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PCR analysis of x- and y-type genes present at the complex *Glu-A1* locus in durum and bread wheat

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Abstract Genes (x-type) corresponding to different high-molecular-weight glutenin subunits encoded at the *Glu-A1* locus present in bread- and durum-wheat cultivars have been selectively amplified by the polymerase chain reaction (PCR). DNA fragments corresponding to an unexpressed x-type gene were also amplified. As unexpressed y-type genes may or may not contain an 8-kb transposon-like insertion, two different sets of primers were designed to obtain amplification of DNA fragments corresponding to these genes. Amplified DNA fragments were also digested with restriction enzymes. The digestion patterns of amplified fragments corresponding to unusual x-type subunits showed similarities with genes encoding the most common subunits 2* and 1. The unexpressed amplified x-type gene showed a restriction pattern similar to the one obtained with the allelic gene encoding high-molecular-weight glutenin subunit 1; homologies were also found within the repetitive region of the linked y-type genes. On the basis of these observations it is postulated that an ancestral active x-type gene, most likely corresponding to subunit 1, was silenced following the insertion of the 8-kb transposon-like fragment into the linked y-type gene.

Key words Wheat · High-M_r glutenin genes · *Glu-A1* locus · Silent genes · Polymerase chain reaction

Introduction

High-M_r glutenin subunits of wheat have been thoroughly investigated because of their direct impact on flour technological properties (Payne 1987; Shewry et al. 1992). Allelic variation of high-M_r glutenin subunits, as identified by SDS-PAGE, is controlled by the *Glu-1* loci (*Glu-A1*, *Glu-B1*, *Glu-D1*) present on the long arm of homoeologous group-1 chromosomes. Each locus contains two tightly linked genes, one encoding a subunit of higher, and the other of lower, M_r designated as x- and y-types, respectively. Molecular studies have indicated that some high-M_r glutenin subunit genes are silent; bread-wheat cultivars contain in fact from three to five high-M_r glutenin subunits which can be detected by SDS-PAGE analysis; usually the *Glu-D1* locus encodes two subunits, the *Glu-B1* two or one, and the *Glu-A1* locus one or none. When one subunit is present at the *Glu-B1* and *Glu-A1* loci it is invariably an x-type (Payne et al. 1981; Harberd et al. 1986). Studies carried out on the silenced *Glu-A1y* gene have, so far, provided no clear explanation for its lack of expression (D'Ovidio et al. 1996).

The *Glu-A1* locus is less polymorphic than the *Glu-B1* and *Glu-D1* loci and is characterized by three main alleles, namely, *a*, *b* and *c* (Payne and Lawrence 1983). The alleles *a* and *b* code for glutenin subunits 1 and 2*, respectively, while *c* is a null allele (Thompson et al. 1983).

At the *Glu-A1*y locus, molecular studies have led to the identification of two different types of genes present in the bread-wheat cultivars Cheyenne and Chinese Spring. Although neither gene is expressed, they differ by the presence of a transposon-like sequence, of about 8 kb, within the region corresponding to the repetitive domain in the *Glu-A1*y gene of Chinese Spring (Forde et al. 1985; Harberd et al. 1987); moreover, the sequence of the repetitive region of the *Glu-A1y* gene of Chinese Spring differs from that of Cheyenne by the presence of one extra repeat unit. Two repeat units of 18 bp are found between nucleotides 1009–1044 in *Glu-A1y* of Chinese Spring, compared with only one of these at the same position in *Glu-A1y* of Cheyenne (Forde et al. 1985; Harberd et al. 1987). Recently, additional information on this important group of proteins has been obtained by polymerase chain reaction (PCR) analysis of their encoding genes; D'Ovidio et al. (1994, 1995), using published nucleotide sequences of high-M_r

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glutenin subunit genes and making use of the differences existing among them, have developed different sets of primers which can selectively amplify x-type genes (homoeoallelic genes) of the A-, B- and D-genomes.

Specific primers have been used in the present work in order to study polymorphism of expressed and silent x- and y-type genes present at the complex *Glu-A1* locus.

Materials and methods

Plant materials

Cultivars and germ plasm accessions of both durum and bread wheat were used.

