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An approach to the genetics of nitrogen use efficiency in maize

A. Gallais[1,*] and B. Hirel[2]

[1] *Station de Génétique Végétale, INRA-UPS-INAPG, Ferme du Moulon, 91190 Gif/Yvette, France*

[2] *Unité de Nutrition Azotée des Plantes, INRA route de St Cyr, 78026 Versailles Cedex, France*

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Abstract

To study the genetic variability and the genetic basis of nitrogen (N) use efficiency in maize, a set of recombinant inbred lines crossed with a tester was studied at low input (N–) and high input (N+) for grain yield and its components, grain protein content, and post-anthesis nitrogen uptake and remobilization. Other physiological traits, such as nitrate content, nitrate reductase, glutamine synthetase (GS), and glutamate dehydrogenase activities were studied at the level of the lines. Genotype×nitrogen (G×N) interaction was significant for yield and explained by variation in kernel number. In N–, N-uptake, the nitrogen nutrition index, and GS activity in the vegetative stage were positively correlated with grain yield, whereas leaf senescence was negatively correlated. Whatever N-input, post-anthesis N-uptake was highly negatively related to N-remobilization. As a whole, genetic variability was expressed differently in N+ and N–. This was confirmed by the detection of QTLs. More QTLs were detected in N+ than in N– for traits of vegetative development, N-uptake, and grain yield and its components, whereas it was the reverse for grain protein content and N-utilization efficiency. Several coincidences between genes encoding for enzymes of N metabolism and QTLs for the traits studied were observed. In particular, coincidences in three chromosome regions of QTLs for yield and N-remobilization, QTLs for GS activity and a gene encoding cytosolic GS were observed. This may have a physiological meaning. The GS locus on chromosome 5 appears to be a good candidate gene which can, at least partially, explain the variation in nitrogen use efficiency.

Key words: Glutamate dehydrogenase, glutamine synthetase, maize, nitrate content, nitrate reductase, nitrogen uptake, nitrogen use efficiency, remobilization.

Introduction

The absorption of nitrogen by plants plays an important role in their growth. Consequently, nitrogen fertilization has been a powerful tool for increasing the yield of cultivated plants, such as cereals. Nowadays, both to avoid pollution by nitrates and to maintain a sufficient profit margin, farmers have to reduce the use of nitrogen fertilizer. These objectives can be met through efficient farming techniques, but also by using plant varieties that have a better nitrogen use efficiency (NUE). The development of such varieties will be more efficient with a better knowledge of the physiological and genetic bases of NUE.

For grain maize NUE has been defined as the grain yield per unit of nitrogen available from the soil, including nitrogen fertilizer (Moll *et al*., 1987). It is the product of nitrogen uptake efficiency (N-uptake/N from soil), and nitrogen utilization efficiency (NUtE, i.e. yield/N-uptake). For NUE, genetic variability and genotype×nitrogen fertilization level interactions reflecting differences in responsiveness have been observed in several studies on maize (Beauchamp *et al*., 1976; Pollmer *et al*., 1979; Balko and Russell, 1980*a*; Reed *et al*., 1980; Russell, 1984; Moll *et al*., 1987; Landbeck, 1995; Bertin and Gallais, 2000). In addition, it has been found that correlations among various agronomic traits such as grain protein yield and its components are very different according to the level of nitrogen fertilization (Balko and Russell, 1980*b*; Di Fonzo *et al*., 1982; Rizzi *et al*., 1993; Bertin and Gallais, 2000). At high N-input, genetic variation in NUE was explained

* To whom correspondence should be addressed. Fax: +33 1 6933 2340. E-mail: Gallais@moulon.inra.fr

by variation in N-uptake, whereas at low N-input, NUE variability was mainly due to differences in nitrogen utilization efficiency. This suggests that the limiting steps in N-assimilation may be different when plants are grown under high or low levels of nitrogen fertilization.

Differences in N-uptake are likely to be related to the quantity and the quality of the root system. However, while experiments have shown a variability in the architecture of the root system (Hébert *et al*., 1992), it has not been related to variability in N-uptake. It remains therefore to find which traits control N-uptake. Nitrogen uptake at silking determines kernel number (Di Fonzo *et al*., 1982; Muruli and Paulsen, 1981; Sherrard *et al*., 1986). This can be explained by the high demand for nitrogen of embryos just after fertilization (Czyzewicz and Below, 1994). As a consequence, kernel number is very susceptible to a N-stress in comparison to kernel weight (Uhart and Andrade, 1995; Reed *et al*., 1988; Below, 1995). Furthermore, Di Fonzo *et al*. (1982) and Moll *et al*. (1987) have shown that the role of post-anthesis N-uptake in grain filling can be related to leaf senescence. Indeed, by increasing leaf longevity, thus prolonging the capacity of the plant to absorb mineral nitrogen, better yields were obtained in modern hybrids (Tollenaar, 1991; Ma and Dwyer, 1998; Racjan and Tollenaar, 1999*a*, *b*).

Although the agronomic studies on maize have demonstrated that there is genetic variability for NUE, present knowledge on the corresponding physiological traits is still limited. Several studies have attempted to assign a role for the different proteins and enzymes involved in mineral N-uptake, assimilation and recycling (Lea and Ireland, 1999). However, most of these approaches involving either whole plant physiology or the use of transgenic plants or mutants have not contributed to an understanding of the physiological and genetic basis of NUE in a more integrated manner.

Nowadays, quantitative genetic studies associated with the use of molecular markers may be a way of identifying Quantitative Trait Loci (QTL) involved in the genetic variation of a complex character such as NUE. Coincidences between QTLs for agronomic traits and QTLs for physiological traits related to NUE will give a physiological meaning to the QTLs for the agronomic traits. In addition, if there is co-mapping with genes encoding enzymes involved in N-assimilation, this will give a genetic meaning to these QTLs, thus allowing the identification of so called 'candidate' genes, i.e. genes for which allelic variation could be responsible for a part of the observed variation. It is also possible to identify new genes as potential candidates by the studies of gene expression. Having identified a good candidate gene, to validate it, the favourable allele can be transferred to a genotype with an unfavourable allele to test whether there is the expected effect. Although QTLs for adaptation to environmental stresses such as drought resistance (Agrama and Moussa, 1996; Ribaut *et al*., 1997; Tuberosa *et al*., 1998), and tolerance to phosphorus stress (Reiter *et al*., 1991) have already been detected in maize, few studies have been published on the identification of QTLs for adaptation to low N-input. Agrama *et al*. (1999) found common and specific QTLs for high and low N-input whereas Bertin and Gallais (2001) clearly showed that QTLs detected at high N-input were different from those detected at low N-input. With the same material as Bertin and Gallais, coincidences between QTLs for agronomic traits and QTLs for some physiological traits related to nitrogen assimilation were studied by Hirel *et al*. (2001). The observed coincidences of QTLs for yield and kernel weight with QTLs for glutamine synthetase (GS) activity led to the proposal that GS plays an important role in the determination of yield.

