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ORIGINAL PAPER

# The detection of a de novo allele of the *Glu-1Dx* gene in wheat–rye hybrid offspring

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**Abstract**

***Key message*** **This study provides a link between a de novo gene and novel phenotype in wheat–rye hybrids that can be used as a model for induced de novo genetic variation.**

*Abstract* Wide hybridization can produce de novo DNA variation that may cause novel phenotypes. However, there is still a lack of specific links between changed genes and novel phenotypes in wide hybrids. The well-studied high-molecular-weight glutenin subunit (HMW-GS) genes in tribe Triticeae provide a useful model for addressing this issue. In this study, we investigated the feasibility of a wheat–rye hybridization method for inducing de novo phenotypes using the *Glu-1Dx2.2* subunit as an example. We developed three hexaploid wheat lines with normal fertility and a *Glu-1Dx2.2* variant, named *Glu-1Dx2.2*$^{v}$, derived from three $F_1$ hybrids. The wild-type *Glu-1Dx2.2* has two direct repeats of 295 bp length separated by an intervening 101 bp in its central repetitive region. In the mutant *Glu-1Dx2.2*$^{v}$, one copy of the repeats and the intervening sequence were deleted, probably through homology-dependent illegitimate recombination (IR). This study provides a direct link between a de novo allele and novel phenotype. Our results indicate that the wheat–rye method may be a useful tool to induce de novo genetic variations that broaden the genetic diversity for wheat improvement.

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## Introduction

Inter-specific hybridization can combine divergent genomes into one nucleus. It is very important for speciation, as chromosome doubling of wide hybrids is responsible for the origin of many allopolyploid species. Also, repeated backcrossing of wide hybrids to their parental species has contributed to the evolution of some species by gene introgression (Stebbins 1971; Arnold 1997). Wide hybridization has been used as an important tool for chromosome manipulation (or chromosome engineering) in crop improvement to incorporate alien chromosomes or chromosome fragments (Liu et al. 2014). Most pioneering efforts in chromosome engineering have involved the *Triticum* species in Triticeae, with the greatest emphasis being placed on improving common wheat (*T. aestivum* L., $2n = 6x = 42$, AABBDD) (Crouch et al. 2009; Qi et al. 2007; Reynolds et al. 2009; Wang 2009). Common wheat is an important cereal grain crop that provides nearly 20 % of the calories and protein consumed by the world's population (Hawkesford et al. 2013). It originated about 8,500 years ago via natural wide hybridization of *T. turgidum* L. ($2n = 4x = 28$, AABB) with *Aegilops tauschii* Cosson ($2n = 2x = 14$, DD) (Feldman 2001; Kihara 1944; McFadden and Sears 1946). In plants, it has been reported that wide hybrids may be associated with rapid genomic changes in comparison with their parents (Chen 2007; Feldman and Levy 2009; McClintock

1984). In tribe Triticeae, it was suggested that hybridization of wheat with rye (*Secale cereale* L., $2n = 2x = 14$, RR) might induce a high rate of DNA sequence changes (Ma and Gustafson 2008; Schwarzacher et al. 2011). This implies a new use for wide hybridization since de novo DNA variation may alter gene expression and produce novel phenotypes for wheat breeding. However, there is still a lack of specific links between changed genes, proteins and novel phenotypes in wide hybrids (Finigan et al. 2012). Providing such a link is difficult because of the complex polyploid structure of wheat hybrids, which have many orthologous (highly similar but non-allelic) sequences in their homoeologous genomes. Distinguishing orthologous genes among hybrid genomes from each other is complicated.

The well-studied high-molecular-weight glutenin subunit (HMW-GS) genes provide a useful model for the study of genetic variation induced by wide hybridization (Yuan et al. 2011). HMW-GSs are storage proteins specifically expressed in the endosperm of bread wheat and related species that are important determinants of the processing and bread-making quality of wheat flour (Shewry et al. 1995). In wheat, HMW-GSs are encoded by the loci *Glu-1A, Glu-1B* and *Glu-1D* on chromosomes 1A, 1B and 1D, respectively (Payne 1987; Shewry et al. 1992). Their duplicate origins have led to the presence of two tightly linked paralogous genes at each locus (x-type and y-type) that are separated by the insertion of retrotransposons (Kong et al. 2004). Although the six HMW-GS genes (*Glu-1Ax*, *Glu-1Ay*, *Glu-1Bx*, *Glu-1By*, *Glu-1Dx* and *Glu-1Dy*) in wheat have similar primary structures, alleles can be differentiated by separation of gene products by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) or DNA sequence comparison. Genes of HMW-GSs are co-dominant, which allows SDS-PAGE analysis to discriminate heterogeneous and homogeneous protein forms. A previous study on wheat–rye hybrids detected novel HMW-GS phenotypes and indicated that DNA changes via illegitimate recombination were responsible for the production of a novel HMW-GS at the *Glu-1Ax* locus (Yuan et al. 2011). However, this preliminary work only involved $F_1$ and $F_2$ hybrids, and the sterility of the analyzed plants prevented subsequent detailed studies (Yuan et al. 2011).

