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Nitrogen uptake and remobilization in tetraploid 'Langdon' durum wheat and a recombinant substitution line with the high grain protein gene *Gpc-B1*

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With 2 figures and 4 tables

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Abstract

Tetraploid wheat (*Triticum turgidum* L. var. *durum*) cv. 'Langdon' (LDN) and its near-isogenic recombinant substitution line no. 68 (RSL no. 68) carrying the high grain protein gene *Gpc-B1* from emmer wheat, were compared in three greenhouse experiments to establish in which way *Gpc-B1* increases grain protein concentration (GPC). At anthesis, RSL no. 68 had higher soluble protein and amino acids concentrations in the flag leaf than LDN. At maturity, both lines presented a similar above ground biomass and grain yield. However, RSL no. 68 showed a higher total N content in ears, grain and chaff than LDN; N harvest index (NHI) was also higher because of a lower straw N concentration and higher grain N concentration. When both lines were grown with a low N supply, and when N supply was interrupted before anthesis, similar trends were observed but the differences in GPC were smaller. It is concluded that RSL no. 68 accumulates a higher GPC than LDN mainly because of a more efficient N remobilization from the leaves to the ears during grain filling.

Key words: *Triticum turgidum* var. *dicoccoides* — *Triticum turgidum* var. *durum* — grain protein concentration

Higher grain protein concentration (GPC) in durum wheat (*Triticum turgidum* L. var. *durum*) generally improves pasta products because semolina protein concentration alone can account for 30–40% of the variability in pasta cooking quality (Dexter et al. 1980). In addition, the increase of grain nitrogen (N) concentration in the wheat grain is associated with an increased nutritional value of the end-use products.

Progress in the improvement of GPC by traditional breeding has been slow because of the high influence of the environment on GPC and the negative correlation between GPC and yield (reviewed by Lawlor 2002, Triboi and Triboi-Blondel 2002). As yield is one of the most important targets of agricultural production, direct selection for GPC was often inefficient because it generally led to a decrease in yield (Lawlor 2002). There are, however, exceptional varieties that combine excellent yields with high levels of GPC, indicating that in some cases it is possible to break this correlation (Cox et al. 1985). Efforts to improve GPC can be accelerated by the identification of genes that increase GPC without reducing yield, followed by direct selection for the high GPC alleles using molecular markers.

The discovery of a new source of high GPC in wild *T. turgidum* var. *dicoccoides* (accession FA15-3; Avivi 1978), designated hereafter as *dicoccoides* (DIC), and the mapping of the main chromosome region contributing to the increase in

GPC (Joppa and Cantrell 1990, Joppa et al. 1997) opened the possibility to experimentally test this new strategy. The gene for GPC was first assigned to chromosome 6B by using substitution lines of each of the DIC chromosomes into the cultivated durum cultivar 'Langdon' (LDN) (Joppa and Cantrell 1990). The consistently higher GPC of LDN (DIC-6B) substitution lines compared with LDN suggested the presence of one or more genes controlling GPC on chromosome DIC-6B. The LDN (DIC-6B) lines were superior to LDN for many processing and pasta quality traits (Joppa et al. 1991, Steiger et al. 1996, Kovacs et al. 1998). Different studies have shown equivalent grain yields between LDN (DIC-6B) substitution lines and LDN (Cantrell and Joppa 1991, Elias et al. 1996) and between lines segregating for the gene affecting GPC (Humphreys et al. 1998). However, an additional study found a negative correlation between GPC and grain yield in a segregating population in one of the three locations tested (Chee et al. 2001).

A population of recombinant substitution lines (RSLs) from a cross between the LDN (DIC-6B) substitution line (from DIC accession FA15-3) and LDN was used to map a quantitative trait locus (QTL) for high GPC near the centromere of chromosome arm 6BS within the *Xabg387–Xmwig79* interval. The *QGpc.ndsu.6Bb* QTL accounted for approximately 66% of the total phenotypic variation in GPC (Joppa et al. 1997). This same DIC-6B chromosome region was associated with an average increase in GPC of 15 g/kg across environments in a segregating population from the cross between LDN (DIC-6B) and the adapted durum cultivar 'Vic' (Chee et al. 2001). This chromosome region explained up to 72% of the phenotypic variance in GPC in this cross.

