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Genetic Analysis of low M_r Glutenin Subunits Fractionated by Two-dimensional Electrophoresis (A–PAGE × SDS–PAGE)

R. Redaelli*, M.-H. Morel†, J.-C. Autran† and N. E. Pogna‡

*Istituto Sperimentale per la Cerealicoltura, via Mulino 3, 20079 S. Angelo Lodigiano, Italy, †INRA, Laboratoire de Technologie des Céréales, 2 Place Viala, 34060 Montpellier, France and ‡Istituto Sperimentale per la Cerealicoltura, via Cassia 176, 00191 Roma, Italy.

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ABSTRACT

Alkylated glutenin subunits of F_2 progenies from the cross between the Italian bread wheat cultivar Costantino and the Canadian cultivar Neepawa were fractionated by one-dimensional A–PAGE and SDS–PAGE and by two-dimensional A–PAGE × SDS–PAGE. Each gliadin allele at the *Gli-1* loci of the parental cultivars was shown to be associated with a specific allele at each of the *Glu-3* loci, at which low M_r glutenin subunits are encoded. The *Glu-A3* locus was found to code for two low M_r subunits in Neepawa and three in Costantino. In this latter cultivar, eight low M_r subunits were assigned to each of the *Glu-B3* and *Glu-D3* loci, whereas seven subunits were attributed to the *Glu-B3* locus and seven to the *Glu-D3* locus in Neepawa. A–PAGE × SDS–PAGE can be employed for a detailed description of low M_r subunits of glutenin in different cultivars following a genetic approach based on the correspondence between the alleles at the *Gli-1* and *Glu-3* loci.

Keywords: low M_r glutenin subunits, bread wheat, two-dimensional electrophoresis, recombinant inbred lines.

INTRODUCTION

Glutenin represents about 45% of the total protein in the endosperm of bread wheat (*Triticum aestivum* L.). It has been shown to have an important effect on wheat technological quality[1]. Glutenin is a polymeric protein, ranging in M_r from 80k to several millions[2], in which many subunits are cross-linked by disulphide bonds. These subunits can be fractionated, after reduction, by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). They subdivide into high M_r glutenin subunits (M_rs of 80–120k) and low M_r glutenin subunits (M_rs of 30–50k)[3].

Low M_r glutenin subunits have been studied intensively in the past few years in view of their possible contribution to breadmaking quality[4–10]. The aim of these studies has been to obtain a description of their allelic variability and to determine their relationship with the viscoelastic properties of dough. Unfortunately, low M_r glutenin subunits have proved to be difficult to analyse because they co-migrate with some of the gliadin polypeptides in one-dimensional SDS–PAGE under reducing conditions.

Different techniques have been developed to differentiate gliadin polypeptides and low M_r glutenin subunits in electrophoretic patterns. One of these consists of a two-step SDS–PAGE fractionation: polymeric glutenin is trapped in the first centimeter of a gel run first under unreduced

ABBREVIATIONS USED: A–PAGE = acid. polyacrylamide gel electrophoresis; SDS–PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; IEF = isoelectric focusing; NEPHGE = non-equilibrium pH-gradient electrophoresis.

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conditions, and the low M_r subunits are fractionated subsequently, after reduction, in a SDS–PAGE gel[11]. In another approach, a selective extraction procedure has been used: gliadins are extracted away from the sample with propan-1-ol or dimethyl sulphoxide (DMSO), then the polymeric glutenin is reduced and alkylated before fractionation by one-dimensional SDS–PAGE[7,8] or A–PAGE[9]. Two-dimensional electrophoresis has also been applied to the fractionation of low M_r glutenin subunits[10,12,13].

In this work we present an improved two-dimensional method (A–PAGE × SDS–PAGE) for low M_r glutenin subunit separation. It has been applied to a group of recombinant inbred lines derived from the cross between two spring bread wheat cultivars. This method allowed us to identify low M_r subunits encoded at each of the three *Glu-3* loci in the parental cultivars.

