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Detection and characterization of a glutenin subunit with unusual high *Mr* at the *Glu-A1* locus in hexaploid wheat

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Abstract A hexaploid wheat landrace collected from the Baluchistan province of Pakistan was found to possess a novel high-molecular-weight glutenin subunit (HMW-GS). The subunit has a very slow electrophoretic mobility as revealed by SDS-PAGE, and its molecular weight is comparable to that of the highest molecular weight glutenin subunit ("2.2" encoded in the D-genome) reported so far in hexaploid wheat varieties and landraces of Japanese origin. Evidence obtained from (PCR) gene amplification studies using the primers specific for *Glu-1* loci proved that the gene coding for this novel subunit belongs to the *Glu-A1* locus located on the long arm of chromosome 1A. Digestion of the amplified gene (PCR product) with restriction enzymes indicated that the novel gene differs from prevailing *Glu-A1* alleles (null, 1 and 2*) by an extra DNA fragment of approximately 600 base pairs. The results also indicated that the novel subunit is most probably a derivative of subunit 2* that has very likely incorporated the 600-bp fragment following a process of unequal crossing over. The present findings were further substantiated by reserved phase high performance liquid chromatography (RP-HPLC) analysis.

Key words Wheat · HMW glutenin genes · *Glu-A1* · PCR · RP-HPLC

Introduction

A series of research efforts during the past 15 years (Holt et al. 1981; Lawrence and Shepherd 1981; Payne and Corfield 1979; Payne et al. 1980, 1981; 1982, 1985) have clearly demonstrated the extent of allelic variation for high-molecular-weight glutenin subunits (HMW-GS) in wheat (*T. aestivum*) as revealed by SDS-PAGE. A typical hexaploid genotype usually contains from three to five HMW-GS. Allelic variation of these subunits is controlled by genes found at three *Glu-1* loci (*Glu-A1 Glu-B1* and *Glu-D1*) located on the long arm of the homoeologous group 1 chromosomes which correspond to the A, B, and D genomes of hexaploid wheat, repectively. The *Glu-B1* and *Glu-D1* loci usually code for two subunits (*1Dx* and *1Dy*) and one or two subunits (*1Bx* or *1Bx* and *1By*), respectively. Only one subunit (*1Ax*) or no subunit at all is encoded at the *Glu-A1* locus.

Early electrophoretic data showed less polymorphism at the *Glu-A1* locus that at the *Glu-B1* and *Glu-D1* loci, and three alleles designated as *a*, *b* and *c* were reported (Payne and Lawrence 1983). Alleles *a* and *b* code for glutenin subunits termed 1 and 2*, respectively, while allele *c* corresponds to a silent gene (Thompson et al. 1983) which does not code for a protein. Waines and Payne (1987) reported four new allelic variants at the *Glu-A1* locus (*d*, *e*, *f*, *g*) in hexaploid wheat that showed faster mobility on SDS-PAGE than the normal alleles. Allelic variants at the *Glu-A1* locus with a faster mobility on SDS-PAGE than the normal alleles were also found by Cross and Guo (1993) in a diverse wheat germ plasm collection. In this report, we describe a new variant at the *Glu-A1* locus that was found in hexaploid wheat germ plasm collection from Pakistan.

Materials and methods

Electrophoretic and chromatographic analyses

The hexaploid wheat used in this study is a landrace belonging to the Baluchistan province of Pakistan. The seeds for this landrace (PK-15684) were obtained from Plant Genetic Resources Institute, National Agriculture Research Center Islamabad, Pakistan, as a part of the wheat germ plasm collection from Pakistan. The seed for reference standards was provided by the Department of Agrobiology and

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Total protein extract from the brush-half of the single kernels was fractionated by SDS-PAGE on 10% gels (C = 1.28%) according to the procedure described by Payne et al. (1980). SDS-PAGE urea gels (C = 2.67%) were prepared by incorporating 4*M* urea into the main gel. RP-HPLC analyses of HMW-GS were conducted following the methods described by Lafiandra et al. (1993). Fractions corresponding to peaks were collected, freeze-dried and identified by SDS-PAGE.

