-D1* (8), *Glu-A1* and *Glu-B1* (1), or *Glu-A1* and *Glu-D1* (2), with the remaining 5 being dominant for either *Glu-A1* (4) or *Glu-B1* (1) (Table 1). The 16 syntenic genes spanned approximately 2.06 Mb on chromosome 2 of *B. distachyon*, 15 of which were also conserved on rice chromosome 5 (Online Resource 3).

The *Glu-1* deletions in DLGluA1, DLGluB1 and DLGluD1 were estimated using the 16 markers. Judging from the absence of specific markers, the *Glu-1* deletions in the three lines lied between STS^{Glu-1}*-7* and STS^{Glu-1}*-11* (DLGluA1), STS^{Glu-1}*-8* and STS^{Glu-1}*-11* (DLGluB1) or STS^{Glu-1}*-8* and STS^{Glu-1}*-10* (DLGluD1) (Table 2). Based on the sequence of *B. distachyon* chromosome 2 and rice chromosome 5, the *Glu-A1* deletion corresponded to about 340 kb in *B. distachyon* and 345 kb in rice, which carried 55 and 67 annotated genes, respectively. The *Glu-B1* deletion corresponded to about 170 kb in *B. distachyon* and 206 kb in rice, which harbored 34 and 45 annotated genes, respectively. Finally, the regions orthologous to *Glu-D1* deletion were approximately 160 kb in *B. distachyon* and 156 kb in rice, which hosted 31 and 38 annotated genes, respectively.

Accumulation profiles of HMW-GSs, LMW-GSs and gliadins in *Glu-1* deletion lines

Before investigating the gluten and GMP parameters of *Glu-1* deletion lines, we compared the accumulation profiles of HMW-GSs, LMW-GSs and gliadins between Xiaoyan 81 and DLGluA1, DLGluB1 or DLGluD1 using RP-HPLC. The composition and expression levels of HMW-GSs, LMW-GSs and gliadins were analyzed for the grain samples of the four genotypes harvested from both environments. As anticipated, in both environments, DLGluA1, DLGluB1 and DLGluD1 lacked 1Ax1, 1Bx14 and 1By15, and 1Dx2 and 1Dy12, respectively, but their composition of LMW-GSs or gliadins did not differ significantly from that of Xiaoyan 81 (Online Resource 4).

Subsequently, the total amounts of HMW-GSs, LMW-GSs and gliadins in each grain sample were estimated

Table 2 Estimation of approximate borders of *Glu-1* deletions in DLGluA1, DLGluB1 and DLGluD1 using 16 STS markers

Marker	*STS Glu-1_1*	*STS Glu-1_2*	*STS Glu-1_3*	*STS Glu-1_4*	*STS Glu-1_5*	*STS Glu-1_6*	*STS Glu-1_7*	*STS Glu-1_8*	*STS Glu-1_9*	*STS Glu-1_10*	*STS Glu-1_11*	*STS Glu-1_12*	*STS Glu-1_13*	*STS Glu-1_14*	*STS Glu-1_15*	*STS-Glu-1_16*
Xiaoyan 81	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DLGluA1	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+
DLGluB1	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+
DLGluD1	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+

"+" and "−" indicate positive and negative amplifications, respectively

by integrating the relevant RP-HPLC peaks presented in the chromatograms. To facilitate comparisons between Xiaoyan 81 and DLGluA1, DLGluB1 or DLGluD1, the quantitative HMW-GS, LMW-GS and gliadin data of the three deletion lines were all normalized against those of Xiaoyan 81. As shown in Fig. 2, relative to Xiaoyan 81, the total amounts of HMW-GSs or LMW-GSs in DLGluA1, DLGluB1 and DLGluD1 were generally and significantly decreased. Consequently, the combined amount of HMW-GSs and LMW-GSs (i.e., glutenins) was significantly lower in DLGluA1, DLGluB1 and DLGluD1 than in Xiaoyan 81 (Fig. 2). Moreover, the magnitude of the decrease in HMW-GSs, LMW-GSs and glutenins was generally largest for DLGluD1, followed by DLGluB1 and DLGluA1 (Fig. 2). By contrast, the total levels of gliadins in DLGluA1, DLGluB1 and DLGluD1 were generally increased compared with Xiaoyan 81, with the scale of the increase being highest for DLGluD1 (Fig. 2). The decrease of HMW-GSs and LMW-GSs and the increase of gliadins in DLGluA1, DLGluB1 and DLGluD1 were observed under both field and glasshouse conditions, indicating that these changes were unlikely caused by experimental errors. Owing to reduced accumulation of glutenins and up-regulated expression of gliadins in DLGluA1, DLGluB1 and DLGluD1, the Gli/Glu ratio in the three deletion lines was all significantly elevated compared with that of Xiaoyan 81 in both environments (Fig. 3). The magnitude of the increase in Gli/Glu ratio was relatively higher for DLGluD1 than for DLGluA1 and DLGluB1 (Fig. 3).