In this paper, some of the results of Bertin and Gallais (2000, 2001) and Hirel *et al*. (2001) are reviewed and discussed, with emphasis being given to traits related to NUE and to coincidences between QTLs for agronomic and QTLs for some physiological traits as well as to their coincidence with genes involved in nitrogen metabolism. In addition, new results are given for N-remobilization and post-anthesis N-uptake.

Materials and methods

Plant material and experiments

For QTL detection it is necessary to use a material where correlation among non-homologous genes can only be due to physical linkage. For the field studies, a random set of 99 recombinant inbred lines (RIL) has been used from 145 that were derived from the cross between a French flint and early line (F2) and an iodent late line. For the agronomic study, they were crossed to an unrelated tester (F252) in order to study NUE at the hybrid level, the chosen tester combining well with both parents. Such a population was chosen because its two parents are highly complementary in terms of grain productivity, i.e. heterotic. Furthermore, the agronomic study of the parents revealed differences in their NUE. As described by Bertin (1997) and Bertin and Gallais (2000), two N-levels were used: a normal nitrogen level (N+) with 175 kg N ha^{-1} applied at the time of sowing and no nitrogen fertilization (N–), all the N being supplied by the soil, a supply estimated to be at least 50–60 kg ha^{-1}. The objective was to reduce the yield by about 40%. The experiment was developed in two consecutive years, 1994 and 1995, in the same location ('Le Moulon' Plant Breeding Station) with two-row plots of 5.2 m in length and 0.80 m between rows and an average of 95 000 plants ha^{-1}. Several traits were measured at flowering and grain harvest. In the present study, traits used for both correlation studies and QTL detection were grain yield and its components (kernel number $plant^{-1}$ and kernel weight, i.e. thousand kernel weight), grain nitrogen content and grain nitrogen yield. For more details about the procedures used to measure these agronomic traits, see Bertin and Gallais (2000). Furthermore, traits related to NUE were considered in particular. At flowering they are nitrogen uptake, nitrogen content, and nitrogen nutrition index (NNI, see below), and nitrogen uptake in the whole plant (aerial part). At grain harvest they are nitrogen uptake allocated to the grain and the stover, grain and stover nitrogen content, nitrogen harvest index, and apparent N-remobilization from the aerial biomass, the leaf blade and the stem to the grain derived

from N-quantity in the corresponding organ at flowering minus N-quantity at maturity. NNI is defined as the ratio of observed N-content to a critical N-content corresponding to the minimum N-content allowing the maximum growth (Lemaire and Gastal, 1997). This index allows a correction for the dilution effect.

Due to the cost of the studies, physiological studies were developed (Hirel *et al*., 2001) on only 77 RILs randomly chosen from the 99 lines used to perform the agronomic studies. Plants were grown in hydroponic culture with a nutrient solution containing 1 mM NO_3^- which corresponds to a high N-input. The plants were harvested at the 6–7 leaf stage and separated into shoots, stems and roots. The experiment was replicated over two consecutive years (1998 and 1999). Leaf nitrate content, leaf NADH-nitrate reductase (NR) activity, and leaf GS activity were selected as representative marker metabolites and enzyme activities of primary N-assimilation in young developing plants. Furthermore, in 2000, from the set of 99 RILs evaluated in the field at two nitrogen levels, GS and glutamate dehydrogenase (GDH) activities were studied in the leaf below the ear, which corresponds to one of the main origins of carbon and nitrogen assimilates exported to the grain in adult plants (Prioul and Schwebel-Dugué, 1992).

Gene mapping

For the mapping of QTLs, the RFLP genetic map published by Causse *et al*. (1996) containing 152 marker loci corresponding to a total map length of 1813 cM was used. The mean interval between two markers varies from 8 cM to 18 cM. To identify some candidate genes, specific genes involved in N-metabolism were also mapped: a high affinity nitrate transporter, *NTR1* (Trueman *et al*., 1996); two NADH-NR, *NR1* and *NR2* (Long *et al*., 1992); nitrite reductase, *NiR* (Lahners *et al*., 1988); glutamate dehydrogenase, *GDH1* (Sakakibara *et al*., 1995); four cytosolic GS, *gln1*, *gln2*, *gln3*, and *gln4*, plastidic GS, *gln5* (Sakakibara *et al*., 1992*a*); and asparagine synthetase (*AS1* and *AS2*) (Chevalier *et al*., 1996) which was located on two loci**.** The loci corresponding to *gln1*, *2*, *3*, *4*, and *5* correspond to the GS genes named pGS122, pGS134, pGS107, pGS112, and pGS202 by Sakakibara *et al*. (1992*a*) and GS1-1, GS1-2, GS1-4, GS1-3, and GS2 by Li *et al*. (1993).

QTLs were detected using the PlabQTL software (Utz and Melchinger, 1995) following simple interval mapping. Only QTLs with a LOD score greater than 2 were considered. To take into account the error in location, location of a QTL on the map is represented by the chromosome region corresponding to the maximum LOD minus 1, which determines an approximate confidence interval (Lander and Botstein, 1989). Two QTLs of different traits will be declared as coincident when their LOD-1 intervals largely overlap. A coincidence will be said to be positive when there is coincidence of a favourable (or an unfavourable) allele for both traits. The coincidence will be said to be negative when there is coincidence of a favourable allele for one trait with an unfavourable allele for the other trait.