Subunit 1Dx2.2 is a rare *Glu-1Dx* allele in wheat worldwide, and is mainly distributed in Japanese wheat (Nakamura and Fujimaki 2002). It is one of the largest HMW-GSs in wheat because it has an extra-large central repetitive region (Wan et al. 2005). It can be clearly differentiated from other subunits because of its much lower mobility in SDS-PAGE. This makes it a desirable 'marker' to study genetic variation induced by wide hybridization. In this study, we made a systematic genetic study of *Glu-1Dx2.2* variants by analyzing the $F_1$–$F_6$ generations from three independent wheat–rye hybrid families. We found a direct link between a de novo HMW-GS allele and a novel HMW-GS phenotype. We developed new wheat lines with the novel *Glu-1Dx*. Our results indicate that wheat–rye hybrids can be used as a model for induced de novo genetic variation that is helpful for broadening the genetic diversity for wheat improvement.

## Materials and methods

### Plant materials

Japanese common wheat (*T. aestivum* L., $2n = 6x = 42$, AABBDD) cv. Shinchunaga and Chinese rye (*S. cereale* L., $2n = 2x = 14$, RR) landrace Qinling were used in this study. Shinchunaga was kindly provided by Dr. Shin Taketa of the Barley Germplasm Center, Research Institute for Bioresources, Okayama University, Japan. Qinling came from the germplasm collection at Sichuan Agricultural University. In their HMW-GSs, Shinchunaga has a null allele at *Glu-1A*, 1Bx7 + 1By8 at *Glu-1B*, and 1Dx2.2 + 1Dy12 at *Glu-1D* (Nakamura 2001), and Qinling has Rx + Ry at *Glu-1R* (Yuan et al. 2011).

### Hybrid production and selection of 1Dx2.2 variants

$F_1$ hybrids between Shinchunaga wheat as the female parent and Qinling rye were produced as described previously (Yuan et al. 2011). No embryo rescue technique or hormone treatment was applied when producing the $F_1$ hybrids to prevent variation caused by chemical treatment. Hybrid plants were continuously selfed. During this process, hybrids and their parents Shinchunaga and Qinling were planted in a plot for each year, isolated from other wheat materials. Since $F_3$ generations, hybrids with variant 1Dx2.2 subunit and with high seed set of over 100 seeds were retained and descended to following generations until new wheat lines with homozygous HMW-GSs and normal fertility were obtained.

Each of the hybrid seeds from different generations was cut in half. The halves with the embryos were germinated in Petri dishes and the resulting seedlings were transplanted into an experimental plot 20 cm apart with 30 cm row spacing. Leaf samples of individual hybrid plants at the heading stage were collected and used for DNA isolation. The other halves with enough endosperm were used to identify the HMW-GS combinations by SDS-PAGE (Wan et al. 2000; Yuan et al. 2011).

### Cytological observation

Observations of chromosome number in root-tip cells and chromosome pairing in pollen mother cells (PMCs)

in the hybrids were done as described previously (Zhang et al. 2007). Eight plants from each line were used for chromosome counting. About 20 PMCs from each line were observed for chromosome pairing. Genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) were done as described previously (Hao et al. 2011, 2013). Clones containing the repeat sequences pSc119.2 (Contento et al. 2005) and pTa71 (Fujisawa et al. 2006) were used as probes in FISH analysis. Chromosome observation results were recorded with a BX-51 microscope (Olympus, Tokyo, Japan) coupled with a Photometric SenSys Olympus DP70 CCD camera.

### Cloning and sequencing of the HMW-GS genes

DNA extraction from seedlings, PCR amplification and gene cloning were done as described previously (Yuan et al. 2011). A pair of universal primers (PF1: 5′-ATCAC CCACAACACCGAGCA-3′; PR1: 5′-AGCTGCAGAGA GTTCTATCA-3′) was used to amplify the complete coding region of each HMW-GS gene (Xie et al. 2001). The full-length sequence of the coding region was obtained by sequencing a set of subclones, which were made by the nested deletion method (Yan et al. 2002). To avoid sequencing errors, the final sequences were verified using at least three clones.

### Expression of HMW-GS genes in *E. coli*

To remove the signal peptides of the cloned HMW-GS alleles, the primer pair 5′-ACCCATATGGAAGGTGAGG CCTCTGGGC-3′ and 5′-TTCCTCGAGCTATCACTGG CTGGCCAAC-3′ (*Nde*I or *Xho*I restriction site underlined) for *Glu-1Dx* was used. The modified PCR products were cloned into the expression vector pET-30a (Novagen, Beijing, China) and transformed into *E. coli* strain BL21 (DE3) pLsS. Expression of HMW-GSs in *E. coli* was induced by 1 mM IPTG (isopropyl b-D-thiogalactopyranoside) for 5 h when the cell concentration reached $OD_{600} = 0.6$. Protein samples extracted by the general extraction method (Wan et al. 2005) and the acetone sedimentation method specific for HMW glutenins (Verbruggen et al. 1998) were then used for SDS-PAGE analysis.