DNA extraction and polymerase chain reaction

The embryo-half of the seeds saved from above analyses were grown in pots to raise the individual plants. Leaves and/or boots from these plants were used to extract genomic DNA following the procedure of Dellaporta et al. (1983). Polymerase chain reaction, (PCR) analysis was carried out in a total volume of 50 µl using 150 ng of template DNA. The composition of the reaction mixture was 1 × *Taq* PCR buffer (life technologies™), 0.3 m*M* of each dNTP, 0.3 µM of each of the primers (125 ng of 20-mer oligonucleotide) and 2.5 units of *Taq* DNA polymerase (Life Technologies™). Primers used for PCR analyses were as reported by D'Ovidio et al. (1995). For all reactions, a Perkin Elmer Cetus Thermocycler (Model 480) was programmed to obtain amplication conditions of 30 cycles at 94 °C for 1 min 60 °C for 2 min. and 72 °C for 2 min, followed by a final incubation step at 72 °C for 7 min. The amplified products were digested with restriction enzymes (*Hin*dIII and *Hae*III). Agarose gel (1%) electrophoresis in 1 × TBE buffer used to separate the amplified DNA fragments as well as the digestion products of the amplified DNA.

Results

SDS-PAGE

A survey of the hexaploid wheat germ plasm collected from Pakistan was recently conducted with respect to high-molecular-weight glutenin subunit (HMW-GS) composition. Figure 1 (upper) presents the SDS-PAGE separations for HMW-GS in some of the landraces from Pakistan (lanes 4, 5, 6 and 7) as compared to some bread wheat cultivars (lanes 1, 8, 9 and 10) and germ plasm lines (lanes 2 and 3). As indicated by the arrowhead in lane 4 of Fig. 1 (upper), a landrace (PK-15684) that originated from the Baluchistan province of Pakistan was found to possess a very large HMW glutenin subunit. The mobility of this subunit was comparable to that of the highest-molecular-weight *Glu-D1*-encoded subunit, designated as 2.2, which was first reported by Payne et al. (1983) in Japanese cultivars, and later by Margiotta et al. (1993) in a germ plasm line (MG-7249) again of Japanese origin (Fig. 1, upper, slowest moving band in lane 3). The only subunit pair which could be readily identified in the landrace PK-15684 was 7 + 8 at the *Glu-B1* locus. In addition, the landrace possessed two other subunits, a subunit (second slowest band in lane 4) with a mobility slower than that of the *1Dx* subunit 2 (lane 1) and a subunit with a mobility intermediate of that of subunits 9 and 10 corresponding to genes *1By* and *1Dy*, respectively (lane 8 and, fastest moving in lane 4). These two subunits were assigned to the *Glu-D1* locus because we had already encountered

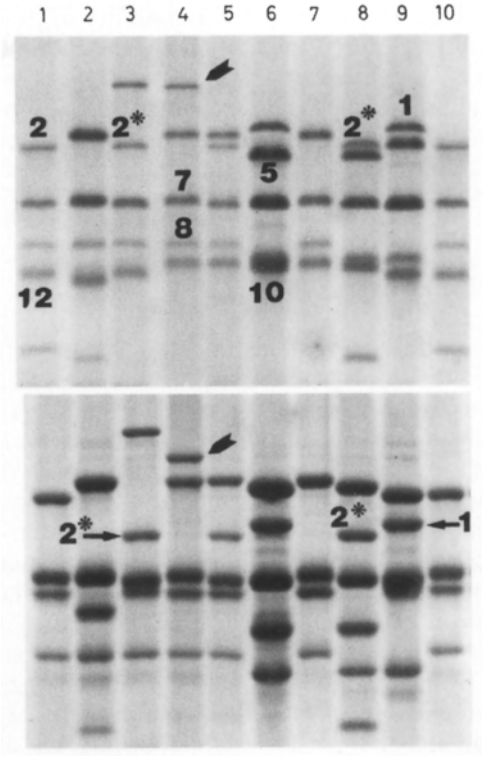


Fig. 1 Upper One-dimensional SDS-PAGE (10%) separation of HMW-GS from hexaploid genotypes *Lane 1* 'Chinese Spring' ("null", 7 + 8, 2 + 12), *2* MG-7631 ("null", 7 + 8, 2** + 12*), *3* MG-7249 (2*, 7 + 8, 2.2 + 12), *4* PK-15684 (2.1*, 7 + 8, Dx + Dy), *5* PK-16437 (2*, 7 + 8, Dx + Dy); *6* PK-16476 (1, 7 + 9, 5 + 10), *7* PK-16475 ('null', 7 + 8, Dx + Dy), *8* 'Cheyenne' (2*, 7 + 9,5 + 10), *9* 'Pandas' (1, 7 + 9, 2 + 12), *10* 'Chinese Spring'. **Lower** One-dimensional SDS-PAGE (4 *M* urea) separations of HMW-GS for the hexaploid wheat genotypes shown in **Fig 1 upper**

