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### The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches

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#### Abstract

In this review, recent developments and future prospects of obtaining a better understanding of the regulation of nitrogen use efficiency in the main crop species cultivated in the world are presented. In these crops, an increased knowledge of the regulatory mechanisms controlling plant nitrogen economy is vital for improving nitrogen use efficiency and for reducing excessive input of fertilizers, while maintaining an acceptable yield. Using plants grown under agronomic conditions at low and high nitrogen fertilization regimes, it is now possible to develop wholeplant physiological studies combined with gene, protein, and metabolite profiling to build up a comprehensive picture depicting the different steps of nitrogen uptake, assimilation, and recycling to the final deposition in the seed. A critical overview is provided on how understanding of the physiological and molecular controls of N assimilation under varying environmental conditions in crops has been improved through the use of combined approaches, mainly based on whole-plant physiology, quantitative genetics, and forward and reverse genetics approaches. Current knowledge and prospects for future agronomic development and application for breeding crops adapted to lower fertilizer input are explored, taking into account the world economic and environmental constraints in the next century.

Key words: Crops, environment, fertilization, low input, nitrogen management, yield.

#### Nitrogen fertilization and sustainable agriculture

The doubling of agricultural food production worldwide over the past four decades has been associated with a 7fold increase in the use of nitrogen (N) fertilizers. As a consequence, both the recent and future intensification of the use of N fertilizers in agriculture already has and will continue to have major detrimental impacts on the diversity and functioning of the non-agricultural neighbouring bacterial, animal, and plant ecosystems. The most typical examples of such an impact are the eutrophication of freshwater (London, 2005) and marine ecosystems (Beman *et al.*, 2005) as a result of leaching when high rates of N fertilizers are applied to agricultural fields (Tilman, 1999). In addition, there can be gaseous emission

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Abbreviations: DHL, doubled haploid line; GS, glutamine synthetase; LAI, leaf area index; N, nitrogen; %Nc, critical nitrogen concentration; NHI, nitrogen harvest index; NUE, nitrogen use efficiency; NupE, nitrogen uptake efficiency; NutE, nitrogen utilization efficiency; QTL, quantitative trait locus; RIE, radiation interception efficiency; RIL, recombinant inbred line; Rubisco, ribulose 1,5-bisphosphate carboxylase; RUE, radiation use efficiency.

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of N oxides reacting with the stratospheric ozone and the emission of toxic ammonia into the atmosphere (Ramos, 1996; Stulen *et al.*, 1998).

Despite the detrimental impact on the biosphere, the use of fertilizers (N in particular) in agriculture, together with an improvement in cropping systems, mainly in developed countries, have provided a food supply sufficient for both animal and human consumption (Cassman, 1999). Production of N fertilizers by the Haber-Bosch process was therefore one of the most important inventions of the 20th century, thus allowing the production of food for nearly half of the world population (Smil, 1999). However, between now and the year 2025, the human population of around 6 billion people is expected to increase to 10 billion. Therefore, the challenge for the next decades will be to accommodate the needs of the expanding world population by developing a highly productive agriculture, whilst at the same time preserving the quality of the environment (Dyson, 1999). Furthermore, farmers are facing increasing economic pressures with the rising fossil fuels costs required for production of N fertilizers. Enhancing productivity in countries which did not benefit from the so-called 'green revolution' will also be required by developing specific cropping strategies and by selecting productive genotypes that can grow under low N conditions (Delmer, 2005).

More recently, the production of biofuel from plant biomass in a variety of crops has been widely seen as an alternative to replace fossil energy and, as such, requires an extensive use of N fertilizers in several species (Boddey, 1995; Giampietro *et al.*, 1997). Since large quantities of fossil fuels are required to produce N fertilizers, selecting new energy crop species such as *Miscanthus* (Lewandowski *et al.*, 2000) or willow (Heller *et al.*, 2004), or genotypes of the already cultivated crops that have a larger capacity to produce biomass with the minimal amount of N fertilizer, could be another interesting economic and environmental challenge.

When an excess of N cannot be totally avoided, it should also be important to search for species or genotypes that are able to absorb and accumulate high concentrations of N. Although it is well known that there is some genetic variability in maximum N uptake in rice (Borrell et al., 1998) and wheat (Le Gouis et al., 2000), the physiological and genetic basis for such variability has never been thoroughly investigated (Lemaire et al., 1996). Such variability could confer on some species or genotypes the ability to store greater quantities of N during periods of abundant N supply, thus avoiding losses into the soil. As described in the review articles of Lemaire et al. (2004) and Hirel and Lemaire (2005), it is possible to develop a framework for analysing the genotypic variability of crop N uptake capacity across a wide range of genotypes, thus allowing the selection of those having the greatest capacity to accumulate an excess of N.

Rice, wheat, maize, and, to a lesser extent, barley, coarse grains in legumes along with root crops are the most important crops cultivated in the world and account for the majority of end-products used for human diets (http://apps.fao.org/), and it is likely that they will still contribute to human nutrition in the next century. Moreover, the high yields of rice, wheat, and maize largely contributed to the total increase in the global supply of food production since 1967 (Cassman, 1999). It is therefore of major importance to identify the critical steps controlling plant N use efficiency (NUE). Moll et al. (1982) defined NUE as being the yield of grain per unit of available N in the soil (including the residual N present in the soil and the fertilizer). This NUE can be divided into two processes: uptake efficiency (NupE; the ability of the plant to remove N from the soil as nitrate and ammonium ions) and the utilization efficiency (NutE; the ability to use N to produce grain yield). This challenge is particularly relevant to cereals for which large amounts of N fertilizers are required to attain maximum yield and for which NUE is estimated to be far less than 50% (Zhu, 2000; Raun and Johnson, 1999). In addition to the improvement of N fertilization, soil management, and irrigation practices (Raun and Johnson, 1999; Alva et al., 2005; Atkinson et al., 2005), there is still a significant margin to improve NUE in cereals by selecting new hybrids or cultivars from the available ancient and modern germplasm collections in both developed and developing countries. Consequently, the effective use of plant genetic resources will be required to meet the challenge posed by the world's expanding demand for food, the fight against hunger, and the protection of the environment (Hoisington et al., 1999).

More recently, the production of oilseed rape (*Brassica napus* L.), an emerging oilseed crop, has been significantly increased, to become second only to soybean in the world supply. This increased interest in this crop is mostly due to the use of the oil in end-products, including biofuel (Rayner, 2002). However, as for cereals, the ratio of plant N content to the N supplied does not exceed 50% whatever the level of N fertilization (Malagoli *et al.*, 2005), which suggests that improvement of NUE in this species is also a possibility.