Recently, the DIC allele for high GPC was mapped 1.5-cM proximal to *Xcdo365* and 1.2-cM distal to *Xucw67* as a single Mendelian locus designated *Gpc-B1* (Olmos et al. 2003). In addition, comparative studies between chromosome segments in rice and wheat showed a conserved microcolinearity between the two genomes in the GPC locus, and further delimited the location of *Gpc-B1* to a 0.3-cM interval flanked by polymerase chain reaction (PCR) markers *Xucw79* and *Xucw71* (Distelfeld et al. 2004).

Contrasting with recent progress in the precise mapping of this gene, there is limited information on the physiological regulation for increased GPC induced by *Gpc-B1*. It is assumed that an increase in the available N for grain protein may derive from either improved direct soil uptake (before or after anthesis)

or through a greater remobilization of N from the vegetative tissues to the ears. Genetic variation for protein content is small compared with the variation because of differences in the environment, and the environmental conditions determine what proportion of the genetically fixed maximum N concentration may be achieved (Barneix et al. 1992).

In order to clarify the physiological processes regulated by the high GPC allele on DIC chromosome 6B, the effect of N fertilization is evaluated on the selected LDN (DIC-6B) no. 68 RSL and durum cv. LDN.

Materials and Methods

Plant materials: Seeds from the homozygous RSLs LDN (DIC-6B) no. 68 (RSL no. 68 hereafter) of durum wheat, *Triticum turgidum* L., were developed from a cross between the LDN (DIC-6B) substitution line and the durum cultivar LDN and were kindly provided by Dr L. R. Joppa. RSL no. 68 has a 30-cM DIC-6BS chromosome segment including restriction fragment length polymorphic (RFLP) loci *Xcmvg642* and *Xcdo534*, and the rest of the genome is isogenic to LDN.

Caryopses of cultivar LDN and the RSL no. 68 were incubated on moist filter paper at 4°C during 48 h and then germinated at 25°C in the dark. Forty-eight hours later, three or four seedlings (depending on the experiment) were transplanted to 5 l-pots filled with a homogenized soil [pH (soil : H₂O 1 : 2.5) 5.6; electrical conductivity 0.60 ds/m; P Bray 1:12.6 mg/kg; cation-exchange capacity 16.5 cmol/kg and total N 0.20%]. Plants were grown in a greenhouse with maximal and minimal temperatures of 25 and 15°C, natural light and a day length that varied during the growing season between 10.8 and 15.2 h. The pots were held near field capacity by watering daily. They were randomly placed and periodically rotated. After sowing, each pot was fertilized twice a week with 50 ml of a nutrient solution (Hoagland and Arnon 1950) modified as indicated for each experiment. All plants per pot (main stems plus tillers) were hand-harvested at different times between anthesis and maturity. In all experiments, anthesis time was judged by the appearance of anthers on approximately 50% of all ears. LDN and RSL no. 68 showed no differences in flowering time or anthesis time. At each sampling, with the exception of maturity, fully expanded flag leaves of all stems per pot were excised, weighed and freeze-dried for further protein and amino acid determination. The remaining parts of the shoot were harvested, dried at 60°C for 48 h. At maturity, grain and chaff were also separated.

Experimental design: Three experiments were performed over 3 successive years. All three experiments were organized in a randomized complete block design with five replicates per treatment in Expts 1 and 2, and 10 replicates per treatment in Expt 3. Each pot was considered as an experimental unit with three or four plants per pot depending on

the experiment. In all experiments, plants of both lines were grown as described above and half of the pots were supplied with 16.0 mM KNO₃ from tillering until maturity. In Expt 1, the other half of the pots received only 1.0 mM KNO₃ from tillering until maturity, whereas in Expt 2, NO₃⁻ was eliminated from the supplied nutrient solution 10 days before anthesis until maturity in the second half of the pots. In Expt 3, all pots (10 replications) were supplied with nutrient solution containing 16.0 mM KNO₃. Samples were collected at anthesis and at maturity for all three experiments and at two additional times between those phenological stages for Expt 2. Sufficient pots were planted to have five replications at each sampling time.

