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Review Paper

IMPROVING NITROGEN USE EFFICIENCY IN CROPS AND ITS EFFECT ON CROP PRODUCTIVITY- A REVIEW

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Abstract

Agriculture is the mainstay of most developing economies across the globe and continues to play a vital role in the sustenance of human society. Most agricultural practices depend heavily on the use of inorganic nitrogenous fertilizers, with global use of nitrogen (N) at the beginning of this decade amounting to 87 million metric tonnes and projected to increase to 236 million metric tonnes by the year 2050. However, the ability of plants to effectively utilize N from the soil depends on a number of variables, which is further compounded by the fact that close to 50–75% of N applied to agricultural land is used by microorganisms or lost through leaching. Naturally, there is growing interest in reducing fertilizer-N inputs by improving plant N-use efficiency (NUE). Although the amount of N available to the plant can be improved through fertilizer–soil–water–air interactions, the innate efficiency of the plant to utilize this available N necessitates biological interventions. They may include biological processes, such as N uptake, distribution and assimilation, and their optimal contribution toward agricultural outputs, such as biomass growth and/or increased grain/leaf/flower/fruit/seed output. The identification of appropriate phenotypes, genotypes, molecular markers, and target candidates for the improvement of NUE pose a formidable challenge. The present article discusses NUE as a concept and different approaches for enhancing NUE and improvement of crop productivity.

Key words: Nitrogen, NUE, N-uptake, improvement of NUE, Crop productivity.

INTRODUCTION

Fertiliser use efficiency by crop is both economically and environmentally highly desirable trait. Plant breeding is traditionally focussed on yield, quality and protection of these characteristics (resistance to pests and diseases). Until legislation limited nitrogen inputs, the application of fertilisers was generally not considered a major limitation and efficient nutrient use was not a priority area. Nitrogen (N) is an essential element in the growth and development of crops. Poor access to N fertilizer is a major limitation to crop productivity in some developing countries, whilst in other more affluent countries increased productivity over recent decades has been associated with a substantial increase in N fertilizer use. However, high N fertilizer use is also environmentally damaging. Approximately half the energy costs of cereal production are associated with the manufacture and transport of inorganic N fertilizer. Cereal crops are inherently inefficient in their use of N with only around 33% of the N available to the crop being recovered in the grain. Excess N may be lost from the soil by leaching or surface runoff to water

courses or by gaseous emissions (including ammonia and nitrous oxide) contributing to eutrophication and global warming. It is now widely recognised that nitrogen use efficiency (NUE) must be improved so that cereal yields can be increased to meet the growing global demand, but with reduced inputs of fertilizer. Nitrogen use efficiency in crops is a complex set of processes and priorities have to be recognised for breeding (Foulkes *et al*., 2009).

Two complementary approaches to this problem are possible: a) to improve agronomic practice and N management strategies and b) to increase the NUE of new varieties. The latter approach requires information on which phenotypic (morphological and physiological) traits govern NUE, and the extent of variation in these traits within the breeding population. A retrospective analysis of the effects of this breeding on NUE is useful for identifying traits associated with NUE and whether there is scope for future improvement.

1. Nitrogen use efficiency

NUE includes N uptake, utilization or acquisition efficiency and expressed as a ratio of output (total plant N, grain N, biomass yield, grain yield) and input (total N, soil N or N-fertilizer applied). Nitrogen use efficiency (NUE) is the product of both nitrogen uptake efficiency (NUpE, see Figure 1) which is a root-associated trait, and nitrogen utilisation efficiency (NUtE), which is a function of canopy activity. NUE is yield per unit of available N. Yield is mainly determined by C fixation in the canopy (which is dependent on N for growth and function); grain also requires N for protein, which is transported directly from the soil or from remobilisation during canopy senescence (major contribution).

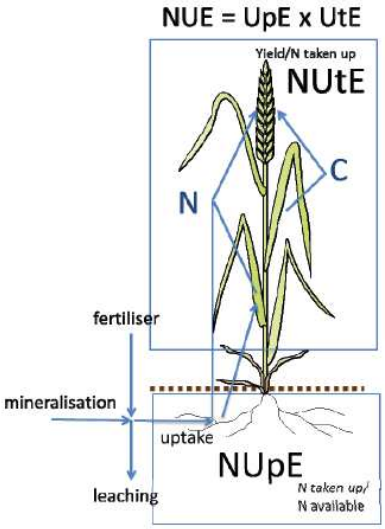

Figure1. Factors affecting Nitrogen use efficiency (NUE).

New improved varieties, with lower requirements for fertilizer, can help increase the sustainability of cropping systems, minimise pollution and assist in legislative compliance. Greater NUE and reduced use of inorganic N fertilizer can help towards meeting energy efficiency targets and contribute to N management strategies for nitrate vulnerable zones (NVZs).

2. Crop traits to improve N use efficiency

N use efficiency may be improved by 1) minimising the amount of N that is required in the crop to capture the majority of the radiation and to use this efficiently to produce dry matter, 2) increase the efficiency with which the crop is able to acquire N that is mineralised from organic residues or from applications of inorganic fertiliser and 3) increasing yield without increasing fertiliser N requirement.

3. Molecular physiology of nitrogen uptake and assimilation

Productive agriculture needs a large amount of expensive nitrogenous fertilizers. Improving nitrogen use efficiency (NUE) of crop plants is thus of key importance. NUE definitions differ depending on whether plants are cultivated to produce biomass or grain yields. However, for most plant species, NUE mainly depends on how plants extract inorganic nitrogen from the soil,

assimilate nitrate and ammonium, and recycle organic nitrogen. Efforts have been made to study the genetic basis as well as the biochemical and enzymatic mechanisms involved in nitrogen uptake, assimilation, and remobilization in crops and model plants. The detection of the limiting factors that could be manipulated to increase NUE is the major goal of such research.

3.1 Nitrate uptake

Nitrate uptake occurs at the root level and two nitrate transport systems have been shown to coexist in plants and to act coordinately to take up nitrate from the soil solution and distribute it within the whole plant (Tsay *et al*., 2007).