Barley (*Hordeum vulgare* L.), besides its importance as a crop, is an established model plant for agronomic, genetic, and physiological studies (Raun and Jonhson, 1999). However, knowledge of the biochemical and molecular mechanisms controlling N uptake, assimilation, and recycling is still fragmentary (Mickelson *et al.*, 2003). Moreover, the influence of N fertilizer levels and timing of application on grain yield and grain protein content was investigated in only a few studies (Penny *et al.*, 1986; Bulman and Smith, 1993). Therefore, although this crop may be of interest for future research, NUE in this crop will not be covered in this review.

In this review, an overview is presented on how understanding of the key steps of N assimilation in some

of the main crop species cultivated in the world has been improved through the use of combined approaches including physiology and molecular genetics in relation to agronomy. This review will focus on crop species that do not fix nitrogen under symbiotic conditions. Symbiotic N fixation will not be covered in this review, although it has been estimated that it contributes approximately half of the amount of N applied in inorganic N fertilizers (Smil, 2006) and it may represent an ecological alternative to inorganic N fertilization in several areas in the world (Shantharam and Mattoo, 1997). A number of reviews focusing on selection criteria, breeding methods, and genetic engineering approaches have covered future improvements in legume crops that will be beneficial not only to the environment and farmers but also to consumers in both developed and developing countries (Hirel et al., 2003; Ranalli, 2003).

Fertilizer recovery is the result of the balance between crop N uptake and N immobilization by microbial processes in soils of different compositions. Therefore, the concept of the NUE of a crop should also be considered as a function of soil texture, climate conditions, interactions between soil and bacterial processes (Walley *et al.*, 2002; Burger and Jackson, 2004), and the nature of organic or inorganic N sources (Schulten and Schnitzer, 1998). However, due to the complexity of these factors and their interactions, this aspect of N assimilation in plant growth and productivity will not be covered here.

Current knowledge and prospects for future plant improvement under various N fertilization conditions are explored, taking into account both the plant biological constraints and the species specificities.

### The main steps in plant N economy and their species specificities

In most plant species examined so far, the plant life cycle with regard to the management of N can be roughly divided into two main phases occurring successively in some species or overlapping in others (Fig. 1). During the first phase, i.e. the vegetative phase, young developing roots and leaves behave as sink organs for the assimilation of inorganic N and the synthesis of amino acids originating from the N taken up before flowering and then reduced via the nitrate assimilatory pathway (Hirel and Lea, 2001). These amino acids are further used for the synthesis of enzymes and proteins mainly involved in building up plant architecture and the different components of the photosynthetic machinery. Notably, the enzyme Rubisco (ribulose 1,5-bisphosphate carboxylase) alone can represent up to 50% of the total soluble leaf protein content in C<sub>3</sub> species (Mae et al., 1983) and up to 20% in C<sub>4</sub> species (Sage et al., 1987). Later on, at a certain stage of plant development generally starting after flowering, the remobilization of the N accumulated



Fig. 1. Schematic representation of nitrogen management in various crops. (A) During vegetative growth, N is taken up by the roots and assimilated to build up plant cellular structures. After flowering, the N accumulated in the vegetative parts of the plant is remobilized and translocated to the grain. In most crop species a substantial amount of N is absorbed after flowering to contribute to grain protein deposition. The relative contribution of the three processes to grain filling is variable from one species to the other and may be influenced under agronomic conditions by soil N availability at different periods of plant development, by the timing of N fertilizer application, and by environmental conditions such as light and various biotic and abiotic stresses. (B) The relative contribution (%) of N remobilization and postflowering N uptake in different crops. Rice utilizes mostly ammonium as an N source, whereas the other crops preferentially use nitrate. Note that in the case of oilseed rape, a large amount of the N taken up during the vegetative growth phase is lost due to the falling of the leaves.

by the plant takes place. At this stage, shoots and/or roots start to behave as sources of N by providing amino acids released from protein hydrolysis that are subsequently exported to reproductive and storage organs represented, for example, by seeds, bulbs, or trunks (Masclaux et al., 2001). However, for N management at the whole-plant or organ level, the arbitrary separation of the plant life cycle into two phases (Masclaux et al., 2000) remains rather simplistic, since it is well known that, for example, N recycling can occur before flowering for the synthesis of new proteins in developing organs (Lattanzi et al., 2005). In addition, during the assimilatory phase, the ammonium incorporated into free amino acids is subjected to a high turnover, a result of photorespiratory activity, as it needs to be immediately reassimilated into glutamine and glutamate (Hirel and Lea, 2001; Novitskaya et al., 2002). Therefore, the photorespiratory flux of ammonium, which at least in C<sub>3</sub> plants can be 10 times higher compared with that originating from the nitrate reduction, is mixed with that channelled through the inorganic N assimilatory pathway (Novitskaya et al., 2002). Furthermore, a significant proportion of the amino acids is released following protein turnover concomitantly with the two fluxes of ammonium (from assimilatory and photorespiratory fluxes) (Malek et al., 1984; Gallais et al., 2006). The occurrence of such recycling mechanisms introduces

another level of complexity in the exchange of N within the pool of free amino acids. The co-existence of these different N fluxes has led to reconsideration of the mode by which N is managed from the cellular level to that of the whole plant (Hirel and Gallais, 2006; Irving and Robinson, 2006).

In wheat (Triticum aestivum L.), 60-95% of the grain N comes from the remobilization of N stored in roots and shoots before anthesis (Palta and Fillery, 1995; Habash et al., 2006). A less important fraction of seed N comes from post-flowering N uptake and N translocation to the grain. After flowering, both the size and the N content of the grain can be significantly reduced under N-deficient conditions (Dupont and Altenbach, 2003). However, it is still not clear whether it is plant N availability (including the N taken up after anthesis and the remobilized N originating from uptake before anthesis) or storage protein synthesis that limits the determination of grain yield in general and grain protein deposition in particular (Martre et al., 2003). In winter wheat grown under agronomic conditions, N applications are performed in a split way and are generally calculated by the total N budget method (Meynard and Sebillote, 1994; Kichey et al., 2007). Until tillering, the plant demand is usually satisfied by the N available from the soil. Therefore, three applications are generally performed: one at tillering (50–80 kg ha<sup>-1</sup>), one at the beginning of stem elongation (around 50 kg  $ha^{-1}$ ), and one at the second node stage (40–50 kg  $ha^{-1}$ ). In wheat, it has been shown that the SPAD meter has potential for predicting grain N requirements. However, its utilization is limited to certain varieties (Lopez-Bellido et al., 2004).

