Theor Appl Genet (2005) 111: 540–550
DOI 10.1007/s00122-005-2045-1

ORIGINAL PAPER

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Genetic analysis of dry matter and nitrogen accumulation and protein composition in wheat kernels

Received: 30 August 2004 / Accepted: 18 April 2005 / Published online: 11 June 2005

Abstract The maximum rate and duration for grain dry matter (DM) and nitrogen (N) accumulation were evaluated in 194 recombinant inbred lines (RILs) from a cross between the two French wheat cultivars Récital and Renan. These cultivars were previously identified as having contrasting kinetics of grain DM and N accumulation. Grain protein composition was analysed by capillary electrophoresis (CE), which enabled quantification of the different storage protein fractions (αβγ-gliadins, ω-gliadins, LMW glutenins, HMW glutenins, and each of their subunits). Correlation analyses revealed that DM and N accumulation rates were closely correlated and repeatable over several years, which was not the case for DM and N accumulation durations, and that protein composition was primarily influenced by the N accumulation rate. This was particularly true for the LMW-glutenins and the αβγ-gliadins, the most abundant protein fractions. A genetic map of 254 molecular markers covering nearly 80% of the wheat genome was used for quantitative trait loci (QTL) analysis. A total of seven QTLs were found. Five QTLs were significantly associated with the kinetics of DM and N accumulation, and two of them also influenced protein composition. Two QTLs affected only the protein composition. One major QTL explained more than 70% of the total variation in HMW-GS Glu1B-x content.

Keywords Kernel growth · Grain filling · *Triticum aestivum* · Gliadins · Glutenins

Communicated by P. Langridge

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Introduction

Grain yield is the major economic trait for wheat. However, end-use quality is of uppermost importance in enhancing the value of the world's wheat harvest. Proteins are the most important determinants of end-use quality. Dough properties, and thus bread and biscuit making ability, are influenced by both the total protein concentration and the composition of storage proteins of the flour, i.e. the relative proportion of the different fractions and subunits (Weegels et al. 1996; Lafiandra et al. 1999; Branlard et al. 2001).

Grain protein concentration is the ratio of grain protein quantity and grain dry weight. Thus, grain protein concentration reflects the accumulation kinetics of both nitrogen (N) and dry matter (DM), the latter consisting of 60–70% starch. The genetic correlation between grain yield and protein concentration has consistently been reported as highly negative (see Oury et al. 2003 for a review). It can thus be postulated that common genetic factors are involved in the control of both sets of kinetics. However, no formal study of the genetics of protein and DM accumulation in wheat kernel has been reported to date.

Although grain protein composition is significantly affected by environmental factors and genetic/environmental interactions (Graybosch et al. 1996; Huebner et al. 1997; Triboi et al. 2000; Zhu and Khan 2001), a genetic basis can also be postulated. The expression of genes encoding storage proteins is regulated by several transcription factors, which bind to the *cis*-regulatory sequences in their promoter regions and act as enhancers

or repressors of transcription. Genetic variability in the promoter sequences of genes of storage protein, including *cis*-element duplication, deletion or point mutation, has been reported (Anderson et al. 1998). In wheat, several transcription factors such as SPA (storage protein activator, Albani et al. 1997) and PBF (prolamine box binding factor, Mena et al. 1998) have been reported to bind to the promoters regions of genes for several storage proteins. Although few sequences for wheat SPA or PBF are available in the genomic databases, a preliminary survey revealed some genetic variability (Guillaumie et al. 2004). Thus, beside the well-known allelic variability in the structure of the storage proteins (Branlard and Dardevet 1985; Payne 1987; Shewry et al. 1995; Shewry and Halford 2002), genetic variability in their molecular regulation pathways, and consequently in the relative quantities of the different fractions, is likely to occur in wheat.

The accumulation of the different protein fractions is highly asynchronous. In particular, gliadins accumulate earlier than glutenins during grain filling (Gupta et al. 1996; Stone and Nicolas 1996; Panozzo et al. 2001). One consequence is that either genetic or environmental factors that affect the duration of grain filling are likely to modify the balance between the different protein fractions. It is generally accepted that changes in protein composition greatly influence gluten properties and thus the end-use quality of most baking products. These changes in the relative distribution of protein fractions have been shown to result from either environmental factors or genotype. Environmentally induced changes have been extensively studied (Blumenthal et al. 1991; Daniel and Triboï 2001; Wardlaw et al. 2002). The most effective factors are high temperature (either moderately high, 25–32°C, or heat stress >35°C) and drought during the grain filling period (Sofield et al. 1977). The environmental factors that tend to shorten grain filling are thus also likely to change the composition of grain protein.

Genotypic differences in the response of DM and N accumulation to environmental variations have also been documented. The rate of DM accumulation is most important as a selection criterion, since the duration of accumulation is influenced to a much greater extent by the environment than by the genotype (Whan et al. 1996; Stone and Nicolas 1995; Robert et al. 2001). In a previous paper (Robert et al. 2001), we analyzed the genetic variability of the kinetics of DM and N accumulation, modelled by a logistic curve, in a set of 16 wheat cultivars that span the full range of variation in bread-making quality and thus most likely also in protein composition. We found that in most environments, the cultivars Renan and Récital had contrasting genotypes with respect to maximum rates and durations of DM and N accumulation. In the present paper, we report a genetic analysis of grain filling parameters and final protein composition using a recombinant progeny from a cross between Renan and Récital.

Materials and methods

Plant materials

The population studied consisted of 194 F_7 recombinant inbred lines (RILs) obtained by single seed descent from a cross between two cultivars of winter bread wheat (*Triticum aestivum* L.), Renan and Récital. These two cultivars were registered in 1989, and are classified as "high bread making" and "strong improving" grades, respectively. Renan has a higher grain protein concentration (GPC) and dough strength than Récital. The recombinant population and the parental lines were sown in 1998 and 1999 (harvest years 1999 and 2000, respectively) at Clermont-Ferrand, France (45°47′N, 3°10′E, elevation 329 m). The experimental field design consisted of a randomized trial with two replications, grown under normal field conditions.