EXPERIMENTAL

Plant material

The spring bread wheat cultivars Costantino (Italian) and Neepawa (Canadian) were crossed in the greenhouse at the Istituto Sperimentale per la Cerealicoltura, S. Angelo Lodigiano, Italy. The F_2 progeny was analysed by electrophoresis of gliadins, and the plants heterozygous at each *Gli-1* locus were multiplied in the field. This procedure was repeated for six self-pollinated generations. Finally, F_7 recombinant inbred lines homozygous at the *Gli-1* loci were selected on the basis of gliadin electrophoretic analysis and used in this study. Bread wheat cultivars from several countries and mutant lines lacking the *Gli-B1/Glu-B3* loci or the *Gli-D1/Glu-D3* loci were also analysed.

Gliadin extraction and fractionation

Grains of each F_7 line were crushed and extracted with 70% ethanol (v/v) for 2 h at 50°C. Gliadins were fractionated in a 15% acrylamide, pH 8·4, SDS–PAGE gel. The gliadin compositions were determined as described previously[14].

Extraction of low M_r glutenin subunits

Low M_r glutenin subunits were extracted according to Singh *et al.*[7] with the following modifications. Single crushed grains were extracted three times with 50% (v/v) propan-1-ol (1·5 ml) for 30 min at 60°C; after centrifugation, the supernatant was discarded. The pellet was then resuspended in 50% (v/v) propan-1-ol, 0·08 M Tris-HCl, pH 8·5, 0·02 M dithiothreitol (DTE) (0·15 ml). The samples were incubated for 30 min at 60°C. After centrifugation, the supernatant was transferred to a new tube and diluted with the same volume of a solution containing 50% (v/v) propan-1-ol, 0·08 M Tris-HCl, pH 8·5, 0·04 M vinylpyridine (VP). Both DTE and VP were added to the buffer just before use.

Acid-PAGE (A–PAGE)

An aliquot (0·15 ml) of reduced and alkylated glutenin was precipitated with cold acetone (1 ml), centrifuged at 12 000 rpm for 15 min at 0°C and dried under a hood for 1 h. The dried pellet was resuspended in 6 M urea, 30% (w/v) glycerol, 0·025 M acetic acid (0·05 ml). Aliquots (7 µl) of the extracts were loaded onto the gel and fractionated according to Morel[9].

SDS–PAGE (one dimensional)

SDS–PAGE running gels were prepared with 15% (w/v) acrylamide ($T=15\%$, $C=0{\cdot}5\%$), 0·375 M Tris-HCl, pH 8·4, 0·1% (w/v) SDS. Stacking gels ($T=5{\cdot}6\%$, $C=10\%$) contained 0·08 M Tris-HCl, pH 6·8, 0·1% (w/v) SDS. Electrophoresis buffer was 0·025 M Tris-glycine, pH 8·3, 0·1% (w/v) SDS. Aliquots (0·01 ml) of the extracts were loaded into each well. The gels (160 × 180 × 1·5 mm) were run at 20 mA/gel at 18°C and stopped 1 h after the tracking dye (pyronin Y) had reached the bottom of the gel. They were stained in 12·5% (w/v) trichloroacetic acid, 0·01% (w/v) Coomassie Brilliant Blue R250, and destained with distilled water.

Two-dimensional A–PAGE × SDS–PAGE

After the first dimension (A–PAGE), the gels were cut into single strips and incubated for 30 min in 0·0625 M Tris-HCl, pH 6·8, 2% (w/v) SDS, 40% (w/v) glycerol. The strips were then loaded onto a SDS–PAGE gel prepared as described above. Gels were run at 40 mA/gel at 18°C and stopped 30 min after the tracking dye had reached the bottom of the gel. They were stained as described above.