Compared with wheat, a similar pattern of N management was observed during the life cycle of rice (Oryza sativa L.), although the plant preferentially utilizes ammonium instead of nitrate. The remobilized N from the vegetative organs accounts for 70-90% of the total panicle N (Mae, 1997; Tabuchi et al., 2007). In the field, the amount of N fertilizer applied in the form of ammonium or urea to sustain the early growth phase and tillering ranges from 40 to 110 kg N ha<sup>-1</sup>. Additional top-dressing N (15–45 kg ha<sup>-1</sup>) is then applied between the panicle primordia initiation stage and the late stage of spikelet initiation, and appears to be the most effective for spikelet production. After this period, N uptake has very little influence on sink size. During the grain-filling stage, it is the N accumulated in leaf blades before flowering that is in large part remobilized to the grain and that contributes to grain N protein deposition (Mae, 1997). Some field trials revealed that it is rather difficult to synchronize N supply with seasonal plant demand (Cassman et al., 1993). However, in some cases, the use of chlorophyll meter (SPAD)-based N fertilization treatments may help to monitor the leaf N status to guide fertilizer-N timing on irrigated rice (Peng et al., 1996).

In maize (Zea mays L.), 45-65% of the grain N is provided from pre-existing N in the stover before silking. The remaining 35–55% of the grain N originates from post-silking N uptake (Ta and Weiland, 1992; Rajcan and Tollenaar, 1999b; Gallais and Coque, 2005). Under field growth conditions, only a single application of N fertilizer is generally performed at sowing, ranging from 100 to 240 kg N ha<sup>-1</sup> to attain optimal yield depending both on the genotype and on soil residual N (Plénet and Lemaire, 2000). However, in some cases, it can be fractionated by applying the N fertilizer at sowing and at the 5-6 leaf stage (Plénet and Lemaire, 2000). In maize, chlorophyll meters provide a convenient and reliable way to estimate leaf N content during vegetative growth (Chapman and Baretto, 1997) and over a large time scale after anthesis (Dwyer et al., 1995), which may be a way to monitor N fertilizer applications. However, the correlation between chlorophyll content and grain yield is not always significant (Gallais and Coque, 2005).

In oilseed rape, the requirement for N per yield unit is higher than in cereal crops (Hocking and Strapper, 2001). Oilseed rape has a high capacity to take up nitrate from the soil (Lainé et al., 1993), and thus to accumulate large quantities of N that is stored in vegetative parts at the beginning of flowering. However, in oilseed rape, yield is half that of wheat, due to the production of oil, which is costly in carbohydrate production. Since oilseed rape N content in the seed is not much higher (3% in oilseed rape and 2% in wheat on average), an important part of the N stored in the vegetative organs is not used. Moreover, a large quantity of N is lost in early falling leaves (Malagoli et al., 2005) and the amount of N taken up by the plant during the grain-filling period apparently remains very low (Rossato et al., 2001). After sowing, to allow maximum growth at the beginning of winter, N fertilizer application may be necessary when there a shortage in soil N availability. Fertilization is necessary again in spring during the full growth period when large amounts of N are required and up to 70% of the plant N requirement must be satisfied. This is achieved by the application of N fertilizers, which may be fractionated according to the size of the plant and yield objectives (Brennan et al., 2000). Peak seed yield usually occurs when 180–200 kg N ha<sup>-1</sup> are applied (Jackson, 2000).

The main steps in N assimilation in rice, wheat, maize, and oilseed rape are summarized in Fig. 1.

## Is productivity compatible with low N fertilization input?

A prerequisite to maintaining high crop productivity under lower N fertilization input is to determine whether it is possible to select for genotypes that are adapted to low or high N fertilization, or that can perform well under both N fertilization conditions.

The majority of the selection experiments at low N input have up to now been performed on maize, for which genetic variability compared with other crops is high in both tropical and temperate genotypes (Wang et al., 1999). This has led to the proposal of the concept of critical N concentration (%Nc), corresponding to the minimum %N in shoots required to produce the maximum aerial biomass at a given time of plant development (Plénet and Lemaire, 2000). In Europe, Presterl et al. (2003) showed that it is possible to select genotypes under low N fertilization conditions, although there was a significant reduction in yield. Despite this reduction in yield, these authors showed that a direct selection under low N fertilization input would be more effective than an indirect selection under high N fertilization input. The same conclusions were drawn by Bänziger et al. (1997), when a panel of tropical maize lines was studied using the condition that the decrease in yield was not lower than 43%. In some cases, it has been reported that the genotypes selected under low N fertilization input are not truly adapted to N-rich soils (Muruli and Paulsen, 1981). Gallais and Coque (2005) suggest that when the plant material performs relatively well under low N input, it should be selected under N deficiency conditions for which yield reduction does not exceed 35-40%.

Obtaining a satisfactory minimum yield under low N fertilization conditions is therefore one of the most difficult challenges for maize breeders. Instead of developing blind breeding strategies, as was carried out in the past, further research needs to be performed to explain, for example, why certain maize varieties originating from local populations have a better capacity to absorb and utilize N under low N fertilization conditions (Toledo Marchado and Silvestre Fernandes, 2001) whereas others do not (Lafitte *et al.*, 1997). This will allow the identification of the morphological (roots traits in particular), physiological, and molecular traits that are associated with adaptation to N-depleted soils.

In parallel, whole-plant physiological studies (Hirel *et al.*, 2005*a*, *b*) combined with <sup>15</sup>N-labelling experiments (Gallais *et al.*, 2006) preferably performed in the field should be undertaken. These experiments will allow the identification of some of the key molecular and biochemical traits, and NUE components that govern the adaptation to N-depleted environments before and after the grain filling in lines or hybrids exhibiting variable capacities to take up and utilize N (Rajcan and Tollenaar, 1999*b*; Martin *et al.*, 2005).

To investigate in more detail the genetic control of maize productivity under low N input, correlation studies between the different components of NUE and yield using different genotypes or populations of recombinant inbred lines (RILs) have been carried out. The aim of these studies was to identify NUE components, chromosomal regions, and putative candidate genes that may control

yield and its components directly or indirectly when the amount of N fertilizers provided to the plant is varied. Such an approach allowed Moll et al. (1982) to show that with high N fertilization, differences in NUE in a range of experimental hybrids were largely due to variation in the NupE, whereas with low N supply it was the NutE. Bertin and Gallais (2001) found that most of the chromosomal regions for yield, grain composition, and traits related to NUE detected at low N input corresponded to quantitative trait loci (QTLs) detected at high N input, whereas Agrama et al. (1999) detected more QTLs at low N input. These quantitative genetic studies confirmed that, in maize, variation in the utilization of N including remobilization at low N input was greater than the variation of N uptake before or after flowering, whereas it was the opposite at high N input (Bertin and Gallais, 2000; Gallais and Coque, 2005). Interestingly, comparison of N uptake capacities of maize and sorghum under contrasting soil N availability showed that under non-limiting N supply, the two crops have similar N uptake, while under severe N limitation the N uptake capacity of sorghum is higher than that of maize (Lemaire et al., 1996). The reason for this difference is unclear, but it could be due to a more developed and branched root system for sorghum as compared with maize. It would therefore be interesting to identify in sorghum which components of the N uptake system are involved and to find out if they can be used to improve N uptake capacity in maize and possibly other crops under N-limiting conditions.

