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Genetic characterization of storage proteins in a set of F_1-derived haploid lines in bread wheat

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Abstract Wheat storage proteins were evaluated by SDS-PAGE in a population of 206 doubled haploid (DH) lines, produced from a cross between bread wheat cvs Chinese Spring (CS) and Courtot (CT). The analysis of gliadins and high- and low-molecular-weight glutenins gave rise to 11 protein markers between parental varieties. Among these, one each was encoded at the *Glu-A1*, *Gli-A1*, *Gli-A2*, *Gli-A5*, *Glu-B3*, *Gli-B1* and *Gli-D1* loci and four were encoded at the *Glu-D3* locus. Only the *Gli-A2* marker showed a distorted segregation. A distance of 1.94 cM was evaluated between the *Gli-A1* locus and the recently found *Gli-A5* locus. Among the DH lines, only nine exhibited an unexpected pattern. The chromosome allocation was determined for almost all the LMW-GS and gliadin bands of CS using nullitetrasomic and ditelosomic lines. Two C LMW-GS were found to be coded by 6DS. Similarly, substitution lines into CT allowed the allelic determination of numerous LMW-GS and gliadin bands. A correspondence between gliadin markers separated in SDS-PAGE and in A-PAGE revealed that the common allele *Gli-Aa* between CS and CT determined in A-PAGE was able to be separated into two alleles when SDS-PAGE was used.

Key words Bread wheat · Allelic diversity · Gliadins · Glutenins · DH lines

Introduction

Among the wheat storage proteins, glutenins are polymeric structures which, when treated with reducing agents, give rise to two subgroups termed high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS). HMW-GS and LMW-GS are encoded at the *Glu-1* and *Glu-3* loci on the long and short arms respectively of the homoeologous group-1 chromosomes (1A, 1B and 1D). HMW-GS, encoded by pairs of linked genes on each chromosome, are constituted by a low- and high-molecular-weight y-type and x-type subunit respectively (Payne 1987; Shewry et al. 1992). The LMW-GS were subdivided into groups B, C on the basis of their molecular weight (Payne and Corfield 1979) and group D in the two-dimensional electrophoresis employed by Jackson et al. (1983).

The other main storage proteins, gliadins, are monomeric proteins separated into four groups, denoted as α, β, γ and ω when fractioned by gel electrophoresis at low pH (Woychick et al. 1961). Gliadin synthesis is mainly controlled by the *Gli-1* and *Gli-2* loci on chromosomes of homoeologous groups 1 and 6 (Payne et al. 1984).

Doubled haploid plants have many uses in genetic studies and plant breeding. Since haplo-diploidisation could considerably improve the efficiency of breeding, numerous authors have reported on the breeding value of doubled haploids as compared to lines developed by conventional selection methods (De Buyser et al. 1987; Picard et al. 1988; Baenziger et al. 1989).

Few reports mention studies of storage proteins in DH lines of bread wheat. Some authors have used HMW-GS to control the homogeneity of DH lines at different steps of breeding (Henry et al. 1980; Suenaga and Nakajima 1993). Others have investigated the relationships between HMW-GS and agronomic traits (Bjornstad et al. 1993; Inagaki and Egawa 1994). But, as far as we know, no paper has reported a genetic study of storage proteins, and especially LMW-GS and gliadins, using DH lines.

The aim of the present study was to establish the allelic assignment of LMW-GS and gliadins between cvs Chinese Spring and Courtot by SDS-PAGE (sodium dodecyl sulphate polyacrylamide-gel electrophoresis) in a large population of DH lines derived from the F_1 hybrid. Although the parental varieties were low-

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responding genotypes with regard to in vitro androgenesis, they have often been involved in academic genetic research. In addition, a number of unexpected patterns were described among the DH progeny. The A-PAGE (acid polyacrylamide-gel electrophoresis) method was employed in conjunction with SDS-PAGE to increase the fractionation of α, β and γ gliadins.

Materials and methods

Plant material

Two hundred and six DH lines were produced from F_1 hybrids (Chinese Spring × Courtot and reciprocal cross) of bread wheat through in vitro androgenesis following a protocol described previously (Martinez et al. 1994)

This material was developed in 1991 and 1992 and multiplied by selfing in the greenhouse at the INRA plant breeding station of Clermont-Ferrand, France.