Changes of FPC, SDS-SV and SIG in *Glu-1* deletion lines

In both field and glasshouse environments, the three deletion lines did not differ substantially from Xiaoyan 81 in FPC (Fig. 4a). However, compared with Xiaoyan 81, the three deletion lines generally showed significant decreases in SDS-SV and SIG, and for both environments, the decreases exhibited by DLGluD1 were the strongest (Fig. 4b, c).

Changes of GMP content and the elastic and viscous moduli of GMP gel in *Glu-1* deletion lines

Under both field and glasshouse conditions, DLGluA1, DLGluB1 and DLGluD1 displayed significant reductions in GMP content as well as G′ and G″ of GMP gel relative to Xiaoyan 81 (Table 3). In general, the degree of the reduction was strongest for DLGluD1, intermediate for DLGluB1 and relatively low for DLGluA1 (Table 3). Specifically, the GMP content of DLGluD1 was reduced by 71.1–81.2 %, whereas the corresponding reduction ranges for DLGluB1 and DLGluA1 were 32.5–41.6 and 7.8–23.3 %, respectively. For DLGluD1, G′ was lowered

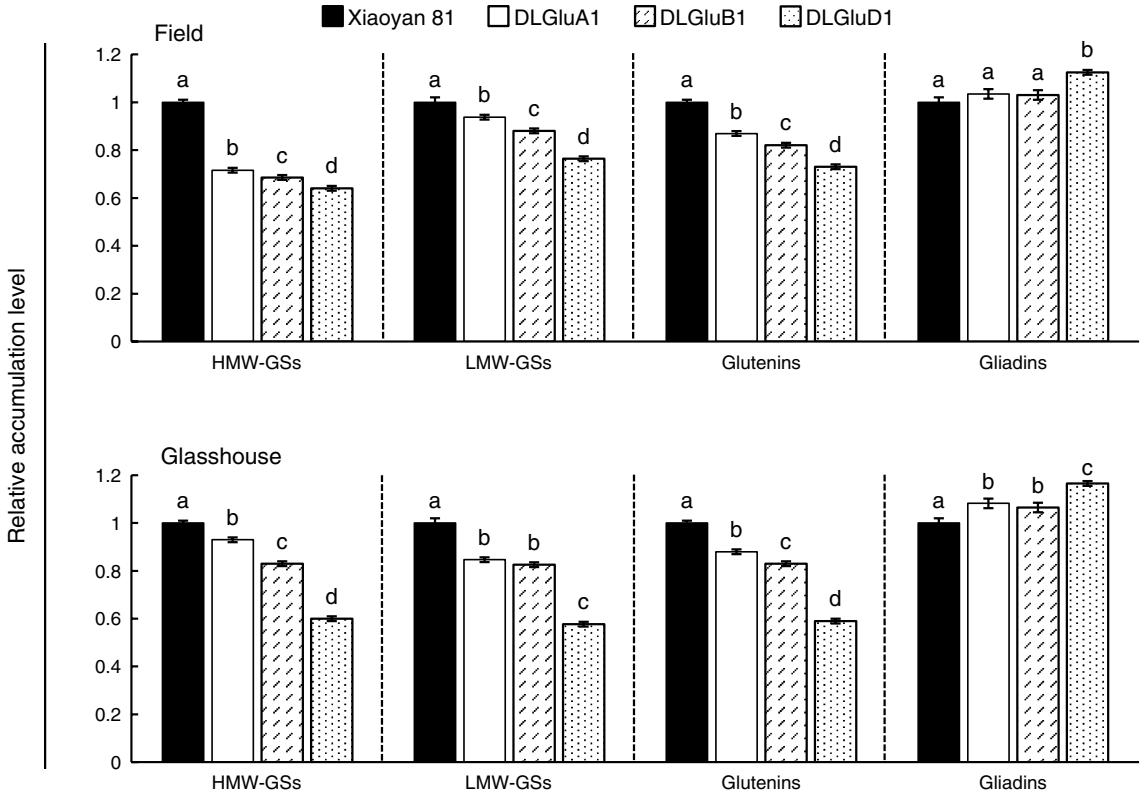

Fig. 2 Relative accumulation levels of HMW-GSs, LMW-GSs, total glutenins (HMW-GSs+LMW-GSs) and gliadins in Xiaoyan 81 and three *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGlu-D1) harvested from the field or glasshouse environments. The data from the deletion lines were normalized against those of Xiaoyan 81. Each data point was the mean (±SE) of three separate assays. *Different letters on top of the columns* indicated significant difference ($p < 0.05$)

by 75.5–80.8 %, but for DLGluB1 and DLGluA1, G′ was decreased by 35.2–62.5 % and 16.6–45.3 %, respectively. In the case of G″, the reduction shown by DLGluD1 was 53.0–68.6 %, whereas those recorded for DLGluB1 and DLGluA1 were 35.2–55.2 and 8.8–33.5 %, respectively (Table 3).

Correlation between gluten protein fractions and gluten and GMP parameters