It is generally assumed that the NRT1 gene family mediates the root low affinity transport system (LATS), with the exception of the AtNRT1·1, which is both a dual affinity transporter (Liu et al., 1999) and a nitrate sensor (Ho et al., 2009). In Arabidopsis, 53 genes

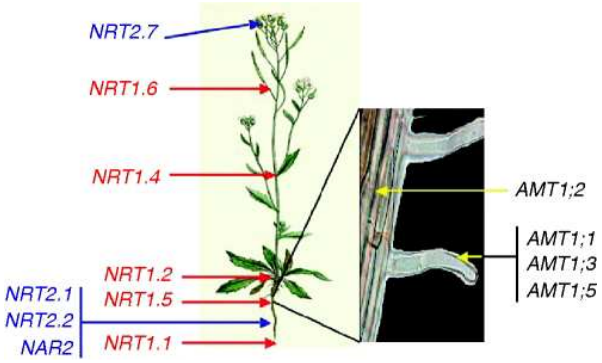

Fig.2. Schematic presentation of the known localisation of NRT1, NRT2 and AMT genes in Arabidopsis. Two nitrate transport systems have been shown to coexist in plants and to act coordinately to take up nitrate from the soil solution and distribute nitrate within the plant.

belong to the NRT1 family. Among them 51 genes are expressed and exhibit different tissue expression patterns in the whole plant (Tsay *et al*., 2007), suggesting a specialized and unique function for at least some of them. AtNRT1·1 (formerly Chl1) is the most extensively studied gene and was the first to have been isolated (Tsay *et al*., 2007). AtNRT1·2 is constitutively expressed only in the root epidermis and participates in the constitutive lowaffinity system (Huang *et al*., 1999). Once taken up by root cells, nitrate must be transported across several cell membranes and distributed in various tissues. AtNRT1·5, located on the plasma membrane, is involved in longdistance transport of nitrate from the root to the shoot (Lin *et al*., 2008). The AtNRT1·4 gene is only expressed in the leaf petiole, and the AtNRT1·6 gene, expressed in the vascular tissue of the silique and funiculus, is thought to deliver nitrate from maternal tissue to the developing embryo (Almagro *et al*., 2008).

The high affinity transport system (HATS), acting when the external nitrate concentration is low, relies on the activity of the socalled NRT2 family (Williams and Miller, 2001). Although the functional characterization of almost all higher plant NRT2 transporters remains to be done, it is now well documented that AtNRT2·1, in interaction with an NAR2 protein (Orsel *et al*., 2006), is a major component of the HATS in Arabidopsis, as shown by the fact that a mutant disrupted for the AtNRT2·1 gene has lost up to 75 % of the highaffinity NO uptake activity and showed a lower leaf nitrate content (Filleur *et al*., 2001). As a consequence, growth of these mutants is severely impaired at low NO concentration (Orsel *et al*., 2004, 2006). A lower nitrate content was also found in a clce mutant, affected in a protein belonging to the Arabidopsis AtCLC (ChLoride Channel) family. De Angeli (2006) have demonstrated that another member of the family, the vacuolar AtCLCa protein, behaves as a NO /H exchanger allowing the accumulation of nitrate within the vacuole. Insertion mutants within the AtCLCa gene exhibit normal development but show a reduced capacity to store nitrate but not other anions. This phenotype was also recently found when the expression of AtNRT2·7 was affected. This AtNRT2 gene is expressed in aerial organs and also highly induced in dried seeds. In two allelic atnrt2·7

mutants, less nitrate is accumulated in the seed. In contrast, seeds from plants overexpressing the AtNRT2·7 coding region accumulate more nitrate and as a consequence are less dormant than the corresponding wildtype seeds (Chopin *et al.,* 2007).

3.2 Physiology of Ammonium uptake

An additive contribution of each protein to ammonium transport was shown, AMT1·1 and AMT1·3 conferring a similar capacity of 30–35 % while AMT1·2 conferred a lower capacity of 18–25 %. A second saturable transport system with a low K of 4·5 mM and a very low capacity is thought to be coded by the AMT1·5 gene. A complex picture is now emerging from these studies. There is a spatial organization of AMT1 proteins (Fig. 1), the transporters possessing the highest ammonium affinities being located in outer root cells or root hairs where they can take up ammonium from the soil solution (AMT1·1, AMT1·3, AMT1·5). The lower affinity of AMT1·1, and its location in the endodermis along the root hair zone, suggests a function in the retrieval of ammonium that is released from the cortex or that enters the root via the apoplastic route. The electrochemical gradient between the vacuole and cytosol would drive NH import to and NH export out of the vacuole. Indeed, tonoplast intrinsic proteins from the TIP family were shown to play a role in NH_3 transport into the vacuole (Loque *et al*., 2005). Vacuolar loading with NH_3 should require an electrogenic ammonium transporter, which is not yet identified.

3.3 Nitrogen assimilation

The nitrogen sources taken up by higher plants are nitrate or ammonium as inorganic nitrogen sources and amino acids under particular conditions of soil composition. Nitrogen assimilation requires the reduction of nitrate to ammonium, followed by ammonium assimilation into amino acids (Fig. 3A).

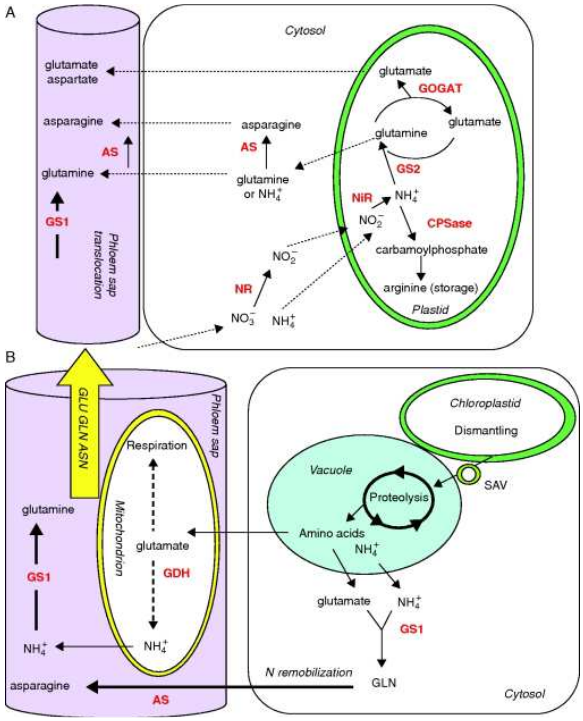

Fig.3. Schematic presentation of key enzymes involved in nitrogen management in (A) young and (B) senescing leaves. (A) Nitrate reductase (NR) and asparagine synthetase (AS) are localized in the cytosol, and nitrite reductase (NiR), glutamine synthetase 2 isoenzyme

Nitrate reduction takes place in both roots and shoots but is spatially separated between the cytoplasm where the reduction takes place and plastids/chloroplasts where nitrite reduction occurs. Nitrate reduction into nitrite is catalysed in the cytosol by the enzyme nitrate reductase (NR) (Meyer and Stitt, 2001). This enzyme is a homodimer, each monomer being associated with three prosthetic groups: flavin adenine dinucleotide (FAD), a haem and a molybdenum cofactor (MoCo). Characterization of mutants resistant to chlorate, which can be

reduced into toxic chlorite by NR, identified two classes of genes, the Nia genes encoding the NR apoenzyme and the Cnx genes encoding the MoCo cofactor. Since 1993, considerable work has been done to characterize the NR in different species (reviewed by Meyer and Stitt, 2001). Although the NR enzyme is thought to be localized in the cytosol, an association with the plasma membrane (PMNR) has been found in corn roots and barley (Ward *et al*., 1989).

After nitrate reduction, nitrite is translocated to the chloroplast where it is reduced to ammonium by the second enzyme of the pathway, the nitrite reductase (NiR). The Nii genes encoding the NiR enzyme have been cloned from various species, the number of genes varying from one to two copies (Meyer and Stitt, 2001).