Glu-D1-encoded subunits of identical mobilities in other germ plasm lines of the same origin along with the *1A* subunit 2* (Fig. 1 upper, lane 5) or "null" (lane 7). Moreover, the mobility of the *1Dx* subunit in PK-15684 was similar to that of the *1Dx* subunit 2** (Fig. 1, upper, slowest moving band in lane 2) found in a germ plasm line MG-7631. This evidence led us to believe that the slowest moving glutenin subunit possessed by PK-15684 was most probably a novel subunit encoded at the *Glu-A1* locus. We will refer to this novel subunit as 2.1* from here on.

It has been reported that SDS gels containing 4 *M* urea can be used to further discriminate and substantiate the results obtained in SDS gels without urea (Lafiandra et al. 1993). In particular the mobility of HMW glutenin subunits 2* and 1, encoded at the *Glu-A1* locus, is faster than that of the *Glu-D1*-encoded subunits 2 and 5 when this type of gel is used. To further strengthen our belief that the novel subunit 2.1* possessed by PK-15684 is encoded at the *Glu-A1* locus, we separated the HMW-GS in SDS-PAGE including 4*M* urea (Fig. 1, lower). It is obvious from Fig. 1 (lower) that novel subunit 2.1* (indicated by the arrowhead in lane 4) migrates faster than the *1Dx* subunit 2.2 (slowest moving

band in lane 3) excluding the possibility of its being a *1Dx* subunit. Further, the mobility of the *1Dx* subunit in PK-15684 (Fig. 1, lower, second slowest band in lane 4) is equivalent to that of the *1Dx* subunits of slow mobility found in other germ plasm lines (Fig. 1 lower) along with alleles "null" (lane 2 and 7) and 2* (lane 5) at the *Glu-A1* locus.

RP-HPLC analysis

The separation of proteins according to their surface hydrophobicities by reversed phase high performance liquid chromatography (RP-HPLC) is a powerful tool by which to complement the information provided by electrophoresis. Figure 2 (A, B, C, D) shows the RP-HPLC separation of reduced only and both reduced and alkylated (Margiotta et al. 1993) high-molecular-weight glutenin subunits extracted from the landrace (PK-15684) possessing the novel subunits 2.1* as well as from a mixture of 'Cheyenne' and 'Mec' possessing the 1A subunits 2* and 1, respectively. The elution times of various *x*-type and *y*-type glutenin subunits possessed by these genotypes are presented in Table 1. Peak collection after RP-HPLC and electrophoretic analysis confirmed that the fastest moving glutenin subunit possessed by PK-15684 as seen in SDS-PAGE corresponds to the *Dy* subunit. The difference in molecular size as reflected by its slow mobility did not affect significantly the surface hydrophobicity compared to that of subunits 10 and 12 both when reduced and reduced/alkylated (Fig. 2, A and C vs. B and D). Similarly, comparative chromatographic and electrophoretic analysis confirmed that the peak eluting at 77.7 min when reduced and at 32.7 min (*Dx* in Fig. 2, B) when reduced/alkylated corresponded to the subunit showing a slower mobility than subunit 2 on SDS-PAGE (Fig.1, lower, second-slowest band in lane 4). Its similarity in surface hydrophobicity to the *Dx* subunits further confirmed that it is a *Dx*-type subunit. In fact, as seen in Table 1, its elution times are fairly similar to those of subunits 2 and 5. Finally, the peak collection indicated that the slowest moving band present in landrace PK-15684 has a surface hydrophobicity very similar to that of subunits 1 or 2* (Table 1).