Statistical analysis

To understand how the plant functions at high and low N-input, phenotypic correlations (r_P), i.e. correlations among genotypic means, between agronomic traits in each situation and between agronomic traits and physiological traits were calculated. The phenotypic correlations given in this paper were highly significant (greater than 0.26 or 0.29, the thresholds at 0.01, and noted with two asterisks **). It must be underlined that, due to environmental errors, the correlation can be much lower than genotypic correlation which would be calculated on true unknown genotypic values. When the measurements are independent, as with an agronomic and a physiological trait, then it is only necessary to divide r_P by the square root of the product of the heritabilities (Becker, 1984). Such genotypic correlations (r_G) have been derived for some pairs of traits and were given in detail by Bertin and Gallais (2000). Broad-sense heritabilities were derived from the ratio of genotypic variance to phenotypic variance among lines estimated from the analysis of variance, considering genotypic effects as random.

Results and discussion

Understanding genetics and physiology of N-utilization from the study of N-stress on trait means and correlations between traits

Effect of nitrogen deprivation on trait means: The effect of nitrogen deprivation on traits related to growth and development gives preliminary information for understanding plant reaction to nitrogen fertilization. In the experimental conditions used by the authors the reduction in yield from high to low N-input was 38%. Among the yield components, kernel number was the most affected (32%) while kernel weight was reduced by only 9%. The reduction in kernel number is due to ovule abortion after fertilization, since the number of ovules is only slightly affected by nitrogen stress (Lemcoff and Loomis, 1986; Uhart and Andrade, 1995; Below, 1995; Below *et al*., 2000). Such abortion could be the result of a limitation in the source of photosynthetic products, which also affects post-anthesis growth (29% reduction) much more than vegetative development (14% reduction), as already shown by McCullough *et al*. (1994). This suggests that, just after fertilization, the sink demand must be too high compared with the availability of resources, thus leading to embryo abortion in a genotype-dependent manner.

Genetic variation in NUE in relation to fertilization: At a given level of nitrogen, differences in yield means that there are differences in NUE among different genotypes. Genetic variance for NUE has been observed both at low and high nitrogen fertilization levels. The genetic correlation between the two levels was high for both years (0.85). On average, the variance of genotype×nitrogen interaction represented about 25% of the total genotypic variation. Surprisingly, this result is comparable to that already observed by various authors on more diverse plant material (Balko and Russell, 1980*a*; Landbeck, 1995). Kernel number per ear was the yield component explaining the most about such an interaction, underlining once again the role of embryo abortion just after ovule fertilization. Responsiveness of yield and kernel number was negatively related to traits at low N-input: yield or kernel number, N-content at flowering, nitrogen nutrition index (NNI), N-uptake at harvest, and post-anthesis N-uptake. This observation means that genotypes exhibiting low agronomic performance at low N-input, i.e. those having a low NUE, were those reacting more to nitrogen fertilization. Thus, genotype×nitrogen interaction appears to be essentially due to variation in the adaptation of the plant to low

N-input rather than to variation in the adaptation to high N-input. The absence of such interactions for traits relative to vegetative growth means that they are due to grain development.

N-uptake is the product biomass×N-content. Its variation depends on the correlation between the two components. At flowering, under low N-input, there was a strong negative correlation between yield and N-content (dilution effect), resulting in a strong reduction of genetic variance of N-uptake. Until flowering, maize functions like a typical forage grass for which a negative correlation between dry-matter yield and protein content is generally observed (Lemaire and Gastal, 1997). At maturity, the observed low variation in whole plant N-uptake under low N-input suggests that there was a limiting factor in nitrogen availability in the soil and in the capacity of the plant to absorb nitrogen. Unlike low N-input, at high N-input there was a strong variation in N-uptake, with no negative correlation between yield and N-content.

Since only the aerial biomass is usually taken into consideration in the definition of NUE, nitrogen utilization efficiency (NUtE) can be expressed as the ratio of harvest index/N-content of the aerial parts. In agreement with such an expression, these results showed that NUtE was highly negatively related to the plant N-content and positively related to the harvest index. An increase in NUtE corresponds with a decrease in the N-content and with an increase in the harvest index. These results were similar to that obtained by Di Fonzo *et al*. (1982) who showed that under a low level of N fertilization, grain yield was related to the nitrogen harvest index.

Relationships between nitrogen remobilization and post-flowering absorption: It appeared that, regardless of the level of N fertilization, there was strong opposition between N-remobilization and post-anthesis N absorption (r_P= –0.80**). Remobilization from the stem appeared to be moderately correlated to remobilization from leaf blades (r_P=0.46**) and, as expected, the amount of nitrogen remobilized was always related to the quantity of nitrogen present at flowering. Whatever the organ, the relative remobilization (or rate of remobilization) was highly related to the absolute remobilization (r_P=0.88** under high N input). Grain yield and protein grain yield were positively correlated with absorption at high N-input, confirming again the major role of N-uptake under these growth conditions whereas, as a consequence of the strong negative correlation between remobilization and absorption, they were negatively correlated to remobilization.

The opposition between N-remobilization and N-absorption can be explained by the fact that N-remobilization comes essentially from leaf protein degradation (Rubisco in particular) in senescing leaves while photosynthetically efficient leaves (with an active Rubisco) are required for an efficient N absorption. Therefore, it can be assumed that nitrogen remobilization takes place when absorption is reduced or stopped following various biotic or abiotic stresses, including water stress, or during natural senescence.

Relationships between grain yield, NUE and some specific traits. (1) Relationships with vegetative development and NNI at flowering: Whatever the N-input, high vegetative development at flowering was favourable to high nitrogen uptake efficiency. As post-anthesis N-uptake explains a great part of the genetic variability of the total absorption, it can be assumed that high vegetative development, including roots, is necessary to have high nitrogen absorption during grain filling. Such vegetative development allows remobilization to be greater if soil-N availability is restricted. At both low and high N-input, grain yield was significantly correlated with N-uptake at flowering, and more intensively at low N-input where it determines kernel number. In this condition, it was also related to NNI at flowering (r_P=0.45**). Interestingly, at low N-input, NNI at flowering was related to leaf senescence although this process occurred about 3–4 weeks later (r_G=0.80). These observations led to the proposal that NNI reflects the physiological status of the leaf, such as its potential photosynthetic activity. In other words, with active chloroplasts, leaf N-content is higher and thus delays leaf senescence. Consequently, a low NNI limiting the flux of photosynthesis products at flowering can lead to embryo abortion just after fertilization. Such a conclusion is also supported by the results obtained by Uhart and Andrade (1995), Reed *et al*. (1988) and Czyzewicz and Below (1994), who showed that nitrogen is necessary for kernel development, perhaps through the supply of carbon assimilates. Genetic variation in responsiveness of kernel number could mean a resistance to abortion, due for example to the ability to remobilize stalk reserves.