In order to determine the chromosome allocation of LMW-GS and gliadin loci in the parental varieties, two sets of materials were used: the intervarietal chromosome substitution lines for groups 1 and 6 from cvs Prinqual, Magdalena, Cappelle, Vilmorin 23, Magnif 27 and Azteca into cv Courtot (CT) developed at INRA, Clermont-Ferrand (Bouguennec 1993), and the Chinese Spring (CS) nullisomic-tetrasomic and ditelosomic lines for chromosomes 1 and 6 (Sears 1954).

Protein extraction and electrophoresis

The sequential method of Marchylo et al. (1989), as modified by Singh et al. (1991), was used for protein extraction.

First, the unreduced gliadins were obtained by treating crushed endosperm halves (at least two kernels per genotype) with 50% propanol solution. The supernatant was divided into two equal parts and dried at 60 °C. The residue was dissolved either in sample buffer (SDS, Tris HCl 1M pH = 8; Singh et al. 1991) or in 25% chloro-2 ethanol solution (NFV03-723) and was fractioned in 10% T SDS-PAGE and 8% T A-PAGE, respectively.

The HMW-GS and LMW-GS present in the pellet were reduced and alkylated in 50% propanol solution with Dithiothreitol (1%) and 4-vinylpiridine (0.7%) and separated in 12% T SDS-PAGE according to the method of Laemmli (1970) as modified by Payne et al. (1980).

When an abnormal pattern was detected, ten more grains were analysed.

Band, allele and locus symbols

The nomenclature of subunits and alleles of HMW-GS (*Glu-1* locus) corresponds to the terminology of Payne and Lawrence (1983).

Some alleles of gliadins were denoted according to Metakovsky (1991) and some gliadins bands according to Khelifi et al. (1992).

For CS, the allelic nomenclature of LMW-GS and of gliadins is that of Gupta and Shepherd (1990) and Metakovsky (1991) respectively. The *Gli-1* alleles are generally easier to determine than the *Glu-3* alleles. As their respective loci are tightly linked, the nomenclature determined for gliadin alleles by Metakovsky (unpublished data) was employed for alleles of CT at the *Glu-3* locus.

The different protein markers were numbered according to their mobility order in SDS-PAGE, prefixed with the letter S for bands from CS and the letter T for bands from CT.

Cytology

Standard Feulgen squashes of roots at mitosis metaphase were used to analyse the chromosome structure of particular doubled haploids.

Genetic analysis

The recombination ratio (p) was calculated directly by dividing the observed number of recombinants by the total number of progenies analysed (n) excluding lines with abnormal patterns. The standard error (Sp) of the recombination fraction was calculated using the formula for the binomial distribution (Mather 1951):

$$S_p = [p(1-p)/n]^{1/2}$$

Where no recombinants were detected between two protein markers, the upper limit (at the 95% confidence level) for the recombination fraction (p) was calculated using the method of Hanson (1959): $p = [1 - (0.05)^{1/n}]$ where n = number of euploids.

Map distances (cM) and their standard errors were calculated from recombination frequencies using the Kosambi function (Kosambi 1944): $cM \pm SE = 25 \times \ln[(100 + 2p)/(100 - 2p)] \pm 2500\, S_p/(2500 - p^2)$.

