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# Concurrent Expression and Regulation of Genes Involved in Carbon and Nitrogen Metabolism in Relation with Nitrogen Use Efficiency

**Anamika Kashyap[1*], Arnab Saha[1], I.N. Sanyal[2] and B.R. Singh[1]**

*[1]Department of Molecular Biology and Genetic Engineering, College of Basic Science and Humanities, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar- 263145 (India)*
*[2]Plant Transgenic Lab, CSIR-National Botanical Research Institute, P.O. Box 436, Rana Pratap Marg, Lucknow 226 001, India*

**Corresponding author*

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**A B S T R A C T**

Nitrogen use efficiency (NUE) for the crop plants is of great concerns throughout the world. The burgeoning population of the world needs crop genotypes responding to higher nitrogen and showing a direct relationship to yield with the use of nitrogen inputs i.e. high nitrogen-responsive genotypes. However, for fulfilling the high global demand of organic produce, it requires the development of low nitrogen-responsive genotypes with greater nitrogen use efficiency and grain yields. Nitrogen is the most important inorganic nutrient for plant growth. Its effects have been directed to understand the molecular basis of plant responses to nitrogen and to identify nitrogen-responsive genes in order to manipulate their expression and enable the plant to use nitrogen more efficiently. Nitrogen use efficient crops can be produced by manipulating the genes existing in pathways relating to nitrogen uptake, assimilation, amino acid biosynthesis, C/N storage and metabolism, signaling and regulation of nitrogen metabolism and translocation, remobilization and senescence.

## Introduction

Nitrogen (N) is one of the crucial plant macronutrients and required in greatest amount than all another mineral element. It comprises 1.5–2.0 percent of plant dry matter and approximately 16 percent of total plant protein (Frink *et al.,* 1999). Even healthy plants contain 3 to 4 percent nitrogen in their above-ground tissues.

Different plant genotypes of a species sense and respond differentially to the available N in the soil giving rise to differential N responsiveness which is an important agricultural trait. Most of the high yielding varieties of the major crops developed in the last several decades have high demands of N and other nutrients, as well as optimal cultivation conditions (Socolow, 1999).

Nitrogen is most widely used important mineral nutrient, responsible for plant growth and biomass production, synthesis of amino acids, nucleic acids, proteins, lipids, chlorophyll, and various other N-containing compounds (Kusano *et al.,* 2011).

The purpose of this review article is to understand the molecular aspects expression and regulation of genes involved in carbon and nitrogen metabolism with respect to N uptake, assimilation and transportation to different parts and the areas for increasing NUE through frontier science.

## Nitrogen use efficiency

Nitrogen use efficiency (NUE) is defined as grain yield obtained per unit of applied or available nitrogen in the soil. NUE was also defined as the product of nitrogen uptake efficiency (NUPE) and nitrogen utilization efficiency (NUTE) (Moll *et al.,* 1982). It mainly helps in the quantification of apparent Nitrogen recovery using physiological and agronomic parameters (Lochab *et al.,* 2007). NUPE [%] can be delineated as all N present in biomass at maturity divided by the sum of the N applied as fertilizer and Nitrogen present in soil ie. available Nitrogen and NUTE is a ratio of grain yield (in kg) to total N uptake in biomass (NUP in kg). Nitrogen uptake efficiency can be improved through split applications of fertilizers, other nutrient management, and crop management practices thereby minimizing fertilizer losses. The most suitable way to asses NUE depends on the crop, its harvest product and the processes involved in it. But the Nitrogen Utilization Efficiency could only be tackled biologically for higher productivity (Abrol *et al.,* 1999) that includes a balance between storage and current use at the cellular and whole plant level.

$$NUE = NUPE \times NUTE$$

$$NUPE = \frac{\text{N present in biomass at maturity}}{\text{Fertilizer N + Soil N}}$$