Relationships between grain yield, NUE and some specific traits. (2) Relationships with anthesis–silking interval: Anthesis–silking interval (ASI) is defined as the difference between silking date and anthesis date. From a physiological point of view, the observed negative relationship between grain yield and ASI (r_G= –0.81), also observed in other experiments (A Gallais, B Hirel, unpublished results) and by Lafitte and Edmeades (1995), is interesting to note. When maize plants are subjected to various stresses such as drought or nitrogen deficiency, there results an increase in ASI (Lafitte and Edmeades, 1995). The consequence is that in monogenotypic stands there could be a deficit in ovule fertilization. In the authors' experiment, this expected effect was suppressed by a continuous pollen production during silking except for late genotypes. ASI could then have a physiological meaning in relation to stress tolerance. In other words, genotypes for which ASI

does not increase would have a more efficient nitrogen metabolism, or a physiology leading to greater yield at low N-input. It is well known that a short ASI is related to a prolific physiology. At the extreme, true prolificacy leads to protogyny whereas normal maize shows protandry. Such genotypes have two favourable traits. First, they have a high degree of translocation from the stover to the grain, a characteristic which favours yield in stress conditions; this explains why, with such material, NUtE is more important than N-uptake, unlike normal maize (Jackson *et al*., 1986). Second, Bertin *et al*. (1976) and Boyat and Robin (1977) have shown that they have a higher NR activity, which is already induced in the leaf at low light intensity.

Relationships between grain yield, NUE and some specific traits. (3) Relationships with nitrate absorption in young vegetative plants: In many plant species, when nitrate is absorbed in excess it is usually stored in the vacuole and serves both as an osmoticum and as a source of mineral nitrogen when the soil supply becomes depleted (McIntyre, 1997; Crawford and Glass, 1998). During vegetative growth in maize, the vacuolar pool of nitrate constitutes an important source of nitrogen that can be metabolized further and subsequently participates in the grain-filling process (Teyker *et al*., 1989; Plénet and Lemaire, 1999). In this experiment, leaf nitrate content and leaf nitrate quantity in young developing plants was related to grain and protein yield, mainly through a strong relationship with kernel weight (Hirel *et al*., 2001). The correlation between kernel weight and nitrate uptake in young vegetative plants could be due to a greater vigour of the plants from a heavy kernel, a vigorous plant absorbing more nitrates than a non-vigorous one. However, in the field, the effect of seed size on plant development tends to disappear before the 4–5 leaf stage. Therefore, the nitrate content in young developing plants could be considered as a good 'metabolic marker' of nitrate uptake ability in field-grown plants. This hypothesis was confirmed by the positive correlation between leaf nitrate content (and quantity) in young vegetative plants and post-anthesis nitrogen uptake of adult plants under low N-input (r_P=0.29**). Under this condition, leaf nitrate content was negatively related with nitrogen remobilization. This can be interpreted as a consequence of the observed negative correlation between N-remobilization and post-anthesis N-uptake. However, when soil nitrogen availability becomes limiting, such a negative correlation could be the result of a high N-uptake before flowering by efficient absorbing genotypes. At high N-input no correlation was observed between leaf nitrate content or quantity and remobilization or post-anthesis N-uptake. This could mean that, as nitrogen from soil is not limiting, it can be absorbed at any time as long as the plant does not senesce.

Relationships between grain yield, NUE and some specific traits. (4) Relationships with GS at young stage and GDH at mature stage: GS (EC 6.3.1.2) is one of the main enzymes involved in the assimilation and recycling of mineral nitrogen which catalyses the ATP-dependent conversion of glutamine into glutamate utilizing ammonia as a substrate (Lea and Ireland, 1999; Cren and Hirel, 1999). The authors' working hypothesis was that the rate of ammonium assimilation derived from nitrate reduction and/or organic nitrogen recycling is of major importance for plant NUE. The role of GS1 during N-remobilization has already been shown in maize hybrids containing lower amounts of nitrate, suggesting that the active contribution of cytosolic GS during the recycling of nitrogen results from protein hydrolysis (Purcino *et al*., 1998). In these studies (Hirel *et al*., 2001), leaf GS activity was positively correlated with grain yield and kernel number under low N-input and to GNY (r_P=0.28**) at high N-input. As N-remobilization has been shown to play a greater role at low N-input, this could appear to be consistent with this study's assumption. However, N-remobilization throughout the growth period after flowering can affect grain filling, i.e. kernel weight, but not kernel number, which is determined very early just after fertilization, unless a high GS activity allows the synthesis of a high level of glutamine derived compounds just after flowering. In fact, leaf GS activity in young vegetative plants was positively correlated both to the post-anthesis N-uptake and to the percentage of nitrogen in the grain from post-anthesis N-uptake (and then negatively correlated with the percentage of nitrogen in the grain coming from remobilization) at high N-input. This observation is also consistent with the correlation between GS activity and nitrate content or quantity in young developing plants. The relationship with the kernel number is likely to be due to a greater flux of nitrates and of nitrogen compounds in genotypes absorbing higher amounts of nitrate. The whole leaf GS activity (plastidic+cytosolic) measured in young vegetative plants at high N-input then appears to be related to N-uptake ability rather than to N-remobilization. Ammonium recycling during remobilization could be catalysed by another cytosolic GS isoenzyme which becomes the predominant form of the enzyme after flowering (B Hirel, unpublished data).

If GDH (E.C.1.4.1.2) activity is considered, which is another enzyme which is able to aminate 2-oxoglutarate or deaminate glutamate to release ammonium and is induced in senescing leaves (Dubois *et al*., 2003), the results are more difficult to interpret. It is mainly because the exact function of the enzyme *in vivo* is not fully defined. Leaf aminating GDH activity of adult plants measured *in vitro* was positively related to kernel number at low N-input (r_P=0.27**). It can therefore be hypothesized that, under these conditions, the enzyme, in conjunction with GS, may participate in the reassimilation of ammonium released

following protein hydrolysis during the process of N-remobilization. Aminating GDH activity could be interpreted as an adaptation to a shortage of nitrogen. Deaminating GDH activity at low N-input was negatively correlated with GS activity, with the amount of nitrogen accumulated at anthesis, and with the leaf N-content at maturity. Dubois *et al*. (2003) suggest that its function would be more for carbohydrate replenishment rather than N-assimilation. However, one cannot completely exclude the dual function of the enzyme: depending on the nitrogen status of the plant it could act as a signal to control the homeostasis of glutamate (the substrate of GS) and thus the flux of reduced N.

