Theor Appl Genet (2009) 119:645–662
DOI 10.1007/s00122-009-1076-4

ORIGINAL PAPER

A quantitative genetic study for elucidating the contribution of glutamine synthetase, glutamate dehydrogenase and other nitrogen-related physiological traits to the agronomic performance of common wheat

Jean-Xavier Fontaine · Catherine Ravel · Karine Pageau · Emmanuel Heumez · Frédéric Dubois · Bertrand Hirel · Jacques Le Gouis

Received: 6 April 2009 / Accepted: 15 May 2009 / Published online: 10 June 2009

Abstract To better understand the genetic variability for nitrogen use efficiency in winter wheat is a necessity in the frame of the present economic and ecological context. The objective of this work was to investigate the role of the enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH), and other nitrogen (N)-related physiological traits in the control of agronomic performance in wheat. A quantitative genetics approach was developed using the Arche × Récital population of doubled haploid lines grown for 3 years in the field. GS and GDH activities, ammonium, amino acid and protein contents were measured at different stages of plant development in different organs after flowering. Significant genotypic effects were observed for all measured physiological and agronomical traits. Heading date was negatively correlated with ammonium, amino acid, protein contents and GS activity in the flag leaf lamina. Grain protein content was positively correlated with both ammonium and amino acid content, and to a lesser extent with soluble protein content and GS activity. A total of 148 quantitative trait loci (QTLs) were detected, 104 QTLs for physiological traits and 44 QTLs for agronomic traits. Twenty-six QTLs were detected for GDH activity spread over 13 chromosomes and 25 QTLs for GS activity spread over 12 chromosomes. We found only a co-localization between a QTL for GS activity and *GSe*, a structural gene encoding cytosolic GS on chromosome 4B. A coincidence between a QTL for GDH activity and a gene encoding GDH was also found on chromosome 2B. QTL regions combining both physiological and agronomical QTLs were mainly identified on linkage groups 2A, 2B, 2D, 5A, 5B and 5D. This approach allowed us to propose possible functions of physiological traits to explain the variation observed for agronomic traits including yield and its components.

Communicated by X. Xia.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-1076-4) contains supplementary material, which is available to authorized users.

J.-X. Fontaine · K. Pageau · F. Dubois
Faculté des sciences, UPJV EA3900 BioPI, Nitrogen Metabolism, 33 rue Saint Leu, 80039 Amiens Cedex, France

E. Heumez
Abiotic Stress and Differentiation of Cultivated Plants, INRA/USTL UMR 1281, BP50136, Chaussée Brunehaut, Estrées-Mons, 80203 Péronne Cedex, France

B. Hirel
Plant Nitrogen Nutrition, INRA UR 511, RD 10, Route de Saint-Cyr, 78026 Versailles Cedex, France

C. Ravel · J. Le Gouis (✉)
Genetics, Diversity and Ecophysiology of Cereals, INRA/UBP UMR 1095, Site de Crouël, 234 avenue du Brézet, 63100 Clermont-Ferrand, France
e-mail: jlegouis@clermont.inra.fr

Abbreviations

14DAF	14 days after flowering
28DAF	28 days after flowering
DHL	Doubled haploid line
DM	Dry matter
DW	Dry weight
FL	Flowering
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
LOD	Logarithm of the odd ratio
N	Nitrogen

NUE	Nitrogen use efficiency
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism

Abbreviations of the 16 traits studied

A	Flag leaf lamina area
AA	Amino acids
C	Carbon in flag leaf lamina
CN	Carbon nitrogen ratio in flag leaf lamina
DTH	Heading date
DW	Flag leaf lamina dry weight
FLS	Flag leaf lamina senescence
GDH_{DM}	Glutamate dehydrogenase activity expressed per dry matter
GDH_{PR}	Glutamate dehydrogenase activity expressed per protein
GPC	Grain protein content
GPS	Grain number per spike
GS_{DM}	Glutamine synthetase activity expressed per dry matter
GS_{PR}	Glutamine synthetase activity expressed per protein
N	Nitrogen content of the flag leaf lamina
NH_4^+	Ammonium
PROT	Protein content of the flag leaf
QPG	Quantity of protein per grain
TKW	Thousand kernel weight

Introduction

Nitrogen (N) fertilizers have been and are still extensively used to increase both grain yield and grain protein content (GPC) in cereals in general and bread wheat in particular. However, the current agricultural situation requires that growers must optimize the use of N fertilizers to avoid pollution, while maintaining reasonable profit margins. Therefore, selecting new crop varieties exhibiting improved nitrogen use efficiency (NUE), being defined as the yield of grain per unit of available N in the soil, and adapting agricultural practices to reduce the use of N fertilizers represents a challenge for both breeders and farmers (Hirel et al. 2007).

NUE is a complex polygenic trait. Therefore, quantitative genetic studies were undertaken to decipher the genetic basis of N uptake and utilization in several crops including barley (Kjaer and Jensen 1995), maize (Agrama et al. 1999; Bertin and Gallais 2001; Hirel et al. 2001; Gallais and Hirel 2004; Coque et al. 2006), rice (Obara et al. 2004; Lian et al. 2005) and wheat (An et al. 2006; Habash et al. 2007; Laperche et al. 2006a, b). Whichever the crop examined, these studies have allowed the identification of chromosomal regions that are either representative of the responsiveness of the plant to the level of N nutrition, or controlling plant root or shoot vegetative growth or yield through the efficiency of N uptake, assimilation and recycling. For example, experiments based on the discrimination of ^{15}N-natural abundance (Coque et al. 2006), or studying the impact of root architecture on NUE through the development of a simplified conceptual model (Laperche et al. 2007), are representative of the variety of approaches that can be used to identify the genetic determinism of NUE. Other studies have allowed the identification of putative candidate genes encoding enzymes involved in N assimilation and recycling (Lea and Azevedo 2007) that were present in chromosomal regions for which co-localizations between agronomic and physiological traits related to N metabolism were identified (Hirel et al. 2001; Obara et al. 2001). In particular, quantitative trait loci (QTLs) for grain yield and its components were found to be coincident with QTLs for glutamine synthetase (GS; EC 6.3.1.2) activity and loci for distinct cytosolic forms of the enzyme (GS1). The functional validation of the role of these different GS1 isoenzymes in the control of grain number or grain size was further validated using either reverse or forward genetic approach both in rice and maize (Obara et al. 2004; Martin et al. 2006). Since all of the N in a plant, whether derived initially from nitrate, ammonium ions, N fixation or generated by other reactions within the plant that release ammonium, is channeled through the reactions catalyzed by GS, it is somehow logical to find that in cereals the enzyme is likely to be a major check point controlling plant growth and productivity (Miflin and Habash 2002). Several other co-localizations between QTLs related to yield, physiological traits related to NUE and enzyme activities involved in the control of N assimilation and recycling were detected for nitrate reductase (NR) and glutamate dehydrogenase (GDH) in maize (Hirel et al. 2001; Gallais and Hirel 2004), cytosolic GS in wheat (Habash et al. 2007) and proteases in barley (Yang et al. 2004). Although the physiological function of the enzyme GDH is still not fully elucidated in higher plants (Dubois et al. 2003), there are strong lines of evidence that the enzyme serves as a major link between carbohydrate and amino acid metabolism in different organs or cell types (Miyashita and Good 2008) and is therefore a key enzyme controlling plant productivity (Lightfoot et al. 2007). However, in all cereals studied so far, neither the identification nor the functional validation of the candidate genes controlling metabolic major agronomic traits other than the genes encoding GS has yet been carried out.

This is particularly relevant to wheat, a plant of major economic importance, for which there is still no clear evidence that the activities of GS and possibly GDH are tightly linked to the control of grain production (Habash et al. 2001). Despite this, it is clear that in wheat GS

activity is one of the best physiological markers to depict the whole plant N status, irrespective of the developmental stage or N fertilization level. Moreover, in wheat the role of GS in controlling the plant N economy was further strengthened by the finding that there was a strong correlation between GS activity and the amount of N re-mobilized from shoots to the grain and grain yield. This was demonstrated by performing correlation studies using five cultivars exhibiting contrasted NUE (Kichey et al. 2006), or using a quantitative genetic approach to show that there was coincidence between QTLs for GS activity and grain N content (Habash et al. 2007).

There are also strong lines of evidence that the GDH enzyme may also be implicated in the control of cereal productivity at least in maize as demonstrated using a QTL (Gallais and Hirel 2004), or a forward genetic approach (Lightfoot et al. 2007). Although the physiological role of GDH still needs to be fully assessed, it is becoming more and more likely that the enzyme plays a pivotal role at the interface between carbon (C) and N metabolism through its metabolic activity or sensing function restricted to the vascular tissues. This pivotal role of GDH by virtue of its tissue-specific expression may be essential for regulating the plant C and N balance and thus resource allocation at particular stages of plant growth and development (Tercé-Laforgue et al. 2004a, b), or under stress conditions (Skopelitis et al. 2006; Miyashita and Good 2008).

Thus, the objective of this work was to further investigate, through a quantitative genetic approach, the role of the enzymes GS and GDH and other N-related physiological traits in the control of the agronomic performance of wheat. Whether the two enzymes can be used as key targets for improving NUE in relation to plant growth and productivity with emphasis on the grain filling period is discussed.

Materials and methods

Plant material and growth conditions

Two bread wheat (*Triticum aestivum* L.) cultivars, Arche and Récital, were chosen as parental lines to produce a DHL population because they exhibit a contrasting response to N deficiency with regard to both N uptake and N utilization efficiency. Arche is more tolerant to N deficiency than Récital as its grain yield is less affected by N deficiency (Le Gouis et al. 2000). The population used in the present work consisted of subsets of the original 241 double haploid lines previously studied by Laperche et al. (2006a, b, 2007).