Results

Chromosome allocation of LMW-GS and gliadin genes present in the parental lines

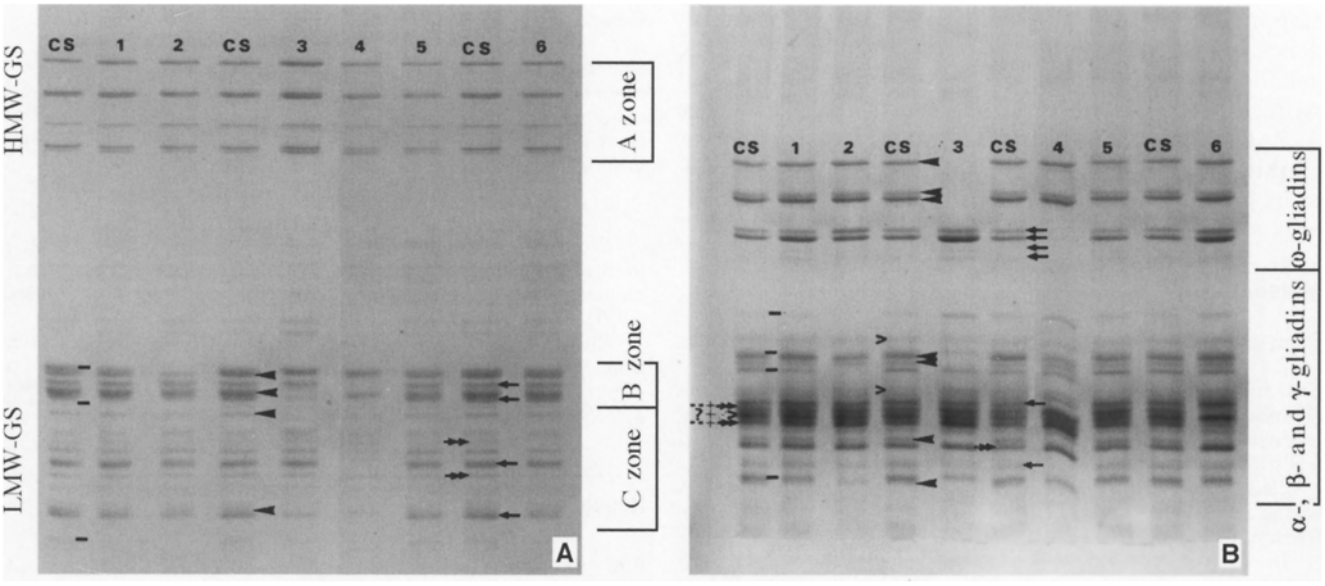
The chromosome allocation of glutenin and gliadin bands of Chinese Spring was almost completed except for minor C LMW-GS and some α, β or γ gliadins bands (Fig. 1). Chinese Spring carried at least seven B and ten C LMW-GS bands. Among these, 3, 4 and 4 bands were found to be controlled by 1AS, 1BS and 1DS respectively (Fig. 1 A). Further, genes of group 6 were also involved in the expression of minor bands fractioned in the C zone. Thus, two bands were controlled by genes on 6DS. CS expressed at least 28 bands of α, β, γ and ω gliadins. Among these, four were encoded at genes on 1AS, seven on 1BS and six on 1DS (Fig. 1 B). On group 6, two bands were encoded by 6BS and one by 6DS. Because of overlapping of bands, analysis with ditelosomic lines revealed that two genes located on group 6 (6AS + 6DS and 6BS + undetermined locus) were involved in the appearance of "one band". Consequently, their precise chromosome assignment could not be determined.

The electrophoretic pattern of Courtot revealed at least 8 and 15 bands of B and C LMW-GS and 30 bands of gliadins (Fig. 2). Among these, CT had two bands of LMW-GS controlled by chromosome 1AS (one each in the B and C zones), four by 1BS and five by 1DS (Fig. 2 A). Among gliadins, five bands were controlled by 1AS, three each by 1BS and 1DS (Fig. 2 B). On group 6, one band was encoded at 6AS, and two at 6BS.

An equivalent number of LMW-GS and gliadin bands were assigned in Chinese Spring and Courtot. Nevertheless, CT surprisingly expressed twice more gliadin bands encoded at the *Gli-A1* locus than did CS.

LMW-GS and gliadin polymorphic markers between Chinese Spring and Courtot and their segregation in DH lines

A high level of polymorphic LMW-GS and gliadin bands was detected between the parental varieties



Chinese Spring and Courtot (Fig. 3) while only one HMW-GS (subunit 2*) differed between them.