Table I Allele compositions at the *Gli-1*, *Glu-1* and *Glu-3* loci of F_7 lines from the cross between cvs Costantino and Neepawa

Cultivar or line	Gliadin[a] and low M_r glutenin subunits			High M_r glutenin subunits[b]		
	Gli-A1 *Glu-A3*	*Gli-B1* *Glu-B3*	*Gli-D1* *Glu-D3*	*Glu-A1*	*Glu-B1*	*Glu-D1*
Costantino	*a*	*m*	*k*	*a*	*a*	*a*
Neepawa	*m*	*d*	*j*	*b*	*c*	*d*
R3	*a*	*d*	*k*	*b*	*c*	*d*
R6	*m*	*m*	*k*	*a*	*c*	*a*
R8	*m*	*d*	*k*	*b*	*a*	*a*
R19	*a*	*d*	*j*	*b*	*a*	*a*
R20	*m*	*m*	*j*	*b*	*a*	*a*
R22	*m*	*m*	*j*	*b*	*a*	*a/d*
R23	*a*	*m*	*j*	*b*	*a*	*a*
R24	*a*	*null*	*j*	*a*	*a*	*d*

Nomenclature of [a]Metakovsky[16] and [b]Payne and Lawrence[25].

RESULTS

The *Gli-1* loci, encoding γ- and ω-gliadins, and the *Glu-3* loci, encoding low M_r glutenin subunits, are linked tightly on the short arms of group 1 chromosomes[1]. The *Glu-B3* locus was found to be 1·8–2·0 cM from *Gli-B1*[11,15]; so far, no recombination has been found between the *Gli-1* and *Glu-3* loci on chromosomes 1A and 1D[11]. Therefore, *Gli-1*-encoded gliadins can be considered as reliable markers of low M_r glutenin subunits.

According to the nomenclature of Metakovsky[16], the cultivar Costantino carries alleles *Gli-A1a*, *Gli-B1m* and *Gli-D1k*, whereas Neepawa carries alleles *Gli-A1m*, *Gli-B1d* and *Gli-D1j*. The allele compositions at the *Gli-1* loci of the genotypes analysed here are reported in Table I.

Reduced and alkylated glutenins from the parental cultivars and their F_7 inbred lines were fractionated by one-dimensional A–PAGE and SDS–PAGE [Fig. 1 (a) and (b)]. On the basis of the gliadin allele compositions of the genotypes analysed, it was possible to attribute some low M_r subunits in the A–PAGE pattern to each of the three *Glu-3* loci on chromosomes 1A, 1B and 1D. For example, the low M_r subunits arrowed in the A–PAGE fractionation of cv. Neepawa [Fig. 1(a), lane N] were attributed to the *Glu-D3* locus because they occurred only in genotypes containing the *Gli-D1j* allele from Neepawa (lanes R19, R20 and R23).

Moreover, a strong band in the A–PAGE pattern of Costantino [Fig. 1(a), lane C, asterisk] was found to comprise two overlapping subunits, one controlled by chromosome 1A, the other by chromosome 1B. In fact, this band was present in lines containing either the *Gli-A1a* or the *Gli-B1m* alleles of cv. Costantino [Fig. 1(a), lanes R3, R6, R19, R20] and absent in lines possessing both the *Gli-A1m* and *Gli-B1d* alleles of cv. Neepawa [Fig. 1(a), lanes R8 and N].

Several low M_r glutenin subunits in the A–PAGE patterns could not be attributed to any specific *Glu-3* locus, however, because of overlap between polypeptides encoded by different alleles.

Another group of proteins, which was found to be present in significant amounts in the A–PAGE patterns, is the group of alkylated high M_r glutenin subunits: these have been numbered in Fig. 1(a). Moreover, a few ω-gliadins, encoded at the *Gli-D1* locus, appeared as faint bands in the anodic region of the gel. The high M_r subunits of glutenin were recognised easily by SDS–PAGE [Fig. 1(b)]. In contrast, only three bands in the B group of low M_r subunits and four bands in the C group were attributed to the *Glu-3* loci.

The one-dimensional electrophoretic systems were combined in a two-dimensional A–PAGE × SDS–PAGE method. Figure 2 shows the two-dimensional maps of reduced and alkylated glutenins from Costantino (a) and Neepawa (b). The high M_r subunits of glutenin were identified easily. Low M_r subunit patterns appeared to comprise 30 to 40 polypeptides with different staining intensities. Despite the extraction with propan-1-ol before reducing the glutenin, some ω-gliadins were found to be present in small amounts in the two-dimensional patterns (Fig. 2, brackets).

