Environmentally-induced changes in protein composition in developing grains of wheat are related to changes in total protein content

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Received 14 November 2002; Accepted 2 April 2003

Abstract

Nitrogen (N) nutrition, post-anthesis temperature and drought-induced changes in the kinetics of accumulation of dry mass, total grain N and protein fractions (albumins-globulins, amphiphils, gliadins, and glutenins) contents were examined for winter wheat (*Triticum aestivum* L.). Crops were grown in controlled environment tunnels in 1994 and 1998. In 1994, five post-anthesis temperatures averaging from 15–25 °C were applied during grain-filling. In 1998 two post-anthesis temperatures averaging 13 °C and 20 °C were applied and factorized with two post-anthesis water regimes. In 1994 crops also were grown in the field, where different application rates and timing of N nutrition were tested. When expressed in thermal time, the kinetics of accumulation of the protein fractions were not significantly affected by post-anthesis temperature or drought; whereas N nutrition significantly increased the rate and duration of accumulation of storage proteins. Albumin-globulin proteins accumulated during the early stage of grain development. The rate of accumulation of that fraction decreased significantly at c. 250 °Cd after anthesis, when the storage proteins (gliadins and glutenins) started to accumulate significantly. Single allometric relationships for the different environmental conditions exist between the quantity of each protein fraction and the total quantity of N per grain. From these results it was concluded that (1) the process of N partitioning is neither significantly affected by post-anthesis temperature or drought nor by the rate and timing of N nutrition and (2) at maturity, variations in protein fraction composition are mainly because of differences in the total quantity of N accumulated during grain-filling.

Key words: Albumins-globulins, drought, grain development, gliadins, glutenins, lipid-binding proteins, nitrogen, protein composition, temperature, wheat.

Introduction

Proteins are the most important components of wheat (*Triticum aestivum* L.) grains governing end-use quality (Weegels et al., 1996). Variations in both protein content and composition significantly modify flour quality for bread-making (Weegels et al., 1996; Lafiandra et al., 1999; Branlard et al., 2001). Although grain protein composition depends primarily on genotype, it is significantly affected by environment factors and their interactions (Graybosch et al., 1996; Huebner et al., 1997; Triboï et al., 2000; Zhu and Khan, 2001).

In wheat, grain proteins are traditionally separated on the basis of their solubility as albumins, globulins, gliadins, and glutenins. From anthesis to 10–15 days after anthesis (daa), the first two fractions consisted of only metabolic and structural proteins, but from 10–15 daa albumin (mainly α-amylase/trypsin inhibitors and β-amylases) and globulin (mainly triticins) storage proteins accumulate in the developing starchy endosperm (Singh et al., 1991; Gupta et al., 1991, 1996). At maturity, triticins account only for about 5% of the total grain protein (Singh et al., 1991), and β-amylases account only for 8–10% of the polymeric protein fraction (Gupta et al., 1991). However, most of the albumin proteins identified from proteomic analyses of mature grain endosperm of wheat (Skylas et al., 2000; Singh et al., 2001) and barley (*Hordeum vulgare* L.; Finnie et al., 2002) belong to a family of α-amylase/trypsin inhibitors, with putative dual storage roles. The gliadin and glutenin fractions consist of storage proteins only. Storage proteins are defined as...
proteins that accumulate during the grain-filling period and are used as nitrogen (N) sources during seed germination (Shewry and Halford, 2002). With the exception of the α-amylase/trypsin inhibitors and β-amylases, storage proteins have no known physiological function during grain maturation. A fifth fraction of lipid-bound proteins, named amphiphil, can be isolated (Marion et al., 1994) and consists of proteins involved in the formation of cell membranes and hydrophobic layers such as cutin and suberin, and of proteins involved in the transport of fatty acid or their CoA derivatives (Douliez et al., 2000). Some of the non-membrane amphiphil proteins have antimicrobial activity in vitro (Douliez et al., 2000). Non-membrane amphiphil proteins have a large effect on grain hardness and dough rheological properties (Dubreil et al., 1998).

Albumins-globulins accumulate from anthesis to approximately 20 daa, and then remain at an almost constant level. Storage proteins accumulate from approximately 6 daa to the end of grain-filling (Gupta et al., 1996; Stone and Nicolas, 1996; Panozzo et al., 2001). It has been reported that gliadins accumulate earlier in grain-filling than glutenins (Stone and Nicolas, 1996; Panozzo et al., 2001). The kinetics of accumulation of the amphiphil fraction have not been reported. The accumulation of the different protein fractions is highly asynchronous, inferring that the protein composition of the grain changes during grain development. One consequence is that conditions that shorten grain-filling, such as high temperature or drought, affect the balance of protein fractions (Jamieson et al., 2001).