Table 1 RP-HPLC retention times (RT) for reduced (upper) and 4-vinylpyridine-alkylated (lower) *x*-Type and *y*-Type HMW glutenin subunits (GS) observed in two cultivars and a landrace

Cultivar/ landrace	GS	RT	GS	RT	GS	RT	GS	RT	GS	RT
		42.7		75.0		79.7		68.4		65.5
Cheyenne	10		9		5		7		2*	
		17.6		27.0		32.3		37.9		51.4
		42.7				77.3		69.9		64.9
Mec	12				2		7*		1	
		17.6				33.2		38.8		50.7
		42.4		95.2		77.7		68.4		66
PK-15684	Dy		8		Dx		7		2.1*	
		17.4		32.7		32.7		38.1		53.7

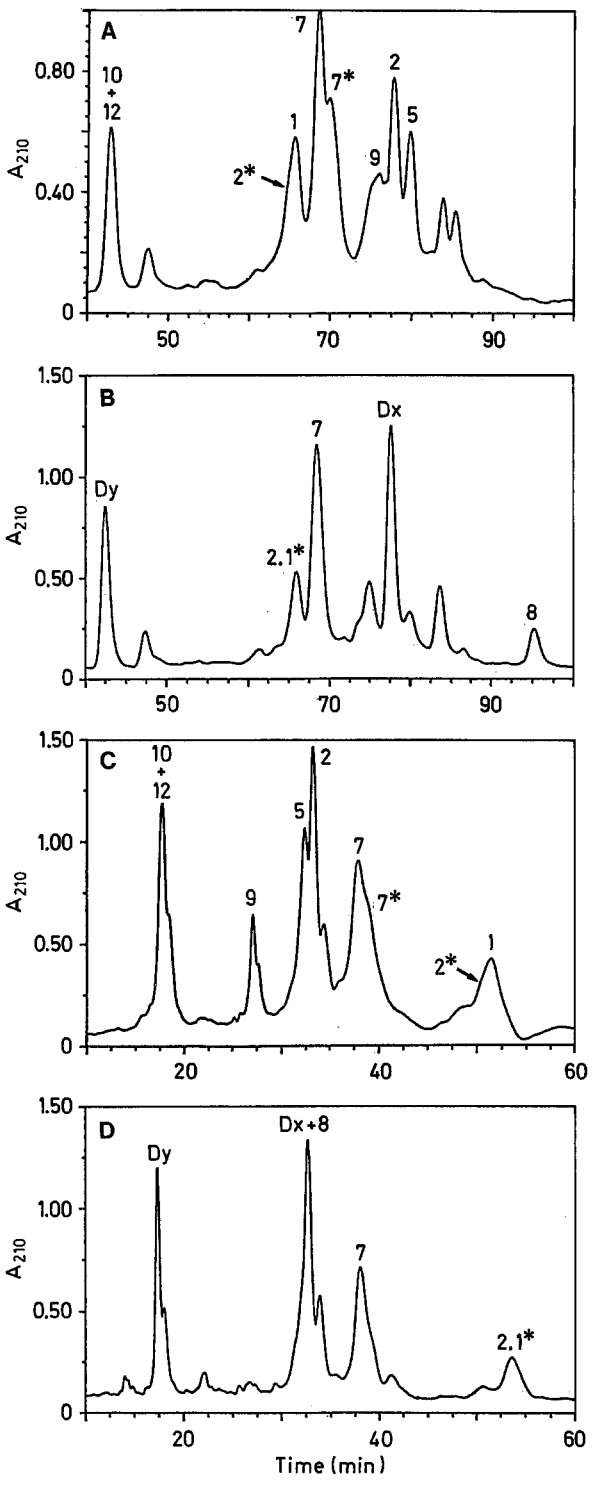


Fig. 2 RP-HPLC of HMW glutenin subunits, reduced (*top*) and reduced and alkylated (*bottom*), present in hexaploid genotypes 'Cheyenne' + 'Mec' (A) and (C) and PK-15684 (B) and (D)

PCR analysis

To further confirm that the novel glutenin subunit (2.1*) detected in landrace PK-15684 is encoded at the *Glu-A1* locus, we carried out PCR analysis using primers specific for the complete coding region of the *Glu-A1x* gene

(D'Ovidio et al. 1994). The amplification products of the genes for allelic variants (null, 1 and 2*) as well as of the gene coding the subunit 2.1* are shown in Fig. 3. The nucleotide length of the genes was expected to be 2490 bp for subunit 1 (Halford et al. 1992) and 2445 bp for the subunit 2* (Anderson and Greene 1989). Our results (Fig. 3, lanes 2–7) showed the same nucleotide length as previously reported for these genes. However, the gene coding for the novel subunit (Fig. 3, lane 9) was distinguished by its larger size, with some 600 bp more than the other *1A* genes. The total nucletoide length of this DNA fragment was approximately 3000 bp which is comparable to the gene length of 3050 bp as reported for the gene corresponding to *1Dx* subunit 2.2 (D'Ovidio et al. 1994).