### DNA, RNA and protein analysis of immature seeds

After removing the embryos, endosperm tissues at 20 days post-anthesis (DPA) were collected from three or four immature seeds for each line and immediately frozen in liquid nitrogen. RNA, DNA and proteins were extracted from the endosperm tissues using TRIzol® reagent (Invitrogen, Beijing, China) according to the user's manual. Contaminating DNA in the total RNA was removed by treatment with DNase. The first-strand cDNA was synthesized using 2 μg RNA and 200 U M-MLV reverse transcriptase (Invitrogen Kit) in a total volume of 20 μl following the manufacturer's instructions. RNase H was used to remove RNA from RNA/DNA hybrid strands as cDNA synthesis proceeded. A pair of primers specific for the complete coding region of the *Glu-1Dx* gene (DxF: 5′-ATGGCTAAGCGG TTAGTCCT-3′; DxR: 5′-CTGGCTGGCCGACAATG CGT-3′) was used for DNA and cDNA analysis as described previously (Yuan et al. 2011).

## Results

### Development of wheat lines with normal fertility and a novel *Glu-1Dx*

Bread wheat cultivar Shinchunaga was used as the female parent in crossing with Chinese rye landrace Qinling. We obtained 2,068 $F_1$ seeds with the co-dominant subunits of their parents, i.e., 1Bx7 + 1By8 at locus *Glu-1B*, 1Dx2.2 + 1Dy12 at *Glu-1D* and Rx + Ry at *Glu-1R* (Fig. 1$F_1$). A total of 136 vigorously growing $F_1$ plants were obtained from random 707 $F_1$ seeds used for germination. Only 26 of them were partially fertile and produced 75 $F_2$ seeds. However, the $F_1$ plant A showed a high seed-set level and produced 26 $F_2$ seeds, thus indicating that it produced a high frequency of functional gametes. Plant B set two and C set one seed. Their three $F_2$ plants A-2, B-1 and C-4 set 117, 35 and 28 $F_3$ seeds, respectively. The $F_3$ plants A-2-23, B-1-2 and C-4-1 showed good fertility and each produced more than 100 seeds. Out of the randomly identified three $F_4$ seeds from plant A-2-23, two had 41 wheat + 1 rye chromosomes and one had 41 wheat + 2 rye + 1 wheat–rye translocation chromosomes. This suggested the quick elimination of rye chromosomes during the selfing process. The loss of chromosomes carrying HMW-GS genes would result in the absence of HMW-GSs in selfed progenies. Therefore, we focused on the appearance of novel subunits rather than subunit loss in subsequent screening.

Out of the 75 $F_2$ seeds, we observed a novel subunit near Rx in seed C-4 (Fig. 1$F_2$). Because of the existence of 1Dy12, we speculated that this new subunit was a variant of 1Dx2.2, so we named it 1Dx2.2$^v$. Out of the analyzed 20 $F_3$ seeds derived from the plant C-4, five had 1Dx2.2$^v$, two had 1Dx2.2, and 13 had both of 1Dx2.2$^v$ and 1Dx2.2. The highly fertile $F_3$ plant C-4-1 had subunit 1Dx2.2$^v$ (Fig. 1$F_3$). Although $F_2$ seeds A-2 and B-1 showed the wild-type HMW-GSs (Fig. 1$F_2$), a new subunit similar to 1Dx2.2$^v$ was observed in their some $F_3$ seeds, such as A-2-23 and B-1-2 (Fig. 1$F_3$). Out of the analyzed 41 $F_3$ seeds derived from plant A-2, 12 had 1Dx2.2$^v$, 16 had 1Dx2.2, and 13 had both of them. Out of 16 $F_3$ seeds from plant B-1, two had

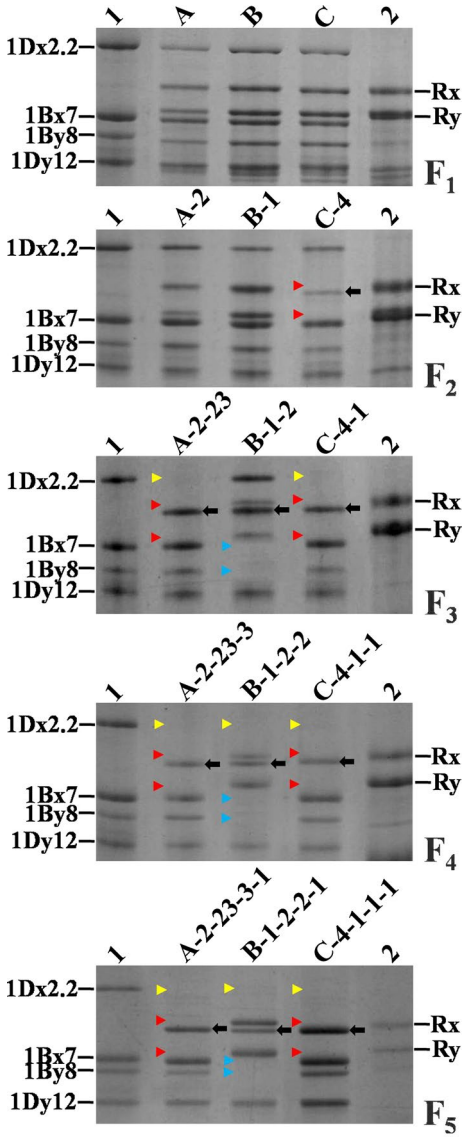


**Fig. 1** HMW-GS identification in $F_1$–$F_5$ seeds of hybrids between Shinchunaga wheat and Qinling rye. *1* Shinchunaga wheat; *2* Qinling rye. *Red*, *yellow* and *blue triangles* show the positions of missing Rx + Ry, 1Dx2.2 and 1Bx7 + 1By8 subunits, respectively; *black arrowheads* show the position of the novel subunit

