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ORIGINAL PAPER

Generation of high frequency of novel alleles of the high molecular weight glutenin in somatic hybridization between bread wheat and tall wheatgrass

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Abstract Somatic hybridization between bread wheat and tall wheatgrass (*Agropyron elongatum*) has generated fertile introgression progenies with novel combinations of high molecular weight glutenin subunits (HMW-GS). Most of these novel HMW-GS alleles were stably inherited. Sixteen HMW-GS sequences were PCR amplified from three introgression progeny lines and sequenced. The alignment of these sequences indicated that five, probably derived from point mutations of the parental genes, whereas eight likely represent the product of replication slippage. Three *Glu-1Ay* sequences appear to have lost the transposon presented in the parental gene. Two subunits carry an additional cysteine residue, which may be favorable to the quality of end-use product. We demonstrate that novel HMW-GS alleles can be rapidly generated via asymmetric somatic hybridization.

Introduction

The high molecular weight glutenin subunits (HMW-GS) of bread wheat, which are the major determinants of dough viscoelasticity (Shewry et al. 2003a), are encoded by the *Glu-1* homoeoloci located on the long arms of the homoeologous group 1 chromosomes (Payne et al. 1980). Each locus comprises two genes, one producing the x-type and the other the smaller y-type subunit (Harberd et al. 1986). Their peptide sequences comprise three domains, two of which are highly conserved (the terminal N and C domains), while the other (the central domain) is variable both with respect to sequence and length. Both the x- and y-type central domains are characterized by highly repetitive hexa- and nonapeptide motifs, while the x-type subunits also contain tripeptide motifs. The structural similarity between the x- and y-types suggests that they evolved from a common ancestral gene (Shewry and Tatham 1990; Shewry et al. 2003b). The simplest mechanism suggested for this evolution involves a combination of single base changes, intra-repeat indels, single repeat changes and deletions or duplications of blocks of repeats (Anderson and Greene 1989). The impact of the HMW-GS on the processing quality of wheat dough is due to both quantitative differences in overall HMW-GS content, and qualitative effects associated with differences in polypeptide structure. A genetic approach has led to an assignment of a "glutenin score" to each of the various subunit combinations, and this is partially predictive of the breadmaking quality of the flour (Shewry et al. 2003a). In particular, the subunit pair 1Dx5 + 1Dy10 is the most favorable gene product of *Glu-D1*, and 1Bx17 + 1By18 is the best-performing *Glu-B1* product (Shewry et al. 2003a). However, even though the HMW-GS are such important determinants of processing quality, the number of subunits associated with good quality remains rather limited.

The tall wheatgrass *Agropyron elongatum* is a decaploid ($2n = 70$) species related to wheat, and possesses a number of traits which would be beneficial for wheat improvement. These include high seed protein content and a range of biotic and abiotic stress tolerance. While the species can be sexually hybridized with wheat, an asymmetric somatic hybridization approach, in which bread wheat protoplasts were fused with UV-irradiated tall wheatgrass protoplasts,

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has proven to be an effective means of obtaining introgression (Xia et al. 2003; Liu et al. 2006). A number of these materials have been stabilized by advancing to late generations, and this process has allowed the effect of the introgression on plant phenotype to be assessed. Type I plants are lax and tall with an enlarged spike and a reduced number of tillers; type II plants are erect and short with smaller spikes and many tillers; and type III plants are erect and compact and have taller stems and larger spikes than type II. The HMW-GS of most types I and III plants are fixed, but in type II plants, it appears to be less stable. Because allelic variation among the HMW-GS is so highly correlated with flour quality, this segregating material is of particular interest, both as a source of new alleles and as a means of investigating the mechanism(s) underlying the creation of allelic variation. The objective of this study was to characterize the novel Glu-1 alleles generated from F_5 to F_8 progeny of a type II line, designated II-11-4.