Relationships between grain yield, NUE and some specific traits. *(5) Nitrate reductase activity:* As expected, a negative correlation was observed between NR activity and nitrate content in young plants (r_P= –0.32**). Whatever N-input, NR activity was related negatively to kernel weight and N reduction efficiency was negatively related to both grain yield and grain protein yield. In other words, high NR activity and nitrogen reduction efficiency characterize the less yielding genotypes (Hirel *et al*., 2001). Similar conclusions were drawn by Reed *et al*. (1980) who showed that higher yields were obtained in genotypes exhibiting low NR activity. This observation is consistent with the negative relationship between NR and GS activity which suggests that, when the rate of nitrate reduction is too high, GS activity becomes limiting to cope with the stronger flux of reduced nitrogen.

Understanding genetics and physiology of N utilization from the study of QTLs

QTLs for N-uptake and N utilization efficiency: QTLs detected for agronomic traits (grain yield and its components, kernel number and kernel weight), N-content, and some other associated traits as senescence, are recalled in Fig. 1. It appears clearly, as shown by Bertin and Gallais (2001) that more QTLs for yield and its components are detected for plants grown under high N-input. By contrast, more QTLs are detected for N-content for plants grown under low N-input. For whole-plant N-uptake at high N-input, five QTLs were detected, three on chromosome 1 and two on chromosome 4, whereas only one QTL was detected at low N-input. This is due to a very low genetic variation in such a condition (Bertin and Gallais, 2000) resulting from a dilution effect (high negative relationship between dry-matter yield and N-content). For grain N-uptake, the same situation was observed, since no QTL were detected at low N-input. Unlike N-uptake, QTLs for whole plant NUtE were detected mainly at low N-input. Under this condition, the five detected QTLs explained 38.9% of the phenotypic variance, whereas at high N-input, only one specific QTL was detected. The same situation was observed with QTLs detected for grain NUtE. Four QTLs were detected under low N-input, three coinciding with those detected for whole-plant NUtE and two under high N-input (one coinciding with one of the previous QTLs, the other being specific to plants at high N-input). As discussed by Bertin and Gallais (2001) differences in heritabilities and effects of N-deprivation on means are not sufficient to explain such results. Therefore, the assumption here is that genes are regulated differently according to the level of N-fertilization. This is also consistent with the conclusion that genetic variability (variances and correlations) was expressed differently under both growth conditions.

QTLs for absorption and remobilization: At low N-input, probably due to the high experimental error expressed by the low heritability of such traits, only one QTL was detected. It concerns the remobilization of nitrogen from the stem. It is located at the top of chromosome 1 and coincides with the *gln1* gene (encoding cytosolic GS). At high N-input, ten other QTLs were detected (Table 1). Five are located on chromosome 1 at 80, 122, 172–182, and 204 cM from its top. The first three were related to N-remobilization (expressed at either absolute or relative values) from the whole plant at flowering, or from the leaf blades or the stem. The first at position 80 cM coincided positively with a QTL of kernel weight under both levels of N fertilization. The second, at position 122 cM coincided negatively with a chromosome region where a QTL for kernel number and three genes are present. Two of these genes encodes enzymes involved in N-assimilation (NR1 and GDH1) while the other encodes ADPGppase, an enzyme involved in C-metabolism. The two QTLs detected in position 172–182 correspond to two types of traits: the relative rate of N-absorption in the grain (expressed as % of the total N-uptake in the shoots or as % of total nitrogen in the grain) and the relative rate of remobilization. For the absorption the favourable allele comes from the parental line Io, while for remobilization it origin is from the other parental line F2. However, it is impossible to conclude whether it is two different QTLs nearly linked or the same QTL with a pleiotropic effect because the two traits are highly negatively correlated (r_P= –0.75**). It is interesting to note that there was coincidence with the *gln2* gene encoding another cytosolic GS isoenzyme. This zone was also involved in the genetic control of grain yield, kernel number and grain protein yield at high N-input. The last QTL on chromosome 1 in position 204 cM was related to absolute remobilization from the whole plant at flowering and coincided with QTLs for grain yield and kernel number at high N-input.

Another QTL located on chromosome 2 at position 64 cM was related to the N-remobilization process. It overlapped positively with a QTL for kernel weight detected at high N-input. On chromosome 4, three QTLs were detected: two (at 104 and 148 cM) were related to N-