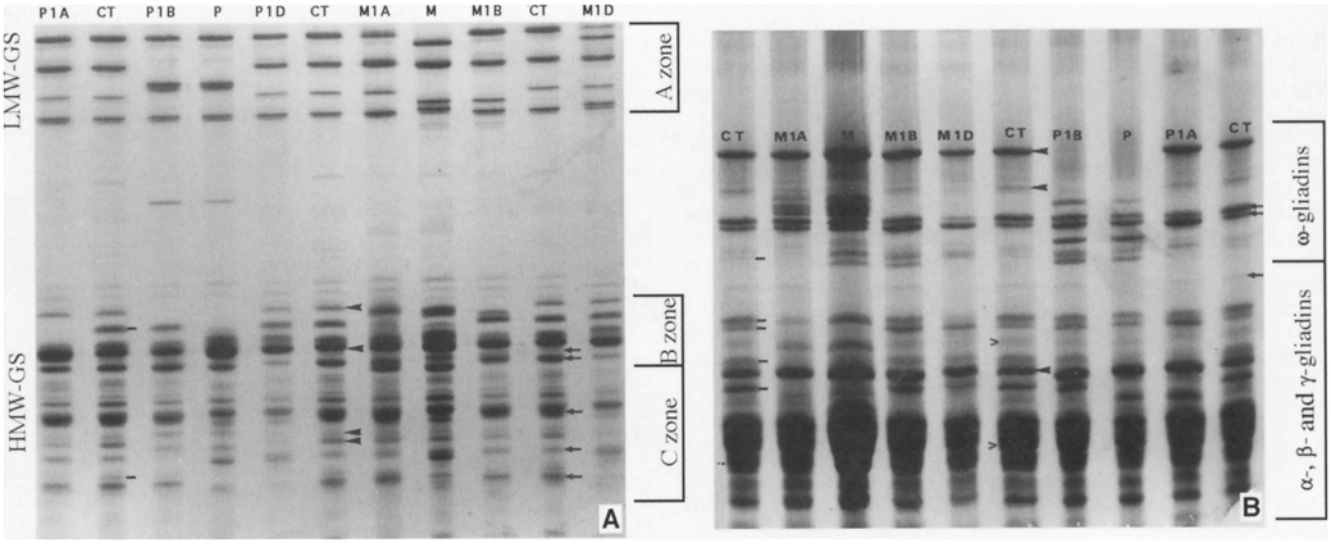
Among the 206 DH lines, some bands of gliadins and LMW-GS constituting a couplet or a triplet were very close to each other and always segregated together. They were considered in this study as a group of bands and were probably inherited from a unique locus.

The polymorphic markers concerned one HMW-GS controlled by the *Glu-A1* locus, nine bands of B and C LMW-GS: two controlled by the *Glu-B3* locus and seven by the *Glu-D3* locus (Fig. 3A).) In addition, ten gliadin markers were detected: three encoded at *Gli-A1*, four at *Gli-B1*, two at *Gli-D1* and one at *Gli-A2* loci (Fig. 3B). At the *Gli-A1* locus, the common allele *Gli-A1a* between CS and CT, determined by A-PAGE (Metakovsky, personal communication), was split into two different alleles (corresponding to bands S18 and T19, see Fig. 3B) when SDS-PAGE was employed. In total, 11 markers, differing between Chinese Spring and Courtot, were located. Their segregation in DH lines, as

Fig. 1A, B SDS-PAGE patterns of glutenin subunits (**A**) and gliadins (**B**) of Chinese Spring (*CS*) ditelosomic (DT) lines. *1*: DT1AS; *2*: DT1AL; *3*: DT1BL; *4*: DT1DL; *5*: DT6AS; *6*: DT6DL. —, ▬, ←, ⇢, >, ⇾, refer to bands controlled by genes on chromosomes 1AS, 1BS, 1DS, 6AS, 6BS and 6DS respectively

estimated by a chi-square test, fitted the expected ratio (Table 1). No distortion segregation ratio was observed except for the gliadin marker (bands T20u/S21, u for unidentified band) encoded at the *Gli-A2* locus. The ratio of this marker also did not correspond to a digenic segregation. A previous study revealed the implication of this band as a marker of the efficiency of embryo production during in vitro androgenesis (data not

Fig. 2A, B SDS-PAGE patterns of glutenin subunits (**A**) and gliadins (**B**) of chromosomes 1A, 1B and 1D substituted from cvs Prinqual (*P*) and Magdalena (*M*) into Courtot (*CT*). —, ▬, ←, – –, > refer to bands controlled by genes on chromosomes 1AS, 1BS, 1DS, 6AS, and 6BS respectively



