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The Contribution of Molecular Physiology to the Improvement of Nitrogen Use Efficiency in Crops

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Abstract

In this review, we discuss the ways in which our understanding of the genetic control of nitrogen use efficiency applied to crop improvement has been increased through the development of molecular physiology studies using transgenic plants or mutants with modified capacities for nitrogen uptake, assimilation, and recycling. More recently, exploiting crop genetic variability through quantitative trait loci and candidate gene detection has opened up new perspectives toward the identification of key structural or regulatory elements involved in the control of nitrogen metabolism for improving crop productivity. All together, these studies strongly suggest that in the near future nitrogen use efficiency can be improved both by marker-assisted selection and genetic engineering, thus having the most promise for the practical application of increasing the capacity of a wide range of economically important species to take up and utilize nitrogen more efficiently.

Key words: crops, moleculor, gentics, nitrogen, physiology, productisity

Introduction

The use of nitrogen (N) fertilizers in agriculture together with an improvement in cropping systems have provided at least in developed countries a food supply sufficient for both animal and human consumption (Cassman 1999). However, due to the detrimental impact of the overuse of N fertilizers on the biosphere such as the eutrophication of both marine and terrestrial ecosystems (Hirel et al. 2007b), the challenge for the next decades will be to accommodate the needs of the expanding world population by developing a highly productive agriculture, while at the same time preserving the quality of the environment (Dyson 1999).

To develop highly productive agriculture, it is therefore of major importance to identify the limiting steps of N recovery including those occurring both in the soil and in the crop, and their interactions (Singh et al. 2005). In this article, we present an overview on how studies combining plant physiology, plant biochemistry, and plant molecular genetics have contributed to the identification of the critical steps controlling plant N use efficiency (NUE). There are several definitions for NUE, depending on whether authors are dealing with agronomic, genetic, or physiological studies (Good et al. 2004). In this review we will use that defined by Moll et al. (1982), representing 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 utilization efficiency (NutE; the ability of the plant to use N to produce grain yield).

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How genetic manipulations contributed to the identification of limiting steps for NUE

The first attempts to identify the limiting steps of plant NUE were largely facilitated following the development of genetic engineering techniques on both model and crop species (Good et al. 2004; Sinclair et al. 2004; Hirel and Lemaire 2005). The general idea behind the use of such techniques originally used for basic research was to manipulate the expression of a protein or an enzyme involved in the nitrate or ammonia assimilatory pathways. This would then indicate which steps of the pathway are limiting NupE or NutE and thereby important in the control of plant growth and development.

For example, it has been known for a long time that nitrate is the principal N source for most wild and crop species.

Therefore, the reaction catalyzed by the enzyme nitrate reductase (NR; EC 1.6.6.1), which reduces nitrate to nitrite and constitutes the first step in the inorganic N assimilatory pathway, was originally though to be one if not the main limiting factor in the control of NUE. A large number of studies have thus been undertaken to characterize the relationship between the structure and the function of the enzyme, and its regulation by environmental factors such as light and nitrate availability (Meyer and Stitt 2001). Several attempts were made to modulate its activity through the use of genetic engineering or mutagenesis (Hirel and Lemaire 2005). Despite all these efforts, most of the authors came up with the conclusion that NR was not the main enzyme involved in the control of NutE (Andrews et al. 2004). Nevertheless, it has been recently reported that higher biomass accumulation in tubers was associated with the deregulation of NR activity in transgenic potato plants exhibiting decreased nitrate concentration in all organs (Djennane et al. 2004). Lea et al. (2004) showed that expressing constitutively high NR can lead to the formation and excretion of nitrite but also to the production of NO which may ultimately influence plant growth and development. These results indicate that in some cases, manipulating NR activity may be beneficial for both plant quality and productivity and thus may open up new perspectives toward the improvement of NUE at least in tuber crops.

In contrast, in plants with down-regulated nitrite reductase (NiR; EC 1.7.7.1) activity, the other enzyme involved in the NO_3^- reduction pathway, growth was more severely affected than in plants exhibiting decreased NR activity. Analysis of the physiological impact of the genetic manipulation showed that a decrease in net CO_2 assimilation was accompanied by a decrease in both NO_3^- uptake and reduction (Quilleré et al. 2001). However, there is still no indication that up-regulating the enzyme may be beneficial in terms of plant growth and development. Since most of the studies involving the manipulation of NR and NiR activities were performed on model species grown under controlled conditions, further work will be required to verify if at least NupE and possibly NutE are controlled by NR or NiR in roots and shoots of crops grown under various N fertilization regimes and subjected to adverse environmental constraints such as salt or water stress (Andrews et al. 2004).