DNA extraction and PCR amplification

DNA was isolated from leaves of single plants according to Dellaporta et al. (1983). Three pair of primers were used for the amplification of DNA fragments corresponding to the y- or x-type genes present at the *Glu-A1* locus. The two pairs of primers used to amplify DNA fragments of genes at the *Glu-A1y* locus were selected from the published sequences of the two allelic genes present in the bread-wheat cultivars Chinese Spring and Cheyenne (Forde et al. 1985; Harberd et al. 1987) and have the following sequences: (a) 5′ACG TTCCCCTACAGGTACTA3′, (b) 5′TATCACTGGCTAGCCGAC AA3′; (c) 5′CCATCGAAATGGCTAAGCGG3′; (d) 5′GTCCAG AAGTTGGGAAGTGC3′. Fragments corresponding to the coding region of the x-type gene present at the *Glu-A1x* locus were amplified using a single set of primers identified by D'Ovidio et al. (1995).

A programmed Perkin Elmer Cetus Thermocycler (model 480) was used in all the PCR experiments to obtain amplification conditions of 30 cycles with the following temperature profiles: 94°C for 1 min, 60°C for 2 min and 72°C for 2 min, followed by a final incubation step at the same temperature for 7 min. For the x-type gene 35 cycles were used with an extension step of 2 min and 30 s.

Each reaction was performed in a total volume of 50 µl using 150 ng of genomic DNA as template. The composition of the reaction mixture was 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.3 mM of each deoxyribonucleotide, 1.75 mM $MgCl_2$, 0.3 µM of each primer (125 ng of 20-mer oligonucleotide), and 2.5 units of *Taq* DNA Polymerase (Life Technologies™).

Amplified fragments were digested with *Hae*III or *Dde*I restriction endonucleases (Life Technologies™). Each reaction was performed using 2 µg of amplified DNA in 1 reaction buffer containing 4 U/µg of endonuclease.

DNA electrophoresis

PCR products were analyzed by electrophoresis using a 1% agarose (NUSIEVE) gel in a TBE buffer system (90 mM Tris-borate, pH 8.0, and 2 mM EDTA). Digested fragments were analysed by polyacrylamide-gel electrophoresis using a discontinous buffer system (Davis 1964) with an 8% acrylamide gel concentration.

PCR product cloning and sequencing

A fragment amplified by PCR with primers c and d from the genomic DNA of Pandas was purified after agarose electrophoresis and ligated into a pGEM-T plasmid vector (Promega). The vector was used to transform *E. coli* competent cells of the DH5a strain. The cloned fragment was partially sequenced using the method of Sanger et al. (1977).

Extraction of high-M_r glutenin subunits and electrophoresis

Single seeds were crushed and extracted with 50% propanol. The residue remaining after propanol extraction was re-suspended (1/7 w/v) in 0.125 M Tris-HCl pH 6.8 buffer containing 2.75% SDS, 10% glycerol, 10% dimethylformamide and 0.2% DTT and incubated for 1 h at 70°C. Samples were centrifuged and 10 µl of the supernatant was used for electrophoresis. Proteins were analysed in 1D-gradient SDS-PAGE (T=8–15%, C=2.67). Gels were run at 9 mA constant current for about 20 h, stained overnight with 12% trichloroacetic acid solution containing 0.05% Coomassie brillant blue R-250 in absolute ethanol (1% w/v) and de-stained in water.

Results and discussion

SDS-PAGE and PCR analysis of x- and y-type genes at the *Glu-A1* locus

SDS-PAGE separation of high-M_r glutenin subunits extracted from a set of bread- and durum-wheat cultivars is reported in Fig. 1. The materials used possess the three main alleles detected at the *Glu-A1x* locus in bread and durum wheat termed 2*, 1 and null (Payne and Lawrence 1983; Branlard et al. 1989).

In order to investigate the silent y-type genes present on the same chromosome, two pairs of primers were selected from the published sequences of the two different *Glu-A1y* alleles detected in Cheyenne and Chinese Spring. The first pair of primers (a and b) was designed to obtain specific amplification of the region that lies downstream from the transposon-like insertion in the Chinese Spring allele. To obtain this, the sense primer was selected within, but at the distal end of, the transposon, whereas the antisense primer was located at the end of the coding sequence. The second pair of primers (c and d) was selected to amplify a fragment formed by the N-terminal and the repetitive domain of the *Glu-A1y* gene present in Cheyenne and at the same time to avoid the amplification of other y-type genes present on chromosomes 1B and 1D. This was possible using a cysteine codon difference existing in the repetitive domain, close to the C-terminal region, between the *Glu-A1y* gene of Cheyenne and those present at the *Glu-B1* and *Glu-D1* loci (Forde et al. 1985; Shewry et al. 1989).