Three separate experiments were carried out in 2004, 2006 and 2007 at Estrées-Mons INRA experimental station (Somme, Northern France, 03°00′N lat., 49°08′E long.). The soil, which is classified as a deep loam soil (Orthic Luvisol, FAO classification), contained an average of 190 g clay; 730 g silt; 52 g sand; and 19 g organic matter per kg, with a pH of 8.1. Soil characteristics were very similar over the 3 years of field trials. In the 2004 experiment, the population was composed of a subset of 137 DHLs that were sown on 29 October 2003 at a density of 240 plants m^{-2} in 6.5 m^2 plots and received 160 kg N ha^{-1}. The DHLs were distributed in four incomplete blocks, the two parental lines Arche and Récital being grown in each block as controls. In the 2006 experiment, the DHL population was composed of 166 lines sown on the 18 October 2005 at a density of 240 plants m^{-2} in 6.5 m^2 plots that received 180 kg N ha^{-1}. The DHLs were distributed in four incomplete blocks with 25 DHLs repeated twice, the two parental lines being grown in each block as controls. In the 2007 experiment, the population was composed of 221 DHLs. They were sown on the 17 October 2006 with two 2 m rows for each line at a density of 280 plants m^{-2} and received 180 kg N ha^{-1}. On the 3 years, N was applied as a liquid solution (15% uric acid, 7.5% ammonium, 7.5% nitrate) in three applications at mean tillering (GS21), stem elongation (GS30) and 2nd node (GS32) growth stages. One hundred and eight DHLs randomly selected within the DHL population were common to the 3 years of experimentation. Fungicide, herbicide and insecticide treatments were applied each year to achieve total control of parasites. To reduce lodging, a growth regulator was sprayed as 0.92 kg ha^{-1} chlormequat chloride + 2 g ha^{-1} imazaquine. These substances are known to have effects beside those on height; however, their use is necessary on unselected material grown at high level of N fertilization. Main meteorological data (rainfall, incoming radiation and mean temperature) are presented for each growing season in Table ESM S1.

Agronomic traits

On the 3 years, DTH, TKW, GPC, QPG and GPS were measured. Grain protein concentration was measured with a near infrared reflectance analyzers (NIRS 6500, Foss NIRSystems Inc., Laurel, MD, USA). In 2006, flag leaf senescence was scored visually 28DAF on a scale spanning from 0 to 100% with 0% representing no visible symptoms of senescence (green leaves) and 100% complete senescence (yellow leaves). In 2007, the leaf area was measured 14DAF on the flag leaves of five individual plants. The area of the flag leaf was measured using the software ImageJ® (http://rsb.info.nih.gov/ij/). Plant samples were harvested on the basis of the flowering date to avoid variations within the DHL population due to precocity.

Physiological traits

The plant material used for physiological studies was essentially the same as that used for agronomic studies. For measurements of the physiological markers (N metabolite contents and enzyme activities), five individual plants were selected for each parental line and for each DHL. In 2004, the flag leaf was harvested 14DAF and 28DAF, which corresponds approximately to the beginning and the end of the linear grain filling period, respectively (Robert et al. 2001). In 2006, the flag leaf samples were collected at the same two dates with an additional sampling at flowering (FL). In 2007, the flag leaf samples were harvested only 14DAF. For the five flag leaves, their sheaths and adjacent stem section were also collected. All the samples were collected twice. Fresh plant material was frozen in liquid N and then reduced to a homogeneous powder using a grinder (MM301, Retsch, Germany). The powder was stored at −80°C and used for all further physiological studies. Harvesting was performed between 1:00 and 3:00 p.m.

Metabolite extractions and analyses, enzymatic assays

Enzymes were extracted at 4°C. GS was measured according to the method of O'Neal and Joy (1973). Aminating NADH-dependent GDH was measured as described by Turano et al. (1996), except that the extraction buffer was the same as that used to measure GS activity. Soluble protein was determined using a commercially available kit (Coomassie Protein assay reagent; Bio-Rad, München, Germany) using bovine serum albumin (BSA) as a standard (Bradford 1976). Ammonium concentration was determined by the phenol-hypochlorite assay (Berthelot reaction) that provides reliable data for comparative studies when the concentration is low (Husted et al. 2000). Total free amino acids were determined by the Rosen colorimetric method, using glutamine as a standard (Rosen 1957). Elemental analysis (N; C ratio) was determined on 4–7 mg of dried plant material using a Flash EA 1112 Elemental Analyzer manufactured by Thermo Electron (Milano, Italy).

Candidate gene mapping

We used the genetic map of the Arche × Récital DHL population described in the study of Laperche et al. (2007), with a few additional SSR markers. In addition, the gene encoding *GSe* (Habash et al. 2007) was mapped on chromosome 4B using the gpw7026 microsatellite (Sourdille et al. 2004) that amplifies the region corresponding to the primers designed by Habash et al. (2007). The *GSr* gene was mapped on chromosome 4D (Habash et al. 2007) using the gpw6001 microsatellite marker that was designed using a GS genomic sequence of *Triticum tauschii*. The map consisted finally of 197 markers spread over 31 linkage groups with a total size of 3,285 cM and a mean distance between markers of 19.9 cM. The genetic map of the Récital × Renan population is described by Groos et al. (2003).

For *GDH* gene mapping, SNP was searched between the wheat varieties Arche, Renan and Récital using the method described by Ravel et al. (2006). Genome-specific primers designed from the sequences obtained by Boisson et al. (2005) that were used to amplify the homoeologous genes encoding NAD(H)-GDH are presented in Table 1. Genomic DNA was extracted from fresh leaves of 3-week-old seedlings from the parental lines (Arche, Renan and Récital), grown in greenhouse, as described by Tixier et al. (1998). Physical assignment of the DNA fragments corresponding to NAD(H)-GDH genes on the chromosomes was

Table 1 Specific oligonucleotides and PCR conditions used for amplification and sequencing of fragments from NAD(H)-GDH genes

Fragment name[a]	Primer sequence		Annealing temperature (°C) maximum[b]	Fragment size (bp)
	Forward (5′-3′)	Reverse (5′-3′)		
A1	GGGGGGATTTCGTCGG	GCGTTGGTGCCCATATCT	68	≈1,300
A2	GCTTGTTTCATTCATGGATTC	GTTCTGGTCTTGCTCCCTT	65	≈600
B1	GGAACTTCAAGCAGGCGG	GGAACTTTGAGTACTCATCCAGA	70	≈1,400
B2	GGAACTTCAAGCAGGCGG	GTAGAAAAAGGAAAACAACATACT	65	≈910
D1 D2[c]	GGAACTTCAAGCAGGCGG	CCATGCCATTGTCTGAAAG	65	≈1,200
D3	GCACAGGTATTTATTGTCCA	GTCAAGAACAGCCTTTTTG	65	≈1,150

[a] The first letter corresponds to the wheat genome (A, B or D). The following number indicates the fragment from the 5′ to the 3′ region of the gene. These fragments were assembled with sequences obtained by Boisson et al. (2005)

[b] Indicates the Tm maximum used for the touch-down program for amplification

[c] The quality of the forward sequence of the PCR product obtained with these primers was poor. Thus, this sequence was too short to overlap the reverse sequence. This led to two non-overlapping fragments called D1 and D2

performed using a set of Chinese Spring aneuploid lines (Sears 1966; Endo and Gill 1996). Cycling conditions in a touch-down experiment consisted of an initial denaturation at 94°C for 4 min, 10 cycles at 94°C for 1 min, Tm max for 1 min (Table 1), and in subsequent cycles decreasing the annealing temperature by 1°C/cycle, 72°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by a final extension at 72°C for 5 min. The Staden software (Staden et al. 2000) was used to assemble all the GDH sequences obtained either with specific primers or using the DNA fragments cloned by Boisson et al. (2005) and to search for SNP within these sequences. The SNPs for GDH sequences were genotyped using 155 DH lines of the Arche × Récital DHL population and the 144 lines of the DHL Récital × Renan population.

Statistical analyses

For each year, sampling dates and organs, means for the traits measured in the two parental lines and each DHL were calculated using a SAS proc GLM (SAS Institute Inc. 1999) and the lsmeans option. To achieve this, the following ANOVA model was used:

$$X_{ij} = \mu + G_i + E_j + e_{ij}$$

where X_{ij} is the value of genotype i in environment j, G_i the random genotype effect and E the random environmental effect. Each combination of year, sampling dates and organs were considered as being different environmental conditions. Broad sense heritability was calculated as $h^2 = (\sigma_g^2/(\sigma_g^2 + \sigma_e^2/E))$, where σ_g^2 and σ_e^2 are the genetic and environmental variances and E the number of environments. Heritability and its confidence interval were calculated using the SAS program developed by Holland et al. (2003). Coefficients of correlation and partial correlation were calculated with the proc CORR of SAS.

QTL detection

The mean of each line for each year was considered for QTL detection. For data measured on the flag lamina at 14DAF, a QTL detection was also performed on the mean over the 3 years. QTL analyses were carried out using the Unix version of QTL cartographer 1.17d (Basten et al. 1994, 2002). Model 6 was used to carry out composite interval mapping (CIM). The maximum cofactor number involved in model 6 was set at 5, and the window size was 10 cM. We used the 'experimentwise' threshold defined at the 10% error level. It was estimated from the 1,000 permutation test analyses (Churchill and Doerge 1994). In our case, the LOD threshold corresponded to 2.50. Confidence intervals were defined by a LOD drop-off of one unit.

Map projection

To simplify the analysis of QTLs, we decided to represent QTLs in regions. QTLs are considered to co-localize in the same region when their confidence intervals overlap. When QTLs for the same trait are detected several years in the same region, we represented only one QTL at a mean position and with a confidence interval encompassing all individual confidence intervals. To look for coincidences between QTL position and the localization of structural genes, we used the consensus wheat map of Somers et al. (2004) and the genetic-physical map relationships established by Sourdille et al. (2004).

Results

Sequence analysis and genes mapping

Ta.Gdh-A1, *-B1* and *-D1* correspond to chromosomes A, B and D homoeologous copies of the genes encoding NAD(H)-GDH genes, respectively. The PCR products for *Ta.Gdh-A1*, *-B1* and *-D1* were assigned to chromosome deletion bins 5AL-10-0.57-0.78, 5BL-16-0.79-1.00 and 5DL 5-0.76-1.00, respectively, in agreement with Habash et al. (2007), who assigned a GDH gene to 5BL based on comparative mapping with maize. The alignment for the A genome gave a total length of 2,784 bp including a gap between the 5′ contig (2,033 bp) comprising PCR fragments 1, 2 and 3 and the last one. The alignment for the B genome gave a unique contig of 3,111 bp. We obtained three contigs for the D genome that represent a sequence of 2,753 bp: the forward sequence of the fragment D1 (312 bp) did not overlap the second contig (955 bp), which comprises the reverse sequence of D1 and the sequence of fragments D2. The third contig contained the sequences of D3 and sequences obtained by Boisson et al. (2005) (1,504 bp).