$$NUTE = \frac{\text{Grain yield}}{\text{Total N in biomass}}$$

## The fate of nitrogen in the plant

Irrespective of the source of organic or inorganic N provided to the plant, the principal source of N is Nitrate for most crops and wild species, (Salsac *et al.,* 1987; Näsholm *et al.,* 2009), which is taken up by means of specific transporters (high and low affinity) located in the cell membrane of root cells (Miller *et al.,* 2007; Dechorgnat *et al.,* 2011). After the uptake of nitrogen in the form of Nitrate, it is then reduced to form Nitrite with the help of nitrate reductase enzyme (NR; EC 1.6.6.1), (Kaiser *et al.,* 2011). Nitrate Reductase was the first substrate induction system seen in plants (Tang and Wu, 1957). Nitrite is further gets reduced to form ammonia catalyzed by the nitrite reductase enzyme (Nir; EC 1.7.7.1) (Sétif *et al.,* 2009). Exceptions to this pathway are also present which under circumstantial environments, ammonia transporters in roots (Ludewig *et al.,* 2007) can facilitate a direct uptake of ammonia, if available in the soil, an example in paddy fields of rice or in acidic forest habitats (Mae *et al.,* 1997). Ammonia can also be produced inside the plant by an array of metabolic pathways such as phenylpropanoid metabolism, photorespiration, amino acids catabolism and utilization of N transport compounds. Another important source of N is symbiotically fixed N which is readily available to herbaceous woody or plants species that forms a symbiotic relationship with N fixing microorganisms (Hirel *et al.,* 2011). Some plants to a lesser extent use proteins, peptides or amino acids as a source of Nitrogen under low Nitrogen conditions (Good *et al.,* 2007; Rentsch *et al.,* 2007; Nasholm *et al.,* 2009). Few types of research

have been done on the uptake of organic N by crops like corn (Biernath *et al.,* 2008), clover (Nasholm *et al.,* 2000) and wheat (Nasholm *et al.,* 2001) under organic farming conditions but the importance and significance have not been yet established. Plants growing on mature forests or arctic tundra (low pH and reduce soils) take up Ammonium or amino acids as a source of Nitrogen although plants adapted to aerobic soils prefer Nitrate (Maathius, 2009).

## Optimum nitrate uptake: Preeminent requirement fir nitrogen use efficiency

This process occurs at the root level and two nitrate transporters coexist in plants to act coordinately to take up nitrate from the soil and allow its distribution in the whole plant (Daniel-Vedele *et al.,* 1998).

Two nitrate transport systems have been shown to coexist in plants and to act co-ordinately to take up nitrate from the soil solution and distribute nitrate within the whole plant (Masclaux-Daubresse *et al.,* 2010).

This transporter system can be divided into two types, Firstly, The low-affinity transport system (LATS) is used when nitrate is present at a higher concentration ie., above 1 mM. Secondly, the high-affinity transport system (HATS) works at low concentrations nitrates (1 µM–1 mM). Among the two transporters, LATS is constitutively expressed and act as a signal molecule to induce the expression of HATS and nitrate assimilatory genes (Pathak *et al.,* 2008). There are mainly two types of HATS namely inducible High-Affinity Transport System (or iHATS) which is strongly induced in presence of nitrate while the second High-Affinity Transport System is constitutively expressed.

Km values of iHATS, cHATS, and LHATS for nitrate are in the ranges of 13-79uM, 6-20uM and >1mM respectively. Nitrate transport through LATS is mediated by the NRT1 gene family. NRT1.1, which is a dual transporter participating in both low and high-affinity $NO^{3-}$uptake is an exception of this family. (Wang *et al.,* 1998). iHATS is a multicomponent system of NRT2 family partly encoded genes or nitrate-nitrite porter family of transporters. The HATS relies on the activity of the NRT2 family (Miller *et al.,* 2001) when the $NO^{3-}$ concentration in the external medium is low. Other ion transport systems such as phosphates, sulfates etc. cannot act as a regulator for its own uptake while the nitrate does. If the cells are exposed to prolonged nitrate content, a lag period of 0.5 to 1.5 hours can be seen followed by increasing uptake capacity and finally reaches to a new steady state after 4 to 6 hours (Figure 1).

For transport of ammonia, both HATS and LATS are found in plant roots for its uptake (Glass *et al.,* 2002). HATS, a saturable transport system for $NH^{4+}$ uptake, is operated only when the concentration of $NH^{4+}$ is present in less than 0.5 mM (Marschner, 2012). Physiological and ammonium influx studies were carried out on single, double, triple and quadruple mutants in order to develop the function of each of the AMT. It is mainly obtained through T-DNA insertion or by complementing the quadruple mutant by single genes (Yuan *et al.,* 2007). Among different AMTs, AMT 1.1 and AMT 1.3 have similar $NH^{4+}$ uptake capacity of around 30-35% while AMT 1.2 contributes 18-25%. AMT 1.5 is having a low Km of 4.5 mM with a low uptake capacity.