Later on, the hypothesis arose that the capacity of the plant to take up N was the main checkpoint in the control of NUE. This idea shifted a large part of the research activities towards the characterization and regulation of the nitrate uptake system. At the origin, physiological studies showed that three main root nitrate transport systems exist in plants, allowing uptake of the ion from the soil (Glass and Siddiqi 1995). A low capacity, high affinity constitutive system (cHATS) allows plants that have never been exposed to nitrate to take up the ion when the external concentration is low (< 200μM). After the first exposure to nitrate, a low capacity, high affinity system (iHATS) is then induced. When the external nitrate concentration is high, it is taken up by means of a high capacity transport system exhibiting a low affinity for the ion (LATS) (Forde and Clarkson 1999; Forde 2000). Following this, the identification and functional characterization of the two main families of genes encoding putative nitrate transporters named NRT1 (LATS) and NRT2 (HATS) was undertaken (Orsel et al. 2002). For this aim, several groups exploited extensively the use of knock-out mutants and transgenic plants to assess the role of the different components of the nitrate transport system (Glass et al. 2002; Orsel et al. 2002). However, these groups soon realized that the regulatory mechanisms involved in the control of NupE were rather complex, notably following the discovery that there were compensatory mechanisms between the different classes of nitrate transporters (Gansel et al. 2001) and following the identification of a tight relationship existing between N availability, N uptake, and root development (Zhang *et al.* 1999; Remans et al. 2006; Walch-Liu et al. 2006; Lea and Azevedo 2006). Since NupE is one of the most critical NUE components under N-limiting or non-limiting conditions in a number of crops, further work is still required before the knowledge gained from these fundamental studies performed mostly on the model species *Arabidopsis* can be transferred to crops such as maize (Quaggiotti et al. 2003), which may have a different pattern of root development.

In parallel with the studies on the regulation of nitrate uptake and reduction, intensive research was undertaken by other groups to determine whether the ammonia assimilatory pathway was of major importance in the control of NUE. The discovery of the glutamine synthetase (GS, EC 6.3.1.2)/glutamate synthase pathway (GOGAT; EC 1.4.7.1) in the seventies, was at the origin of a large number of investigations to better understand the control of ammonia assimilation through the activity of the two enzymes (Miflin and Lea 1976). Since ammonia can originate not only from nitrate reduction but also internally within the plant by a variety of metabolic pathways such as photorespiration, phenylpropanoid metabolism, utilization of N transport compounds, and amino acid catabolism, from symbiotically fixed N (Hirel and Lea 2001), it was logical to think that the efficiency of ammonia assimilation could significantly contribute to the overall plant NUE and especially NutE. One of the main milestones in this field of research was the discovery of the various GS and GOGAT isoenzymes located in different cellular compartments and differentially expressed in a particular organ or cell type according to the developmental stage of the plant (Hirel and Lea 2001). Later on, a number of studies using whole plant physiology, biochemistry, and transgenic plants demonstrated that the GS isoenzymes play specific roles during the life cycle of the plant either at the organ or the cellular level, due to their differential mode of expression. In addition to the complexity found for GS and GOGAT gene and protein expression, a number of studies showed that there is a tissue-specific specialization for ammonia assimilation and recycling which can be modulated during plant development and may be in several cases, specific to the species.

This level of complexity is largely illustrated by the recent work performed on rice (Tabuchi et al. 2007), wheat (Kichey et al. 2005; 2007) and maize (Martin et al. 2006), where it appears that even though all three species are grasses, the mode of N management from the vegetative stage until the grain filling period is unique to each plant (Hirel et al. 2007b). Nevertheless, genetic manipulations and the use of mutants have provided strong evidence that in most crops examined so far, cytosolic GS isoenzymes (GS1) are involved in the efficiency of N mobilization from senescing leaves to the grain and therefore are key targets for yield improvement (Andrews et al. 2004) in a number of crops including oilseed rape (Schjoerring et al. 2001), rice (Tabuchi et al. 2005) and maize (Martin et al. 2006). Only in rice has the importance of NADH-GOGAT also been emphasized with respect to NUE, however the role of the enzyme seems to be confined to the recycling of N towards developing organs (Tabuchi et al. 2007).