The sequences were annotated using TA69991_4565 as a putative transcript. The structure contained eight exons with the coding sequence spanning from exon 1 to exon 8. Sequence for exon 1 was incomplete and starts downstream of the start codon. Sequences for exon 8 were also incomplete although they contained the stop codon.

Contrary to the introns, the length of the exons did not differ between each homoeologous gene. For the A genome, the GDH sequences from Arche and Récital were identical, while differences were found between these two varieties and the variety Renan (3 deletions of 4, 2 and 84 nucleotides and 2 mutations, A/G and C/T changes). The 4-nucleotide indel was present in the first intron, whereas the modifications in the nucleotide sequence were located in the second intron. For the B genome, a T/A mutation

was detected in exon 5 that substituted a glutamic acid for allele A to an aspartic acid for allele T in the amino acid sequence, the variety Récital having the T allele. For the D genome, sequences obtained for the three varieties were identical. The C/T substitution was used for the genotyping of the A genome and for mapping *Ta.Gdh-A1* using the Renan × Récital genetic map. The A/T substitution was used to map the *Ta.Ggh-B1* copy using both the Arche × Récital and the Renan × Récital genetic maps. In the Renan × Récital population, the C/T substitution was mapped on chromosome 5AL between the markers gwm271b and mta13 (Fig. 1). The marker for *Ta.Gdh-B1* was mapped in a position homoeologous to that of chromosome 5BL and located between markers gwm271 and fbb35. In the population Arche × Récital, this marker was the most distal marker of the chromosome 5BL, at 47.1 cM from the gwm217 marker.

Genotypic effect on physiological traits

Analyses of variance showed significant genotypic effects for all physiological traits (Table 2). Broad sense heritabilities ranged from 0.33 for ammonium content to 0.65 for amino acid content. Similarly, significant genotypic effects were also observed for agronomic traits (ESM S2), with heritabilities ranging from 0.92 for heading date to 0.71 for the number of grains per spike.

The changes observed for the physiological characters between the sampling dates were consistent over the 3 years of experimentation (Table 2) and with previously published data obtained on the two parental lines grown in the same location in 2003 (Kichey et al. 2005). In 2004 and 2006, we observed a decrease of leaf protein, amino acid, ammonium contents and GS_{DM} activity between 14DAF and 28DAF. GDH activity (expressed either on a DM or soluble protein basis) increased during the same period. In 2006, at flowering, leaf protein, amino acid and ammonium contents, GS_{DM} and GDH_{DM} activities were higher at flowering compared to 14DAF. In 2007, metabolite contents and enzyme activities were higher in the flag leaf lamina than in the flag leaf sheath, or the peduncle, except for amino acid content.

Correlations studies

Correlation between sampling dates

Physiological traits measured on the flag leaf lamina can be compared between 14DAF and 28DAF in 2004 and between FL, 14DAF and 28DAF in 2006. In 2004, only the correlation between 14DAF and 28DAF for ammonium content was significant (0.40***). In 2006, all the correlations were significant between 14DAF and 28 DAF (from 0.23** GDH_{PR} to 0.58*** for GS_{DM}). Between FL and 14DAF and between FL and 28DAF, all the correlations were significant (from 0.21** for GS_{PR} to 0.44*** for ammonium content) except for GDH_{DM} and GS_{DM}.

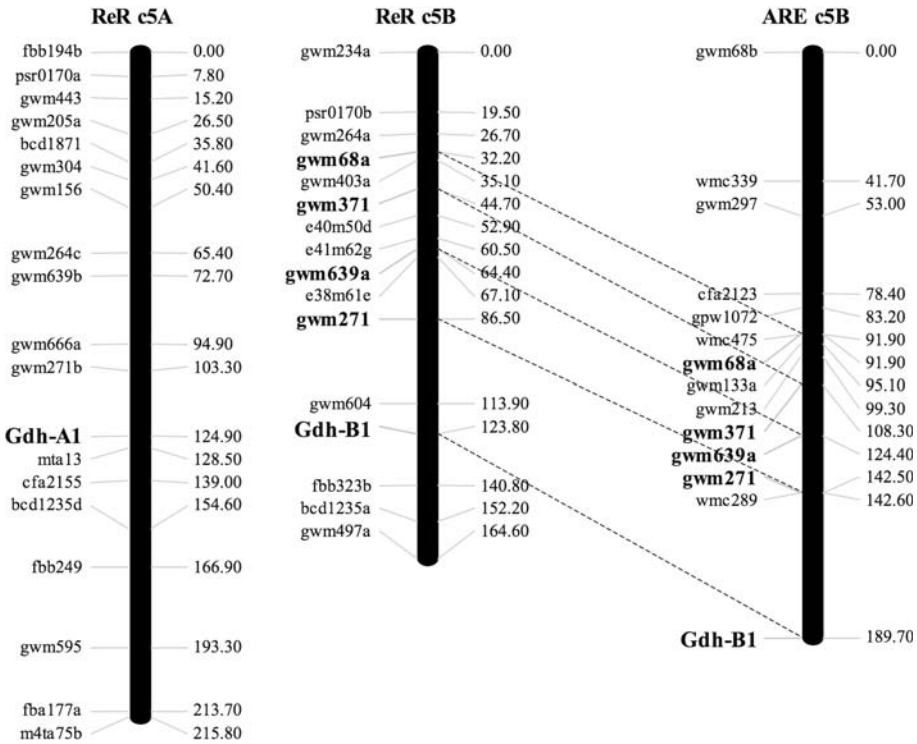

Fig. 1 Genetic map of chromosomes 5A and 5B of the Récital × Renan (ReR) population and of chromosome 5B of Arche × Récital (ARE) population showing the position of the NAD(H)-GDH (*Ta.Gdh1*) genes

Table 2 Significance of the genotype effect, coefficient of variation, broad sense heritability and mean values in each environment (combination of a year, sampling date and organ) for seven physiological traits measured on a DHL wheat population

	Prot (%)	GS_{PR} (μmol min^{-1} $gProt^{-1}$)	GDH_{PR} (μmol min^{-1} $gProt^{-1}$)	GS_{DM} (μmol min^{-1} gDW^{-1})	GDH_{DM} (μmol min^{-1} gDW^{-1})	AA (μmol gDW^{-1})	NH_4 (μmol gDW^{-1})
Genotype effect	0.0001	0.0050	0.0076	0.0001	0.0001	0.0001	0.0004
CV (%)	15.8	23.3	47.2	18.7	23.0	19.4	31.0
Heritability (%)	0.52 ± 0.05	0.41 ± 0.07	0.35 ± 0.07	0.53 ± 0.05	0.41 ± 0.07	0.65 ± 0.04	0.33 ± 0.08
2004-Lamina-14DAF	17.8b	132.6c	11.3d	23.1c	1.87d	84.4f	23.5a
2004-Lamina-28DAF	10.2c	138.2b	21.4b	13.9d	2.02c	57.5 g	14.7b
2006-Lamina-F	25.6a	110.4 g	12.1d	28.2a	3.02a	109.8c	14.2bc
2006-Lamina-14DAF	17.9b	127.8d	11.9d	22.8c	2.09c	89.1e	11.0d
2006-Lamina-28DAF	7.6e	109.1 g	35.5a	8.7e	2.43b	55.6 g	4.9e
2007-Lamina-14DAF	18.0b	144.2a	8.9e	25.9b	1.55e	121.3b	13.5c
2007-Sheath-14DAF	8.7d	121.5e	8.4e	10.6	0.72f	105.3d	10.1d
2007-Peduncle-14DAF	4.5f	117.4f	17.9c	5.3f	0.79f	147.4a	10.9d

For a given trait, two mean values that are followed by the same letter are not significantly different according to the standard Newman–Keuls multirange test ($p < 0.05$)

Correlation between organs

All the correlations between organs (including flag leaf lamina, sheath and peduncle) concerning the physiological traits measured in the 2007 experiment were significant. On average, the highest correlations were observed between peduncle and sheath (from 0.42*** for GDH_{DM} activity to 0.64*** for GS_{DM}). On average, the lowest correlations were obtained between flag leaf lamina and peduncle (from 0.15* for GDH_{DM} to 0.53*** for GS_{PR}).

Correlation between physiological traits

Correlations were computed between all physiological traits in the eight different combinations of years, sampling dates and organs, representing all together eight different environmental conditions. AA, NH_4^+ and PROT contents were positively correlated [on average r(AA, NH_4^+) = 0.35, r(AA, PROT) = 0.33, r(NH_4^+, PROT) = 0.35]. GS and GDH activities were also positively correlated in most environments [r(GS_{DM}, GDH_{DM}) = 0.32 and r(GS, GDH per protein) = 0.24]. GS_{DM} was positively correlated with amino acid content (0.32), ammonium content (0.31) and protein content (0.69). The correlation was much lower between GDH_{DM} and amino acid (0.04), ammonium (0.17) and protein contents (0.21). For GDH and GS activities, the correlations obtained with soluble protein content were consistent with those already obtained in a previously published work (Kichey et al. 2007), thus validating the reliability of our physiological approach over several years in different field trials.

Correlation between physiological and agronomic traits

Correlations between the physiological traits and agronomic traits were computed using the eight possible different combinations of years, sampling dates and organs (Table 3; ESM S3). Heading date was generally negatively correlated with ammonium, amino acid, protein contents and GS_{DM} activity in the flag leaf lamina. On the contrary, positive correlations were found for heading dates in 2007 with ammonium, and amino acid contents, both in the sheath and in the peduncle. GPC was in general positively correlated with both ammonium and amino acid content, and to a lesser extent with soluble protein content and GS_{DM} (2 combinations). The number of grains per spike was generally negatively correlated with the flag leaf amino acid content, and with GDH_{DM}. The significant positive correlations concerning the number of grains per spike (protein content, GS activities) were always reported for sheath and peduncle measurements in 2007. The TKW and QPG were generally positively correlated with the flag leaf lamina metabolite content and to GS activities.