## Genes involved in Nitrogen assimilation

A small portion of nitrate that is taken up by the roots is assimilated in the roots itself, but the larger part is transported to the shoot. In the shoot, NAD/NADP dependent nitrate is

reduced to reductase (NR) in the cytoplasm (Meyer and Stitt, 2001). NR is mainly thought to be localized in the cytosol, although the association with the plasma membrane is seen on corn roots and barley (Ward *et al.,* 1989). It is a homodimer where each monomer associated with a 3 prosthetic groups FAD, Haem, and Molybdenum cofactor. Characterization and identification of genes have ben done of NR in different species since 1993 (Reviewed by Meyer and Stitt, 2001). There are mainly two classes of genes namely Nia genes encoding NR apoenzyme and Cnx genes encoding Molybdenum Cobalt (Mo-Co) cofactor. Increase in NR gene expression did not improve NUE of cereal crops under low Nitrogen conditions (Good *et al.,* 2007). Although patents have been issued utilizing NR genes from red algae showed increased maize yield under limiting Nitrogen conditions (Loussaert *et al.,* 2011). nitrite by nitrate. (Figure. 2)

The ultimate source of inorganic N available to the plant is ammonium, which is incorporated into organic molecules in the form of Glutamine and Glutamate through the combined action of the two enzymes *GS* and *GOGAT*. Carbon originating from photosynthesis through the tricarboxylic acid cycle (TCA cycle) provides the α–ketoglutarate needed for the reaction catalyzed by the enzyme GOGAT. Amino acids are further used for the synthesis of proteins, nucleotides and all N-containing molecules (Hirel *et al.,* 2011).

In higher plants, two forms of protein are representing the glutamine synthetase (GS)-Cytosolic and Plastidic forms. (Hirel B *et al.,* 1993) Decameric structure of Maize GS was described by Unno *et al.,* 2006. Studies on both monocot and dicot plant species showed that cytosolic GS (GS1) is encoded by complex GLN1 gen family (Lam H-M *et al.,* 1995). It mainly involves in ammonium recycling during development stages such as leaf senescence and also in Glutamine synthesis for transports it to phloem sap (reviewed by Bernard and Habash, 2009). Whereas, plastidic GS2 is encoded by single nuclear gene GLN2. It is thought to be involved in assimilation of $NH_4^+$ coming from nitrate reduction in both $C_3$ and $C_4$ plants (Keys *et al.,* 1978).. The GS fixes ammonium with glutamate to form glutamine which reacts with 2-oxoglutarate to form 2 molecules of Glutamate. The latter reaction is catalyzed by Glutamine-2-oxoglutarate aminotransferase (or Glutamate synthase, GOGAT). 2 forms of Glutamate synthase are present namely Fd-GOGAT and NADH-GOGAT which uses Fd and NADH as the electron donor respectively (Vanoni *et al.,* 2005). Fd-GOGAT is primarily found on leaf chloroplast whereas NADH-GOGAT predominantly located in plastids of nonphotosynthetic tissues such as roots, companion cells. Structures, properties, regulatory mechanism and role in amino acid metabolism by this enzyme was reviewed by Suzuki and Knaff (2005). Cross genome-ortho-meta-QTL studies in cereals identified GOGAT genes, assuming that it may be a major candidate for cereal NUE (Vitousek *et al.,* 2009). In primary assimilation of ammonia, prevailing GS/GOGAT isoenzymes are chloroplastic GS2 and Fd-GOGAT and cytosolic GS1 and NADH-GOGAT (Lam *et al.,* 1998). Secondary assimilation of ammonia is executed by its incorporation in glutamine/glutamate amino acids using carbon-containing intermediates which are produced via metabolic pathways. Three enzymes participate in this reaction namely-Cytosolic Asparagine Synthetase (AS), Plastidic Carbamoyl phosphate synthase (CPSase) and Mitochondrial NADH-Glutamate dehydrogenase (NADH-GDH). AS transfers the amido group of Glutamine to aspartate to form glutamate and asparagines in an ATP catalyzed reaction (Lam *et al.,* 2003). Asparagine has higher N/C ratio than