Plant analysis: Total N concentration and GPC were determined through micro-Kjeldahl distillation after wet digestion in concentrated H₂SO₄ and H₂O₂. Soluble proteins were determined on 500 mg of flag leaves (Bradford 1976). **Amino acids were extracted overnight at -18°C from 50 mg of the freeze-dried leaf tissue with 50 ml of a mixture of ethanol : chloroform : water (12 : 5 : 3, v/v/v) (Barneix et al. 1984).** A lithium citrate-based Beckman 6300 (Beckman Coulter Inc., Fullerton, California, USA) amino acid analyzer that uses ion-exchange chromatography (Molecular Structure Facility, UC Davis, USA) was used for the determination of individual amino acids (50 µl injection volume). Analyses of variance of the data were performed using STATGRAPHICS® software.

Results

Consistent results were observed among the three experiments for the plants treated with 16.0 mM KNO₃ in spite of differences in plant growth during the 3 years. At anthesis, the plants of both lines showed similar aerial biomass, ear weight, straw weight, N concentration in the ear, straw N concentration, total N in biomass and total N in ears and straw (Table 1). However, even at this early stage of the grain development, **RSL no. 68 showed a higher soluble protein concentration in the flag leaf compared with LDN, and a higher soluble amino acids concentration,** although with a significant interaction among experiments (Table 1).

At maturity, both lines still presented a similar above ground biomass, ear weight, straw weight and grain yield, individual grain weight and HI (Table 2). However, RSL no. 68 showed a higher total N content in ears, grain and chaff than LDN. It also showed a higher concentration of grain protein and chaff N (Table 2). The N concentration in the straw, on the other hand, was higher in LDN than in RSL no. 68. This last line also presented a higher NHI with a significant interaction between experiment and N concentration in the straw (Table 2).

Anthesis	Units	LDN	RSL no. 68	Significance (P-values)		
				Line	Experiment	Interaction
Total biomass	g/plant	5.86 ¹	5.84	0.95	< 0.01	0.52
Ear weight	g/plant	0.66	0.68	0.60	< 0.01	0.19
Straw weight	g/plant	5.18	5.16	0.91	< 0.01	0.41
Ear N concentration	%DM	1.59	1.64	0.16	< 0.01	0.34
Straw N concentration	%DM	0.87	0.91	0.22	0.32	0.96
Total N/biomass	mg/plant	53.2	56.7	0.71	< 0.01	0.87
Total N/ear	mg/plant	9.74	11.2	0.51	< 0.01	0.60
Total N/straw	mg/plant	44.8	47.2	0.81	< 0.01	0.48
Flag leaf soluble protein	µg/g FW	36.0	48.4	< 0.01	0.08	0.64
Soluble amino acids	µg/g FW	375.2	422.0	0.04	< 0.01	< 0.01

LDN, Langdon; RSL no. 68, substitution line with gene *Gpc-B1*; DM, dry matter; FW, fresh weight; N, nitrogen.

¹Numbers are mean values of the three experiments, including only the plants grown at 16.0 mM KNO₃.