Further to the experiments described above, correlation analysis was conducted to investigate if the changes in gluten protein fractions and those in gluten and GMP parameters may be correlated. Judging from the Pearson's coefficients displayed in Table 4, HMW-GSs, LMW-GSs and glutenins (HMW-GSs+LMW-GSs) were each positively correlated with SDS-SV, SIG, GMP content, G′ and G″, whereas gliadins and Gli/Glu ratio exhibited negative correlation with the five gluten and GMP parameters. In general, the level of glutenins showed the strongest positive correlation with SDS-SV, SIG, GMP content, G′ and G″, whereas Gli/Glu ratio displayed the highest negative correlation with the five parameters (Table 4). Within the three gluten protein fractions, the levels of HMW-GSs and LMW-GSs were positively and highly significantly correlated, whereas HMW-GSs, LMW-GSs and glutenins all showed significant negative correlation with gliadins (Table 4). Finally, positive correlations were also detected among SDS-SV, SIG, GMP content, G′ and G″ (Table 4).

Discussion

Efficient screening and mapping of ion beam-induced mutations

Prior to this work, many studies have shown that ion beam irradiation has strong mutagenesis effects in plants, which can be exploited for crop improvement (Shikazono et al. 2005; Magori et al. 2010; Ishikawa et al. 2012). However, only in a few cases have ion beam mutants been used for functional investigations of important genes (Hase et al. 2000, 2006; Rahman et al. 2006; Shitsukawa et al. 2007), and no efforts have been reported on efficient identification and mapping of ion beam deletions for homoeologous loci in the crop species with polyploid genomes. Here, we

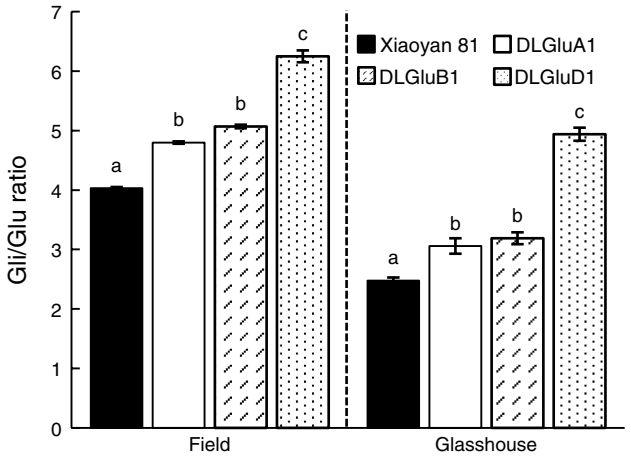

Fig. 3 Comparison of Gli/Glu ratio among Xiaoyan 81 and three *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGlu-D1) harvested from the field or glasshouse environments. The data points (mean ± SE) were each calculated using the measurements from three separate assays. *Different letters on top of the columns* indicated significant difference ($p < 0.05$)

showed that fragment analysis of the PCR products amplified from six HMW-GS genes with a pair of conserved primers permitted simultaneous identification of ion beam mutants for three *Glu-1* loci. This approach is likely generally applicable for identifying ion beam mutants for homoeologous loci in common wheat or other polyploid crop species, providing that conserved primers can be designed for the target loci.