Glutamine. So it can be used as a long storage compound and for long-range transport in case of legumes (Rochat and Boutin, 1991; Lam *et al.,* 2003).Small gene family encodes AS in case of higher plants (Lam H-M *et al.,* 1998). While in Arabidopsis it is mainly encoded by three genes (ASN1, ASN2, ASN3). Overexpressing ASN1 using constitutive promoter causes enhanced soluble seed protein content, total protein content and better growth on N limiting medium(Lam H-M *et al.,* 2003). While ASN2 gene overexpression effects less endogenous ammonium compared to wild-type plant on 50mM $Nh_4^+$ medium (Lam H-M *et al.,* 2003). NADH-GDH incorporate $NH4^+$ to 2-oxoglutarate to form glutamate to a high level of $NH4^+$ under stress condition (Skopelitis *et al.,* 2006). It is the main enzyme involved in inorganic N assimilation in plants (Lea *et al.,* 2011). The physiological role of GDH has not yet fully understood (Dubois F *et al.*,2003). But a number of experiments using $^{15}N$ labeling followed by GCMS or NMR spectroscopy showed that it helps in glutamate deamination to provide organic acids in C-limited conditions (Aubert *et al.,* 2011; Labboun *et al.,* 2009) although the rate is far lower than GS-GOGAT pathway (Skopelitis *et al.,* 2006). GDH activity in N management and in whole plant physiological properties has been done on Tobacco (Terce-Laforgue *et al.,* 2004) and Maize (Hirel *et al.,* 2005)

## Genes involved in Transport of Nitrogen and its remobilization

During senescence, leaf proteins, particularly photosynthetic proteins of plastids are extensively degraded, provides an enormous source of nitrogen to plant. Plants can use this nitrogen as a supplement of nutrition to grow organs such as new leaves and seeds. (Figure. 3) In oilseed rape and *Arabidopsis*, it has been shown that nitrogen can be remobilized from senescing leaves to seeds at the reproductive stage as well as from senescing leaves to expanding leaves at the vegetative stage (Lemaitre *et al.,* 2008). At the reproductive stage experiments of $^{15}N$ tracing showed that the rate of nitrogen remobilization from the rosettes to the seeds and to the flowering organs was similar in early and late senescing lines (Diaz *et al.,* 2008).

Some studies in maize, wheat, and barley show that grain nitrogen content is correlated with flag leaf senescence. It shows that flag leaf senescence plays a special role in nitrogen availability for grain filling. For NRE, the onset and the speed of flag leaf senescence are essential (Uauy *et al.,* 2006). Delaying leaf senescence results in increases grain yield and carbon filling in seeds due to the prolongation of photosynthesis but it also responsible for decreasing protein content.

During senescence chloroplasts show the first symptoms of deterioration, whereas other organelles are degraded later, the mechanisms involve for chloroplast degradation are unclear. Chloroplasts contain a high number of proteases like DegP, FstH proteases, and FstH6 protease that responsible for degradation of chloroplast proteins within the organelle during. In senescence, DegP and FstH proteases degrade D1 protein and FstH6 protease degrade LHCII protein (Martinez *et al.,* 2008).

## Genes for Carbon Metabolism

The ability of the plant to take up and bestow nitrogen cannot result in increased nitrogen use efficiency alone. The other important aspect to be considered for increasing NUE is the link between C and N. If there is the insufficient availability of carbon, plants capability to utilize N can be compromised and vise versa (Reich *et al.,* 2006). For example, upregulation of nitrate transporters (AtNRT2.1 and At NRT1.1) was related to

Glucose-6-Phosphate concentration (Wirth *et al.,* 2007). In spite of this, it was shown that increase in nitrate supply causes a decrease of starch synthesis and produces more amino acids from organic acids through carbon diversion. On the other hand, nitrate deficiency causes a decrease in many amino acids along with increasing carbohydrates, phosphoesters and secondary metabolites (Fernie t al., 2004). Studies on global gene expression showed that nitrate responsive gene required the presence of both N and sugar, with carbon modulating effect and vice versa (Price *et al.,* 2004). Nitrogen is stored in large quantities in photosynthetic proteins such as Rubisco and phosphoenolpyruvate carboxylase (PEPc); also crucial to plant C:N ratios are the products of the GS-GOGAT assimilatory pathway. Overexpressing Rubisco (rbcs) gene in a rice plant showed increase rubisco-N to leaf-N although there was no change in photosynthesis (Suzuki *et al.,* 2007). Using native PEPc promoter to overexpress PEPc gene showed increasing PEPc transcript level but photosynthetic rates were limited by phosphate (Ku *et al.,* 1999; Hausler *et al.,* 2002). PEPc involved in N metabolism but not play a direct role in NUE (Figure 4).