To further elucidate the structure of the gene encoding for the novel 1A subunit (2.1*), we digested its corresponding amplification product with two restriction endonuclease enzymes, *Hin*dIII and *Hae*III. Considering the structure of high-molecular-weight glutenin subunit genes *Hin*dIII could cut close to the boundary between the N-terminal unrepetitive domain and the central repetitive domain; consequently, fragments obtained would consist largely of the complete central repetitive region and the C-terminal sequence. Electrophoretic separation of amplified and *Hin*dIII-digested DNA produced the same pattern as the one reported in Fig. 3 except that the fragment that resulted was smaller due to the elimination to the N-terminal region (results not shown). As it has been reported that the C-terminal domains are highly conserved in the HMW glutenin sequences (Harberd et al. 1986) the increase in size displayed by the *Hin*dIII fragment corresponding to the subunit 2.1*, compared to allelic subunits null, 1 or 2*, may be due to differences in the number of repeats in the central domain. *Hae*III could also cut the amplified gene fragments in the central repetitive region, differently from *Hin*dIII. In particular, the gene coding for the novel subunit 2.1* after digestion with this restriction enzyme showed an extra DNA fragment of approximately 600 bp (Fig. 4, indicated by arrow in lane 5); moreover the restriction pattern of the gene corresponding to subunit 2.1* showed a strong similarity with the one corresponding to the gene coding subunit 2* (Fig. 4, lanes 4 and 5). These analyses provided further evidence that the "null" allele shows a similar digestion pattern (Fig. 4, lanes 2 and 3) to subunit 1.

Discussion

The presence of multiple allelic forms at the *Glu-A1* locus located on the long arm of chromosome *1A* has been reported in recent years. The most commonly found alleles at this locus are 1, 2* or "null" (absence of a protein or subunit). The novel or rare allelic forms at this locus reported in hexaploid (Cross and Guo 1993; Waines and Payne 1987) and tetraploid (Branlard et al. 1989; Vallega and Waines 1987) wheat species are either

Fig. 3 Agarose gel showing the PCR products of the gene fragment coding for the *1Ax* glutenin subunits in the hexaploid genotypes. *Lane 1* 1-kb DNA size marker, *2* 'Chinese Spring', *3* 'Pandas', *4* 'Cheyenne', *5* PK-16475, *6* PK-16476, *7* PK-16437, *8* MG-7249, *9* PK-15684

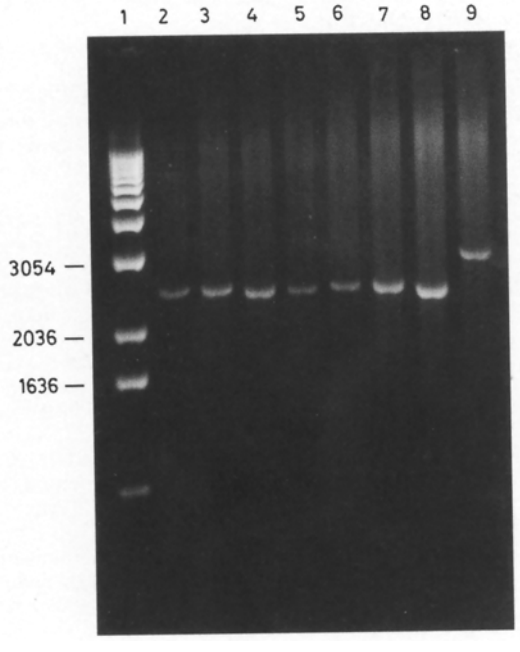
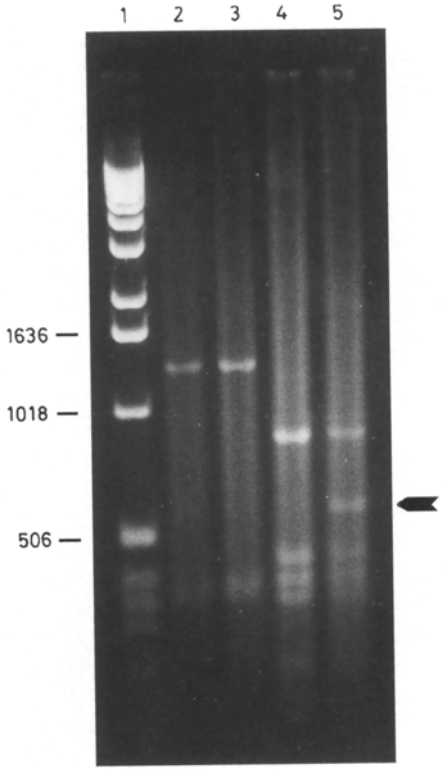