Materials and methods

Plant material

The HMW-GS of F_5–F_8 progeny of line II-11-4 was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the following lines were selected: II-11-4-1 (1Bx7 + 1By8, 1Dx2 + 1Dy12), II-11-4-3 (1Ax1, 1Bx13 + 1By16 + 1By8, 1Dx5 + 1Dy12), II-11-4-6 (1Ax1, 1Bx7 + 1By8, 1Dx5 + 1Dy12), II-11-4-10 (1Ax2*, 1Bx7 + 1By8, 1Dx5 + 1Dy12) and II-11-4-16 (Null, 1Bx13 + 1By16, 1Dx5 + 1Dy12). The panel also included three bread wheat lines: JN177 (the parent of the introgression lines) with glutenin composition null, 1Bx7 + 1By9, 1Dx2 + 1Dy12; Chinese Spring (null, 1Bx7 + 1By8, 1Dx2 + 1Dy12), and 4072 (1Ax1, 1Bx13 + 1By16, 1Dx5 + 1Dy10).

Protein extraction from the grain and SDS-PAGE

Protein was extracted from the grain following the procedure described by Feng et al. (2004). A 20-µl sample was loaded onto an SDS-PAGE gel, formed by a 7.5–10% gradient separating gel (pH 8.5) and a 4% stacking gel (pH 6.8). Separation was carried out at a constant current of 6 mA for 14–16 h. The gel was stained in 0.1% w/v Coomassie Brilliant Blue R250, 10% v/v carbinol and 50% v/v acetic acid for 20 min with gentle shaking, and destained by boiling in distilled water for 1 h.

Cloning the HMW-GS genes

Genomic DNA was extracted from introgression line seedlings using the CTAB method of Murray and Thompson (1980). The full-length DNA sequences of genes were amplified using a pair of degenerate primers. The sequence of the forward primer (P1) was 5′-ATGGCTAAGCGGc/tTa/gGTCCTCTTTG, and that of the reverse primer P2 5′-CTATCACTGGCTAa/gGCCGACAATGCG. P1 contains the HMW-GS start codon, and P2 contains two conserved stop codons. As the target sequence is GC-rich, LA *Taq* polymerase (TaKaRa Biotechnology) was used for the PCR, which consisted of an incubation at 95°C for 5 min, followed by 28 cycles of 94°C/40 s and 68°C/4 min, and a final incubation at 72°C for 7 min. Amplified fragments were recovered from 1.0% agarose gels, cloned into the pUCm-T vector, and transformed into *E. coli* DH10B competent cells. Five to six sub-clones of these fragments were prepared using the nested deletion method of Sambrook et al. (1989), and then submitted for commercial sequencing (Invitrogen). Both cloning and sequencing were repeated at least three times to exclude sequencing errors. Sequence analyses were performed with the software packages DNAMAN, MEGA (Kumar et al. 2004) and the ClustalW program from the EBI networks.

Results

The inheritance of HMW-GS in the F_5-F_8 progeny of line II-11-4

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of 50 F_5–F_8 plants derived from the hybrid line II-11-4 showed that HMW-GS profile of most of the F_5 plants differed from that of JN177, although the electrophoretic mobility of the non-parental HMW-GS was similar to certain already documented bread wheat subunits (Table 1; Fig. 1). Among the F_6 plants derived from five progenies of II-11-4 single seed, it was clear that most progeny lines (i.e., II-11-4-1, -6, -10 and -16) inherited the HMW-GS profile of their parent, but some (i.e., II-11-4-3) continued to segregate. Some of the subunits presented in the F_5 generation were not transmitted to any of the subsequent generations (Table 2).

Cloning and sequence analysis of HMW-GS genes

The agarose gel profile of the amplification products from the template of II-11-4-3, II-11-4-10 and II-11-4-16 of F_5 consisted of, respectively, six, five and five bands (Fig. 2). The fragments corresponding to each of these bands were designated H3-1 to H3-6, H10-1 to H10-5 and H16-1 to H16-5, respectively. After cloning and sequencing each of these 16 products, it was possible to define how they differed from one another at the sequence level (Table 3). Aligning the sequences showed that H10-4 was almost

Table 1 Composition of HMW-GS alleles among F_5–F_8 progeny lines of the somatic hybrid line II-11-4