In legumes, following the finding that shoot biomass production was improved in alfalfa plants treated with a molecule that specifically inhibited root GS activity (Knight and Langston-Unkeffer 1988), a number of research programs were further developed to manipulate the expression of either the root or the root nodule GS isoenzymes in both model (Hirel et al. 2003) and crop legumes (Fei et al. 2002). In some of these studies, there were some strong lines of evidence that it is possible under certain growth conditions to improve the utilization of ammonium arising from symbiotic N fixation. However, these fundamental studies were not pursued, probably because extending such a research program on a field scale would have been labor intensive, costly, and possibly unacceptable by the public.

The reaction catalyzed by the enzyme glutamate dehydrogenase (GDH; EC 1.4.1.2), which has the potential capacity to assimilate ammonia, was originally thought to be the main source of glutamate in plants until the discovery of the GS/GOGAT assimilatory pathway (Sims et al. 1968). Although the vast majority of the experiments have shown that GDH operates in the direction of glutamate deamination to provide organic acids when the cell is C-limited, the exact physiological function of the enzyme remains to be fully elucidated (Skopelitis et al. 2006). Nevertheless, improved stress tolerance has been reported in corn plants over-expressing the bacterial NADPH-dependent GDH enzyme (Lightfoot et al. 2007). It is therefore attractive to think that the enzyme may be an interesting breeding target for stabilizing crop productivity through a better tolerance to various stresses (Schmidt and Miller 1999; Dubois et al. 2003).

There are strong lines of evidence that at least in the model plant *Arabidospis*, the enzyme asparagine synthetase (AS; EC 6.3.5.4), which catalyzes the synthesis of asparagine, a molecule used for N long-distance transport from source to sink organs in a number of higher plants (Lea et al. 2007), plays a major role in controlling both the quality and quantity of N resources in the seed (Lam et al. 2003). However, it remains to be demonstrated that AS plays the same role in grain crops, in which the amount of asparagine transported to sink organs appears to be determinant in the control of storage protein content (Lohaus et al. 1998). A few years ago, a US patent was released by Good et al. (2000) describing the use of oilseed rape plants transformed with alanine amino transferase (AaT; EC 2.6.1.1). These plants showed increased yield under N-limited conditions in both controlled and field conditions indicating that the role of the enzyme during seed filling (Murooka et al. 2002) needs to be further investigated in other crops such as cereals.

Exploiting genetic variability for improving NUE

Exploiting interspecific and intraspecific genetic variability is another way to validate the performance of crops as well as to identify key physiological functions involved in the control of NUE.

Concerning the exploitation of the inter-specific variability for NUE, introducing the ability to fix atmospheric N_2 into non-legume crops has always been a dream for a large number of plant biologists. However, such attempts have so far been unsuccessful due to the complexity and the specificity of the interactions occurring during the establishment of the legume-*Rhizobium* symbiosis (Kolchinsky et al. 1994; de Bruijn et al. 1995). More reasonably, the inter-specific variability found in N uptake (Hirel and Lemaire 2005) or primary N assimilation between C_3 and C_4 plants (Oaks 1994) could possibly be exploited in the future. However, mostly due to the leaf structural differences and specific carbon and N metabolic compartmentation between the two groups of species (Nelson and Langdale 1992; Becker et al. 1993), there is still a long way to go before we can transfer to a C_3 plant the capacity of a C_4 plant to use N more efficiently, at least in the shoots. In roots, the N-uptake capacities of sorghum are higher than in maize and many other cereals (Lemaire et al. 1996). It would therefore be interesting to identify in sorghum which components of the N-uptake system are involved in the control of root architecture and to establish if they can be used to improve N-uptake capacity in maize and possibly other crops under N-limiting conditions.