Ammonium, originating from nitrate reduction, and also from photorespiration or amino acid recycling, is mainly \assimilated in the plastid/chloroplast by the so called GS/GOGAT cycle (Lea and Forde, 1994). The glutamine synthetase (GS) fixes ammonium on a glutamate molecule to form glutamine. This glutamine reacts subsequently with 2oxoglutarate to form two molecules of glutamate, this step being catalysed by the glutamine 2oxoglutarate amino transferase (or glutamate synthase, GOGAT). The decameric structure of maize GS was described by Unno et al. (2006). Two classes of nuclear genes code for GS: the GLN2 and GLN1 genes. GLN2, present as a single nuclear gene in all the species studied so far, codes for the chloroplastic GS2, thought to be involved in the primary assimilation of ammonium coming from nitrate reduction in both C and C plants and in the reassimilation of ammonium produced from photorespiration in C plants. The magnitude of the ammonium flux through the photorespiration pathway in the leaves of C plants was indeed estimated to exceed that produced from nitrate reduction by fiveto tenfold (Keys *et al*., 1978).

Conversely, the GLN1 gene family codes for cytosolic GS1 isoforms, present in different organs such as roots or stems and thought to be involved in ammonium recycling during particular developmental steps such as leaf senescence and in glutamine synthesis for transport into the phloem sap (reviewed by Bernard and Habash, 2009). Two different forms of glutamate synthase are present in plants: FdGOGAT and NADHGOGAT use ferredoxin and NADH as the electron donors, respectively (Vanoni *et al*., 2005). FdGOGAT is predominantly localized in leaf chloroplasts whereas NADHGOGAT is primarily located in plastids of nonphotosynthetic tissues, such as roots, etiolated leaf tissues and companion cells. The structural, mechanistic and regulatory properties of GOGAT enzymes and their role in amino acid metabolism have been reviewed by Suzuki and Knaff (2005).

In addition to the GS/GOGAT cycle, three enzymes probably participate in ammonium assimilation. Cytosolic asparagine synthetase (AS) catalyses the ATPdependent transfer of the amido group of glutamine to a molecule of aspartate to generate glutamate and asparagine (Fig. 2A; Lam *et al*., 2003). MasclauxDaubresse *et al*., (2006) provided evidence that AS can also use ammonia as a substrate. Indeed, providing NH to leaf discs in the presence of azaserine, the authors blocked glutamate biosynthesis but not asparagine biosynthesis, thus showing that asparagine production is not amino transferase dependent. In Arabidopsis, three genes encode AS (ASN1, ASN2 and ASN3). Asparagine has a higher N/C ratio than glutamine and can be used as a longrange transport and storage compound, especially in legumes (Lam *et al*., 2003). AS could in certain situations compensate for the reduced GSdependent ammonium assimilatory activity.

Finally, the mitochondrial NADHglutamate dehydrogenase could alternatively incorporate ammonium into glutamate in response to high levels of ammonium under stress (Skopelitis *et al*., 2006). However, the major catalytic activity for GDH in plant cells has been reported to be glutamate deamination (MasclauxDaubresse *et al.,* 2006). GDH activity was shown to be essential for plant survival in dark conditions (Miyashita and Good, 2008). NR, NiR and GOGAT require reducing power as either NADH or ferredoxin (Fdx) according to the enzyme. Glutamine synthetase and asparagine synthetase need ATP. In addition, carbon skeletons and especially ketoacids are essential to form organic nitrogen as amino acids. The availability of carbon skeletons for ammonium condensation and the supply of ATP, Fdx and NADH as products of photosynthesis, respiration and photorespiration pathways are thus essential for nitrogen assimilation.

3.4 Nitrogen remobilization: a key factor for nitrogen use efficiency

Nitrogen remobilization and yield dilemma Leaf proteins and in particular photosynthetic proteins of plastids are extensively degraded during senescence, providing an enormous source of nitrogen that plants can tap to supplement the nutrition of growing organs such as new leaves and seeds. Nitrogen remobilization has been studied in several plant species through the 'apparent remobilization' method, which is the determination of the amount of total nitrogen present in the different plant organs at different times of development and through N longterm labelling, which allows the determination of fluxes (Gallais *et al*., 2006). In Arabidopsis and oilseed rape, it has been shown that nitrogen can be remobilized from senescing leaves to expanding leaves at the vegetative stage as well as from senescing leaves to seeds at the reproductive stage (Lemaître *et al*., 2008). In Arabidopsis, for which sequential (at vegetative stage) and monocarpic (after flowering) senescence phases can be distinguished easily, it was shown that the N remobilization rate was correlated with leaf senescence severity at the vegetative stage only (Diaz *et al*., 2008). Experiments of N tracing at the reproductive stage showed that the rate of nitrogen remobilization from the rosettes to the flowering organs and to the seeds was similar in early and late senescing lines (Diaz *et al*., 2008). At the reproductive stage, NRE is mainly related to harvest index.

Studies using N tracing in cereals, oilseed rape and legumes showed that the onset of grain filling was a critical phase because N uptake and N fixation declined during plant maturation and seed filling (Salon *et al*., 2001). Nitrogen fluxes and N remobilization experiments performed by Cliquet *et al*. (1990) showed that leaf blades, stalks, cob, husk and shank serve successively as Nsinks and Nsources. Nitrogen uptake and assimilation during the grain filling period is generally insufficient for the high demand of the seeds, and the progressive and numerous remobilization steps, occurring successively in the different plant organs, are needed to route nitrogen to the seeds. The contribution of leaf N remobilization to rice, wheat or maize grain N content is cultivar dependent, varying from 50 to 90 % (Masclaux *et al*., 2001). N remobilization is also environment dependent and favoured under limiting nitrate supplies (Lemaître *et al.,* 2008). Although N remobilization is a stepbystep mechanism that involves the different plant organs, evidence shows that grain nitrogen content is correlated with flag leaf senescence in maize, wheat and barley. Flag leaf senescence seems then to have a special role in nitrogen availability for grain filling. The onset and the speed of flag leaf senescence are likely to be essential for NRE. Leaf senescence is not only essential for nitrogen mobilization. Evidence has shown that leaf senescence is also important for yield. Delaying leaf senescence results in a prolongation of photosynthesis that increases grain yield and carbon filling into seeds. Breeding plants have then to cope with the dilemma that delayed senescence could lead to higher yields but also to a decrease in NRE and to a decrease in grain protein content. On the other hand, increasing nitrogen remobilization has the advantage of reusing nitrogen from the vegetative parts and of lowering nitrogen loss in the dry remains.

1. **Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4^+ cycling in roots:**

Ammonium ($NH4^+$), one of the two inorganic nitrogen sources used by plants ($NH4^+$ and $NO3^-$), is beneficial for plant growth under many circumstances and, indeed, serves as a ubiquitous intermediate in plant metabolism (Glass *et al*., 1997). Its assimilation furthermore entails lower energy costs compared to $NO3^-$. Additionally, studies have shown $NH4^+$ can improve the capacity to tolerate water stress in rice in comparison with $NO3^-$ (Guo *et al*. 2007), and has been shown to act as an inducer of resistance against salinity conditions in other species (Fernandez-Crespo *et al*. 2012).