Determination of grain DM and N accumulation kinetics

In both harvest years, 100 and 50 ears were collected from each plot at two stages, namely 200–250 and 500–550 degree-days after anthesis. These stages are approximately the bounds of the linear filling phase for most genotypes (see Fig 1 in Robert et al. 2001). Since there is variation for flowering time in the RIL population and temperatures fluctuate, these stages correspond to between 9 and 13 and 20 and 30 days after anthesis, respectively. The ears were oven-dried at 80°C for 48 h and the average grain dry mass was determined by weighing 200 grains. Grain N concentration was determined using the Kjeldahl method. The quantity of N per grain was calculated as its average dry mass times its N concentration. Grain protein concentration was calculated from the percentage of total N multiplied by a conversion factor of 5.7.

The maximum rates of DM and N accumulation were calculated as the difference in grain dry weight or N quantity per grain between the two sampling dates divided by the cumulative thermal time, based at 0°C, between the two dates. The duration of DM and N accumulation was calculated as the mature grain dry mass or N content per grain divided by the rate of DM or N accumulation. This approach enabled a good approximation of our previously developed logistic model (Robert et al. 1999), which cannot be used for large populations involving hundreds of lines.

Evaluation of storage protein fractions

For both harvest years, $\alpha+\beta+\gamma$ gliadins, ω gliadins, low (LMW-GS) and high (HMW-GS) molecular weight glutenin subunits were quantified using capillary electrophoresis (CE). These analyses were carried out from

grain samples of a single replicate to save costs and labour. The procedure has been described in detail elsewhere (Werner et al. 1994, Bietz and Schmalzried 1995; Bean and Lookhart 1997, 1999; Carceller and Aussenac 1999). Gliadins were specifically extracted in 50% (v/v) ethanol. Glutenins were precipitated with 50% (v/v) propan-2-ol (final concentration 70%) then resuspended in the presence of 1% (v/v) SDS and 1% (v/v) 2-mercaptoethanol. The extractions were performed on 0.5 g representative samples of whole meal flour.

Glutenins were then separated according to their molecular mass, for 15 min at 15kV and 20°C, in a BioRad polyacrylamide gel (reference 148–5032) eluted by 5% methanol (capillary gel electrophoresis). An uncoated silica capillary (total length 24 cm, separation length 18 cm and 50 µm internal diameter; BioRad reference 148–3060) was used. Gliadins were separated in the free zone using capillary zone electrophoresis, in 0.1 M phosphate buffer (pH 7.2; BioRad reference 148–5011), for 20 min at 22 kV and 45°C. An uncoated silica capillary (total length 26 cm, 20 µm internal diameter; Thermo Finnigan TSP 20375) was used. Electropherograms were integrated and the surfaces of the peaks evaluated (PC 1000 software, Thermo Finnigan) to obtain ratios of protein subclasses. These ratios were then compared to the total amount of proteins in the extracts assayed by the Lowry method (BioRad reference 5000116) modified to be used in the presence of a reducing agent, i.e. after its neutralisation by the addition of 1 M iodoacetamide.

Results were expressed in mg per gram of grain dry weight. After fresh weight was determined for approximately 1 g of material, the grains were oven dried at 133°C for 2 h and dry weight was determined. The water content of samples was calculated as the difference between fresh and dry weight. Protein concentration was expressed in % as the ratio between protein and DM content.

Genetic map

The construction of the genetic linkage map has been described by Groos et al. (2002). The map used for our quantitative trait loci (QTL) analysis consisted of 265 loci for a total length of 2,722 cM (reference ITMI map 3,500 cM). The markers were quite evenly distributed over the 21 wheat chromosomes, except for chromosome 4D, which lacked polymorphic markers. Some unlinked markers that did not deviate from the expected ratio (1:1) were also integrated for the QTL analysis.

Statistical analysis and QTL detection

Pearson correlation coefficients between phenotypic data were calculated using Splus software (Statistical Sciences, 1993). Correlations were calculated between the 1999 and 2000 harvests for each trait and among the traits for each year. For those traits which were evaluated on the two replicates (i.e. not for CE data), a two-way analysis of variance was carried out to yield *F*-tests for both genotype main effect and G × E (genotype × year) interaction. The QTL analysis was performed using a home-made Splus routine (Groos et al. 2002). First, one-way analyses of variance (ANOVA) were used to detect significant differences between genotypic classes for each marker. In order to select a subset of non-redundant markers for further use as covariates, significant markers were then used as candidates in a multiple regression model. To locate the QTLs more precisely and to estimate their additive effects, a marker regression analysis (Kearsey and Hyne 1994) was then carried out on every linkage group with at least one significant marker. This program enabled the detection of two QTLs on the same chromosome using a two dimensional scanning of the chromosome (Hyne and Kearsey 1995). Confidence intervals (at 95%) of the QTL locations and additive effects were established using a bootstrap technique (Visscher et al. 1996) with 1,000 replicates for the one-QTL model and 400 for the two-QTL model.

Results

Description of phenotypic variation

Table 1 shows the range of variation observed in the parental lines and their progeny for the 18 traits analysed for the 1999 harvest (a similar figure was obtained for the 2000 harvest). As previously indicated, the two parental lines have contrasting single grain dry weights, protein contents and protein concentrations, with the population mean being close to the mid-parent value. Relatively few transgressive lines were observed, suggesting that the genetic determinants of these traits were mostly additive. A similar shape was observed for DM and N accumulation rates. For the two parental lines, the rate values estimated by linear approximation fitted the mid-range of values estimated through logistic regression in a previous study (DM rate: 0.056–0.072 mg/°C day for Récital, 0.077–0.101 for Renan; N rate: 0.00075–0.00108 mg/°C day for Récital and 0.00133–0.00186 for Renan over nine environments). Conversely, the duration of DM and N accumulation were similar in the two parental lines and were, as expected, lower-bound estimates compared to those obtained by the logistic regression (DM duration 478–708 °C days; N duration 538–792 °C days), whereas significant variation was observed in the progeny, suggesting either a more complex control or that favourable alleles for these traits are distributed equally between the two parents. For grain protein composition, several traits showed non-Gaussian distributions. In these cases the distribution shape was either asymmetric, flat or bimodal. The bimodal shape strongly suggests that a major gene or QTL was the cause of the distribution