For certain varieties, flour, dough, and baking quality parameters were reported to be altered in response to a short period of heat stress (>35 °C, Blumenthal et al., 1993), and some of these effects have been linked to an increased gliadins-to-glutenins ratio (Blumenthal et al., 1991) and decreases in the proportion of the larger molecular size glutenins (Wardlaw et al., 2002). However, moderately high temperatures of 25–32 °C have a positive effect on dough properties (Finney and Fryer, 1958; Randall and Moss, 1990; Wrigley et al., 1994), and have been reported to lead to variation of the composition of the gliadin fraction (Daniel and Triboí, 2000). With current knowledge, the effect of moderately high temperature during grain-filling on the accumulation of protein fractions has only been reported for the gliadin fraction (Daniel and Triboí, 2001).

Although water deficits severely affect end-use quality for several cultivars of wheat (Guttiere et al., 2000, 2001), little is known about the effect of water deficit and its interaction with temperature on the accumulation of protein fractions. Analysis of the kinetics of accumulation of gliadins and high- and low-molecular weight glutenins in irrigated and non-irrigated fields did not show a significant effect of drought (Panozzo et al., 2001). Similarly, post-anthesis drought did not affect the rate of accumulation of SDS-soluble and SDS-insoluble polymers (gliadins; Daniel and Triboí, 2002), however, post-anthesis drought shortened the period of grain-filling before the onset of polymer insolubilization (Daniel and Triboí, 2002).

N nutrition increases the total quantity of protein per grain at harvest ripeness and this is correlated with an increase in the quantity of gliadin and glutenin storage proteins for wheat (Pechanek et al., 1997; Wieser and Seilmeyer, 1998; Triboí et al., 2000) and hordeins for barley (Hordeum vulgare; Shewry et al., 2001). For wheat, increasing N supply usually leads to an increase of the percentage of gliadins while that of glutenins is not changed (Jia et al., 1996; Doekes and Wennekes, 1982; Gupta et al., 1992); although, more recent work shown that this result depends on the genotype considered (Pechanek et al., 1997; Wieser and Seilmeyer, 1998). The quantity of albumins-globulins is scarcely influenced by N nutrition (Pechanek et al., 1997; Wieser and Seilmeyer, 1998; Johansson et al., 2001). Amino acid composition also changes with the total quantity of N per grain (Mossé et al., 1985).

Despite the fact that the response of protein composition to environmental factors in mature wheat grain results from changes in protein deposition during grain-filling, very few studies have examined separately the effects of environmental factors and their interactions on the accumulation of protein fractions during grain-filling. In the present study, the effect of post-anthesis temperature and drought, and N nutrition on the kinetics of accumulation of the different protein fractions have been studied in separate experiments in controlled environment closed-top chambers and in the field.

Materials and methods

Plant material and culture conditions

All experiments were at Clermont-Ferrand, France (45°47’ N, 3°10’ E, 329 m elevation) with the winter wheat (Triticum aestivum L.) variety Thésée.

Temperature and drought experiments

Crops were grown outside in 2 m² containers 0.5 m deep, filled with a 2:1 mixture of black soil:peat, in order to control the water and N supply and temperature. Nitrogen was supplied as ammonium-phosphate (N:P, 18:46); 30 kg N ha⁻¹ was applied about 1 week after the beginning of tillering, 100 kg N ha⁻¹ when the stem started to elongate, 100 kg N ha⁻¹ at meiosis, and 100 kg N ha⁻¹ at anthesis. Seeds were sown at a density of 500 seed m⁻², resulting in 382–478 plant m⁻² at anthesis. The high plant density inhibited the development of axillary tillers which co-ordinated the development of the crops within and between the containers. The crops were rain-fed from sowing to anthesis, and received 328 and 226 mm of rainfall during that period in 1994 and 1998, respectively. In order to control and monitor the air temperature, water supply, and gas exchange, the containers were covered with transparent closed-top chambers under natural light 5 daa. Details of the controlled
environment closed-top chambers are given elsewhere (Triboi et al., 1996). At anthesis, all the containers were irrigated to field capacity by applying 90 mm of water, they then received 25–50 mm of water every 4–7 d until harvest in order to replace measured crop evapotranspiration. Spikes were tagged at anthesis in order to allow the accurate determination of the developmental stage when harvesting.