These two pairs of primers were used on DNA prepared from the same materials analysed by SDS-PAGE for their composition in the high-M_r glutenin subunits reported in Fig. 1. All the genotypes possessing an expressed x-type subunit in SDS-PAGE (either 2* or 1) produced an amplified fragment of similar size (approximately 1500 bp) to the one obtained from the wheat cultivar Cheyenne (lane 1) only when primers c and d were used (lanes 1–8 on Fig. 2). No amplification was obtained with primers a and b. A small size difference was, however, observed between fragments of cultivars possessing subunit 2* (lanes 1–4) compared to those possessing subunit 1 (lanes 5–8), with the former slightly smaller than the latter.

By contrast, when genotypes without any expressed subunit at the *Glu-A1x* locus (null allele) were analysed

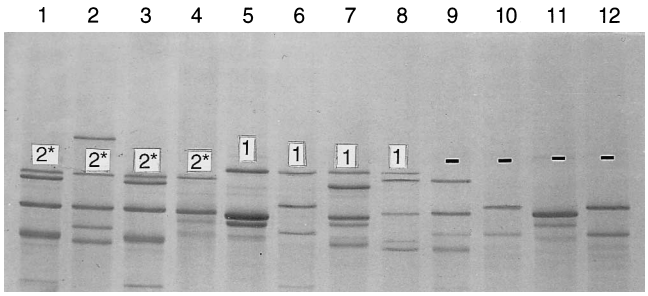

Fig. 1 SDS-PAGE separation of high-M_r glutenin subunits present in durum- and bread-wheat cultivars possessing alleles 2*, 1 and null at the *Glu-A1* locus. *Lanes*: *1* Cheyenne, *2* MG 7249, *3* Fiocco, *4* Duramba, *5* Lobeiro, *6* Fenix, *7* MG 2272, *8* Pandas, *9* Chinese Spring, *10* Langdon, *11* Durati, *12* Creso

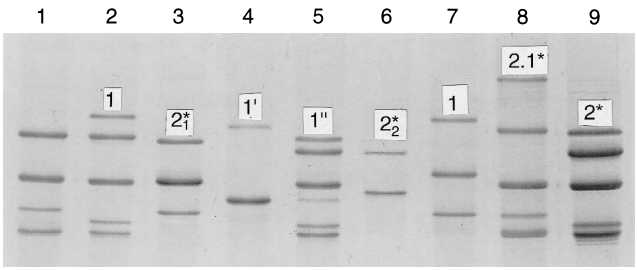

Fig. 3 SDS-PAGE separation of high M_r glutenin subunits present in durum- and bread-wheat cultivars with novel allelic subunits at the *Glu-A1* locus. *Lanes*: *1* Chinese Spring, *2* Pandas, *3* MG 68, *4* Belfuggito, *5* BT 2288, *6* MG 2984, *7* MG 2272, *8* PK 15684, *9* Cheyenne