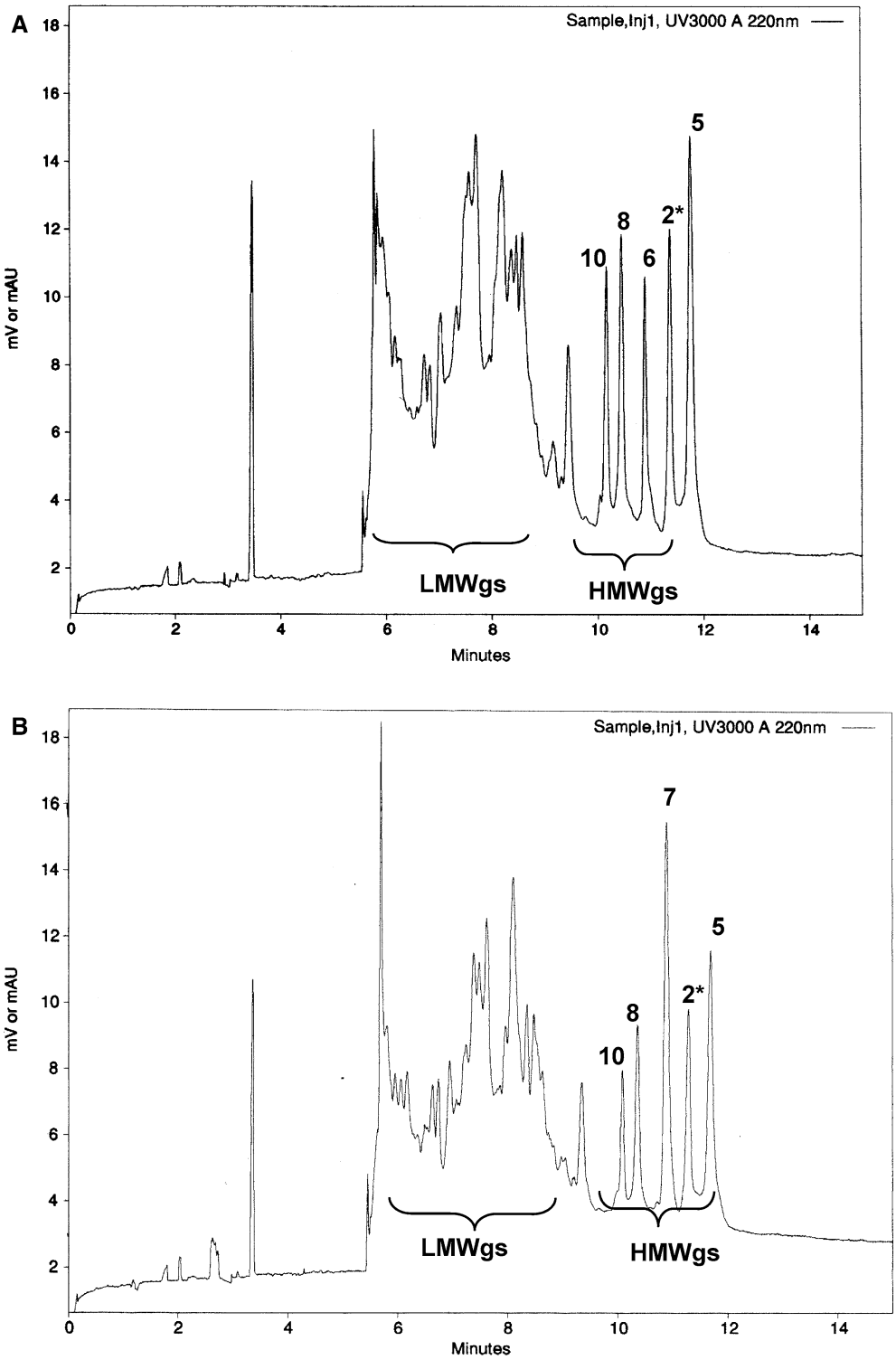

Fig. 1 Example of capillary gel electrophoresis of the glutenins of two contrasted RILs. **a** ReR146 recombinant line (GluB1x6); **b** ReR152 recombinant line (GluB1x7). The *numbers* above the peaks indicate the name of the different HMW-GS allele. *X-axis* Migration time in minutes, *Y-axis* standardized arbitrary units (same Y-scale)

observed. The same bimodal distribution was observed for the quantity of HMW-Glu subunits 1-A, 1-Bx, 1B-y and 1D-y. Figure 1 shows the capillary electropherograms of glutenins for two RILs with contrasting composition. Subunit glu-B 1x-7 was more strongly expressed than subunit glu-B 1x-6. Similar shapes were observed for the 2000 harvest.

Correlations between phenotypic traits

The relationships between the rate and the duration of DM and N accumulation and the percentage of the different protein fractions were analyzed by correlation analysis (Table 2). It should be noted that most single trait correlations between the 1999 and 2000 harvests

Table 1 Mature grain dry weight and protein content, maximum rates and duration of DM and N accumulation, quantities of glutenins, gliadins and their different subunits for parental and progeny lines for the 1999 harvest. For Récital and Renan, the data are the means of eight replications. For the RILs, minimum and maximum values and the shape of the distribution are shown

Trait	Recital	Renan	RIL mean	RIL minimum	RIL maximum	Shape
Grain dry weight (mg DM/grain)	30.53	42.425	35.826	26.91	45.98	Gaussian
Grain protein content (mg N*5.7/grain)	2.96	5.022	3.89	2.890	5.560	Gaussian
Grain protein concentration (%)	10.18	12.18	11.18	9.00	13.30	Gaussian
Maximum rate of DM accumulation (mg/°C day)	0.0638	0.0831	0.0735	0.0586	0.09085	Gaussian
Duration of DM accumulation (°C days)	483.3	509.3	490.3	442.3	572.5	Gaussian
Maximum rate of N accumulation (mg/°C day)	0.00087	0.00145	0.00113	0.00078	0.00160	Gaussian
Duration of N accumulation (°C days)	597.3	609.7	603.9	514.1	736.6	Gaussian
Glutenins (mg/grain)	2.360	3.247	2.718	1.897	3.651	Asymmetric
Gliadins (mg/grain)	1.179	1.962	1.497	1.118	2.304	Gaussian
LMW-GS (mg/grain)	2.070	2.739	2.345	1.672	3.173	Gaussian
HMW-GS (mg/grain)	0.288	0.5149	0.3718	0.181	0.593	Gaussian
Glu-A1 (mg/grain)	0.0529	0.0790	0.06092	0.030	0.127	Bi-modal
Glu-B1x (mg/grain)	0.0529	0.1539	0.0843	0.029	0.196	Bi-modal
Glu-B1y (mg/grain)	0.0529	0.0790	0.0676	0.029	0.130	Bi-modal
Glu-D1x (mg/grain)	0.0832	0.13289	0.1109	0.058	0.174	Gaussian
Glu-D1y (mg/grain)	0.0529	0.0422	0.0467	0.029	0.087	Bi-modal
$\alpha+\beta+\gamma$-gliadins (mg/grain)	1.0352	1.7380	1.3020	0.932	2.043	Asymmetric
ω-gliadins (mg/grain)	0.1481	0.2229	0.1955	0.032	0.397	Gaussian