For reliably using ion beam deletions in functional studies, it is necessary to map the chromosomal fragments deleted in the target loci. This represents a strong challenge to common wheat that carries a highly complex and unsequenced polyploid genome. Fortunately, molecular markers can be developed based on the genes syntenically conserved among wheat, *B. distachyon* and rice (Ishikawa et al. 2009; Gasperini et al. 2012), and these markers have been found very useful in mapping the chromosomal position of common wheat genes (Qin et al. 2011; Gasperini et al. 2012). In this work, we succeeded in developing 16 such markers based on the genes conserved between *Glu-1* region and its orthologous counterpart in *B. distachyon*. Using these markers, we mapped the approximate borders of the deleted fragments in DLGluA1, DLGluB1 and DLGluD1. The *B. distachyon* and rice chromosomal regions corresponding to the *Glu-1* deletions in DLGluA1, DLGluB1 and DLGluD1 varied from 160 to 340 kb or 156 to 345 kb. The precise size of the *Glu-1* deletions is unknown at present. Judging from the multiple genes located in the *Glu-1* orthologous regions in *B. distachyon* and rice (Online Resource 3), DLGluA1, DLGluB1 and DLGluD1 lacked not only the target HMW-GS genes but also a number of

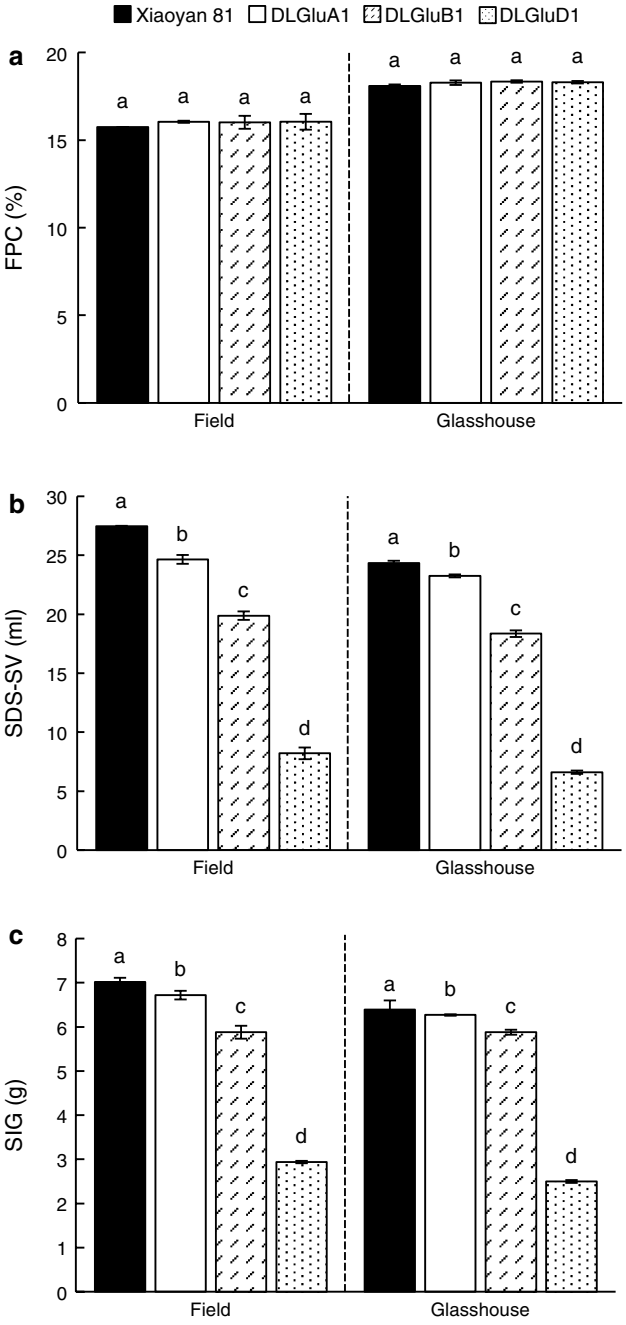

Fig. 4 Comparison of FPC (**a**), SDS-SV (**b**) and SIG (**c**) among Xiaoyan 81 three *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGlu-D1) harvested from the field or glasshouse environments. Each data point (mean ± SE) was calculated using the measurements from three separate tests. *Different letters on top of the columns* indicated significant difference ($p < 0.05$)

other genes. Nevertheless, the three deletion lines did not differ significantly from WT control (Xiaoyan 81) in their growth performance and yield related traits under either glasshouse or field conditions. The high tolerance of the three mutant lines to large deletion in their *Glu-1* region is probably associated with functional compensation among