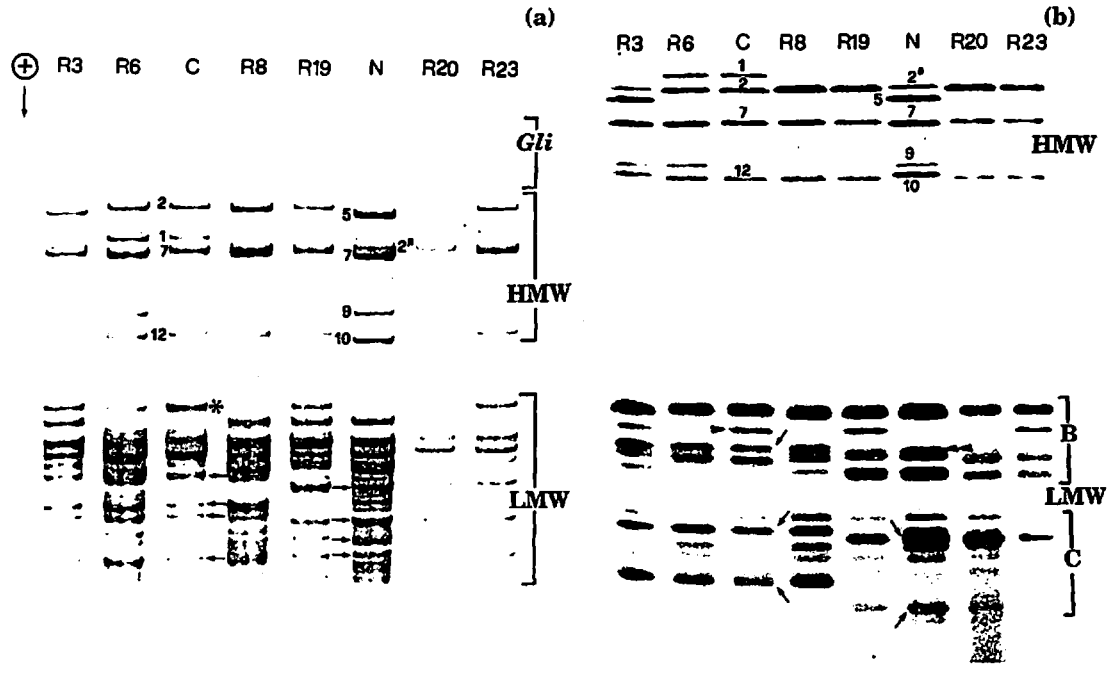

Figure 1 One-dimensional A–PAGE (a) and SDS–PAGE (b) of F_7 lines derived from the cross between cv. Costantino (lane C) and cv. Neepawa (lane N). Low M_r glutenin subunits encoded at *Glu-A3* (arrowheads), *Glu-B3* (double arrowheads) and *Glu-D3* (arrows) are indicated. Asterisk in (a) shows a band comprising two low M_r subunits encoded at *Glu-A3* and *Glu-B3*. High M_r glutenin subunits are numbered according to Payne and Lawrence[25].

Figure 3 shows the two-dimensional maps of reduced and alkylated glutenins from six F_7 lines. To attribute low M_r glutenin polypeptides to the *Glu-A3* locus, the pattern of cv. Costantino [Fig. 2(a)] was compared with that of line R6, which carries the *Gli-A1m* gliadin allele of cv. Neepawa in a cv. Costantino background [Fig. 3(a)], whereas the pattern of cv. Neepawa [Fig. 2(b)] was compared with that of line R19, which contains the *Gli-A1a* allele of cv. Costantino in a cv. Neepawa background [Fig. 3(b)]. Three main components in cv. Costantino [Fig. 3(b), arrows] and two components in cv. Neepawa [Fig. 3(a), arrows] were shown to be encoded at the *Glu-A3* locus. The location of genes coding for these low M_r subunits was confirmed by A–PAGE × SDS–PAGE of alkylated glutenins from cultivars Pricama, Giuliana, Liocorno, Hope, Thatcher, Ilves and Ruso, which contain the *Gli-A1m* allele as in cv. Neepawa, and cultivars Chiarano and Loreto, which carry the *Gli-A1a* allele as in cv. Costantino.