Nevertheless, $NH4^+$ frequently reaches levels in soils that affect plant growth negatively. These negative effects manifest in stunted root growth, yield depression, and chlorosis of leaves (Li *et al*. 2011). However, higher plants display widely differing responses to $NH4^+$ nutrition (Marschner 1995) and, accordingly, can be divided into tolerant and sensitive species (Britto and Kronzucker 2002). Based on a series of comparative studies, more than 18 kinds of plants or plant species, including eight kinds of wild plants, have been classified as highly adapted to NH^+ as a nitrogen source (Omari *et al*. 2010). Further, more than 22 kinds of plants or plant

species, eight of which wild, have been classified as sensitive to the NH_4^+ source (Britto and Kronzucker 2002). Rice is regarded as unique in its high degree of NH_4^+ tolerance (Wang et al. 1993a, b). Studies have suggested that NH_4^+ -tolerant plants generally possess higher glutamine synthetase (GS) activity and less accumulation of free NH_4^+ in plant tissues (Balkos *et al*. 2010).

Several important hypotheses have been proposed, such as carbon depletion in roots induced by NH_4^+ assimilation NH_4^+ -induced pH reduction in the root zone (Chaillou *et al*. 1991), deficiencies of mineral cations (Siddiqi *et al*. 2002), impairments in the N-glycosylation of proteins (Barth et al. 2010) and futile and energy-costly NH_4^+ cycling at the plasma membrane of both root and shoot cells (Britto and Kronzucker 2002). However, to date, no single mechanism has been able to fully elucidate NH4 + toxicity (Roosta and Schjoerring 2008). On the basis of the fact that all of the hypothesized mechanisms with regard to NH_4^+ toxicity are linked to the permeation of NH_4^+ (or perhaps NH3) into the cell, useful clues can be obtained by studying transmembrane NH_4^+ fluxes. Britto *et al.* (2001) studied NH_4^+ fluxes across the root plasma membranes of barley and rice by using a high precision positron tracing technique and found, at elevated levels of NH_4^+ , a significantly larger NH_4^+ efflux, accounting for up to 80 % of primary influx, in barely cells, which carried a high energetic cost and was independent of N metabolism. Britto *et al*. (2001) furthermore suggested that rice, unlike barley, was resistant to the respiratory drain induced by futile NH_4^+ cycling.

Despite its reputation as an NH_4^+ -tolerant species, rice can be affected negatively by elevated NH_4^+ , particularly at low K^+ (Balkos *et al*. 2010), which, in turn, may be relieved by elevated K^+ , similar to conclusions reached in Arabidopsis (Li *et al*. 2010). Several studies have shown declines in K^+ -bearing clay minerals over extended cultivation periods in many ricegrowing areas of China . In fact, K^+ deficiency has been observed in about 70 % of rice paddies in southeastern China (Yang *et al.* 2005). Similar declines have been noted in other parts of Asia.

2. **Deciphering the genetic basis of nitrogen use efficiency in crops**:

There have been an increasing number of studies only performed on the model species Arabidopsis, in an attempt to link plant physiology to whole genome expression in order to obtain an integrated view on how the expression of genes can affect overall plant functioning (Coruzzi *et al*., 2009). When a structural or regulatory gene putatively involved in the control of a metabolic pathway or a developmental process or both is identified, information can then be obtained by producing overexpressors or selecting deficient mutants of the gene in question. By studying the impact of the genetic modification or the mutation on the phenotype or the physiology of the plant, it is often possible to determine whether the expression of this specific gene is a limiting step in the development of a particular organ or of a metabolic pathway. In general, this targeted approach, which allows the identification of a single limiting reaction, or a co-limiting/ non-limiting reaction does not adequately take into account the variation in complex traits such as those controlling NUE, which involves multiple genes and thus multiple enzyme reactions and regulatory factors.

Over the last ten years, quantitative genetics, through the detection of quantitative trait loci (QTL), has become an important approach for identifying key regulatory or structural genes involved in the expression of complex physiological and agronomic traits in an integrated manner and for the study of plant responses to environmental constraints(Xu 1997). When QTLs for agronomic and phenotypic traits are located on a genetic map, it is possible to look for their genetic significance by establishing the co-location of QTLs for physiological or biochemical traits with genes putatively involved in the control of the trait of interest (candidate genes). Validation of candidate genes can then be undertaken using transgenic technologies (forward genetics) or mutagenesis (reverse genetics) or by studying the relationship between allelic polymorphism and the trait of interest (association genetics; Figure 3) either at a single gene or genome-wide level (Yu and Buckler, 2006). Positional cloning is another alternative strategy that can be used to focus on the chromosomal region controlling the trait of interest and that ultimately allows access to a single gene (Salvi *et al*., 2007).

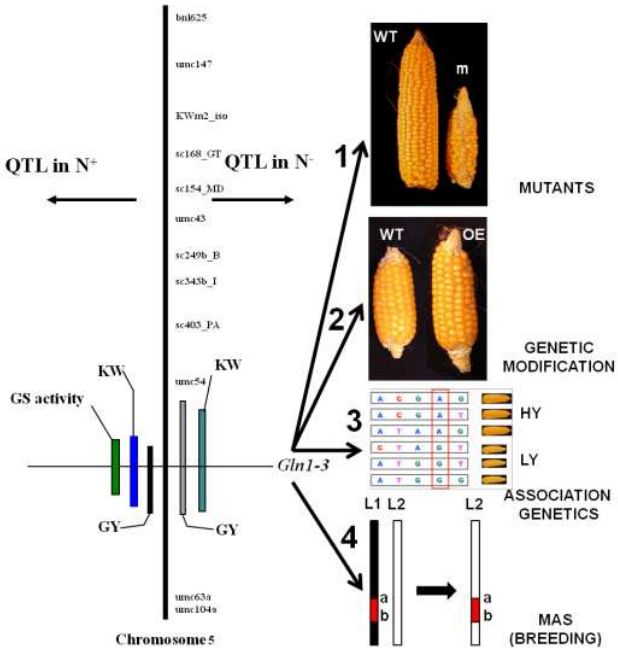

Figure 4. Example of identification and validation of a candidate gene involved in the control of NUE and yield in maize. On the left is shown a chromosomal colocation of QTLs for different yield traits (KW = kernel weight and GY = grain yield) and for glutamine synthetase (GS) activity at the level of the Gln1-3 locus (encoding a cytosolic GS involved in ammonia assimilation; see paragraph 4 and Figure 2). N^+ means with high N fertilization, Nwith low N fertilization. Such a result shows that the Gln1-3 gene is a good candidate gene for explaining variation in NUE. Validation of the candidate gene Gln1.3 was then performed using: (1) mutants {reduction of grain yield in the mutant (m) compared to the wild type (WT)}; (2) genetic modification by overexpressing the Gln1.3 in transgenic maize plants {increase in grain yield in the trangenics (OE) compared to the untransformed plant (WT); see (Martin *et al.,* 2006); (3) association genetics linking Gln1.3 gene nucleotide polymorphism to the increase in yield (HY = high yield, LY = low yield) to identify the best performing Gln1.3 allele among a population covering maize genetic diversity; (4) marker assisted selection (MAS) can be then undertaken by breeders where a trait of interest (yield associated to the presence of the Gln1.3 locus) is selected not based on the trait itself, but on a marker or markers linked (marker a and b)to it and introduced in the desired elite line (L2) from the donor line (L1) containing the best performing Gln1.3 allele in terms of yield.