1Dx2.2$^v$, six had 1Dx2.2, and eight had both of them. The $F_3$ plant C-4-1, A-2-23 and B-1-2 with the novel 1Dx2.2$^v$ showed high fertility and their novel subunits were further advanced to $F_4$ (Fig. 1$F_4$) and $F_5$ (Fig. 1$F_5$). At the $F_5$ generation, the three plants A-2-23-3-1, B-1-2-2-1, and C-4-1-1-1 had normal fertility and 42 chromosomes (Fig. 2a–c) that usually paired into 21 bivalents at meiotic metaphase I (Fig. 2d–f). A-2-23-3-1 and C-4-1-1-1 had subunit constitutions of 1Bx7 + 1By8 and 1Dx2.2$^v$ + 1Dy12, while B-1-2-2-1 had 1Dx2.2$^v$ + 1Dy12 and Rx + Ry (Fig. 1$F_5$), inherited from their $F_4$s (Fig. 1$F_4$). The HMW-GS loci in these $F_5$ plants were homologous since no genetic segregation was observed in 80 randomly analyzed $F_6$ seeds derived from each of these three $F_5$ plants.

Cytological analysis indicated that the absence of Rx + Ry at locus *Glu-1R* in A-2-23-3-1 and C-4-1-1-1, and 1Bx7 + 1By8 at *Glu-1B* in B-1-2-2-1 was caused by the loss of 1R and 1B, respectively. No rye chromosome was detected in A-2-23-3-1 or C-4-1-1-1 by GISH analysis. The absence of Rx + Ry was first observed in the $F_2$ seed C-4 (Fig. 1$F_2$) and the $F_3$ seed A-2-23 (Fig. 1$F_3$). However, B-1-2-2-1 had a pair of rye chromosomes that usually formed a rod bivalent at MI (Fig. 2g, h). FISH analysis indicated that it was a 1B/1R substitution line because of the presence of FISH signals for rye chromosome 1R and the absence of signals for wheat chromosome 1B (Fig. 2i). The absence of 1Bx7 + 1By8 was first found in the $F_3$ seed B-1-2 (Fig. 1$F_3$).

## Genetic basis for the appearance of the novel subunit 1Dx2.2$^v$

To clarify the genetic basis for the appearance of the novel subunit 1Dx2.2$^v$ near Rx, we amplified the HMW-GS genes from the genomic DNA of the three $F_5$ plants, their corresponding $F_1$–$F_4$ plants and their parents using a pair of universal primers for HMW-GS genes. The PCR products were then cloned and sequenced. Based on similarity comparison conducted with the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov), we determined the gene sequences for *Glu-1Ax null*, *Glu-1Ay null*, *Glu-1Bx7*, *Glu-1By8*, *Glu-1Dx2.2*, and *Glu-1Dy12* in the wheat parent Shinchunaga and *Glu-Rx* and *Glu-Ry* in the rye parent Qinling (Table 1). The homologous HMW-GS sequences from the wheat–rye derivatives were then compared with the HMW-GSs from their parents. Except for *Glu-1Dx*, there was no difference in the HMW-GS genes between the wheat–rye derivatives and their parents. Compared with *Glu-1Dx2.2* of Shinchunaga, *Glu-1Dx2.2$^v$* in the three $F_5$ plants A-2-23-3-1, B-1-2-2-1 and C-4-1-1-1 had a 396 bp deletion at positions 953–1,348 bp of the central repetitive region (Fig. 3). This deletion fragment was flanked by two direct repeat sequence (DR) units with a length of 295 bp at 953–1,247 bp and 1,349–1,643 bp (Fig. 3). The novel *Glu-1Dx2.2$^v$* had a complete open reading frame (ORF) that was same for the analyzed three $F_5$ plants A-2-23-3-1, B-1-2-2-1 and C-4-1-1-1 (GenBank number KC881262).