Table 1: Measurements at anthesis of various parameters of the two lines treated with 16.0 mM KNO₃

Table 2: Measurements at maturity of various parameters of the two lines treated with 16.0 mM KNO₃

Maturity	Units	LDN	RSL no. 68	Significance (P-values)		
				Line	Experiment	Interaction
Total biomass	g/plant	9.07 ¹	8.82	0.53	< 0.01	0.29
Ear weight	g/plant	3.24	3.27	0.88	< 0.01	0.78
Straw weight	g/plant	5.83	5.47	0.23	< 0.01	0.10
Grain yield	g/plant	2.33	2.27	0.74	< 0.01	0.54
Grain weight	mg/grain	27.5	28.8	0.10	< 0.01	0.93
HI		25.4	25.7	0.74	< 0.01	0.61
Grain protein	%DM	10.25	12.23	< 0.01	0.07	0.11
Chaff N concentration	%DM	0.698	0.822	0.01	< 0.01	0.85
Straw N concentration	%DM	0.365	0.330	0.03	< 0.01	0.53
Total N/biomass	mg/plant	67.4	74.1	0.01	< 0.01	0.99
Total N/ear	mg/plant	46.9	55.8	0.01	< 0.01	0.92
Total N/straw	mg/plant	20.3	18.4	0.11	< 0.01	0.02
Total N/chaff	mg/plant	6.22	8.03	0.01	< 0.01	0.93
Total N/grain	mg/plant	40.7	47.7	0.03	< 0.01	0.90
Grain N	mg/grain	0.49	0.62	< 0.01	0.03	0.45
NHI		59.7	64.7	0.04	0.02	0.26

LDN, Langdon; RSL no. 68, substitution line with gene *Gpc-B1*; DM, dry matter; HI, harvest index; N, nitrogen.

¹Numbers are mean values of the three experiments, including only the plants grown at 16.0 mM KNO₃.

In Expt 1, significant differences in the concentration of grain protein and chaff N were observed between lines grown at 16 mM KNO₃ but not between lines grown at 1 mM KNO₃ (Table 3). Even though the differences were not significant at 1 mM KNO₃, the trend was in the same direction as in the 16 mM KNO₃ treatment, with RSL no. 68 showing a 1.06% more GPC than LDN. As expected, the concentration of grain protein and chaff N was also significantly higher in the 16 mM KNO₃ treatment compared with the 1 mM KNO₃ treatment (Table 3).

In the second experiment (Fig. 1), when plants were supplied with 16.0 mM KNO₃ until maturity, both lines showed similar ear N concentration increases until 9 days after anthesis. After this date, ear N concentration showed no significant increases in LDN but continued increasing for another 13 days in RSL no. 68 (Fig. 1). When N supply was interrupted 10 days before anthesis, the ears of LDN increased their N concentration until 9 days after anthesis, when the concentration started to decrease (Fig. 1), likely due to starch accumulation. As a consequence, the GPC at maturity showed a significant interaction between line and N supply, with no significant differences in protein concentration between lines when N supply was interrupted (Table 4). A similar effect was observed in the chaff N concentration (although in the border of significance), and no significant differences were detected in straw N concentration (Table 4). As in Expt 1, even though the differences were not significant when N supply was interrupted

10 days before anthesis, the trend was in the same direction as in the 16 mM KNO₃ with RSL no. 68 showing a 0.6% more GPC than LDN.

When the amino acids concentration in the flag leaf was followed during grain filling (Expt 2), higher total amino acids concentration was detected in RSL no. 68 compared with LDN at anthesis but not 22 days after anthesis (Fig. 2a). When the individual soluble amino acids composition was analysed, higher amino acids concentration in RSL no. 68 were found for alanine (Fig. 2b), serine (Fig. 2c) and threonine (Fig. 2d). All other amino acids showed no differences between lines (data not shown).

Discussion

Grain N accumulation is the result of N absorption from soil during vegetative growth, and its subsequent remobilization to the ear during grain filling, plus the N absorption from the soil after anthesis (Gastal and Lemaire 2002). The grain N concentration also depends on the amount of photosynthates accumulated as starch in the grain, for it is expressed as percentage of dry matter (DM). Thus, any variation in grain yield affects N and protein concentrations (Lawlor 2002).