Therefore, quantitative genetic approaches were developed first in maize for which recombinant inbred lines (RIL) populations were used to build-up genetic maps and then study QTLs. The aim of such studies was to identify chromosomal regions involved in the control of yield and its components at high or low N fertilization input, and to determine whether or not some of these regions were specific for one of the two nutrition regimes. In one study, a limited number of QTLs for yield was detected only at low N-input . In another study, it was found that most of the chromosomal regions for grain composition and traits related to NUE detected at low N-input, corresponded to QTLs detected at high N-input (Bertin *et al*., 2001). These contrasting results suggest that depending on the RIL population, the response of yield to various levels of N fertilization could be different and thus controlled by a different set of genes.

In a more detailed investigation by Bertin and Gallais in 2001 using maize RILs, agronomic traits, NUE and physiological traits were associated with DNA markers (Hirel *et al.,* 2001). Interestingly, coincidences were detected between QTLs for yield and two genes encoding cytosolic GS (Gln1-3 and Gln1-4) and whole leaf enzyme activity. As a result of which, the hypothesis that cytosolic GS activity could be a major element controlling grain yield was put forward. Since a QTL for a thousand kernel weight was coincident with the Gln1-4 locus and QTLs for a thousand kernel weight and yield were coincident with the Gln1-3 locus (Figure 3), further work was undertaken to validate the function of these two putative candidate genes. In

another study also performed in maize, fine QTL mapping of C and N metabolism enzymes activities was performed on a different RIL population. These QTLs did not colocalize with those reported by other authors [180], which indicates that there are large differences in diversity traits in maize (Zhang *et al*., 2010).

Hirel *et al*. (2001) developed a quantitative genetic approach by associating maize metabolic functions and agronomic traits to DNA markers. In their study, coincidences of QTLs for yield and its components with genes encoding cytolistic gultamine synthestase (LGS-H) and leaf $N0_3$- content (LNC-H) were located on the genetic map of maize (Figure 3). These studies revealed several RFLP markers mapped in regions of the maize genome which have been shown to carry QTLs for yield under nitrogen stress and/or other components. QTLs for yield components were clustered mainly on chromosome 5, with other significant effects under low N, high N or both conditions on every chromosome except 8. However, this cluster region has QTLs coincidence for grain yield, GS and nitrate reductase (LNR-H) activities. Hirel *et al*. (2001) hypothesize that leaf nitrate accumulation and the reactions catalyzed by NR and GS are co-regulated and represent key elements controlling NUE in maize.

The impact of the knockout mutations gln1-3 and gln1-4 on kernel yield and its components were examined in plants grown under controlled 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 the leaves, there was an increase in kernel number, thus providing further evidence that the cytosolic GS isoenzyme GS1-3 plays a major role in controlling kernel yield Figure 3). The hypothesis that GS is one of the key steps in the control of cereal productivity was strengthened by a study performed on rice, in which a co-localization of a QTL for the GS1;1 locus and a QTL for one-spikelet weight was identified (Obara *et al*., 2001). As a confirmation, a strong reduction in growth rate and grain yield was observed in rice GS1;1 deficient mutants (Tabuchi *et al*., 2005).

The role of the GS enzyme and other N-related physiological traits in the control of agronomic performance in wheat still remains to be clearly established. Using a quantitative genetics approach, Fontaine and coworker in 2009 found only a co-localization between a QTL for GS activity and GSe, a structural gene encoding cytosolic GS, but no obvious colocalization with a QTL for yield, in agreement with previous work published by Habash et al. [158]. In contrast, in recent work, physical mapping, sequencing, annotation and candidate gene validation of an NUE QTL on wheat chromosome 3B suggested that the NADH-dependent GOGAT enzymes contribute to NUE in wheat and other cereals (Fontaine *et al*., 2009) in agreement with work previously performed on rice (Yamaya *et al*., 2002).

Interestingly, in a woody species such as maritime pine that is far away from cereals on an evolutionary point of view, a protein QTL for GS co-localized with a GS gene and a QTL for biomass (Plomion *et al.,* 2009). Functional validation of the pine GS gene in transgenic poplars (see above), which can be considered as a crop for wood production, shows once again that quantitative genetics represent one of the most powerful approaches for identifying NUE candidate genes that may be involved in the control of plant productivity.

To date, there are only a few reports reporting specific breeding for organic input systems and especially N (Lammerts *et al*., 2009). A question that could be addressed is whether the genetic control of NUE under organic or conventional fertilization conditions is similar or if there are specific genes or combinations of genes that are more adapted to one mode of fertilization compared to the other, taking into account that organic material can be directly taken up by the plant (Hawkesford *et al*., 2010). Moreover its appears that using appropriate selection environments is important for breeding crops adapted to organic farming systems (Messmer *et al*., 2009).

1. Improving NUE through breeding:

Conventional breeding procedures have been performed empirically over the last two decades by selecting the most appropriate traits in terms of yield or technological characteristics to improve plant productivity. Many plant-breeding programs have relied on the

ability to select desired individuals or populations that are enriched for the traits of interest. Usually this selection has been performed either by "eye" or by elaborate testing techniques based on statistical inference. While there have been considerable improvements in crop varieties over the years, these have been achieved with substantial investment in time and money. Ifefforts of breeders are to continue to be successful in developing improved varieties, it is highly probable that they will need additional selection techniques. Not only will increases in yield be necessary, the new varieties will require improved levels of resistance to biotic and abiotic stresses.

A retrospective analysis of the effects of nearly 75 years of breeding on the nitrogen use efficiency (NUE) of spring barley (Hordeum vulgare L.) was undertaken to identify physiological mechanisms governing NUE and targets for future improvement. Fifteen varieties, selected to be as genetically dissimilar as possible and to represent the breeding period from 1931 to 2005, were grown at three site year combinations in the NE of Scotland. Varieties were grown with zero or 110 kg N ha-1 supplied as ammonium nitrate. Averaged across site years, breeding was shown to increase yield and NUE (grain yield N supply-1) by 1 and 1.2% per year respectively in the presence of fertilizer. Mean grain weight was larger in modern compared to old varieties grown both with and without N fertilizer. The greater response of yield to fertilizer in modern varieties was associated with the production of more grains m-2. Significant variation was found between genotypes in efficiencies of N uptake (NupE; N offtake per unit N supply) and N utilization (NutEg, grain yield per unit N off take). Differences in NutEg contributed 60% to the variation in NUE, whilst NupE contributed 40%. The improvement in NutEg was mostly the result of increases in harvest index (HI) and was stable across environments. NupE was positively correlated with postanthesis, but not preanthesis, dry matter accumulation and N uptake, which suggests that greater NupE of some varieties was the result of an increased N demand from a large grain sink. Across all varieties NupE was not related to NutEg suggesting that improvements in each may be selected independently. The results also indicated possible opportunities for improving the efficiency of preanthesis N uptake.