Fig. 4 Agarose gel showing the PCR products of the gene fragment coding for the *1Ax* glutenin subunit after digestion with restriction enzyme *Hae*III. *Lane 1* 1-kb DNA size marker, *2* 'Chinese Spring', *3* 'Pandas', *4* 'Cheyenne', *5* PK-15684



slower or faster in mobility on SDS-PAGE than the common alleles (1 or 2*). However, none of these novel subunits possess a molecular weight such as one displayed by the novel *Glu-A1* encoded subunit (2.1*) detected in landrace PK-15684 from Pakistan, although glutenin subunits (2.2 and 2.2* encoded at the *Glu-D1* locus) of a very high molecular size have been detected previously in hexaploid wheats (Payne et al. 1983; Margiotta et al. 1993). While we found the frequency of 2.1* to be very low – only 1 accession out of 30 possessed this subunit – the actual frequency of 2.1* can only be determined when other accessions (approximately 300) from the Baluchistan province of Pakistan are characterized for glutenin subunits. The germ plasm collected from this region of Pakistan appears to have a high potential in terms of multiallelism at the *Glu-1* loci as revealed in our survey by SDS-PAGE (data not published).

The structure of HMW glutenin genes consist of unrepetitive domains (C- and N-terminal regions) and a central repetitive domain. It is considered (Shewry et al. 1989) that the repetitive block structure of the central domain provides the basis for major and rapid structure changes in the *Glu-1* genes by duplication and/or delection of large gene segments as a result of unequal crossing over while the terminal regions are conserved and characterized by minor changes as a result of insertion, delection or substitution of single nucleotides/amino acids. According to Margiotta et al. (1993), the structural changes in the central repetitive domain of *Glu-1* genes do not affect surface hydrophobicity as detected by RP-HPLC analysis. These researches did not find significant changes in elution times among the allelic variants (2, 2.2 and 2.2*) at the *Glu-D1* locus. The results obtained in our study are in agreement with these since the surface hydrophobicity of novel allele 2.1* was similar to that of the commonly found alleles (1 and 2*) at the *Glu-A1* locus.

Moreover, our findings from PCR analysis and subsequent analysis by digestion of PCR products with restriction enzymes confirmed that the novel allele (2.1*) belonging to the *Glu-A1* locus possesses an extra fragment of DNA (approximately 600 bp) which is most probably present in the central repetitive domain. This novel subunit has very likely evolved as a result of unequal crossing over as has also been indicated for the *1Dx* subunits 2.2 and 2.2* (Payne et al. 1983; Margiotta et al. 1993). The present results also indicate the usefulness of PCR to gain further information on structural features of active and silenced genes. In fact, the present results indicate that the "null" allele at the *Glu-A1* locus has a structural similarity with the subunit 1 and that the novel subunit 2.1* is very likely to be a derivative of subunit 2*.

In bread wheat, it is now well-known (Payne 1987) that HMW subunits of glutenin confer the strength and elasticity to doughs that are required to make the bread. The *1A* subunits 1 and 2* are related to good bread-making quality in comparison to the "null" allele at this locus, which has detrimental effects on bread-making properties. It will also be interesting to study the relationship of novel *1A* subunit 2.1* to bread-making properties.

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