Table 3 Comparison of GMP content and elastic (G′) and viscous (G″) moduli among Xiaoyan 81 and three *Glu-1* deletion lines (DLGluA1, DLGluB1 and DLGluD1)

Environment	Genotype	GMP content		G′		G″	
		Mean (% of flour)	Percentage of decrease (relative to Xiaoyan 81)	Mean (Pa)	Percentage of decrease (relative to Xiaoyan 81)	Mean (Pa)	Percentage of decrease (relative to Xiaoyan 81)
Field	Xiaoyan 81	3.69 ± 0.11a	na	17.17 ± 0.26a	na	3.88 ± 0.09a	na
	DLGluA1	2.83 ± 0.10b	23.3	9.40 ± 0.35b	45.3	2.58 ± 0.08b	33.5
	DLGluB1	1.80 ± 0.05c	41.6	6.44 ± 0.55c	62.5	1.74 ± 0.12c	55.2
	DLGluD1	0.89 ± 0.02d	71.1	3.30 ± 0.60d	80.8	1.22 ± 0.18d	68.6
Glasshouse	Xiaoyan 81	3.08 ± 0.05a	0	15.82 ± 0.15a	0	3.64 ± 0.09a	0
	DLGluA1	2.84 ± 0.03b	7.8	13.19 ± 1.05b	16.6	3.32 ± 0.26b	8.8
	DLGluB1	2.08 ± 0.11c	32.5	10.26 ± 0.23c	35.2	2.36 ± 0.11c	35.2
	DLGluD1	0.58 ± 0.03d	81.2	3.88 ± 0.39d	75.5	1.71 ± 012d	53.0

Each mean (and associated SE) was calculated with the measurements from three separate tests. Different letters after the means mark significant difference ($p < 0.05$)

na not applicable

Table 4 Correlation coefficients between gluten protein fractions and gluten and GMP parameters investigated in this work

	HMW-GS	LMW-GS	Glutenin	Gliadin	Gli/Glu	SDS-SV	SIG	GMP content	G′	G″
HMW-GS	na									
LMW-GS	0.88**	na								
Glutenin	0.96**	0.97**	na							
Gliadin	−0.73**	−0.85**	−0.83**	na						
Gli/Glu	−0.91**	−0.98**	−0.98**	0.90**	na					
SDS-SV	0.85**	0.95**	0.93**	−0.84**	−0.96**	na				
SIG	0.79**	0.93**	0.90**	−0.84**	−0.94**	0.98**	na			
GMP content	0.93**	0.94**	0.97**	−0.82**	−0.96**	0.97**	0.93**	na		
G′	0.95**	0.92**	0.97**	−0.78**	−0.94**	0.91**	0.86**	0.96**	na	
G″	0.97**	0.88**	0.94**	−0.73**	−0.89**	0.88**	0.81**	0.94**	0.97**	na

HMW-GS high molecular weight glutenin subunit, *LMW-GS* low molecular weight glutenin, *Glutenin* HMW-GSs+LMW-GSs, *Gli/Glu* ratio of gliadins/glutenins, *SDS-SV* SDS-sedimentation volume, *SIG* swelling index of glutenin, *G′* elastic modulus, *G″* viscous modulus, *na* not applicable

** Statistically significant at $p < 0.01$

homoeologous genes, which often exists among the A, B and D genomes of common wheat (Sears 1954; Chandler and Harding 2013).