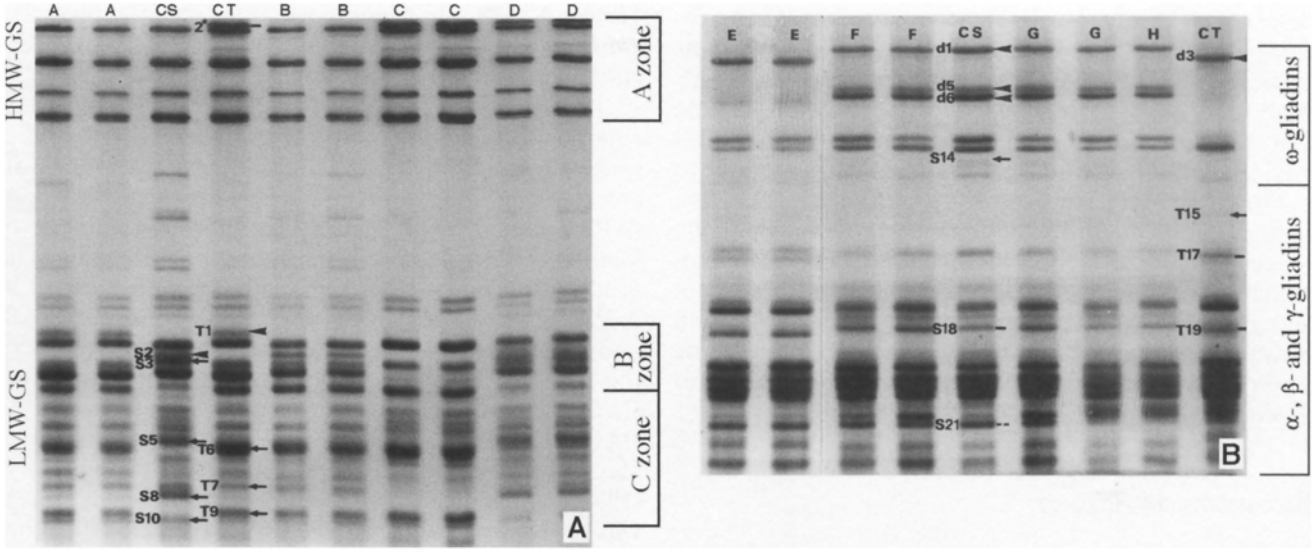


Fig. 3A, B SDS-PAGE patterns of glutenin subunits (**A**) and gliadins (**B**) of DH lines (*A–H*) derived from a cross between Chinese Spring (*CS*) and Courtot (*CT*). *S2–S20* correspond to the protein markers from *CS* and *T1–T19* to the markers from *CT*(see Table 1). —, ➡, ←, – –, refer to bands controlled by genes on chromosomes 1AS, 1BS, 1DS, and 6AS respectively

shown). For the following study, this band was not taken into account.

On chromosome 1A, 1.94% recombination was estimated between the gene coding for gliadin bands S16u/T17 and gene coding for gliadin bands S18/T19 (Fig. 3B, Table 1). This ratio was similar to that obtained by Pogna et al. (1993), and suggested the existence of two loci: the *Gli-A1* locus (bands S18/T19) and another locus (bands S16u/T17) probably the *Gli-A5* locus, the new locus discovered by Pogna et al. (1993, 1995). The gliadin band T17 of CT, determined in SDS-PAGE, gave rise to one major and one minor band in A-PAGE. This association between T17 and these two bands was confirmed without exception in the analysis of the 206 DH lines. These bands were attributed by Metakovsky to the *Gli-5* locus (personal communication).

Sixteen different patterns (2^4, 4 = number of distinct loci), corresponding to all the allelic combinations between loci, were observed. No significant difference between the expected and observed frequency of the 16 genotypes (3% to 8.6%) was detected among the DH population. In addition, two different patterns were determined corresponding to recombination between *Gli-A1* and *Gli-A5*.

Deviant lines

Nine out of the 206 DH lines showed abnormal patterns. For two of them, all proteins encoded at the *Gli-B1* and *Glu-B3* loci on the short arm were not expressed. Feulgen staining revealed the deletion of the satellite on chromosome 1B for only one of these two lines. In another line, among 12 grains analysed, one of them expressed only the 1Bx and 1Dx HMW glutenin subunits and not 1By and 1Dy. Moreover, although their loci are closely linked, the LMW-GS were not

Table 1 Correspondence between protein bands and alleles of the parental varieties Courtot (CT) and Chinese Spring (CS) for the glutenins (HMW-GS and LMW-GS) and gliadins separated in SDS-PAGE. Nomenclature of Payne and Lawrence (1983)[a]; Metakovsky (1991) for CS[b], personal communication for CT; and Khelifi et al. (1992)[c]. *Gli-5* is the new locus detected by Pogna et al. (1993). A band followed by u corresponds to an unidentified band in SDS-PAGE. "*Gli-Aa*" corresponds to the common allele *Gli-A1a* of CS and CT determined by Metakovsky (1991) in A-PAGE, which appeared different when SDS-PAGE was used (Fig. 2B)