Due to increasing fertiliser costs as well as environmental concerns, N-efficiency became an attractive breeding topic. Genotypes can be considered as N-efficient because it realises an above average yield at suboptimal N level (Graham, 1984). On the other hand a genotype can be called N-efficient if it converts high N input into yield comparatively better than other genotypes (Sattelmacher *et al*., 1994). Moll and coworker in 1982 defined nitrogen use efficiency as grain yield per unit N supply. Efficiency of utilisation of N can be defined as grain yield per unit N uptake (Muchow, 1998). Genetic variation in nutrient efficiency is based on two components (Sattelmacher et al., 1994; Moll et al., 1982): (i) on differences in efficiency of nutrient uptake (uptake efficiency) and (ii) on differences in efficiency to use absorbed nutrients for yield formation (utilisation efficiency).

To evaluate potential of Oryza sativa L. in nitrogen use efficiency compared to Oryza glaberrima Steudt a two years field experiment was conducted by Gueye and Becker, 2011. Twelve O. sativa genotypes were tested in a split-plot design with two N-levels (without N fertiliser and with 150 kg N/ha). For comparison, one genotype of African rice, O. glaberrima, was included in the experiment. Variability about grain yield at harvest and N-uptake was observed between O. sativa and O. glaberrima and within O. sativa genotypes. O. glaberrima had lower yield as O. sativa.They observed that in low N-level, a close relationship between total N-uptake in plant and grain yield was observed. In high N-level, no correlation was observed. An effect of O. glaberrima genotype on the variance component GN was demonstrated. Variation in N uptake and in N utilisation efficiency depends on N fertilisation. At low N level variation in N uptake were higher than at high N level. At the opposite, variations in utilisation efficiency were lower without fertilisation than with fertilisation.

Oilseed rape has a high requirement for nitrogen (N) fertiliser relative to its seed yield. Berry *et al*., 2007 that if the concentration of N in the stem and pod wall at crop maturity could be reduced from 1.0% to 0.6%, the root length density increased to 1cm/cm3 to 100 cm soil depth and the post flowering N uptake increased by 20 kg N/ha then the fertiliser requirement could be reduced from 191 to 142 kg N/ha and the N use efficiency could be increased from 15.2

to 22.4 kg of seed dry matter per kg N . Genetic variation was found for all of the traits that were estimated to be important for N use efficiency. This indicates that there is significant scope for plant breeders to reduce N use efficiency in oilseed rape.

Genetic variation in nitrogen (N) use efficiency, N uptake, and N utilization was analyzed in a doubled haploid (DH) population derived from winter oilseed rape cultivars by Nyikako *et al*., 2014. The aim was to analyze the relative importance of uptake and utilization efficiency and to identify parameters that allow an easy selection of N efficient genotypes. Significant genotypic variation was observed for both uptake and utilization efficiency. At low N supply, variation in N efficiency was mainly the result of differences in uptake efficiency. Seed yield was correlated positively with N uptake and N utilization efficiencies at low N supply and with N uptake at high N supply. The interaction between genotypes and N supply for grain yield was highly significant, and the correlation between low N and high N was of only medium size, suggesting the possibility of selecting genotypes with specific adaptation to low N supply.

Diverse germplasm collections, including older varieties and landraces, and multiple mapping populations are being analysed for both grain and biomass production and performance at lower nutrient inputs. Screening wheat varieties has enabled the identification of processes involved in nitrogen use efficiency, and indicated a range of variation amongst modern varieties, which is available to breeders. Furthermore, screening more diverse germplasm may facilitate recovery of lost alleles and genes which may contribute to major improvements in yield and nutrient use efficiency.

6.1 **Transgenic efforts to improve nue**

NUE is a multigenic trait spread across hundreds of genes that extends beyond primary nitrate assimilation and metabolism. Naturally, transgenic efforts have concentrated on diverse targets that include genes belonging to uptake, translocation, remobilization, and carbon metabolism. In addition, signaling targets and regulatory elements have recently emerged as prospective candidates for biotechnological interventions. The following sections summarize various transgenic attempts to improve NUE based on the processes targeted.

Biotechnological interventions to improve NUE have largely revolved around manipulation and overexpression of many crucial candidate genes apart from using knockout mutations to assess its effects on biomass and plant N status and overall yield (Goods *et al*., 2004, Masclaux-Daubresse *et al*., 2010, Pathak *et al*., 2008). N assimilatory pathway is one of the most widely chosen targets for improvement of NUE. The primary nitrate assimilation pathway that involves nitrate transporters, nitrate reductase (NR), nitrite reductase (NiR), plastidic glutamine synthetase (GS2), and ferredoxin glutamate synthase (Fd-GOGAT) along with secondary N assimilation and remobilization that includes cytosolic glutamine synthetase (GS1), reduced nicotinamide adenine dinucleotide glutamate synthase (NADH-GOGAT), glutamate dehydrogenase (GDH), and various aminotransferases; offers myriad opportunities of intervention at the uptake, assimilation, translocation, and remobilization stages.

Emerging evidences from these efforts reveal that attempts to improve NUE in plants need to target different organs for individual plants. For example, in cereals, the total grain biomass or grain N content would be most suitable indicators of NUE, rather than any other organ. Thus, areas where NUE can be targeted is to improve the distribution of N between canopy (leaves and stem) and roots, better photosynthetic rate/unit leaf N, reduced leaf senescence, transgenics developing C_4 options for rice and wheat, or otherwise increasing the efficiency of net photosynthesis in warmer environments by modifying Rubisco, Rubisco activase, and the enzymes that modulate photorespiration in C3 plants.

6.2 Manipulating genes of N uptake

N uptake is the first step in nitrate assimilation, which can be manipulated to enhance NUE. Two types of nitrate uptake systems: low-affinity transport system encoded by NRT1 gene family and high-affinity transport system encoded by NRT2 gene family. In addition, a number of ammonium transporters and putative amino acid transporters have also been identified in Arabidopsis. In the last decade, several attempts of overexpression of high-affinity nitrate/nitrite transporters in tobacco and Arabidopsis (Pathak *et al*., 2008) reported enhanced nitrate uptake, but concomitant increase in assimilation and NUE was not observed. Though the

various nitrate and ammonium transporters in plants are very well characterized and their differential regulation mechanisms are well known, overexpression studies involving their genes have not been conclusive .