Table 3 Correlations and partial correlations taking into account the possible interaction between the heading date and the physiological and agronomic traits measured on the Arche × Récital DHL wheat population

	PROT (%)	GS_{PR} (μmol min^{-1} $gProt^{-1}$)	GDH_{PR} (μmol min^{-1} $gProt^{-1}$)	GS_{DM} (μmol min^{-1} gDW^{-1})	GDH_{DM} (μmol min^{-1} gDW^{-1})	AA (μmol gDW^{-1})	NH_4 (μmol gDW^{-1})
Correlations							
GPC (%)	+0.26 (2)	+0.20 (1)		+0.29 (2)	+0.16 (1)	+0.30 (5)	+0.22 (4)
		−0.15 (1)	−0.22 (1)				
GPS	+0.19 (1)	+0.18 (2)		+0.19 (2)			
	−0.19 (1)				−0.23 (3)	−0.27 (3)	−0.18 (1)
TKW (g)	+0.24 (3)	+0.31 (2)		+0.30 (3)	+0.34 (1)	+0.20 (3)	+0.25 (2)
	−0.23 (1)		−0.17 (1)	−0.22 (1)	−0.22 (1)	−0.24 (1)	−0.16 (1)
QPG (mg)	+0.27 (4)	+0.31 (2)		+0.35 (3)	+0.36 (1)	+0.29 (5)	+0.28 (3)
	−0.15 (1)	−0.17 (1)	−0.17 (3)	−0.18 (1)	−0.23 (1)	−0.16 (1)	
DTH (days)	+0.50 (2)	+0.28 (3)	+0.30 (4)	+0.52 (3)	+0.26 (4)	+0.34 (2)	+0.35 (3)
	−0.46 (4)	−0.31 (1)	−0.20 (3)	−0.38 (5)	−0.35 (2)	−0.37 (6)	−0.34 (4)
Partial correlations							
GPC (%)	+0.20 (4)			+0.20 (3)	+0.14 (1)	+0.26 (6)	+0.24 (3)
			−0.18 (2)				
GPS		+0.18 (1)					
					−0.25 (3)	−0.25 (3)	
TKW (g)	+0.22 (3)	+0.29 (2)	+0.20 (1)	+0.23 (4)	+0.24 (2)	+0.25 (1)	+0.27 (1)
			−0.22 (1)		−0.18 (1)		
QPG (mg)	+0.21 (2)	+0.26 (2)	+0.16 (1)	+0.26 (3)	+0.24 (2)	+0.25 (5)	+0.30 (2)
			−0.20 (3)		−0.18 (2)		

Correlations were calculated within each of the eight available environments (combinations of years, sampling dates and organs; Tables ESM S3). Significant positive and negative correlations were then averaged separately and they appear on the top and bottom lines, respectively. Between parentheses is indicated the number of correlations used to calculate the average

The heading date was significantly correlated with most traits, which may explain why we frequently obtained significant correlations between physiological and agronomical traits. To overcome the effect of the heading date, partial correlations were computed (Table 3; ESM S3). This analysis confirmed that most of the significant correlations obtained were independent of the heading date, notably for those found between GPC, metabolite content and GS_{DM}. Although lower or higher for some traits, the positive correlation observed between physiological traits and agronomic traits was on average consistent with those already obtained with the varieties Arche and Récital (Kichey et al. 2007). Negative correlations were always determined between peduncle and sheath in 2007 with the TKW and in most cases with the GPC (5 out of 8).

QTL detection

A total of 157 QTLs were detected using the Arche × Récital population tested over 3 years (ESM S4, ESM S5). One hundred and thirteen QTLs were detected for physiological traits and 44 were detected for agronomic traits. Twenty-eight QTLs were detected for GDH activity spread over 14 chromosomes and 27 for GS activity spread over 12 chromosomes. All the QTL LOD and confidence intervals are indicated in Table ESM S4.

QTL detection for GDH and GS activities

Twenty-eight QTLs were detected for NADH-GDH activity. They were grouped in 19 regions spread over chromosomes 1A, 1D, 2A, 2B, 2D, 3B, 3D, 4B, 5A, 5B, 5D, 6A, 7A and 7B (ESM S4). For these 28 QTLs, the percentage of phenotypic variance (R^2) ranged from 5.0 to 18.2 %. The allele from the parental line Arche was favorable for 20 QTLs that were spread over all chromosomes except for chromosomes 2A, 2B and 5A2. The allele from the parental line Récital was favorable for 8 QTLs detected on chromosomes 2A, 2B, 4B, 5A1 and 5A2. The alleles from Arche and Récital were favorable for GDH activity in the peduncle and in the flag leaf lamina, respectively, in two QTL regions located on chromosomes 4B and 5A1.

Twenty-seven QTLs were detected for GS activity. They were grouped in 16 regions spread over chromosomes 1D, 2A, 2B, 2D, 3B, 3D, 4A, 4B, 5A, 5B, 5D and 7A (ESM S4). The R^2 ranged from 5.4 to 20.4%. The allele from Arche was favorable for the expression of this trait in eight QTLs detected on chromosomes 2A, 2B, 2D and 5D. Conversely, the allele from Récital was favorable for the expression of this trait for the remainder of the QTLs (19 out of 27) on chromosomes where QTLs for GS activity were detected, except on chromosome 5D. In addition, regions on chromosomes 2A, 2B and 2D included QTLs for which the positive alleles came from both parents. On linkage group 2A2, the two QTLs for flag leaf GS activity with the favorable alleles derived from either Arche or Récital were not located in a different position on the chromosome. We also found that the two QTLs for GS activity on the same region of chromosome 2B corresponded to the enzyme activity measured in different organs, and the allele derived from Récital increased GS activity in the flag leaf lamina, while the allele derived from Arche increased the enzyme activity both in the peduncle and in the flag leaf sheath. On linkage group 2D1, six QTLs for GS activity were detected. The allele from Récital increased the value of this trait in the flag leaf lamina in 2006 and the allele from Arche increased the value in the peduncle, flag leaf sheath and lamina in 2007.

Co-localization between GDH and GS activities and the corresponding structural genes

GDH genes were identified on homoeologous groups 1, 2 and 5. The wheat Expressed Sequence Tag (EST) BG314430 corresponded to a gene encoding GDH was mapped on homoeologous chromosomes 1B and 1D in deletion bins 1BL1-0.47-0.69 and 1DL2-0.41-1.00 (graingenes, http://wheat.pw.usda.gov/westsql/). The EST BE404371 was mapped on homoeologous group 2 in deletion bins C-2AL-0.85-1, 2BL2-0.36-0.50 and 2DL3-0.49-076 (graingenes, http://wheat.pw.usda.gov/westsql/). In this work, we mapped wheat GDH genes on chromosome 5A using the population Renan × Récital and on chromosome 5B using the populations Arche × Récital and Renan × Récital. Another gene encoding GDH was mapped on chromosome 5DL, 5-0.76-1.00 deletion bin. Seventeen QTLs for GDH activity were detected on homoeologous groups 1, 2 and 5. We used the consensus map of Somers et al. (2004) and the genetic-physical map relationships of Sourdille et al. (2004) to look for co-localization between QTL and GDH loci. Two QTLs were detected on group 1 (on linkage group 1A2 and 1D) but neither of them coincided with a GDH locus. Co-localizations were also not observed between the six QTLs detected on chromosomes 2A and 2D and a GDH locus. In contrast, the QTL region for GDH activity located on chromosome 2B coincided with a GDH locus. Finally, we did not find any co-localization between the GDH structural genes and the eight QTLs for GDH activity detected on homoeologous group 5.

Several members of the GS multigene family were located on homoeologous chromosome groups 2, 4 and 6. A wheat gene encoding plastidic GS (*GS2*) was previously mapped on chromosome 2AL, and two other monomorphic bands were assumed to be 2B and 2D homoeologs, located in the corresponding regions (Habash et al. 2007). *GSr*

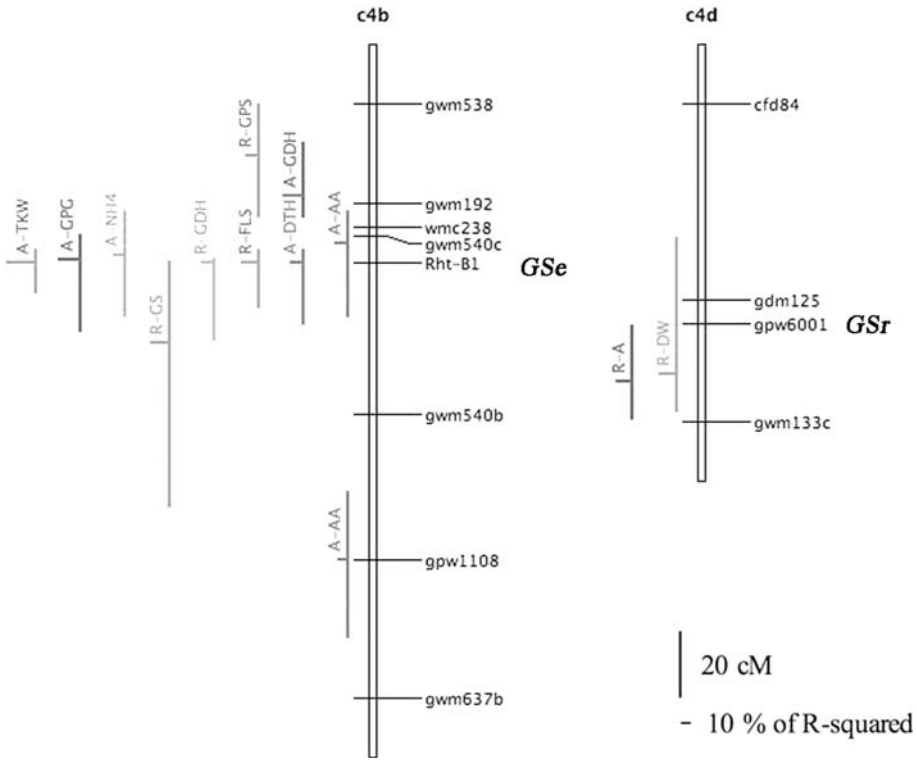

Fig. 2 Genetic map of chromosome 4B and 4D of Arche × Récital population showing the position of two GS genes (*GSe* and *GSr*, respectively) and of the clusters of QTLs for various physiological and agronomic traits. A QTL is represented by a *vertical bar* corresponding to its confidence interval (LOD drop-off of ±1 unit) and a *horizontal bar* positioned at the peak LOD value and proportional to the % of the phenotypic variance explained. The name of the QTL is composed of two parts: (1) the favorable allele (A for Arche and R for Récital) and (2) the abbreviated name of trait