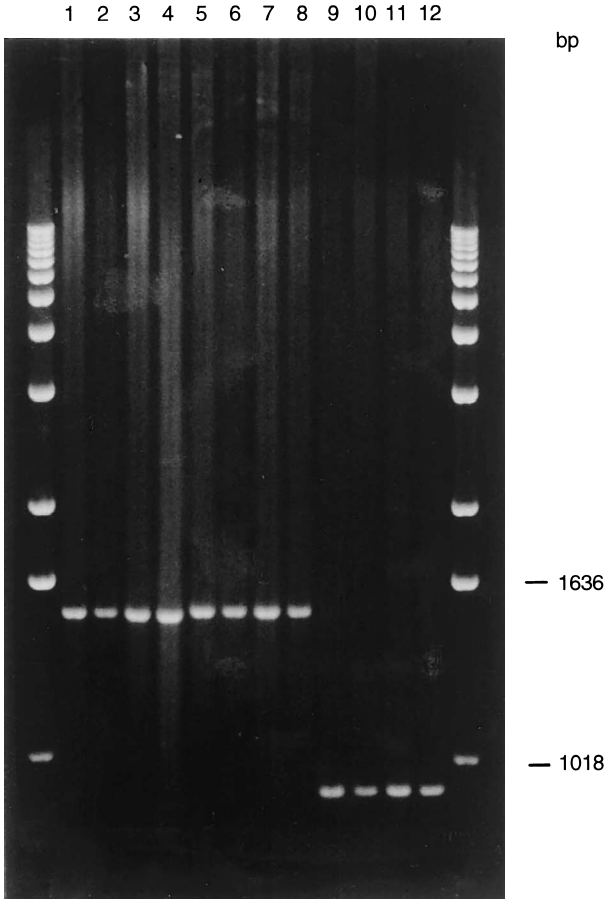

Fig. 2 Agarose-gel separation of amplified fragments corresponding to the x-type gene present at the *Glu-A1* locus in the same materials shown in Fig. 1. Primers c and d were used for samples in *lanes 1–8* and primers a and b for samples in *lanes 9–12*. A 1-Kb DNA marker is included on both sides of the gel

(lanes 9–12) a fragment (approximately 920 bp) similar to the one obtained in cultivar Chinese Spring (lane 9) was obtained with primers a and b, whereas no amplification was obtained when the c and d were used. Further analyses carried out on several durum- and bread-wheat cultivars, possessing the three mentioned alleles, allowed the extension of these results; in general, when a protein subunit is present at the *Glu-A1x* locus (either 2* or 1) the y-type gene is similar to the one present in Cheyenne, whereas when no Ax subunit is present the y-type gene is similar to the one present in Chinese Spring and possesses an 8-kb transposon-like insertion, confirming linkage between the x- and y-type genes for the *Glu-A1* locus.

In addition to the three major alleles detected at the *Glu-A1x* locus (Payne and Lawrence 1983), SDS-PAGE analyses of primitive cultivars and land races of durum and bread wheats have revealed a few additional allelic variants at this locus (Waines and Payne 1987; Branlard et al. 1989; Margiotta et al. 1993). Some of these new variants, designated 1′, 1″, 2^*_1, 2^*_2 and 2.1*, are shown in Fig. 3 together with subunits 2* of Cheyenne (lane 9) and 1 of Pandas and MG 2272 (lanes 2 and 7 respectively) for reference; Chinese Spring (null allele) is also included (lane 1). All of the new allelic variants have electrophoretic mobilities similar to subunits 2* and 1, except for subunit 2.1* (Tahir et al. 1996), which has a much reduced mobility (lane 8).

When the primers developed by D'Ovidio et al. (1995) for allelic *Glu-A1x* genes were used on genomic DNA prepared from the genotypes reported in Fig. 3, amplification products were obtained in all cases and estimates of the size of corresponding genes were deduced. Except for the fragment corresponding to subunit 2.1*, which had a size of 3000 bp, all of the fragments corresponding to the other subunits were of similar size (approximately 2500 bp) with small but detectable differences among them (data not shown). Bread-wheat cultivar Chinese Spring which, as mentioned, possesses an x-type gene not expressed at the *Glu-A1* locus, also gave an amplified fragment of similar size (2500 bp).

Linked y-type genes were also investigated and, as observed for those associated with genes encoding subunits 2* and 1, an amplified fragment of approximately 1500 bp was obtained with primers c and d (Fig. 4).