Different air temperatures relative to the outside air temperature were applied under the controlled environment closed-top chambers. Therefore, as under field conditions, there was a gradual increase in the air temperature during the grain-filling period. In 1994, four air temperatures were applied relative to the outside air temperature: −5 °C (treatment termed −5, average temperature of 14.9 °C); 0 °C (average temperature of 19.5 °C); +5 °C (+5, average temperature of 22.5 °C); +5 °C until 300 °Cd, base 0 °C, after anthesis then +10 °C until harvest maturity (+5/+10, average temperature of 24.7 °C); and +10 °C until 300 °Cd after anthesis then +5 °C until harvest maturity (+10/+5, average temperature of 23.7 °C). In 1998, two air temperatures were applied relative to the outside air temperature: −5 °C (average temperature of 12.6 °C) and +5 °C (average temperature of 19.9 °C). In order to avoid extreme temperatures, the temperature under the closed-top chambers was limited to ensure that the air temperature did not fall below −5 °C and did not exceed +35 °C.

In 1998, in order to study the interaction between post-anthesis temperature and drought, one container for each temperature treatment was irrigated, as described previously, to replace measured crop evapotranspiration, whereas the other one received only 5–15% of the measured crop evapotranspiration from anthesis to harvest maturity. Total irrigation amounts over the period of differential irrigation were 220 mm and 13 mm for the wet and drought −5 treatments (−5W and −5D, respectively) and 243 and 38 mm for the wet and drought +5 treatments (−5W and −5D, respectively).

In order to study the dynamic accumulation of dry mass, total N, and protein fractions, three replicates of 20 plants (approximately 0.25 m²) were collected every 50–130 °Cd from approximately 50 °Cd after anthesis to grain maturity.

**Nitrogen experiment**

The effect of N availability at anthesis in relation to the level of N nutrition before anthesis was studied in 1994 in a field experiment. Seeds were sown at a density of 300 seeds m⁻². Average temperature from sowing to anthesis and from anthesis to ripeness harvest were 7.8 °C and 19.6 °C, respectively. Crops were rain-fed. Accumulated rainfall from sowing to anthesis and from anthesis to ripeness harvest was 344 mm and 61 mm, respectively.

Three levels of N supply before anthesis were applied. Low-N treatments (treatments termed L) were established on plots that had not received N since 1948. Moderate-N nutrition treatments (M) were established on the same plots as the L treatments, but received 50 kg N ha⁻¹ at the beginning of tillering. High-N nutrition treatments (H) were on plots where leaves of sugar beet from a previous cultivation and a cut of alfalfa had been buried. The H treatments resulted in an uptake by the crops of 63.7 kg N ha⁻¹ from sowing to anthesis, compared with 23.0 ± 0.8 and 47.8 ± 3.3 kg N ha⁻¹ for the L and M treatments, respectively (P < 0.001; df = 24). At anthesis, each plot was split into three subplots to which 0 kg N ha⁻¹ (treatments termed L0, M0, and H0), 30 kg N ha⁻¹ (L30, M30, and H30) or 150 kg N ha⁻¹ (L150, M150, and H150) were applied.

Samples of 0.20 m² were taken in each subplot at 0, 290, 505, 712, and 900 °Cd after anthesis. Three replicates were used per treatment.

**Plant sampling and protein extraction**

Stems, leaves, chaffs and grains were separated, and their dry mass was determined on subsamples after oven-drying at 70 °C to a constant mass. The remaining grains were frozen in liquid N, freeze-dried, and stored at 4 °C before analysis.

The protein fractions albumin-globulin, amphiphil, gliadin, and glutenin were sequentially extracted from 833 mg of flour (Marion et al., 1994; Triboi et al., 2000). Briefly, grains were ground to wholemeal flour using a Quadramat Jr mill (Brabender, Duisburg, Germany). During each extraction step, the samples were continuously stirred on a magnetic stirrer for 60 min. Soluble and insoluble fractions were separated by centrifugation at 8 000 g for 30 min at the extraction temperature. Albumins-globulins were extracted at 4 °C with 25 ml 0.05 M NaCl, 0.05 M sodium phosphate buffer pH 7.8. Amphiphilic proteins were extracted at 4 °C from the previous pellet with 25 ml 2% (v/v) Triton X-114, 0.1 M NaCl, 0.05 M sodium phosphate buffer pH 7.8. Gliadins were extracted at 20 °C from the previous pellet with 25 ml 70% (v/v) ethanol. Glutenins were extracted at 20 °C from the previous pellet with 25 ml 20 g l⁻¹ SDS (sodium dodecyl sulphate), 2% (v/v) 2-mercaptoethanol (2-MH), 0.05 M tetraborate buffer pH 8.5. After centrifugation the glutenins were recovered in the supernatant.