Since NUE is a complex agronomic trait controlled by a large number of genes, this has prompted a number of groups to exploit its intra-specific genetic variability in a more targeted way taking advantage of the recent developments in quantitative genetics. Moreover, relying only on the results obtained by modifying the expression of a single gene or a limited number of genes at a particular stage of plant development has for most of the time not been totally satisfactory, as plant growth and N nutrition interact in a complex way and are constantly changing from the vegetative stage to the grain-filling period.

Deciphering the genetic basis of complex traits such as NUE requires the linking of physiological function and agronomic traits to DNA markers (Prioul et al. 1997; Hirel et al. 2007a). This is usually achieved in two steps, first consisting of mapping segregating lines with DNA markers by linking each marker to

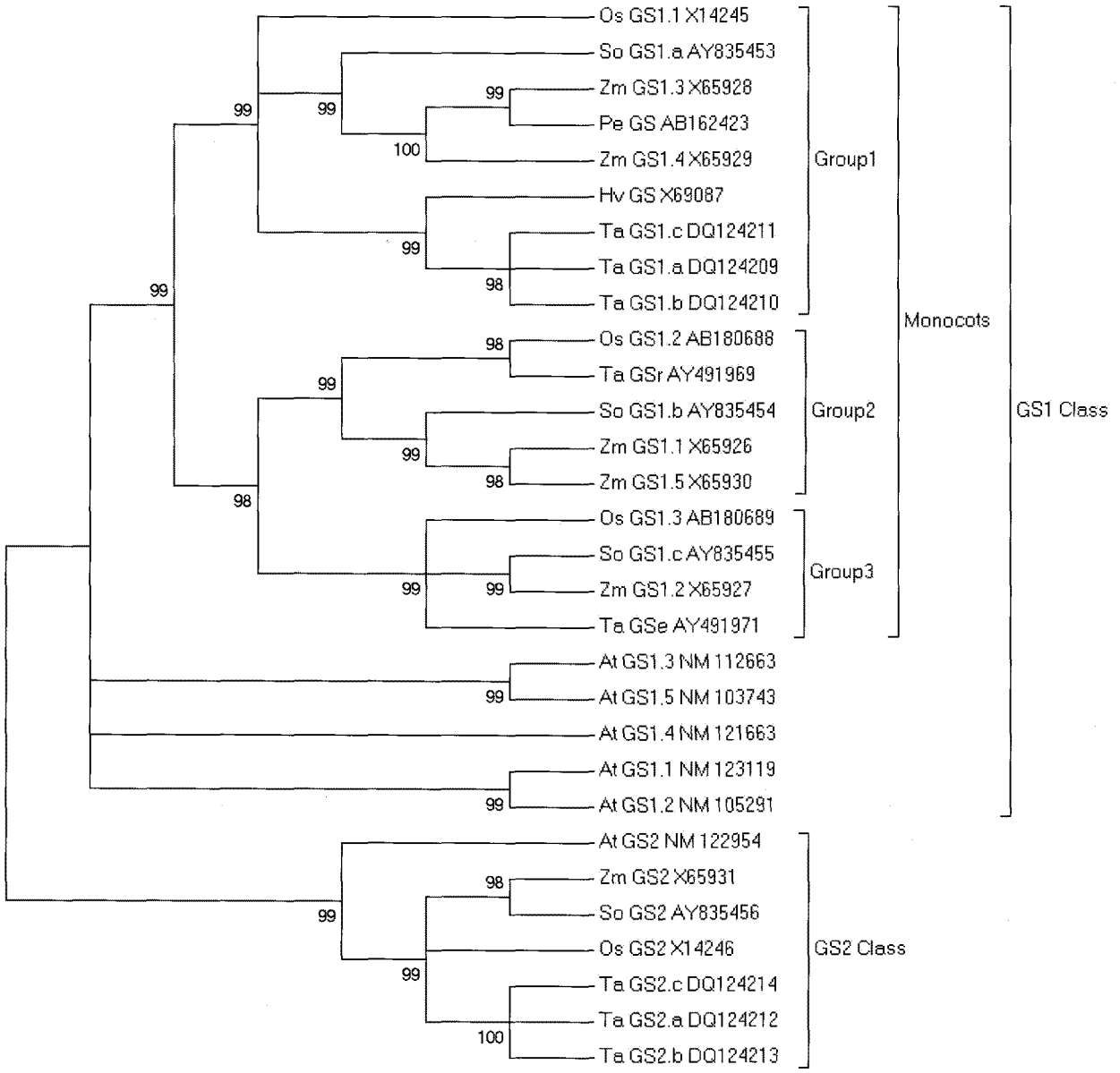

Fig. 1. Bayesian inferred tree of the GS1 monocot nucleotide sequences obtained by using Mr. Bayes software (Ronquist and Huelsenbeck 2003), (500,000 generations and burning period = 100,000). Abbreviations for species: *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm), *Hordeum vulgare* (Hv), *Triticum aestivum* (Ta), *Saccharum officinarum* (Sa), *and Phyllostachys edulis* (Pe). Accession numbers are indicated at the right of the gene nomenclature.

at least another one to produce a saturated genetic map and then in finding a statistical relationship between the quantitative trait value at each marker (Quantitative Trait Loci, QTL). A significant statistic relationship means that in the vicinity of the markers there is at least one gene controlling part of the trait variability. When the DNA marker corresponds to a gene of known function, co-locations with a QTL related to the same function can be investigated. In such a case, the gene becomes a candidate gene related to the same function that can then be validated using several complementary approaches such as forward and reverse genetics, association genetics, or positional cloning (Hirel et al. 2007a).

The first attempt to identify QTLs for NUE in maize in relation to plant development and grain production was performed on maize recombinant inbred lines (RILs) grown in the field under low- and high-N input. A significant genotypic variation was found for several phenotypic traits related to plant growth and yield, such as leaf area, plant height, grain number and production, allowing the detection of several genomic regions corresponding to these traits. In addition, a number of QTLs for traits determining the response of maize to low-N conditions were located on the RFLP map (Agrama et al. 1999; Bertin and Gallais 2001).