6.3 Manipulating genes of N assimilation

Several attempts at transgenic manipulation of the enzymes of primary and secondary assimilation have been made . Primary nitrate assimilation involves NR, NiR, plastidic GS (GS2), and Fd-GOGAT, while cytosolic GS (GS1) and NADH-GOGAT are a part of secondary ammonia assimilation and remobilization. NR is considered as a rate-limiting step in nitrate metabolism. There have been reports of overexpression of two genes nia1 and nia2 for NR in Arabidopsis, tobacco, potato, and lettuce, without any specific improvement in NUE. Similarly, little improvement in phenotype and NiR activity levels were observed when nii gene was overexpressed in tobacco and Arabidopsis under CaMV 35S promoter, though there was an increase in the NiR transcript level. Overexpression of GS2 has also been reported in rice and tobacco, but no significant improvement in terms of NUE was observed. Though, transgenic tobacco plants overexpressing GS showing improved capacity for photorespiration and an increased tolerance to drought while transgenic rice plants showed only better growth rate. The potential of transgenic plants with overexpressed Fd-GOGAT gene has not been tested yet, although barley mutants with reduced Fd-GOGAT revealed changes in various nitrogenous metabolites, decreased leaf protein, rubisco activity, and nitrate contents.

The genes of secondary ammonia assimilation have also been overexpressed in a variety of crops for developing transgenic with enhanced NUE. GS1 has emerged as a potential candidate from among all the genes that have been tested so far. Overexpression of GS1 gene has been tried in several plants such as wheat, tobacco, and maize have resulted in higher grain yield and biomass with improved N content. Transgenic overexpression and antisense technology has been employed to modulate the expression of NADH-GOGAT gene in rice and alfalfa plants. Though the genes of secondary ammonia assimilation appear to be good candidates for improving NUE in short run, the results may vary with crop to crop variation.

6.4 Manipulating genes of N-translocation and remobilization

N remobilization is proposed as one of the key steps in improving NUE in plants (Masclaux-Daubresse *et al*., 2006). In cereals, the main source of N for the grains is N remobilized from the vegetative parts. This source accounts for 60–92% of the N accumulated in the grains at maturity. The amount of N remobilized depends on N remobilization efficiency and the amount of N available. It is also well known that genotype and many environmental factors are known to affect N translocation, which makes the genes involved in remobilization and translocation attractive targets for improvement of NUE.

There are several reports of transgenic overexpression of AS genes that are actively involved in remobilization and translocation of amino acids resulting in enhanced seed protein content and total protein content. Molecular manipulation of asparagine synthetase (encoded by Aln1 gene) has been attempted recently along with reports of genetically engineered plants overexpressing alanine aminotranferase (Good *et al*., 2007). Taking a cue from some of these studies, genetically engineered rice (Oryza sativa L.) was developed by introducing barley alanine aminotranferase complementary DNA (cDNA) driven by tissue-specific OsAnt1 promoter (Shrawat *et al*., 2008). These plants showed improved biomass and grain yield along with significant change in key metabolites and nitrate content confirming increased NUE. Further, Beatty and co-worker in 2009 reported the involvement of some candidate genes through root and shoot transcriptome analysis in engineered rice overexpressing alanine aminotransferase (AlaAT) under the control of a tissue-specific promoter showing a strong NUE phenotype. Though the importance of GDH in higher plant N remobilization is still controversial, transgenic plants overexpresing gdhA gene were shown to have improved amino acid content, higher yields in maize and wheat (Lightfoot 2009). More recently, Arabidopsis NRT1transporter NRT1.7 has been implicated in nitrate remobilization from source to sink tissues by mutant analysis (Fan *et al*., 2007).

6.5 Manipulating genes of C metabolism

Sugars play an important role in plant growth and metabolism by providing carbon skeletons and energy for cellular metabolism. However, sugar metabolism and signaling influences a number of processes involved in plant growth and development, such as seed germination, embryogenesis, flowering, and senescence, and have also been implicated in hormone signaling. The genes involved in N metabolism and nitrate signaling are also tightly regulated by sugar signaling mechanisms. A coordination between N and C metabolism is required at the amino acid synthesis level due to the requirement of carbon skeletons for their synthesis. SnRK1, a principal regulator in carbon signaling, is known to be linked to N and amino acid metabolism. Similarly to CDPK, GCN2 directly act on NR in plants. A mutant lacking GCN2 showed decreased expression of nitrate reducatse (nia1) gene in Arabidopsis.However, whether this had any direct implication on improvement of NUE remains to be validated. Recently, transgenic Arabidopsis plants overexpressing STP13, a member of sugar transporter family, showed increased rates of glucose uptake, higher internal sugar levels, and more total C per plant. STP13OX seedlings also displayed improved N use, with the induction of a nitrate transporter and higher total N per plant.

6.6 Manipulating signaling targets

Nitrate is a potent signal that affects N and carbon metabolism as well as organ growth and development in plants. These effects are mediated at least in part by changes in gene expression that are elicited by nitrate. Identification of nitrate-responsive gene expression profiles of plants would offer potential targets for improvement of NUE. The list of nitrate responsive genes in Arabidopsis runs into several hundreds, as revealed by microarray analyses. Recently, we found more than 1000 genes in rice to be nitrate responsive in green and etiolated leaves, with 159 genes being exclusively nitrate responsive (Gene Expression Omnibus accession ID: GSE 12940). Transcription factors (TFs) are master regulators that coordinate the expression of entire response networks of target genes and a number of attempts have been made to indentify TFs that regulate nitrate-responsive gene expression. Recently, bioinformatic approaches have been employed to search for TFs in nitrate-responsive genes identified by microarray in Arabidopsis and rice, although no consensus candidate has been revealed yet (Das *et al*., 22007). Transgenic Arabidopsis lines overexpressing Dof1, a maize protein that belongs to the Dof family of plant-specific TFs known to activate the expression of several C-metabolizing genes associated with organic acid metabolism have been generated. The genes upregulated by Dof1 overexpression clearly belong to the list of known nitrate-responsive genes, opening up attractive possibilities of improving NUE through coordinated expression of N and C metabolizing genes. A few other attempts to manipulate signaling/regulatory proteins have been made without significant advantage in terms of NUE. TFs such as AtNF-YB1 from Arabidopsis thaliana and ZmNF-YB2 from maize improved NUE and water use efficiency (WUE) in transgenic plants. (Other attempts, such as the one to manipulate a MADS box protein that controls nitrateinduced changes in root architecture, have not been assessed for their impact on NUE.)

2. Improvement of nitrogen utilization using genetically modified crops:

Nitrate reduction is rarely limiting for optimal grain yield or biomass production. For example the work of Fuente and co-worker in 2001 showed that, in tobacco, overexpression of a gene encoding cytosolic glutamine synthetase (GS1) from alfalfa, causes an increase in photosynthesis and growth under a low N fertilization regime. These results suggest that the transgenic tobacco plants overexpresing GS1 are able to utilize N more efficiently under N stress conditions. Interestingly, Oliveira and co-worker in 2002 also showed that in tobacco, the overexpression of a gene encoding a pea GS1 lead to increased biomass production both under limiting and non-limiting N feeding conditions.

In wheat, the overexpression of a gene for GS1 from French bean led to an increase in grain yield and therefore of NUE (Habash *et al*., 2001). Similar work was conducted in maize consisting in the overexpression of a native gene encoding GS1 (*Gln1-3*) of maize. Grain yield of

the maize transgenic plants grown under greenhouse conditions was increased by about 30%. However, grain N content and biomass production of the transgenic plants were not modified at maturity (Martin *et al*., 2006). More recently, transgenic rice lines overexpressing GS1 showed improved harvest index, N harvest index and N utilization efficiency.