**N content determination**

Total N content for grains was determined on freeze-dried samples by the Kjeldhal method using a Kjeltec 2300 analyser (Foss Tecator AB, Hoejgaard, Sweden). After the supernatant solutions were evaporated to approximately 5 ml in a forced-draught oven at 50 °C, total N content for the different protein fractions and the residue of the extraction was determined using the same method. The sum of the quantity of N for each protein fraction plus the residue was strongly correlated with the total quantity of N determined independently (r² = 0.99, df = 98, P < 0.001), and the slope of the reduced-major-axis linear regression between these two dependent variables was very close to unity (0.99 ± 0.01). Protein content was calculated from the percentage of total N by multiplying by a conversion factor of 5.62 for grains of wheat (Mossé et al., 1985).

**Statistics**

Percentages of the different protein fractions were analysed using one-way ANOVA (α = 0.05) on log transformed data, followed by a Tukey’s test. Statistically significant differences were judged at P < 0.05.

To determine the rate and duration of accumulation of dry mass, total N and protein fractions, data were fitted with a 3-parameter logistic function equation:

\[ Q(t) = \frac{Q_{\text{max}}}{1 + 0.05 \exp \left(\frac{4(t-t_0)}{b_{\text{max}}} \right)} \]

Where \( Q \) is the quantity of dry mass or N, \( t \) is the number of days or degree-days after anthesis, and \( Q_{\text{max}} \) is the final value of \( Q \) approached as \( t \to \infty \). \( r \) is the maximum rate of accumulation defined as the derivative of the point of inflexion, and \( t_0 \) is the duration of accumulation defined as the duration, from anthesis, in which 95% of \( Q_{\text{max}} \) is accumulated. For each experiment, the accumulation of the different grain components were analysed simultaneously using a parallel curve analysis (Ross, 1984) using GenStat statistical package (5th edn, VSN International Ltd, Oxford, UK).

The partitioning of the total quantity of N per grain between the different protein fractions was analysed using the broken-stick procedure of the GenStat statistical package with the constraint for the two lines to intercept at the change point (\( \hat{k} \)):

\[ Q_i = \begin{cases} p_{\text{II}} N + b_{\text{II}} & N \leq \hat{k}_i \, , \quad \text{with} \, \hat{k}_i = b_{\text{II}} - b_{\text{II}} \\ p_{\text{I}} N + b_{\text{I}} & N > \hat{k}_i \end{cases} \]
where $Q_i$ is the quantity of N in the fraction $i$, $p_{1,i}$ and $b_{1,i}$ are the partitioning coefficient and the intercept for the initial stage of grain development (termed stage I) for the fraction $i$, respectively, and $p_{II,i}$ and $b_{II,i}$ are the partitioning coefficient and the intercept for the second stage of grain development (stage II) for the fraction $i$, respectively, and $k_i$ is the intercept between the lines for the two stages of grain development for the fraction $i$, and represents the quantity of total N accumulated in the fraction $i$ at the end of the stage I.

**Results**

**Accumulation of dry mass and total N**

In order to characterize the effect of the different treatments the kinetics of accumulation of dry mass and total N per grain are presented. The effects of temperature and drought were examined using non-limiting fertilizer treatments. Figures 1 and 2 present the kinetics of accumulation of dry mass and total N per grain in real time and thermal time, respectively, for different post-anthesis temperatures and watering regimes when soil N supply is not limiting.

An increase in the average post-anthesis temperature of 7°C (in 1998) and 9°C (in 1994) increased the rate of dry mass accumulation per day and per grain by 20% and 22%, respectively (ranging from 1.95±0.15 to 2.76±0.16 mg d$^{-1}$ grain$^{-1}$; Fig. 1A, B), which did not compensate for the decrease of 60% in the duration of dry mass accumulation, as determined from anthesis. When expressed as a function of accumulated thermal time above 0°C, the rate of dry mass accumulation decreased by 26% when the post-anthesis temperature increased by 7–9°C (ranging from 0.12±0.01 to 0.16±0.01 mg (°Cd)$^{-1}$ grain$^{-1}$), whereas the duration of accumulation was not affected, and averaged 625±20°Cd (Fig. 2A, B). The rate of dry mass accumulation per day or per degree-day was decreased by 17% and 26% by post-anthesis drought for the −5D and +5D treatments as compared to the −5W and +5W treatments, respectively. Post-anthesis drought decreased the duration of dry mass accumulation by 17% and 13% for the −5D and +5D treatments as compared to the −5W and +5W treatments, respectively.