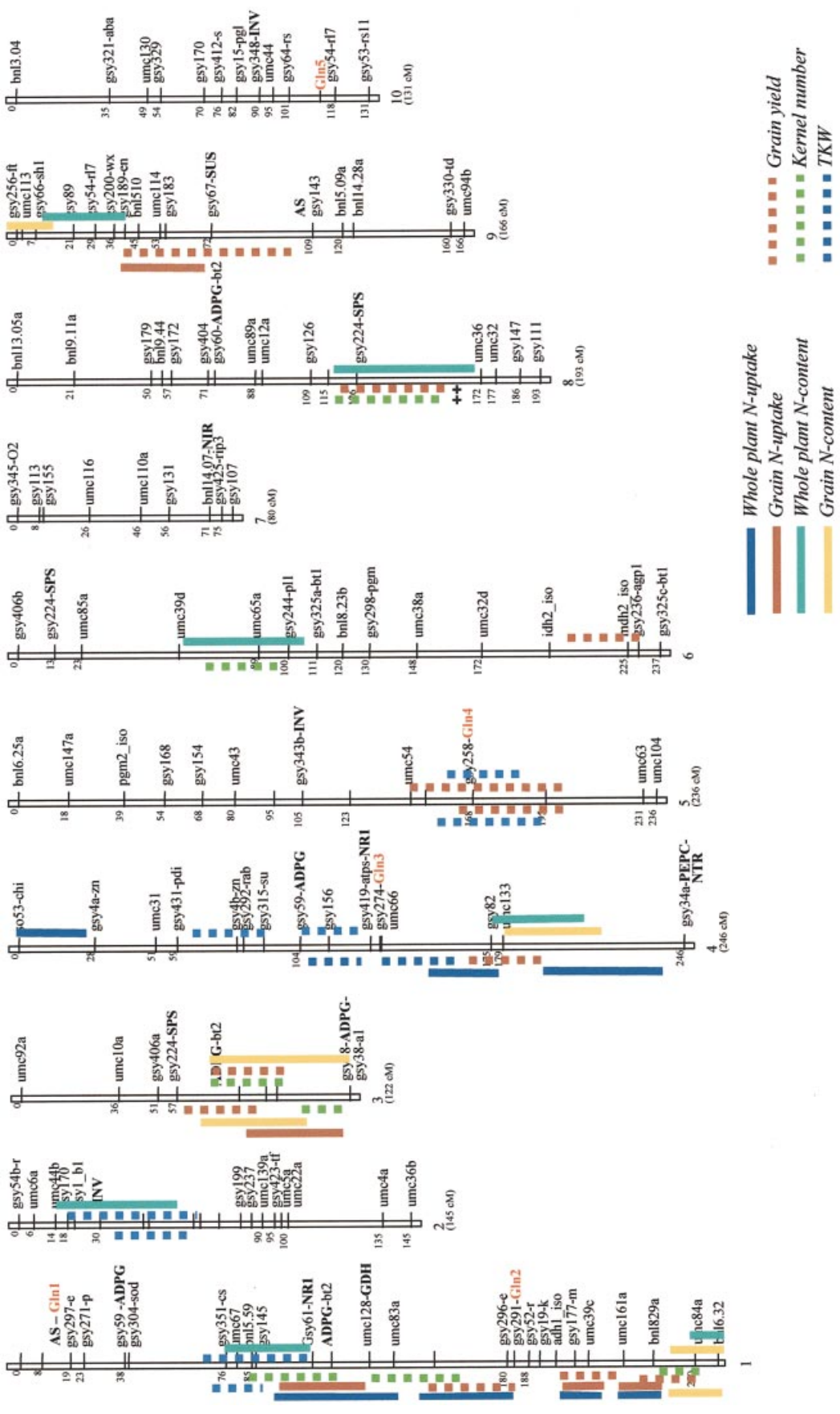

Fig. 1. Locations of the detected QTLs for agronomic traits with a population size of 99 recombinant inbred lines crossed to the tester F252. A QTL was shown when it was detected in one year or in the average of the two years. QTLs detected at high N-input are on the left of the chromosome whereas those detected at low N-input are on the right of the chromosome. For more details on the parameters of these QTLs see Bertin and Gallais (2001). Note that nitrogen whole-plant content is the reciprocal of nitrogen use efficiency at the whole plant level.

Table 1. *QTLs detected for N-remobilization and post-anthesis N-uptake with a population size of 99 recombinant inbred lines crossed to the tester F252*

Trait[h]	Chro[a]	Position[b]	Marker[c]	Interval[d]	LOD[e]	R^{2f}	Allele effect[g]
Stem remobilization 94N–	1	8	UMC11	2–24	1.91	9.7	+
Leaf blade remobilization 94N+	1	80	SC351	56–94	2.30	10.2	–
% Remobilization from leaf 94N+	1	80	SC351	60–94	2.95	13	–
Total remobilization (94+95)N+	1	122	SC60B	110–138	2.27	10.1	–
% Remobilization from leaf 94N+	1	168	SC282A	154–188	2.51	11.1	–
Total remobilization (94+95)N+	1	172	SC282A	160–198	3.46	15	–
% Absorption in grain (94+95)N+	1	182	SC296	160–198	2.45	10.9	+
Total remobilization (94+95)N+	1	204	ADH1	198–210	3.28	14.4	–
% Remobilization from leaf 94N+	2	58	SC108	30–72	2.10	9.3	–
Total remobilization (94+95)N+	2	68	SC136	60–74	3.78	16.4	–
Remobilization from stem 94N+	4	104	SC59C	94–112	2.67	11.8	–
Remobilization from stem (94+95)N+	4	148	UMC66	116–168	2.03	9.2	–
% Remobilization from leaf 94N+	4	236	UMC133	202–244	2.00	13.8	+
% Remobilization from stem 94N+	6	228	MDH2	204–236	1.94	10.3	–

[a] Chromosome number.
[b] Distance in cM from the chromosome top of the maximum LOD.
[c] Nearest marker on the genetic map (Causse *et al*., 1996) from the chromosome top.
[d] LOD-1 interval.
[e] Maximum LOD greater than 2 except for the first and last line of the table.
[f] Percentage of phenotypic variance explained by the variation at the QTL.
[g] + when the favourable allele derives from the Io parent whereas – when it derives from F2 parent.
[h] 94 means observation in the year 1994, whereas 94+95 means average of the two years of study 1994 and 1995. N+ (N–) refers to high (low) N-input.

remobilization expressed either as absolute or relative values. The first one overlapped positively with a QTL of kernel weight detected at low N-input, whereas the second one overlaps, also positively, with a QTL of kernel weight detected in both nitrogen fertilization conditions. Moreover this second QTL coincided with two genes encoding enzymes involved in nitrogen metabolism (NR2 and *gln3*). The last QTL on chromosome four at position 236 cM was linked to the relative rate of leaf N-remobilization and is located near the genes encoding PEPC and NTR1.

Finally, among the ten QTLs detected for N-remobilization, three coincided with QTLs for kernel weight and one with a QTL for grain yield. This finding stresses the role of N-remobilization during grain filling, despite the lack of correlation between N-remobilization and grain yield or kernel weight. Considering the role of post-anthesis absorption at high N-input for grain filling, it is surprising that not more QTLs were found for N-absorption. This could mean that there are many genes involved, each having a relatively low effect. Furthermore there were three coincidences of QTLs for remobilization with a gene encoding cytosolic GS which suggests that such genes are involved in the control of remobilization. However, taking into account the high negative correlation between post-anthesis N-uptake and N-remobilization, a QTL for remobilization could be considered as a QTL for N-uptake, with an allelic effect of opposite sign. Following results on physiological traits will shed some light on the meaning of such QTLs.