The low M_r subunits encoded at the *Glu-B3* locus were identified by comparing cv. Costantino [Fig. 2(a)] with line R3, which contains the *Gli-B1d* allele of cv. Neepawa in a cv. Costantino background [Fig. 3(c)]. Eight polypeptides in cv. Costantino and seven in cv. Neepawa were attributed to the *Glu-B3* locus [Fig. 3(c) and 3(d)]. All these polypeptides belong to the B group of low M_r glutenin subunits. These results were confirmed when the pattern of cv. Neepawa [Fig. 2(b)] was compared with that of line R22, which contains the *Glu-B1m* allele of cv. Costantino in a cv. Neepawa background [Fig. 3(d)].

Figure 4 shows the A–PAGE × SDS–PAGE pattern of glutenins from progeny R24, which lacks the *Gli-B1* locus because of the spontaneous deletion of the short-arm satellite of chromosome 1B (data not shown). As expected this line does not contain any of the low M_r subunits assigned to the *Glu-B3* locus, *Gli-B1* and *Glu-B3* both being located on the 1B satellite[17,18].

Finally, the two-dimensional map of cv. Costantino [Fig. 2(a)] was compared with that of line R23, which carries the *Gli-D1j* allele of cv. Neepawa in a cv. Costantino background [Fig. 3(e)]. The *Glu-D3* locus was found to encode seven main low M_r subunits in cv. Costantino and six in cv. Neepawa, only one band for each allele belonging to the B group of low M_r subunits [Fig. 3(e) and (f)]. Comparison of cv. Neepawa with line R8, which contains the *Gli-D1k* allele of cv.