Chromosome	Proteins	Locus	Bands		Alleles		Occurrence		χ^2 value ($\alpha = 0.05$)
			CS	CT	CS	CT	1:0	0:1	
1A	HMW-GS	*Glu-1*	Null	2*	*Glu-A1c*[a]	*GluA1b*[a]	99	102	0.044
	ω gliadins	*Gli-1*	S18	T19	*Gli-A1a*[a]	"*Gli-A1a*"	106	95	0.60
	γ gliadins	*Gli-5*	S16u	T17	*Gli-A5a*[a]	*Gli-A5b*	104	97	0.24
1B	LMW-GS /	*Glu-3* /	S2 /	T1 /	*Glu-B3a* /	*Glu-B3b* /	96	105	0.40
	ω gliadins	*Gli-1*	d1d5d6[c]	d3[c]	*Gli-B1a*[b]	*Gli-B1b*[b]			
ID	LMW-GS /	*Glu-3* /	S3, S5, S8, S10 /	T4u, T6, T7, T9 /	*Gli-D3a* /	*Glu-D3b* /	107	94	0.60
	ω. γ gliadins	*Gli-1*	S14	T15	*Gli-D1a*[b]	*Gli-D1b*[b]			
6A	α, β, γ gliadins	*Gli-2*	S21	T20u	*Gli-A2a*[b]	*Gli-A2t*[b]	120	81	7.9

expressed whereas the gliadins were present. The chromosome structure revealed by Feulgen staining was normal. The progeny from that particular seed (64 seeds analysed) did not show such an anomaly, suggesting that the previous absence of expression was probably a transient event in the original endosperm attributable to in vitro culture. Four other lines each possessed two distinct homozygous genotypes, as confirmed by progeny analysis. In two other lines, a partial heterozygozity were detected on chromosome 1AS or 1DS but the cytological study did not indicate the presence of an additional chromosome. In addition, the line hererozygous for chromosome 1AS appeared heterogeneous in height.

Discussion and conclusion

Compared to the results obtained by Australian authors in cv Chinese Spring (Gupta and Shepherd 1987, 1988, 1993; Gupta et al. 1994), the resolving power of the electrophoretic method used in the present work allowed a better separation of LMW-GS, and consequently the identification of the corresponding genes. Thus, the intermediate mobility LMW-GS of the B zone, in our study, were shown alternatively coded by genes on the 1BS and 1DS chromosomes (Fig. 1 A). A faint minor LMW-GS coded by the gene at the *Glu-A3* locus was detected for the first time in the B zone. An equivalent faint 1AS band was detected for cvs Langdon and Edmore by Liu and Shepherd (1995) using wheat-rye translocation lines which facilited the identification of this band because of the absence of co-segregation with the 1BS bands. In the C zone, two bands controlled by genes on 6DS were clearly revealed in SDS-PAGE. In previous studies, some prolamins with similar molecular weight to C subunits have been assigned to chromosome 6BS (Kasarda et al. 1987; Gupta and Shepherd 1993). Several authors reported the presence of C subunits as being controlled by genes on group 6. Some α- and γ-type gliadins (coded at the *Gli-1* and *Gli-2* loci) in C zone were detected by various approaches based on the N-terminal amino-acid sequencing of spots obtained in two-dimensional electrophoresis (Tao and Kasarda 1989), the sequencing of fractions obtained by RP-HPLC (Lew et al. 1992), and the use of antisera specific for the different gliadins and for LMW-GS (Denery-Papini et al. 1994).

In cv Courtot, the genetic determination of LMW-GS was more difficult to assess because 1A, 1B, 1D substitution lines from cvs Cappelle, Magnif 27, Vilmorin 23, and 1B from cv Azteca had patterns close to that of CT. Nevertheless, compared to the results of Morel (1994) using A-PAGE, which should increase the resolution of LMW-GS, the SDS-PAGE method we used revealed the same number of assigned protein bands. In addition, the mobility of HMW-GS was not modified as it was with A-PAGE. LMW-GS of Chinese Spring and Courtot were mostly controlled by genes on 1BS and 1DS, as in many other varieties, which was confirmed by two-dimensional electrophoresis for other cultivars by Redaelli et al. (1995).