QTLs for leaf nitrate content and NR activity: Five QTLs for leaf nitrate content explaining 28% of the phenotypic variation were detected: two were located on chromosome 2, both with the favourable allele from the parental line F2 and three on chromosome 5 with the favourable allele from the parental line Io. On chromosome 2 one of the QTLs for leaf nitrate content, coincided positively with a QTL for kernel weight when plants were grown under high N-input. One of the QTLs for leaf nitrate content located on chromosome 5 was also positively coincident with a QTL for grain yield and kernel weight, regardless of the N-fertilization level. These results are in agreement with the positive correlation observed between leaf nitrate content of young developing plants, grain yield and kernel weight in field-grown mature plants independent of the level of fertilization. Furthermore, the observed coincidences with QTLs for kernel weight support the conclusion that nitrate content (and quantity) at a young stage is an indicator of post-anthesis N-uptake ability.

For maximal leaf NADH-NR activity (with measurements performed only on a one-year experiment), two main QTLs were found on chromosome 5 explaining 36.2% of the observed phenotypic variation, which is very high for only two QTLs. One of the QTLs for NADH-NR activity located in the region of the *gln4* locus, was negatively coincident with a QTL for nitrate content and a QTL for yield, both detected under low or high N-input. These results are consistent both with the observed negative correlation between grain yield or kernel weight and leaf NR activity and the expected negative correlation between NR activity and nitrate content. The other QTL was positively coincident with a QTL for nitrate content.

QTLs for GS activity: Six QTLs for total leaf GS activity were detected explaining 52.5% of the phenotypic vari-

ation: three were located on chromosome 1, two on chromosome 5, and the other on chromosome 9. Interestingly, out of these six QTLs, three coincided with genes encoding cytosolic GS quoted as *gln1*, *gln2* and *gln4*. This result suggests that for these three genes the final leaf cytosolic enzyme activity is mostly regulated at the transcriptional level. By contrast, for the other cytosolic GS gene *gln3* located on chromosome 4 and the gene encoding plastidic GS (*gln5*) located on chromosome 10, other regulatory mechanisms acting at the post-transcriptional and/or translational levels are likely to be involved in controlling the corresponding enzyme activity (Cren and Hirel, 1999). The detection of four QTLs for leaf GS activity which did not coincide with GS structural genes indicates that some loci located on different chromosome segments may be partly involved in the regulation of both cytosolic and plastidic GS activity.

Two QTLs for GS activity were coincident with QTLs for yield and its components (kernel weight and kernel number) and a GS gene. One was located on chromosome 1 coincident with *gln2* locus and with a QTL for yield and kernel number at high N-input, and one on chromosome 5 coincident with *gln4* locus and with QTLs for yield and kernel weight whatever N-input. Such positive coincidences are consistent with the positive correlation observed between grain yield and GS activity, particularly at low N-input. However, QTLs for yield on chromosome 5 can be considered as common to both nitrogen levels, because the favourable allele was detected at both low and high N-inputs. By contrast, the QTL for yield, coinciding with QTL for leaf GS activity on chromosome 1, was detected only under high N level. This could mean that *gln2* and *gln4* translation products have a different role. In the same manner, the QTL for N-remobilization in the region of the *gln1* locus (on chromosome 1), which coincides positively with a QTL of GS activity, could be considered as a QTL of true remobilization, whereas the QTL for N-remobilization in the region of the *gln2* locus, which coincides negatively with a QTL of GS activity, could be considered as a QTL of post-anthesis N-uptake. The *gln1* locus could code for an isoenzyme involved in N-remobilization whereas the *gln2* locus could code for an isoenzyme involved in nitrogen assimilation. This supports the conclusion that the different GS genes appear to have non-overlapping functions in different organs or tissues and according to the plant developmental stage (Sakakibara *et al*., 1992*b*; Li *et al*., 1993; Rastogi *et al*., 1998). The relative contribution of the corresponding GS isoenzyme activity in either synthesizing or recycling organic nitrogen necessary for grain filling would be finely balanced, not only depending on the plant developmental stage but also on soil N availability.

The coincidence between the *gln4* locus and several QTLs for grain yield, kernel weight, leaf GS activity, NR activity, and leaf nitrate content (Fig. 2) suggests that both nitrate availability and the reactions catalysed by NR and GS are key steps in the NUE for seed production. However, the coincidence is negative between the QTL for NR activity and all the others co-localizing with it, whereas it is positive between them. This suggests that complex interactions between GS and NR activities are likely to occur. As already discussed in the previous section, this result is consistent with the negative impact of nitrate reduction capacity on yield and its components. By contrast, the positive coincidence between QTLs for grain yield, kernel weight, leaf nitrate content, and leaf GS activity, confirm the positive effect of the last two traits on yield found in the correlation studies. Furthermore, the coincidence between QTLs for leaf GS activity, leaf NR activity and leaf nitrate content found in two regions on chromosome 5, is in favour of the hypothesis that signals derived from the ammonia assimilatory pathway interact with nitrate uptake and reduction (Scheible *et al*., 1997). Finally *gln4* appears as a good candidate gene controlling NUE and influencing yield.

It can also be underlined that, on chromosome 10, there was coincidence between another GS locus (*gln5*) and QTLs for ASI, leaf senescence, and NNI. Interestingly, this locus corresponds to the plastidic GS. Therefore, taking into account coincidences shown previously between QTL for remobilization and the different members of the GS multigene family, it appears that coincidences observed with the five GS loci are quite consistent with their possible role. It is well known that the different genes encoding GS can be differentially expressed according to both the physiological status and the developmental stage of the plant (Cren and Hirel, 1999).

QTLs for GDH: Three QTLs for GDH activity were detected. Two corresponding to GDH aminating activity were located on chromosome 3 and 8 while the other corresponding to GDH deaminating activity was located on chromosome 6. The QTL for GDH deaminating activity was found in plants grown in the field under a high level of N-fertilization whereas the two QTLs for GDH aminating activity were found in plants grown under low N-input. The two QTLs for GDH aminating activity coincided positively with QTLs for grain yield and kernel number whereas the QTL for GDH deaminating activity coincided negatively with a QTL for kernel number. This result suggests that the GDH aminating activity may be an important factor controlling plant productivity as already found using transgenic plants overexpressing the enzyme (Ameziane *et al*., 2000). GDH deaminating activity could be involved in controlling the translocation of assimilates during the remobilization phase, when it is induced, as suggested by the negative correlation between GDH activity and leaf N-content at maturity. This possible role of GDH is strengthened by the recent finding that GDH protein is mostly concentrated in the vascular tissue of a