The rate of N accumulation per day was increased by 40% and 60% with an increase in post-anthesis temperature of 7°C (in 1998) and 9°C (in 1994), respectively, whereas the rate per degree-day was not significantly modified (Figs 1C, D and 2C, D), and averaged 2.4±0.1 μg N (°Cd)$^{-1}$ grain$^{-1}$. Similarly the duration of N accumulation was reduced from 48 d for the −5 treatments to 28 d for the +10/−5 treatments, whereas the duration in degree-days was not significantly modified, and averaged 746±57 °Cd. Post-anthesis drought had only slight effects on the rate and duration of N accumulation, as a function of thermal time.

These results confirm that deposition of dry mass is more sensitive to moderately high temperature (<30°C) or drought post-anthesis than is the deposition of protein (Jenner et al., 1991; Triboi and Triboi-Blondel, 2002). This probably reflects the relative insensitivity of starch
production, which accounted for 60–72% of the grain dry mass (data not shown), to moderate post-anthesis temperature (Jenner et al., 1991). Consequently, the percentage of protein increased from 8.9% to 13.8% and from 11.8% to 14.6% when the average post-anthesis temperature increased by 7 °C (in 1998) and 9 °C (in 1994), respectively. Post-anthesis drought had a similar effect on the percentage of protein, though at the higher temperature, the drought decreased the quantity of protein per grain by 18%.

Figure 3 presents the kinetics of accumulation of dry mass and total N in thermal time for different rates and timing of N nutrition. By contrast with post-anthesis temperature and drought, N nutrition had no significant effect on the rate (2.5±0.1 mg DW (°Cd)^{-1} grain^{-1}) and duration (642±18 °Cd) of dry mass accumulation (Fig. 3A). However, the rate of N accumulation was significantly increased by N applied either before or after anthesis (Fig. 3B). Specifically, the rate of N accumulation for the treatments L0, H0, and H150 were 1.28±0.16, 1.50±0.24, and 2.14±0.19 μg N (°Cd)^{-1} grain^{-1}. Consequently, N applied at anthesis at a rate of 150 kg N ha^{-1} increased the grain protein concentration by 89, 55, and 37% compared to the L0, M0, and H0 treatments, respectively. Similar protein concentrations were obtained for the L150, M150, and H150 treatments, averaging 13.8±0.2%. The rate of N accumulation per degree-day and per grain was similar for the L150, M150, and H150 treatments. The duration of N accumulation increased from 646±60 and 594±52 °Cd for the L0 and M0 treatments, respectively, to 772±16 °Cd for the L150, M150, and H150 treatments. These results confirm that deposition of dry mass is less sensitive to N nutrition than is the
Fig. 4. Relationship of the percentages of N at harvest ripeness in the albumin-globulin (Alb-Glo), amphiphil (Amp), gliadin (Gli), and glutenin (Gln) fractions as a function of the total quantity of N per grain. Wheat crops were grown in controlled environment closed-top chambers at different post-anthesis temperatures and watering regimes, and in the field where different rates and timing of N fertilization were applied. Data are means ±1 SE for n=3–6 replicates.

Protein fractions composition

The residue from the sequential protein extraction accounted for 1.5–9.0% of the total quantity of N per grain. This fraction is mostly composed of polymeric storage proteins: glutenins, β-amylases, α-amylase/trypsin inhibitors, and triticins (Singh and Shepherd, 1987; Gupta et al., 1991). In the results presented here the residue fraction has been pooled with the glutenin fraction.

Figure 4 summarizes for the different temperature, drought and N fertilization experiments the trends of the percentage of the different protein fractions versus the total quantity of N per grain at harvest ripeness. Interestingly, unifying patterns were clearly visible (Fig. 4). The experimental treatments applied induced a range of variation for the total quantity of N per grain at harvest ripeness of 740 µg N grain⁻¹ (varying form 560 to 1300 µg N grain⁻¹). The percentage of gliadins was positively correlated with the total quantity of N per grain for values of the total quantity of N per grain between 560 and c. 1000 µg N grain⁻¹, with no further variation for higher total quantity of N per grain. The opposite trend was observed for the albumins-globulins and amphiphils, where percentages decreased by 26% and 18%, respectively, when the total quantity of N per grain increased from 560 to 1000 µg N grain⁻¹. The percentage of glutens at harvest ripeness was not modified by the variations of the total quantity of N per grain, and averaged 39.5±0.4%.

Accumulation of protein fractions

Under conditions of non-limiting soil-N supply, the rate of accumulation of the different protein fractions was not markedly modified by post-anthesis temperature and drought (Fig. 5), and averaged 0.88±0.08, 0.31±0.03, 0.80±0.06, and 1.03±0.06 µg N (°Cd)⁻¹ grain⁻¹, for the albumins-globulins, amphiphils, gliadins, and glutenins fractions, respectively. However, the duration of accumulation of the different protein fractions expressed in degree-days decreased by 21–25% when the post-anthesis temperature increased by 7–9 °C.