encoding a cytosolic form of GS was mapped on chromosomes 4A and 4D (Habash et al. 2007). *GSe* encoding another cytosolic GS isoform was located on chromosome 4B (Habash et al. 2007). In each case, monomorphic bands were assumed to correspond to the other homoeologous copies of the same gene. In our study, *GSe* and *GSr* were mapped on chromosome 4B (cosegregating with the gene *Rht-B1*) and 4D (marker gpw6001), respectively. The other homoeologous copies were assumed to be located in the corresponding chromosomal regions. The third gene encoding cytosolic GS (GS1) was mapped on chromosome 6BL and a monomorphic homoeologous band was assigned to chromosome 6A and 6D (Habash et al. 2007). We detected 13 QTLs for GS activity located on 2 and 4 homoeologous groups. On chromosomes 4B, the confidence interval of the QTL for GS activity encompassed the gene *GSe* (Fig. 2). For the other regions on 2A, 2B, 2D and 4A, the QTL regions controlling the enzyme activity did not co-localize with those containing a GS locus. We did not detect any QTL for GS activity on homoeologous group 6 corresponding to the position of the *GS1* locus (Habash et al. 2007).

Analysis of chromosomal regions where QTLs for GDH or GS activities coincided with QTLs for agronomic and physiological traits

Only the QTLs for GS and GDH activities found in at least 2 years of experimentation will be considered. This concerns six regions on chromosomes 2A2, 2B1, 2D1, 5A1, 5B and 5D (Fig. 3).

On chromosome 2A2, the allele from Récital was favorable for GDH activity, whereas it was the allele from Arche that increased GS activity. In 2007, a QTL for GPC co-localized with the two QTLs for the two enzyme activities.

On linkage group 2B1, two QTLs for GS activity, at marker gwm429, were detected. The first one was detected in 2007 at 14DAF in the flag leaf sheath, the allele from Arche being favorable for the expression of this trait. The second one was detected in 2004 at 28DAF in flag leaf lamina, with a positive allele coming from Récital. Two QTLs for heading date positively controlled by the allele derived from Arche were detected in 2006 and 2007, in the vicinity of the same marker.

On linkage group 2D1, three QTLs for GDH activity and six QTLs for GS activity were detected near marker gwm484. In 2006, the alleles from Récital were favorable for GS activity, whereas in 2007 it was the allele from Arche. In both years, only the allele from Arche favored GDH activity. In the chromosomal region where QTLs for GS and GDH activity were detected, co-localization with other physiological traits in the flag leaf lamina such as soluble protein (two QTLs), amino acid content (four QTLs), flag leaf area and dry weight was also detected with the favorable allele for both traits coming from Récital. In contrast, total C content in the flag leaf lamina, soluble protein (one QTL) and amino

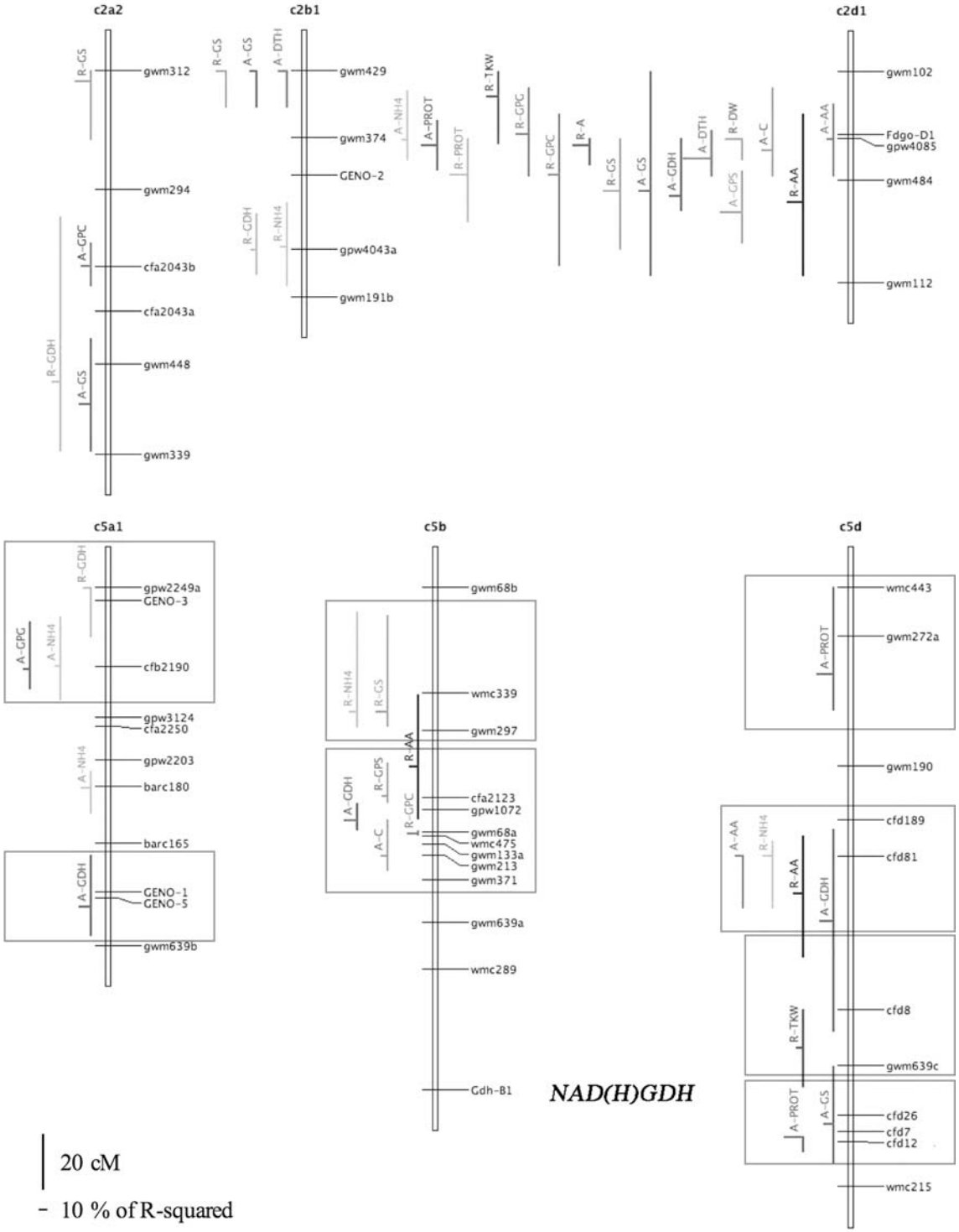

Fig. 3 Genetic map of chromosome 2A, 2B, 2D and 5A, 5B, 5D of the Arche × Récital population showing the position of the clusters of QTLs for various physiological and agronomic traits. A QTL is represented by a *vertical bar* corresponding to its confidence interval (LOD drop-off of ±1 unit) and a *horizontal bar* positioned at the peak LOD value and proportional to the % of the phenotypic variance explained. Genome regions with QTL clusters are represented by *rectangles*. The name of the QTL is composed of two parts: (1) the favorable allele (A for Arche and R for Récital) and (2) the abbreviated name of trait

acid content (one QTL) in the flag leaf sheaths were detected, with the allele from Arche controlling positively these three traits. QTLs for agronomic traits were also detected in this region. They include GPC, TKW and QPG with the positive allele coming from Récital. For DTH and GPS, the positive allele was derived from Arche.

On chromosome 5A1, two QTL regions were detected. In the first region, a QTL for peduncle GDH activity was detected in 2007, with the positive allele coming from Récital. This QTL overlapped with a QTL for ammonium content of the flag leaf lamina and a QTL for QPG with the positive allele coming from Arche. In the second region, a QTL for flag leaf lamina GDH activity was detected in 2004 and 2006 with the positive allele coming from Arche.

Two QTL regions were detected on chromosome 5B. In the first region, where Récital provided the favorable allele, we detected two QTL for GS enzymatic activity (one for peduncle in 2007 and one for lamina in the detection carried out on the mean over the 3 years) and one QTL for ammonium content in the flag leaf sheath. In the second region, three QTLs for flag leaf lamina GDH activity were detected in 2004 and 2006 with the positive allele coming from Arche. These QTLs co-localized with physiological traits including total C leaf content (positive allele from Arche), and amino acid content (positive allele from Récital). QTLs for agronomic traits in this region included a QTL for GPC detected in 2004 and one QTL for GPS detected in 2007. Récital carried the favorable allele in this region.

On chromosome 5D, four main QTL regions were identified. In the last region (at the end of the chromosome), two QTLs for GS activity were detected in the flag leaf lamina in 2004 and in the peduncle in 2007, which overlapped with one QTL for flag leaf protein content detected in 2004 (positive allele coming from Arche).

Discussion

Impact of environmental year effects

In this study, QTL detection for physiological traits including GS and GDH activities and N metabolites has been performed over 3 years on a range of wheat DHLs grown in the field. Such an experimental design has established that there is a year effect interacting with a genotypic effect, as shown by the large variation in the QTL detection across environments. Part of these variations may arise from the difference in total number of DH lines tested each year (from 137 in 2004 to 221 in 2007). However, more than a hundred randomly selected lines were common to the 3 years of experimentation. It is, therefore, unlikely that variations in the total number of lines tested each year had a significant impact on the correlations between traits. The finding that several QTLs for the different physiological traits were detected only in 1 year also highlighted this interaction. It is also known that the size of the population has an influence on the power of QTL detection and false discovery rate (e.g. Soller et al. 1976; Bernardo 2004). However, in our study, the number of QTLs detected each year was very similar (Table S4).