F5 Generation			F6 Generation			F7 Generation			F8 Generation		
Glu-A1	*Glu-B1*	*Glu-D1*	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-A1*	*Glu-B1*	*Glu-D1*
2*	13 + 16	5 + 12	2*	13 + 16	5 + 12	2*	13 + 16	5 + 12	2*	13 + 16	5 + 12
2*	7 + 8	5 + 12	2*	7 + 8	5 + 12	1	13 + 16	5 + 12		13 + 16	5 + 12
1	13 + 16	5 + 12	1	13 + 16	5 + 12		13 + 16	5 + 12		13 + 16	2 + 12
1	7 + 8	5 + 12	1	7 + 8	5 + 12		13 + 16	2 + 12			
1	7 + 8	2 + 12		13 + 16	5 + 12		7 + 8	2 + 12			
1	13 + 16 + 8	5 + 12		13 + 16	2 + 12		7 + 8	5 + 12			
	13 + 16 + 8	5 + 12		7 + 8	2 + 12						
	13 + 16	5 + 12		7 + 8	5 + 12						
	13 + 16	2 + 12									
	7 + 8	2 + 12									
	7 + 8	5 + 12									

identical to JN177 *1Bx7*, H10-5 was very similar to JN177 *1Dx2*, and H3-2, H10-2 and H16-2 were all very similar to JN177 *1Dy12*. Because of this high similarity, it was assumed that the sequences had evolved from the parental ones via a small number of point mutations. The H3-3 and H10-3 sequences differ from JN177 *1By9* by the presence of an additional block of repeated peptides, and H3-4 along with H16-3 each carry a second different repetitive block. These sequences most probably were generated from *1By9* by slippage during replication (Fig. 3a). Compared with JN177 *1Bx7*, H3-5 and H16-4 both contain an additional repeat motif in their central repetitive region (Fig. 3b), while H3-6 and H16-5 each lack one repetitive block compared to JN177 *1Dx2* (Fig. 3c). Thus it seems likely that H3-5 and H16-4 are the result of replication slippage from *1Bx7*, while H3-6 and H16-5 arose in the same way from *1Dx2*. Interestingly, the coding sequence of the additional repeat motif was always starting from CCAGG, which is a target of DNA methyltransferase (Fig. 3).

Three *Glu-1Ay* alleles are known in bread wheat, typified by the cultivars Cheyenne, Chinese Spring and Pandas. These three cultivars contained, respectively, the HMW-GS 2*, null and 1, encoded by *Glu-1Ax* (Forde et al. 1985; Harberd et al. 1987; Lafiandra et al. 1997). The Chinese Spring and Pandas *Glu-1Ay* alleles differ from one another by the presence of a ~8-kbp transposon-like sequence in the repeti-

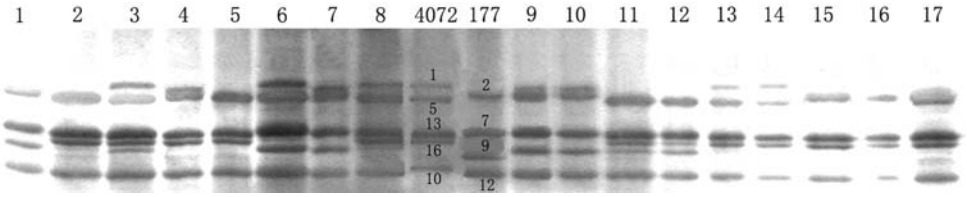

Fig. 1 SDS-PAGE separations of HMW-GS. *Lanes* 1–17: F_5 seeds of the somatic hybrid line II-11-4. 1: subunits 7 + 8, 2 + 12; 2 and 5: 13 + 16, 5 + 12; 3: 1, 13 + 16 + 8, 5 + 12; 4: 2*, 13 + 16, 5 + 12; 6: 1, 7 + 8, 5 + 12; 7, 9 and 10: 2*, 7 + 8, 5 + 12; 8, 13 and 14: 1, 13 + 16, 5 + 12; 11, 12 and 17: 13 + 16 + 8, 5 + 12; 15 and 16: 13 + 16, 2 + 12. 4072 and 177 indicate, respectively, wheat cultivars 4072 and JN177

Table 2 Segregation of HMW-GS alleles among progeny lines between F_5 and F_6 derivatives of the somatic hybrid line II-11-4

Hybrid line	F_5 sample	Subunit composition			F_6 sample	Subunit composition		
		Glu-A1	*Glu-B1*	*Glu-D1*		*Glu-A1*	*Glu-B1*	*Glu-D1*
II-11-4-1	1		7 + 8	2 + 12	22		7 + 8	2 + 12
II-11-4-3	1	1	13 + 16 + 8	5 + 12	17	1	13 + 16 + 8	5 + 12
					5	1	13 + 16	5 + 12
II-11-4-6	1	1	7 + 8	5 + 12	25	1	7 + 8	5 + 12
II-11-4-10	1	2*	7 + 8	5 + 12	19	2*	7 + 8	5 + 12
II-11-4-16	1		13 + 16	2 + 12	17		13 + 16	2 + 12