These agronomic QTL approaches were soon followed by more detailed investigations in which, in addition to agronomic traits, physiological functions were associated to DNA markers

in maize (Hirel et al. 2001; Gallais and Hirel 2004), rice (Obara et al. 2001) and wheat (Habash et al. 2007). Such studies were undertaken because in previous investigations genetic variation for metabolites and enzymes activities related to N assimilation and recycling were detected (Masclaux et al. 2001).

Several co-localizations between physiological traits, agronomic traits, and candidate genes were identified in the three species all related directly or indirectly to the capacity of the plant to take up or utilize N at a particular stage of its developmental cycle. The most exciting results were found in the three cereals, where QTLs for yield and its components coincided with either genes encoding cytosolic GS or leaf GS activity, which could, at least partially, explain variations in yield. In maize, Hirel et al. (2001) found that one QTL for thousand kernels weight was coincident with GS1.4 (*Gln1-4* locus) and two QTLs for thousand kernel weight and yield were coincident with GS1.3 (*Gln1-3* locus). Such strong coincidences are consistent with the positive correlation observed between kernel yield and GS activity (Gallais and Hirel 2004). In rice, a co-localization of a QTL for GS activity and a QTL for one-spikelet weight was identified (Obara et al. 2001). In wheat, QTLs for GS activity were co-localized with those for grain N content (Habash et al. 2007). These three studies confirmed previous hypotheses on the key role of the enzyme GS in plant productivity that arose from either whole plant physiological studies, or genetic manipulations (Andrews et al. 2004; Good et al. 2004).

Both in rice and maize, the role of cytosolic GS during grain filling was confirmed by studying the molecular and physiological properties of insertion mutants. In maize, the phenotype of the two mutant lines was characterized by a reduction in kernel size in the *gln1-4* mutant and by a reduction in kernel number in the *gln1-3* mutant. In the *gln1-3/1-4* double mutant, a cumulative effect of the two mutations was observed. The role of *Gln1-3* in controlling grain number production was further established in transgenic plants over-expressing *Gln1-3* (Martin et al. 2006). In rice, a severe reduction in grain filling was also observed in a mutant deficient in the gene encoding cytosolic GS, *OsGS1;1* (Tabuchi et al. 2005).