Therefore, it can be considered that the 3 years of experimentation correspond to three different environments. Despite this environmental variability, it was possible to identify four QTL regions for GDH activity and three regions for GS activity at least over 2 years of experimentation, thus indicating that they represent solid constitutive QTLs for physiological traits. The other regions may be classified as environmentally dependent QTLs that were detected depending on various external conditions such as climatic conditions, N mineralization and soil N availability. However, the nature of such a type of interaction is poorly documented (Quarrie et al. 2005; Coque et al. 2008) and will require further experimentation and analysis to establish, possibly through modeling approaches, the influence of these interactions on plant productivity (Reymond et al. 2004).

In contrast, traits related to yield appeared to be less sensitive to variation in the environmental conditions as shown by the heritabilities measured in this study. Our interpretation of such differences between agronomic and physiological traits may be explained by the fact that agronomic traits are somehow the result of several factors controlled by a large number of loci whose global effect over a long period of time allows yield to be stabilized within a certain threshold. In contrast, physiological traits taken individually may be subject to a large variability as a function of plant development and environmental conditions. This would allow over a short period of time the maximum flexibility of compensation and bypass mechanisms of given metabolic pathways due to the occurrence of multigene families and isoenzymes, in order to ensure optimal productivity. The variability of both the GS and GDH isoenzyme complement according to the developmental stage of the plant, the organ and the nutrition conditions perfectly illustrates the flexibility of the metabolic adaptation of a plant for optimal growth and development as a function of N fertilization, both qualitatively and quantitatively (Cren and Hirel 1999; Turano et al. 1997). In addition, the polyploid nature of a crop such as wheat gives an additional level of complexity, since interactions may occur between homoeologous genes as a function of external conditions to improve their fitness to a particular environment (Chantret et al. 2005; Chen 2007).

To illustrate the flexibility of the interaction genotype × environment in some QTLs, we observed, e.g., on chromosome 2D that the allele favorable for GDH activity originated from the parental line Récital in 2006, whereas in 2007 it was the allele derived from Arche. This result suggests that the regulation of the expression of the GS isoenzymes may be allelic as a function of external

conditions, especially when plants are grown in the field. Up to now, most of the studies aiming to identify QTLs for physiological traits have been conducted for 1 year only under controlled conditions, rather than under agronomic conditions. This is mostly because physiological traits are largely influenced by the environment and are difficult to measure accurately, which in turn limit both the size of the experimental design and the timing of harvesting of the various vegetative and reproductive organs for detailed metabolic profiling analysis (Hirel et al. 2001; Obara et al. 2001; Habash et al. 2007). The difficulty of clearly analyzing genotype × environment interactions was also encountered when QTLs for yield and components in wheat were examined using a combination of site and year treatments (Quarrie et al. 2005; Coque et al. 2008). In our work, although the experimentation was conducted over 3 years, most of the QTLs for physiological traits such as GS and GDH activities and most of the other metabolic traits were detected for only 1 year. Nevertheless, most of these QTL regions co-localized with QTLs for agronomic traits that had already been identified in the same population using an experimental design composed of 14 different environments (Laperche et al. 2007). For 19 QTL regions spread over 15 chromosomes, several coincidences were found between agronomical and physiological traits. These results indicate that, in the framework of future marker-assisted selection, these QTL regions must be taken into consideration as they reflect the flexibility of the plant to adapt to a particular environment. Although QTLs for GS and GDH activities co-localizing with agronomic traits appear to be important in the control of wheat plant productivity, we will consider in the following discussion only the physiological QTLs that have been detected for at least 2 years.

Compensatory metabolic and inter-organ regulatory mechanisms

Although the physiological function of the GS isoenzymes in several plant species (including those of wheat, Bernard et al. 2008) is well established, there are still some large uncertainties concerning GDH with respect to its metabolic and/or signaling function (Tercé-Laforgue et al. 2004b; Skopelitis et al. 2006). Despite this, it is noteworthy that the parental line Arche provided most of the favorable alleles for GDH activity (12 out of 18), while the favorable allele for GS activity originated from Récital (13 out of 15). This puzzling observation is in line with previous findings that when GS activity is impaired, it is accompanied by an increase in GDH activity (Harrison et al. 2003). Conversely, when GS is predominantly active in the cytosol during certain phases of plant development such as leaf N remobilization, GDH activity is often much lower (Tercé-Laforgue et al. 2004a). The finding that five QTLs for GS and GDH activities co-localized also supports the hypothesis that the two enzymes play a non-redundant role in the control of NUE (Melo-Oliveira et al. 1996) and that their regulation is tightly linked (Tercé-Laforgue et al. 2004a). Interestingly, for three out of these five QTLs, the same parent did not carry the same favorable allele for GS and GDH activities. In another cereal such as maize, GS and GDH activities are also likely to play a non-redundant function, since the corresponding QTLs were found to co-localize with distinct QTLs for yield-related traits (Gallais and Hirel 2004).

We also found that several QTLs for the two enzyme activities co-localized with the leaf ammonium content, which further support the hypothesis of a non-redundant function of GS and GDH with respect to the cellular concentration of the ion, which can be either a substrate or a product of the two enzymatic reactions. Moreover, in one of these QTL regions, co-localization with other physiological traits (AA, senescence) and agronomic traits (GPS, QPG, TKW) was also found. This confirms the tight relationship existing between tissue ammonium concentration and the relative activity of the GDH enzyme during the process of N assimilation and recycling, during which time cytosolic GS is also actively involved (Tercé-Laforgue et al. 2004a, b).

Interestingly, in some QTL regions, we found that the allelic control of GS and GDH enzyme activities was organ-dependent and influenced by the year of experimentation highlighting the flexibility of both enzymatic systems in different parts of the plant and as a function of environmental conditions.

Coincidences between QTL and structural candidate genes

In maize, out of six QTLs detected for leaf GS activity, three co-localized with genes encoding a cytosolic form of the enzyme (Hirel et al. 2001). In rice, Obara et al. (2001) found a co-localization between NAD(H)-GOGAT protein content and the corresponding structural gene. More recently, Habash et al. (2007) reported that out of the 22 QTLs detected for leaf GS activity in wheat, only two co-localized with GS structural genes. One was located on chromosome 2AL (plastidic *GS2*) and one on chromosome 4AL (cytosolic *GSr*).

In our study, although numerous QTLs were detected for both GS and GDH activities, we found for each enzyme only one co-localization with the corresponding structural gene. There is always the possibility that there are further copies of genes encoding either GS or GDH, which have not yet been identified. However, it is very unlikely that this is the case at least for GS, for which orthologs of the

four rice GS genes have been identified (Bernard et al. 2008). In the present study, using the DHL population also used for QTL detection, we mapped two genes encoding cytosolic GS (*GSe* on chromosome 4B and *GSr* on chromosome 4D) and one gene encoding GDH (*Gdh-B1*) on chromosome 5B. The putative locations of the other members of the GS and GDH families were determined using the consensus map of Somers et al. (2004) and the relationships existing between the physical and the genetic maps established by Sourdille et al. (2004). Based on the assumption that enough markers are common to the three genetic maps, it is likely that the position of the different genes may be estimated with sufficient accuracy. However, the uncertainty linked to both the projection method and the QTL confidence interval may lead to difficulties in determining either the presence or absence of coincidence. A direct mapping of the gene of interest on the genetic map established with the population used for QTL detection may however be difficult in wheat due to the lack of polymorphism of several structural genes encoding enzymes involved in N metabolism (Boisson et al. 2005).

On chromosome 4B, we found a coincidence between a QTL for GS activity and *GSe*, a gene encoding one of the GS isoenzymes. Using primers that did not discriminate between homoeoalleles, *GSe* has been found to be expressed in most wheat organs at rather a low level compared to other genes encoding cytosolic GS (Bernard et al. 2008). Interestingly, 17 QTLs for both agronomic and physiological traits had a confidence interval that encompassed the position of *GSe*. We observed that the dwarfing gene *Rht-B1* is also located in the same QTL region with no detectable segregation between *Rht-B1* and *Gse*; however, despite this, there was segregation between plant height and the whole plant N budget (Laperche et al. 2006a, b). This finding suggests that the function of *GSe* is not directly linked to a true N metabolic process independent of plant growth and development. The cultivar Récital that carries the dwarf allele also exhibited the highest GS and GDH activities. Four QTLs for GDH activity were also detected close to the dwarfing gene *Rht-B1* on linkage group 4B explaining from 5.9 to 16.6 % of the total variation of the physiological trait. This may be linked to a pleiotropic effect of the *Rht-B1* gene that is known to affect many characters besides plant height. However, in the Chinese Spring × SQ1 population that also segregated for *Rht-B1* with large impacts on agronomic characters associated with variation in grain number per spike (Quarrie et al. 2005), Habash et al. (2007) did not detect any QTLs for GS activity on chromosome 4B. In the present investigation, no QTLs for GS activity were identified on any chromosomes of linkage group 6, where the *GS1* locus is located. But the current ARE map does not cover the 6BL and 6DL regions where the GS1 locus lies. The absence of QTL for GS activity on group 6 is however in agreement with that previously published by Habash et al. (2007), which led these authors to suggest that the activity of both enzymes is mostly regulated at the post-transcriptional level.