In other species, the overexpression of GS1 had a rather negative impact on growth and yield of the plant. For example, overexpression of a GS1gene from tobacco in the legume birds foot trefoil (*Lotus corniculatus* L.) grown on nitrate led to an acceleration of senescence, which was apparently detrimental to the overall plant developmental process (Vincent *et al*., 1997). When the transgenic *L. corniculatus* plants were grown under symbiotic N-fixing conditions an increase in plant biomass production was unexpectedly observed. In rape (canola), the overexpression of a gene encoding the enzyme alanine aminotransferase (AlaAT) from barley, directed by a rape root-specific promoter, led to a dramatic increase in biomass production and seed yield (Good *et al*., 2007). Improvement of plant productivity was only observed under low N fertilization conditions and was attributed to a higher flux of nitrate, associated or induced by a decrease in the content of glutamine and glutamate in the stem. In the field when the applied N fertilizer rate was reduced by 40%, the agronomic performance of the transgenic rapeseed plants overexpressing AlaAT was similar to that of untransformed control plants grown under higher optimal N fertilizer rates.

Overexpression of the same gene in rice led to increased biomass production and N content of stems (Shrawat *et al*., 2008). Unlike in rapeseed, there was an increase of glutamine and asparagine content both in the stems and in the roots. The genetically modified rice plants had a finer, denser and more branched root system, which was presumably more favorable for the absorption of N. This result indicates that genetic modification targeted to improve N utilization efficiency also had an impact on plant development, although the effect of AlaAT overexpression was variable from one species to another in terms of both plant growth and metabolic activity.

There are a few other examples of successful genetic modification of N metabolism using either structural or putative regulatory genes. When the bacterial enzyme glutamate dehydrogenase (GDH A) from *E. coli* was constitutively overexpressed in tobacco, biomass production of the transgenic plants was increased by about 10–15%. In addition to the increase in biomass production GDHA overexpressors had more leaves and their free amino acid content was higher, suggesting that both N metabolism and C metabolism were modified (Ameziane *et al*., 2000). The transgenic tobacco plants were also more tolerant to water stress.

In rice, overexpression of a gene of unknown function OsENOD93-1, a N-responsive gene identified following genome-wide gene expression profiling, led to an increase in grain yield, under limiting and non-limiting N nutrition conditions respectively (Bi *et al*., 2009). When a gene encoding NAD(H)-dependent GOGAT from alfalfa was constitutively expressed in tobacco, a significant increase in biomass production was observed(Chichkova *et al*., 2001). Overexpression of the native NAD(H)-dependent GOGAT in rice led to an increase in grain weight (Yamaya *et al*., 2002). These results suggest that the GOGAT enzyme plays a major role with respect to organic N management and is used either for growth or for grain production depending on the species examined.

There are fewer studies in which the importance of regulatory genes has been clearly demonstrated (Yanagisawa *et al*., 2004). When a *Dof1* gene encoding a transcription factor from maize was overexpressed in Arabidopsis (*Arabidopsis thaliana* L.), an increase in amino acid content and of N uptake was observed, especially when plants were grown at a low level of N supply. In addition, the transgenic plants produced more biomass under low N supply and they did not exhibit symptoms of N deficiency in comparison to the untransformed control plants, which developed much earlier symptoms of senescence. When the *Dof 1* gene was overexpressed in potato, transgenic plants accumulated more amino acids especially glutamine and glutamate (Yanagisawa *et al*., 2004). These two sets of experiments suggest that this gene could be used to improve the uptake and utilization of N in several species. Thus, overexpressing regulatory genes rather than structural genes, such as genes encoding GS,

GOGAT or AlaAT appears to be an interesting alternative to improve plant NUE and overall plant growth and development in a more stable and balanced way across species.

When vegetable crops such as lettuce or spinach are grown under greenhouse conditions they can accumulate substantial amounts of nitrate in the leaf cell vacuoles. The threshold of nitrate accumulation often exceeds the limits permitted by law, even when N fertilization is reduced because mineralization of soil organic matter always provides a surplus of nitrate to the plant. In human food, when nitrate is absorbed in excess, its reduction to nitrite during digestion can oxidize hemoglobin, causing a kind of anemia. Moreover, nitrites can be converted to carcinogenic nitrosamines. Conventional methods of selection have led to the development of varieties able to reduce the absorbed nitrate more efficiently instead of storing it, but these varieties are not able to completely eliminate any risk of toxic accumulation. Studies were therefore undertaken to limit nitrate accumulation by increasing the capacity of a plant to reduce nitrate by increasing nitrate reductase (NR) activity in genetically modified plants, by overexpressing a gene that allows the deregulation of the synthesis of the enzyme (Quilléré *et al*., 1994). In tobacco a 50% reduction in leaf nitrate content was observed after introduction of the native structural NR gene (*Nia2*) placed under the control of the 35S strong constitutive promoter. Using the same approach, encouraging results were obtained in a variety of potato that showed a 95% decrease in the amount nitrate in the tubers. The more effective reduction of nitrate probably allowed a better allocation of N to the photosynthetic apparatus and to enzymes involved in C metabolism, which was demonstrated by higher leaf chlorophyll content in the transgenic potato plants (Djennane *et al*., 2004).

In lettuce transformed with the same 35S-*Nia2* construct, a problem of post-transcriptional regulation of the NR enzyme was encountered (Curtis *et al*., 1999). The transgenic lettuce accumulated 21% less nitrate after 22 days. However, the nitrate content was only 4% lower in 84 days-old transgenic plants. The hypothesis that the strength of the 35S promoter decreases during plant ageing was put forward, suggesting that a way to maintain NR activity at a high level regardless of plant age needs to be found. Such a strategy to reduce the nitrate content in vegetable crops requires further research before the use of the *Nia2* transgene can be efficiently mastered.

CONCLUSION

Improving global plant productivity and product quality together with taking care of environmental quality and human wellbeing are the main challenges for the immediate future. Such a goal depends on agricultural development and policy and can be achieved by providing the right nutrient source at the right rate, the right time and the right place. To improve sustainable agricultural production, it is also necessary to grow crops that can remove the nutrient applied to soil efficiently, and therefore require less fertilizer. Such global 'resource use efficiency' necessitates having a global view of plant physiology, plant uptake capacity, plant metabolism and plant response to restrictions, as well as a view of soil physical and chemical properties. This review gives an overview of the different metabolic and physiological clues that agronomical research has provided. The enzymes and regulatory processes that can be manipulated to control NUE are presented. The last results obtained from natural variation and QTL studies show the complexity of NUE and open new perspectives. With regard to the complexity of the challenge we have to face and with regard to the numerous approaches available, the integration of data coming from transcriptomic studies, functional genomics, quantitative genetics, ecophysiology and soil science into explanatory models of whole plant behaviour in the environment has to be encouraged.

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