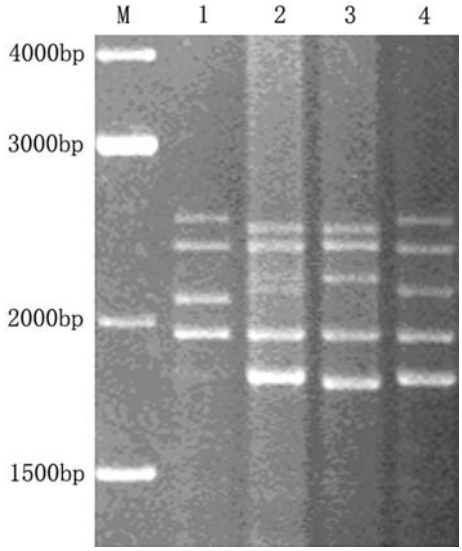

Fig. 2 PCR analysis of HMW-GS sequences amplified from F_5 progeny lines of the somatic hybrid line II-11-4. *Lane* 1: Wheat cultivar JN177; 2: II-11-4-3; 3: II-11-4-16; 4: II-11-4-10; M: 2-Log Ladder (NEB, USA)

tive region, while both differ from Cheyenne by the presence of an 18 bp repeat motif in the repetitive region. The H3-1, H10-1 and H16-1 sequences were compared with those of Cheyenne and Chinese Spring *Glu-1Ay* (ignoring the transposon sequence). This showed that both H3-1 and H10-1 were very similar to the Chinese Spring *Glu-1Ay* allele, as both included the characteristic 18 bp repeat that was absent in the Cheyenne *Glu-1Ay* allele, while H16-1 was similar to the Cheyenne *Glu-1Ay* allele (Fig. 3d).

Phylogenetic analysis of the II-11-4 *Glu-1* alleles

The phylogenetic tree, constructed on the basis of the full length sequences, was divided into two subtrees, equivalent to the x- (lower subtree) and y-(upper subtree)type genes (Fig. 4). The 13 y-type sequences were arranged into three clades, with the first containing H3-1, H10-1, H16-1; the second including H3-2, H10-2, H16-2 and JN177 *1Dy12,* and the third comprising H3-3, H10-3, H3-4, H16-3 and JN177 *1By9*. Similarly, the eight x-type alleles fell into two clades, with the first comprising H3-5, H10-4, H16-4 and JN177 *1Bx7*, and the second H3-6, H10-5, H16-5 and JN177 *1Dx2*.

Discussion

Allelic variation at *Glu-1* among the II-11-4 lines

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed the presence of a number of non-parental HMW-GS among the introgression lines derived from somatic hybridization between wheat and tall wheatgrass. Of the six novel subunits, three resembled (and therefore were probably derived from) the *Glu-B1* locus, two the *Glu-A1* locus and one the *Glu-D1* locus. Most of these novel alleles were genetically stable (Table 1). The ease with which HMW-GS sequences can be isolated, and therefore sequenced, made it possible to show that sequence changes had been induced among the progenies of the

Table 3 Comparison of 16 HMW-GS sequences isolated from three progeny lines of the somatic hybrid line II-11-4

Subunit	Possible subunit type	ORF	Signal peptide	N-terminal region		Repetitive region		C-terminal region	
		Size/bp	Size	Size	Cys	Size	Cys	Size	Cys
H3-1	1Ay	1,830	21	104	5	440	0	42	1
H3-2	1Dy12	1,980	21	104	5	491	1	42	1
H3-3	1Dy8	2,166	21	104	5	553	1	42	1
H3-4	1By16	2,220	21	104	5	571	1	42	1
H3-5	1Bx13	2,391	21	81	3	651	1	42	1
H3-6	1Dx5	2,478	21	89	3	672	0	42	1
H10-1	1Ay	1,830	21	104	5	440	0	42	1
H10-2	1Dy12	1,980	21	104	5	491	1	42	1
H10-3	1By8	2,166	21	104	5	553	1	42	1
H10-4	1Bx7	2,373	21	81	3	645	0	42	1
H10-5	1Dx2	2,523	21	89	3	687	1	42	1
H16-1	1Ay	1,812	21	104	5	434	0	42	1
H16-2	1Dy12	1,980	21	104	5	491	1	42	1
H16-3	1By16	2,220	21	104	5	571	1	42	1
H16-4	1Bx13	2,391	21	81	3	651	1	42	1
H16-5	1Dx5	2,478	21	89	3	672	0	42	1