We traced the novel gene *Glu-1Dx2.2$^v$* from the earlier generations ($F_1$–$F_4$) of the three $F_5$ plants by sequence comparison. Subunit 1Dx2.2$^v$ was first observed in the C-4 $F_2$ seeds (Fig. 1$F_2$), while its DNA sequence was first detected in leaf DNA samples from the C-4 $F_2$ plant at the heading stage, co-existing with the wild-type *Glu-1Dx2.2*. This suggested that the new allele occurred in the $F_1$ generation of plant C, either during meiosis or mitosis during

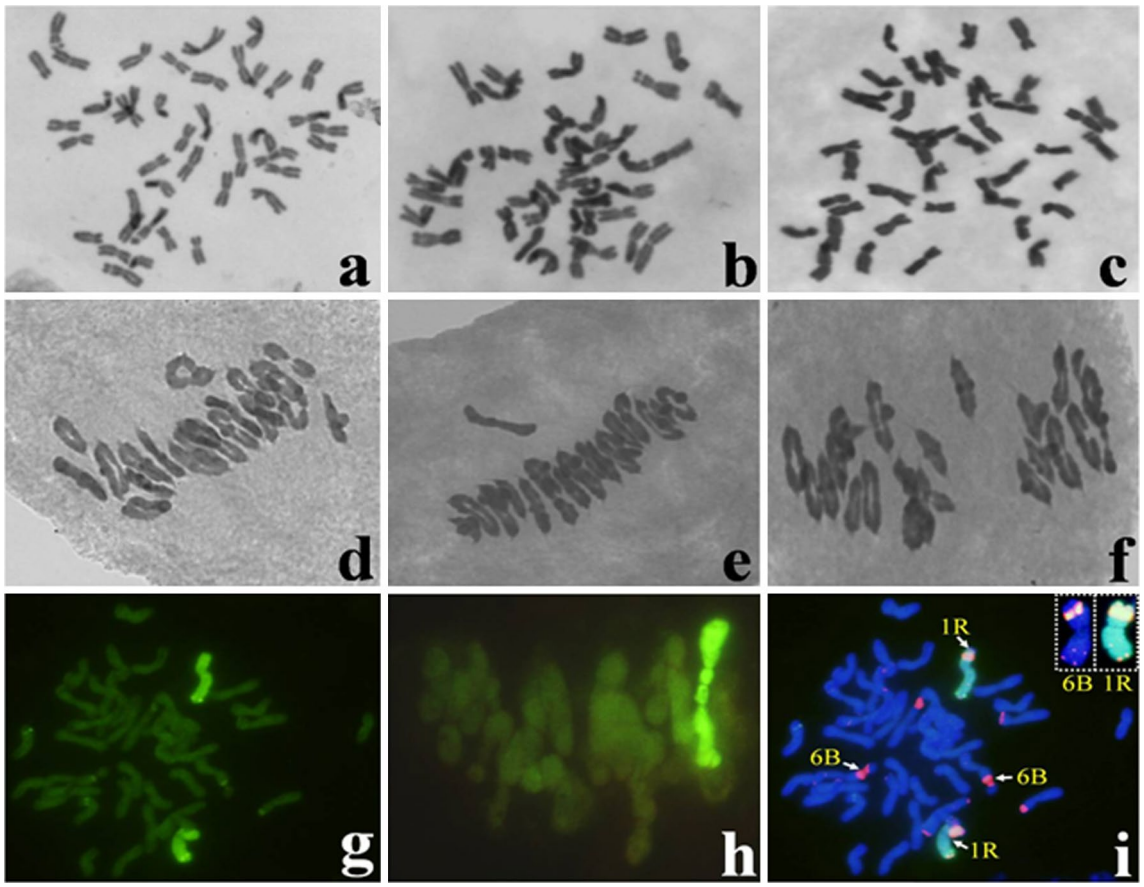


**Fig. 2** Cytological observations. The 42 chromosomes in root-tip cells of lines A-2-23-3-1 (**a**), B-1-2-2-1 (**b**) and C-4-1-1-1 (**c**). The 21 pairs of chromosomes in pollen mother cells at meiotic metaphase I of A-2-23-3-1 (**d**), B-1-2-2-1 (**e**) and C-4-1-1-1 (**f**). Pairs of rye 1R chromosomes in B-1-2-2-1, revealed by GISH on a root-tip (**g**) and pollen mother cell (**h**), and FISH analysis (**i**)

**Table 1** Sequence information for HMW-GS genes obtained from the parents

| HMW-GS gene | Length (bp) | GenBank number |
|---|---|---|
| *Glu-1Ax null* | 2,496 | HQ613179 |
| *Glu-1Ay null* | 1,752 | JF736012 |
| *Glu-1Bx7* | 2,391 | JF736013 |
| *Glu-1By8* | 2,166 | JF736014 |
| *Glu-1Dx2.2* | 2,919 | JF736015 |
| *Glu-1Dy12* | 1,980 | JF736016 |
| *Glu-Rx* | 2,268 | GU373813 |
| *Glu-Ry* | 2,286 | GU373814 |

gametogenesis. Although the *Glu-1Dx2.2*$^{v}$ gene also co-existed with *Glu-1Dx2.2* in the $F_2$ plant A-2, subunit 1Dx2.2$^{v}$ was first observed in its some $F_3$ seeds (Fig. 1$F_3$). This suggested that the new allele might originate in its $F_2$ plant. Considering the complex of inter-generic hybrids, however, we cannot exclude the possibility of out-crossing for the appearance of the new allele. If the new allele in $F_2$ plant A-2 was obtained by out-crossing between $F_1$ plant A and another hybrid with *Glu-1Dx2.2*$^{v}$ (such as plant C), the failure detection of subunit 1Dx2.2$^{v}$ in the $F_2$ seed A-2 might be caused by changes at the epigenetic level that have been indicated in some inter-specific hybrids (Chen 2007). Subunit 1Dx2.2$^{v}$ was also observed in $F_3$ seeds from its $F_2$ plant B-1 (Fig. 1$F_3$), but its DNA sequence was first detected in $F_3$ generation. This suggested that the new allele occurred in the $F_2$ generation of plant B-1 although we cannot totally exclude the possibility of out-crossing for the appearance of the new allele in this lineage.