Photosynthetic rate controls N uptake and assimilation as well as remobilization (Zheng 1996), thus leading to a plateau in NUE unless the photosynthetic rate is also increased. Photosynthetic Nitrogen Use Efficiency (PNUE) is calculated by the rate of carbon assimilation per unit leaf nitrogen (Kumar *et al.,* 2001).$C_4$ plants have a greater PNUE than $C_3$ plants, owing to the $C_4$ concentrating mechanism that leads to $CO_2$ saturation of Rubisco. Further evaluation of the key components of photosynthesis and interactions of C/N metabolites might offer avenues for improving N utilization by optimizing N content in respect to photosynthetic demand.

## Transcription factors and other regulatory proteins

Nitrate is not only a nutrient but also a signal for the regulation of hundreds of nitrate-responsive genes, which include N and C metabolizing enzymes, redox enzymes and a whole range of signaling proteins and transcription factors.

The transcriptional regulation of nitrate-responsive genes could involve *cis*-acting regulatory sequences or nitrate response elements (NRE) (Raghuram *et al.,* 2006). Identification of such regulatory elements might provide an end-point for nitrate signaling and open up avenues for characterizing/manipulating the rest of the signaling pathway to enhance NUE.

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 identify TFs that regulate nitrate-responsive gene expression. Dof1, a plant-specific transcription factor, is involved in the activation of non-photosynthetic, C4-related PEPc, as well as other organic acid metabolism proteins, and is up-regulated during drought stress. Dof1 over-expressing rice and Arabidopsis showed increased induction of the gene encoding PEPc.

When Dof1 over-expressing rice lines were grown in N deficient conditions, both the N and C amounts in the seedlings were increased. Transgenic plants also showed increases in root N, root biomass, and rate of photosynthesis under N limiting condition (Kurai *et al.,* 2011). More experimentation, particularly field trials, is necessary for relation to Dof1 and its role in NUE (Figure. 5).

**Figure.1** Schematic presentation of the known localisation of NRT1, NRT2 and AMT genes in *Arabidopsis*

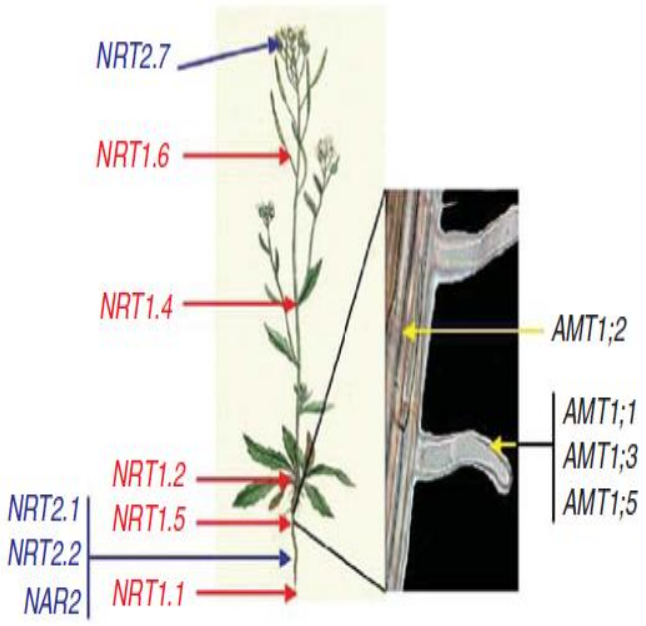



**Figure.2** Main reactions involved in nitrogen assimilation in higher plants. $NO_3^-$ = nitrate; $NO_2^-$ = nitrite; $NH_4^+$ = ammonium, $N_2$ = atmospheric dinitrogen. The main enzymes involved in nitrate reduction and ammonia assimilation are indicated in italics: *NR* = nitrate reductase; *NiR* = nitrite reductase; *Nase* = nitrogenase; *GS* = glutamine synthetase; *GOGAT* = glutamate synthase