In other species, such as bread wheat and rice, studies are currently being performed to identify key traits related to plant performance at low N input (Kichey *et al.*, 2006, 2007) and to localize chromosomal regions and genes involved in tolerance to N deprivation (Laperche *et al.*, 2007). When the adaptation and performance of bread wheat were evaluated under conditions of low N fertilization, it was found that modern cultivars were more responsive to N in terms of economic fertilizer rates compared with old cultivars (Ortiz-Monasterio *et al.*, 1997).

Le Gouis *et al.* (2000) confirmed that there is a genetic variability for grain yield at a low N level and that the genotype×N level interaction is significant. They also showed that N uptake explained most of the variation for NUE at low N and of the interaction for grain yield. As for maize, it has been shown that a direct selection under low N fertilization would be more efficient in wheat (Brancourt-Hulmel *et al.*, 2005). In more recent studies performed on wheat double haploid lines (DHLs), direct selection under low N fertilization conditions was also proposed (Laperche *et al.*, 2006) as well from a wide range of soil N availability (An *et al.*, 2006). Correlation studies revealed that under low N availability, it would be easier to select for traits related to plant or grain N protein content rather than yield *per se*. In another recent report,

the finding that specific QTLs for yield were detected under low N fertilization conditions suggests that it may be possible to improve yield stability by combining QTLs related to yield that are expressed in low N environments (Quarrie *et al.*, 2005).

As for other cereals, significant differences were obtained for N uptake and efficiency of use in different rice genotypes, N uptake being one of the most important factors controlling yield (Singh *et al.*, 1998). The potential importance of non-symbiotic N fixation in rice, together with the possibility of enhancing nitrification efficiency in rice paddy fields, has also been emphasized (Britto and Krunzucker, 2004). A preliminary analysis of a rice RIL population for tolerance to low amounts of N fertilization showed that most of the QTLs related to shoot and root growth at the seedling stage were different under low and high N fertilization conditions (Lian *et al.*, 2005).

In oilseed rape, there is a paucity of data on the genetic variability for NUE at low N fertilization input. In spring rape, it has been shown that cultivars with the lowest yields at the lowest N concentration generally responded more markedly to increased N application rates than cultivars with a higher yield at high N supply (Yau and Thurling, 1987). This is presumably due to a greater ability for uptake and translocation of N (Grami and LaCroix, 1977). More recently, in spring canola differences in NUE were found resulting in a greater biomass production (Svecnjak and Rengel, 2005) and due to differences in the root to shoot ratio and harvest index. However, no major impact on plant biomass, N uptake, and seed yield were found across two contrasting N treatments (Svecnjak and Rengel, 2006). These observations confirmed earlier findings showing that there was no interaction between QTLs for yield and N treatments (Gül, 2003). As recently reviewed by Rathke et al. (2006), it is clear that to improve seed yield, oil content, and N efficiency in winter oilseed rape the use of N-efficient management strategies is required, including the choice of variety and the form and timing of N fertilization adapted to the site of application.

Although more work is required to understand better the genetic basis of NUE in crop plants, attempts have been made to identify individual genes or gene clusters that are responsible for the variability of this complex trait. A limited number of candidate genes have already been identified using maize (Gallais and Hirel, 2004; Martin *et al.*, 2006) and rice (Obara *et al.* 2001; Tabuchi *et al.* 2005) as a model species.

In maize, Hirel *et al.*, (2001) have highlighted the putative role of glutamine synthetase (GS) in kernel productivity using a quantitative genetic approach, since QTLs for the leaf enzyme activity have been shown to coincide with QTLs for yield. One QTL for thousand kernels weight was coincident with a GS (*Gln1-4*) locus, and two QTLs for thousand kernel weight and yield were

coincident with another GS (Gln1-3) locus. Such strong coincidences are consistent with the positive correlation observed between kernel yield and GS activity (Gallais and Hirel, 2004). In higher plants, all 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 channelled through the reactions catalysed by GS (Hirel and Lea, 2001). Thus, an individual N atom can pass through the GS reaction many times (Coque et al., 2006), following uptake from the soil, assimilation, and remobilization (Gallais et al., 2006) to final deposition in a seed storage protein. As such, the hypothesis that in cereals the enzyme is one of the major checkpoints in the control of plant growth and productivity has been put forward on a regular basis (Miflin and Habash, 2002; Hirel et al., 2005b; Tabuchi et al., 2005; Kichey et al., 2006). However, whether this checkpoint may be more efficient under low and high N fertilization regimes has never been assessed, since all the experiments for candidate gene detection were performed at high N input (Hirel et al., 2001; Obara et al., 2001).

Very recently the roles of two cytosolic GS isoenzymes (GS1) in maize, products of the Gln1-3 and Gln1-4 genes (Li et al., 1993), were further investigated by studying the molecular and physiological properties of *Mutator* insertion mutants. The impact of the knockout mutations on kernel yield and its components was examined in plants grown under suboptimal N feeding conditions (Martin et al., 2006). The phenotype of the two mutant lines was characterized by a reduction of kernel size in the gln1-4 mutant and by a reduction of kernel number in the gln1-3 mutant. In the gln1-3/1-4 double mutant, a cumulative effect of the two mutations was observed. In transgenic plants overexpressing *Gln1-3* constitutively in leaves, an increase in kernel number was observed, thus providing further evidence that the GS1-3 isoenzyme plays a major role in controlling kernel yield under high N fertilization conditions. The ear phenotype of the three GS mutants and the GS-overexpressing lines was examined when the plants were grown under N-limiting conditions. As expected, a strong reduction in kernel number was observed in the wild type when N was limiting (Below, 2002). The three mutants, grown under the same Nlimiting conditions (N<sup>-</sup>), did not produce any kernels (Fig. 2A). In N<sup>-</sup>, the two GS-overexpressing lines still produced more kernels but, compared with the corresponding null segregants, did not perform any better than when N was not limiting (N<sup>+</sup>) (Fig. 2B). These results therefore strongly suggest that, in maize, GS controls kernel yield whatever the N application conditions. The constitutive nature of the enzyme whatever the N nutrition was also highlighted by the identification of the N-responsive chromosomal region following recurrent selection (Coque and Gallais, 2006). The finding that in both maize (Hirel