were quite high, particularly for the grain weight and accumulation rates of both N and DM, with the exception of the durations of accumulation, which were the least repeatable traits. These high correlations indicate that most traits are highly heritable, which was confirmed, whenever possible, by the higher values of *F* tests for genotype-main effect compared to those F for G × E, which are often non-significant. The maximum rates of DM and N accumulation were closely correlated ($r=0.81$), suggesting that they share some common physiological mechanisms. As expected, the rate and the duration of accumulation for both DM and N were negatively correlated, though the correlation coefficient was rather low. The percentage of the different protein fractions determined by CE was positively correlated with both the rate and the duration of the DM and N accumulations and also with the grain dry weight. The correlation coefficient between the different protein fractions and the rate of DM and N accumulation increased with their proportion in the total protein (data not shown).

QTL analyses

Seven chromosome regions were identified as having significant effects on either grain filling parameters or protein composition (Table 3 and Fig. 2). The QTL for grain filling parameters were found in five regions on chromosomes 2B, 3A, 6A, 7A and 7D. The region on chromosome 3A displayed only QTLs for grain filling parameters, while the other four also explained variations in the composition of grain protein. The strongest QTLs, on chromosomes 2B and 7A, explained about 21 and 19% in the variation of the rates of DM and N accumulation, respectively, and were detected in both harvest years. Positive additive effects for the rates of DM and N accumulation, grain dry weight and quantities of total protein, LMW-GS and gliadins ware associated with the presence of the Recital allele at the 2B QTL, although this parent had the smallest grain dry weight and protein content. A similar picture was found on chromosome 7A, but as expected the allele from Renan increased the rate of N accumulation grain dry weight and the quantities of total protein, as well as those of LMW-GS. The QTL for grain filling parameters and protein composition found on chromosomes 6A and 7D were less robust for most traits i.e. detected in less than 75% of boostrap samples, with the exception of the quantity of LMW-GS.

Finally, two QTLs were associated only with variation in protein composition as measured by CE: one on chromosome 5A where the presence of Renan allele was associated with an increase in the quantity of HMW-Glu, particularly the Glu-D1x subunit, and $\alpha+\beta+\gamma$-gliadins, and one on chromosome 1 B where the Renan allele increased the quantities of Glu-B1x and the Récital allele increased Glu-A1x, Glu-D1x and Glu-D1y. The latter major QTL was detected in both years. The highest *F* value of single marker ANOVA (data not shown) was clearly that of *XgluB1*, the locus encoding HMW-GS.

Discussion

In the present study of 194 sister lines in two environments, significant genotypic variation for the parameters DM and N accumulation were observed. The genotypic coefficients of variation for the accumulation rates were 9.5 and 12.1% for DM and N, respectively, while the corresponding values for the duration of accumulation were 4.2 and 6.4%. Moreover, the durations values were loosely correlated over the two years of the study,

Table 2 Matrix of Pearson correlations between the rates and durations of DM and N accumulation and the quantities of glutenins, gliadins and their different subunits, determined from the data for the 1999 harvest The *first column* contains the correlation between values of 1999 and 2000 for the same traits. Figures greater than 0.5 are in *bold* type. The last *two columns* are Fisher's *F* values for genotype main effect and G × E interaction when replicated data enabled statistical analysis. *NS* Not significant

Trait	Correlation 1999–2000	DM rate	N rate	DM duration	N duration	$F_{genotype}$	$F_{G \times E}$
Maximum rate of DM accumulation (mg/°C day)	0.63	–	**0.81**	−0.41	−0.12	9.80**	2.46*
Duration of DM accumulation (°C days)	**0.07**	**0.81**	–	−0.33	−0.28	1.45 NS	1.12 NS
Maximum rate of N accumulation (mg/°C day)	0.69	−0.41	−0.33	–	**0.57**	**15.26*** **	**2.13***
Duration of N accumulation (°C days)	−0.13	−0.12	−0.28	**0.57**	–	1.83 NS	1.20 NS
Grain dry weight (mg DM/grain)	**0.75**	**0.82**	**0.70**	−0.02	0.27	16.9***	2.37*
Grain protein content (mg N*5.7/grain)	**0.73**	**0.76**	**0.87**	−0.05	0.23	17.6***	1.81*
Grain protein concentration (%)	**0.59**	0.19	−0.09	**0.57**	0.03	5.92**	1.24 NS
Glutenins (mg/grain)	**0.58**	**0.69**	**0.66**	−0.04	0.24		
Gliadins (mg/grain)	**0.49**	**0.59**	**0.71**	−0.07	0.11		
LMW-GS (mg/grain)	**0.39**	**0.68**	**0.64**	−0.01	0.26		
HMW-GS (mg/grain)	0.56	0.46	0.47	−0.08	0.09		
Glu-A1 (mg/grain)	0.19	0.35	0.37	−0.11	−0.01		
Glu-B1x (mg/grain)	0.65	0.19	0.18	0.02	0.16		
Glu-B1y (mg/grain)	0.31	0.47	0.47	−0.06	0.06		
Glu-D1x (mg/grain)	0.29	0.46	0.47	−0.08	0.02		
Glu-D1y (mg/grain)	0.44	0.24	0.25	−0.04	0.03		
α + β + γ-gliadins (mg/grain)	**0.56**	**0.56**	**0.70**	−0.09	0.05		
ω-gliadins (mg/grain)	0.47	0.37	0.37	0.01	0.23		

*$P = 0.05$; **$P = 0.01$; ***$P = 0.001$

indicating that genotypic effects are lower than environmental effects and G × E interactions. These results agree well with previous studies (Stone and Nicolas 1995; Robert et al. 2001). Compared to the results of Stone and Nicolas (1995), who studied wheat accessions of worldwide origin, the lower values for filling rates observed in this study can be attributed to the narrower genetic base, which is derived from a single bi-parental cross. Our results showed significant genetic variability in the rate of grain DM and N accumulation, and considerably higher variability for rate than for duration; these results are in accordance with the observations made on fewer genotypes by Campbell et al. (1990).