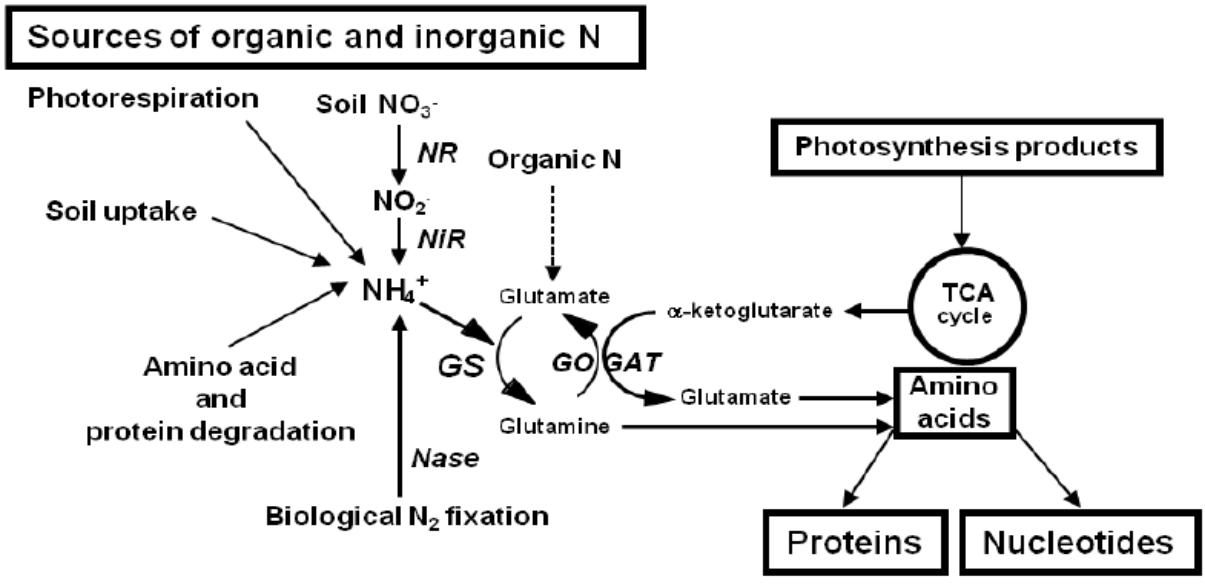


**Figure.3** Schematic representation of nitrate transport steps within the plant

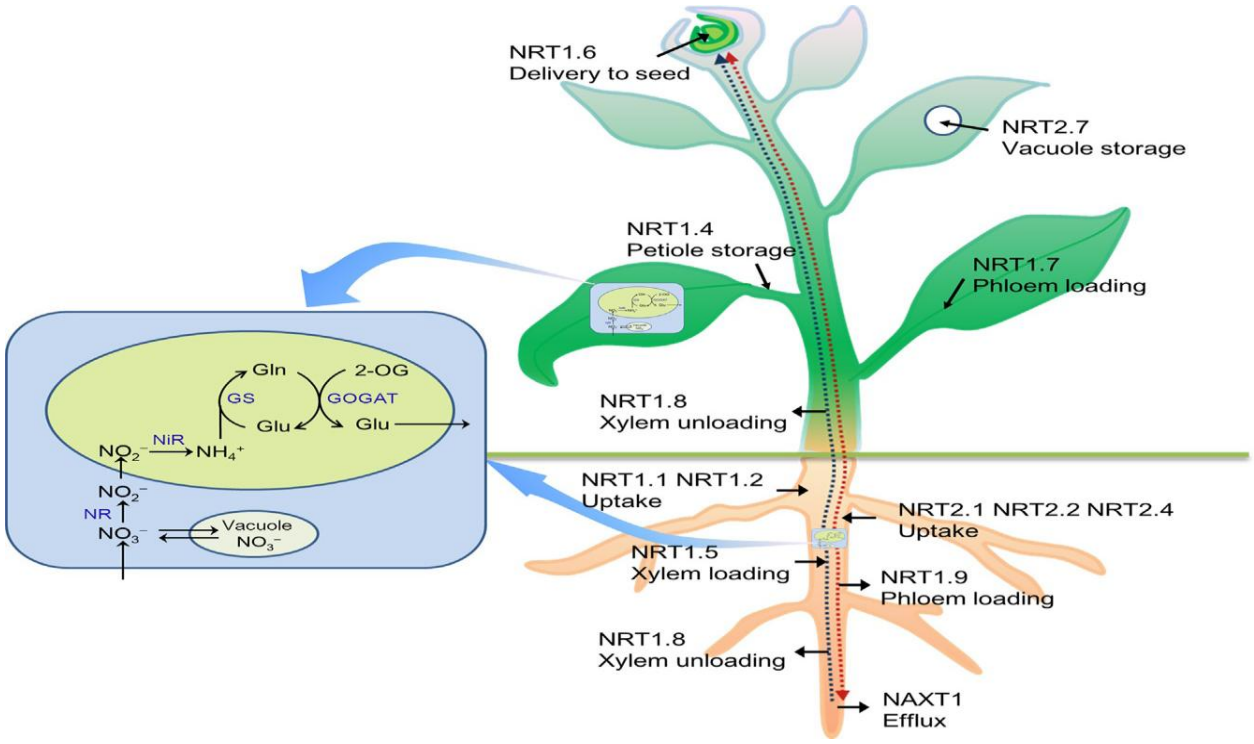



**Figure.4** Enzyme pathways important in the balance of C and N