Under conditions of non-limiting soil-N supply, drought increased the rate of accumulation of albumins-globulins by 60% at the lower temperature, but had no significant effect at the higher temperature (Fig. 5B). Post-anthesis drought had no significant effect on the rate of accumulation of the amphiphils at both temperatures (Fig. 5D). With the exception of the amphiphils at the lower temperature, whose duration of accumulation was not modified, the duration of accumulation of the albumins-globulins and amphiphils was decreased by 40–62% by post-anthesis drought. By contrast, post-anthesis drought decreased the rate of accumulation of gliadin by 40% and 30% at the lower and higher temperature, respectively, and that of glutenins by 30% and 60% at the lower and higher temperature, respectively (Fig. 5F, H). The duration of accumulation of gliadins and glutenins was not significantly modified by post-anthesis temperature, regardless of the post-anthesis temperature.

By contrast with the effect of post-anthesis temperature and drought, the rates of accumulation of albumins-globulins, gliadins, and glutenins were markedly increased in response to N fertilization (Fig. 6), and this effect was stronger for the storage proteins than for the structural proteins. For the L treatments, N fertilization at anthesis increased the rate of gliadins and glutenins accumulation by 161% and 68%, respectively, and for the H treatments by 48% and 51% for the gliadins and glutenins, respectively. The rate of accumulation of the amphiphils was not significantly affected by N nutrition. The duration of accumulation of the different protein fractions was not significantly modified by N nutrition.

Quantity of protein fractions versus the total quantity of N per grain

The relationship between the quantity of each protein fraction and the total quantity of N per grain for developing and mature grain was not influenced by post-anthesis temperature and drought or by the rate and timing of N nutrition (Fig. 7). The partitioning coefficients of N
between the protein fractions are equal to the first derivatives of this relationship. Two different linear relationships can be identified for each protein fraction. This indicates a change in the ratio of the relative rates of accumulation of total N to the different protein fractions (Table 1). As expected, the sum of the slopes for the different protein fractions for each stage of grain development equalled one, whereas the sum of the intercept for each stage of grain development was not significantly different from zero.

These changes in the partitioning coefficients for the different protein fractions were synchronized and the decrease in the partitioning coefficients for the albumins-globulins corresponded to an increase in the partitioning coefficients for the storage protein (i.e. gliadins and glutenins). The intercept of the two linear relationships...
averaged $275 \pm 30 \, \mu g \, N \, grain^{-1}$, which corresponded approximately to 240 °Cd after anthesis. For the second stage of grain development (i.e. from 240 °Cd after anthesis to grain physiological maturity) the partitioning coefficients were four times higher for the storage proteins compared with the structural proteins. Also for the second stage of grain development, the partitioning coefficient was 30% higher for the glutenins compared with the gliadins, and the intercept was significantly higher for the glutenins compared with the gliadins. The direct implication of the former result is that the gliadin-to-glutenin ratio increased during the grain-filling period, despite a higher rate of accumulation and partitioning coefficient for the glutenins compared to the gliadins.

**Discussion**

The treatments applied in this study produced a wide range of dry mass and total quantity of N per grain. Variations in protein concentration were due mainly to variations of grain dry mass for the post-anthesis temperature and drought treatments or to variations in the total quantity of N per grain for the N-nutrition experiments. This agrees well with previous studies (Triboi and Triboi-Blondel, 2002). Variations in the kinetics of accumulation of total N into the grain resulted in important variations in the kinetics of accumulation and final composition of protein fractions. However, stable relations were found between the different protein fractions and the total quantity of N per grain.

Assuming that the rate of total N accumulation per grain and per degree-day ($F_N$) is constant, then the total quantity of N per grain ($N$) could be viewed as the equivalent of a developmental time that integrates the effect of temperature (as thermal time, $T_t$), and of N availability ($F_N$):

$$N = T_t \times F_N$$  \hspace{1cm} (3)