As shown by previous studies (Campbell et al. 1990; Mou and Kronstadt 1994; Mou et al. 1994; Van Sanford 1985; Robert et al. 2001), the maximum rate of DM accumulation was positively correlated with kernel dry weight ($r = 0.82$) while the duration of accumulation was not. The same pattern was obtained for the total quantity of grain protein (GPC), which was even more strongly correlated with the maximum rate of N accumulation ($r = 0.87$). In the case of specific protein fractions, again the rate of N accumulation rather than its duration appears to be the major determinant of the amounts in the mature kernel. The more abundant the fraction, the closer its correlation with the rate of N accumulation; $r = 0.64$ for the LMW-glutenins and $r = 0.70$ for the α + β + γ-gliadins. In contrast to environmental factors that affect protein composition by shortening the filling period, genotypic factors apparently differentially affect the relative rates of accumulation of the different fractions. A dynamic evaluation of protein content at different stages of grain filling would be required to confirm this hypothesis, but this was not practicable on such a large progeny. The correlations between the amounts of the different protein fractions and the rates of accumulation of DM and N and the high correlation between the rates of DM and N accumulation indicated that the two grain filling phenomena are closely related. As all correlations were calculated within a single environment (similar values were found for both harvest years), a large proportion of the variation for the traits considered was likely due to genetic differences, although the influence of G × E interactions cannot be rejected. We can therefore assume that a genetic relationship exists between DM grain filling and N grain filling.

This close genetic relationship was confirmed by co-location of several QTLs for DM rate and grain dry weight, and QTLs for N rate and protein content or the quantities of their different fractions. Such co-locations were observed on chromosomes 2B, 3A, 6A, 7A and 7D. The regions on 3A and 7D have previously been identified as bearing QTLs for GPC in the same plant material (Groos et al. 2003). The QTL on chromosome 7D was also reported by Zanetti et al. (2001) in a totally different background, namely a progeny from a cross between a winter wheat and a spelt (*Triticum aestivum spelta* L.). The only other QTL analysis of grain filling parameters involving the ITMI population [RILs from the cross between the spring wheat variety Opata 85 and the synthetic hexaploid wheat W7984 generated using *T. tauschii* accession CIGM86.940 (DD) with the tetraploid wheat Altar 84 (AABB) by Dr A Mujeeb-Kazi at CIMMYT, Mexico] was reported by Börner et al. (2002), who found one major (i.e. LOD > 3) QTL for grain filling duration (DM) on chromosome arm 5AL, different from those found in our study. This illustrates the fact that different recombinant populations are likely to segregate for different QTL, and that an overall picture of the genetic architecture of a complex trait can only be

Table 3 QTLs locations and effects for mature grain dry weight and protein content, maximum rates and durations of DM and N accumulation, and the quantities of glutenins, gliadins and their different subunits. In each cell, the data are arranged in the following order: QTL location (confidence interval) in cM from the chromosome origin, year (1999 or 2000) and h^2%, h^2 being the proportion of trait phenotypic variance accounted for by the additive effect of the QTL

Trait	Chromosome						
	1B	2B	3A	5A	6A	7A	7D
Grain dry weight (mg DM/grain)		82 (71–85) Rc 99–11%; 00–20%			15 (0–32) Rn 99–11%	122 (82–148) Rn 99–7%; 00–7%	
Grain protein content (mg/grain)		92 (78–109) Rc 99–10.5%			14 (0–32) Rn 99–12.5%; 00–13%	129 (82–151) Rn 99–11%; 00–26%	63 (39–75) Rn 99–8%
Maximum rate of DM accumulation (mg/°C day)		78 (68–84) Rc 99–21%; 00–17%	27 (20–33) Rn 00–11%				60 (35–73) Rn 99–6%
Duration of DM accumulation (°C days)			2 (16–30) Rc 00–6.7%				
Maximum rate of N accumulation (mg/°C day)		77 (62–84) Rc 99–13%; 00–11%	30 (23–34) Rn 99–7%; 00–9%		10 (0–35) Rn 99–7.2%	110 (92–142) Rn 99–19%; 00–16%	61 (47–71) Rn 99–9%
Duration of N accumulation (°C days)							
Glutenins (mg/grain)						129 (86–136) Rn 99–7%; 00–12%	61(45–72) Rn 99–7%
Gliadins (mg/grain)		81 (62–115) Rc 99–7.2%					60 (30–70) Rn 99–7%
LMW-GS (mg/grain)		90 (80–126) Rc 99–8%; 00–11%			17 (3–54) Rn 99–16%	132 (85–145) Rn 99–10%; 00–12%	54 (43–73) Rn 99–8%
HMW-GS (mg/grain)				55 (21–88) Rn 99–16%			
Glu-A1 (mg/grain)	88 (69–96) Rc 99–6.5%						
Glu-B1x (mg/grain)	79 (77–83) Rn 99–73%; 00–68%						
Glu-B1y (mg/grain)					29 (15–40) Rn 99–12%		
Glu-D1x (mg/grain)	88 (71–97) Rc 99–14%; 00–12%			64 (39–105) Rn 99–7.4%			
Glu-D1y (mg/grain)	88 (81–91) Rc 99–34%; 00–28%						
α + β + γ-gliadins (mg/grain)				76 (45–117) Rn 99–10.4%			63 (54–73) Rn 99–6%
ω-gliadins (mg/grain)							46 (21–62) Rn 99–6%