**Fig. 2.** Phenotypes of maize ears in GS1-deficient mutants and overexpressing lines. (A) Ears of wild type (WT), *gln1-3*, *gln1-4*, and *gln1-3/gln1-4* mutants harvested at maturity and grown under suboptimal N conditions (N<sup>+</sup>) or under N-limiting conditions (N<sup>-</sup>). (B) Ears of WT null segregants and T4 transgenic lines (lines 1 and 9) overexpressing the *Gln1-3* cDNA. Maize plants (line FV2) were harvested at maturity and grown under suboptimal N conditions (N<sup>+</sup>) or under N-limiting conditions (N<sup>-</sup>). Plant growth conditions (N<sup>+</sup>) or under N-limiting conditions (N<sup>-</sup>). Plant growth conditions were essentially the same as those previously described by Hirel *et al.* (2005*a*) and Martin *et al.* (2006).

*et al.*, 2005*b*) and wheat (Kichey *et al.*, 2006), GS enzyme activity is representative of the plant N status regardless of the developmental and N fertilization conditions further supports this conclusion.

Although a large number of studies have been devoted to GS because of its central role in N assimilation and

recycling, further work is necessary to identify whether other root and shoot enzymes or regulatory proteins (Yanagisawa et al., 2004) could play a specific role under low N availability. These include those directly related to N metabolism or intervening at the interface between carbon and N metabolism during plant growth and development (Krapp and Truong, 2005). In a recent report by Coque and Gallais (2006), strategies to achieve this task have been envisaged, although it appears that most of the genes expressed under stress conditions (including N stress) are constitutive but may be differentially regulated under adverse conditions. Altogether, the studies performed on maize suggest that some of the genes involved in the control of yield and its components may be different from those related to the adaptation to N deficiency. It will therefore be necessary to identify genomic regions responding specifically to an N stress and isolate, via positional cloning, the gene(s) involved in the expression of the trait, as was achieved for tolerance to drought stress in maize (Tuberosa and Salvi, 2006). It is very likely that the occurrence of epistatic interactions between genes (Li et al., 1997) under low or high N input and possibly the presence of non-shared genes within the genome of different genotypes (Brunner et al., 2005) will complicate gene identification and cloning. However, the recent progress made in sequencing and mapping of large genomes will probably help to decipher part of this complexity (http://www.maizegenome.org/; http:// www.wheatgenome.org/; http://www.brassica.info/). Whether the populations available for different crop species are appropriate to identify these genes also remains open to discussion. The fact that most of the lines used to produce populations for QTL studies or cultivated hybrids were selected under high N fertilization input (Gallais and Coque, 2005) needs to be carefully considered. Therefore, to circumvent this problem, it may be necessary to develop specific breeding programmes and QTL approaches using parental lines and populations originating from different areas of the world that have been adapted to a wide range of environments (climate, photoperiod, water availability, flowering precocity, soil properties, etc.).

#### Importance of the root system

The roots are central to the acquisition of water and mineral nutrients including N. Therefore, improving our understanding of the relationship between plant growth, plant productivity, and root architecture and dynamics under soil conditions is of major importance (Whu *et al.*, 2005). Among the morphological traits associated with the adaptation to N-depleted soils, the qualitative and quantitative importance of the root system in taking up N under N-limiting conditions has been pointed out in several

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studies (Guingo et al., 1998; Kamara et al., 2003; Coque and Gallais, 2005).

One of the main difficulties in evaluating the influence of the size, the volume, and the root architecture system on NupE and traits related to yield or grain N content is to remove the entire intact root system from soil when plants are grown under agronomic conditions (Guingo et al., 1998; Kondo et al., 2003). To solve this problem, alternative techniques have been developed under controlled environmental conditions using either 'rhizotrons' (Devienne-Barret et al., 2006; Laperche et al., 2007), artificial soil (Wang et al., 2004), or hydroponic culture systems (Tuberosa et al., 2003). Consequently, there are only a limited number of reports describing the response of the root morphology of cereals to different levels of N fertilization (Kondo et al., 2003; Wang et al., 2004), and there are even fewer studies in which the importance of the root system was investigated in relation to N supply, biomass production, and vield (Mackav and Barber, 1986). For example, it has been shown that the morphology of the root system may be influenced by a locally restricted nitrate supply (Sattelmacher and Thoms, 1995; Zhang and Forde, 1998) and that N application rates affect various essential components of root morphology such as length, number of apices, and frequency of branching (Drew and Saker, 1975; Maizlich *et al.*, 1980). However, it is important to bear in mind, as pointed out by Wiesler and Horst (1994), that N uptake conditions in the field may be non-ideal due to the irregular distribution of roots and nitrate and to limited root-soil contact and differences between root zones in uptake activity. Consequently, studies of the response of roots to soil physical conditions should be undertaken in parallel in order to develop realistic models to describe the mechanisms controlling growth in response to soil structure and N availability (Bengough et al., 2006).

The first study in which the genetic analysis of root traits was investigated showed that in a maize RIL population used to study the genetic basis of NUE (Gallais and Hirel, 2004), there is a weak but significant genetic correlation between some root traits, biomass production, and yield under suboptimal N feeding conditions (Guingo et al., 1998). However, further analysis of these data revealed that there is a negative correlation between yield and root number particularly at low N input (Gallais and Coque, 2005). This observation can be interpreted as there being a competition between the two sinks represented by the roots and the shoots when N resources are limited, an hypothesis previously put forward concerning the competition between N assimilation in roots and N processing in shoots (Oaks, 1992). In more recent studies, a similar approach was carried out on both wheat and maize in order to identify genomic regions involved in root architecture and the relationship with N assimilation under low N fertilization input. Coincidences with QTLs for traits related to NUE were detected (Laperche *et al.*, 2007), indicating that such a quantitative genetic approach holds promise for further identification of genomic regions involved in the control of plant adaptation to N deficiency under agronomic conditions. A recurrent selection programme for the adaptation of maize at low N input showed that root architecture would be of major importance for grain yield setting, whatever the amount of N fertilizer applied (Coque and Gallais, 2006).