The relationship between the quantity of each protein fraction and the total quantity of N per grain strongly suggests that the processes of N partitioning within the grain were not altered by post-anthesis temperature, drought, and the rate and timing of N fertilization. Single linear relationships have been reported between the total quantity of gliadins and the total quantity of N per grain and between the quantities of $\alpha$, $\beta$, $\gamma$, and $\omega$-gliadin subunits and the total quantity of gliadins per grain for crops grown at different temperatures and with different rates of N nutrition (Daniel and Triboi, 2000, 2001). Other studies have found close correlations between the percentage of gliadins or glutenins in the flour and the protein content of the flour for mature grains of wheat (Jia et al., 1996; Pechanek et al., 1997). For maize (Zea mays L.), single linear relationships apply between the quantities of the different protein fractions (albumins-globulins, zeins, and glutelins) and the total quantity of N for crops grown in the field and for detached grains grown in vitro (Landry, 2002). Linear relationships were also found between the percentages in the flour of the 20 protein amino acids and protein content for samples of mature grains of wheat from 12 different genotypes, and one genotype grown in 11 locations with a range of protein content ranging from 7.9–18.5% (i.e. 650–1700 $\mu g \, N \, grain^{-1}$; Mossé et al., 1985).
An important implication of these results is that the amino acid and protein fractions composition of wheat grains at harvest ripeness merely reflect differences in the total quantity of N accumulated during grain-filling. Practically, the protein fractions composition and amino acid composition of wheat grains can be deduced directly from the total quantity of N per grain. Herein lies a mechanistic explanation for the belief that higher protein content is generally equated with higher protein quality for bread making (Weegels et al., 1996).

The coefficients of partitioning of N within the grains changed markedly at approximately 240 °Cd after anthesis for the albumins-globulins, gliadins and glutenins. This time also corresponds to the transition between the cell division and the cell expansion stages for grains (Briarty et al., 1979; Chojecki et al., 1986). Similarly, the coefficients of partitioning of the albumins-globulins and the storage proteins change concomitantly at the beginning of the cell expansion stage for grains of maize (Landry and Moureaux, 1976). This is similar to results for pea (Pisum sativum), where, in embryos, the expression of RNA of storage proteins is restricted to regions of the embryo that lacked mitotic activity (Hauxwell et al., 1990). However, a significant amount of N was detected in the gliadin and glutenin fractions as early as 50 °Cd after anthesis, about 60 °Cd earlier than previous reports using SDS-Page

Table 1. Partitioning coefficients ($p_1$ and $p_2$), and intercepts ($b_1$ and $b_2$), for the relationships between the different protein fractions and the total quantity of N per grain

<table>
<thead>
<tr>
<th>Protein fractions</th>
<th>Stage I</th>
<th>Stage II</th>
<th>$k$ (μg N grain$^{-1}$)</th>
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<tr>
<td></td>
<td>$p_n$</td>
<td>$b_1$ (μg N grain$^{-1}$)</td>
<td>$p_n$</td>
</tr>
<tr>
<td>Albumins-globulins (239)</td>
<td>0.59±0.04</td>
<td>10±8</td>
<td>0.15±0.01</td>
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<tr>
<td>Amphiphils (238)</td>
<td>0.16±0.01</td>
<td>-4±2</td>
<td>0.05±0.00</td>
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<tr>
<td>Gliadins (239)</td>
<td>0.06±0.03</td>
<td>-6±5</td>
<td>0.34±0.01</td>
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<tr>
<td>Glutenins (239)</td>
<td>0.22±0.04</td>
<td>-4±7</td>
<td>0.46±0.01</td>
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<tr>
<td>$\Sigma$</td>
<td>1.03±0.06</td>
<td>-4±12</td>
<td>1.00±0.01</td>
</tr>
</tbody>
</table>

Fig. 7. Relationships between the quantity of albumins-globulins (A), amphiphils (B), gliadins (C), and glutenins (D) and the total quantity of N for developing and mature grains of wheat. Crops were grown in controlled environment closed-top chambers and in the field. Treatments are denoted as outlined in Materials and methods. Solid lines are linear regressions using the broken stick regression model; results for the linear regressions are given Table 1. Each point represents an independent measurement.
electrophoresis for gliadins (Tercé-Laforgue and Pernollet, 1982), and SDS-Page electrophoresis and SE-HPLC for gliadins and low molecular weight glutenin subunits (Gupta et al., 1996). This may be due to contaminants in this study’s gliadins and glutenin fractions, particularly in the glutenin fractions which have been pooled with the residue of the sequential protein extraction.

It has been clearly established that the primary control of the synthesis of storage proteins in barley and wheat grains is at the level of gene transcription (Rahman et al., 1983; Giese and Hopp, 1984; Bartels and Thompson, 1986). A regulatory element called the ‘prolamin box’ comprising up- and down-regulatory motifs, named the ‘endosperm motif’ and the ‘GCN4 motif’, respectively, have been found in the promoter region of several genes of storage proteins (Hammond-Kosack et al., 1993; Müller and Knudsen, 1993); and their role in the regulation by the level of N of storage protein gene expression has been clearly demonstrated (Müller and Knudsen, 1993). This and the results reported here together suggest that the synthesis of storage proteins is limited mainly by the availability of N, and provide a mechanistic explanation for the better estimates of the accumulation of total N from simulation modelling when N demand by the grain is set in response to N supply rather than grain number per unit area (sink determined; Jamieson and Semenov, 2000).