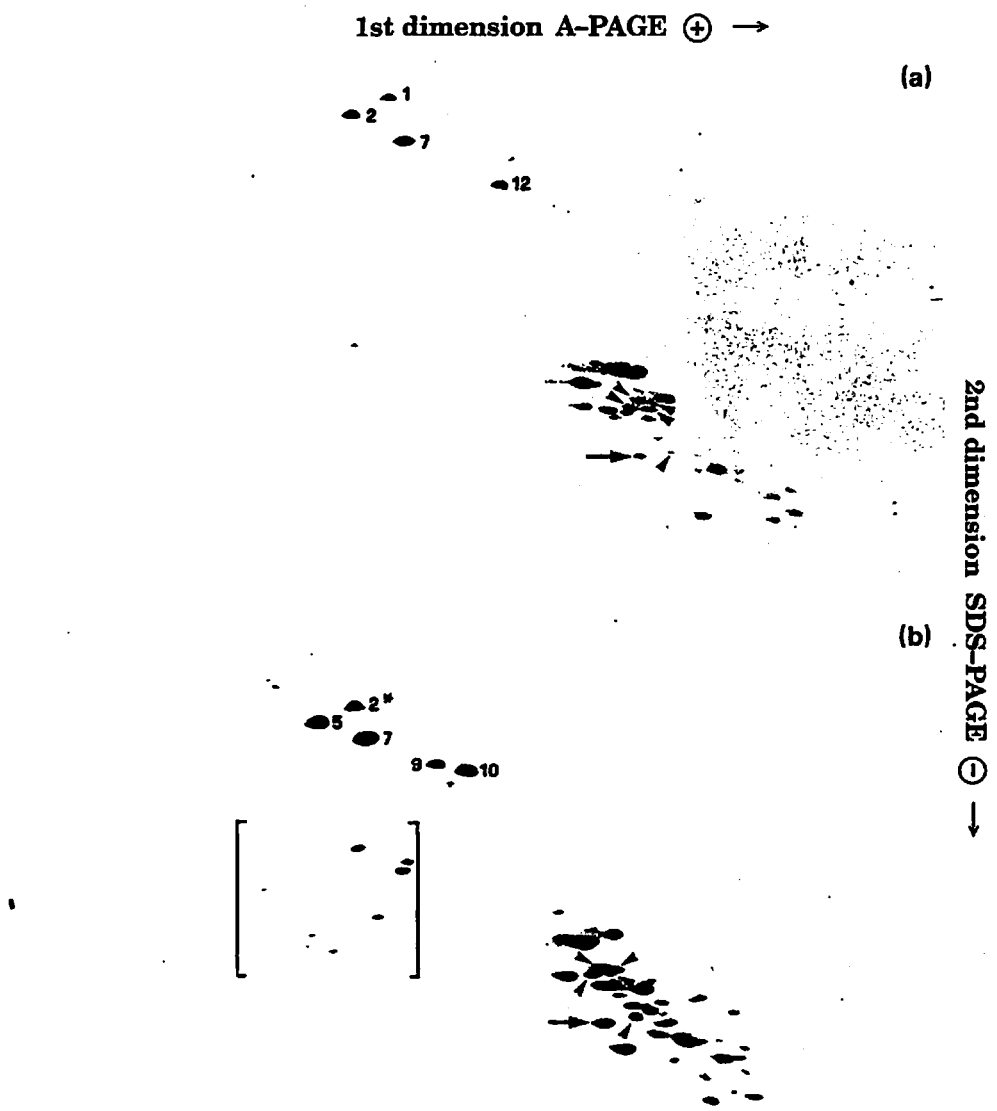

Figure 2 Two-dimensional A-PAGE × SDS-PAGE of glutenin subunits from cv. Costantino (a) and cv. Neepawa (b). High M_r glutenin subunits are numbered according to Payne and Lawrence[25]. Arrows and arrowheads indicate low M_r subunits that are present in both cultivars. Gliadins are shown in brackets.

Costantino in a cv. Neepawa background [Fig. 3(f)], confirmed these conclusions.

Several low M_r subunits have identical or very similar positions in the two-dimensional maps of cvs Costantino and Neepawa (Fig. 2). These subunits occurred in all the F_7 lines analysed and, therefore, could not be assigned to any *Glu-3* allele. Five of these subunits (Fig. 2, arrowheads) were found to be present in the deletion line R24, however, suggesting that they are not encoded at the *Glu-B3* locus. Furthermore, the low M_r subunit arrowed in Fig. 2 was found to occur in both parents, as well as in all the bread wheat cultivars fractionated here by A-PAGE × SDS-PAGE. This component is likely to be encoded at the *Glu-D3* locus because it is absent, along with other *Glu-D3*-encoded subunits, in the mutant line Alpe 3, which lacks the *Gli-D1* locus because of deletion of a small segment of chromosome 1DS[19].

Several minor spots were difficult to attribute to any *Glu-3* locus because their behaviour was inconsistent. In some lines their presence was clear, in some others they were present as very faint spots if at all. Our data are not sufficient to infer