Quantitative changes in gluten protein fractions in *Glu-1* deletion lines

As anticipated, deletion of *Glu-1* loci led to the absence of target HMW-GSs and significant decrease in the accumulation level of HMW-GSs in DLGluA1, DLGluB1 and DLGluD1. However, these lines also displayed quantitative alterations in the overall levels of LMW-GSs and gliadins. Taking consideration of the data from both field and glasshouse environments, several features can be summarized on the alterations in gluten protein fractions in the *Glu-1* deletion lines. First, the level of LMW-GSs is decreased in all three lines, with the decrease being strongest in DLGluD1. Second, the level of gliadins is increased in all three lines, with the magnitude of the increase being highest in DLGluD1. Third, the total amount of glutenins is significantly down-regulated in all three mutants, with the scale of the down-regulation being most severe in DLGluD1. Finally, in line with the changes in glutenins and gliadins, Gli/Glu ratio is substantially up-regulated for all three lines, with the order being DLGluD1 > DLGluB1 > DLGluA1.

Previous studies have suggested that there are strong compensatory interactions among the accumulation levels of glutenins and gliadins. In general, decreasing the level of HMW-GSs leads to increased accumulation of gliadins (Galili et al. 1986; Dumur et al. 2004), and reducing the

level of gliadins up-regulates the amounts of HMW-GSs and LMW-GSs (Gil-Humanes et al. 2010, 2012; Pistón et al. 2011; Becker et al. 2012). Increased expression of HMW-GSs suppresses the level of gliadins (León et al. 2010) and over-accumulation of a LMW-GS down-regulates the expression of HMW-GSs and gliadins (Scossa et al. 2008). Thus, the increased level of gliadins in the three deletion lines may be, at least partly, due to compensation for the lack of the target HMW-GSs. However, it is surprising to observe that the level of LMW-GSs was significantly decreased in all three deletion lines, particularly for DLGluD1. Since this decrease was found for the grain samples harvested from two separate environments, it is unlikely caused by experimental artifacts. One possibility is that there may be co-regulation in the accumulation of HMW-GSs and LMW-GSs in the developing grains, although the mechanisms involved are not understood at present. Finally, we observed that the much greater decrease in LMW-GSs shown by DLGluD1 paralleled with a stronger increase in gliadins in this deletion line, indicating that the reduction of LMW-GSs may also provoke compensation by gliadins.

Despite multiple changes in the levels of HMW-GSs, LMW-GSs and gliadins, the three deletion lines did not differ significantly from Xiaoyan 81 in FPC (Fig. 4a). A similar phenomenon has been recorded by previous studies (Rakszegi et al. 2005; León et al. 2010; Gil-Humanes et al. 2012). The compensatory increase of gliadins is probably an important reason for keeping FPC in the three *Glu-1* deletion lines, although this increase led to the rising of Gli/Glu ratio and contributed to the lowering of the examined gluten and GMP parameters (Table 4, see also below).

Usefulness of *Glu-1* deletion lines in understanding the contributions of HMW-GSs to gluten functionality

Since 1970s, *Glu-1* loci and their encoded HMW-GSs have been studied for more than 30 years (Payne et al. 1979; Shewry 2009). A wealth of information has been obtained on the effects of *Glu-1* loci on gluten and dough properties and end-use qualities by the studies using recombinant lines, variety population or NILs (Lawrrence et al. 1988; Uthayakumaran et al. 2002; Beasley et al. 2002; Liu et al. 2005; Zhang et al. 2009; Jin et al. 2013). However, to our knowledge, there is still no report of functional comparison of *Glu-1* loci using a complete set of well-defined locus deletion lines derived from a single bread wheat variety. In this work, we developed three *Glu-1* deletion lines sharing a highly similar genetic background with their WT parent, elucidated the quantitative changes of different types of gluten proteins in DLGluA1, DLGluB1 and DLGluD1, and investigated the consequences of *Glu-1* deletions on multiple gluten and GMP parameters. Based on the test data obtained using the grain samples collected from two separate environments, we can conclude that the genetic effects of *Glu-1* loci on gluten functionality follow the order of *Glu-D1* > *Glu-B1* > *Glu-A1*. This conclusion is consistent with the findings made by previous studies (Lawrrence et al. 1988; Uthayakumaran et al. 2002; Beasley et al. 2002; Liu et al. 2005; Zhang et al. 2009; Jin et al. 2013) and demonstrates that DLGluA1, DLGluB1 and DLGluD1 can be used reliably to reveal functional difference among *Glu-1* loci.