metabolism. AAT, aspartate amino transferase; AS, asparagine synthetase; GS, glutamine synthetase; GOGAT, glutamate synthase. (Miflin *et al.*, 2002)

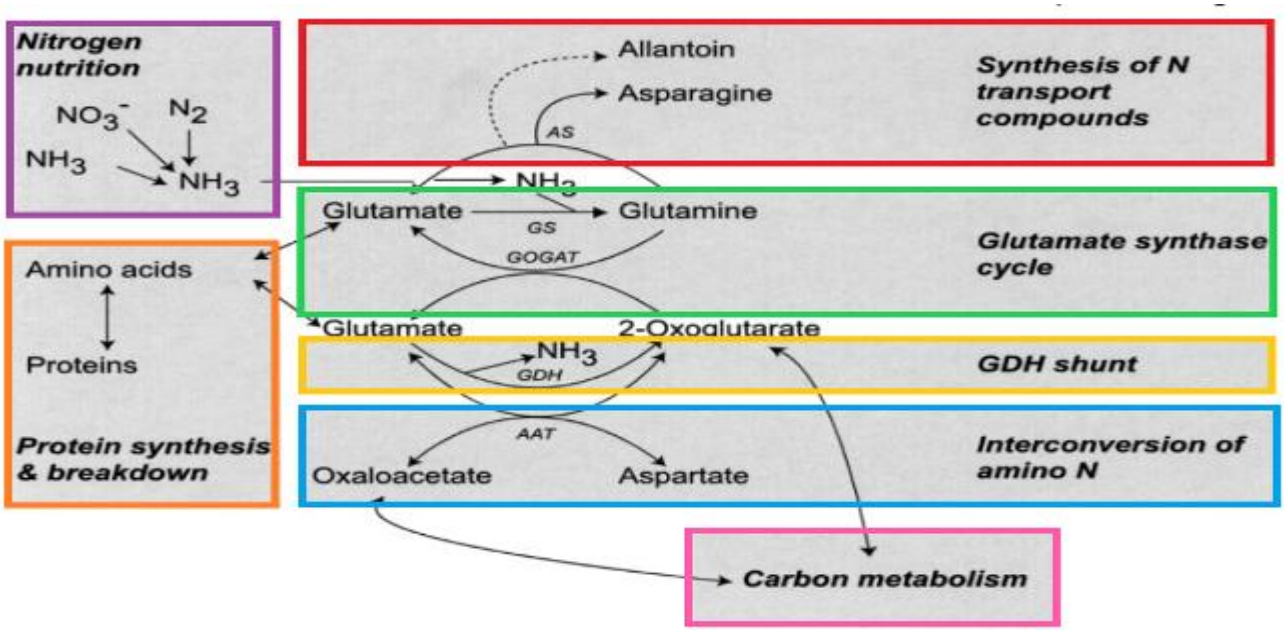
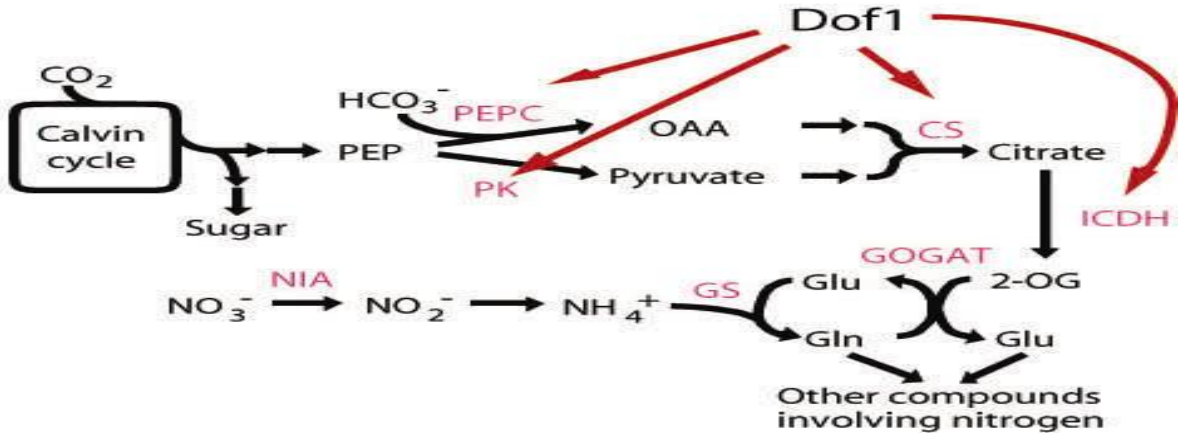