## Expression of subunit 1Dx2.2$^{v}$ in *E. coli* and developing endosperms

The authenticity of the cloned *Glu-1Dx2.2*$^{v}$ was confirmed by bacterial expression of its coding region in *E. coli*. After removing the signal peptides, *Glu-1Dx2.2*$^{v}$ was inserted into the expression vector pET-30a, and the resultant plasmid construct *pET-30a-Glu-1Dx2.2*$^{v}$ was obtained.

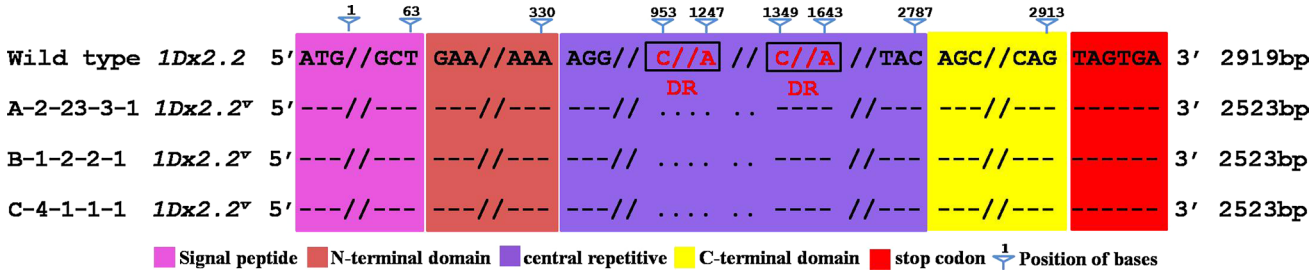


**Fig. 3** Sequence comparison between *Glu-1Dx2.2* and its mutant *Glu-1Dx2.2*$^{v}$. Deleted nucleotides are indicated with a *dot* and identical nucleotide sequences are shown with a *dash*. Direct repeat (DR) sequences are marked by *black boxes*

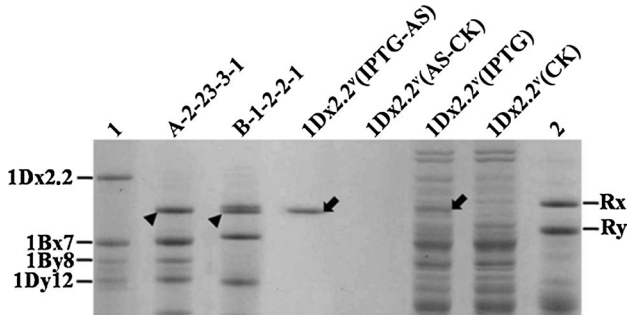


**Fig. 4** *E. coli* expression analysis of *Glu-1Dx2.2*$^{v}$. *1* Shinchunaga wheat; *2* Qinling rye. *1Dx2.2*$^{v}$ *(IPTG-AS) E. coli* cultured with isopropyl β-Δ-thiogalactopyranoside (IPTG) and protein extracted using acetone sedimentation (AS). *1Dx2.2*$^{v}$ *(IPTG) E. coli* cultured with IPTG and protein extracted using general extraction. *1Dx2.2*$^{v}$ *(CK) E. coli* cultured without IPTG. *Black arrowheads* the expressed *Glu-1Dx2.2*$^{v}$ protein from *E. coli* culture; *black triangles Glu-1Dx2.2*$^{v}$ extracted from endosperm

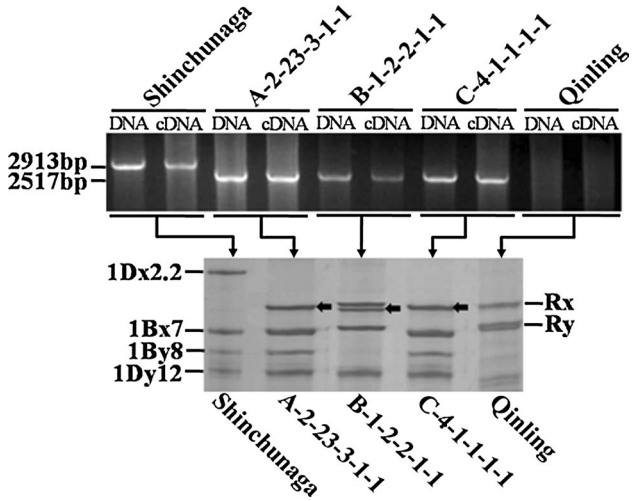


**Fig. 5** Expression and protein analysis of *Glu-1Dx2.2*$^{v}$ in $F_6$ seed endosperm. *Black arrowheads* 1Dx2.2$^{v}$

Protein samples from cultures of the transformed *E. coli* with and without inducer IPTG and from hybrid seeds were assayed by SDS-PAGE (Fig. 4). This assay showed that *Glu-1Dx2.2*$^{v}$ encoded a similar protein to the speculated novel Dx2.2$^{v}$ in the endosperm of the hybrid seeds (Fig. 4).