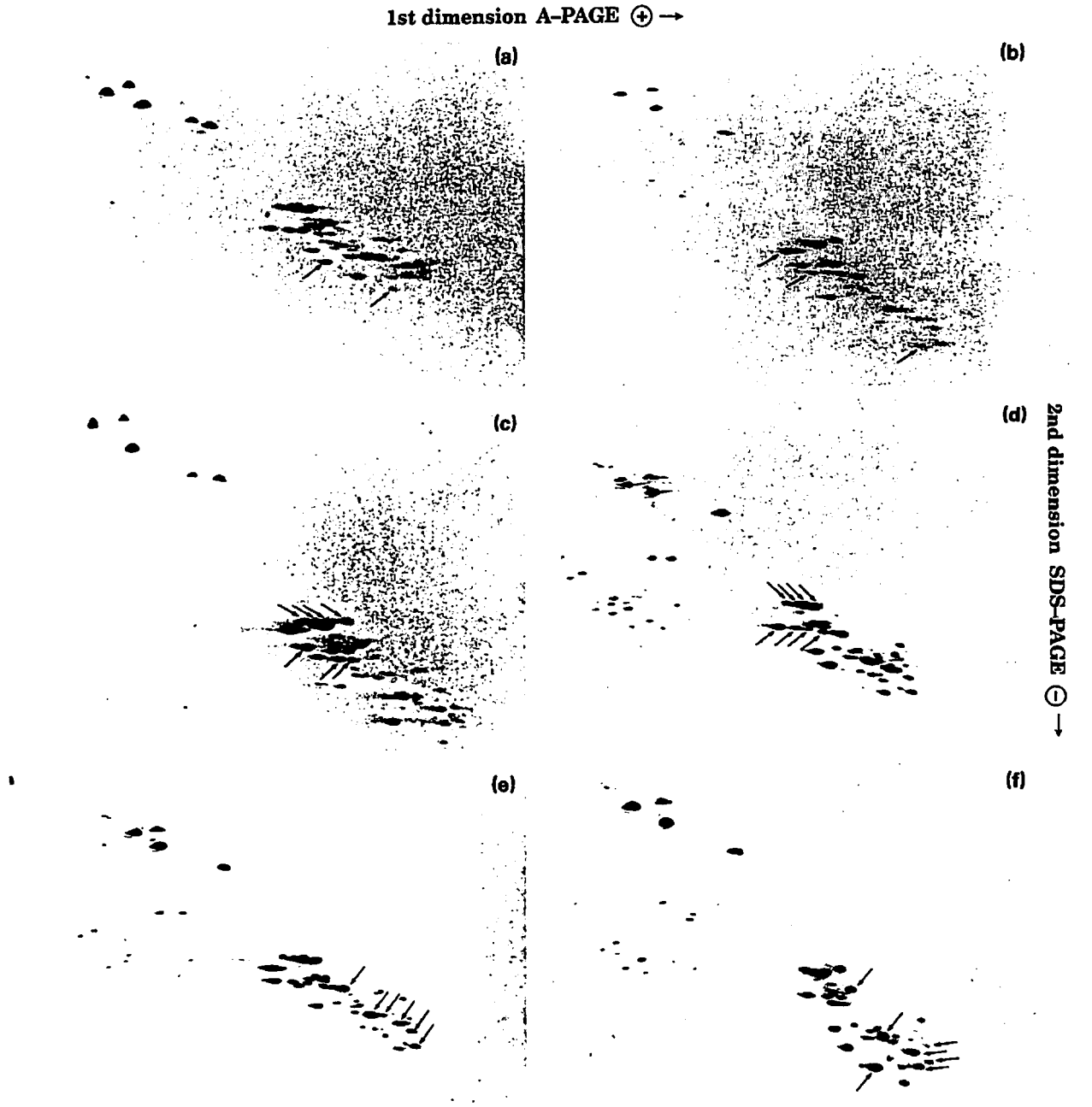

Figure 3 Two-dimensional A-PAGE × SDS-PAGE of glutenin subunits from F_7 lines: (a) R6; (b) R19; (c) R3; (d) R22; (e) R23; (f) R8. Arrows indicate low M_r subunits encoded by each *Glu-3* allele.

which chromosome or locus is involved in their genetic control.

DISCUSSION

Several electrophoretic techniques have been applied to separate low M_r subunits in bread wheat cultivars. The method proposed by Gupta and Shepherd[5] consists of a two-step, one-dimensional fractionation by SDS–PAGE in which glutenin is reduced before being loaded onto a gradient gel for the second step. A great number of bread wheat cultivars have been analysed by this method. Three different groups of electrophoretic patterns were identified and attributed to genomes A, B and D. A similar approach was proposed by Khelifi

Figure 4 A–PAGE × SDS–PAGE fractionation of low M_r glutenin subunits from the *Glu-B3*-null line R24. Arrowheads indicate low M_r subunits that are present in both cvs Costantino and Neepawa.

and Branlard[6], the first separation being A–PAGE. Furthermore, Singh *et al.*[7] and Gupta and MacRitchie[8] have described an extraction procedure to remove gliadin from flour before fractionation of reduced glutenin by one-step SDS–PAGE. Some low M_r subunits have also been analysed by different two-dimensional electrophoresis methods, such as IEF or NEPHGE × SDS–PAGE[10,12] and two-pH electrophoresis[13].