Thirty QTLs for both agronomic and physiological traits were detected very close to each other on chromosome 2D1. The *Fdgo-D1* gene encoding ferredoxin-dependent glutamate synthase, another enzyme involved in conjunction with GS, in the synthesis of key N containing molecules, was mapped in this region (Boisson et al. 2005). Notably, the QTL region includes three QTLs for GDH activity that explains between 9.9 and 14.7 % of the total variation of the trait. A strong QTL for heading date was also detected over 3 years positioned at marker gpw4085, explaining from 25.7 to 29.3% of the total variation of the phenotypic trait. This region contains the *Ppd-D1* gene controlling photoperiod sensitivity (Hanocq et al. 2003). It is, therefore, possible that photoperiod sensitivity leading to later heading dates indirectly controls the expression of both physiological and grain quality traits. The occurrence of such a control has already been suggested by Habash et al. (2007) for the response to vernalization, which was controlled by the *Vrn-A1* and *Vrn-D1* genes in their population. We observed other co-localizations between QTL for heading date and physiological traits on linkage groups 2B1, 4A1 and 4B, always with opposite allelic effects. The negative correlation between heading date and a number of flag leaf lamina physiological traits (ammonium, amino acid, protein contents and GS activity) did not seem to be associated to a dilution effect at the leaf level, since both in our study and in the study of Habash et al. (2007) plants that had a delayed developmental cycle also had smaller flag leaves. However, these developmental characteristics could be related to a N dilution effect observed at the whole plant level (Justes et al. 1994). Plants exhibiting delayed development produce generally more biomass at flowering as it was observed in near-isogenic lines differing for photoperiod genes (González et al. 2005). This dilution effect is likely to occur in the model plant *Arabidospis*, if we consider the negative genetic correlations existing between biomass and several metabolites, including glutamine and phenylalanine (Meyer et al. 2007). After flowering, changes in both GS and GDH activities always occur during leaf aging (Habash et al. 2001; Kichey et al. 2005, 2006). Therefore, the negative correlation observed between the heading date and a number of physiological traits could be related to an interaction between the rate of plant development, leaf aging and N metabolism.

Co-localizations with agronomic traits

One of the most interesting results was the significant numbers of QTL coincidences on linkage groups 2D1, which were detected in the same genomic region at least over the 2 years of experiments. They included most of the agronomic and physiological traits evaluated in the present study except leaf ammonium content. In this region, we can divide these QTLs into two main groups with respect to the favorable allele provided by either of the two parental lines. The first group contained QTLs for GS and GDH activities, PROT and GPS with the favorable allele coming from Arche, which suggests that the reaction catalyzed by the two enzymes and the associated metabolic traits are important in the control of grain setting. Récital provided the favorable allele in the other group of QTLs including GS activity, TKW, GPC and QPG indicating that this chromosomal region is important in controlling both grain size and grain quality. Traits related to the availability of C and N resources such as the flag leaf area (A), flag leaf lamina C (C) and amino acid content (AA) further support the hypothesis that this region is tightly linked to the supply of assimilates to fill the grain. Two different QTLs for GS activity controlling either grain number or grain filling have already been identified and validated in maize (Martin et al. 2006), thus suggesting that the same regulatory control mechanism may occur in wheat through the activity of two different GS isoenzymes. However, as discussed earlier, the finding that the genes controlling photoperiod sensitivity and heading dates are located in the same region could be related to the complex interaction existing between metabolism and the rate of plant development as pointed out by Habash et al. (2007). Nevertheless, these authors also found in the 2D region QTLs related to grain weight per plant, grain N, leaf soluble protein content and GS activity, thus confirming the strength of our QTL detection in this chromosomal region.

Although less obvious than for the QTL regions detected on linkage group 2D, physiological and agronomic QTL regions were also identified in linkage groups 5A, 5B and 5D including those controlling GS activity, leaf amino acid content and traits related to grain yield and quality. QTLs with several physiological and agronomic traits were previously identified by Habash et al. (2007) on the same linkage groups, thus confirming that these genomic regions are important in regulating several steps of N metabolism likely to be also involved in controlling grain yield and grain quality.

Conclusion and perspectives

Our study shows that, in wheat, genetic variability for NUE in relation to yield and its components can be studied in a more targeted and integrated manner by means of a quantitative genetic approach using molecular markers and combining agronomic, metabolic and biochemical profiling studies. Our approach together with those recently developed in parallel on the same species (An et al. 2006; Habash et al. 2007) will be extensively developed in the future. This will allow the identification of new genes or *loci* involved in the regulation of N uptake, assimilation and recycling and their intricate relationships, which will assist the selection of genotypes which assimilate or remobilize N more efficiently in a given environment, or adapted to a particular environment. Moreover, our study has highlighted the fact that, in addition to environmental variation, organ specificities should be taken into account, as inter-organ compensatory mechanisms are likely to be involved in the control of N assimilation and partitioning during plant growth and development. The occurrence of such compensatory mechanisms has already been proposed since the physiological status of a particular plant section may reflect what is occurring at the whole plant level (Kichey et al. 2006). The results of the correlation studies obtained between organs in the present study further support this hypothesis.

In the future, it would also be interesting to complete our understanding of the roles of GS and GDH and additional N-related traits to the agronomic performance of common wheat by integrating the knowledge obtained on different genetic populations grown under diverse environmental conditions. Then, through map projection using common markers or reference maps, followed by meta-QTL analysis (Chardon et al. 2004; Hanocq et al. 2007; Coque et al. 2008), it will be possible to group the QTLs common to different mapping populations, for which physiological and agronomic QTLs have been associated. For the most promising regions, the construction of near-isogenic lines will enable the validation of the QTLs. It is, however, a rather long process for winter wheat starting from DH lines as it requires several backcrosses. Thus, there are only a few examples of such validation concerning agronomic characters in wheat (Quarrie et al. 2006). The recent development of genome sequencing and mapping projects in species with large and complex polyploid genomes as wheat (Paux et al. 2008) will be a valuable tool allowing the precise location of QTLs associated with NUE traits. In addition, genetic characterization of the identified QTLs through sequence analysis will certainly allow the identification of possible structural or regulatory genes controlling NUE at particular phases of plant growth and development according to different environmental conditions such as low and high N fertilization. Subsequently, the functional validation of these genes can be undertaken using the forward and reverse genetic approaches that have been successfully used to validate the role of GS for grain production both in rice and maize.

Acknowledgments We thank Dr. Dimah Habash and Professor Peter Lea for their valuable comments and suggestions on the manuscript. We thank Damien Bouthors, Dominique Brasseur and Jean-Pierre Noclerc for their technical assistance. Financial support by the Conseil Régional de Picardie (IBFBio project no. 2005.2) is greatly acknowledged.

References

Agrama HAS, Zakaria AG, Said FB, Tuinstra M (1999) Identification of quantitative trait loci for nitrogen use efficiency in maize. Mol Breed 5:187–195

An D, Su J, Liu Q, Zhu Y, Tong Y, Li J, Jing R, Li B, Li Z (2006) Mapping QTLs for nitrogen uptake in relation to the early growth of wheat (*Triticum aestivum* L.). Plant Soil 284:73–84

Basten CJ, Weir BS, Zeng ZB (1994) Zmap-a QTL cartographer. In: Proceedings of 5th congress on genetics applied to livestock production, vol 22, Guelph, Ontario, pp 65–66

Basten CJ, Weir BS, Zeng ZB (2002) QTL Cartographer Version 1.16

Bernard SM, Moller ALB, Dionisio G, Kichey T, Jahn TP, Dubois F, Baudo M, Lopes MS, Tercé-Laforgue T, Foyer CH, Parry MAJ, Forde BG, Araus JL, Hirel B, Schjoerring JK, Habash DZ (2008) Gene expression. cellular localisation and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.). Plant Mol Biol 67:89–105

Bernardo R (2004) What proportion of declared QTL in plants are false? Theor Appl Genet 109:419–424

Bertin P, Gallais A (2001) Genetic variation for nitrogen use efficiency in a set of recombinant inbred lines. II. QTL detection and coincidences. Maydica 46:53–68

Boisson M, Mondon K, Torney V, Nicot N, Laine AL, Bahrman N, Gouy A, Daniel-Vedele F, Hirel B, Sourdille P, Dardevet M, Ravel C, Le Gouis J (2005) Partial sequences of nitrogen metabolism genes in hexaploid wheat. Theor Appl Genet 110:932–940

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Chantret N, Salse J, Sabot F, Rahman S, Bellec A, Laubin B, Dubois I, Dossat C, Sourdille P, Joudrier P, Gautier M-F, Cattolico L, Beckert M, Aubourg S, Weissenbach J, Caboche M, Bernard M, Leroy P, Chalhoub B (2005) Molecular basis of evolutionary events that shaped the hardness locus in diploid and polyploid wheat species (*Triticum* and *Aegilops*). Plant Cell 17:1033–1045

Chardon F, Virlon B, Moreau L, Falque M, Joets J, Decousset L, Murigneux A, Charcosset A (2004) Genetic architecture of flowering time in maize as inferred from quantitative trait loci meta-analysis and synteny conservation with the rice genome. Genetics 168:2169–2185

Chen ZJ (2007) Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Annu Rev Plant Biol 58:377–406

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Coque M, Bertin P, Hirel B, Gallais A (2006) Genetic variation and QTLs for ^{15}N natural abundance in a set of maize recombinant inbred lines. Field Crops Res 97:310–321

Coque M, Martin A, Veyrieras J, Hirel B, Gallais A (2008) Genetic variation for N-remobilization and postsilking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. Theor Appl Genet 117:729–747

Cren M, Hirel B (1999) Glutamine synthetase in higher plants regulation of gene and protein expression from the organ to the cell. Plant Cell Physiol 40:1187–1193

Dubois F, Tercé-Laforgue T, Gonzalez-Moro MB, Estavillo JM, Sangwan R, Gallais A, Hirel B (2003) Glutamate dehydrogenase in plants: is there a new story for an old enzyme? Plant Physiol Biochem 41:565–576

Endo TR, Gill BS (1996) The deletion stock of common wheat. J Hered 87:295–307

Gallais A, Hirel B (2004) An approach to the genetics of nitrogen use efficiency in maize. J Exp Bot 55:295–306

González FG, Slafer GA, Miralles DJ (2005) Pre-anthesis development and number of fertile florets in wheat as affected by photoperiod sensitivity genes Ppd-D1 and ppd-B1. Euphytica 146:253–269

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

Habash DZ, Massiah AJ, Rong HL, Wallsgrove RM, Leigh RA (2001) The role of cytosolic glutamine synthetase in wheat. Ann Appl Biol 138:83–89

Habash DZ, Bernard S, Schondelmaier J, Weyen J, Quarrie SA (2007) The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield. Theor Appl Genet 114:403–419