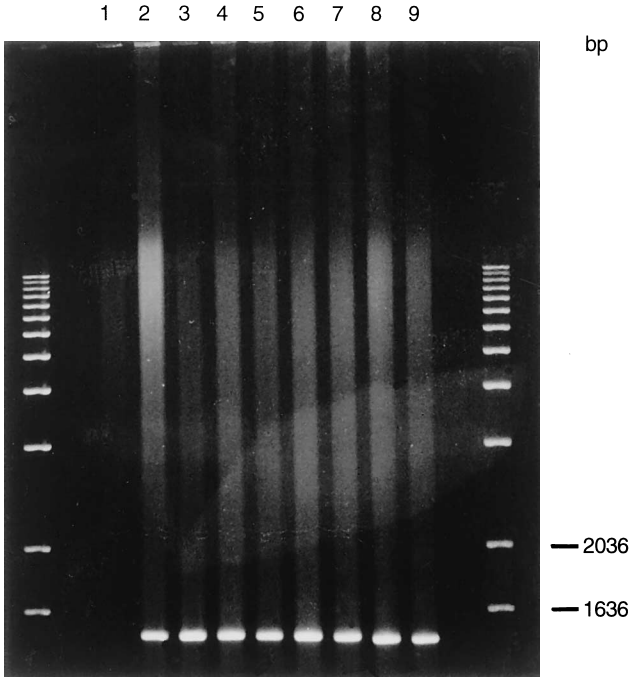

Fig. 4 Agarose-gel separation of amplified fragments, obtained using primers c and d, corresponding to the y-type gene present at the *Glu-A1* locus in the same materials shown in Fig. 3

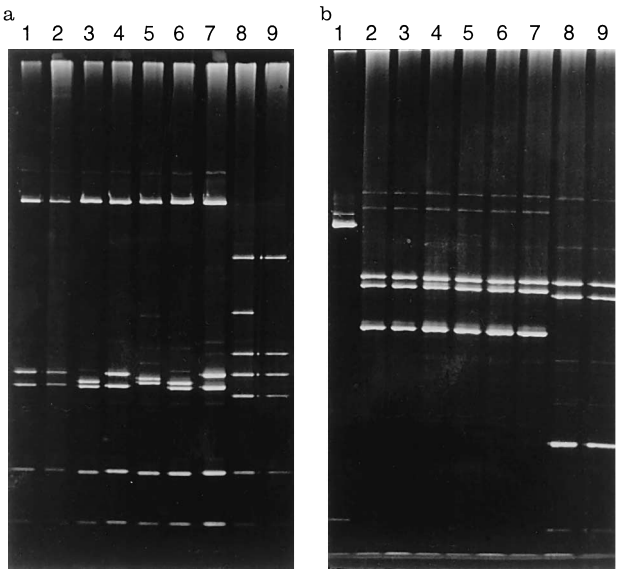

Fig. 5 a, b Polyacrylamide-gel separation of digested amplified fragments from the materials reported in Fig. 3. **a** Fragments corresponding to x-type genes digested with *Hae*III; **b** fragments corresponding to y-type genes digested with *Dde*I

Digestion patterns of amplified fragments corresponding to x- and y-type genes

Further information on the structure of genes corresponding to the different alleles present at the *Glu-A1x* locus and the linked unexpressed y-type genes were obtained by the digestion of amplified fragments with restriction enzymes.

When amplified fragments corresponding to x-type genes were digested with *Hae*III, two main types of patterns were clearly observed (Fig. 5 a). The first one corresponded to genes for subunits 2.1* and 2* (lanes 8 and 9) and the second one to the remaining subunits, including the unexpressed gene present in Chinese Spring (lanes 1–6). Within the first pattern, the gene corresponding to subunit 2.1* produced an extra fragment of approximately 600 bp compared to the digestion pattern of the gene corresponding to subunit 2*. The null allele present in Chinese Spring showed a digestion pattern identical to the one obtained from the digested fragment corresponding to subunit 1 present in Pandas and MG 2272 (lanes 1, 2 and 7).

Amplified fragments of linked y-type genes were digested with *Dde*I and, similarly to that observed for x-type genes, two types of patterns were evident (Fig. 5 b). The first one includes y-type genes linked with x-type genes encoding subunits 2* and 2.1*, while the second group includes those linked with the remaining x-type genes. In lane 1 of the same figure the undigested fragment amplified from Chinese Spring, corresponding to the region downstream from the transposon-like insertion, is shown.