An elegant way to increase the value of a quantitative genetic approach is to determine whether there is any relationship between the phenotypic evaluation of agronomic traits and the phylogeny of a putative candidate gene. This approach is particularly relevant when we are dealing with a multigene family, which allows transfer of information concerning the function of a member of the multigene family in question, from a model to a crop species. The closer the homologous gene sequences are across species, the more likely the function of the gene will also be conserved across species. To illustrate this approach, we have analyzed the phylogeny of the *GS1* multigene family in grasses using twenty-three sequences found in the NCBI database (http://www.ncbi.nlm.nih.gov/Entrez/). Sequences exhibiting a close homology to *Arabidopsis GS1* were found in different monocots species such as rice, maize, wheat, barley, sugarcane, and bamboo. In addition, monocot sequences homologous to *GS2* from *Arabidopsis* were also used in the analysis for rooting the phylogenetic tree. The nucleic sequences have been aligned manually using the BioEdit software (Hall 1991). By using the Mr. Bayes software based on the Bayesian interference method (Ronquist and Huelsenbeck 2003), a phylogenic tree of the plastidic and cytosolic multigene families in monocots was obtained. The topology of the tree confirms the organization of *GS* sequences into two classes corresponding to *GS1* and *GS2* genes (Fig. 1). Monocot *GS1* genes are grouped into a cluster in which the Arabidopsis gene is not included. This observation indicates that *GS1* genes in cereals and Arabidopsis have evolved independently. In addition, we observed that *GS1* genes from monocots could be classified into three distinct groups, depending on the grass species examined. This observation is consistent with the occurrence of three members in the *GS1* multigene family in an ancestor common to all grasses. Group 1 is represented by *GS1.3* and *GS1.4* from maize, and *GS1.1* from rice. In the two species, these three genes are mainly expressed in leaf blades and play a major role in the control of grain yield (Tabuchi et al. 2005; Martin et al. 2006). *GS1.2* from rice (Sakamoto et al. 1989), *GS1.1* from maize (Martin et al. 2006), and *GS1.2r* from wheat

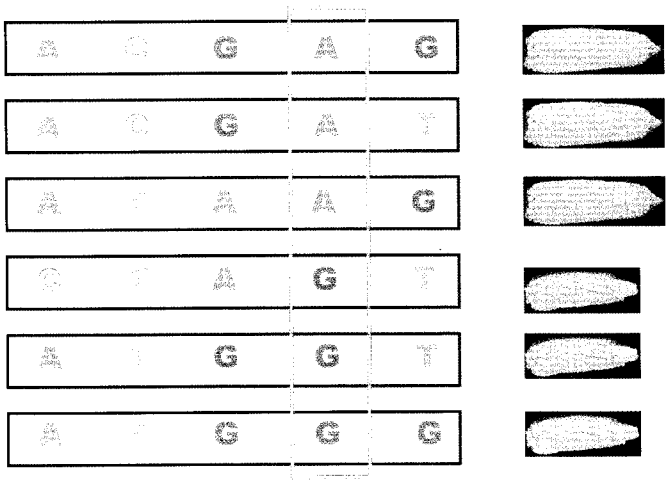

Fig. 2. Association genetics exploits the ancestral recombination events involving the nucleotidic diversity from the whole species to establish the association of *Gln3* gene polymorphism to a phenotype. In the case depicted above, on position 4, A is associated with large ear, G with small cobs, whatever the genotype at the other positions.

(Habash et al. 2007) belong to group 2 which represents a class of *GS* genes highly expressed in the root cortical cells. We found that *GS1.5* from maize belongs to group 2, although the gene appears to be only expressed in the leaf epidermis (Martin et al. 2006). This suggests that the *GS* proteins encoded by this group of genes may have a similar function in the outermost cells layers of both root and shoots. Group 3 is represented by *GS1.3* from rice, a *GS* gene mainly expressed in spikelets (Tabuchi et al.

2005), and by *GS1.2* from maize, a *GS* gene expressed in the phloem (Martin et al. 2006) and in the pedicel of the kernels (Muhitch 2003). One can therefore hypothesize that there is an ancestral *GS* gene expressed in leaf and root cortical cells and an ancestral *GS* gene expressed in the vasculature of both vegetative and reproductive organs. If we consider the most parsimonious hypothesis of a conservation in the *GS* gene expression patterns in each group, by analyzing the phylogenetic relationship between the different sequences of a gene encoding *GS1* in monocots, it is possible to determine to which group it belongs and therefore to have an idea of its putative physiological function in other cereals.