The A-PAGE was used in the present study to test whether the polymorphism of gliadins coded by *Gli-2* was, or was not, revealed (data not shown). An equivalent number of protein markers were identified with A-PAGE and with SDS-PAGE but the number of identified bands differed depending on the locus. The unidentified band T20 in SDS-PAGE was detected in A-PAGE. In contrast, two different bands between CT and CS, encoded at the *Gli-A1* locus (corresponding to the *Gli-A1a* allele, see Table 1) were detected in SDS-PAGE, whereas no difference could be found with A-PAGE. Consequently, this allowed a new allele to be identified. This result is in agreement with Metakovksy (1991) who noticed that the detection of *Gli-A1* alleles was slightly more difficult in A-PAGE than for the other alleles. In this particular case, SDS-PAGE improved the determination of these alleles. The use of A-PAGE in our study did not increase the polymorphism of gliadins from group 6.

Despite the use of nulli-tetrasomic and ditelosomic lines of Chinese Spring, and substitution lines into Courtot, some minor bands never disappear, suggesting overlapping of bands encoded at homoeologous groups 1 and 6 or groups other than 1 and 6.

Doubled haploid lines are of particular interest to clearly establish the allelic relationships of storage proteins between two varieties. The genetic study of these proteins was made easier due to the homozygozity of the progeny in an immortalized F_2 segregation. In the present study, clusters of LMW-GS and gliadin alleles could be detected in chromosome arms 1AS, 1BS and 1DS and also in 6DS. No recombination was detected between genes of markers at the *Gli-B1* or *Gli-D1* loci and the *Glu-B3* or *Glu-D3* loci respectively, making it possible to assess an upper limit of 1.4% recombination between the above-mentioned loci (Hanson 1959). This percentage is close to the 1.7% recombination reported by Singh and Shepherd (1988) between genes at the *Gli-B1* and *Glu-B3* loci. A recombination of 1.94% (1.94 cM $\pm$ 0.01 cM) was found between gliadin genes on chromosome 1A, indicating the presence of two loci. This result is in agreement with that obtained by Pogna et al. (1993, 1995), who identified two new loci: *Gli-A5* (1–5% recombination with the *Gli-A1* locus) and *Gli-B5* (average of 1.4% recombination with the *Gli-B1* locus), suggesting that the two loci found on chromosome 1A were indeed *Gli-A1* and *Gli-A5*. Bands coded by genes at the *Gli-A5* locus were detected only in CT with both the SDS-PAGE and A-PAGE techniques. The corresponding allele in CS may be a null allele. This assumption was supported by the fact that CT expressed a greater number of gliadin bands controlled by chromosome 1A.

Almost 5% of abnormal patterns could be detected in the DH progeny. Several hypotheses can account for the different cases observed. The loss of all the electrophoretic bands controlled by a given locus could

result from a chromosomal deficiency (Pogna et al. 1985; D'Ovidio et al. 1991; Sabelli et al. 1992), the presence of regulatory mutations (Lafiandra et al. 1987), partial deletion, or possible retrotransposition (Moore et al. 1991). Molecular analysis is in progress to determine which hypothesis can be substantiated. As was reported in maize (Murigneux et al. 1993) and triticale (Gonzalez et al. 1993), a chimeric structure of the regenerated plant could result from the fusion of two different pro-embryos during the initial stages of microspore division which could lead to the emergence of two distinct genotypes. The heterozygozity could also occur following an abnormal meiosis leading to unreduced gametes (Hermsen 1984). In this case the heterozygozity of the F_1 could not be recovered.

The resolving power of the one-dimensional method is not sufficient for a complete determination of LMW-GS and gliadins alleles. The use of two-dimensional electrophoresis might be better for that purpose. Nevertheless, several wheat storage proteins, and their corresponding alleles, were revealed for the first time by using SDS-PAGE. This type of material, and the large number of DH lines analysed, allowed protein markers and clusters of alleles at the *Glu-3* and *Gli-1* loci to be clearly characterised. RFLP analysis with probes specific for these loci should provide a clear view of the gene complexes involved in the synthesis of storage proteins. The diversity revealed through these markers is also a useful too for analysing the genetic and biochemical factors involved in end-use quality. Although some unexpected deviant lines can be found, haploid material makes genetic studies easier and, thus it would certainly be useful to develop such material from other crosses.

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