Hanocq E, Sayers EJ, Niarquin M, Le Gouis J, Charmet G, Gervais L, Dedryver F, Duranton N, Marty N, Dufour P, Rousset M, Worland AJ (2003) A QTL analysis for earliness under field and controlled environment conditions in a bread wheat doubled-haploid population. In: Börner A, Snape J (eds) Proceedings of 12th international European wheat aneuploid cooperative, Norwich, UK, pp 57–59

Hanocq E, Laperche A, Jaminon O, Lainé A-L, Le Gouis J (2007) Most significant genome regions involved in the control of earliness traits in bread wheat, as revealed by QTL meta-analysis. Theor Appl Genet 114:569–584

Harrison J, de Crescenzo M-AP, Sené O, Hirel B (2003) Does lowering glutamine synthetase activity in nodules modify nitrogen metabolism and growth of *Lotus japonicus* L. Plant Physiol 133:253–262

Hirel B, Bertin P, Quilleré I, Bourdoncle W, Attagnant C, Dellay C, Gouy A, Cadiou S, Retailliau C, Falque M, Gallais A (2001) Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. Plant Physiol 125:1258–1270

Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58:2369–2387

Holland JB, Nyquist W, Cervantes-Martinez CT (2003) Estimating and interpreting heritability for plant breeding: an update. Plant Breed Rev 22:9–111

Husted S, Hebbern CA, Mattsson M, Schjoerring JK (2000) A critical experimental evaluation of methods for determination of NH_4^+ in plant tissue, xylem sap and apoplastic fluid. Physiol Plant 109:167–179

Justes E, Mary B, Meynard J-M, Machet J-M, Thelier-Huches L (1994) Determination of a critical nitrogen dilution curve for winter wheat crops. Ann Bot 74:397–407

Kichey T, Le Gouis J, Sangwan B, Hirel B, Dubois F (2005) Changes in the cellular and subcellular localization of glutamine synthetase and glutamate dehydrogenase during flag leaf senescence in wheat (*Triticum aestivum* L.). Plant Cell Physiol 46:964–974

Kichey T, Heumez E, Pocholle D, Pageau K, Vanacker H, Dubois F, Le Gouis J, Hirel B (2006) Combined agronomic and physiological aspects of nitrogen management in wheat highlight a central role for glutamine synthetase. New Phytol 169:265–278

Kichey T, Hirel B, Heumez E, Dubois F, Le Gouis J (2007) In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and

remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. Field Crops Res 102:22–32

Kjaer B, Jensen J (1995) The inheritance of nitrogen and phosphorus content in barley analysed by genetic markers. Hereditas 123:109–119

Laperche A, Brancourt-Hulmel M, Heumez E, Gardet O, Le Gouis J (2006a) Estimation of genetic parameters of a DH wheat population grown at different N stress levels characterized by probe genotypes. Theor Appl Genet 112:797–807

Laperche A, Devienne-Barret F, Maury O, Le Gouis J, Ney B (2006b) A simplified conceptual model of carbon/nitrogen functioning for QTL analysis of winter wheat adaptation to nitrogen deficiency. Theor Appl Genet 113:1131–1146

Laperche A, Brancourt-Hulmel M, Heumez E, Gardet O, Hanocq E, Devienne-Barret F, Le Gouis J (2007) Using genotype × nitrogen interaction variables to evaluate the QTL involved in wheat tolerance to nitrogen constraints. Theor Appl Genet 115:399–415

Le Gouis J, Béghin D, Heumez E, Pluchard P (2000) Genetic differences for nitrogen uptake and nitrogen utilisation efficiencies in winter wheat. Eur J Agron 12:163–173

Lea PJ, Azevedo RA (2007) Nitrogen use efficiency. 2. Amino acid metabolism. Ann Appl Biol 151:269–275

Lian X, Xing Y, Yan H, Xu C, Li X, Zhang Q (2005) QTLs for low nitrogen tolerance at seedling stage identified using a recombinant inbred line population derived from an elite rice hybrid. Theor Appl Genet 112:85–96

Lightfoot DA, Mungur R, Ameziane R, Nolte S, Long L, Bernhard K, Colter A, Jones K, Iqbal M, Varsa E, Young B (2007) Improved drought tolerance of transgenic *Zea mays* plants that express the glutamate dehydrogenase gene (*gdhA*) of *E. coli*. Euphytica 156:103–116

Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C, Dubois F, Balliau T, Valot B, Davanture M, Tercé-Laforgue T, Quilleré I, Coque M, Gallais A, Gonzalez-Moro MB, Bethencourt L, Habash DZ, Lea PJ, Charcosset A, Perez P, Murigneux A, Sakakibara H, Edwards KJ, Hirel B (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18:3252–3274

Melo-Oliveira R, Oliveira IC, Coruzzi GM (1996) Arabidopsis mutant analysis and gene regulation define a non-redundant role for glutamate dehydrogenase in nitrogen assimilation. Proc Natl Acad Sci USA 93:4718–4723

Meyer RC, Steinfath M, Lisec J, Becher M, Witucka-Wall H, Törjék O, Fiehn O, Eckart A, Willmitzer L, Selbig J, Altman T (2007) The metabolic signature related to high plant growth rate in *Arabidospis thaliana*. Proc Natl Acad Sci USA 104:4759–4764

Miflin BJ, Habash DZ (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J Exp Bot 53:979–987

Miyashita Y, Good AG (2008) NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidospis thaliana* during dark-induced carbon starvation. J Exp Bot 59:667–680

O'Neal D, Joy KW (1973) Glutamine synthetase of pea leaves. I. Purification, stabilization, and pH optima. Arch Biochem Biophys 159:113–122

Obara M, Kajiura M, Fukuta Y, Yano M, Hayashi M, Yamaya T, Sato T (2001) Mapping of QTLs associated with cytosolic glutamine synthetase and NADH-glutamate synthase in rice (*Oryza sativa* L.). J Exp Bot 52:1209–1217

Obara M, Sato T, Sasaki S, Kashiba K, Nagano A, Nakamura I, Ebitani T, Yano M, Yamaya T (2004) Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice. Theor Appl Genet 110:1–11

Paux E, Sourdille P, Salse J, Saintenac C, Choulet F, Leroy P, Korol A, Michalak M, Kianian S, Spielmeyer W, Lagudah E, Somers D, Kilian A, Alaux M, Vautrin S, Bergès H, Eversole K, Appels R, Safar J, Simkova H, Dolezel J, Bernard M, Feuillet C (2008) A physical map of the 1-Gigabase bread wheat chromosome 3B. Science 322:101–104

Quarrie SA, Steed A, Calestani C, Semikhodskii A, Lebreton C, Chinoy C, Steele N, Pljevljakusic D, Waterman E, Weyen J, Schondelmaier J, Habash DZ, Farmer P, Saker L, Clarkson DT, Abugalieva A, Yessimbekova M, Turuspekov Y, Abugalieva S, Tuberosa R, Sanguineti MC, Hollington PA, Aragués R, Royo A, Dodig D (2005) A high-density genetic map of hexaploid wheat (*Triticum aestivum* L.) from the cross Chinese Spring × SQ1 and its use to compare QTLs for grain yield across a range of environments. Theor Appl Genet 110:865–880

Quarrie SA, Quarrie SP, Radosevic R, Ransic D, Kaminska A, Barnes JD, Leverington M, Ceoloni C, Dodig D (2006) Dissecting a wheat QTL for yield present in a range of environments: from the QTL to candidate genes. J Exp Bot 57:2627–2637

Ravel C, Praud S, Murigneux A, Canaguier A, Sapet F, Samson D, Balfourier F, Dufour P, Chalhoub B, Brunel D, Beckert M, Charmet G (2006) Single-nucleotide polymorphism frequency in a set of selected lines of bread wheat (*Triticum aestivum* L.). Genome 49:1131–1139

Reymond M, Muller B, Tardieu F (2004) Dealing with the genotype × environment interaction via a modelling approach: a comparison of QTL of maize leaf length or width with QTLs of model parameters. J Exp Bot 55:2461–2472

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

Rosen H (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67:10–15

SAS Institute Inc. (1999) SAS/STAT user's guide, version 8. SAS Institute Inc., Cary, NC

Sears ER (1966) Nullisomic–tetrasomic combinations in hexaploid wheat. In: Riley R, Lewis KR (eds) Chromosome manipulations and plant genetics. Oliver and Boyd, Edinburgh, London, pp 29–45

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 18:2767–2781

Soller M, Genizi A, Brody T (1976) On the power of experimental designs for the detection of linkage between marker loci and quantitative loci in crosses between inbred lines. Theor Appl Genet 47:35–59

Somers DJ, Isaac P, Edwards K (2004) A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). Theor Appl Genet 109:1105–1114

Sourdille P, Singh S, Cadalen T, Brown-Guedira GL, Gay G, Qi L, Gill BS, Dufour P, Murigneux A, Bernard M (2004) Microsatellite-based deletion bin system for the establishment of genetic-physical map relationships in wheat (*Triticum aestivum* L.). Funct Integr Genom 4:12–25

Staden R, Beal KF, Bonfield JK (2000) The Staden package, 1998. Methods Mol Biol 132:115–130

Tercé-Laforgue T, Mäck G, Hirel B (2004a) New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown under different nitrogen regimes. Physiol Plant 120:220–228

Tercé-Laforgue T, Dubois F, Ferrario-Méry S, de Crecenzo MAP, Sangwan R, Hirel B (2004b) Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion

cells when ammonia is provided either externally or released during photorespiration. Plant Physiol 136:4308–4317

Tixier MH, Sourdille P, Charmet G, Gay G, Jaby C, Cadalen T, Bernard S, Nicolas P, Bernard M (1998) Detection of QTLs for crossability in wheat using a doubled-haploid population. Theor Appl Genet 97:1076–1082

Turano FJ, Dashner R, Upadhyaya A, Caldwell CR (1996) Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. Plant Physiol 112:1357–1364

Turano FJ, Thakkar SS, Fang T, Weisemann JM (1997) Characterization and expression of NAD(H)-dependent glutamate dehydrogenase genes in Arabidopsis. Plant Physiol 113:1329–1341

Yang L, Mickelson S, See D, Blake TK, Fischer AM (2004) Genetic analysis of the function of major leaf proteases in barley (*Hordeum vulgare* L.) nitrogen remobilization. J Exp Bot 55:2607–2616