To increase the value of the candidate gene approach, association genetics studies (Wilson et al. 2004) are currently being performed to identify intra-specific gene polymorphisms in the *GS1* multigene family in maize using germplasm collections composed of different ancient and modern genotypes originating from various areas in the world. This approach will allow the linkage of the molecular diversity of these genes to phenotypical traits related to NUE and yield and thus identification of the best performing allele for the trait of interest. Figure 2 illustrates the principle of the approach currently being developed to find the best performing *Gln1-3* alleles for grain filling (Martin et al. 2006). It is likely that such an approach will be extended to other crop species, not only for the genes encoding *GS1*, but also to a number of other candidate genes when the demonstration of their role in the control of NUE has been established. Although the use of association genetics to dissect complex traits is relatively recent, the results obtained on both crop and other plant species using this type of approach look very promising. For an important adaptive trait such as flowering time, associating it with allelic variation in the two *Arabidopsis genes CRY2* (Olsen et al. 2004), and *FRI* (Shindo et al. 2005) was successfully achieved. Due to reduced linkage disequilibrium in maize (Tenaillon et al. 2001), it was possible not only to ascertain the role of *Dwarf8* in the induction of flowering (Thornberry et al. 2001) but also to further uncover the role of a 6-bp insertion/deletion sequence in a key domain of its coding region (Camus-Kulandaivelu et al. 2006). In the same species, both the identification of useful alleles and their validation through functional analysis has also been successfully achieved for economically important agronomic traits. For example, associations between the composition of maize kernel food processing properties and the variation in allelic sequences of *sh1* (sucrose synthase), *SH2* (large subunit of ADP-glucose pyrophosphorylase), and *BT2* (small subunit of ADP-glucose pyrophosphorylase) alleles have been identified (Wilson et al. 2004). Similarly, the role in cell wall digestibility of a *MITE* insertion element in the second exon of a maize peroxidase gene (*ZmPox3*) was recently demonstrated (Guillet-Claude et al. 2004). The occurrence of a highly significant association of two sequence polymorphisms in the promoter of the maize gene encoding the NADP-dependent dihydroflavanol reductase with plant maysin content was also shown recently (Szalma et al. 2005). Interestingly, association genetic studies have been successfully applied to a wide range of species including those having a large genome such as the forest tree Eucalyptus. Thumma et al. (2005) found two single nucleotide polymorphisms in the gene encoding cinnamoyl CoA reductase associated with variation in microfibril angle, a trait important for wood property. In *Pinus taeda*, a non-synonymous single nucleotide polymorphism in the gene encoding 4-coumarate CoA ligase was found to be associated with the percentage of latewood (Gonzalez-Martınez et al. 2007). These authors also found a strong association between allelic variation in the sequence of an α-tubulin gene involved in the formation of cortical microtubules, and in early wood microfibril angle. In light of these different studies, one can conclude that association genetic studies holds the most promise for having a practical application for a wide variety of complex traits including NUE in a wide range of economically-important plant species. Favorable alleles controlling the traıt can be then introduced into elite lines through the development of breeding programs based on maker-assisted selection (Mohan et al. 1997).

Combining whole plant and crop molecular physiology for identifying markers of NUE

The adaptation of plants to various N levels in soils is a systemic and quantitative process that proceeds from growth to development. Because it is constitutive, it integrates many traits and hence it is polygenic. Therefore, tools to diagnose differential responses either for varied inputs or for genotype differences have to combine many elements that are defined as indicative of function and N economy at a particular stage of development.

A first step for developing these tools was to perform whole plant molecular physiology studies using crops grown in the field. These studies have depicted in a dynamic and integrated manner the changes in various physiological and biochemical markers representative of N uptake, N assimilation, and N recycling in both model (Terce-Laforgue et al. 2004; Diaz et al. 2005) and crop species (Hirel et al. 2005b; Kichey et al. 2006). For example, in both maize and wheat the changes in metabolite concentrations and enzyme activities involved in N metabolism within a single leaf, at different stages of leaf growth and at different periods of plant development during the grain-filling period were investigated (Hirel et al. 2005b; Kichey et al. 2006). Interestingly, in both species it was found that total N, chlorophyll, soluble protein content, and GS activity are strongly interrelated. It has therefore been proposed that these four physiological traits are indicators that mainly reflect the metabolic activity of individual leaves with regards to N assimilation and recycling, regardless of the level of N fertilization (Hirel et al. 2005a,b; Kichey et al. 2006).