Application of two-dimensional electrophoresis to genetic studies of prolamin genes was discussed by Shewry *et al.*[20] Although generally this approach is not followed in recombination studies because of the great number of progenies required to obtain statistically significant results, it provides high resolution of individual polypeptides. For example, genes coding for gliadins have been located to chromosomes by A–PAGE × SDS–PAGE of aneuploid lines of Chinese Spring[21].

In the present study we describe an improved two-dimensional A–PAGE × SDS–PAGE for fractionation of low M_r subunits of glutenin. Acid PAGE in the first dimension, buffered with acetic acid, provided better resolution of low M_r subunits in one-dimensional electrophoresis than a sodium lactate system. On the other hand, the use of pH 8·4 in the SDS–PAGE running gel gave improved resolution of certain high M_r glutenin subunits with similar mobilities, especially subunits 2 and 2* (unpublished results).

Two-dimensional A–PAGE × SDS–PAGE maps showed reproducible fractionations of low M_r subunits: the alleles at each *Glu-3* locus were shown to control the synthesis of specific polypeptides. As expected from the tight linkage between the *Gli-1* and *Glu-3* loci, there was a close correspondence between gliadin and low M_r subunit alleles. Our results are in agreement with those by Gupta *et al.*[22] These authors compared the A–PAGE patterns of gliadins with the SDS–PAGE fractionation of low M_r subunits and found a strong parallelism between alleles coding for gliadins and those coding for low M_r subunits. In several cases, apparently identical low M_r subunits occurred in cultivars containing different alleles at the gliadin-encoding loci, however. The converse, that is two or more different low M_r subunit patterns for the same *Gli-1* allele, was also found. According to these authors, these deviations are due to differences in the resolving powers of A–PAGE and SDS–PAGE, as well as to rare recombination between *Gli-1* and *Glu-3*.

Each of the *Glu-3* alleles in cvs Costantino and Neepawa are indicated here by the letter of the corresponding gliadin allele as catalogued by Metakovsky[16] (Table I). The *Gli-A1a* allele of cv. Costantino and the *Gli-A1m* and *Gli-B1d* alleles of cv. Neepawa have been found to correspond, respectively, to the *Glu-A3c*, *Glu-A3e* and *Glu-A3h* alleles, as described by SDS–PAGE[22]. On the contrary, alleles *Gli-B1m*, *Gli-D1k* and *Gli-D1j* (Table I) have not been associated with any known allele at the *Glu-B3* or *Glu-D3* loci.

Low M_r glutenin subunits are important in determining the dough viscoelastic properties of hexaploid and tetraploid wheat flours[15,23]. However, the basis of differences in effects of different low M_r subunit alleles on dough properties are still largely unknown. Gupta and MacRitchie[24] have shown recently that the *Glu-A3e* allele (*Glu-A3m* in our nomenclature) produces no major B subunit, whereas allele *c* (allele *a* in our nomenclature) codes for one major B subunit. This allelic difference was found to be responsible for variation in both the size distribution of the glutenin polymers and dough strength. Our results show that the *e* (= *m*) allele codes for two C subunits, whereas its counterpart allele *c* (= *a*) produces one major plus one minor B subunit as well as one C-type polypeptide. Therefore, the main difference between these two alleles is in their effects on the ratio of B and C subunit quantities, as suggested previously[24]. On the other hand, *m* and *d* alleles at *Glu-B3* produce subunits in similar amounts, all of them

belonging to the B group. Finally, alleles *k* and *j* at *Glu-D3* code for one major B-type subunit plus six and seven C-type subunits, respectively, and, therefore, do not show significant differences in the amounts of low M_r subunits.

Two-dimensional A–PAGE × SDS–PAGE fractionations of the low M_r glutenin subunits of different wheat cultivars are in progress following a genetic approach based on the correspondence between the alleles at the *Gli-1* and *Glu-3* loci. The information gained from this type of study should prove valuable as a basis for explaining the contrasting effects of the *Glu-3* alleles on the viscoelastic properties of dough.

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