In the present experiments no differences were observed at maturity in grain yield or biomass production or partitioning (HI) between lines. However, RSL no. 68 presented consistently higher GPC and grain N content as expected from the

Table 3: Grain protein concentration, and chaff and straw N concentration at maturity at two levels of fertilization (Expt 1)

Line	Grain protein (%DM)		Chaff N (%DM)		Straw N (%DM)	
	16 mM	1 mM	16 mM	1 mM	16 mM	1 mM
RSL no. 68	12.42	11.01	0.71	0.38	0.32	0.33
'Langdon'	10.50	9.95	0.50	0.42	0.33	0.35
Difference	1.92	1.06	0.21	-0.04	-0.01	-0.02
Significance (P-values)						
Line		< 0.01		0.05		0.28
N		< 0.01		< 0.01		0.47
L × N		0.10		< 0.01		0.69
LSD		1.20		0.16		—

DM, dry matter; N, nitrogen.

Fig. 1: Total N concentration in the ears of plants of recombinant substitution line (RSL) no. 68 and 'Langdon' (LDN) grown with 16.0 mM KNO_3 supply until maturity ($\blacktriangle$, $\triangle$), or with 16.0 mM NO_3^- supply until 10 days before anthesis and no N supply from then on ($\blacksquare$, $\square$). Closed symbols: RSL no. 68; open symbols: LDN. Bars represent the mean $\pm$ SE ($n = 5$). The first sampling point coincides with anthesis

presence of the high GPC allele in this line (Table 2). The biomass production and growth of both lines were similar, despite variation among experiments, so no major effect other than the change in GPC can be attributed to the 30 cM segment from DIC chromosome 6B. The absence of differences in grain yield observed in our three greenhouse studies parallel the results from previous field experiments (Cantrell and Joppa 1991, Elias et al. 1996, Humphreys et al. 1998). The only exception is the negative correlation observed between GPC

and grain yield in one of the three locations tested by Chee et al. (2001). Preliminary results from 1 year field trials performed at two locations in California (Davis and Madera, five replications using large plots in a split plot design) confirmed the absence of significant differences in grain yield between isogenic lines (BC_6F_3) for the *Gpc-B1* gene in three different genetic backgrounds (unpublished results). Although a second year of yield trials would be necessary to validate our first year results, taken together with the published studies, these results suggest that *Gpc-B1* is not associated with a significant reduction in yield at least in some environments.

Grain N content is the result of N uptake from soil and remobilization from the vegetative organs to the grain. It is accepted that 70–90% of the grain N is absorbed before anthesis, and is remobilized to the ear during grain filling (Dalling 1985). The present experiments indicate that the differences in GPC between the lines were mainly due to a more efficient remobilization in RSL no. 68 than in LDN, as indicated by the significant differences in NHI. RSL no. 68 showed both, significantly lower N concentration remaining in the straw and higher N concentration in the chaff and grain than LDN (Table 2). The increase in GPC by a more efficient N remobilization provides a tentative explanation for the limited negative effect of the *Gpc-B1* gene on yield. A more efficient remobilization of the N available at anthesis probably imposes a small energetic toll to the plants carrying the *Gpc-B1* gene, resulting in a limited impact on yield.

The process of N remobilization from the leaves to the grain is not completely understood, but it is most probably regulated at leaves and ears rather than at the grain level (Barneix and Guitman 1993, Martre et al. 2003). The leaf proteins, mostly Rubisco, are hydrolysed to amino acids during senescence by proteolytic enzymes, and are then exported to the phloem (Hörtensteiner and Feller 2002). It has been shown that GPC is related to the soluble amino acids in the flag leaf (Barneix and Guitman 1993) and to the amount of amino acids exported to the phloem (Caputo et al. 2001). The present experiments showed for the first time that at anthesis the flag leaves of the RSL no. 68 plants have higher amino acid concentrations than LDN (Fig. 2a), specifically serine, alanine and threonine (Fig. 2b–d), and that this difference decreased rapidly during grain filling. This difference in amino acids concentration could explain the differences in N remobilization between the lines.