At harvest ripeness, the composition of the protein fractions was much more affected by the N treatments than the temperature treatments, as concluded earlier (Wieser and Seilmeier, 1998; Daniel and Triboï, 2000). However, the results presented here show that the different effects of temperature and N nutrition on protein composition is mostly due to different quantities of N per grain, but not to different effects on the partitioning of N within the grain. The percentage of glutenins in the total N was not significantly influenced by post-anthesis temperature, drought or N nutrition, so that the percentage of glutenin in the total N is nearly totally genotype-dependent (Graybosch et al., 1996).

As far as is known, the kinetics of accumulation for the amphiphil fraction has not been reported before. As with albumins-globulins, amphiphils accumulated during the early stage of grain development, although their rate of accumulation did not decrease as much as for the albumins-globulins. This pattern of accumulation is in good agreement with their putative functions in the formation of membrane and cell wall, but also as a protein associated with lipid storage in the endosperm (Douliez et al., 2000).

The gliadin fraction represented 15–27% of the total protein, which is significantly lower than values previously reported (30–45%; Bénétrix et al., 1994; Jia et al., 1996; Stone and Nicolas, 1996). In the present study, amphiphil proteins were extracted prior to storage proteins, which may explain the lower percentage of gliadins reported here. Indeed the quantity of protein extracted from the flour in 70% ethanol is decreased by approximately 40% if the flour has been defatted before the alcohol extraction (Tercé-Laforgue and Pernollet, 1982). A significant amount of lipids, and thus probably of lipid-binding proteins, is extracted by the ‘classical’ procedure, involving 70% ethanol, used to extract the gliadin fraction (Ponte et al., 1967). Thus purer gliadins are extracted on defatted flour, as in this study (Marion et al., 1994). Although, the Triton X114, used to extract the amphiphil proteins, also extracts some γ-gliadins (Blochet et al., 1991).

The rates and durations of accumulation of both gliadins and glutenins expressed in thermal time were not influenced by moderately high temperature (<35 °C), confirming earlier results (Stone and Nicolas, 1998; Daniel and Triboï, 2001). Also the rates of accumulation of the different protein fractions were not significantly modified by drought post-anthesis, but their durations of accumulation were significantly reduced, confirming earlier results (Panozzo et al., 2001).

The observation that the quantity of gliadins and glutenins in mature grain increased in response to N corroborates previous reports (Jia et al., 1996; Pechanek et al., 1997; Triboï et al., 2000). In addition, new information on the kinetics of accumulation of protein fractions was obtained. The rate of accumulation of albumins-globulins, gliadins, and glutenins increased in response to N. However, no significant effect of N nutrition on the duration of accumulation of the different protein fractions was found.

### Conclusions

The separate study of the effects of the major environmental factors on protein fractions accumulation gave new insights into the regulation of protein accumulation. At least for the cultivar studied, the relationships between the total quantity of N accumulated and the quantity of the different protein fractions were modified neither by post-anthesis temperature or drought nor by the rate or timing of N nutrition. Thus the protein fractions composition depended mostly on the total quantity of N per grain. Structural proteins (albumins-globulins and amphiphils) accumulated in the grain mainly during the cell division stage, whereas the storage proteins (gliadins and glutenins) accumulated mainly during the filling period. The next step in the study of the environmental determination of grain protein fractions will be to transcribe these results and hypotheses into a simulation model. This will allow not only the effect of varied environmental conditions to be analysed, but also the effect of the genotypic variability of N remobilization on the accumulation of the different protein fractions and the synthesis of storage proteins.
Acknowledgements

The authors thank Professor Dr John R Porter (Department of Agricultural Sciences, Royal Veterinary and Agricultural University, Taastrup, Denmark) and Drs Jean-François Soussana (Unité d’Agronomie, INRA Clermont-Ferrand, France), and Peter D Jamieson (New Zealand Institute for Crop and Food Research Ltd., Christchurch, New Zealand) for helpful discussions and comments on the manuscript, and Ruth Butler (New Zealand Institute for Crop and Food Research Ltd., Christchurch, New Zealand) for her excellent statistical assistance, and Lucette Leblevenec, Joëlle Messaud, and François Gerbe for their technical assistance. PM gratefully acknowledges the support of the French National Institute for Agricultural Research (INRA) for financial support during his visit to New Zealand.

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