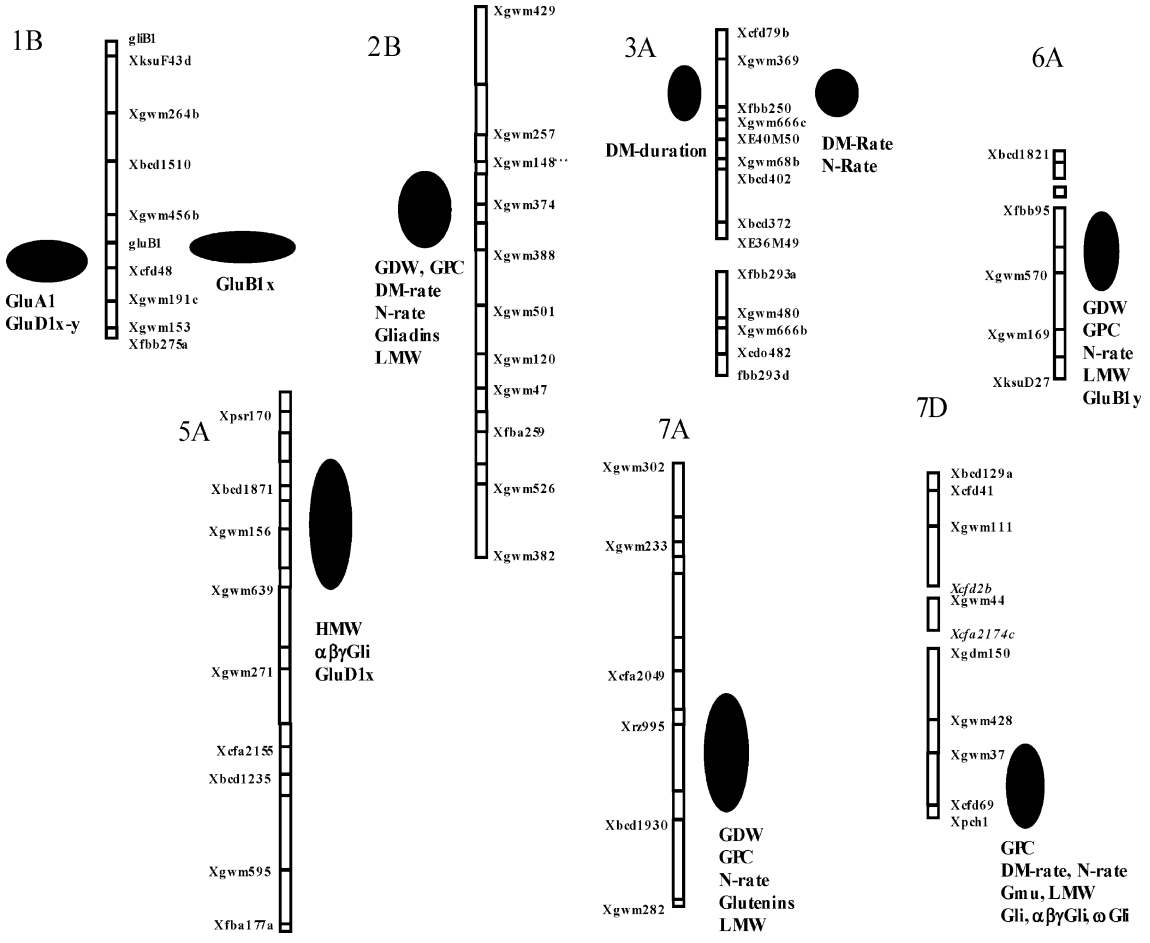

Fig. 2 Locations of QTLs for mature grain dry weight (*GDW*), protein content (*GPC*), maximum rates and duration of DM and N accumulation, the quantities of glutenins, gliadins and their different subunits. The *vertical rectangles* reprensent the chromosomes with anchor markers of the Renan × Récital map. The *ellipses* on each side represent the detected QTLs. The height and the width of the ellipse are proportional to the confidence interval and the intensity (h^2 in Table 3, maximum value over traits and years) of the QTL, respectively. The favourable allele is brought by Renan when the ellipse is on the *right* of the chromosome, and by Récital when on the *left*

achieved by synthesising results from many bi-parental populations (meta-analysis, Goffinet and Gerber 2000) or by analysing QTL in complex, multiparental pedigree (e.g. Crepieux et al. 2004). In our study, no region was identified as having only either a QTL for the rate of DM accumulation or a QTL for the rate of N accumulation. This result is not encouraging with respect to the efficiency of marker assisted selection in the improvement of GPC, as pyramiding QTLs whose only effect is on N accumulation would be more efficient. In fact, more subtle allelic differences may exist, with some QTLs having stronger effects on N rate than on DM rate (e.g. on 7A) or vice-versa (on 2B). However the use of such QTL is more risky, as the degree of precision of QTL effects is often not very high. Moreover, in the QTL region on chromosome 3A opposite effects have been observed for both rate and duration of DM accumulation. This possible pleiotropic effect may explain the negative, although moderate, correlation between rates and durations of DM and N accumulation. However it should be kept in mind that both durations are loosely correlated over years and thus less heritable than accumulation rates.

The proposed genetic relationship between the rate of N accumulation and protein composition was confirmed by co-location of several QTLs on chromosomes 2B, 6A, 7A and 7D. The QTLs for protein composition mostly affect the most abundant fractions such as LMW-GS (2B, 6A, 7A and 7D) and gliadins (2B and 7D). These last two regions harboured QTL alleles that have similar effects on gliadins as on glutenins, thereby explaining the overall positive correlation between all the protein fractions. However the effect of two other chromosome regions was limited to protein composition, independent of the overall N accumulation dynamics. These regions were found on chromosomes 1B (long arm, a major QTL found both years) and 5A (QTL found only in 1999) for protein fractions estimated by CE, and notably for the quantities of HMW-GS, a class of storage proteins known to be involved in gluten formation. On chromosome 1 BL, the major QTL for the quantity of GluB1-x subunit is located in the vicinity of the gene which encodes this protein. The observed effect on the quantity of protein implies that either the transcription