More recently, the use of ^{15}N-labeling techniques performed in the field to estimate the genetic variability for N uptake, N assimilation, and N recycling in different wheat cultivars combined with the measurement of physiological traits revealed that GS

and NR activities are potential markers to estimate the proportion of N taken up or N remobilized. The N taken up or remobilized is further invested in grain yield elaboration or grain N content, respectively (Kichey et al. 2007). Therefore, ^{15}N-labelling techniques combined with the use of simple physiological markers may be a way to assist breeders to estimate crop performance under different levels of N nutrition, since both methods allow scoring relatively easily and cheaply when using a large number of genotypes.

In order to increase the potential value of the physio-agronomic indicators identified using whole plant and organ biochemical profiling, it will be necessary to monitor in parallel the changes in the whole spectrum of proteins and genes under different N nutrition conditions in different organs, harvested at various periods of plant development. Although this type of approach will require a huge computational analysis when developed on a large set of genotypes, it will be the only way to identify not only genes and proteins involved in the control of the dynamics of N management throughout the whole plant life cycle but also regulatory genetic and metabolic networks (Hirel et al. 2007b). When the value of these physiological and molecular indicators is verified on a large panel of genotypes by performing multiple field trials in different soils and climatic conditions, these indicators will hopefully help breeders when screening the best performing lines under lower N fertilization input.

The next step in the development of diagnostic tools will be to fit them into a precision agriculture framing system combining soil testing, fertilizer application, and projected fertilizer requirement to determine fertilizer rate applications under different environmental conditions. This would obviously rely on the possibility of developing easy-to-use diagnostic tools, either based on kits to measure metabolites, enzyme activity, or micro arrays containing a set of marker genes representative of the N physiological status of the plant.

In parallel, the development of crop models will be required to simulate the plant morphogenetic response to contrasting N nutrition and thus to understand in a more integrated way the constraints linked to increasing crop NUE on both the side of the plant and the environment. The development of these models will enable breeders to identify crop ideotypes and agronomists to optimize N management practices.

Developing a framework based on crop simulation models to improve our ability to analyze complex traits such as NUE will become not only a viable tool for genetics and subsequent genomics research, but will also provide a physiological interpretation in the variation and interactions of key traits representative of NUE. The use of these models may also be a way to link plant model parameters with simple physiological and biochemical traits and thus facilitate genetic and genomic research to identify the key regulatory or structural genes involved and their individual or interacting regulation.

Conclusions and perspectives

In the last two decades, combined physiological and genetic approaches have allowed researchers to make significant progress in the understanding of plant N economy in an agronomic context. These combined approaches have also allowed the identification of a limited number of key elements involved the control of NUE in relation to crop productivity in general and crop yield in particular, that could provide new tracks for breeders to improve or at least maintain plant productivity under varying N environments.

However, further research is still required to extend these approaches to monitor the changes in a broader spectrum of both biochemical and genetic markers in the various organs and tissues of the plant during a close range of stages of development. For example, more specific characteristics should be taken into consideration, such as leaf growth rate and expansion, root development and architecture as well as the interaction with the rhizosphere. All these processes are of major importance for maintaining a relatively high rate of N uptake and N-utilization efficiency in mineral N-rich or N-depleted soils (Hirel et al. 2007b).

An improved and integrated view of NUE and its regulation both at the physiological and molecular level, should also maximize the possibilities of integrating the key parameters representative of N uptake and N-utilization efficiency within dynamic plant or crop models for developing agronomic and monitoring tools that can be used in precision agriculture.

All these approaches will certainly be greatly facilitated by the exponential development of large-scale genomic, proteomic, and metabolomic studies. However, it will first be necessary to integrate the huge amount of data generated by these studies and then decipher interplays occurring between the different biological steps spanning from basic gene expression to final physiological function both at the plant and cellular levels. In the future, these investigations will also allow, *via* marker assisted selection, identification of crops with high yield at low N-input, thus offering an alternative to the dissemination of transgenic plants and keeping the sustainability of agriculture in a more friendly environment.

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