Further work is necessary to ascertain the role of root architecture in the expression of yield and its components, taking into account the species specificities in terms of NupE and duration of N uptake before and after flowering. The finding that, in maize, N uptake is less important at low N supply, whereas it is the reverse in wheat, needs to be considered. Taking into account the capacity of a given genotype to absorb N before or after flowering will also be essential. Figure 3 illustrates the genetic variability existing for root architecture in selected maize lines representative of American and European diversity (Camus-Kulandaivelu et al., 2006) which could be exploited for a better understanding of the control of NupE. In addition, the use of mutants specifically affected in root development like those isolated in maize will probably help to expand further our knowledge of N acquisition by root crops (Hochholdinger et al., 2004). The availability of a limited supply of N during these two periods will also be important since, whatever strategy is developed by the plant for capturing the maximum amount of N, its availability and accessibility in the soil will in turn become a limiting factor.

In parallel, it will be necessary to take into account the genetic control of nitrate uptake by the roots at different levels of N fertilization through the activity of the different components of the nitrate transport system in relation to root and shoot development (Zhang et al., 1999). This will establish whether or not there are common factors determining the genetic variability of root development and N uptake regardless of the requirement of the plant (Gastal and Lemaire, 2002; Harrison et al., 2004). Such adaptive regulatory control mechanisms allowing a response to a shortage in N availability may, under certain conditions, be directly controlled through the activity of the nitrate transport system itself, in a given environment (Remans et al., 2006). During the last decade, both physiological and molecular genetic studies have already demonstrated the importance of the nitrate and ammonium high affinity (HATS) and low affinity (LATS) transport systems (Glass et al., 2002; Orsel et al., 2002) in the control of N acquisition in relation to plant demand and to  $NO_3^-$  and  $NH_4^+$  availability. Although most of our present knowledge on the regulation of inorganic N absorption arose from studies performed on Arabidospis, it is likely that similar regulatory control also occurs in crops such as rice (Lin et al., 2000; Tabuchi et al., 2007), maize (Santi et al., 2003), and barley (Vidmar et al., 2000).



Fig. 3. The root architecture of different maize lines. The maize lines were selected to represent the genetic diversity of the crop (Camus-Kulandaivelu *et al.*, 2006). Note the genetic variability in the density and length of lateral roots in both the primary and lateral roots.

Interestingly, it was proposed that in maize the inducible  $NO_3^-$  transport system could be a physiological marker for adaptation to low N input (Quaggiotti *et al.*, 2003). Thus, more research is required to study the regulation of the  $NO_3^-$  and  $NH_4^+$  uptake system and further exploit its genetic variability in relation to crop demand under low or high N fertilization input. In addition, the use of models that integrate interaction of below- and above-ground plant growth as a function of N availability, taking into account the contribution of the N uptake system, will

certainly be very useful in order to understand better the interaction between roots and their environment (Wu *et al.*, 2007).

# Nitrogen use efficiency, grain composition, and grain filling

In addition to agronomic NUE (Good *et al.*, 2004; Lea and Azevedo, 2006), the N harvest index (NHI), defined as N in grain/total N uptake, is an important consideration

in cereals. NHI reflects the grain protein content and thus the grain nutritional quality (Sinclair, 1998). However, studies on identifying the genetic basis for grain composition showed that breeding progress has been limited by an apparent inverse genetic relationship between grain yield and protein or oil concentration in most cereals (Simmonds, 1995) including maize (Feil et al., 1990), wheat (Canevara et al., 1994), and oilseed rape (Brennan et al., 2000; Jackson, 2000). It is possible, however, to identify wheat lines that have a higher grain protein content than predicted from the negative regression to grain yield (Oury et al., 2003; Kade et al., 2005). It has also been demonstrated that both grain yield and grain protein respond positively to supplemental N fertilizer, and such a paradox suggests that studying the interactive effect of genotype and N availability should provide insights into the genetic and physiological mechanisms that underline the negative yield-protein relationship.

Recently, in an interesting study performed on maize hybrids derived from the Illinois high and low protein strains, it has been shown that the strong genetic control of grain composition can be modulated by the positive effect of N on reproductive sink capacity and storage protein synthesis (Uribelarrea *et al.*, 2004). This finding opens new perspectives towards breaking the negative control existing between yield and grain protein content by performing the appropriate crosses between high yielding and high protein varieties. This will also allow a better understanding of the relative contribution of N uptake and N use for grain protein deposition under low and high N fertilization conditions (Uribalarrea *et al.*, 2007).

Another aspect of grain filling in relation to N availability concerns the period before anthesis (Fig. 1), which, for example in maize, is known to be critical for translocation of carbon assimilates and kernel set (Neumann Andersen *et al.*, 2002). Moreover, the N status of the plant around 2 weeks before anthesis appears to be a determinant for the number of kernels, since it is strongly dependent on the amount of N available during this period of plant development (Below, 1987). However, there is a paucity of data on both the physiological and molecular control of this process in relation to N availability and its translocation during this critical period of ear development (Seebauer *et al.*, 2004).

Therefore, it will be necessary to identify the critical steps associated with NUE during the formation of the ear and the reciprocal regulation between the vegetative plant and the seed. This will allow the identification of physiological QTLs and genes controlling kernel set under low and high N fertilization input to evaluate their impact on grain filling (translocation of carbon). In both spring wheat (Demotes-Mainard *et al.*, 1999; Martre *et al.*, 2003) and rice (Mae, 1997), grain number is reduced in plants affected by N deficiency around anthesis and is highly dependent on the intensity and duration of N deficiency.

This observation indicates that, as in maize, N availability during the flowering period is a determinant for yield, and its genetic variability should be investigated (Martre *et al.*, 2003).

#### Photosynthesis and nitrogen use efficiency

N nutrition drives plant dry matter production through the control of both the leaf area index (LAI) and the amount of N per unit of leaf area called specific leaf N (SLN). There is therefore a tight relationship between N supply, leaf N distribution, and leaf photosynthesis and, as such, an effect on radiation use efficiency (RUE), to optimize light interception depending on N availability in individual plants or in the entire canopy (Gastal and Lemaire, 2002). Moreover, the photosynthetic NUE (PNUE), which is dependent on the level of CO<sub>2</sub> saturation of Rubisco, is another factor that needs to be taken into consideration when  $C_3$  or  $C_4$  crop species are studied. At low N availability,  $C_3$  plants have a greater PNUE and NUE than C<sub>4</sub> plants, whereas at high N, the opposite is true (Sage et al., 1987). Consequently, identifying the regulatory elements controlling the balance between N allocation to maintain photosynthesis and the reallocation of the remobilized N to sink organs such as young developing leaves and seeds in C3 and C4 species is of major importance, particularly when N becomes limiting. Therefore, the complexity of the ubiquitous role of the enzyme Rubisco in primary CO<sub>2</sub> assimilation, in the photorespiratory process, and as a storage pool for N needs further investigation to optimize NUE and particularly NupE under low fertilization input in both C<sub>3</sub> and C<sub>4</sub> species (Sage et al., 1987; Esquivel et al., 2000; Lawlor, 2002). The physiological impact of plant N accumulation with respect to an increased photosynthetic activity requires critical consideration as a supplemental investment of N in the photosynthetic machinery may be detrimental to the transfer of N to the grain and thus to final yield (Sinclair et al., 2004). In addition, the recent finding that the synthesis, turnover, and degradation of Rubisco are subjected to a complex interplay of regulation renews the concept of the importance of N use and recycling by the plant (Hirel and Gallais, 2006).