into mRNA or its translation level is higher for allele 7 (Renan) than allele 6 (Récital). This overexpression of GluB1x 7 has already been reported (Marchylo et al. 1992). It has been shown that transcription levels of coding genes are controlled by the DNA sequence located upstream from the start codon, the so-called promoter (Sorensen et al. 1989). It is also widely recognised that specific DNA motifs or boxes in the promoter bind proteins called transcription factors, which act either as repressors or as activators of transcription (Forde et al. 1985; Bevan et al. 1993; Hammond-Kosack et al. 1993; Washida et al. 1999). These transcription factors (TF) are obviously coded by genes, which can themselves be regulated by other TF and/or by environmental factors (Müller and Knudsen 1993). At least three families of TF have been shown to bind with the promoter of cereal storage proteins; the bZIP family (Albani et al. 1997), the DOF family (Mena et al. 1998) and the MYB family (Diaz et al. 2002). Variations in transcription level from one HMW-GS allele to another could thus be due to variations in specific TF binding boxes. Polymorphism among HMW-GS promoter sequences has been reported by Anderson et al. (1998). Notably, the GluB1-x 7 promoter differs from those of other HMW-GS alleles by duplication of the so-called cereal box, a 50 base pair motif that may be involved in endosperm-specific expression (Forde et al. 1985). This duplication was postulated to enhance the transcription level of the gene, however Anderson et al. (1998) reported no further evidence and discarded this hypothesis. More recently, Juhasz et al. (2003) found a duplication of a 43 bp element in different versions of the Glu-B1x 7 promoter to be systematically associated with its over-expression. Unfortunately the promoter sequence of GluB1-x 6 is not yet available for comparison. More recently a matrix attachment region (MAR) was also found in the region upstream from GluB1-x 7 (Anderson et al. 2002). The MARs are known to stabilize and improve expression of transgenes, and although their role in vivo is still largely unclear, they are also candidates to explain the overexpression of the GluB1-x 7 allele. This major QTL on chromosome 1 B has no effect on the overall content of HMW-GSs, since parental alleles have opposite effects on either GluB1x or other HMW-GS (GluA1, GluD1x, GluD1y Table 3), which compensate each other. However in a recent study based on a larger genetic base, we found variation at GluB1x to be statistically associated with the overall quantity of HMW-GSs and even to the overall GPC (Ravel et al, unpublished results). Paradoxically, the QTL found on chromosome 5A for GluD1x does has an effect on the overall HMW-GS quantity.

Conclusion

This is the first study to report simultaneous QTL analysis for grain filling parameters and grain protein composition using a RIL population. QTL were found mostly for the rates of accumulation and were associated with both DM and N, suggesting that the genotypic determinants of starch and N grain filling are either pleiotropic and/or tightly linked to each other. Unlike the effect of environmental factors, genotype influences grain protein composition essentially through the N-filling rate, as suggested by many QTL co-locations. However, two QTLs affected only the protein composition, particularly that of HMW-GS. From a practical point of view, this means that the balance between protein fractions and their aggregation status can be manipulated, using markers, independently from starch and N accumulation, i.e. from the average GPC. This may offer breeders the opportunity to improve both yield and quality despite the strong negative correlation between yield and protein concentration.

Acknowledgements This study was supported by the French Ministry of Research and Technology within the framework of the National Plant Genomics Programme "Génoplante". The authors wish to thank the staff of the experimental farm and its director B. Debote for the excellent management of the experiments, and all workers who helped with sample collection and laboratory analyses, particularly P. Bérard for organizing the work.

References

Albani D, Hammond-Kosack MCU, Smith C, Conlan S, Colot V, Holdsworth MJ, Bevan MW (1997) The wheat transcriptional activator SPA: a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. Plant Cell 9:171–184

Anderson OD, Abraham-Pierce FA, Tam A (1998) Conservation in wheat high-molecular-weight glutenin gene promoter sequences: comparisons among loci and among alleles of the *GLU-B1-1* locus. Theor Appl Genet 96:568–576

Anderson OD, Larka L, Christoffers MJ, McCue KF, Gustafson JP (2002) Comparison of orthologous and paralogous DNA flanking the wheat high molecular weight glutenin genes: sequence conservation and divergence, transposon distribution, and matrix-attachment regions. Genome 45:367–380

Bean SR, Lookhart GL (1997) Separation of wheat proteins by two dimensional reversed phase high performance liquid chromatography plus free zone capillary electrophoresis. Cereal Chem 74:758–765

Bean SR, Lookhart GL (1999) Sodium dodecyl sulfate capillary electrophoresis of wheat proteins. 1. Uncoated capillaries. J Agric Food Chem 47:4246–4255

Bevan M, Colot V, Hammond-Kosack M, Holdsworth M, Torres de Zabala M, Smith C, Grierson C, Beggs K (1993) Transcriptional control of plant storage protein genes. Philos Trans R Soc Lond B Biol Sci 342:209–215

Bietz JA, Schmalzried E (1995) Capillary electrophoresis of wheat gliadin: initial studies and application to varietal identification. Lebensm Wiss U Technol 28:174–184

Blumenthal CC, Batey IL, Bekes EWR, Wrigley CW, Barlow EWR (1991) Seasonal changes in wheat-grain quality associated with high temperature during grain filling. Aust J Agric Res 42:21–30

Borner A, Schumman E, Furste A, Coster H, Leithold B, Röder MS, Weber WE (2002) Mapping of quantitative trait loci determining agronomic important characters in hexaploid wheat (*Triticum aestivum* L). Theor Appl Genet 105:921–926

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weigh subunits of glutenin and flour quality characteristics. J Cereal Sci 3:345–354

Branlard G, Dardevet M, Saccomano R, Lagoutte F, Gourdon J (2001) Genetic diversity of wheat storage proteins and bread wheat quality. Euphytica 119:21–30

Campbell CA, Cutforth HW, Selles F, Depauw RM, Clarke JM (1990) Dynamics of DM, N and P accumulation in the developing kernels of four spring wheat cultivars for irrigation and dryland. Can J Plant Sci 70:1043–1056

Carceller JL, Aussenac T (1999) Accumulation and changes in molecular size distribution of polymeric proteins in developing grains of hexaploid wheats: role of the dessication phase. Aust J Plant Physiol 26:301–310

Crepieux S, Lebreton C, Servin B, Charmet G (2004) Quantitative trait loci (QTL) detection in multi-cross inbred designs: recovering QTL identical-by-descent status information from marker data. Genetics 168:1737–1749

Daniel C, Triboï E (2001) Effects of temperature and N nutrition on the accumulation of gliadins analysed by RP-HPLC. Aust J Plant Physiol 28:1197–1205

Diaz I, Vicente-Carbajosa J, Abraham Z, Martinez M, Isabel-La Moneda I, Carbonero P (2002) The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. Plant J 29:453–464

Forde BG, Heyworth A, Pywell J, Kreis M (1985) Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. Nucleic Acids Res 13:7327–7339

Goffinet B, Gerber S (2000) Quantitative trait loci: a meta-analysis. Genetics 155:463–473

Graybosh RA, Peterson CJ, Shelton DR, Baenziger PS (1996) Genotypic and environmental modification of wheat flour protein composition in relation to end-use quality. Crop Sci 36:296–300