To determine whether the novel gene *Glu-1Dx2.2*$^{v}$ could be transcribed into messenger RNA (mRNA) and then translated into a protein in plant organs, we extracted DNA, mRNA, and protein from the endosperm of immature seeds at 20 days post-anthesis (DPA), when the HMW-GS genes have a high transcript level (Wegel et al. 2005). A pair of primers specific for *Glu-1Dx* was used to amplify DNA and cDNA from $F_6$ seeds A-2-23-3-1-1, B-1-2-2-1-1, and C-4-1-1-1-1, derived from the corresponding $F_5$ plants A-2-23-3-1, B-1-2-2-1, and C-4-1-1-1. The DNA and cDNA sequences from the $F_6$ immature seeds were the same, thus showing that the novel gene *Glu-1Dx2.2*$^{v}$ could be transcribed into mRNA (Fig. 5). We also observed subunit 1Dx2.2$^{v}$ in these immature $F_6$ seeds by SDS-PAGE analysis.

## Discussion

### The fertility of $F_1$ hybrids and following elimination of rye chromosomes

Inter-specific hybridization brings divergent genomes from different species together in amphihaploid (analogous to haploid) $F_1$ hybrids. Because only one set of homologous chromosomes is present, amphihaploids are usually sterile due to irregular meiosis. However, functionally unreduced gametes can be produced in amphihaploids by first division restitution (FDR) that occurs because of an equational division with segregation of sister chromatids of univalents (Ramanna and Jacobsen 2003), and their union immediately generates an amphidiploid. Silkova et al. (2011) found that in some anthers of bread wheat–rye $F_1$ hybrids, 80–100 % of microsporocytes displayed equational division. The percentage of dyads can also reach 80–100 % although these hybrids still showed a low seed-set rate. Meanwhile, they also observed a high frequency

of equational + reductional pathway in some anthers, in which some chromosomes divided in an equational manner while others were segregated reductionally within the same microsporocyte. If this pathway results in functional gametes, the resultant seeds should be partial amphidiploids. In the present study, we obtained 75 $F_2$ seeds from 136 $F_1$ plants, ranged from 0 to 26 with a mean of 0.55 seed per plant. Since data on chromosome constitution of $F_2$ seeds are unavailable, it is unclear if the production of some $F_2$ seeds were related to equational + reductional pathway.

Some primary octoploid triticales show meiotic instability and low fertility. Hexaploid wheat can spontaneously appear in the selfing progenies of octoploid triticale (see review by Gupta and Priyadarshan 1982). In octoploid triticales, since many lines exhibiting univalents tend to revert back to hexaploid wheat, it was speculated that the univalents should predominantly belong to rye chromosomes that are eliminated (Gupta and Priyadarshan 1982). The data on chromosome constitutions of the three $F_2$ seeds chosen in this study are unavailable. If they were octoploid, a question is how long they took to revert back to hexaploid wheat since we obtained hexaploid wheat in $F_5$. Although the strong selection on fertility may accelerate the process, the reversion from octoploid triticale to hexaploid wheat is expected to need more generations. However, if the hybrids were further out-crossed with wheat, rye chromosomes became monosomes that are more prone to elimination in selfed offspring. A quick reversion may also have occurred if equational + reductional pathway resulted in $F_2$s that contained all or most of wheat chromosomes and monosomic rye chromosomes. A further study is still needed to illustrate if equational division mainly occurs in wheat chromosomes for equational + reductional pathway in wheat–rye $F_1$ hybrids.

## Genetic distance may be critical for inducing novel HMW-GSs in hybrids

Wheat HMW-GSs share two conserved, non-repetitive domains at both their N and C termini, separated by an extensive central repetitive region. The repetitive domain is composed of repeat motifs including tripeptide, hexapeptide and nonapeptide. The difference of molecular masses among HMW-GSs is mainly resulted from variation in the number of repeat motifs in the repetitive domain (Shewry et al. 1995). To our best known, however, there is no study focusing on the rate of spontaneous mutation in repetitive domain of HMW-GSs in wheat although this kind of mutation is responsible for the origination of different subunits. In our a previous study on analyzing 89 bread wheat landraces native to Sichuan, China, no variation at locus *Glu-D1* was observed although these landraces had been planted in Sichuan for a long history (Wei et al. 2000). All these landraces, including Chinese Spring that is thought to originate from Sichuan (Sears and Miller 1985), have 2 + 12 at this locus. This result suggested that in natural population of bread wheat, the spontaneous mutation by the number change of repeat motifs in the repetitive domain that results in new HMW-GSs is very low.