**Figure.5** Dof 1 controlling the genes involved in metabolic pathway for nitrogen assimilation in plants. PEP, Phosphoenolpyruvate; OAA, Oxaloacetate; GOGAT, Glutamate synthase; NIA, Nitrate reductase. (Yanagisawa *et al.*, 2004)




Another transcription factor that has been implicated in NUE is HAP3, a member of protein family haeme activator proteins (HAP). It is involved in regulating flowering time in plants (Cai *et al.*, 2007) and implicated in yeast for increasing NUE (Herna´ ndez *et al.*, 2011). In mammalian systems, HAP proteins are also referred to as NF-Y; NF-YB is used to designate HAP3 (Kumimoto *et al.*, 2008). HAP is a protein complex, which also includes HAP2 and HAP5 (Cai *et al.*, 2007). Initial studies on HAP proteins suggested that the overexpression of HAP5a in tomato caused early flowering (Ben-Naim *et al.*, 2006; Cai *et al.*, 2007). However, over-expression of the same protein, as well as HAP3a, in Arabidopsis resulted in delayed flowering (Wenkel *et al.*, 2006; Cai *et al.*, 2007). In yeast the Hap2-3-5-Gln3 complex has been shown to act as a transcriptional activator of both GDH1 and ASN under N-limiting conditions (Herna´ ndez *et al.*, 2011), suggesting that plant HAP protein/complexes may interact with N assimilation enzymes as well.

HY5 and its homolog HYH, two transcription factors from the bZIP family, are essential for phytochrome-dependent light-activated expression of NR (Lillo, 2008). Despite having a negative effect on transcription the NRT1.1 promoter also has three binding sites for HY5 (Lillo, 2008).

PII is an N sensing and regulatory protein. While a central role for this protein is well documented in bacteria and archaea, its role in N sensing and signaling in plants is less well understood.

In both Arabidopsis and castor bean, a PII-like protein / homolog, GLB1, has been studied in relation to its role in N metabolism. Constitutive over-expression in Arabidopsis of this protein resulted in the accumulation of anthocyanins and a decreased ability to sense or metabolize glutamine (Hsieh *et al.*,1998). PII also regulates the activity of arginine biosynthesis and may act as a sensor of internal N levels (Ferrario-Me´ ry *et al.*, 2006). In the early to late stages of seed development, Plant PII transcripts have been shown to increase approximately ten-fold, a period in which much of the plant N is stored as arginine, suggesting a link between PII and protein storage (Uhrig *et al.*, 2009).

It is concluded that, for economically and environmentally friendly use of valuable N resources, developing high- NUE cultivars is more challenging than targeting N applications as part of an integrated nutrient management. So for the production of high NUE crops, we can target several genes either individually or in a combination. There are several individual genes which are being characterized for defining their role in NUE but there is a need for considering such approaches in which two or more genes are analyzed simultaneously but in a combinatorial way. This review presented the enzymes and regulatory processes that can be manipulated for controlling NUE. 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 behavior in the environment have to be encouraged.
Conflict of Interest:

## Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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