From the analysis conducted in this work, it becomes clear that *Glu-A1*, *Glu-B1* and *Glu-D1* contribute to gluten functionality through at least three means: (1) *Glu-A1*, *Glu-B1* and *Glu-D1* contribute directly to gluten functionality by promoting the formation of GMP, the elastic and viscous moduli of GMP gel, and thereby the strength and quality of gluten. This is supported by substantial decreases of GMP content, G′ and G″ in DLGluA1, DLGluB1 and DLGluD1, and highly significant correlation coefficients among HMW-GSs, GMP content, G′, G″, SDS-SV and SIG (Table 4). Because the decrease in GMP content in the deletion lines followed the order DLGluD1 > DLGluB1 > DLGluA1 (Table 3), the direct contribution to gluten functionality is largest for *Glu-D1*, intermediate for *Glu-B1* and relatively low for *Glu-A1*. (2) *Glu-A1*, *Glu-B1* and *Glu-D1* are required for repressing excessive accumulation of gliadins. In the absence of *Glu-1* loci, the amount of gliadins increased in the three deletion lines. This led to substantial up-regulation of Gli/Glu ratio, which was negatively and significantly correlated with all of the five examined gluten functionality indicators (Table 4). Therefore, the repression of excessive gliadin accumulation represents an indirect contribution of *Glu-1* loci to gluten functionality. Since gliadins were more highly up-regulated in DLGluD1 than in DLGluA1 and DLGluB1 (Fig. 2), the indirect contribution to gluten functionality by repressing gliadin accumulation is higher for *Glu-D1* and comparatively lower for *Glu-A1* and *Glu-B1*. (3) *Glu-A1*, *Glu-B1* and *Glu-D1* are involved in keeping the level of LMW-GSs. Since LMW-GSs were positively and significantly correlated with gluten and GMP parameters (Table 4), the maintenance of LMW-GS level by *Glu-A1*, *Glu-B1* and *Glu-D1* may indicate yet another indirect contribution of *Glu-1* loci to gluten functionality. Since the level of LMW-GSs was more severely reduced in DLGluD1 than in DLGluA1 and DLGluB1 (Fig. 2), the capacity to maintain LMW-GS level, and the resultant indirect contribution to gluten functionality, is likely to be stronger for *Glu-D1* than for *Glu-A1* and *Glu-B1*.

In past studies, the direct contribution of *Glu-1* loci and their encoded HMW-GSs to quality-related parameters through promoting GMP formation has been well analyzed (Weegles et al. 1996a, b; Don et al. 2006), and it has been

shown that the more HMW-GSs expressed the higher GMP content reached (Don et al. 2006). An obvious difference among the three *Glu-1* loci of common wheat is that both *Glu-B1* and *Glu-D1* specify two HMW-GSs, whereas *Glu-A1* encodes only one HMW-GS. This difference may partly explain the different levels of direct contribution to gluten functionality by *Glu-A1*, *Glu-B1* and *Glu-D1* found in this work and previously (Lawrrence et al. 1988; Uthayakumaran et al. 2002; Beasley et al. 2002; Liu et al. 2005; Zhang et al. 2009; Jin et al. 2013). In contrast, the indirect contributions of *Glu-1* loci to quality-related parameters via interacting with the loci encoding other types of gluten proteins (gliadins and LMW-GSs) have not been well investigated in the past. In this work, we uncovered that *Glu-1* loci differed in their indirect effects on gluten functionality and that this difference appeared to correspond to the different capacity of the three loci to affect the accumulation levels of gliadins and LMW-GSs (see above). This may represent an important direction for more systematically studying the indirect contributions of *Glu-1* loci to gluten functionality in further research.

In summary, this study illustrates that ion beam mutations can be efficiently identified, mapped and deployed for comparative functional analysis of homoeologous loci in common wheat. As genomic information is obtained for more and more polyploid plants (Feuillet et al. 2011; Brenchley et al. 2012; Paterson et al. 2012), the screening and utilization of ion beam mutants may become a valuable approach for revealing functional difference among homoeologous loci. The set of *Glu-1* deletion lines described here are useful genetic stocks for more advanced investigations of *Glu-1* loci in the future, especially for dissecting the molecular and biochemical mechanisms underlying the indirect contributions of *Glu-1* loci to gluten functionality and end-use quality.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments described in this work were all conducted in compliance with the current laws of the Chinese government.

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