Interestingly, in maize, a number of QTLs for NUE were found to co-localize with candidate genes encoding enzymes involved in carbon assimilation, thus supporting the finding that N facilitates the utilization of carbon used for grain filling (Gallais and Coque, 2005). Whether the function of some of these genes may be important at high and low N input needs further investigation by developing a physiological quantitative genetic approach similar to that used for N metabolism in both vegetative and reproductive parts of the plant. Identifying epistatic interactions between QTLs and genes for NUE, PNUE,

and carbon metabolism should also provide a route for deciphering the complex interplay between the two major plant assimilatory pathways (Krapp and Truong, 2005).

In addition, the relationship between plant photosynthetic capacities, chlorophyll degradation during leaf senescence, and the shift from N assimilation to N remobilization (Fig. 1) has also been investigated in a number of crops by studying the impact of prolonged green leaf area duration on yield of maize (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999a, b) and other major crops (Thomas and Smart, 1993; Borrell et al., 2001; Spano et al., 2003). Attempts have also been made to identify some of the components responsible for the physiological control of the 'stay-green' phenotype particularly in relation to NUE. For example, in both Sorghum and maize, delayed leaf senescence allowed photosynthetic activity to be prolonged, which had a positive effect on the N uptake capacity of the plant. In Sorghum this enabled the plant to assimilate more carbon and use more N for biomass production (Borrel et al., 2001), whilst in maize yields were higher (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999a, b). Despite attempts to improve the characterization of the control of N uptake, N assimilation, and N recycling in plants that are stay-green, our knowledge of the fine regulatory mechanisms that control this trait still remains fragmentary (Martin et al., 2005; Rampino et al, 2006). Whether the stay-green character is beneficial in terms of NUE and yield in different crop species still remains a matter of controversy (Borrell et al., 2000; Martin et al., 2005). This is probably because most studies conducted on stay-green genotypes have not been performed on plants grown under agronomic conditions and under varying N supply. However, in a recent report, Subedi and Ma (2005) showed that the stay-green phenotype in maize was exhibited only when there was an adequate supply of N. Therefore, further investigation is required to characterize better the physiological and molecular basis of the stay-green phenotype (Verma et al., 2004) in relation to N supply, root N uptake capacity, root architecture, and leaf structure, and to determine whether such a phenotype can be beneficial when N fertilization is reduced and when water resources are limited (Borrell et al., 2000).

### Influence of nitrogen nutrition on plant development

In plants, it is well known that N availability influences several developmental processes. According to the species, the number of leaves and their rate of appearance, the number of nodes (Snyder and Bunce, 1983; Mae 1997; Sagan *et al.*, 1993), and the number of tillers (Vos and Biemond, 1992; Tràpani and Hall 1996) are reduced under N-limiting conditions. Moreover, both in spring wheat (Demotes-Mainard *et al.*, 1999; Martre *et al.*, 2003)

and in rice (Mae 1997), grain number decreases under N deficiency conditions, a process occurring during the period bracketing anthesis, which is highly dependent on both the intensity and the duration of the N stress (see section: Nitrogen use efficiency, grain composition, and grain filling). The availability of N for yield determination is also important through its direct influence on the sources (leaf area), and consequently the sinks (reproductive organs). Generally, the reduction in photosynthesis of the canopy following N starvation is due to the reduction of the leaf area (radiation interception efficiency, RIE), rather than a decrease of RUE (Lemaire et al., 2007). In grasses, the reduction of leaf area extension is due to a lower cell division in the proximal zone rather than to the final size of the cell (Gastal and Nelson, 1994). In many crops, the relationship between leaf area index (LAI) and N uptake was found to be directly proportional, whatever the environmental conditions.

In contrast, the respective contribution of RIE and RUE in the adaptation to N starvation is variable among species. For example, potato and maize have two different strategies in their response to N-limiting conditions. In potato, the leaf area is reduced and adjusted to the rate of N uptake, keeping the plant leaf-specific nitrogen ( $g \text{ Nm}^2$ ) and RUE unchanged ('potato strategy'). In maize, leaf area is almost not affected, while photosynthesis and RUE decrease ('maize strategy') (Vos and van de Putten, 1998; Vos et al., 2005). In potato, the adaptation to N limitation results exclusively in a decrease in the amount of light intercepted, the RUE remaining constant, while in maize both leaf area and RUE are decreased. Classifying species and genotypes according to both strategies merits further investigation as it may be another way for selecting crops more adapted to low N fertilization conditions.

#### What did we learn from model species?

During the two last decades, a large number of programmes have been developed worldwide using predominantly Arabidopsis thaliana as a model species to cover most of the biological facets of plant growth and development from the seed to seed (Meinke et al., 1998). To identify key components of NUE was one of the objectives of several research groups, taking advantage of the physical map and of the large genetic diversity of the species (Yano, 2001). To achieve this, transcriptome studies were undertaken in order to identify possible genes that were responsive to long-term or short-term nitrate deprivation (Wang et al., 2000; Scheible et al., 2004) and the interaction with carbon metabolism (Gutiérrez et al., 2007). A large number of differentially expressed genes were identified which may play central roles in co-ordinating the response of plants to N nutrition. However, even though a number of genes encoding plant homologues of bacterial and yeast proteins known to participate in C and N signal transduction pathways such as PII, SNF1, and TOR have been isolated, neither transgenic technology nor mutants have allowed a clear demonstration that these proteins play a similar role in plants (Hirel and Lemaire, 2005). Recently, the overexpression of DOF1, a transcription factor involved in the activation of several genes encoding enzymes associated with organic acid metabolism, revealed that both plant growth and nitrogen content are enhanced under low nitrogen conditions (Yanagisawa *et al.*, 2004). These results demonstrate that manipulating the level of expression of regulatory proteins may be a good alternative for improving NUE in crop plants, although there is no clear evidence that the transcription factor DOF1 plays the same regulatory function in cereals (Cavalar *et al.*, 2007).