In the present experiments we could not detect differences in total N accumulation between the lines, suggesting no differences in total uptake. The soluble amino acids and protein

Table 4: Grain protein concentration, and chaff and straw N concentration at maturity when KNO_3 (16 mM) was eliminated from the supplied nutrient solution 10 days before anthesis in half of the plants (Expt 2)

Line	Grain protein (%DM)		Chaff N (%DM)		Straw N (%DM)	
	Continued (N)	Interrupted (N)	Continued (N)	Interrupted (N)	Continued (N)	Interrupted (N)
RSL no. 68	11.12	8.77	0.94	0.79	0.38	0.37
'Langdon'	10.07	8.20	0.74	0.80	0.43	0.35
Difference	1.05	0.57	0.2	–0.01	–0.05	0.02
Significance (P-values)						
Line		< 0.01		0.02		0.65
N		< 0.01		0.17		0.04
L $\times$ N		0.03		0.02		0.04
LSD		0.89		0.21		0.09

RSL no. 68, substitution line with gene *Gpc-B1*; DM, dry matter; N, nitrogen.

Fig. 2: Total soluble amino acids (a), alanine (b), serine (c) and threonine (d) from anthesis until 22 days after anthesis in the flag leaf of plants of recombinant substitution line (RSL) no. 68 and 'Langdon' (LDN) grown with continued 16 mM KNO_3 supply until maturity. Closed symbols: RSL no. 68; open symbols: LDN. Bars represent the mean $\pm$ SE (n = 3). The first sampling point coincides with anthesis

concentration in non-senescent leaves is dependent on the rate of nitrate uptake and reduction (Barneix et al. 1984, Caputo and Barneix 1997). When high soil N was available, RSL no. 68 showed a higher protein concentration in the grain than LDN across the experiments (18.3% increase, Table 3).

However, when the N supply was limited to fertilization with 1.0 mM KNO_3 the increase in grain protein content (%DM) in RSL no. 68 relative to LDN was reduced to 10.7% and was no longer significant (Table 3). This result suggests that *Gpc-B1* might be more efficient at higher levels of N.

When N supply was interrupted 10 days before anthesis (Table 4, Fig. 1) the relative increase in GPC (%DM) in RSL no. 68 relative to LDN was reduced from 10.7% to a non-significant 7.0%. This result suggests that, even though the major contribution to the differences in GPC was due to differences in N translocation efficiency, part of the observed differences might have resulted from increased N uptake during grain filling. Alternatively, the reduced increase might reflect the lower efficiency of *Gpc-B1* at lower levels of N.

Our results are in agreement with those from Deckard et al. (1996), who showed that the LDN (DIC-6B) chromosome substitution lines had a more efficient N remobilization and N uptake relative to LDN. However, it should be pointed out that the differences in N uptake (accumulation ratio) observed by Deckard et al. (1996) were significant only in one of the three LDN (DIC-6B) lines analysed, and the higher accumulation ratios were the result of a lower total dry weight (a variable parameter) rather than a higher total N in the three LDN (DIC-6B) lines compared with LDN (Deckard et al. 1996). A more precise determination of the contribution of N uptake relative to N remobilization to the final GPC will require additional experiments. Nitrate uptake by plant roots is a highly regulated process, mediated by transporters in the root cell plasma membrane (Crawford and Glass 1998), and at the whole plant level, by the plant N demand for growth (Glass et al. 2002).

The use of a RSL in this study with a smaller region of DIC chromosome 6B compared with the complete substitution indicates that the gene(s) responsible for the increase in GPC is(are) manifested in the flag leaves at anthesis by an increase of the total soluble amino acids, and that the effect is probably associated with the more efficient remobilization in RSL no. 68. These results correspond to plants grown in pots in a greenhouse. However results from field experiments performed in diverse environments of the United States (Joppa et al. 1997, Olmos et al. 2003), Canada (Humphreys et al. 1998) and Argentina (unpublished results) also showed a consistent increase in GPC in lines carrying the *Gpc-B1* allele from DIC. Because most of the additional N incorporated to the grain derives from a more efficient remobilization of N already present in the plant at anthesis, the incorporation of the *Gpc-B1* gene into commercial wheat cultivars has the potential to improve not only the grain quality but also the fertilizer use efficiency.

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