Groos C, Gay G, Perretant MR, Gervais L, Bernard M, Dedryver F, Charmet G (2002) Study of the relationship between preharvest sprouting and grain color by quantitative trait loci analysis in a white × red grain bread wheat cross. Theor Appl Genet 104:39–47

Groos C, Robert N, Bervas E, Charmet G (2003) Genetic analyses of grain protein content, grain yield and thousand kernel weight in bread wheat. Theor Appl Genet 106:1032–1040

Guillaumie S, Linossier L, Robert N, Bénard V, Charmet G, Ravel C (2004) Co-location between the gene encoding for the bZip factor SPA and a PQL for a high-molecular-weight glutenin subunit in wheat. Genome 47:705–713

Gupta RB, Masci S, Lafiandra D, Bariana HS, Mac Ritchie F (1996) Accumulation of protein subunits and their polymers in developing grains of hexaploid wheats. J Exp Bot 47:1377–1385

Hammond-Kosack MC, Holdsworth MJ, Bevan MW (1993) In vivo footprinting of a low molecular weight glutenin gene (*LMWG-1D1*) in wheat endosperm. EMBO J 12:545–554

Huebner FR, Nelsen TC, Chung OK, Bietz JA (1997) Protein distributions among hard red winter varieties as related to environment and baking quality. Cereal Chem 74:123–128

Hyne V, Kearsey MJ (1995) QTL analysis: further uses of 'marker regression'. Theor Appl Genet 91:471–476

Juhasz A, Gardonyi M, Tamas L, Bedõ Z (2003) Characterization of the promoter region of *Glu-1Bx7* gene from everexpressing lines of an old Hungarian wheat variety. In: Pogna N (ed) Proceedings of the 10th international wheat genetics symposium, 1–6 September, Paestum, Italy, pp 1348–1350

Kearsey MJ, Hyne V (1994) QTL analysis: a simple 'marker-regression' approach. Theor Appl Genet 89:698–702

Lafiandra D, Masci S, Blumenthal CS, Wrigley CW (1999) The formation of glutenin polymers in practice. Cereal Foods World 44:572–578

Marchylo BA, Lukow OM, Kruger JE (1992) Quantitative variation in high molecular weight glutenin subunit 7 in some Canadian wheats. J Cereal Sci 15:29–37

Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P (1998) An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. Plant J 16:53–62

Mou B, Kronstadt WE (1994) Duration and rate of grain filling in selected winter wheat populations: I. Inheritance. Crop Sci 34:833–837

Mou B, Kronstad WE, Saulescu NN (1994) Grain filling parameters and protein content in selected winter wheat populations: II. Associations. Crop Sci 34:838–841

Müller M, Knudsen S (1993) The N response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J 4:343–355

Oury FX, Berard P, Brancourt-Hulmel M, Depatureaux C, Doussinault G, Galic N, Giraud A, Heumez E, Lecomte C, Pluchard P, Rolland B, Rousset M, Trottet M (2003) Yield and grain protein concentration in bread wheat: a review and a study of multi-annual data from a French breeding programme. J Genet Breed 57:59–68

Panozzo JF, Eagles HA, Wooton M (2001) Changes in protein composition during grain development in wheat. Aust J Agric Res 52:485–494

Payne PI (1987) Genetics of wheat storage proteins and the effects of allelic variation on breadmaking quality. Annu Rev Plant Physiol 38:141–153

Robert N, Huet S, Hennequet C, Bouvier A (1999) Methodology for choosing a model for wheat kernel growth. Agronomie 19:405–417

Robert N, Hennequet C, Bérard P (2001) Dry matter and N accumulation in wheat kernel: genetic variation in rate and duration of grain filling. J Genet Breed 55:297–305

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53:947–958

Shewry PR, Napier JA, Tatham AS (1995) Seed storage proteins: structures and biosynthesis. Plant Cell 7:945–956

Sofield I, Evans LT, Cook CG, Wardlaw IF (1977) Factors influencing the rate and duration of grain filling in wheat. Aust J Plant Physiol 4:785–797

Sorensen MB, Cameron-Mills V, Brandt A (1989) Transcriptional and post-transcriptional regulation of gene expression in developing barley endosperm. Mol Gen Genet 217:195–201

Statistical Sciences (1993) Splus programmer's manual, version 3.2. MathSoft, Seattle

Stone PJ, Nicolas ME (1995) A survey of the effects of high temperature during grain filling on yield and quality of 75 wheat cultivars. Aust J Agric Res 46:475–492

Stone PJ, Nicolas ME (1996) Varietal differences in mature protein composition of wheat resulted from different rates of polymer accumulation during grain filling. Aust J Plant Physiol 23:727–737

Triboï E, Abad A, Michelena A, Lloveras J, Ollier JL, Daniel C (2000) Environmental effects on the quality of two wheat genotypes. 1. quantitative and qualitative variation of storage proteins. Eur J Agron 13:47–64

Van Sanford DA (1985) Variation in kernel growth characters among soft red winter wheats. Crop Sci 25:626–630

Visscher PM, Thompson R, Haley CS (1996) Confidence intervals in QTL mapping by bootstrapping. Genetics 143:1013–1020

Wardlaw IF, Blumenthal C, Larroque O, Wrigley CW (2002) Contrasting effects of chronic heat stress and heat shock on kernel weight and flour quality in wheat. Funct Plant Biol 29:25–34

Washida H, Wu CY, Suzuki A, Yamanouchi U, Akihama T, Harada K, Takaiwa F (1999) Identification of cis-regulatory elements required for endosperm expression of the rice storage protein glutelin gene *GluB-1*. Plant Mol Biol 40:1–12

Weegels PL, Hamer RJ, Schofield JD (1996) Critical review: functional properties of wheat glutenin. J Ceral Sci 23:1–18

Werner WE, Wiktorowicz JE, Kasarda DD (1994) Wheat varietal identification by capillary electrophoresis of gliadins and high molecular weight glutenin subunits. Cereal Chem 71:397–402

Whan BR, Carlton GP, Anderson WK (1996) Potential for increasing rate of grain growth in spring wheat. I. Identification of genetic improvements. Aust J Agric Res 47:17–31

Zanetti S, Winzeler M, Feuillet C, Keller B, Messmer M (2001) Genetic analysis of bread-making quality in wheat and spelt. Plant Breed 120:13–19

Zhu J, Khan K (2001) Effects of genotype and environment on glutenin polymers and breadmaking quality. Cereal Chem 78:125–130