As McClintock (1984) speculated, putting distinct genomes into the same nucleus via wide hybridization can create a major 'genome shock', leading to significant genomic restructuring in the hybrid. In the present study, we used the wheat *Glu-1Dx2.2* gene as an example to demonstrate that wide hybridization of common wheat with rye induced de novo genetic variation. The appearance of novel HMW-GSs in wide hybrids has been reported before. For instance, Ko et al. (2004) found a novel HMW-GS in common wheat line Yw62-11 that was derived from a hybrid of Olmil wheat and Paldanghomil rye and possessed the 1BL/1RS translocation. Han et al. (2004) found a novel HMW-GS subunit in partial amphiploid lines derived from wheat × *Thinopyrum intermedium* hybridization. Jiang et al. (2014) recently found a novel HMW-GS in amphiploids between tetraploid wheat and *Aegilops sharonensis* Eig. Novel HMW-GS alleles were also generated by asymmetric somatic hybridization (Gao et al. 2010). However, the HMW-GS patterns of a large number of newly synthesized hexaploid wheat lines were investigated, but no novel subunit was found (Hu et al. 2013; Zhang et al. 2012; unpublished data). Synthetic hexaploid wheat has the same genome constitution as bread wheat and was remade into bread wheat by artificial crosses of tetraploid *Triticum* wheat with *Ae. tauschii*, the two parent species of bread wheat. This suggested that genome combination or genetic distance between the parental genomes may play an important role in inducing changes of HMW-GS genes in hybrids. It has been reported that wheat–rye hybrids have a higher rate of sequence change than *T. turgidum–Ae. tauschii* hybrids. For example, microsatellite (SSR) sequences are highly conserved in *T. turgidum–Ae. tauschii* hybrids (Luo et al. 2012; Mestiri et al. 2010), while they are prone to mutation in wheat–rye hybrids (Tang et al. 2009). Methods used to assess DNA variation based on methyl-sensitive or non-sensitive restriction enzymes also revealed higher variation in wheat–rye (Ma and Gustafson 2008; Schwarzacher et al. 2011) than in *T. turgidum-Ae. tauschii* hybrids (Liu et al. 1998; Ozkan et al. 2001; Shaked et al. 2001). It seems that the "wheat–rye" method provides a useful tool for de novo variation.

## Homology-dependent illegitimate recombination may lead to novel HMW-GSs

Illegitimate recombination (IR) is an important recombination mode that has been largely reported in angiosperms. It is

considered to have played an important role in genome evolution in nature (Bennetzen 2002, 2007; Chantret et al. 2005; Devos et al. 2002; Wicker et al. 2003). Our results indicated that a spontaneous deletion in wheat–rye hybrids resulted in the production of the novel gene *Glu-1Dx2.2*$^{v}$. The wild-type *Glu-1Dx2.2* has two direct repeats of 295 bp in length that are separated by an intervening 101 bp. The presence of flanking direct repeat units is typical evidence or a ''signature'' of a homology-depended IR event (Wicker et al. 2007). IR occurred between the direct repeats resulting in the novel gene *Glu-1Dx2.2*$^{v}$ through the deletion (396 bp) of one copy of the repeat plus the intervening sequence. In a previous study, we found that IR led to the mutation of a *Glu-1Ax* gene, although the mutant could not be passed to progenies because the $F_2$ hybrids were sterile (Yuan et al. 2011).

Since we cannot totally exclude the possibility of outcrossing for the appearance of a same mutant in three lineages, it is unclear if the 396-bp deletion via IR was repeatable. Previous studies have also demonstrated that nonrandom, repeatable and directed sequence elimination can occur in *Aegilops–Triticum* (Liu and Wendel 2002) and *Nicotiana tabacum* (Renny-Byfield et al. 2012), although the underlying mechanisms are still unknown. In this study, however, we should point out that we only focused on analyzing HMW-GSs with obvious changes in electrophoretic mobility compared with the wild type. Therefore, we might have ignored subunit variations with slight changes. More variations with short sequence changes might be expected since HMW-GS genes have a long central repetitive region that is composed of repeated units. The occurrence of illegitimate recombination requires only a few base pairs of direct repeat sequence identity to trigger.

## The potential value of wide hybridization: a tool for enhancing genetic diversity

In the past, common wheat breeders have essentially utilized variations provided by wheat's original hybridization and subsequent natural mutations that occurred over the millennia as it spread around the globe, as well as gene flow from *T. turgidum* to bread wheat (Dvorak et al. 2006). To develop new wheat cultivars, breeders combine variations dispersed among different wheat parents into the offspring. However, breeders are finding less and less appropriate variations with desirable traits among currently existing wheat with which to make the improvements needed to meet increasing worldwide demand. One strategy for solving this problem is to transfer desirable genes from ancestral or related species into modern wheat (Crouch et al. 2009; Qi et al. 2007; Reynolds et al. 2009; Wang 2009). However, the process for transferring useful alien chromosome fragments is cumbersome and usually combined with undesirable genes. This study indicates that wheat–rye hybridization presents a potential method to broaden the genetic diversity of wheat. The novel allelic variation at the *Glu-1Dx* locus induced by wheat–rye hybridization was kept in new wheat lines. However, it is unclear at the present time how efficient the 'wheat–rye' method may be in inducing de novo genes controlling other agronomic traits.

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**Ethical standard** The experiments reported here comply with the current laws of the countries in which they were performed.

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