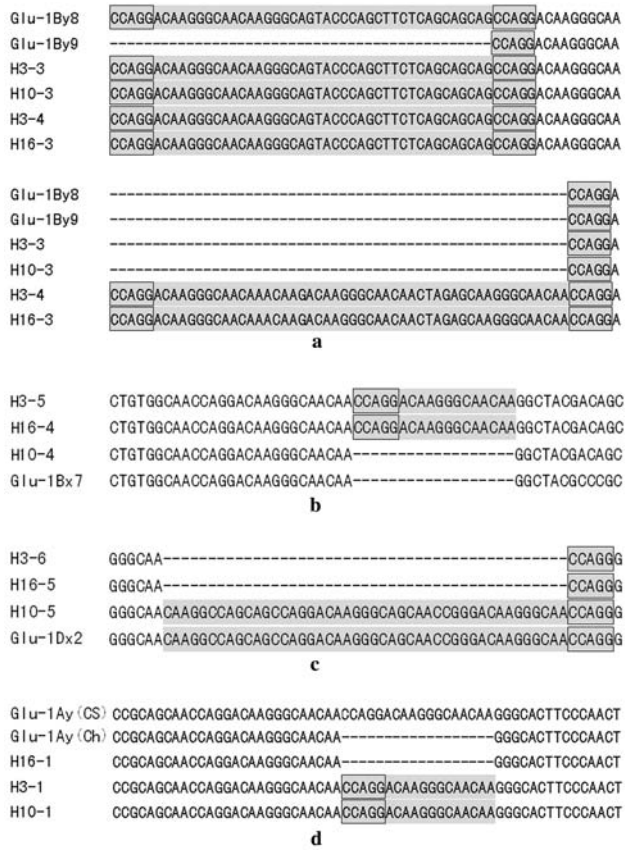

Fig. 3 *Glu-1* sequences of progeny lines of the somatic hybrid line II-11-4. **a** Sequences *H3-3*, *H3-4*, *H10-3*, *H16-3* compared to those of Chinese Spring *Glu-1By8* and JN177 *Glu-1By9*; **b** Sequences of *H3-5*, *H10-4*, *H16-4* compared to that of JN177 *Glu-1Bx7*; **c** Sequences of *H3-6*, *H10-5*, *H16-5* compared to that of JN177 *Glu-1Dx2*; **d** Sequences of *H3–1*, *H10–1*, *H16–1* compared to those of Chinese Spring (CS) and Cheyenne (Ch) *Glu-1Ay*. Added and deleted repeat motifs are shaded and the six nucleotides (CCAGG) are *shaded* and *boxed*. "-" shows the deletion region

introgression line (Fig. 1; Table 3). The results of the simple grain heredity experiments showed that we can accelerate the filtration of stable subunits in the progeny of hybrid lines using this method and make them for wheat quality improvement.

A possible mechanism for inducing variation at the *Glu-1* loci

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the offspring regenerated from tissue-cultured JN177 callus has shown that HMW-GS can be altered by somaclonal variation, although the rate of mutation is low (Feng et al. 2004). Thus it seems unlikely that the much higher frequency of variation for HMW-GS found among the offspring of the somatic hybridization process can be fully explained by somaclonal variation. Instead, it appears that the asymmetric somatic hybridization itself encourages