Quantitative genetic studies were undertaken in parallel on the model plant Arabidopsis to identify some of the key structural and regulatory genes that may be involved in the global regulation of NUE. A number of loci associated with NUE, total, mineral, and organic N content, and biomass production under different levels and modes of N nutrition were identified (Rauh et al., 2002; Loudet et al., 2003). The fine-mapping and positional cloning of the major loci identified in these studies should provide, in the near future, a more comprehensive view of the key genes involved (Krapp et al., 2005). Whether the function of these genes will be equivalent in cereals or closely related crop species such as oilseed rape, which have during certain periods of their developmental cycle a totally different mode of N management compared with Arabidopsis (Shulze et al., 1994), needs to be carefully considered before embarking on long-term and costly field experiments. In spite of this, one of the most significant contributions of the Arabidopsis research community has been the improvement in our understanding of the relationship between N availability, N uptake, and root development (Zhang et al., 1999; Remans et al., 2006; Walch-Liu et al., 2006). Since N uptake is one of the most critical NUE components under N-limiting conditions in a number of crops, the transfer of knowledge should be relatively straightforward when the experimental procedures have been adapted to larger or structurally different root systems (Hochholdinger et al., 2004) grown under agronomic conditions.

### **Future prospects**

An approach that integrates genetic, physiological, and agronomic studies of the whole-plant N response will be essential to elucidate the regulation of NUE and to provide key target selection criteria for breeders and monitoring tools for farmers for conducting a reasoned fertilization protocol. This prospective conclusion outlines the main points that will need to be considered in order to develop an integrated research programme for discovering genes by means of a complete and extensive phenotyping, comprising agronomical, physiological, and biochemical studies on crops grown under low and high N fertilization applications. The main research tasks that will be necessary to develop can be summarized in the following way.

- A functional genomic approach consisting of a meta-(i) analysis of agronomic, physiological, and biochemical (including possibly proteomic) OTLs combined with the data obtained on large-scale transcriptome studies designed to identify N-responsive genes for further location on genetic maps. After gathering all the available data sets in the various crop species concerning the genomic regions that are specific or not specific to the response of reduced N fertilization, it will be necessary to identify the underlying candidate genes controlling the expression of traits related to NUE in relation to agronomic traits (growth and biomass production), grain yield, and possibly other traits, for example, related to water use efficiency. Taking advantage of the synteny between grasses (Ware et al., 2000; http://www.gramene.org/) or between Arabidopsis and closely related species may help to refine genetic maps and find common key genes involved in the control of NUE. Such an approach can be carried out initially with the already existing populations, although in certain cases they are not truly adapted to perform quantitative genetic studies at low N input. In the future, development of new populations with exotic strains adapted to a specific environment will probably be necessary. Another solution would be to use whole-genome scan association mapping based on linkage disequilibrium (Rafalski, 2002), using a large collection of adapted and nonadapted material with a sufficient agricultural meaning to permit a field evaluation. The functional validation of the candidate genes can then be undertaken by reverse and forward genetic approaches (Tabuchi et al., 2005; Martin et al., 2006), supplemented by candidate gene association genetics studies (Wilson et al., 2004) to identify the most favourable alleles controlling the expression of the trait of interest prior to being used for marker-assisted selection (Mohan et al., 1997). Moreover, since NUE is a complex trait, it is likely that the interaction of genes not necessarily linked to N metabolism but involved in the control of carbon assimilation and of development in more or less complex networks will have to be deciphered.
- (ii) A whole-plant molecular physiology approach should depict in a dynamic and integrated manner the regulation of N uptake, N assimilation, and N recycling, and their progression during the growth and development under varying N fertilization treatments (Hirel *et al.*, 2005*b*; Kichey *et al.*, 2006). Such integrated studies will need to be extended by

monitoring in parallel the changes in the whole spectrum of proteins and genes under different N nutrition conditions (Gutiérrez et al., 2007) in different organs harvested at various periods of plant development to increase the potential value of the physio-agronomic indicators previously identified (Hirel et al., 2005a). Although this type of approach will be time-consuming, costly, and will require a huge computational analysis when developed on populations, it will be the only way to identify genomic regions and therefore genes that control the dynamics of N management throughout the whole plant life cycle. This may be achieved by using robot-based platforms to measure multiple enzyme activities and metabolites (Gibon et al., 2004) and integrating metabolites with transcript and enzyme activity profiling (Gibon et al., 2006). Such integrated studies could be completed by employing more sophisticated techniques using, for example, <sup>15</sup>N labelling (Gallais et al., 2006; Kichey et al., 2007) to follow the genetic variability of the dynamics of N distribution within the plant, an aspect which will not be possible to attain even using the most sophisticated metabolomic techniques (Goodace et al., 2004). The recent development of microdissection techniques will also constitute an opportunity to extend these studies at the cellular level by monitoring the changes in metabolites and gene expression in the specialized organs or tissues of root and shoots (Nakazono et al., 2003). Although these types of studies have been performed on a small scale, they have provided a better understanding as to why some genotypes differ in their mode of N management in order to achieve a similar yield (Martin et al., 2005).

(iii) More sophisticated crop simulation models already used in basic and applied research should be developed. These models have already been produced by a number of groups to predict the changes in plant growth, development, and productivity in a given environment and thus to help in the management of resources such as fertilizers and water. These were restricted to the root system (Robinson and Rorison; 1983; King et al., 2003) or to the seed (Martre et al., 2003), or extended to the whole plant system in cereals (Jamieson et al., 1999; Brisson et al., 2002; David et al., 2005) and even to a range of crop and woody species (McCown et al., 1996). The use of these models may also be a way to link model cultivar parameters with simple physiological traits such as those described in the previous paragraph and thus facilitate genetic and genomic research to identify the key genes involved (Semenov et al., 2006). This may also be a way to identify simple physiological markers that are easy to measure to evaluate the physiological status of the plant under given environmental conditions (Hirel *et al.*, 2005*b*; Kichey *et al.*, 2006), thus allowing sustainable fertilizer management practices. Moreover, an interesting challenge for physiologists, agronomists, and farmers will be to set up collaborative efforts to develop easy to use diagnostic kits based on the detection of physiological or even molecular markers by taking advantage of the relatively cheap electronic and computer technology.

It has also been proposed to detect QTLs of model parameters (Reymond *et al.* 2004; Quilot *et al.*, 2005; Laperche *et al.*, 2007). The main advantages are that model parameters are less sensitive to genotype×environment interaction and more easily related to physiological processes, and it is possible to simulate the behaviour of allelic combinations that are not present in the original population.

In addition to this, modelling NUE through system biology approaches will provide in the near future an avenue to enhance integration of molecular genetics technologies in plant improvement (Hammer *et al.*, 2004), thus allowing the re-establishment of fundamental and practical research in an intimate and meaningful way (Sinclair and Purcell, 2005).

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