Comparison of x- and y-type genes of Chinese Spring and those of cultivars expressing subunit 1

Electrophoretic separation of amplified and *Hae*III-digested DNA fragments, corresponding to subunit 1 present in Pandas and the allelic null gene of Chinese Spring, produced the same pattern (Fig. 5 a), suggesting that these two genes are very similar. A comparison of the digestion patterns of fragments corresponding to linked y-type genes present in these two cultivars could not be performed due to the presence of the transposon-like insertion in the Chinese Spring gene. As stressed earlier, the y-type gene of Chinese Spring contains one extra 18-bp repeat unit (Harberd et al. 1987). The presence of this extra repeat unit in the *Glu-A1y* gene of the bread-wheat cultivar Pandas was verified; the amplified fragment corresponding to the y-type gene present in this cultivar was cloned and partially sequenced. Comparison of the sequence determined for Pandas with homologous DNA regions of Chinese Spring and Cheyenne (Fig. 6) show that Pandas possesses the same extra repeat of 18 bp as Chinese Spring, though a substitution (G rather than C) is present at the beginning of the second repeat. This result further substantiates the strong similarities of linked x- and y-type genes present at the *Glu-A1* locus in Chinese Spring with those present in cultivars possessing an expressed x-type subunit 1. The presence of the extra repeat unit in Pandas accounts for the slightly larger size of the amplified fragments observed in cultivars possessing subunit 1 compared to those with subunit 2*, as reported in Fig. 2.

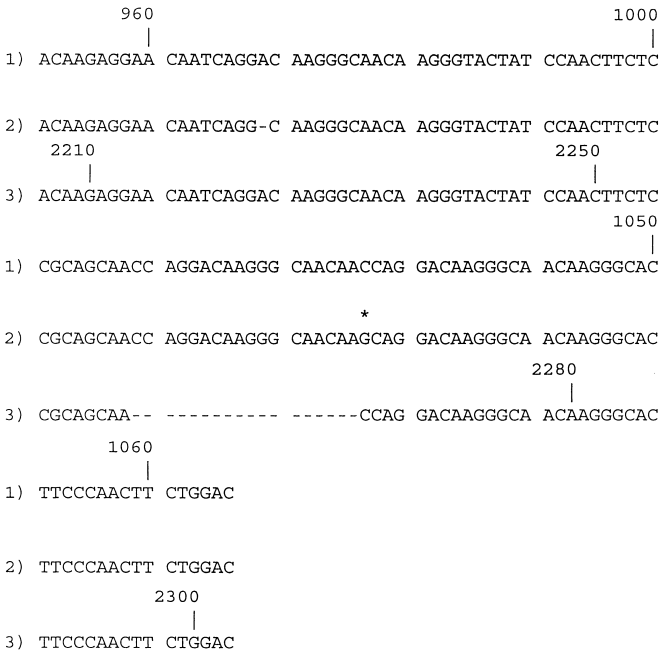

Fig. 6 Comparison of the partial DNA sequences of the *Glu-A1y* gene of Chinese Spring (*1*), Pandas (*2*) and Cheyenne (*3*) in the region where a duplicated unit of 18 bp is present. *Arrows* mark the boundaries of the two repeat units in Chinese Spring, and the *asterisk* indicates a base substitution

Conclusions

Comparative SDS-PAGE and PCR analyses of several bread- and durum-wheat cultivars allowed a close genetic linkage to be established between x- and y-type genes at the *Glu-A1* locus, as already demonstrated by protein analysis for the x- and y-type genes present at the *Glu-B1* and *Glu-D1* loci (Payne 1987).

The usefulness of PCR in a wide range of genetical studies has already been stressed (D'Ovidio et al. 1990, 1994; Inoue et al. 1994; Smith et al. 1994; Talbert et al. 1994). Additionally, we have now extended use of this technique to the study of silenced genes and determined their structural relationships with expressed ones. The use of a primer within the transposon-like sequence of Chinese Spring has made it possible to identify this particular insertion in several bread- and durum-wheat genotypes and to assess close linkage with the null allele at the *Glu-A1x* locus.

Previous results of RFLP analysis of bread-wheat cultivars (Harberd et al. 1986; Margiotta et al. 1993), showed that the *Glu-A1x* alleles could be divided into two groups according to the restriction fragments they contain. The members of the first group are identical on the basis of their restriction fragments and either encode subunit 1 or else are null alleles. The second group contains a smaller *Hin*dIII fragment than is found in the first group and encodes subunit 2*. Digestion patterns observed for the silent x-type gene and the one corresponding to subunit 1 confirm RFLP results; furthermore, the detection of an 18-bp extra repeat unit, also in the linked y-type gene of Pandas, suggests that the *Glu-A1y* allele, associated with the x-type gene expressing subunit 1, could represent a progenitor into which the 8-kb transposon-like insertion has given rise to a Chinese Spring-like *Glu-A1y* allele.

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