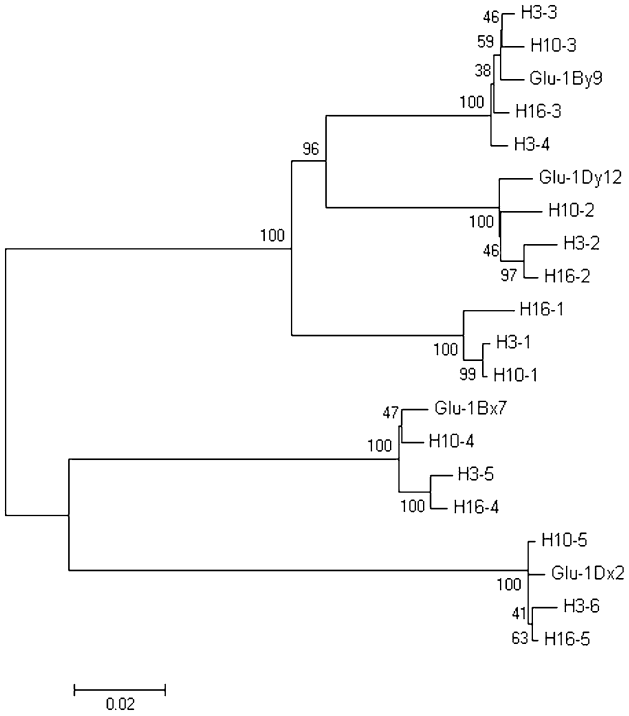

Fig. 4 A phylogenetic analysis of HMW-GS sequences of progeny lines of the somatic hybrid line II-11-4 and their wheat parent JN177

sequence alterations in the HMW-GS genes. The most common event was the addition or deletion of repetitive motifs, a phenomenon which also affects microsatellite loci in these materials (unpublished data). In general, the stability of repetitive sequences is much lower than that of non-repetitive ones, and this observation led Robert (1996) to suggest that repetitive sequences are more susceptible to replication slippage, DNA mismatch repair and gene conversion. The types of sequence change most frequently identified among the somatic hybridization progenies are consistent with the occurrence of replication slippage and point mutation (due to failed mismatch repair).

As it is mentioned above, the repeat motifs deleted or added in the altered *Glu-1* alleles all began with the sequence CCAGG (Fig. 3). This sequence is known to be a target of DNA methyltransferase, and CmC (a/t)GG methylation has been considered to be an epigenetic mark of DNA (Lorincz and Groudine 2001). MSAP analysis has shown that the methylation pattern of 18.6% detected loci in the introgression lines has been changed (data not shown). However, whether or not the variation at the *Glu-1* loci is related to the methylation status of the CCAGG sequences remains unexplored.

The JN177 *1Ax* allele is not expressed, and since no *Glu-1Ay* amplicon was obtained from template of this line, we believe that it carries the same allele as Chinese Spring, in which the coding sequence is interrupted by a transposon-like sequence. However, the *H3-1*, *H10-1* and *H16-1* sequences

all lack this transposon, suggesting that it has been activated by the somatic hybridization process. Re-activation of transposons is a known response to tissue culture (Brettell and Dennis 1991; Hirochika et al. 1996), and we have noted examples of this phenomenon in the introgression material using genome-wide transposon display techniques (unpublished data); whether the higher variation frequency of HMW-GS in the progeny of somatic hybrids is correlated with the activation of transposon need more consideration. According to the results, we can conclude that point mutation and addition or deletion of repeat motifs could be the main drive for the formation of novel *Glu-1* alleles in the somatic hybrid introgression lines, which was coincident with the mechanism about the evolution of HMW-GS genes mentioned by Anderson and Greene (1989); however, we also recognized that the transposon might have some influence on the variation of *Glu-1* alleles.

The possible contribution of novel *Glu-1* alleles to the flour processing quality

An additional cysteine residue was present in the repetitive region of the subunits encoded by the H3-5, H16-4 and H10-5 sequences (Table 3). The same difference distinguishes the 1Dx5 subunit from other *Glu-D1-1* products, and its presence has been correlated with good breadmaking quality (Lafiandra et al. 1993; Gupta and MacRitchie 1994). Thus H3-5, H16-4 and H10-5—if they are expressed—could be beneficial for the functionality of the glutenin polymer. A number of novel HMW-GS combinations which have arisen among the introgression material have been shown to be correlated with improved processing quality (Feng et al. 2004, Liu et al., 2006). A particular advantage of this germplasm is that it is essentially isogenic, and thus the functional performance of different alleles at the *Glu-1* loci can be readily evaluated. Among segregants of II-11-4, for example, are lines with a range of HMW-GS composition (Tables 1, 2).

In conclusion, we have demonstrated that asymmetric somatic hybridization can rapidly generate novel HMW-GS alleles. Thus the technique should represent a valuable means of extending the variation at these functionally important genes.

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