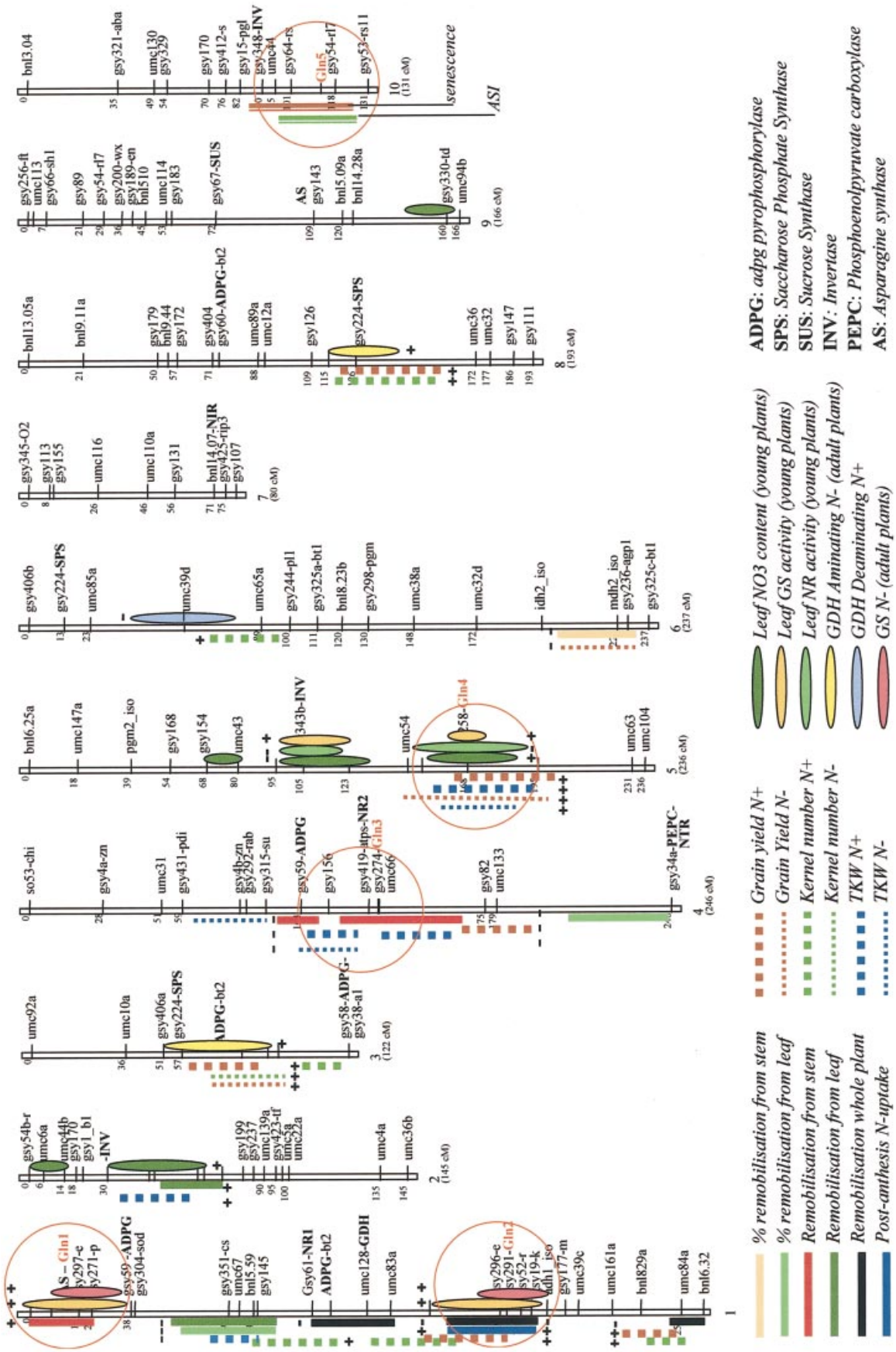

Fig. 2. Locations of the detected QTLs for physiological traits (leaf nitrate content, leaf GS and NR activities of young plants at high N-input; GDH and GS activities of adult plants at both high and low N-input) and their coincidences with mapped genes and QTLs for grain yield, kernel number, thousand kernel weight (TKW), remobilization, and post-anthesis N-uptake at both high (N^+) and low-input (N^-). QTLs for remobilization and post-anthesis N-uptake were detected only at high N-input, except the one at the top of chromosome 1 detected at low N-input. The genetic map was published in detail by Causse *et al.* (1996). The plus and minus signs below or above the segment representing the QTL location shows from what parent the favourable allele comes: plus from Io, minus from F2. For more details on QTLs for nitrate content and GS activity, see Hirel *et al.* (2001).

number of higher plants including maize (Becker *et al*., 2000).

Conclusion

Bertin and Gallais (2000) concluded from both the study of genetic correlations among traits and the detection of QTLs for various agronomic traits that genetic variability was differently expressed under high and low N-inputs. Further physiological studies associated with the detection of QTL confirm such a conclusion. It appears that nitrogen stress may allow the expression of variability controlled by specific genes involved in N-remobilization, unlike high N-input which would favour the expression of variability controlled by specific genes involved in post-anthesis N-uptake.

One of the major breakthroughs from these studies, concerns the role of the gene encoding cytosolic GS (*gln4* locus) located on chromosome 5 as a candidate gene for which the corresponding enzyme activity influences grain filling. Now, experiments are in progress to overexpress the gene and to transfer the favourable allele in a genotype with the unfavourable allele in order to verify whether grain filling and grain yield is improved. Other candidates genes among genes encoding enzymes involved in N–metabolism are the two GS genes (*gln1* and *gln2*) on chromosome 1 and the GS gene on chromosome 4 (*gln3*). The corresponding enzyme activity of all these three genes could be involved either in N-remobilization or in N-translocation during the process of grain filling. It remains, however, to determine whether each metabolic process involved either in the assimilation or in remobilization of nitrogen is controlled by a single GS gene or a combination of genes which can give rise to an homo- or an hetero-octameric form of the enzyme (Hirel and Lea, 2001).

In conclusion, these results clearly show that genetic and physiological bases of NUE can be studied in a integrated manner by means of a quantitative genetic approach using molecular markers, genomics, and combining both agronomic and physiological studies. Such an approach leads to the identification of candidate genes to validate other approaches such as gene transfer or mutagenesis.

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